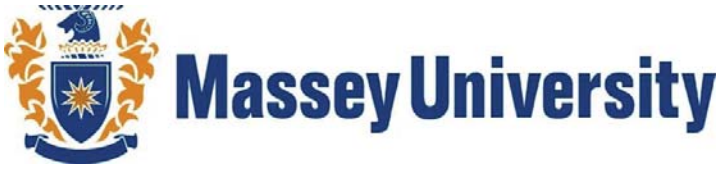
MAXIMIZING VIABILITY OF *LACTOBACILLUS PARACASEI* SUBSP. *PARACASEI L. CASEI* 431 DURING PROCESSING AND AMBIENT STORAGE



A THESIS PRESENTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF TECHNOLOGY IN FOOD TECHNOLOGY

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ABSTRACT

In the present study, fluidized bed drying has been examined as a low-energy alternative to more expensive freeze-drying of the probiotic commercial strain *Lactobacillus paracasei* subsp. *paracasei L. casei* 431. The aim of this study was to maximize the viability of *L. casei* 431 during laboratory and industrial scale processing and storage. The study proceeded in three stages: a) Optimizing the growth conditions and medium composition for maximizing cell growth and desiccation tolerance b) Standardizing the harvesting conditions (harvesting time and techniques) and mixing conditions (mixing of cells with protective carrier) to minimize the mixing and drying loss c) Investigating the effect of various parameters during fluidized bed (FB) drying (initial moisture content, drying time and drying temperature) through Plackett-Burman (PB) and factorial design with the objective of identifying the ideal combination for maximizing the viability of *L. casei* 431 during drying and ambient storage.

The preliminary experiments were performed to optimize growth medium composition under controlled pH in a bioreactor. The effect of supplementing de Man, Rogosa and Sharpe (MRS) media with glucose and yeast extract on viable cell count during batch and fed-batch fermentation was compared. The pH controlled fed-batch fermentation resulted in a 5 fold increase in the viable cell count when compared to batch fermentation. But when the cells obtained from this pH controlled media showed huge drying and storage losses as compared to the uncontrolled pH media, a sequential PB design followed by central composite design matrix was used to screen and optimize the factors that could maximize cell growth under uncontrolled pH conditions. The supplementation of yeast extract and meat extract at a high concentration of 0.6-0.8% nitrogen in MRS media increased the viable cells of *L. casei* 431 by more than 2 fold and biomass by more than 1.5 fold as compared to control (MRS media). The cells from uncontrolled pH fermentations were then harvested by high speed centrifugation and collected cells were mixed with protective carrier (whole milk powder) of different water activity under different mixing conditions. Once growth and mixing conditions were standardized, another PB design followed by factorial design was used to illustrate the effect of various parameters such as harvesting at different growth phases, total solids of harvested cells, initial moisture content, and drying conditions such as drying temperature and drying time, on residual moisture content in combination with their effect on drying and storage stability of *L. casei* 431 under ambient and accelerated storage (37 °C) conditions.

The results showed that the pH during growth, the growth phase, the harvested cell solids/moisture content, the mixing techniques, the drying temperature and the final moisture content were important factors affecting cell stability during drying and storage. Fermentation with acid stress and controlled fluidized bed drying were able to keep the *L. casei* 431 cells relatively stable for 3 months at 25 °C in heat sealed aluminum bags (with inner polyethylene layer) during laboratory and pilot/industrial scale preservation. It was further observed that the shelf-life of FB dried *L. casei* 431 cells at 25 ˚C was 6 times longer than at 37 ˚C.

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CHAPTER 1 INTRODUCTION

Probiotics are generally associated with beneficial health effects, and may be selected for prevention and treatment of certain diseases. Such research has stimulated interest in dairy products containing beneficial bacteria for general population, children and high risk groups (FAO/WHO, 2001). Lactic acid bacteria (LAB), specifically lactobacilli and bifidobacteria are the principal representatives of probiotics in the functional food industry. Within the lactobacilli group *Lactobacillus casei* is commonly used in dairy fermentations owing to its probiotic properties. The human derived *Lactobacillus paracasei* subsp. *paracasei L. casei* 431 (isolated from infant faeces) used in the present study is a commercial probiotic strain with recognized health benefits and has been exploited for use in the development of functional foods. Human studies have shown that *L. casei* 431 alone or in combinations has a beneficial effect on gastrointestinal health (Larsen et al., 2006; Vlieger et al., 2009) and immune health (de Vrese et al., 2005; Rizzardini et al., 2012).

The manufacture of probiotic foods containing viable cells at a recommended level of 10^6-10^7 per millilitre or gram of product at the time of consumption represents a major technical challenge for the industrial producers (Fu and Chen, 2011). Freeze drying is the most popular method of preservation of lactic acid probiotic and starter cultures; however, it involves high operational costs. Spray drying would allow an inexpensive production of large amounts of LAB, as the costs of spray drying can be six times lower per kilogram water removed than the cost of freeze drying. However, commercial production of spray-dried cultures has not been widely successful because many bacterial strains are killed by spray drying due to the high operating temperatures involved (Teixeira et al., 1996). By contrast, the optimal heat and mass transport as well as the equal temperature distribution in a fluidized bed (FB) dryer allow gentle drying of sensitive compounds. In addition, the FB process consumes less time and energy than spray drying and freeze drying (Santivarangkna et al., 2007) and therefore is a cost-effective alternative for preserving bioactive compounds like heat-labile micro-organisms (Strasser et al., 2009). The FB drying process is used extensively in the yeast industry for producing commercial dry yeast. Only a few reports describe use of FB drying for the stabilization of LAB for industrial applications and in these reports a serious problem of shelf-life stability with dried cultures has been presented. The survival rate of the cells during FB drying depends upon a number of factors, including the species and the strain of culture, the inoculum and the growth medium composition, preadaptation of the culture to acquire resistance to processing conditions, protective carrier agents and drying conditions. At the low

temperatures of FB drying thermal inactivation is negligible but the dehydration inactivation may pose serious problems (Lievense et al., 1992). The loss of water from both the cell membranes and the proteins during dehydration is responsible for this damage. The dehydration stress mainly affects the cytoplasmic membrane by changing its fluidity or the physical state as well as causing lipid peroxidation (Crowe e al., 1992; Lievense and van't Riet, 1994).

The industrial applications of probiotics require the maintenance of high viabilities of bacterial populations not only during the preservation process but also during storage. The storage stability of dried cultures is highly dependent on the storage air temperature and the relative humidity. The viability is generally considered inversely related to the storage temperature (Corcoran et al., 2004; Silva et al., 2002; Teixeira et al., 1995), with the dried cultures maintaining longer viability at low refrigeration temperatures. However, refrigeration (4-7 °C) is expensive to both the suppliers and retailers of the probiotic products and thus one of the main obstacles in the commercialization of the probiotic cultures is the development of storable formulated products that retain the viability and the activity of the initial population at ambient storage (Fu and Chen, 2011). The health promoting properties of the probiotic foods thus not only depends on the resistance of bacteria to harsh conditions existing in the gut but also on the viability of probiotic bacteria in the product during its shelf-life. From an industrial point of view, the development of protocols for the preparation of shelf-stable probiotic and starter cultures containing highly active, viable cells tolerant to adverse conditions would be advantageous.

Following this line, the aim of the present study was to maximize the viability and shelf-stability of the probiotic strain *Lactobacillus paracasei* subsp. *paracasei L. casei* 431 through fluidized bed drying. The study began with investigating the effect of growth media composition and growth conditions in maximizing cell growth and desiccation resistance. Once the growth conditions were optimized, harvesting and mixing of cells with the protective carrier were standardized. Because the objective of this study was to also define best processing conditions for scale-up process, the next sets of experiments were performed in which laboratory and industrial scale preservation was compared. On a laboratory scale, the cells are usually harvested by a high speed centrifuge (like Sorvall). The dense pellet of cells thus obtained is then washed with the peptone water to reduce the level of entrained metabolite. Harvesting cells by Sorvall centrifugation is a batch process but in industry, bacterial cells are usually continuously concentrated using some type of separator. The

resulting bacterial concentrate from the separator is more dilute and contains toxic growth medium components. A washing step is also not feasible on this concentrate. Hence the stability of bacteria in FB dried powders made with (i) washed and unwashed concentrate (ii) concentrate of different initial solids/moisture content (iii) cells harvested at different growth phase was investigated. At the same time, experiments were performed to optimize the drying conditions i.e. initial moisture content, drying time and drying temperature with the objective to outline the best combination for maximizing shelf-stability during laboratory and industrial scale processing. The Plackett-Burman (PB) design was used to identify important factors affecting cell viability during fermentation, harvesting and drying. In some studies PB design was followed by factorial design in order to define the optimum levels of factors studied. The storage stability of dried powders was also tested under accelerated storage conditions (37 °C) in order to predict the shelf-stability of powders during long term storage.

CHAPTER 2 LITERATURE REVIEW

2.1 Probiotics

The existence of probiotics has been known for over a century and their health benefits have been studied and investigated over many years. A number of definitions of the term "probiotic" have been used over the years but the one derived by the Food and Agricultural Organisation of the United Nations/World Health Organisation and endorsed by the International Scientific Association for Probiotics and Prebiotics best exemplifies its meaning is 'live microorganisms, which when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2001).

Probiotics are known for improving gut health since the beginning of 19^{th} century. In addition to the gut health benefits such as alleviation of constipation and protection against traveller's diarrhoea, probiotics have also been documented to exert other health-promoting effects such as strengthening of the immune system, antihypertensive effects, reduction of hypercholesterolaemia, alleviation of lactose intolerance, protection against colon and bladder cancer, antioxidative effects, reduction of dermatitis symptoms, facilitation of mineral absorption, amelioration of arthritis, reduction of allergic symptoms and prevention of urogenital disease (Ooi and Liong, 2010; Soccol et al., 2010). Increasingly, clinical evidence supports some of the proposed health benefits of probiotics (Mattila-Sandholm et al., 2002). However, the probiotic potential of different bacterial strains, even within the same species differs, hence strains of the same as well as different species may have differing areas of adherence (site-specific), and their specific immunological effects and actions on a healthy vs. an inflamed mucosal milieu may be distinct from each other (Veld and Havenaar, 1991).

Probiotics are gaining more and more interest as alternatives for antibiotics or anti-inflammatory drugs. But with the suggested daily dose of 10^6 or 10^7 bacteria compared to up to 10^{14} bacteria present in the colon it is to some extent surprising that this minority of bacteria is able to kill or inhibit pathogens successfully. The mode of action of probiotics is still poorly understood but non-specific terms such as colonization resistance or competitive exclusion are often found in literature describing their mode of action. "Colonization resistance or competitive exclusion describes a phenomenon whereby the indigenous anaerobic flora limits the concentration of potentially pathogenic (mostly aerobic) flora in the digestive tract" (Vollaard and Clasener, 1994). This action is supported by various alterations such as modifying gut pH, antagonizing pathogens through production of antimicrobial and antibacterial

compounds such as cytokins and butyric acid, competing for pathogen binding and receptor sites as well as immunomodulatory cells and producing lactase which aids in lactose digestion (Kopp-Hoolihan, 2001). The effects of probiotics may be grouped in three modes of action as described by Oelschlaeger (2010): (i) probiotics might be able to modulate the host's defences including the innate as well as the acquired immune system. This mode of action is most likely important for the prevention and therapy of infectious diseases but also for the treatment of (chronic) inflammation of the digestive tract or parts thereof. In addition this action could be important for the treatment of neoplastic host cells; (ii) probiotics can also have a direct effect on other microorganisms, commensal and/or pathogenic ones. This principle is in many cases of importance for the prevention and therapy of infections and restoration of microbial equilibrium in the gut; (iii) finally, probiotic effects may be based on actions affecting microbial products like toxins and host products, e.g. bile salts and food ingredients. Such actions may result in inactivation of toxins and detoxification of host and food components in the gut.

There has been tremendous interest in the probiotic industry as probiotics have been classified as functional food ingredients. But good viability and stability of probiotics has been both a marketing and technological challenge for industrial producers. In order to exert beneficial effects in probiotic foods, the probiotic cultures must fulfill a range of requirements not only functionally but also technologically and economically. For probiotics to be functional, they have to be viable and available at a high concentration, typically 10^6-10^7 CFU per millilitre or gram of product at the time of consumption. To achieve that they need to survive the manufacturing process, storage conditions and entire shelf-life of the product in which they are added. All of these factors need to be taken into account in the choice of a probiotic strain (Kosin and Rakshit, 2006). Some strict criteria have been proposed for a given microorganism to be considered as a probiotic such as: total safety for the host, resistance to gastric acidity and pancreatic secretions, bile tolerance, adhesion to epithelial cells, antimicrobial activity, inhibition of adhesion of pathogenic bacteria, evaluation of resistance to antibiotics, tolerance to food additives and stability in food matrix. Additionally, they must be able to survive (but not growing) when incorporated into other foods without producing off-flavours or textural changes (Kopp-Hoolihan, 2001). The most extensively studied and widely used probiotics are the strains of Lactic Acid Bacteria (LAB). Some of LAB are thermophilic bacteria having an advantage of withstanding higher temperature during the drying process required for their prolonged storage. These thermophiles are well known for their biotechnological importance in the production of cheeses and fermented milk, which all require incubation of milk or curd at a relatively high temperature (45 °C or above) during their production.

2.2 Lactic acid bacteria

Lactic acid bacteria (LAB) constitute a group of Gram-positive, catalase-negative bacterial species which are able to produce lactic acid as the main end product of fermentation of carbohydrates. Ingestion of LAB has been suggested to confer a range of health benefits including immune system modulation and increased resistance to malignancy and infectious diseases. The most popular LAB used as probiotics are the strains of genera *Bifidobacterium* and *Lactobacillus,* which are known to be resistant against gastric acid, bile salts and pancreatic enzymes, can adhere to colonic mucosa and can readily colonize in the intestinal tract (Soccol et al., 2010). The health benefits of consuming food containing probiotic cultures particularly *L. casei, L. acidophilus*, and *Bifidobacterium* have been documented and their applications in commercial food products like yoghurt and fermented milk are now well accepted (Soccol et al., 2010).

2.2.1 The genus *Bifidobacterium*

Bifidobacteria are generally characterized as rod shaped Gram-positive, non-spore forming, non-motile and catalase-negative anaerobes. They have various shapes including short, curved rods, club-shaped rods and bifurcated Y-shaped rods. The optimum pH for growth is 6–7, with virtually no growth at pH 4.5–5.0 or below or at pH 8.0–8.5 or above. Optimum growth temperature is 37–41 °C, with maximum growth at 43–45 °C and virtually no growth at 25–28 °C or below. Presently, 30 species are included in the genus *Bifidobacterium*, ten of which are from human sources (dental, faecal and vaginal), seventeen from animal intestinal tracts or rumen, two from waste water and one from fermented milk (Gomes and Malcata, 1999). The indigenous microflora of infants is dominated by Bifidobacteria, which are established shortly after birth. The number of Bifidobacteria decreases with increasing age of an individual, and eventually this becomes the third most abundant genus accounting for 25 % of the total adult gut flora.

2.2.2 The genus *Lactobacillus*

Lactobacilli are Gram-positive, non-spore forming microorganisms. Considering cellular shape, they can occur as rods or coccobacilli. The genus *Lactobacillus* is by far the largest of the genera included in LAB. Presently 56 species of *Lactobacillus* have been recognised (Gomes and Malcata, 1999).They are either aero tolerant or anaerobic or facultative anaerobic and require rich media to grow. Glucose is fermented predominantly to lactic acid in the

homofermentative case or equimolar amounts of lactic acid, carbon dioxide and ethanol (and / or acetic acid) in the heterofermentative counterpart.

$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH$ (homolactic fermentation)

$C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + C_2H_5OH + CO_2$ (heterolactic fermentation)

Lactobacilli are widespread in nature. They are found rapidly colonising in the mammalian mucosal membranes such as oral cavity, gastrointestinal (GI) and genital tracts of humans and animals, and constitute an important part of the indigenous microflora of man and higher animals. In general, they are well scattered in places where rich carbohydrate sources are available such as plants and materials of plant origin e.g. sewage and fermenting and spoiled food. Various environmental factors such as pH, oxygen availability, level of specific substrates, presence of secretions and bacterial interactions affect their distribution (Soccol et al., 2010).

Lactobacilli have gained major food and pharmaceutical industry interest nowadays with huge attention focussed on increasing the yield of their biomass and the end products of their metabolism. Application in food industries as starter cultures include fabrication of dairy and meat products, fermentation of plants and vegetables, brewing and wine making and production of lactic acid. Their ability to synthesize enzymes and natural antimicrobial substances called bacteriocins has gained them popularity in the pharmaceutical industry. They are highly acid-tolerant species of LAB and can easily survive gastric acidity and pancreatic secretions and colonise GI tract either permanently or temporarily, thus filling in the prerequisite selection criteria of a probiotic. Other probiotic properties of lactobacilli include *in vitro* adherence to intestine cells, ability to work in multiple hosts and non-pathogenic nature. The consumption of lactobacilli in suggested amounts has been reported to improve intestinal inflammation, maintenance of remission in Crohn's disease, treatment of infections during pregnancy and prevention of urinary tract infections (Axelsson, 1998; Zacharof et al., 2010).

2.2.3 *Lactobacillus casei* group

The commercial probiotic products usually incorporate intestinal species of *Lactobacillus* such as *Lactobacillus casei* not only because of their long tradition of safe use as the starter cultures but also because of their high tolerance to the acidic conditions and thus exerting beneficial effects by balancing the intestinal flora and eventually competing with pathogens for gut colonization (Schiraldi et al., 2003). The probiotic *L. casei* strain Shirota is commonly found in commercial probiotic drink "Yakult". *L. casei* has received attention as a probiotic due to

evidence of anti-microbial, anti-diarrheal and anti-mutagenic effects. In addition, some studies have pointed to their positive effects on cholesterol levels, immune system and blood sugar levels (Aguirre-Ezkauriatza et al., 2010). This *L. casei* group comprises the recently revived *L. zeae* and *L. casei*, *L. paracasei* and *L. rhamnosus.* The latter three are used as probiotics in man and animal (Klein et al., 1998). Recently a number of research groups have focussed their attention towards improving the biomass production of *L. casei* by fermentation, as it is not only a probiotic ingredient that can be added to various food products but also due to its distinctive ability to ferment carbohydrate into lactic acid, which is a valuable product on the market for its widespread field of applications.

2.3 Carbohydrate metabolism in LAB

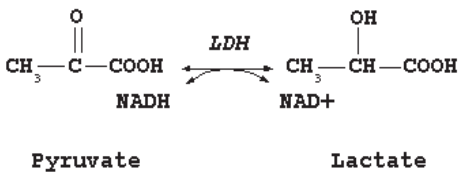
The purpose of Lactobacilli fermenting carbohydrate is primarily to achieve energy in the form of ATP. Two main sugar fermentation pathways can be distinguished among LAB. The glycolysis (Embden-Meyerhof-Paranas (EMP) pathway) results in lactic acid as the end product and the metabolism is referred to as homolactic fermentation. The 6-phosphogluconate/phosphoketolase pathway results in significant amounts of other end products such as ethanol, acetate and CO_2 in addition to lactic acid and the metabolism is referred to as heterolactic fermentation (Axelsson, 1998).

