SURVIVAL AND RECULTURABILITY OF PROBIOTIC CELLS DURING FREEZE DRYING

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ABSTRAK

SURVIVAL AND RECULTURABILITY OF PROBIOTIC CELLS DURING FREEZE-DRYING

Pengeringan beku microorganisma secara komersial telah dijalankan sejak beberapa dekad dahulu. Walaupun terdapat banyak kajian mengenai pengeringan beku microorganisma, tetapi hanya sedikit kajian untuk memahami hubungan antara kemandirian sel dan keupayaan berkultur semula semasa proses pengeringan beku. Tujuan kajian ini adalah untuk menyelidik kemandirian sel dan keupayaan berkultur semula bakteria probiotik semasa proses pengeringan beku. Pada enam jam yang pertama, pengeringan beku primer merupakan tahap yang paling penting kerana kadar kematian sel probiotik adalah paling tinggi pada waktu tersebut. Manakala tidak banyak perbezaan terhadap kemandirian sel pada tahap pengeringan beku sekunder. Akan tetapi, keupayaan berkultur semula sel semasa pengeringan beku sekunder memerlukan masa yang lebih panjang berbanding dengan tahap pengeringan beku primer. Ini disebabkan oleh populasi sel yang tidak cedera atau mati adalah lebih tinggi pada tahap pengeringan beku sekunder berbanding dengan pengeringan beku primer. Satu kaedah telah dihasilkan demi menentukan keupayaan berkultur semula semasa pengeringan beku sel. Secara keseluruhannya, peratus kemandirian sel pada jam keenam semasa proses pengeringan beku ialah 21.21% untuk *Lactobacillus acidophilus ATCC 4356*, 28.27% untuk *Lactobacillus casei 01* dan 34.34% untuk *Lactobacillus casei shirota.* Manakala peratus kemandirian sel pada jam kedua puluh empat semasa proses pengeringan beku ialah: *L. a ATCC 4356* (19.93%), *L. casei 01* (20.82%) and *L. casei shirota* (33.60%). Kesimpulannya, *L. a ATCC 4356* mempunyai kadar kematian sel yang paling tinggi iaitu 0.3186 j^{-1} berbanding dengan *L. casei 01* 0.2432 j^{-1} dan *L. casei shirota* 0.2347j^{-1}.

ABSTRACT

SURVIVAL AND RECULTURABILITY OF PROBIOTIC CELL DURING FREEZE-DRYING

Freeze-drying of microorganisms has been practiced commercially since a few decades ago. Although there have been many studies on the freeze dried microorganisms, there are only limited works to understand the relationship between cell survival and reculturability during freeze-drying. This research was aimed to find out the survival and reculturability of probiotic cells during freeze-drying. The most crucial stage causes the greatest death of probiotic cells was in the first 6 hours of freeze-drying process which is the primary drying. However, there was not much significant effect to the cell survival in the secondary drying stage. In contrast, the reculturability of cells need longer time during the secondary drying compared to primary drying. This was due to the higher population of nonlethally injured cells in the secondary drying stage compared to primary drying stage of freeze-drying. A method was developed in determining the reculturability of freeze-drying cells. Generally the highest percentage of cell survival on the primary drying of freeze-drying were L. casei shirota 34.34%, followed by L. casei 01 28.27% and lastly 21.21% for L. acidophilus ATCC 4356. Meanwhile the highest percentage of cells survival on the secondary drying of freeze-drying were L. casei shirota 33.60%, followed by L. casei 01 20.82% and lastly 19.93% for L. acidophilus ATCC 4356. Finally, L. acidophilus ATCC 4356 has the highest specific death rate which was 0.3186 h^{-1} compared to L. casei 01 at 0.2432 h^{-1} and L. casei shirota at 0.2347 h^{-1}.

ABBREVIATION

CFU	colony forming unit
hPa	Hectopascal
LAB	Lactic acid bacteria
MRS	deMan-Ragosa-Sharpe
MSG	monosodium glutamate
SEM	Scanning electron microscopy
rpm	rotating per minute

LIST OF SYMBOLS

c	Number of colony counted on a plate
D	Dilution factor
k	specific death rate (h^{-1})
n	set of samples
t	time (hr)
R	reliability
X_t	Cell viability at time t (CFU/ml)
X_o	Initial cell viability (CFU/ml)
<	less than
°C	degree Celsius