For sugar to be catabolised, it has to be transferred into the interior of the cell. The majority of sugars are taken up by lactobacillus with the help of specific permeases and are then phosphorylated inside the cell. The major reason for the immediate phosphorylation is to prevent diffusion of the sugars out of the cell. A disaccharide like lactose is taken by a specific permease and is split into glucose and galactose by the enzyme β -galactosidase, prior to being phosphorylated inside the cell. The resulting glucose molecule is fermented via glycolysis into two molecules of pyruvate by the Embden-Meyerhof-Parnas (EMP) glycolytic pathway. This process also generates two molecules of adenosine triphosphate (ATP) as an immediate energy yield and two molecules of NADH.

$$C_6H_{12}O_6 + 2\ ADP + 2\ P_i + 2\ NAD^+ \rightarrow 2\ CH_3COCOO^- + 2\ ATP + 2\ NADH + 2\ H_2O + 2H^+$$

Pyruvate is then reduced to lactic acid by a NAD^+-dependent lactate dehydrogenase (LDH), thereby reoxidising the NADH formed during earlier glycolysis steps. A redox balance is thus

obtained, with lactic acid being the only end product. This metabolism is referred to as homolactic fermentation as depicted in Figure 2.3.1.



The resulting D-galactose obtained after splitting of lactose is converted to glucose 6-phosphate by the Leloir pathway (Figure 2.3.2 B) and can enter the glycolysis pathway and be further converted to pyruvate and finally to lactic acid. However in a few lactobacilli (e.g. *L. casei)* lactose and galactose are taken up by the action of the Phosphoenolpyruvate (PEP) – dependent phosphotransferase system (PTS) which is located in the cellular membrane of the lactobacilli. The lactose phosphate formed is hydrolysed by the enzyme phospho-β-galactosidase, resulting in glucose and D-galactose 6-phosphate. While glucose is fermented via glycolysis, D-galactose 6-phosphate is converted to glyceraldehyde-3-phosphate (GAP) through tagatose-6-phosphate pathway, and then gets converted into lactate through glycolysis (Axelsson, 1998; Kandler, 1983) (Figure 2.3.2 A). Heterolactic fermentation is initiated by oxidation of glucose 6-phosphate to gluconate 6-phosphate followed by decarboxylation as shown in Figure 2.3.1. Thus equimolar amounts of CO_2, lactate and acetate or ethanol are formed from glucose by 6-phosphogluconate/phosphoketolase pathway.

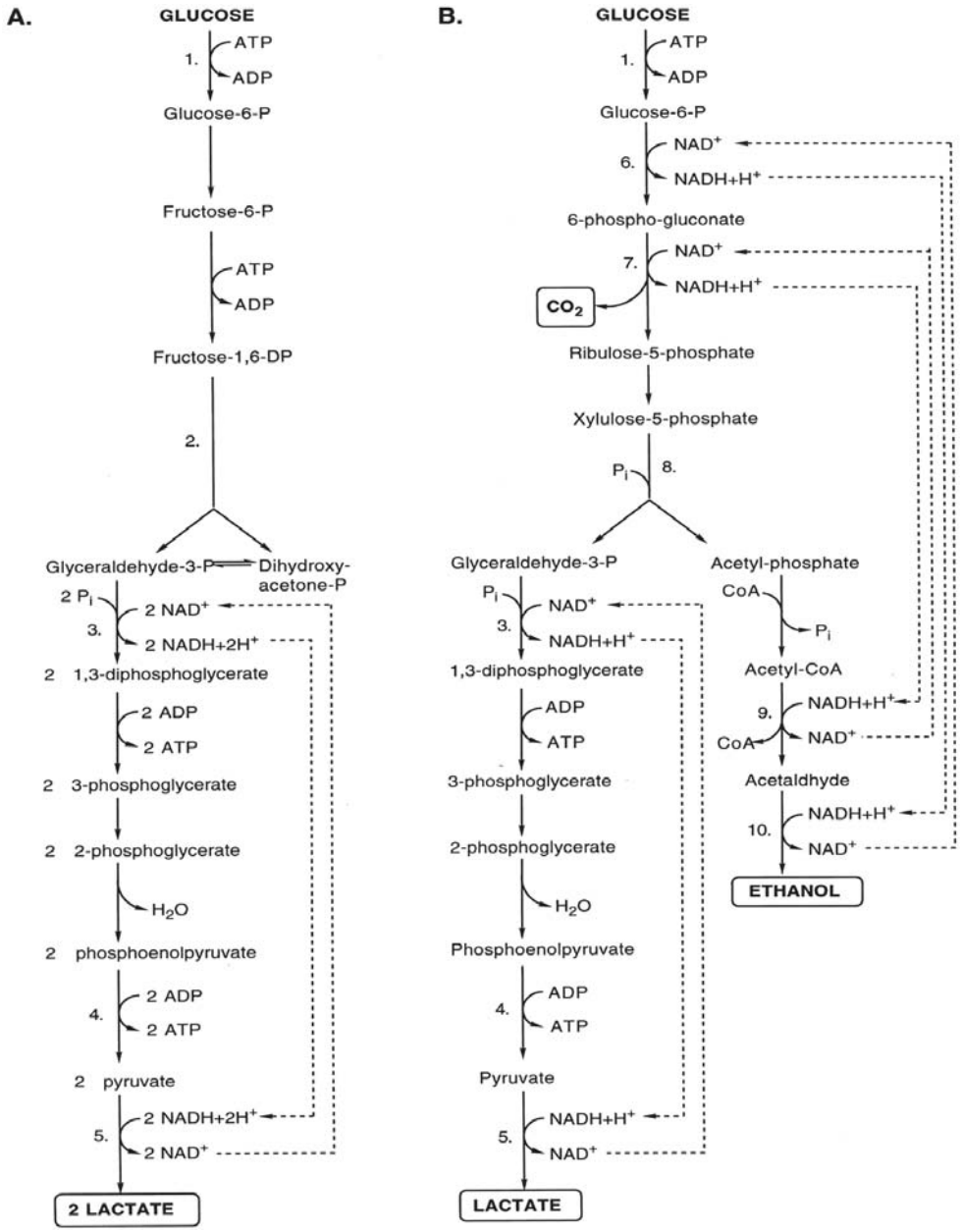


Figure 2.3.1 - Major fermentation pathways of glucose: (A) Homolactic fermentation (glycolysis, Embden-Meyerhof-Parnas pathway); (B) Heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway). Selected enzymes are numbered: 1. Glucokinase; 2. Fructose-1,6-diphosphate aldolase; 3. Glyceradehyde-3-phosphate dehydrogenase; 4.Pyruvate kinase; 5. Lactate dehydrogenase; 6. Glucose-6-phosphate dehydrogenase; 7. 6-Phosphogluconate dehydrogenase; 8. Phosphoketolase; 9. acetaldehyde dehydrogenase; 10. Alcohol dehydrogenase (Axelsson, 1998)

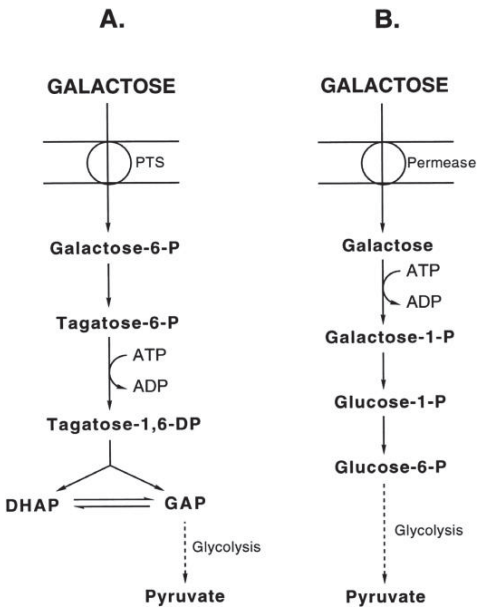


Figure 2.3.2 - Galactose metabolism in lactic acid bacteria: (A) Agatose-6-phosphate pathway; (B) Leloir pathway (Axelsson, 1998)

2.4 Preservation of LAB

The preservation of LAB using different drying methodologies has been used for decades. The whole dehydration process can be divided into three major phases as described by Fu and Chen (2011): "pre-drying, in-drying and post-drying phases" (Figure 2.4.1). At present freeze drying is the most popular industrial method of preservation of probiotic and starter cultures, but it has major drawback of volume limitations. Freeze drying can take days to complete for large product loads, it is lengthy and more expensive than the other drying processes (Table 2.4.1).

Table 2.4.1 - Costs of drying processes referenced to that of freeze drying (adopted from Santivarangkna et al., 2007)

Drying processes	Fixed costs (%)	Manufacturing costs (%)
Freeze drying	100.0	100.0
Vacuum drying	52.2	51.6
Spray drying	12.0	20.0
Drum drying	9.3	24.1
Fluidized bed drying	8.8	17.9
Air drying	5.3	17.9

Therefore, many attempts have been made to develop alternative drying processes with the lower costs. Alternative drying processes can be categorized into two major groups on the basis of drying temperature used (i) processes working at high temperature such as spray drying and (ii) processes working at lower temperatures such as fluidized bed (FB) drying and vacuum drying (Santivarangkna et al., 2007). Spray drying and FB drying are the two commonly used convective drying approaches in industry, whereas vacuum drying could be either convective or conductive, depending on different process designs (Fu and Chen, 2011).

Factors affecting the cell viability in a convective drying process can be further divided into two groups (i) intrinsic factors such as intrinsic stress tolerance of cells and (ii) extrinsic factors such as drying conditions and protective carrier medium used which are summarized in Figure 2.4.1 (Lievense and van't Riet, 1993, 1994; Fu and Chen, 2011).

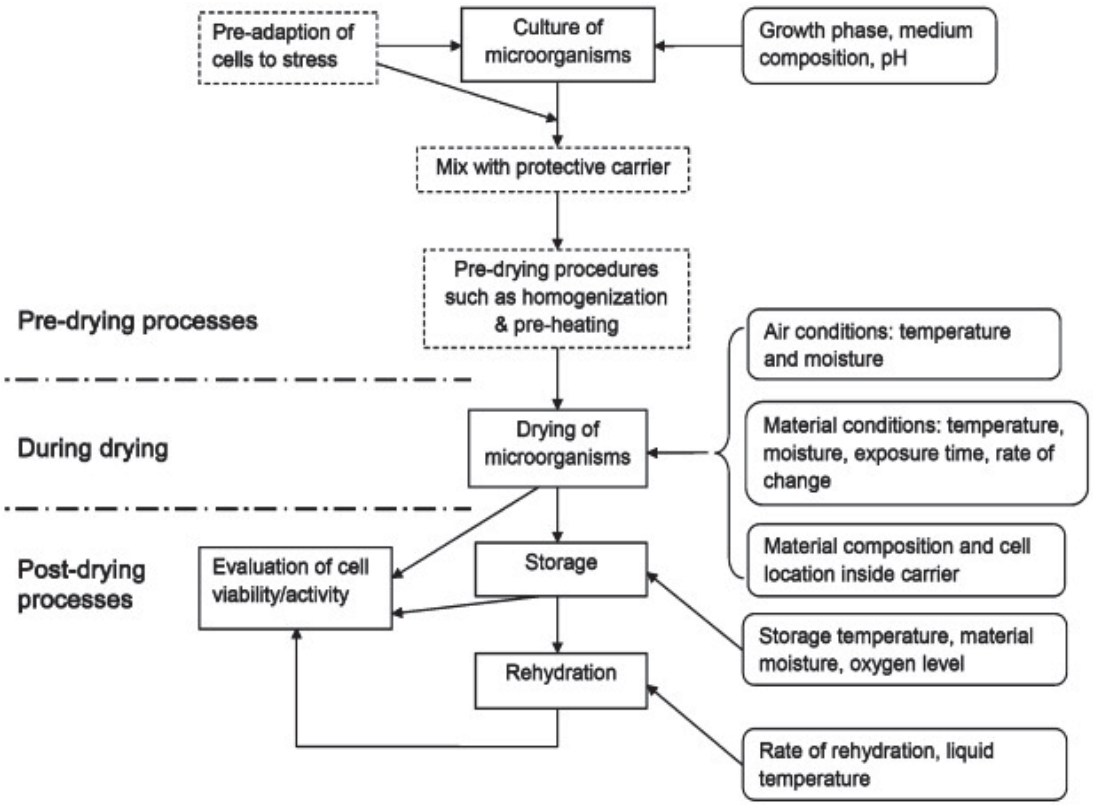


Figure 2.4.1 - Major processing steps during the dehydration of microorganisms and important extrinsic factors in each step. Dotted rectangles represent optional steps and extrinsic factors are shown in round-cornered rectangles (adopted from Fu and Chen, 2011)

2.5 Drying methods

2.5.1 Freeze drying

Freeze drying is the most common method for preservation and culture collections worldwide, including the American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NCTC) (Morgan et al., 2006). Freeze dried cultures are advantageous being easy to handle and having low shipping and storage costs. A typical freeze drying process consists of two steps; firstly cells are frozen at -196˚C followed by dried by sublimation under high vacuum. Even though freeze drying is the most preferred method of preservation, a huge loss of viability during freezing step (Uzunova-Doneva and Donev, 2000) and storage at temperatures above the refrigerated temperatures (Conrad et al., 2000) has been reported in literature. The destabilization of cell membrane and its integral proteins was reported to be the main cause for cell injury during freezing and thawing (Conrad et al., 2000). However, few protectants were found be highly effective over others in achieving higher viability during freezing step and storage when compared to the control (dried without protectant) (Carvalho et al., 2004). But because of high costs and energy consumption during freeze drying, as well as volume limitations within a freeze drying container, spray drying has been widely studied as an alternative industrial process for the preservation of LAB.

2.5.2 Spray Drying

Spray drying is considered to be a good long term preservation method for probiotic and starter cultures. Spray drying is the most popular and widely studied alternative to freeze drying because it is cost effective (Table 2.4.1), readily available, easy to operate, and can be implemented for large-scale throughputs. The spray drying of probiotics however, causes huge decline in viability during and after drying and thus has been less developed commercially. The probiotic and starter cultures are heat sensitive; thus, spray drying must be mild enough to avoid damaging them but sufficiently efficient to yield a powder with moisture content below 4%, which is a requirement for long storage stability (Peighambardoust et al., 2011).

Spray drying produces a dry powder by atomizing of the liquid at high velocity and directing the spray of droplets into a flow of hot air (150-200 ˚C). The atomized droplets have a very large surface area in the form of millions of micrometer- sized droplets (10-200μm), which results in a very short drying time when exposed to hot air in a drying chamber. Various studies investigating the stability of spray dried probiotic bacteria found that the viability of bacteria

was significantly affected by the outlet temperature (or the temperature at which the product leaves the drying chamber). Although, it is reported that increase in the inlet air temperature decreases the cell viability, this is not directly correlated to the inactivation and has only a slight affect. This may be because the extent of inactivation of bacteria during spray drying depends on the temperature-time combinations. The temperature-time history of the particles can be divided into two periods. At the beginning of the drying (constant drying rate period), the temperature of spray dried particles and heat inactivation are limited to the wet bulb temperature by the evaporative cooling effect. Thus, thermal inactivation is limited because the high evaporation rate and the resulting wet bulb temperature protect the cells from the higher air temperature in the dryer. At the next stage (falling drying rate period), the particle surface becomes dry and temperature of the spray-dried particles increases depending on dryer configuration. During this stage, the extent of thermal inactivation depends on the drying parameters such as outlet temperature, residence time and feed rate. Thus the latter stage is the most important for heat inactivation and the optimum residence time is the time required for complete removal of moisture with minimum increase in the temperature of the dried product (Masters, 1991).

Many researchers have obtained higher viability of microbial cells at lower outlet temperatures. However, lowered outlet air temperature could result in dried powder with residual moisture contents higher than 4 %. Desmond et al. (2002) reported higher storage stability of spray dried *Lactobacillus paracasei* manufactured at a lower outlet air temperature. Following spray drying at lower outlet temperature, 100% survival of *L. paracasei* was achievable after one week of storage at 4 ˚C and 15 ˚C; however viability reduced by 20-70 % after four weeks and 99 % after eight weeks of storage at both temperatures. The variable survival rate indicates that cell injury can also occur during storage. Following spray drying, increased susceptibility of damage to cell wall, cell membrane and DNA has been reported (Desmond et al., 2002). The protective carrier used for spray drying could also influence cell stability. Ananta et al. (2005) found that the skim milk powder provided a good protection of spray-dried lactobacilli during storage at 37 ˚C, where a reduction of only 0.5 log CFU in the viable counts occurred in 5 weeks.

Two mechanisms are believed to be responsible for the decline in viability during convective drying of bacterial cells i.e. thermal inactivation and dehydration inactivation. It is not yet clear which of these mechanisms is more damaging, as they generally occur simultaneously. Thermal inactivation involves inactivation of some of the cell critical components such as

ribosomes, cell wall, DNA, RNA and intracellular proteins while at the same time destruction of many other components. The loss of the less critical components does not cause death until their numbers are reduced or the cell is subjected to additional stress. It is not necessary that the same critical component is lost in all cells, and death may be due to destruction of more than one critical component (Peighambardoust et al., 2011). The damage resulting from the dehydration process can be attributed to two primary causes: (1) changes in the physical state of the lipid membrane (Beney and Gervais, 2001) and (2) changes in the structure of sensitive proteins (Texeira et al., 1996). Dehydration inactivation can occur during the drying of cells both at low (during freeze/vacuum drying) and high temperatures (during spray/fluidized bed drying). The cytoplasmic membrane damage is considered to be main factor responsible for the dehydration inactivation process, because the removal of water causes destabilization of cellular components mainly proteins, DNA and lipids. The membrane lipid bilayer structures are thermodynamically unstable and therefore, the lipid membrane is a primary target for dehydration induced damage. A theory about the damage of phospholipid bilayers from dehydration was reviewed by Crowe et al. (1998). According to this theory, the removal of hydrogen-bonded water from the phospholipid bilayer results in the increase in the head-group packing of membrane lipids, leading to increased opportunities for van der Waals interaction among the hydrocarbon chains. Therefore, the lipids change from liquid crystalline to gel phase in dry membranes because of the increase in melting temperature (T_m) of membrane lipid. During rehydration, the dry membrane returns from gel phase to liquid crystalline phase, and in course of this process, there are membrane leakages from packing defects. But it has been reported that after dehydration-rehydration the microorganisms can be recovered even when the cellular membrane is damaged. This indicates that some other bacterial structural parameters besides the membrane integrity affecting the bacterial viability after dehydration-rehydration. The data obtained by differential scanning calorimetry (DSC) revealed that the damages produced in lipids, ribosomes and DNA are reversible whereas the damages produced in proteins are irreversible (Teixeira et al., 1996).

The cell membrane damage was indirectly evidenced by increase in cellular components like enzymes, proteins, UV-absorbing materials in the surrounding medium after rehydration of dried cells as well as by the increase in the sensitivity of cells to chemicals, such as sodium chloride (NaCl), oxygen and lysozyme (Lievense et al., 1992; Selmer-Olsen et al., 1999a; Teixeira et al., 1995). Lievense et al. (1992) concluded that the cell wall and or/cell membrane damage is an important mechanism of dehydration inactivation *of L. plantarum*, but thermal inactivation (up to 60˚C) occurs by a different mechanism. The lipid oxidation during spray

drying can also be fatal for oxygen sensitive LAB due to contact with a large volume of air during drying. Teixeira et al. (1996) reported that the ratio of unsaturated/saturated fatty acids decreased following spray drying and this ratio decreased further during storage in air, presenting evidence of lipid oxidation after prolonged storage.

Considering only the effect of heat, the inactivation of microorganisms upon lethal heat is commonly established as a first-order function:

$$\frac{d(N/N_0)}{dt} = -k \cdot (N/N_0)$$

where N is the survival number of microorganisms (cfu g^{-1} mass) at time t (h), N_0 is the initial number of microorganisms (cfu g^{-1}mass), and k refers to the constant of death rate under constant environmental conditions (h^{-1}). In addition, the death rate constant k is in a log-linear relationship with the temperature. Hence, two factors that have direct effects on the survival number of microorganisms during a lethal heat treatment are the time that cells are exposed to the high temperature, t, and the temperature which determines the death rate constant, k (Fu and Chen, 2011).

2.5.3 Fluidized bed drying

A fluidized bed (FB) is a bed of solid particles with a stream of air or gas blowing upward through the particles at a rate high enough to set them in motion. The feed material is placed in a processing chamber and air travelling through the particle bed imparts fluid-like behavior to the bed causing rapid mixing of solids as shown in Figure 2.5.3.1. This air flow can be conditioned by varying temperature and flow rate. During drying, the moisture content drops, which improves the fluidization. The advantages of a FB dryer over other equipments include: large scale continuous production, lower costs, ease of handling of feed and product, lack of mechanical moving parts, rapid exchange of heat and mass between gas and particles which minimizes overheating and rapid mixing of solids which provides nearly isothermal conditions throughout the fluidized bed (Bayrock and Ingledew, 1997). The FB drying time can be longer than spray drying, but thermal inactivation is minimized by using relatively low air drying temperatures. But some of the drawbacks of FB drying are irregular particle sizes and the sticky nature of the granulated materials, which can lead to an inhomogeneous bed, agglomerated particles and a decreased drying rate. The FB drying process has been

successfully employed in the production of commercial dry yeast (Bayrock and Ingledew, 1997).

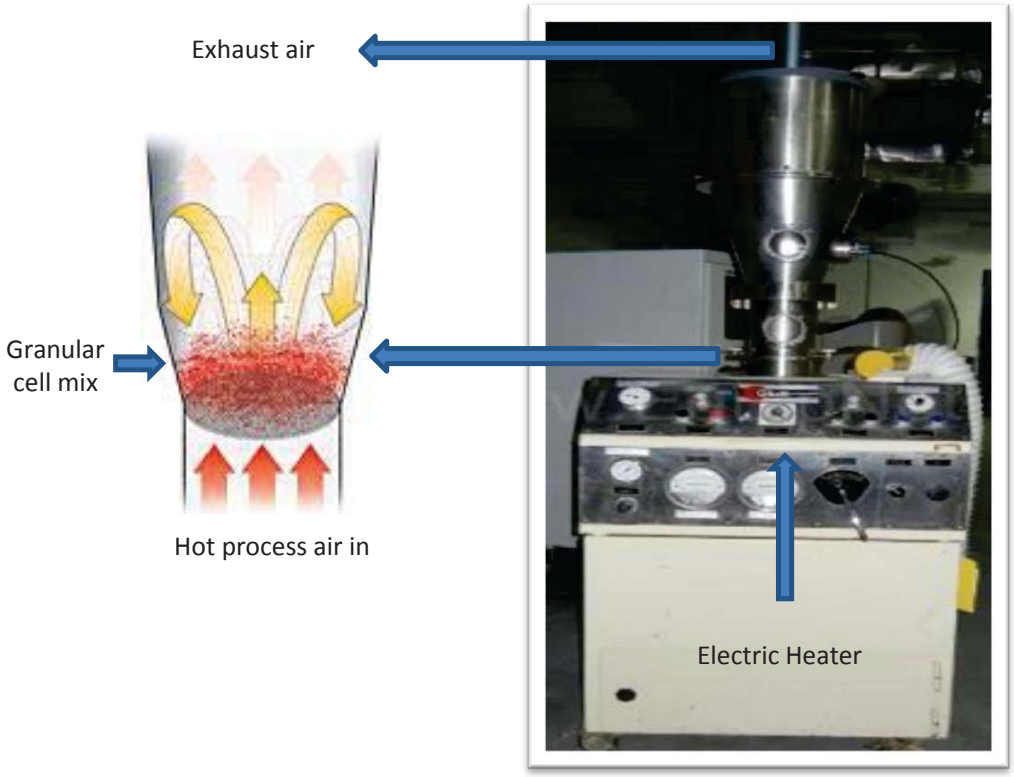


Figure 2.5.3.1 – Mechanism of drying in a Glatt Uni-Glatt laboratory fluid bed dryer

Only a few studies have reported the drying of LAB using FB. Only granular materials can be dried in the FB dryer, and therefore the bacterial cells must be entrapped or encapsulated in a protective carrier before drying. The low water activity of the protective carrier could however pose an osmotic shock leading to a reduction in the viability after mixing. Mille et al. (2004) studied the viability of *L. plantarum* after mixing and FB drying with casein powder of different water activity (a_w). It was found that when *L. plantarum* cells were mixed with extremely desiccated casein powder (a_w of ≤ 0.1), viability of the mix did not exceed 2.5 % and when the a_w of the casein powder was 0.75 (a_w of the wet sample is near 0.95), 100% viability was achieved.

Another advantage of FB drying is that it can incorporate a spray within the FB dryer which can convert the material into a granulated product. This combination of spray and FB drying was

studied by Zimmermann and Bauer (1990); however in this study the product obtained was more hygroscopic than the pure carriers, and the viability of *L. casei* was very low which was thought to be either due to the cell concentration process (microfiltration) or the osmotic effects between the carrier materials and the cells (Zimmermann and Bauer, 1990). Larena et al. (2003) compared the viability of *Penicillium oxalicum* during freeze drying, spray drying and FB drying. The results showed 100% viability after freeze and FB drying but only 20% viability after spray drying. During storage at room temperature for 30 days, viability dropped by at least 50% in freeze dried cells irrespective of protective agents used but FB dried cells remained 100% viable after 30 days of storage. Then viability dropped to 40% after 60 days and remained constant up to 180 days trial.

Lievense et al. (1992) employed a model to describe the change of cell activity of *L. plantarum* during the FB drying process. The inactivation of *L. plantarum* was described as the loss of cell activity rather than the decrease in viable cell number, and was depicted by the glucose fermenting activity. A moisture-related factor, the drying efficiency of *E*, was introduced to correlate the decrease in the cell activity. The total inactivation rate was presented in two parts, one caused by thermal inactivation and another caused by dehydration inactivation, respectively:

$$\left(\frac{dA}{dE}\right)_{tot} = \left(\frac{dA}{dE}\right)_{therm} + \left(\frac{dA}{dE}\right)_{dehydr}$$

where *A* is the activity of *L. plantarum*.

This model assumed that the thermal and the dehydration inactivation were the two main inactivation mechanisms and their effects in inactivating *L. plantarum* cells were additive. By considering the cell inactivation into such two compartments, this model was capable of determining the contribution of each factor to the cell death under specific conditions. The results suggested that for temperatures up to 55 °C, dehydration was mainly responsible for the death of the bacterium, and the major factor affecting the dehydration mechanism was the moisture content rather than the drying rate. However, at this temperature the rate of water evaporation could be low, especially when the environmental moisture level (humidity) is high. Thus under these conditions the actual effect of drying rate is difficult to determine conclusively.

Various models have been proposed describing drying of baker's yeast (*S. cerevisiae)* in FB drier (Debaste and Halloin, 2009; Turker et al., 2006). However, these studies only considered the changes in the moisture concentration and the temperature of the yeast being dried but the kinetics of viability change of the yeast was not considered. The proposed models are thus yet to be combined with the inactivation kinetics of microorganisms in order to establish a model predicting the cell number at any stage of drying.

2.5.4 Vacuum drying

Vacuum drying under conventional conditions (temperature range between 30 and 80 °C) generally leads to a huge cells loss due to heat damage. But the heat stress can be minimized by further reducing the chamber pressure to values just above the triple point of water, which leads to low product temperatures close to 0 °C. This process is referred to as controlled low-temperature vacuum (CLTV) dehydration (Bauer et al., 2012). CLTV dehydration is a well suited dehydration method for heat and oxygen sensitive LAB as temperature could be maintained as low as possible without freezing, and heat and vacuum is applied to promote the evaporation of water. Other advantages include lower drying time and operational cost compared to freeze drying. The basic vacuum dryer consists of a chamber containing heated shelves. Trays containing the wet materials are placed on the shelves, and water is removed by a vacuum pump and condensed at a condenser. The studies on storage stability of vacuum dried LAB are scant in literature (Foerst et al., 2012). Vacuum dried *Lactobacillus paracasei* F19 was found to be stable at refrigeration temperatures for storage period of 3 months but huge decline in its viability was observed when stored at 37 °C (Foerst et al., 2012).

The inactivation mechanism by fluidized bed and vacuum drying is different than spray drying. At the low drying temperatures of FB and vacuum drying, heat inactivation is negligible, but dehydration inactivation may impose serious problems at mild air drying temperatures. It has been reported that between 8 °C and 25 °C, microorganisms exhibit a higher sensitivity to the osmotic pressure caused by dehydration compared to the temperature range higher than 25 °C and lower than 8 °C (Laroche and Gervais, 2003; Mille et al., 2005). The reason for this trend was related to the membrane phase transition at the reported temperature range, which could lead to an increased sensitivity of the lipid bilayer to the water flow caused by diffusion.

2.6 Factors affecting viability during convective drying

2.6.1 Resistance to stress

During industrial food processing, LAB encounters various kinds of stress conditions such as low and high temperatures, low pH and low water activity, which can lead to damage of cell membrane and cell wall, inhibition of active transport, retention of nutrients, morphological changes and loss of viability (Carvalho et al., 2004). The defence mechanisms to cope with different stresses are essential to maintain cell viability both after drying and upon rehydration. The LAB are classified as Gram-positive bacteria and considered to have greater thermal and mechanical stress tolerance as compared to the Gram-negative bacteria which have very thin cell wall structures. Various studies showed that LAB have developed various adaptive mechanisms in response to various stress conditions. A better understanding of these stress resistance mechanisms should allow understanding the basis for the adaptive responses and cross protection (against various stresses) in LAB which should lead to design and manufacture of probiotic cultures with greater viability during preservation and storage.

2.6.1.1 Osmotic Stress resistance

The cell membrane of LAB is readily permeable to water but presents a more effective barrier to most other solutes. Therefore, when the external concentration of water changes because of increase or decrease in the concentrations of extracellular solutes that are excluded by the membrane, water is able to move out of or into these cells (Csonka and Hanson, 1991). A lowering of the external water activity (hyperosmotic conditions) causes a rapid efflux of cellular water and loss of turgor; ultimately, the cells may plasmolyse, i.e. the cytoplasmic membrane may retract from the cell wall. Similarly, upon hypo-osmotic shock, water flows into the cell and increases the cytoplasmic volume and turgor pressure. In order to survive osmotic stress, significant physiological changes have been reported in bacteria which include the induction of specific stress proteins (see section 2.6.1.3), and the accumulation of specific solutes under hyperosmotic conditions and releasing them under hypoosmotic conditions. Such solutes are often referred to as compatible solutes because they can be accumulated to high levels by *de novo* synthesis or transported without interfering with vital cellular processes (Hartke et al., 1996; Poolman and Glaasker, 1998). Such solutes include K^+, amino acids (e.g. glutamate, proline), amino acid derivatives (peptides, N-acetylated amino acids), quaternary amines (e.g. glycine, betaine, and carnitine), sugars (e.g. sucrose, and trehalose) and tetrahydropyrimidines (ectoines). Compounds such as betaine and carnitine have been shown to be protective during drying of LAB (Kets and Debont, 1994; Kets et al., 1996). The exact

mechanism behind these compatible solutes but the possible explanation includes the alteration of cell membrane physical properties and the prevention of aggregation of cellular proteins (Carvalho et al., 2004).

Like many other organisms, when LAB are confronted with a decreased water activity over a long period, they respond by accumulation of compatible solutes such as betaine and carnitine. These organisms have however limited or no mechanism to synthesize compatible solutes (Poolman and Glaasker, 1998) and are also probably not able to accumulate compatible solutes during the short drying process. Thus, these solutes need to be accumulated prior to the drying process in the growth medium (Kets et al., 1996). These compounds are not only thought to be beneficial for LAB during osmotic stress but can also provide cross-protection against high temperature, freeze-thawing and drying (Carvalho et al., 2004; Csonka and Hanson, 1991; Kets et al., 1996; Poolman and Glaasker, 1998).

2.6.1.2 Oxidative Stress resistance

The problem of oxygen toxicity in probiotic bacteria still remains poorly understood. The organisms inhabiting the human gut are generally anaerobic (*Bifidobacterium* spp.) or microaerophilic (*L. acidophilus*) and thus are unable to synthesize ATP by respiratory (electron transport chain) means and have to depend strictly on a fermentative mode of metabolism. Due to the lack of participation of an external electron acceptor (oxygen in aerobic bacteria) in anaerobes, the organic substrate undergoes a balanced series of oxidative and reductive reactions facilitated by pyridine nucleotides such as NADH. As the energy in anaerobes is derived mainly through substrate level phosphorylation, the regeneration of NAD+ from NADH assumes critical importance. The exposure to oxygen in these bacteria causes toxic oxygenic metabolites to accumulate in the cell leading to cell death. This lethal effect of oxygen is termed as oxygen toxicity (Talwalkar and Kailasapathy, 2003) in which the survival mechanisms are unable to deal adequately with the reactive oxygen species (ROS) such as O_2^- (superoxide), and OH^- (hydroxyl radical), in the cells. These ROS can readily diffuse across the cellular membranes and damage the membrane proteins, the lipids and the nucleic acids, thereby constituting one of the major causes of aging and cell death. Apart from the toxic effects of oxygen, aeration can induce important changes in the sugar metabolism of LAB (Talwalkar and Kailasapathy, 2004).

So far, oxidative studies on probiotic bacteria have mainly focused on bifidobacteria with very little known about the effect of oxygen on the physiology of *L. acidophilus*. Various studies showed that bacteria have developed more or less successful ways to cope with oxygen

toxicity by either repairing the damage caused by inducing some oxidant induced proteins, by preventing the formation of these reactive oxygen species, by eliminating them by action of enzymes (such as superoxide dismutases, catalase and pseudocatalase) or by rendering their possible targets less vulnerable. A partial overlap between oxidative and heat damage responses have been described in microorganisms and a correlation between some oxidant induced proteins and heat-shock proteins (described in section 2.6.1.3) have been observed. Certain antioxidant activities were shown to increase due to heat stress while drying may also counteract ROS formation (Guerzoni et al., 2001). Some studies on the aerotolerance of LAB suggested that the ratio and specific activities of the NADH oxidase and NADH peroxidase enzymes determine the elimination of oxygen from the cell. The activities of NADH oxidases in probiotic bacteria give rise to H_2O_2, prompting NADH peroxidase to scavenge H_2O_2 and prevent cell death. It is believed that the intracellular levels of H_2O_2 can block enzymes (such as fructose-6-phosphate phosphoketolase in case of bifidobacteria) required for sugar metabolism of LAB and therefore scavenging H_2O_2 becomes important for cell survival (Talwalkar and Kailasapathy, 2004).

$$\text{NADH} + H^+ + O_2 \xrightarrow{\text{NADH-}H_2O_2\text{ oxidase}} NAD^+ + H_2O_2$$

$$2\text{NADH} + 2H^+ + O_2 \xrightarrow{\text{NADH-}H_2O\text{ oxidase}} NAD^+ + 2H_2O$$

$$\text{NADH} + H^+ + H_2O_2 \xrightarrow{\text{NADH peroxidase}} NAD^+ + 2H_2O.$$

The changes in the fatty acid (FA) composition of cell membrane of some LAB can also help them to survive during oxidative and other environmental stress such as acid stress (see section 2.6.1.4 acid stress response). For instance, in case of *L. helveticus* the increase of an oxygen-consuming desaturase system, with a consequent increase in fatty acid desaturation, is a cellular response to environmental stresses which is able to protect the cells of this anaerobic micro-organism from toxic oxygen species and high temperatures (Guerzoni et al., 2001). Superoxide dismutases (SOD) can also provide a defence against the toxicity of oxygen

by catalytically scavenging O_2^- in many organisms such as *Lactococcus lactis* (Sanders et al., 1995).

$$2O^{2-} + 2H^+ \longrightarrow O_2 + H_2O_2$$

However, *L. plantarum*, which lacks SOD, demonstrated that its high intracellular level of Mn (II) (20-25 mM) takes the place of SOD in scavenging O_2^- (Archibald and Fridovich, 1981). O_2^- scavenging activity of *L. casei* and *L. fermentum* was also believed due to presence of their extremely high manganese (Mn) content (Archibald and Fridovich, 1981). However, *L. acidophilus* and *Bifidobacterium* species do not contain high levels of Mn and are also devoid of catalase or pseudocatalase, which are key enzymes for the breakdown of H_2O_2. These have to rely on enzymes such as NADH oxidase and NADH peroxidase enzymes to scavenge environmental oxygen (Talwalkar and Kailasapathy, 2004). Shimamura et al. (1992) explored the biochemical mechanisms of oxygen sensitivity of several bifidobacteria and based on their results it was concluded that levels of NADH oxidase and NADH peroxidase play an important role in the prevention of oxygen toxicity. Yi et al. (1998) also found high levels of these enzymes in *Bifidobacterium* spp. *L. delbrueckii* ssp. *bulgaricus,* which were assumed to play an important role in oxygen elimination.

2.6.1.3 Heat Shock resistance

Industrial preservation of bacteria through desiccation could result in structural and physiological injury to the bacterial cells resulting in substantial loss of cell viability. Initial investigations carried out on bacteria have demonstrated that they possess an inherent ability to adapt to the unfavourable environments by the induction of various general and specific stress responses. A variety of stress stimuli, other than heat shock , including UV light, ionizing radiation, and oxidative stress, caused cells to accumulate heat-shock proteins (HSPs) mainly proteases (Clp, HtrA, FtsH) and ATP-dependent chaperones proteins (DnaK, DnaJ, GrpE, GroES and GroEL) as a response of these stress conditions (Hwang et al., 2008). The heat shock (HS) and reactive oxygen species may cause accumulation of damaged and misfolded proteins within the cytosol, which is termed as the unfolding protein response. In addition to the damage of intracellular proteins, heat inactivation can also inactivate some of the other critical components of cells such as ribosomes, cell wall, DNA and RNA (Teixeira et al., 1997). The heat shock proteins such as DnaK, GroES and GroEL are known as molecular chaperones. This means that they bind to and stabilize substrate proteins in a transient noncovalent manner, prevent their premature folding, and promote the attainment of the correct state *in vivo*

(Hendrick and Hartl, 1993; Prasad et al., 2003; Walker et al., 1999). The GroEL protein from *E. coli* was also reported to be involved in the protection of mRNA from nuclease degradation, suggesting a new role of GroEL an RNA chaperone (Georgellis et al., 1995). While chaperones proteins stabilize RNA and repair misfolded proteins, proteases are reported to degrade denatured proteins, both leading to an increased heat resistance of cells subjected to heat shock (Corcoran et al., 2008). The HS response has been studied notably in *E. coli* and *Bacillus subtilis.* In *Bacillus subtilis*, a model organism for Gram-positive bacteria, HS response is classified into four categories based on the regulators of the genes. Class I genes are regulated by the HrcA repressor, which binds to the palindromic operator sequence CIRCE (for controlled invert repeat of chaperone expression) (Zuber and Schumann, 1994). Class II genes are regulated by sigma factor σ^B (Petersohn et al., 2001). Class III genes are controlled by the class three stress gene repressor CtrR, which binds to a specific direct repeat referred to as the CtsR-box (Derre et al., 1999). The genes regulated by unknown mechanisms are grouped under class IV.