CONTENTS

LIST OF TABLES

LIST OF FIGURES

GLOSSARY

Anaerobic	Living or acting in the absence of free-form oxygen.
Autoclave	An apparatus in which objects or materials may be sterilize air-free Saturated steam under pressure at temperature above 100°C.
Colony	A group of cells produced from an individual cell when grown on a solid medium such as an agar plate.
Culture	A growth of particular types of microorganisms, such as lactic acid bacteria, bifidobacteria, yeasts, and others in a liquid medium or on a solid medium, formed as a result of the previous inoculation and incubation of that medium.
Facultative	Capable of functioning under varying environmental conditions. Used of certain organisms, such as bacteria that can live with or without oxygen.
Genus	A taxonomic category that includes groups of closely related species.
Gram stain	A set of two stains (crystal violet and safranin) that are used to stain bacteria. The staining depends on the composition and the structure of the bacteria cell wall.
Incubation	The process of development and growth of microorganism after inoculation.
Inoculation	The introduction of specific microorganisms into a nutrient medium.
Inoculum	The microorganism which is inoculated into a nutrient medium.
Lag phase	The time from inoculation to active cell replication in batch culture.
Medium	A solid or liquid substrate that can support the growth of an organism. The medium may consist of pure compounds (defined medium) or crude animal or plant extracts (complex medium).
Mesophilic	An organism that grows at moderate temperature in the range of 20 to 45°C and has an optimum growth temperature in the range of 30 to 39°C.
Metabolism	The physical and chemical processes by which chemical components are synthesized into component elements, complex substances are transformed into simpler ones and energy is made available for use by an organism.
Pure culture	A culture containing a single species of organism.
Species	A taxonomic subdivision of a genus. A group of closely related, morphologically similar individuals which actually or potentially interbreed.
Strain	A group of organisms of the same species having distinctive characteristics but not usually considered as separate breed or variety.
Viable	Describing a cell or an organism that is alive and capable of reproduction.
Viable count	The umber of viable cells in a culture of microorganisms.

KEYWORDS

freeze-drying, probiotic, primary drying, reculturability, secondary drying, specific death rate, survival.

CHAPTER 1

INTRODUCTION

1.1 Introduction

In recent years, there are many attempts of modulating the indigenous intestinal flora by live microbial adjuncts to improve the host health status. According to Fuller (1989), probiotics are described as "live microorganisms which when administered in adequate numbers confer a health benefit on the host". Probiotic containing foods can be categorized as functional foods. The market for this food category continues to expand, in parallel with growing consumer awareness of the role of diet in health maintenance (Stanton *et al.,* 2001), and represents an exciting market opportunity for the Food and Dairy Industries. Currently, probiotics are also being used successfully to improve the quality of feed provided to domestic animals (Dunne *et al.,* 2001). Manufacture of probiotics involves many processing steps. Maintaining the viability of cells during processing has been a challenge or problem to manufacturers, as the efficacy of a probiotic products is directly dependent on the availability of a preparation.

Freeze-drying of microorganisms has been practiced commercially since a few decades ago. This method offers convenience of storing and of transport by mail, as well as keeping the microorganisms viable for long periods (Miyamoto-Shinohara *et al.,* 2005). The industrial use of lactic acid bacteria (LAB) as starter cultures for the food industry depends on the concentration and preservation technologies employed, which

are required to guarantee long-term delivery of stable cultures in terms of viability and functional activity (Carvalho *et al.,* 2003b). Although freeze-drying have commonly been used for this purpose, but these techniques bring about undesirable side effects, such as denaturation of sensitive proteins and decreased viability of many cell types. Damage to biological systems resulting from freeze-drying can be attributed mainly to changes in the physical state of membrane lipids and changes in the structure of sensitive proteins (Leslie *et al.*, 1995). The viability of dried cultures depends also on the method employed to rehydrate them, as survival is increased after slow rehydration (Teixeira, Castro & Kirby, 1995a).

In conjunction to that, cryoprotective additives are employed during freeze-drying. Consequently, a number of studies have examined the potential role of additives in suspensions of microorganisms in their survival throughout freezing and drying such as skim milk, sorbitol, monosodium glutamate (MSG) (Carvalho *et al.,* 2003), polyols, polysaccharides, disaccharides, amino acids, proteins, vitamins, gelatin, xanthan gum, maltodextrins and various sugars (Champagne *et al.*, 1991). Ability of the more effective additives to protect the microorganisms is usually attributed to their capacity to bind water and inhibit either intracellular or extracellular ice crystal formation. Their protective effect can also attributed to their contribution to the structure of the microenvironment around the structural elements of the cell wall. Protective additives have an important role in the conservation of viability. A good protectant should provide cryoprotection of cells during freeze-drying, be easily dried, and provide a good matrix to allow stability and ease of rehydration (Costa *et al.*, 2000).