Reports describing the physiological stress responses in LAB, particularly in *Lactobacillus* species are scant. Furthermore, there is a paucity of information describing the physiological mechanisms underlying the stress-induced improvement in the survival of LAB in relation to industrial processing and storage conditions. Physiological studies have demonstrated that some of the LAB elicit heat shock responses similar to that of other Gram-positive bacteria however no global regulator of the HS response has yet been identified in LAB (van de Gutche et al., 2002). Schmidt et al. (1999) investigated the molecular characterization of the *dnaK* operon in *L. sakei* and reported the regulatory mechanism was similar to *B. subtilis* class I stress gene regulation. The examination of LAB HS responses using 2D electrophoresis revealed variable numbers of induced HSPs. However, many HSPs also belonged to the osmotic stress response. Prasad et al. (2003) found the expression of HS chaperone proteins GroEL and DnaK was up-regulated in *L. rhamnosus* HN001 (DR20) as a result of heat and osmotic pre-shock treatment. A brief pre-shock at temperatures above the normal growth range, could lead to synthesis of chaperones proteins in bacterial cells which could offer cross-protection to the cellular proteins and other macromolecules against other stress like heat (at elevated temperatures) and osmotic stress (Prasad et al., 2003; Walker et al., 1999).

2.6.1.4 Acid stress response

The growth of LAB is characterized by the generation of lactic acid as the major end product of sugar fermentation. The lactic acid produced can easily pass through the cell membrane in

undissociated form into the extracellular medium via a carrier-mediated process. Thus pH within the cytoplasm of fermenting LAB remains more alkaline than the surrounding medium. In addition, the membrane is relatively impermeable to the extracellular protons and the lactate molecules that are produced during fermentation. Accordingly, a pH gradient (ΔpH) is formed between the cytoplasm and the medium. The formation and maintenance of ΔpH is important not only for pH homeostasis but also as a component of the proton motive force (Hutkins and Nannen, 1993). However, as pH continues to decrease, the cells are unable to maintain ΔpH and under high cytoplasmic pH, the acid will rapidly dissociate into protons and charged derivatives to which the cell membrane is impermeable. The intracellular accumulation of protons may then lower the intracellular pH (pH_i), imposing a stress condition on the cells (Silva et al., 2005). The internal acidification can reduce the activity of acid-sensitive enzymes, damage proteins, DNA and cell membrane (Nannen and Hutkins, 1991), and chelate elements essential for growth, such as iron (Presser et al., 1997).

The regulation of the cytoplasmic or internal pH (pH_i) is a fundamental requirement for the survival and viability of LAB. Despite the importance of pH homeostasis in the LAB, relatively little is known about how these microorganisms control their pH_i. Several mechanisms have been proposed unfolding this action such as cytoplasmic buffering capacity, extrusion of protons through the proton motive force and the synthesis of decarboxylases and deaminases which can produce acids and bases (Hutkins and Nannen, 1993). It has been suggested that in many LAB species such as *Lactococcus lactis* subsp. *lactis* and *cremoris*, *L. casei* and *Enterococcus faecalis*, cytoplasmic pH homeostasis is primary maintained by means of a proton-translocating F_0F_1-ATPase (Hartke et al., 1996). Increase in the synthesis and activity of F_0F_1-ATPase is an important element in the response and tolerance to ΔpH. The F_0F_1-ATPase is the main source of cellular ATP which uses a transmembrane proton gradient to drive the synthesis of ATP from ADP and phosphate (Rastogi and Girvin, 1999). The increased activity of this membrane-bound enzyme is believed to expel protons accumulated by the cell as a result of lactic acid production. According to the chemiosmotic theory F_0F_1-ATPase acts as a reversible ion translocating pump, which catalyses the movement of hydrogen ions (H^+) across the cell membrane as a consequence of the hydrolysis or synthesis of ATP (Hutkins and Nannen, 1993). Some cation transport ATPases bacteria such as a K+-ATPase can contribute to pH homeostasis by exchanging intracellular protons for extracellular K^+ at low pHi, thereby increasing pHi (Kashket, 1987). Some LAB are able to synthesise enzymes like deaminases which release ammonia from arginine by arginine deiminase pathway thus alkalizing the environment, thereby helping LAB against acid damage (Casianocolon and Marquis, 1988). The

decarboxylation of the dicarboxylic malic acid and subsequent production of monocarboxylic lactic acid by *L. plantarum* can consume an intracellular proton and elevate pH (Olsen et al., 1991).

The acid tolerance is highly dependent on the cell growth phase, with the stationary phase cultures being much more resistant than the log phase cultures. The changes in protein synthesis and fatty acid composition have been observed as a lactic acid tolerance response (L-ATR) in some bacteria. This inducible survival mechanism against acid stress is referred to as the acid tolerance response (ATR). In LAB, lactic acid tolerance increases in at least two distinct physiological states (i) during logarithmic growth L-ATR , which is activated by protons (ii) during stationary phase L-ATR, which increases as a result of the induction of a general stress response (GSR, see in section 2.6.1.5) (Hartke et al., 1996, 1998). It is not known whether these responses are independent or overlap. The synthesis of some acid-inducible proteins however, overlaps with other stress response and thus can provide cross-protection to LAB against other stresses. A significant overlap of the pre-shock acid-induced polypeptides with heat-inducible (42°C) proteins such as DnaK and GroEL was observed during L-ATR in *Lactococcus lactis* (Hartke et al., 1996).

During the transition of cell growth from the late log to the stationary phase maximal synthesis of cyclopropane fatty acid (CFA) occurred in *Escherichia coli* and many other bacteria which played a major role in protection of *E. coli* from acid shock (Chang and Cronan, 1999).The change in lipid membrane composition as a result of conversion of unsaturated fatty acid (UFA) to CFA formation could help either by decreasing proton permeability of the membranes or by interaction of CFA-containing phospholipids (but not unsaturated fatty acid-containing phospholipids) with membrane proteins. These interactions could passively decrease proton permeability or perhaps actively increase proton efflux (Chang and Cronan, 1999).

2.6.1.5 Starvation response

Bacteria enjoy an infinite capacity for reproduction as long as (1) carbohydrates, amino acids and other nutrients are available; (2) toxic or inhibiting compounds such are H_2O_2 are removed or degraded; and (3) hydrogen ion concentration is maintained below a critical level for that specific strains toleration capability. However, their rapid growth/fermentation ultimately results in the depletion of one or several growth-supporting substrates (with increase in the concentration of toxic wastes) and the population of cells enters a phase defined as the stationary phase of growth. The entry and growth arrest in the stationary phase can trigger

various stress conditions among which nutrient starvation is the most common. Moreover, some extreme environmental stress conditions such as low pH during the stationary phase can indirectly provoke starvation, irrespective of the extracellular amount of the substrate. These multiple stress conditions during stationary phase such as starvation, high osmolarity and low pH due to lactic acid accumulation and non-optimal temperature could trigger general stress response (GSR) which has been exclusively studied in *E. coli* (Hengge-Aronis, 1999) and *Bacillus subtilis* (Bernhardt et al., 1997). The GSR in bacteria could have various consequences including, multi-protection against various stress (such as heat and osmotic stress during drying), altered cell morphology, induction of general stress proteins (GSR) and possession of storage compounds (Bernhardt et al., 1997; Hengge-Aronis, 1999; Nystrom, 2004). The sigma factor σ^B (see section 2.6.1.4 heat shock resistance) is believed to control the expression of GSR in these two bacteria.

Generally, dwarfing i.e. continuous size reduction of cell is triggered by starvation (Nystrom, 2004). During long-term starvation, cells usually reach their minimum size. Lipid, DNA, and RNA contents are diminished during the transition from growth to non-growth (Giard et al., 1996). Whereas, the rate of total protein synthesis decreases under nutrient limitation conditions and the synthesis of some genes that encode proteins with specific roles in protecting the cell against external stresses (e.g. heat, oxidants, osmotic challenge, and exposure to toxic chemicals) increases (Giard et al., 1996; Hartke et al., 1998). Thus, growth-arrested cells could be highly resistant to a variety of secondary stresses, a phenomenon known as stasis-induced cross protection (Nystrom, 2004).

The starvation could take place due to lack of various growth medium components but responses to three types of limiting compounds have largely been studied in bacteria i.e. (1) carbohydrate (sugar) starvation leading to cell energy depletion; (2) phosphate starvation which can be deleterious for both energy supply and DNA/RNA synthesis; and (3) nitrogen (amino acids) starvation which primarily results in the limitation of protein synthesis (van de Gutche et al., 2002). In LAB most studies have been focused on carbohydrate starvation. The factors that can help in survival of LAB during nutrient starvation includes synthesis of "starvation-induced proteins", possession of storage compounds, maintenance of proton motive force and adenylate energy change, and diminution of cell size. In response to the lactose starvation, Stuart et al. (1999) observed that *Lactococcus lactis* remained viable but non-culturable (VBNC) and the cells used arginine for energy (ATP) production by the arginine deiminase pathway after carbohydrate exhaustion. The carbohydrate starvation induced *E. faecalis* cells (Giard et al., 1996; Hartke et al., 1998) and *Lactococcus lactis* (Hartke et al., 1996)

showed multiresistance against heat, acid and oxidative stresses and the stress tolerances increased progressively with the duration of starvation. It was suggested that the synthesis of specific "starvation-induced proteins" in LAB, as revealed by 2-D gel analysis (Giard et al., 1996, Hartke et al., 1998), was necessary for acquisition of maximal resistance towards other stresses. The resistance during first 3 h of starvation was found to be due to these specific protein syntheses, but between 3 h and 24 h of starvation a second mechanism related to protein degradation or alteration was important for survival of *E. faecalis* (Giard et al., 1996). The survival capacity of *Streptococcus cremoris* during lactose starvation was dependent on the amino acid catabolism. Upon loss of glycolytic capacity during lactose starvation, the intracellular pools of several amino acids were depleted with the proton motive force, which suggested use of amino acids as alternative source for energy *S. cremoris* (Poolman et al., 1988). Various studies on LAB have suggested that the starvation-induced mechanisms are different than from *E. coli* and *Bacillus subtilis* due to absence of a σ^B homologue in LAB (van de Gutche et al., 2002).

2.6.1.6 Cold Stress

LAB are exposed to temperatures far below their optimal growth temperatures during various industrial processes such as cheese ripening and refrigerated storage of fermented foods. A sudden downshift in the external temperature could cause problems such as decrease in the cell membrane fluidity, stabilization of the secondary structures of RNA and DNA affecting transcription and translation, slow or inefficient protein folding, reduced ribosome function and increased negative supercoiling of DNA within the bacterial cells. Adaption to this temperature downshift is essential for the survival of cells and this is done by synthesis of a number of cold-induced proteins (CIPs) as a response to cold stress/shock. The most strongly induced proteins include a family of closely related low-molecular weight (~7.5 kDa) proteins termed cold-shock proteins (Csp) (Phadtare and Inouye, 1999). The CSPs are considered as an RNA chaperone and are associated with binding to RNAs and single-stranded DNAs, which may prevent the formation of secondary structures of mRNAs. These might also be involved in sugar metabolism, chromosome structuring and proteolysis of misfolded proteins after cold shock (for references refer to van de Gutche et al., 2002). The change in membrane lipid composition is also a common response to compensate for the decrease of fluidity of the lipid bilayer at low temperature. As the temperature is lowered, the proportion of unsaturated fatty acids (UFAs) in the membrane lipids increases.

The CIPs induced as a result of cold shock response have been studied extensively in *E. coli* (Wang et al., 1999) and *Bacillus subtilis* (Graumann et al., 1997). *E. coli* contains a number of CIPs belonging to Csp family, in which CspA, CspB, CspG and Cspl have been shown to be cold shock inducible whereas CspD has been shown to be induced during stationary-phase and nutrient starvation (Wang et al., 1999). *Bacillus subtilis* has shown to induce CspB, CspC and CspD in response to cold shock (Graumann et al., 1997). Various LAB were also found to induce Csp proteins or their genes as a result of cold-adaptive response. Using 2-D gel electrophoresis, it was found that various CIPs were identified in *E. faecalis* (Panoff et al., 1997), *Lactococcus lactis* (Panoff et al., 1994; Wouters et al., 1999a) and *S. thermophilus* (Wouters et al., 1999b), with a 7 kDa CIP (CspD) overexpressed in *Lactococcus lactis* MG1363 (Wouters et al., 1999a) and *S. thermophilus* (Wouters et al., 1999b) and a 29 kDa CIP (P94) expressed in *Lactococcus lactis* IL1403 (Panoff et al., 1994) along with synthesis of many other CIPs. These cold shock proteins can also provide cross-protection against other stress conditions such as thermotolerance in *Lactococcus lactis* (Panoff et al., 1994).

2.6.2 Factors affecting stress tolerance

The survivability of bacteria during a thermal drying process and thereby storage depends on the heat and dehydration damages that may have occurred during the preservation process. A multitude of factors including pre-adaptation to stress, the intensity of each stress (e.g., temperature and water-potential), the time of cells exposed to each stress and the changing rate of these stresses (e.g., the drying temperature-time combination) could reduce these damages. Even for the same strain, the stress tolerance will be different for cells cultured in different growth medium and harvested at different growth stages. Thus there is a possibility of altering/improving intrinsic stress tolerance of cells by optimizing factors like the growth and harvesting conditions, growth media composition and pre-adaptation process, as discussed in detail below.

2.6.2.1 Growth phase and harvesting time

The growth of bacteria has four distinct phases: lag, log, stationary and death phase when grown in batch culture. The bacterial cells harvested at the stationary phase during a batch culture generally show higher stress tolerance, compared to those harvested at the lag and exponential phases in a batch culture as well those harvested at the chemostat phase of a continuous culture (Fu and Chen, 2011). The exhaustion of nutrients and accumulation of toxic metabolites in stationary phase triggers various stress responses to allow survival of the cell

population (see section 2.6.4.5 starvation response). The survival response also protects the cell in other adverse conditions such as desiccation and adverse temperatures. From comparable cell counts before drying (8.54 and 8.87 log cfu mL-1) of *L. delbrueckii* ssp. *bulgaricus* harvested at exponential and stationary phases, cell counts after the cultures were spray dried were 5.80 and 7.45 log cfu mL^{-1}, respectively (Teixeira et al., 1995). Spray-dried *L. rhamnosus* cells also gave highest recovery when harvested from stationary phase cells (31-50%), whereas early log phase cells exhibited 14% survival with the least survivability in lag phase cells (2% survival) (Corcoran et al., 2004). Prasad et al. (2003) reported higher storage stability of the dried *L. rhamnosus* HN001 cells that were given a heat shock after stationary phase to that of the cells that were heat shocked after log phase. 2-D gel electrophoresis data showed HSPs, DnaK and GroEL were up-regulated after both log and stationary-phase growth, indicating that other stationary-phase related viability factors such as glucose depletion and starvation conditions, were responsible for their improved storage stability (Prasad et al., 2003). Lorca and Valdez (2001) also showed that the ability of *L. acidophilus* CRL 639 to survive low pH conditions depended on the growth phase: stationary phase cell were naturally acid resistant whereas, exponential phase cells needed an adaption step to induce acid tolerance, which is known as the classical log-phase lactic acid tolerance response (L-ATR). The changes in protein profiles with the expression of various proteins (from 6.5 to 70.9 kDa) as result of the stationary phase itself as well as due to acid stress were thought to be involved in increasing resistance of *L. acidophilus*.

2.6.2.2 Growth media composition

During drying, microorganisms undergo an increasing osmotic stress condition as the water activity decreases. One of the ways in which organisms counteract the osmotic stress is by accumulating compatible solutes which could help not only in stabilizing the proteins and the cell membrane during osmotic stress conditions but also exhibit cross-protection effects towards other stresses such as heat stress during drying processes (see section 2.6.1.1). However, LAB have limited or no possibilities to synthesize and accumulate compatible solutes during the short drying process. Most of the LAB are multiple amino acid auxotrophs and reside in environments that contain these amino acids in some form as well as glycine betaine (plant origin) or carnitine (animal origin). Therefore, these compatible solutes need to be added to the growth medium and its accumulation must take place before the drying process.

2.6.2.3 Pre- Adaptation Process

The addition of unutilized components to the culture medium to induce the osmotic stress response will lead to the accumulation of compatible solutes; this is usually referred to as a pre-adaptation process. One of the most commonly used chemicals to induce osmotic stress is sodium chloride (NaCl). Kets et al. (1996) cultured four strains of LAB in the presence of NaCl and observed an enhanced survival ratio in all these bacteria during the subsequent two-stage drying process (two-stage drying involved air drying followed by vacuum desiccation). The presence of betaine in an osmotically stressed medium further protected *L. plantarum, L. haloterans,* and *E. faecium* against drying due to its accumulation by the cells whereas, *L. bulgaricus* was not able to accumulate this specific solute from the medium. However, in media that were not NaCl stressed, addition of betaine did not improve survival after drying. On the other hand a study by Linders et al. (1997b) showed lower glucose fermentation activities of both FB dried and air-convective dried *L. plantarum* grown under osmotic stress (1 mol L^{-1} NaCl) despite having a higher accumulation of the compatible solutes betaine and carnitine in comparison to cells grown without NaCl (Linders et al., 1997 b). The addition of sucrose (20 g l^{-1}) to the culture medium of *L. bulgaricus* improved its survival during the storage period following spray drying, although the survival ratio after drying remained similar to the control (Silva et al., 2002). The enhancement of *L. bulgaricus* survival during vacuum drying when grown in the presence of sucrose (11.3 g l^{-1}) in the growth medium was reported by Tymczyszyn et al. (2007). Since LAB cannot utilize sucrose, the addition of sucrose to the growth medium can be considered as a pre-adaptation effect.

Desiccation tolerance can also be induced by providing certain adverse conditions during microbial growth. It is well documented that when exposed to a low level of stress, most bacteria develop adaptation strategies in order to resist a subsequent exposure to a higher level of the same stress along with a number of other different stresses. Desmond et al. (2002) found that *L. paracasei* NFBC 338 when pre-stressed by either heat (52 °C for 15 min) or salt (0.3 M for 30 min), survived up to 300-fold better during heat stress and 18-fold better during spray drying at outlet temperatures of 100-105 °C. The relatively lower improvement in cell viability after spray drying of pre-adapted cells could be due to the dehydration damage caused by the drying process. Prasad et al. (2003) observed better storage stability of FB dried *L. rhamnosus* cells when heat shocked (50˚C/30min) and osmotic shocked (0.6M NaCl) before drying. At the end of 14 weeks of storage at 30˚C, the heat shocked (stationary phase) and osmotically shocked cells showed decrease in viability of 1.6 and 2 log units with highest

viability losses in unstressed control cells (7.3 log unit reduction) (Prasad et al., 2003). Maus and Ingham (2003) found that acid and cold tolerance was induced in *B. longum and B. lactis* by starving their cells in growth medium at 6 ˚C for 60 minutes (cold stress) or by reducing the pH of growth medium from 6.2 to 5.2 (acid stress). The cold stress was thought to induce genes to encode transcription factors regulating the production of stress proteins whereas acid stress was thought to produce changes in the bacterial cell membrane fatty acid composition and induce the production of acid shock proteins. The carbohydrate starvation can also provide multi-resistance against other stresses (see section 2.6.1.5).

Distinct species of a given genus (or even distinct strains of given species in many cases) can differ in their viability under the same drying conditions due to some genotypical differences. For instance, Kets et al. (1996) found that different strains of LAB differed in their capability to accumulate glycine betaine as a compatible solute, which affected their ability to survive the drying process. O'Callaghan and Condon (2000) also found different strains of *Lactococcus lactis* differed in their capability to accumulate glycine betaine, due to the variations in the genotype.

2.6.2.3 Growth medium pH

The influence of pH on viability of cells after drying is still equivocal and seems to vary with the strains as well as the drying process applied. Palmfeldt and Hahn-Hagerdal (2000) reported higher viability of *L. reuteri* cells grown at pH 5 (90 %) than at pH 6 (65 %) during freeze drying. Silva et al. (2005) also reported higher viabilities of *L. delbrueckii* ssp. *bulgaricus* grown under uncontrolled pH (end fermentation pH was 4) during spray drying and heating as compared to the cells grown under controlled pH (pH 6.5) but no significant differences were observed during storage. The enhanced production of HSPs GroEL, GroES, and Hsp70 due to acid stress uncontrolled pH cultivation was thought to provide cross protection against heat inactivation (Silva et al., 2005). In contradiction to these findings, Linders et al. (1997b) reported lower residual activity of FB dried *L. plantarum* grown under uncontrolled pH.

2.6.3 Initial moisture content/water activity and drying rate

During the period of constant drying rate both inter- and intracellular water is evaporated. When the bound water starts to evaporate, the drying rate decreases. Both drying rate and initial water activity/moisture content are expected to influence the inactivation of bacteria by dehydration during drying (Selmer-Olsen et al., 1999b). The water content of drying air is also

important, as it has been generally accepted that moist heat is more effective in inactivating microorganisms than dry heat. This may be due to the fact that heat is conducted faster when the material is not dry (Fu and Chen, 2011). The water content of drying air along with its temperature also determines the drying time needed to attain desired moisture level (≤ 4%) in the dried culture.

The initial water activity and water content of the material containing microorganism prior to drying, influence the heat resistance of microorganisms during the subsequent drying process. Low initial water content is thought to be more favourable than high initial water content, since there is less water to evaporate, thus a higher processing efficiency especially during FB and vacuum drying, where feed is usually in solid form. However, there is a risk that the osmotic shock caused by the decrease in the water content prior to drying may lead to undesirable cell death (Mille et al., 2004). Santivarangkna et al. (2006) suggested the rapid decrease of survival at the beginning of vacuum drying (at 43°C), when the water activity is quite high (> 0.8), was presumably because of membrane damage following severe passive exit of water from cell as the result of a relatively high drying rate at this period. The cell membranes can be damaged by high water flow from the cell because the rate of water outflow of cell is normally limited by the hydraulic membrane permeability. At the late drying period water content of about 0.3-0.5 g H_2O (g dry weight) $^{-1,}$ survival was drastically decreased. A similar phenomenon was reported by Selmer-Olsen et al. (1999b) with *L. helveticus* dried by FB dryer (5°C and ≈ 55 % RH) at the water content of 0.3–0.4 g H_2O (g dry weight)$^{-1}$. This may be due to physiological damage from large lateral compressive stresses in the plane of the membrane at low water potential.

The high and low temperatures during desiccation could induce not only different inactivation mechanisms on microbial cells (as discussed in Section 2.5.2) but also causes different drying rates. Linders et al. (1996) found no significant influence of the drying rate on cell viability of *L. plantarum* because the slow drying experiment was carried out at the low temperature, i.e., 4 °C. In contrast, Tymczyszyn et al. (2008) found that at a mild drying temperature of 30 °C, slow dehydration made the cytoplasmic membrane more susceptible to dehydration damage than rapid dehydration at 70 °C. At high drying rates (high temperature), the fast diffusion of water out of the cells keeps the membrane components in glassy state. However, at low temperatures (low drying rates), the crystallization of the cytoplasmic components could occur due to the lowered glass transition temperature Tg (see section 2.6.4). As a consequence, the denaturation of cells structures could take place during storage. Another factor related to the

drying rate is the initial size of wet cell droplets/pastes. With the same initial moisture concentration, larger individual sizes of wet samples would require a longer drying time. This makes the change of the moisture content of larger size samples slower than that of samples with smaller size. Thus the actual effect of drying rate on cell survival still remains unquantified. The survival response could be both species-specific and dependent on other factors, such as drying matrix, temperature and process, and thus needs to be experimentally determined for each drying environment.

2.6.4 Protective Carriers

The addition of protective carriers prior to drying is a common means to protect cells during drying and storage. Fu and Chen (2011) grouped the commonly used protective carriers into three categories on the basis of their physical states when mixed with cells and after being dried i.e. carriers in liquid state, carriers in solid state, and carriers used to encapsulate cells. The selection of carrier largely depends on the drying process used but its effectiveness could be strain-specific. For low temperature drying methods such as FB and vacuum drying, the feed in solid form is preferred (Bayrock and Ingledew, 1997; Tymczyszyn et al., 2007). This is mainly because, firstly it the saves the cost and secondly, it improves the drying efficiency because less water needs to be removed. In contrast, high temperature drying methods such as spray drying, the feed solution is in liquid form. The high water content during spray drying helps keeping the temperature of drying material at around the wet-bulb temperature, consequently preventing cells from being overheated by the high inlet temperature of spray drier. Some studies were also performed where cells harvested from the growth medium were dried without supporting materials (Tsaousi et al., 2008) or where cells were dried directly in the original growth medium (Desmond et al., 2002; Wang et al., 2004).

One of the most extensively investigated protectants is trehalose, due to a phenomenon known as anhydrobiosis, in which some organisms in nature survive long and extreme dehydration by accumulating a large amount of disaccharides, especially trehalose (Crowe et al., 1992). Trehalose can be used as a carrier in both solution and solid form. The prominent protective effect of trehalose could be attributed to three factors. Firstly, trehalose molecules are able to act as an alternative to the water molecules in sustaining the original conformation of the lipid bilayer of cell membrane (Crowe et al., 1992) which has been described previously (section 2.5.2). Trehalose can depress the membrane phase transition temperature (T_m) of dry lipids. As a result lipids remain in liquid crystalline phase during dehydration and they do not leak during subsequent rehydration. This mechanism is represented pictorially in Figure 2.6.4.

Secondly, trehalose has a high glass transition temperature Tg, giving it higher tendency to stay glassy compared to other non-reducing disaccharides such as sucrose. When used as a protective matrix in a dry form, the glassy state has high viscosity which slows chemical reactions such as free radical oxidation, in comparison to the crystalline state (Crowe et al., 1998). Lastly, trehalose is also able to act as an effective thermoprotectant against protein denaturation (Eleutherio et al., 1993). But the high price of trehalose inhibits its use in starter culture production on an industrial scale.

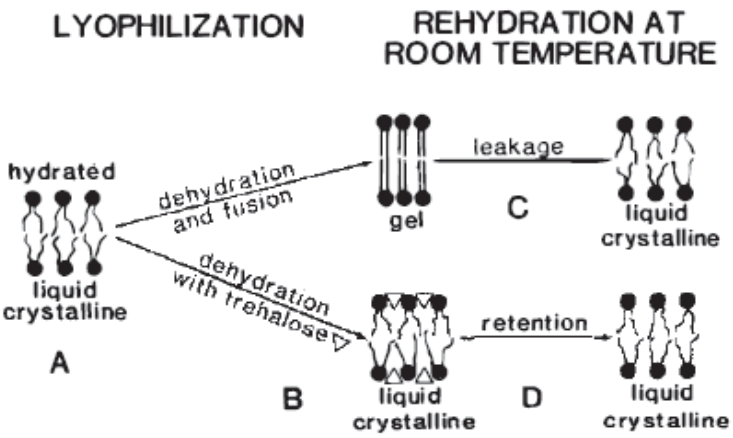


Figure 2.6.4 - Schematic of the potential phase transition of cellular membrane upon dehydration and rehydration in the presence and absence of trehalose (Crowe et al., 1992)

Trehalose was reported to preserve *L. acidophilus* during vacuum drying (Conrad et al., 2000). The presence of borate, which could cross-link the trehalose, thereby raising Tg of the dry matrix, significantly enhanced the protective ability of trehalose during drying and storage (Conrad et al., 2000). The viability of FB dried LAB strains *Enterococcus faecium* and *L. plantarum* increased in presence of trehalose and sucrose during storage at 35 ˚C (Strasser et al., 2009). In contrast, Linders et al. (1997a) reported sorbitol and maltose more effective than trehalose in increasing activity of *L. plantarum* after air drying. The positive effect of maltose and sorbitol was not found to be due to their ability to lower *T*m but was through their free radical scavenging activity (Linders et al., 1997c). Selmer-Olsen et al. (1999a) studied the effect of various protectants other than sugars and found that non-fat milk solids (NFMS) and betaine showed the best protective effect during FB drying and storage of *L. helveticus*, while the

protective effect of glycerol was better during dehydration than during storage indicating different role of protectants during drying and storage. Skim milk powder/reconstituted skim milk powder (RSM) is another carrier matrix that has been shown to improve cell survival during dehydration, especially during spray drying. It was suggested that this effective protection may be related to the lactose in RSM, where lactose interacts with cell membrane and helps to maintain the membrane integrity in a manner similar to trehalose (Corcoran et al., 2004). However, Santivarangkna et al. (2006) found that use of lactose as a protective carrier did not improve the survival of *L. helveticus* during vacuum drying. As major components of skim milk are lactose, casein and whey proteins, whether or not this significant protective effect of RSM was due to the presence of protein remains to be explored. Linders et al. (1997b) used dry potato starch as carrier to dry *L. plantarum* cell pellets in a FB drier. The results did not show any improvement on the cell survival compared to those employing the liquid carrier of potassium phosphate buffer followed by air-convective drying. Mille et al. (2004) mixed casein powder of different water activity in cells obtained after centrifugation and reported that decreased water activity of casein powder can pose osmotic shock on cells while mixing prior to FB drying .

2.7 Rehydration

Rehydration conditions such as rehydration- rate, temperature and time, as well rehydration medium conditions such as solids level, pH and composition, highly affects cells viability after reconstitution. Rehydration can cause osmotic stress on dried cells which could cause rupturing of cytoplasmic membrane with leakage of cellular contents leading to cell death. A slow rehydration rate is therefore recommended to achieve the best cell recovery (Poirier et al., 1999; Teixeira et al., 1995). However, too low rehydration rate can make revival of dried microorganisms less economical. Rehydrating cells in solutions having 10 to 20% solids is usually considered to give favourable results (Champagn et al., 2011). Water is not recommended if powder is to be rehydrated in dilute solutions; however water works well with cultures dried in milk solids because milk not only contains sugars, vitamins, minerals and nitrogenous compounds but it can also provide a very suitable buffered environment when cells are rehydrated in acid environment such as fruit juices (Reid et al., 2007). The rehydration medium temperature is another critical parameter and an optimum rehydration temperature can vary with the drying method used for preservation. Overly warm and overly cold media are both shown to be disadvantageous to cell viability during rehydration. It was reported that for spray-dried LAB, the optimum rehydration temperature was around 20 °C (Wang et al., 2004).

Whereas for FB dried cells, the optimum rehydration temperature was appreciably higher, from 20 °C–30 °C (Mille et al., 2004; Selmer-Olsen et al., 1999b). Champagn et al. (2011) suggested rehydrating powdered cells between 30 to 37 °C for 15 to 30 min before diluting for enumeration. The rehydration conditions could also be strain specific. Poirier et al. (1999) found that improved survival ratio of *S. cerevisiae* could be achieved using a rehydration temperature slightly above the phase transition temperature of cytoplasmic membrane (T_m), from 35 to 50 °C. Thus a multitude of factors affect the viability of probiotic cultures not only during drying but also during rehydration and no single universal rehydration method could be applied.

2.8 Storage and Packaging

The storage stability of probiotic culture until the end of its shelf-life is critical as cell death occurs not only during processing but also during storage. The method of storage and packaging is of huge significance as dried probiotics must be protected from heat, oxygen, light, and moisture. The factors including temperature (Strasser et al., 2009; Teixeira et al., 1995), moisture level (Linders et al., 1997a), protective carrier (Selmer-Olsen et al., 1999a; Strasser et al., 2009), pre-adaption of cells to stresses (Prasad et al., 2003) and oxidative stress (Teixeira et al., 1996) have been investigated for their effects on the maintenance of cell activity during the storage period. The mechanism of cell death during storage is different from that in a thermal drying process. Since there is usually no heat stress during storage, the main stresses that cells suffer are the low water potential and oxidative stress. The loss of cell viability at elevated temperature (above refrigeration temperature) is related to formation of reactive oxygen species (ROS) (see section 2.6.1.2) in the presence of oxygen, fatty acid oxidation and DNA damage (Selmer-Olsen et al., 1999b). Thus, during storage, natural degradation of life-essential macromolecules could occur, considering that *in vitro* lipids and proteins undergo oxidation and denaturation, respectively, during prolonged storage. Teixeira et al. (1996) reported that the ratio of unsaturated-saturated fatty acids decreased following spray drying and this ratio decreased further during storage in air, presenting evidence of lipid oxidation after prolonged storage. The lipid oxidation can cause further increase in membrane permeability and also effect enzymatic activities associated with the membrane (Castro et al., 1995). Another reason could be the continuation of metabolic activities despite at a relatively slower rate. Since the storage environment does not allow reproduction, cells with very little metabolic activity would suffer natural death (Fu and Chen, 2011).

The cell viability during storage is generally considered inversely related to the storage temperature with much higher cell death at ambient and higher storage temperatures as compared with refrigerated temperatures (Corcoran et al., 2004; Silva et al., 2002; Strasser et al., 2009; Teixeira et al., 1995). The moisture level of the dried cells also plays a key role in the maintenance of cell activity, and a high moisture level during storage is disadvantageous (Selmer-Olsen et al., 1999b; Strasser et al., 2009). Under low moisture conditions, micro-organisms can attain an anabiotic state in which metabolic processes are temporarily halted but cells remain viable and can be stored for long periods of time. The type of protective carrier used is also important as protective carriers can stabilize the cellular structures in a glassy state with high viscosity, which restricts molecular movement and inhibits harmful free radicals (Selmer-Olsen et al., 1999a). Pre-adaption of cells could lead to the accumulation of compatible solutes and the production of chaperone proteins, which could alleviate the osmotic stress and stabilize macromolecular (such as proteins, DNA, RNA) structure. Thus the factors that could protect cells during storage are similar to those which exhibit protective effects during drying.

Regarding the role of oxygen during storage, vacuum packaging could retain better storage stability of the cells as compared to without vacuum (i.e., directly exposed to air) (Chavez and Ledeboer, 2007; Hernandez et al., 2007). This could be due to the fact that accumulation of ROS within a cell could cause irreversible damage to the cell components. Teixeira et al. (1995) found evidence of damage to the cell wall, cell membrane and DNA during storage of *L. delbrueckii* ssp. *bulgaricus*. The use of ascorbic acid and monosodium glutamate as antioxidants on storage stability has also been reported by Teixeira et al. (1995). These antioxidants had protective effect at 4 °C, however an increase in cell death of *L. delbrueckii* ssp. *bulgaricus* at 20 °C storage temperature was observed. It was suggested that in addition to its antioxidant property, ascorbic acid could also have pro-oxidant property and could possibly generate hydroxyl radicals which may attack and oxidize the biological molecules. In contrast, the use of oxygen-scavenging agents during storage did not show significant effect in protecting cell viability (Chavez and Ledeboer, 2007; Wang et al., 2004).

2.9 Commercial production of *Lactobacillus*

The lactobacilli are fastidious bacteria requiring rich media containing expensive compounds such as amino acids, peptides, vitamins, and nucleic acids for growth. The most common medium for lactobacilli is the Man Rogosa Sharpe (MRS) medium (Rogosa et al., 1961). The

growth of lactobacilli also depends on the growth conditions such as temperature, pH, and the level of aeration. Various studies have been performed with the aim of optimizing the growth media composition and growth conditions for maximizing viable cell density and biomass in the media. High viable cell concentrations in media are essential as the majority of cells die during drying and long term storage. Thus the higher the initial cell concentration, the longer viable cells will survive in food product (Miao et al., 2008). Liew et al. (2005) found that yeast extract, glucose, vitamins and pH could significantly affect the growth of *L. rhamnosus*. Fung et al. (2008) showed that meat extract, vegetable extract and peptone significantly influenced the growth of *L. acidophilus*. They suggested that meat extract, could be used as a cheap alternative of yeast extract. Moreover meat contains greater amount of total nitrogen (12% w/w) than the yeast extract (10% w/w) (Polak-Berecka et al., 2010). Regarding the effect of carbohydrates, Liew et al. (2005) showed a slight effect of glucose on the viable cell count of *L. rhamnosus*. The biomass production of *L. plantarum* was affected by lactose and maltose, but in a different way. The maltose concentration was found to be linearly related to the biomass value, as its increase resulted in an increase in the OD; but the effect of lactose was not linear as it required a threshold value (20 g l^{-1}) (Bevilacqua et al., 2008). Other authors have recommended use of ammonium salts such as ammonium citrate in the medium as they could increase the biomass yield and specific growth rate.

During fermentation in a batch culture, the upper limit of the concentration of *Lactobacillus* is 10^9 cfu ml^{-1}, because of the lowered pH of the growth medium caused mainly due to the accumulation of lactic acid (Hayakawa et al., 1990). Even if the pH is maintained at the optimum value of around 6.5 by the addition of alkali solution in a bioreactor, the maximum lactobacilli concentration is limited to 10^{10} cfu ml^{-1} because of the high amount of lactate formed during cultivation (Hayakawa et al., 1990). Various studies have reported that increase in cell density by supplementing various nutrients were higher in a fed-batch (pH controlled) fermentation when compared to batch and continuous fermentation in a bioreactor.

The growth medium composition does not only affect the media count but could also influence cell survivability during the subsequent drying process. By providing stressful environments in the growth medium, various stress proteins and compatible solutes could be accumulated within a cell which could provide multi-protection against various stresses during drying (see section 2.6.1).

There are few publications defining the optimal cell concentration for freeze drying, $> 1 \times 10^8$ cells ml^{-1}. In some studies the optimum initial cell concentration was found to be related to protective medium used in freeze drying (Costa et al., 2000). Linders et al. (1997b) found that *L. plantarum* activity after drying was directly correlated to the initial cell density. The ratios of the activity immediately after drying to the activity before drying ranging from 0.1 to 0.83 were achieved using initial cell densities between 0.025 and 0.23 g of cells/ g of sample, respectively.

2.10 Concluding Remarks

Modern consumers are increasingly becoming interested in their personal health, and expect the food that they eat to be healthy or even be capable of preventing illness. Gut health in general has shown to be the key sector for functional foods in Europe. The probiotic yoghurt market is now well established with its beneficial effects on human heath well accepted. New product categories containing probiotic bacteria outside the dairy sector will certainly be the key research and development area for future functional food markets. However, the viability and stability of probiotics has always remained technological challenge for industrial producers. The probiotic foods should contain specific probiotic strains and maintain a suitable level of viable cells during the product's shelf life. But before probiotic strains can be delivered to consumers, they must first be able to be manufactured under industrial conditions, and then survive and retain their functionality during storage as frozen or dried cultures, and also in the food products into which they are finally formulated.