Although there have been many studies of the freeze dried microorganisms, there are only limited works to understand the relationship between cell survival and reculturability during freeze-drying. Literature only reports on the survival of freeze dried probiotic cells whereas not much discovery on the survival of probiotic cells during

freeze-drying (Nei, Araki & Souzu, 1965). The percentage of cell survival after freeze-drying for LAB were very low such as 20% survival for *Lactobacillus bulgaricus* (Champagne, 1991) and 13% survival for *L. acidophilus ATCC 4356*. Therefore, it is crucial to identify a method to improve the survival of cells during the process as can increase the cell survival after freeze-drying. It is interesting to investigate the relationship between the survival and reculturability of probiotic cells during the freeze-drying process as reculturability indicating of cell injury.

The aim of this research was to find out the survival and reculturability of probiotic cells during freeze-drying so that can find out at which stage of freeze-drying cause the deteriorate impact on cellular damage in an effort to increase cell survival in dried products. Finally better understanding in criteria selection of probiotic with good technological properties on technological stress is vital in enhancing the viability of cells in the final product. This would be useful in maintaining sufficiently high numbers of probiotic bacteria in functional food/feed, thereby meeting regulatory standards, and assisting in the delivery of therapeutic benefits to livestock and lastly to consumers.

1.2 Research Objective

This research was aimed to find out the survival and reculturability of probiotic cells during freeze-drying.

1.3 Specific Research Objective

i) To develop a method on cell reculturability during freeze-drying.

ii) To determine the survival of probiotic cells during freeze-drying.

iii) To determine the specific death rate, k of different types of probiotic cells during freeze-drying.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter reviews the literature, theories and data which have been referred or used in this research. Introduction of probiotic was discussed. Then, the freeze-drying process and effect of freeze-drying to the survival of probiotic cells were discussed. Lastly, the response of the probiotic strain to the stress challenge will be look into from the reculturability of cells. Discoveries and problems faced by the previous researcher regarding the field were reviewed. It will be used as guidance for this research.

2.2 What is "Probiotic"?

Probiotics are defined as "live microbial food supplements which benefit the health of consumers by maintaining or improving their intestinal microbial balance" (Fuller, 1989). Generally there are 8 main genera of lactic acid bacteria (LAB) but it does not mean that all LAB is probiotic. The genera: *Lactobacillus, Streptococcus, Lactococcus, Leuconostot, Bifidobacterium, Carnobacterium, Enterococcus* and *Sporolactobacillus* which can be divided into species, subspecies, variants and strains. However, *Lactobacillus* and *Bifidobacterium* are the most commonly used probiotics in foods for human consumption given the significant health benefits associated with ingestion of these microorganisms (Stanton *et al.*, 2003). Due to their perceived health benefits, probiotic bacteria have been increasingly included in yogurts, fermented milks and also

available in the form of dietary supplements where the probiotic is in the form of dried product (Ross *et al.,* 2005).

The large intestine contains over 400 different microbial species. The native microorganisms, which are the dominant microflora in the colon, limit the ability of pathogenic genera including *Escherichia, Clostridium, Salmonella* and *Campylobacter* to attach to the lumen (Ziemer & Gibson, 1998). Once the microbial balance is disturbed, intestinal bloating and diarrhea may occur (Capela, Hay & Shah, 2006).

Clinical studies have shown health improvement associated with consumption of probiotics include reduction in the incidence of childhood atopic eczema (Isolauri *et al.,* 1999; Kalliomaki *et al.,* 2001), decrease in rotavirus shedding in infants (Saavedra *et al.,* 1994) and reductions in antibiotic-associated diarrhea (Plummer *et al.,* 2004). So, in order to be effective as dietary adjunct, it is recommended that the probiotic culture must be present in the product at minimum numbers of 10^7 CFU/ml and even higher numbers have been recommended (Ishibashi & Shimamura, 1993; Lee & Salminen, 1995). Moreover, the probiotic food product should be regularly consumed in sufficient quantities to deliver the relevant dose of live bacteria to the gut, by keeping in mind that losses in cell viability typically encountered during gastric transit. Therefore with sufficient quantities of probiotic in the gut could provide a benefit health to the consumer.