With the increasing popularity of probiotic products among consumers, large-scale industrial fermentation and preservation of lactic acid bacteria (LAB) probiotic strains such as *Lactobacillus* is gaining importance. One of the most widespread *Lactobacillus* species used is *L. casei*, which has high acid tolerance, and is a generally regarded as safe (GRAS) organism. The growth activity of lactobacilli is affected by fermentation conditions such as pH, temperature, medium formulation and others. They comprise fastidious-growing bacteria, requiring rich media for their growth. Thus optimization of growth medium composition and growth conditions is important for enhancing their growth. The industrial preservation of LAB involves processes such as freezing, freeze drying and air drying. Thermal drying by fluidized bed (FB) drying offers a low energy and cost efficient alternative to freeze drying which is currently the most common method of preservation for LAB cultures. Moreover FB drying could be carried out at low temperatures as compared to lethal temperatures of spray drying.

FB drying is extensively used in the yeast industry but very few studies have described its use to preserve LAB. The stability of the dried LAB through FB drying can be achieved by drying it to the appropriate water activity and moisture content; however, the drying process is often harsh. At the low temperatures of FB drying, thermal inactivation is negligible but the dehydration inactivation can affect the viability. The loss of water from both the membranes and the proteins is responsible for this damage.

Studies on the stabilization of LAB through FB drying pointed out the poor efficiency of this process for *Leuconostoc oenos* (Clementi and Rossi, 1984) and recommended the use of protectants such as carbohydrates to improve the survivability of *L. plantarum* (Linders et al., 1997a, c). Mille et al. (2004) observed that the water activity of the protective carrier can affect viability during mixing and drying but the viability of two strains studied (*L. plantarum* and *L. bulgaricus*) were different (80% and 10%) under the same conditions. Prasad et al. (2003) observed prestressing *L. rhamnosus* showed a significant improvement in viability compared with a non-stressed control culture during storage at 30 ˚C. However, there is a paucity of information describing improvement in the survival of *L. casei* in relation to industrial FB processing and during storage at ambient temperature.

Maximization of cell survival during a thermal drying process needs to take into account the combined effect of heat and dehydration damages. This requires a balanced consideration of multiple factors including the intensity of each stress (e.g., temperature and water potential), the time of cells exposed to each stress, and the changing rate of these stresses (e.g., the drying rate and the rate of temperature variation). During drying, microorganisms undergo an increasing osmotic stress condition as the water activity decreases. Various studies showed that the bacteria have evolved stress- sensing systems and defenses against stress, which allow them to withstand harsh conditions and sudden environmental changes. The different responses to different stress treatments e.g. heat, low pH, starvation, osmotic shock, etc. have been reported. These stress responses are characterized by the transient induction of general and specific proteins and by physiological changes that generally enhance an organism's ability to withstand more adverse environmental conditions.

A better understanding of these factors will help in achieving the necessary robustness of bacteria required for various industrial processes during large scale production of shelf- stable commercial cultures and during their application in various food products. Currently, industrial demand for technologies ensuring probiotic stability in foods stored at ambient temperature remains strong. Development of an optimized culture media for probiotics as well as

processing techniques for ensuring probiotic stability are important for commercial scale production of probiotic cultures. The results of these experiments performed in this study will help in providing recommendations for increasing robustness of bacteria in relation to various industrial processes and applications.

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

The peptone water, yeast extract, meat extract, peptone (from casein), ammonium citrate, manganese sulphate (monohydrate), sodium chloride (NaCl) were bought from Merck (Merck, Darmstadt, Germany), MRS broth and MRS agar from Oxoid (Oxoid, Milan, Italy), glycerol, ammonium hydroxide and hydrochloric acid (HCl) from BDH (BDH laboratory supplies, Poole, England), glucose (dextrose monohydrate) from Penford (Penford, Onehonga, NZ). The vitamins used (pyridoxine HCl, calcium-pantothenate, niacin, riboflavin, folic acid) were provided by DSM (DSM Nutritional products, Inc. NJ, USA).

3.2 Strain and inoculum preparation

Lactobacillus paracasei subsp. *paracasei L. casei* 431, (isolated from infant faeces) (Chr. Hansen) was used throughout this study. The strain was stored at -80 ˚C in 10 % glycerol. For inoculum preparation, stock culture was subcultured twice in MRS broth at 37 °C for 20 h with 2 % (v/v) inoculation. For the experimental studies 5 % (v/v) of inoculum was transferred to the cultivation medium and incubated at 37 °C and 160 rpm in a shaking incubator. This procedure was used as the standard inoculum preparation for all the experiments.

3.3 Growth media composition

The base medium in all the experiments consisted of MRS broth containing (g l^{-1}): KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.2; $MnSO_4.H_2O$, 0.04; Tween-80, 1 ml l^{-1}; yeast extract, 4.0 ; peptone from casein, 10; meat extract, 8.0; di-ammonium hydrogen citrate, 2.0; glucose, 20. The amounts of glucose, nitrogen sources and other growth nutrients added to supplement MRS broth varied for different experiments. All the supplemented nutrients were heat sterilized with the base medium except the vitamin solution, which was sterilized separately by filtering through membranes (Minisart mod.16555-Q with 0.45µm pore size, Sartorius, Goettingen, Germany) and was added to the cultivation medium prior to inoculation. The pH of growth medium was adjusted when required with ammonium hydroxide (NH_4OH) and hydrochloric acid (HCl).

3.4 Fermentation experiments

3.4.1 Uncontrolled pH fermentation

All uncontrolled pH fermentation experiments were performed in 1L Durham bottle, containing 1 kg cultivation medium kept in shaking incubator (Infors HT Multitron 2) set at 160 rpm and 37 °C temperature for 16 to 20 h. No pH control was employed during cultivation experiments, but initial pH was set at different values ranging from 6.0 to 7.0. Growth (Log_{10} cfu m l^{-1}) and other growth characteristics of *L. casei* 431 in uncontrolled pH MRS (control) were compared with MRS media supplemented with various amounts of nitrogen sources (yeast extract, meat extract, peptone), glucose and other growth factors.

3.4.2 Controlled pH batch fermentation

The pH controlled batch fermentation was carried out in a 2 L stirred bioreactor (New Brunswick Scientific Co. Inc., USA, Model Multi Gen F-1000). No air was introduced and 160 rpm agitating speed was employed to keep fermentation broth homogenous. The culture temperature and pH were set at 37 ± 0.1 °C and 6.5 ± 0.1, respectively. The pH was controlled by automatically adding 4 M NH_4OH. Growth (Log_{10} cfu ml^{-1}) and other growth characteristics of *L. casei* 431 in controlled pH MRS (control) were compared with MRS supplemented with 30 g l^{-1} glucose and 40 g l^{-1} yeast extract.

3.4.3 pH Feedback-Controlled Fed-Batch fermentation

The base medium for fed-batch experiment consisted of MRS broth supplemented with 40g l^{-1} yeast extract. Feeding solution was a mixture of glucose and NH_4OH. Except for the substrate fed in the fed-batch culture, the other cultivation conditions were the same as the batch experiment. As the pH limits were set at 6.4 and 6.6, the peristaltic pump coupled to pH controller was activated when the pH was below the lower limit. Therefore, the glucose and NH_4OH were added to the fermentation broth proportionally at the same time with one peristaltic pump.

In all these fermentation experiments, about 25 ml samples were withdrawn at time intervals for various analyses.

3.5 Harvesting and mixing of cells with protective carrier (whole milk powder)

Cells were harvested by centrifugation (Sorvall RC 4 centrifuge) at 10000 rpm for 8 min after required growth time. The washing of precipitate when required was done by re-suspending the harvested cells in peptone water and centrifuging them again at the same speed. In the initial experiments the harvested (washed) cells were mixed with whole milk powder (WMP) of initial water activity a_w 0.3 and 0.7 in the ratio 1:9. Water activity of 0.7 was achieved by adding required water to WMP. Mixing of harvested cells with WMP was done either through hand or mechanical mixing. Mechanical mixing methods included slow mixing Kenwood planetary mixer (flat beater attachment) and high shear Kenwood food processor (300w Power, 2-speed mini chopper with SS blades). Cells were added in small amounts in WMP in both hand and mechanical mixing. Mixing was done manually for 5 minutes and mechanically for 15 minutes. Mixing of cells with WMP was then standardized for further experiments with one standard mixing method i.e. 7.5 of harvested cells were mixed with 100g of WMP of a_w 0.3 in Kenwood planetary mixer for 15 min and then ground in Kenwood food processor for 2 min.

3.7 Drying

Harvested cells mixed with WMP, were dried in a fluidized bed (FB) drier (Glatt GmbH, Germany, Model Uni-Glatt) shown in figure 3.7.1. A dehumidifier (Munters Dehumidifier, Aachen, Germany, Model MD 300) was attached to FB drier in later experiments with the aim of reducing moisture content in the dried powders.

Figure 3.7.1 - Glatt Uni-Glatt laboratory fluid bed dryer

3.8 Packaging and storage

FB dried probiotic powders were packed and heat sealed in aluminium pouches with or without inner polythene layer and stored at 25 °C and 37 °C for the required time.

3.9 Powder Rehydration

Viability in powders was determined in duplicates before and after drying and also after required storage time. Sample (4 g) was dissolved in Milli Q water (36 g) at 37 ˚C and was mixed in stomacher (Colworth stomacher 400) for 10-15 min. The rehydration conditions were held constant for all the samples.

3.10 Experimental design

Response surface methodology (RSM) was used to investigate the effect of various parameters during fermentation, cell harvesting and drying with the objective of identifying the ideal combination for maximizing the viability during ambient storage. RSM is a collection of statistical techniques for designing experiments for exploring nonlinear relationships between studied factors and the dependent variables. It provides information about optimal values of these factors to determine the expected largest (or smallest) values for the dependent

variables of interest. This methodology includes factorial design and regression analyses and is commonly preceded by primary screening with the "one-variable-at-time" approach or a Plackett-Burman (PB) design application (Polak-Berecka et al., 2010).

Experimental designs such as Plackett–Burman designs are good methods for screening and optimization of media compositions and culture conditions in fermentation processes through a minimal number of experiments (Brinques et al., 2010). The Plackett-Burman design is a very useful tool that enables us to screen n variables using only n + 1 experiments (Montgomery, 2013; Myers et al., 2009). At this early problem solving stage, the methodology assumes that important main effects will be much larger than two-factor interactions. This technique can be used to identify the more important independent variables and which could be selected further to design a complete factorial design in order to determine the optimum level of factors studied.

3.10.1 Experimental design for uncontrolled pH fermentation experiments

A PB design (Appendix 1) was used for initial screening of factors affecting *L. casei* viable cell count in uncontrolled pH medium in Chapter 6. Seven variables (initial pH, glucose, yeast extract, meat extract, peptone, ammonium sulphate, NaCl, vitamins) at two levels and two dummy variables were screened in 13 trials as shown in Appendix 1 in which each column represents an independent variable and each row represents a trial. Factors were varied over two levels, namely, the high concentration (+1) and the low concentration (-1), with one run at central level (0). The dummy variables were used as the measure of variability. They give a direct estimate of the standard error of a factor effect (Kalil et al., 2000). Variables with confidence levels >90% were considered to be significant. Growth (Log_{10} cfu/ml) of *L. casei* in media was used as the response in all the experiments. In addition, the other properties of *L. Casei* such as OD, biomass, pH, production of lactic acid and utilization of glucose in media were also evaluated.

Central composite design (CCD) (Appendix 2) was then used to further optimize the growth conditions with glucose and yeast extract as independent variables, and pH was set up according to the results of the PB experiment. The variables and the coded and uncoded values of the variables at various levels are given in Appendix 2, which shows 13 trials of the two variables, each at five levels. The design was a central composite design ($k = 2$) with one central point (all factors at level 0) and the four axial points, which have, for one factor, an

axial distance to the centre of ± α, whereas the other factor is at level 0. The axial distance α was chosen to be 1.41 to make this design orthogonal.

When the CCD results did not show any significant effects of factors studied on viable cell count, another PB design (Appendix 3) was then used to identify the most important nitrogen sources effecting *L. casei* viable cell count.

3.10.2 Experimental design for making FB dried powders

A PB design (Appendix 4) was used to illustrate the effect of harvesting time, total solids/moisture content of harvested cells and washing of cells in stabilization of *L. casei* 431 during laboratory and industrial scale preservation in Chapter 7. Once harvesting conditions for making powders were standardized another PB design (Appendix 5) was used in Chapter 8 to identify the best drying conditions (drying time and drying temperature) under laboratory processing conditions. This was followed by a CCD design (Appendix 6) to optimize the two factors; drying time and initial moisture content with drying temperature set up according to results obtained in PB design with response as *L. casei* 431 viable cell count after drying and storage.

3.11 Statistical data analyses

All experimental designs and results analyses were carried out using Minitab 16 (Minitab Inc. International, USA) to test the significance of each factor on the response factors. The experimental data were entered in Minitab worksheet and an analysis of the significance of factors and/or their interaction was obtained. The *P* value gave an indication of significance of a factor or interaction between two or more factors. The effects of independent variables was indicted by a positive or negative sign. A positive coefficient indicated that the variables were positively linearly correlated and a negative coefficient indicated there was inverse linear relationship between the variable.

Statistical verification of the model was performed by analysis of variance (ANOVA). The significance of the obtained model was checked by R^2 value, which indicated how well the data fit the model, i.e. the percentage of variability that the model could explain.

Tukey tests were occasionally carried out to test the significance between levels of the factors. A significant effect between levels of factors was indicated by P values for the tests, as above.

3.12 Analytical determination

3.12.1 Viable cell counts

The number of viable cells was determined as colony forming units (cfu). Serial decimal dilutions of each sample were plated in duplicate onto MRS agar and incubated at 37 ˚C for 72 h before enumeration.

The loss in viable count (log cfu g^{-1} powder) after drying and storage was calculated as follows: Drying/Storage loss = N_o - N, where N_o represents the number of bacteria (log cfu g^{-1}) in the powder before drying and N is the number in the dried powder immediately after drying or after required storage time. Both N and N_o were expressed per gram of powder.

3.12.2 Biomass

Biomass was quantified gravimetrically as the dry weight of cells. Samples of 20 ml of the growth medium after fermentation were centrifuged at 10000 rpm for 8 min at 4 ˚C, twice washed with cold distilled water, and dried in preweighed plastic tubes at 108 °C to constant weight in oven. The cell-free supernatant was used for the estimation of lactic acid and glucose.

3.12.3 Moisture content and water activity

Percent moisture content was calculated by drying around 2 g of FB dried powder to equilibrium moisture in oven set at 105 ˚C. Water activity was measured with a water activity meter (Pawkit, Decagon Devices, Inc., WA, USA).

3.12.3.1 Comparison of moisture content obtained after conventional oven drying (at 105 ˚C) and vacuum oven drying (at 80 ˚C)

Some variability in moisture content was observed during storage, and it was suspected that some decomposition of the sample was occurring during oven drying. So vacuum oven drying (at 80 ˚C) was used as alternate method in Chapter 8 for moisture determination and their results were compared with oven drying method (at 108 ˚C) as shown in Table 3.12.3.1. The correlation between the two methods was poor. Drying sugary powders is notoriously difficult, and it is difficult to choose a fool proof method. The vacuum drying method is probably to be slightly preferred as it generally does result in a constant weight after drying, whereas oven drying continues to show a slight loss due to decomposition.

Table 3.12.3.1 - Comparison of moisture content determined by oven drying and vacuum oven drying

Sample No.	Vacuum oven drying (80 ˚C)	Conventional oven drying (105 ˚C)
1	3.74	4.69
2	3.73	4.64
3	3.76	4.61
4	4.07	5.07
5	3.88	4.61
6	3.90	4.42
7	3.97	4.50
8	3.99	5.39
9	3.33	4.50
10	3.89	5.71
11	3.83	4.68
12	3.40	4.69
13	3.38	4.63
14	4.20	5.36

3.12.4 Optical Density (OD)

OD was evaluated by measuring the absorbance at 600 nm through a spectrophotometer (Ultrospec 2000 UV/ Visible Spectrophotometer, Pharmacia biotech).

3.12.5 Glucose and lactic acid

Lactic acid and glucose concentrations were measured on the supernatant obtained after centrifugation by high pressure liquid chromatography (HPLC) (Agilent Technology 1200 series, CA, USA) coupled with a refraction index detector (model G1362A RID). An Aminex HPX 87H (Biorad®) column and a 5 mM sulphuric acid mobile phase were used with a flow rate of 0.6 ml/min, and temperatures of 50 °C and 60 °C in the detector and oven, respectively.

3.12.6 pH

pH was evaluated through a pH-meter Crison mod 2001 (Crison Instruments, Barcelona, Spain).

3.12.7 X-ray diffraction (XRD)

X-ray diffraction of powder samples was conducted using a Philips X-ray powder diffractometer (Philips, The Netherlands) with a Rigaku Rotaflex 2000 X-ray diffractometer using Co-Kα radiation generated at 40 kV and 30 mA. The dried powder was broken into smaller pieces with pestle and mortar. The broken particles were placed on a single-crystal quartz holder for XRD analysis. The scanning range was 10–35°, the step size was 0.02°, and the scanning rate was 0.5° min^{-1}.

CHAPTER 4

TO ESTABLISH AN EFFICIENT CULTURE METHOD FOR *L. CASEI* 431 TO OBTAIN HIGH DENSITY VIABLE CELLS IN GROWTH MEDIA

4.1 Introduction

This chapter compares batch and fed-batch fermentation of *L .casei* 431 in pH controlled supplemented MRS media in terms of its growth (log cfu ml^{-1}). The aim was to establish an efficient culture method for *L. casei* 431 to obtain high density viable cells in the growth media. From two successive overnight MRS cultures, a 5 % (v/v) inoculum was used to seed 1 L growth media in the fermentor. pH was maintained at 6.5 with 4 N NH_4OH in all the experiments. Growth (log cfu ml^{-1}) of *L. casei* 431 as well as other properties such as OD, biomass, pH, production of lactic acid, and utilization of glucose in media were evaluated.

4.2 Batch fermentation of *L. casei* 431 in MRS media at controlled pH 6.5

Table 4.2 describes the typical characteristics of *L. casei* 431 in MRS media (initial glucose concentration of 20 g L^{-1}) during batch fermentation at controlled pH 6.5. The maximum viable cell count of 9.5 log cfu ml^{-1} was achieved after just 8 h of fermentation. The viable cell count remained unchanged with increase in the fermentation time; however biomass and optical density (OD) data indicated that these parameters kept on increasing for another 2 h, after which their values were constant. The reason for the increase in biomass and OD even when the viable cell count was constant could be due to the fact that even though cells were growing, at the same time a lot of cells were dying as well. Lactic acid concentration also increased with cell growth and kept on increasing even when there was no residual glucose at 10 h. The increase in lactic acid could be due to the presence of intermediate metabolites which were slowly converting into lactic acid (Schepers et al., 2002). It is well known that *Lactobacillus* spp. growth is inhibited by lactic acid and the main challenge of increasing viable count and biomass production by LAB is to circumvent lactic acid inhibition. In a typical batch fermentation process, it is expected that, once lactic acid concentration increases, the cell growth rate is severely diminished (Aguirre-Ezkauriatza et al., 2010; Alvarez et al., 2010; Yoo et al., 1996). Our results were in agreement with findings of Avonts et al. (2004) who reported that pH controlled (pH 6.5) MRS media fermentation resulted in maximal cell count and biomass of *L. casei* complex strains above 9 log cfu ml^{-1} and 3 g L^{-1} respectively with all glucose exhausted between 10-17 h of fermentation and maximal cell counts reached within 12 h or

less. On the other hand none of *L. acidophilus* complex strains reached these high cell counts or biomass values (Avonts et al., 2004).

Six experiments were performed to get a correlation between OD and biomass (cell dry weight) which showed that they had linear relationship. One OD unit corresponded to 0.367 g kg^{-1} of biomass (Fig. 4.2). The maximum biomass and OD obtained for *L. casei* 431 in controlled pH batch fermentation were 2.6 g kg^{-1} and 7.5 respectively as shown in Table 4.2.

Table 4.2 - Viable cell count, OD biomass yield and other properties of *L. casei* 431 during batch fermentation at controlled pH 6.5 in MRS media

Time (h)	OD	Biomass, g kg^{-1}	log cfu ml^{-1}	% Glucose	% Lactic Acid
0	0.31 (0.07)	0.0 (0.03)	7.78	1.98 (0.1)	0.085 (0.0)
4	1.25 (0.18	0.4 (0.18)		1.61 (0.5)	0.66 (0.7)
6	3.27 (1.34)	1.4 (0.43)		0.96 (0.7)	1.36 (1.0)
8	5.85 (2.83)	2.1 (0.83)	9.36 (0.15)	0.48 (0.6)	1.6 (0.7)
10	6.84 (1.79)	2.6 (0.19)	9.42 (0.19)	0.00	1.97 (0.3)
12	7.51 (0.34)	2.6 (0.31)	9.50 (0.17)	0.00	2.03 (0.4)

Values in brackets represent SD

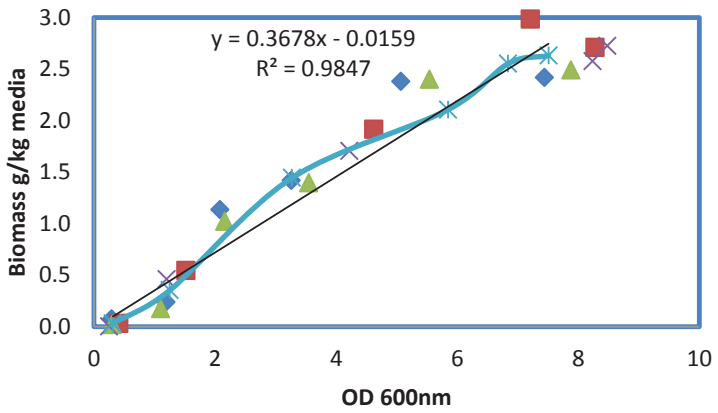


Fig 4.2 - Relation between OD and Biomass of *L. casei* 431 during batch fermentation at controlled pH 6.5 in MRS media

4.3 Batch fermentation of *L. casei* 431 in supplemented MRS media

The effect of supplementing MRS media with glucose (30 g l^{-1}) and yeast extract (40 g l^{-1}) during batch fermentation is shown in Table 4.3. The maximum viable cell count and OD of *L. casei* 431 obtained in supplemented MRS media were 9.5 log cfu ml^{-1} and 7.4 respectively. The biomass (g kg^{-1}) was determined based on biomass-OD_{600} calibration curve obtained in section 4.2. Results showed that the OD/biomass and the viable cell count remained unaffected when compared to the batch fermentation of control MRS media. Similar results were obtained by Aguirre-Ezkauriatza et al. (2010) who reported that the biomass production profiles of *L. casei* BPG4 were not affected by the variation of substrate (lactose 35-50 g L^{-1}) or inoculum concentrations (0.5-1 g L^{-1}) in the range of conditions explored during controlled pH batch fermentation. Only a few studies investigated the biomass yield of *L. casei* in bioreactors, but these studies used whey medium as a substitute for more expensive MRS media. Mondragon-Parada et al. (2006) reported biomass yield of 2 g L^{-1} at 44 h of incubation in the whey-based medium supplemented with ammonium salt and minimal amounts of yeast extract (0.25 g L^{-1}). Increasing yeast extract supplementation to 20 g L^{-1} increased biomass yield to 3.2 g L^{-1} but fermentation time was still very high (30h) (Mondragon-Parada et al., 2006). Aguirre-Ezkauriatza et al. (2010) compared biomass production of *L. casei* BPG4 in non-supplemented goat milk whey during batch, continuous and fed-batch bioreactors and recommended use of fed-batch fermentation for commercial production of probiotic *L. casei* biomass (Aguirre-Ezkauriatza et al., 2010).

Table 4.3 - Viable cell count and OD of *L. casei* 431 in supplemented MRS broth (with glucose 30 g l^{-1} and yeast extract 40 g l^{-1}) at controlled pH 6.5 in batch culture

Time (h)	OD	log cfu ml^{-1}
0	0.45	7.78
4	0.73	
6	1.76	
8	3.57	9.3
10	6.66	9.5
11	7.36	9.5

4.4 Fed Batch Fermentation: pH feedback-controlled fed-batch fermentation

In order to eliminate substrate inhibition, glucose was added into the fermentation media in pH feedback-controlled manner. Feeding solution was a mixture of glucose and ammonium hydroxide (NH_4OH). The residual concentration of glucose in media was controlled at a low level to alleviate any inhibition which might result from a high concentration of glucose. The ratio of glucose to NH_4OH was chosen as 5.7g g^{-1} as described in Zhang et al. (2010). According to the ratio of glucose to NH_4OH (5.7 g g^{-1}), the concentrations of glucose and NH_4OH in feeding solution were 387.6 and 68 g L^{-1}, respectively.

Table 4.4 shows the course of pH feedback-controlled fed-batch fermentation. The growth medium was MRS media supplemented with 40 g l^{-1}yeast extract. The feeding was started from 8 h of fermentation and was stopped at 17 h. During the feeding course, the concentration of residual glucose was in the range of 4–7 g L^{-1}, which demonstrated that the ratio of glucose to NH_4OH in the feeding solution was appropriate to keep the glucose concentration at a relative low level in the fermentation broth. The maximum viable cell count was obtained after 13 h of fermentation, 10.2 log cfu ml^{-1}, which was around 5 times higher as compared to batch fermentation. The biomass and OD kept on increasing with time till the end of fermentation (17 h) even though viable count remained unchanged after 13 h. The OD, lactic acid and biomass yield after 17 h of fermentation were 15.96, 83.5 g L^{-1} and 9.8 g kg^{-1} respectively. When compared to batch fermentation, there was a 5 fold increase in viable cell count, 4 fold increase in biomass and 2 fold increase in OD and residual lactic acid. This was consistent with the results of Zhang et al. (2010) in which pH-feedback controlled fed-batch fermentation of *L. lactis* resulted in higher lactic acid and biomass yield, but its effect on viable cell count was not discussed. Aguirre-Ezkauriatza et al. (2010) also suggested that due to the strong lactic acid inhibition, fed-batch cultures rendered higher viable cell counts and biomass productivity of *L. casei* (2.43 X 10^{10} cfu g^{-1}) than batch (5.17 X 10^{9} cfu g^{-1}) and continuous cultures (1.95 X 10^{10} cfu g^{-1}).

Table 4.4 - Viable cell count, OD and biomass yield of *L. casei* 431 in pH feedback-controlled fed-batch fermentation in MRS media supplemented with 40 g L^{-1} yeast extract

Time (h)	OD	% Glucose	% Lactic Acid	Biomass, g kg^{-1}	log cfu ml^{-1}
0	0.28	2.04	0.1	0.00	7.5
8	5.55	0.8	1.4	2.66	9.5 (0.09)
11	11.10	0.52	3.34	5.61	10.0 (0.02)
13	13.00	0.4	5.33	7.62	10.2 (0.04)
15	14.46	0.47	6.71		10.2 (0.03)
17	15.96	0.69	8.35	9.79	10.2 (0.08)

Values in brackets represent SD

4.5 Comparison of different glucose and ammonium hydroxide ratio in the feeding solution during pH feedback-controlled fed-batch fermentation

The effect of changing the glucose to NH_4OH ratio of feeding solution from 5.7:1 to 7.6:1 and 4.6:1 was also studied. The viable cell count, OD and biomass were unaffected as shown in Table 4.5.

Table 4.5 - Comparison of different glucose and ammonium hydroxide ratio in the feeding solution during pH feedback-controlled fed-batch fermentation

Glucose : NH_4OH	Time (h)	OD 600	log cfu ml^{-1}	Biomass, g kg^{-1}
5.7 : 1	11	11.1	10.0	5.61
7.6: 1	11	11.5	10.1	6.93
4.6:1	11	12.6	10.03	6.1

4.6 Conclusions:

The following general conclusions may be drawn from the results discussed in this chapter:

(i) Supplementing MRS media with yeast extract and glucose did not improve *L. casei* 431 viable cell counts during batch fermentation. The maximum viable cell count obtained was 9.5 log cfu ml^{-1} media.

(ii) pH feedback-controlled fed-batch fermentation resulted in a 5 fold increase in viable cell counts, 4 fold increase in biomass, 2 fold increase in OD as well as residual lactic acid. The maximum viable cell count, biomass, lactic acid and OD observed in fed-batch fermentation were 10.2 log cfu ml^{-1}, 9.7 g kg^{-1}, 93.8 g L^{-1} and 15.96 respectively.

(iii) Changing the ratio of glucose to ammonium hydroxide (NH_4OH) solution in feeding solution of fed-batch fermentation did not affect the viable cell count nor the other growth properties of *L. casei* 431.

CHAPTER 5

OPTIMISING THE MIXING AND DRYING CONDITIONS TO GET THE MAXIMUM VIABLE COUNT OF *L. CASEI* 431 DURING DRYING AND STORAGE

5.1 Introduction

In this chapter preliminary fluidized bed (FB) drying experiments were performed by drying the *L. casei* 431 cells harvested from the high cell density growth media obtained by pH feedback-controlled fed-batch fermentation in Chapter 4. This was followed by comparison between FB dried powders made from cells harvested from pH controlled and uncontrolled fermentation media. In all these experiments performed, the collected cells were mixed with protective carrier whole milk powder (WMP) before drying in a FB drier. The preliminary FB dried powders made showed occasional huge losses in viability after mixing, drying and storage. Various factors can affect *L. casei* 431 survivability under these conditions such as osmotic shock caused by mixing of wet cells with dry protective carrier (Beney et al., 2001; Gervais and Marechal, 1994; Mille et al., 2004), crystallization of disaccharides during drying and storage (Miao et al., 2008), mixing techniques and growth media conditions such as pH during fermentation (Linders et al., 1999b; Silva et al., 2005). Thus various experiments were performed in this chapter with the aim of providing a direct comparison of probiotic powders made with (i) protective carrier (WMP) of different initial water activity (a_w) (ii) different mixing techniques; hand and mechanical (iii) controlled and uncontrolled pH growth media in terms of survival of *L. casei* 431 during mixing, drying and long term storage. The crystallization in some of the stored powders was also determined through XRD analysis at various time intervals.

5.2 Powders made from pH-based feedback-controlled fed batch media

Bacterial cells were harvested after 11 h of pH-based feedback-controlled fed-batch fermentation by high speed centrifugation (10000 rpm for 8min). Peptone water washed bacterial pellets (10^{10}-10^{11} cfu g^{-1}) were then mixed with WMP of different a_w in the ratio 1:9 (w/w). The mixing methods used were either hand mixing or mechanical mixing (as described in Chapter 3). After required mixing time, powders were dried in the FB drier at 50 °C for 30 – 45 min to bring the final a_w of the dried powders to between 0.25 –0.3. The probiotic powders were then packed and heat sealed in aluminium pouches and stored at 25 °C for 30 days.

5.2.1 Results and Discussion

Viability data of the probiotic powders made under different conditions are shown in Table 5.2.1. Results showed that all the powders made with controlled pH fed-batch media had an average drying loss of 1.3 log units and storage loss of 2.5 log units when stored at 25 ˚C for 30 days. The viable cell count of the harvesting media was very high (10.1 log units), thus the collected cells when mixed with powder should have an average expected count of 10.7 log units after mixing. However, the count after mixing (or the count before drying) was found much lower (9.6 ± 0.3 log units) than the expected count, indicating that there was huge loss of viability on mixing with WMP in these powders. Total average viable count after mixing, drying and 30 days of storage was 9.63, 8.33 and 7.07 log cfu g^{-1} powder respectively. Powders mixed with high shear Kenwood chopper had much higher mixing and drying losses when compared to other mixing techniques which could be due to the fact that both high shear rate and heat generated during mixing would have caused huge stress on cells, resulting in their death during mixing and drying.

Table 5.2.1 - Viability data of FB dried powder made from controlled pH fed-batch media after drying and 30 days of storage at 25 °C

	Glucose: NH_4OH ratio used in feeding solution	Mixing	a_w of WMP added	a_w after drying	Media count log cfu ml^{-1}	Mean log cfu g^{-1} powder ± SD		
						Before drying	Day 0	Day 30 @25°C
1	5.7:1	Hand	0.3	0.27	10.1	10.1	9.1	7.4
2				0.25		9.8	8.4	7.6
3		Kenwood food processor		0.22		9.3	< 7	7.0
4				0.18		9.4	< 7	7.4
5	7.6:1	Kenwood planetary mixer	0.7	0.26		9.4	8.6	6.1
6	4.6:1		0.7	0.31		9.7	7.8	7.5
7			0.7	0.2		9.6	7.6	6.4
8			0.3	0.25		9.8	8.6	7.0
				Average	10.1	9.6 ± 0.3	8.3 ± 0.6	7.1 ± 0.5

Thus the preliminary probiotic powders made with controlled pH (pH 6.5) fed-batch media showed huge mixing, drying and storage losses. Since the mixing, drying and storage losses depend on growth conditions (see section 2.6.2.2) as well as a_w and type of the protective carrier used (see section 2.6.4), it was interesting to compare viabilities of *L. casei* 431 in powders made with WMP of different initial a_w under different mixing conditions and under different growth conditions (controlled pH vs uncontrolled pH fermentation). Only hand mixing and planetary mixer were chosen for further experiments as high shear Kenwood chopper mixing showed huge mixing and drying losses.

5.3 Comparing the mixing, drying and storage losses in FB dried probiotic powders made from (1) WMP of initial a_w 0.3 and 0.7 under different mixing conditions (hand mixing and Kenwood planetary mixer) (2) cells obtained from pH controlled (@6.5) and uncontrolled media (end pH 4.0 ± 0.1)

Table 5.3 shows viability of *L. casei* 431 in probiotic powders made under different conditions. The shelf life of these powders at 25 °C was investigated for the time interval of half a year. Comparisons of viabilities between dried bacterial species were performed by Student's t-test (two-tail paired) in MINITAB 16 with significance measured at a probability level of $P \leq 0{\cdot}1$.

Table 5.3 - Viability of FB dried powder containing *L. casei* 431 made under different conditions

	Mixing	a_w of WMP added	a_w after drying	Moisture content %	Mean log cfu g^{-1} powder ± SD					
					Before drying	Day 0	Day 30 @25°C	Day 60 @25°C	Day 120 @25°C	Day 180 @25°C
Powders made with pH controlled media										
1	Hand	0.7	0.21	5.1	10.3 ± 0.2	8.8	8.5	7.3		
2	Planetary mixer	0.7	0.21	5.2		9.1	8.5	7.3		
3		0.7	0.27	5.2		9.3	7.6	7.5	3.8	< 3
4		0.7	0.19	4.6		8.9	8.5	7.9	6.5	
5		0.3	0.23	4.7		9.3	8	7.1	6.1	
6		0.3	0.25	5.0		9	8.6	7.4	6.5	
				Average		9.1± 0.2	8.3 ± 0.3	7.4 ± 0.3	5.7 ± 1.3	<3
Powders made with pH uncontrolled media										
7	Hand	0.75	0.21	4.7	10.3 ± 0.2	9.9	9.2	8.4	8.0	8.0
8	Planetary mixer	0.3	0.25	5.0		9.6	9.1	8.6	8.0	8.0
9		0.3	0.24	5.1		9.9	9.5	8.0	8.2	8.0
10		0.7	0.21	4.9		9.5	9.5	8.1	8.2	8.0
				Average		9.7± 0.2	9.4 ± 0.2	8.1 ± 0.3	8.1 ± 0.1	8.0

5.3.1. Effect of mixing harvested cells with WMP of different water activity (a_w) through various mixing techniques

Statistical analysis (Table 5.3.1) showed no significant difference (*P* value >0.1) in viabilities between powders made with WMP of initial a_w 0.3 and 0.7, after mixing, drying and storage for a period of 120 days. Thus our results showed that initial a_w of WMP did not affect *L. casei* 431 viable counts after mixing, drying and storage. This was in contrast with the results of Mille et al. (2004) who reported that low a_w of casein powder (0.3) induced an osmotic shock when mixed with *L. plantarum* cells, resulting in less than 8.2 % viability in casein-cell mix; however, when a_w of casein powder was 0.75, 100 % and 80% viability of *L. plantarum* was achieved after mixing and FB drying. Under similar conditions, the survival ratio of *L. bulgaricus* after

mixing stayed very high (96%), but it decreased dramatically to less than 2% after FB drying (Mille et al., 2004). The difference in the cells resistance observed by Mille et al. (2004) suggested that osmotolerance could be species specific. Thus the difference between these results and our experiments could also be due to different strains as well as due to different drying conditions. In the study performed by Mille et al. (2004) the powders were dried to much lower final a_w of 0.17 in a shorter time with dry air of relative humidity (RH) 1% and temperature ≤ 35 °C, whereas the temperature and RH of air used in our experiments for drying *L. casei* 431 were 50 °C and 55 % respectively with comparatively longer drying time.

High water activity a_w of the mix before drying resulting from using protective carrier of high initial a_w could however lower glass transition temperature Tg of disaccharides present, resulting in their crystallization during drying and storage which could result in lower survivability of *Lactobacillus* during storage (Mioa et al., 2008). So XRD analysis of powders was performed after 30 and 60 days of storage (Fig 5.3.1), which showed that powders made with WMP of a_w 0.7 had lactose crystals whereas powders made with WMP of a_w 0.3 had lactose in amorphous form. As both powders showed similar survivability, it appears that the lactose in crystalline form as a result of higher initial a_w of WMP did not harm bacteria during long term storage.

Powders made with hand mixing when compared with planetary mixing showed similar drying and storage losses (data not shown). So the mixing of cells with WMP was standardized for further experiments with one standard mixing process i.e. mixing of cells with WMP in a planetary mixer for 15 min and then chopping this mixture in a food processor for 2 min to get a mixture of homogenous small particle size. Particle size of powder before drying is also important as larger particle size could increase the time of drying (Fu and Chen, 2011).