2.3 Selection of Probiotic strains for technological performance

Several aspects, including safety, functional and technological characteristics, have to be taken into consideration in the selection process of probiotic microorganisms. Before a probiotic can benefit human health it must fulfill several criteria such as:

- Non-pathogenicity and antibiotic resistance characteristics which must be regarded as safe (GRAS) status;
- It must be host origin;

- it must have good technological properties so that it can be manufactured and incorporated into food products without loosing viability and functionality or creating unpleasant flavors or textures;
- it must survive passage through the upper gastrointestinal (GI) tract and arrive alive at its site of action;
- it must be able to function in the gut environment (Saarela *et al.*, 2000).

The characterization of probiotic bacteria is important for food industries, as the manufacture of some products requires a particular strain. Again, it must be stressed that the health effects are sometimes related to given strains. Therefore, it is not only necessary to determine if the population of a given species is adequate, but also to ascertain if the product contains the strain it claim to have (Champagne & Gardner, 2005).

2.4 Characteristic of *Lactobacillus* genus

According to Hammes (1995), *lactobacillus* are generally characterized as gram positive, non-sporeforming and non-flagellated rods or coccobacilli. There are either aerotolerant or anaerobic and strictly fermentative (Gomes *et al.*, 1999). Moreover, these organisms are straight or curved bacilli or coccobacilli which occur in chains. Lactobacilli commonly grown on MRS broth (deMan, Ragosa, Sharpe) or agar due to it is specially formulated to sustain the growth of lactobacilli as they have complex nutrient requirement. The surface colonies formed on MRS agar are opaque, white, compact or feathery and small (Robinson *et al.*, 1990). The optimum growth temperature is between 30 to 40°C. Lactobacilli are aciduric and grow best in pH range from 5.5 to 5.8 but in general they can grow at a pH < 5 (Batt, 1999).

There are 64 species recognized under *Lactobacillus* genus. Based on their metabolism of sugars, they are divided into three groups which were Group A (obligately

homofermentative lactobacilli), Group B (facultatively heterofermentative lactobacilli) and Group C (obligately heterofermentative lactobacilli).

2.4.1 *Lactobacillus casei*

Figure 2.1: *Lactobacillus casei*

(Source: http://www.genomenewsnetwork.com/articles/11_02/keepers_art.shtml)

Lactobacillus casei is characterized as group B lactobacilli and is a mesophilic starter. *L. casei* are gram-positive, facultatively anaerobic, non-motile and non-spore-forming, rod shaped with the cell size range = 0.7-1.1 x 2.0-4.0 micrometer. Like other LAB, *L. casei* are acid tolerant, cannot synthesize porphyrins, and posses a strictly fermentative metabolism with lactic acid as the major metabolic end product (Axelsson, 1998; Kandler & Weiss, 1986). Therefore, *L. casei* is also characterized as a homofermentative that only produce lactic acid as its sole product.

L. casei are a remarkably adaptive species, and may be isolated from raw and fermented dairy products, and the reproductive and intestinal tracts of human and animals (Kandler & Weiss, 1986). Industrially, *L. casei* have application as human probiotics (health promoting live culture), and as acid-producing starter cultures for milk fermentation.

2.4.2 *Lactobacillus acidophilus*

Figure 2.2: *Lactobacillus acidophilus*

(Source: www.schudnij.com.pl/pliki/acidophilus.jpg)

Lactobacillus acidophilus and other lactic acid bacteria are important in the fermentation of many foods from dairy products to fruits and vegetables. According to Vela (1995) fermentation occur when bacteria break down sugars and carbohydrates to produce alcohol, carbon dioxide and lactic acid (Jones, 1999). These by-products are responsible for the unique taste of fermented foods.

Currently new research found that there are alternative ways of using lactic acid bacteria, most notably the species *L. acidophilus*. Wood (1992) stated that from the research shows that *L. acidophilus* can also be used as a probiotic or living organism, which upon ingestion in certain numbers; exert health benefits beyond inherent basic nutrition. There is still need for more research in this area before their claims are substantiated, but *L. acidophilus* is linked to decreased instances of vaginal yeast infection, gastrointestinal dysfunction and even boosting immune function.

L. acidophilus is probably the best well-known species of *Lactobacillus*. *L. acidophilus* is characterized as being rod shaped motile bacteria in or without the presence of oxygen. *L. acidophilus* is also characterized as a homofermentative that only produce lactic acid as its sole product.

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