Table 5.3.1 - Student's t-test results for comparing FB dried powders containing *L. casei* 431 made with WMP of different initial a_w

WMP initial water activity	log cfu g^{-1} powder				
	Before drying	Day 0	Day 30	Day 60	Day 120
0.3	10.3	9.60	8.87	8.17	7.43
0.7	10.2	9.43	9.07	7.97	7.57
P value	0.21	0.27	0.16	0.18	0.21

n=4

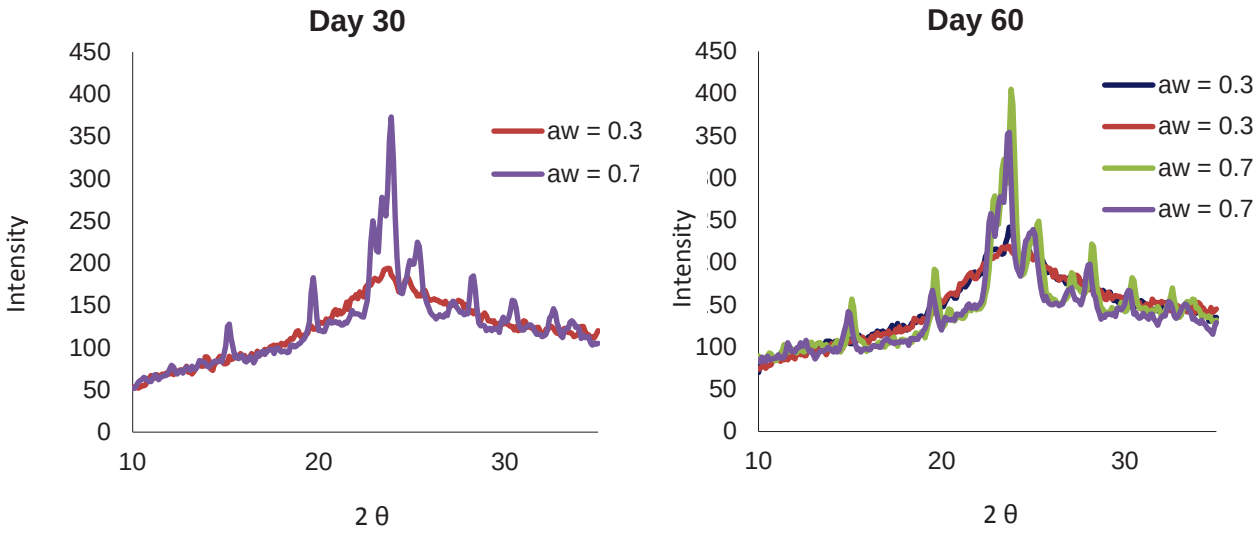


Fig 5.3.1 – XRD analysis of FB dried powders containing *L. casei* 431 at day 30 and day 60

5.3.2 Effect of growth media pH on *L. casei* 431 stability during drying and storage

Since powders made with WMP of different a_w under different mixing conditions had similar mixing, drying and storage losses, it was decided to study effect of pH of fermentation media on these losses. Statistical analysis results (Table 5.3.2) showed that the powders made with uncontrolled pH MRS media had significantly higher (*P* value =0.008) mean viable count after FB drying (9.73 log units) as compared to powders made with pH controlled MRS (9.13 log units). Viability declined with increase in storage time but powders made with uncontrolled pH media had a significantly lower (*P* value < 0.05) storage loss. After 30 and 60 days of storage, viability decreased to 9.3 and 8.3 log units in powders made with uncontrolled pH media and 8.2 and 7.5 log units in powders made with pH controlled media (Table 5.3.2). Powders made with uncontrolled pH media were stable after 60 days of storage until the end of assay. The total mean count in controlled and uncontrolled pH media after 6months of storage was <3 and 8.0 log units respectively. Thus, the stored *L. casei* 431 cells grown under uncontrolled pH conditions showed 5 log units increase in survival when compared to cells grown under controlled pH conditions. Therefore this study demonstrated that growth of *L. casei* 431 under controlled pH resulted in cells more sensitive to drying and storage in the dried state, stresses frequently encountered by the cells during culture preparation, storage and utilization. This increased resistance of uncontrolled pH cells is probably a result of the cross protection

conferred by the low pH attained during growth under uncontrolled pH conditions (see section 2.6.1.4).

In the literature, contradictory results have been reported about the effect of pH. While some suggested that an uncontrolled pH during the culture process led to higher survival ratio than a controlled pH (Silva et al., 2005), others reported the opposite (Linders et al., 1997b). Linders et al. (1997b) results showed that pH control during growth of *L. plantarum* resulted in a higher residual activity after FB drying (37%) compared to growth without pH control (19 %) but the effect on viable cell count was not reported. Silva et al. (2005) reported increased resistance of *L. bulgaricu*s during spray drying and subsequent storage at 20 °C when grown under uncontrolled pH as compared to under controlled pH of 6.5. Survival of *L. acidophilus* CRL 639 under different stress conditions (ethanol, H_2O_2, freezing, lyophilisation) was also higher for cells grown with uncontrolled pH than for those grown at pH 6.5 (Lorca and de Valdez, 2001). The higher resistance when cells were grown under uncontrolled pH was related to the enhanced expression of stress proteins mainly heat shock proteins (HSP) such as GroES and DnaK (an HSP70) (Silva et al., 2005). The discrepancies in effect of pH found in literature may be attributed to the different species and different drying conditions and processes used.

Table 5.3.2 - Student's t-test results for comparing FB dried powders containing *L. casei* 431 made from pH controlled and uncontrolled growth media

	log cfu g^{-1} powder				
Growth media	Day0	Day30	Day 60	Day 120	Day 180
pH controlled	9.1	8.2	7.5	5.73	<3
pH uncontrolled	9.7	9.3	8.3	8.1	8.0
P value	0.008	0.003	P<0.001	0.02	<0.001

n=4

5.4 Effect of maintaining growth media pH at 5.3 instead of 6.6 in the fermentor

Maintaining high pH (6.5) in the fermentor resulted in high drying and storage loss, so powders were made from media in which low pH (5.3) was maintained. During first hours of incubation, the pH of MRS was not controlled (initial pH was 5.86); as soon as the pH reached around 5.3, it was maintained thereafter by the automatic addition of 4N NH_4OH. Results showed similar

higher drying (1.1 log units) and storage loss (4.1 log units) when pH was maintained at 5.4 instead of 6.6 in the fermentor.

Table 5.4.1 - Effect of controlling growth media pH at 5.3 instead of 6.6 in the fermentor on *L. casei* 431 cell count after drying and storage

Powder no.	Initial a(w) of WMP added	Final a(w) after drying	% Moisture content	log cfu g^{-1} powder		
				Before drying	Day 0	Day 30
9b	0.7	0.25	5.6	10.4	9.3	6.3

5.5 Conclusions

The following general conclusions may be drawn from the results discussed in this chapter:

(i) *L. casei* 431 cells when grown in pH controlled growth media had significantly higher (*P* value < 0.05) drying and storage losses when made into powders with WMP, compared to cells made from pH uncontrolled media. The total mean viable count of *L. casei* 431 in pH controlled and uncontrolled media after drying was 9.1 and 9.7 log cfu g^{-1} powder and after 180 days of storage was <3 and 8.0 log cfu g^{-1} powder.

(ii) The loss in cell counts of *L. casei* 431 grown in uncontrolled pH media was higher during first 2 months of storage and after that the counts were stabilized.

(iii) Initial water activity of protective carrier, whole milk powder, had no significant effect (*P* value >0.1) on mixing, storage and drying loss of *L. casei* 431.

(iv) The powders made with hand mixing as well as planetary mixer were similar in terms of viability of *L. casei* 431 after mixing, drying and storage, however powders with high shear Kenwood mixer had much higher mixing and drying loss.

CHAPTER 6

ENHANCEMENT OF GROWTH OF *L. CASEI* 431 DURING BATCH CULTIVATION IN UNCONTROLLED PH GROWTH MEDIA THROUGH RESPONSE SURFACE METHODOLOGY (RSM)

6.1 Introduction

Powders made from uncontrolled pH media (end fermentation pH was 4±0.1) showed higher viabilities during Fb drying and storage as compared to controlled pH media (pH 6.5). Thus, increasing the viable cell count in growth media under uncontrolled pH conditions would increase the chance of having a sufficient number of viable cells after long term storage. Therefore the aim of this chapter was to optimize the production of *L. casei* 431 using response surface methodology (RSM) in uncontrolled pH supplemented MRS media. In the initial screening experiments, culture conditions and nutrients that could most influence cell growth were first investigated using PB methodology. Experimental factorial design was then used in order to optimize the process through the combination of the effects of glucose and yeast extract. When the factorial design did not show any significant effect (*P* value >0.1) of factors studied, another PB design was used to study the effect of yeast extract, meat extract, peptone, glucose and harvesting time on growth of *L. casei* 431 under uncontrolled pH conditions.

6.2. Growth curve of *L. casei* 431 in MRS media under uncontrolled pH conditions

Fig 6.2.1 shows typical growth curve of *L. casei* 431 during uncontrolled pH fermentation in MRS media. The maximum viable cell count of 9.27 log cfu ml^{-1} and OD of 6.6 were obtained after 16 h of fermentation and these values remain unchanged till the end of fermentation time studied (36 h). The growth phases at 16 h, 24 h and 36 h were thereby chosen as early, mid and late stationary phase for *L. casei* 431 for further experiments performed in Chapter 7, where effects of growth phase on drying and storage losses of *L. casei* 431 were compared.

Table 6.2.1 shows the growth characteristics of *L. casei* 431 grown in MRS media under uncontrolled pH conditions. The results presented in Table 6.2.1 are average of individual experiments performed in triplicate. The viable cell count, OD and biomass yield reached maximum at 16 h of fermentation (9.27 log cfu ml^{-1}, 6.6 and 1.7 respectively) and did not change with increase in fermentation time, however lactic acid yield was higher at 24 h when compared to 16 h of fermentation, which was expected. The initial culture pH (6.0) dropped to

a final pH of 4.18 after 16 h and 3.98 after 24 h. When compared to controlled pH batch fermentation (Chapter 4, section 4.2), cultivation of *L. casei* 431 in uncontrolled pH media yielded significantly lower viable cell counts and biomass. The rapid and uncontrolled decrease in pH during uncontrolled pH fermentation with consequent accumulation of inhibitors like lactic acid may partly explain the results obtained in this study.

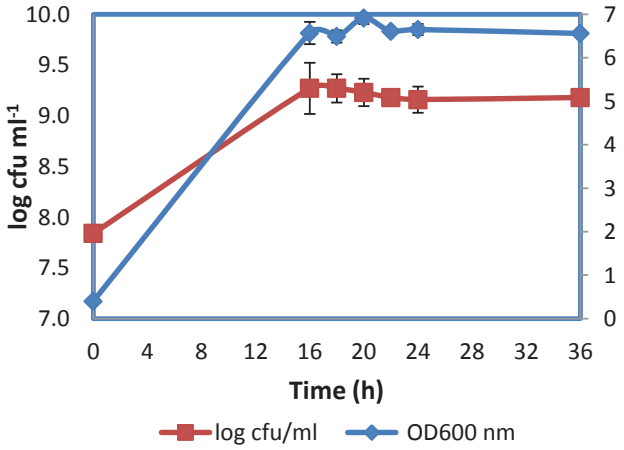


Fig 6.2.1 - Growth curve of *L. casei* 431 in pH uncontrolled MRS media

Table 6.2.1 - Viable cell count, OD and biomass yield of *L. casei* 431 in uncontrolled pH MRS media

Time (h)	pH	Glucose %	Lactic Acid %	OD	Biomass g kg^{-1} media	log cfu ml^{-1} media
0	6	1.9	0.02	0.04		
16	4.18	0.62	1.23	6.57 (0.25)	1.70 (0.02)	9.27 (0.04)
24	3.98	0.32	1.48	6.65 (0.13)	1.8 (0.11)	9.16 (0.02)

Values in brackets represent SD

Experiments were also performed to study the growth of *L. casei* 431 during uncontrolled pH fermentation in presence of manganese ($MnSO_4.H_2O$) present at a concentration of 0.03 g l^{-1} in MRS media. Manganese is considered an essential growth factor for *L. casei* 431, because of its

role as a constituent of lactate dehydrogenase (Fitzpatrick et al., 2000). In addition manganese could act as a possible antioxidant during *L. casei* 431 drying and storage. Results showed that viable cell count and other growth properties remained unaffected by the presence of manganese in growth media (data not shown). Some authors have recommended ammonium salts into the medium as they can increase the biomass yield (Bevilacqua et al., 2008; Sreekumar and Krishnan, 2010). Moreover the use of citrate could result in an increase of specific growth rate. But the effect of supplementing vitamins and ammonium salts was found insignificant on the cell growth of *L. casei* 431 in our study (data not shown).

6.3 Initial screening through Plackett -Burman (PB) Design

6.3.1 Experimental Design

A Plackett-Burman (PB) design was used to evaluate the effects of glucose, yeast extract, meat extract, peptone, vitamins, ammonium citrate and sodium chloride (NaCl) on *L. casei* 431 viable cell count. Seven variables and two dummy variables were screened in 13 trials as shown in Appendix 1. The factors were varied over two levels, namely, the high concentration (+1) and the low concentration (-1), with one run at central level (0): glucose (10, 20, 30 g l^{-1}) (w/v), yeast extract (10, 25, 40 g l^{-1}) (w/v), meat extract (8.3, 20.65, 33 g l^{-1}) (w/v), peptone (18.5, 46.25, 74 g l^{-1}) (w/v), vitamin solution (0.5, 1, 1.5 %) (v/v), ammonium citrate (0.5, 1.25, 2.0 g l^{-1}) (w/v), sodium chloride (NaCl) (0, 15, 30 g l^{-1}) and initial pH (6.0, 6.5, 7.0). Vitamin solution contained (g l^{-1}): pyridoxine HCl, 2.0; calcium-pantothenate, 1.0; niacin, 1.0; riboflavin, 1.0 and folic acid, 1.0 (Liew et al., 2005). Growth (log cfu ml^{-1}) of *L. casei* 431 in media was used as a response of the PB experiment. In addition, other properties of the *L. casei* 431 during growth such as OD, biomass, pH, production of lactic acid, utilization of glucose in media were also evaluated after 20 h of fermentation.

6.3.2 Screening of nitrogen sources

The total nitrogen content in MRS medium is 1.94 g L^{-1} (Fung et al., 2008). Each of the nitrogen sources selected to supplement MRS varied in nitrogen (N) content: peptone (5.4 % N), meat extract (12 % N) and yeast extract (10 % N). All the nitrogen sources were standardized to provide similar total nitrogen contents prior to use. Therefore growth would be influenced only by the type of nitrogen source, each containing equal amounts of nitrogen. The highest level was set at 0.4% (w/v) nitrogen, whereas the low level was set at 0.1% (w/v) nitrogen for

each factor. When all the levels were at their high concentration (+1), the total nitrogen content added would be approximately 1.2 %.

6.3.3 Results and discussion

Table 6.3.1 presents the results of PB experimental design. The maximum viable cell count was 9.5 log cfu ml^{-1} media which was achieved even when the added nutrients were present at lowest level (Run 10) and with initial pH as 6.0 but biomass yield was much lower at these levels (2.2 g kg^{-1}media). The total supplemented nitrogen and glucose values under these conditions were 0.3 % and 1 % respectively. Table 6.3.2 presents the statistical analysis of the studied variables on *L. casei* 431 growth. The complete statistical analysis results are shown in Appendix 7. Variables with confidence levels > 90% were considered to have a significant effect on cell growth.

Table 6.3.1 - PB design matrix for increasing *L. casei* 431 cells count in pH uncontrolled growth media

Std Order[1]	Run Order[2]	MRS broth ($g\ l^{-1}$)	Glucose ($g\ l^{-1}$)	Yeast extract ($g\ l^{-1}$)	Meat extract ($g\ l^{-1}$)	Peptone ($g\ l^{-1}$)	Vitamin solution ($ml\ l^{-1}$)	Ammonium citrate ($g\ l^{-1}$)	Salt ($g\ l^{-1}$)	Initial pH	Final pH	Final OD	Initial Glucose (%)	Final Glucose (%)	Final Lactic Acid (%)	Biomass ($g\ kg^{1}$ media)	log cfu g^{1} media
5	1	55	30	40	8.3	74	1.5	0.5	30	6	4.9	2.1	3.37	2.33	1.1	0.9	8.28
9	2	55	10	10	8.3	74	1.5	2	0	7	4.5	9.0	2.18	0.07	2.4	4	9.48
6	3	55	30	40	33	18.5	1.5	2	0	7	4.3	9.1	3.70	0.97	2.9	4.8	9.56
3	4	55	10	40	33	18.5	1.5	0.5	0	6	4.19	10.8	2.28	0.15	2.5	3.9	9.51
4	5	55	30	10	33	74	0.5	2	0	6	4.27	9.9	3.30	0.98	2.5	3.9	9.54
8	6	55	10	10	33	74	1.5	0.5	30	7	5.2	2.3	2.05	1.18	1.2	1.6	8.09
10	7	55	30	10	8.3	18.5	1.5	2	30	6	5.19	5.0	4.39	2.67	1.7	1.8	8.49
13	8	55	20	25	20.65	46.25	1	1.25	15	6.5	4.22	9.3	3.00	0.66	2.5	3.9	9.0
2	9	55	30	40	8.3	74	0.5	0.5	0	7	4.38	11	3.22	0.30	3.1	4.2	9.64
12	10	55	10	10	8.3	18.5	0.5	0.5	0	6	4	9.0	2.60	0.51	2.2	2.2	9.57
1	11	55	30	10	33	18.5	0.5	0.5	30	7	4.52	4.3	4.09	2.42	1.6	1.3	8.32
11	12	55	10	40	8.3	18.5	0.5	2	30	7	4.58	3.5	2.45	1.06	1.6	1.2	8.27
7	13	55	10	40	33	74	0.5	2	30	6	5.1	1.6	1.73	1.08	0.9	0.7	7.91

[1]Standard order (STDOrder) is the order designed by Minitab

[2]Run Order is the order in which the experiment is conducted

Table 6.3.2 - Estimated effects and coefficients for *L. casei* 431 viable cell count (log cfu ml^{-1} media)

Variable	Effect	*P* value
Glucose	0.1667	0.030
Yeast Extract	-0.0533	0.301
Meat Extract	-0.1333	0.053
Peptone	-0.1300	0.056
Vitamin soln	0.0267	0.577
Ammonium citrate	-0.0267	0.577
Salt	-1.3233	0.000
Initial pH	0.0100	0.830

6.3.3.1 Effect of sodium chloride (NaCl) - osmotic stress

NaCl was the most important variable effecting cell growth and all other growth properties of *L. casei* 431 with its effect highly negative (Table 6.3.2). Viable counts were highest (≥ 9.5 log cfu ml^{-1} media) when NaCl was present at zero level. The maximum cell count achieved when NaCl was present at centre (1.5 %) and highest level (3 %) was 9.0 and 8.4 log cfu ml^{-1} respectively with corresponding biomass as 3.9 and 1.8 g kg^{-1} media. Thus increasing NaCl concentration had highly significant negative effect (*P* value <0.001) not only on cell count but also on biomass. The biomass yield varied markedly in a range of 2.2 – 4.8 g kg^{-1} media in the absence of NaCl and between 0.9 - 3.3 g kg^{-1}in the presence of NaCl. These findings are in agreement with previously reported results which suggested that increase of NaCl had a negative effect on biomass of *L. bulgaricus* PEN (Polak-Berecka et al., 2010). Rao et al. (2004) reported that stressful conditions like a high salt (NaCl) concentration could decrease the biomass yield of *L. plantarum*, without affecting the yield of lactic acid relative to the substrate, because lactic acid bacteria would use some ATP to synthesize the stress proteins like heat shock proteins (DnaK, GroES, GroEL). Therefore they suggested that an uncoupling between growth and energy production could be observed when the cells are stressed and that a population could be metabolically active without showing a significant increase in cell number or in biomass. Our results partially agreed with finding of Rao et al. (2004) as our findings showed that NaCl was slightly significant for lactic acid yield (*P* value=0.064) but effect

of NaCl on growth properties (biomass, OD and cell count) was highly significant (*P* value ≤0.01). Particular care must be taken in selecting strains to be used as starter cultures in high salt containing food systems such as sea food fermentation, because of intra-species variability in response to stress induced by NaCl (Rao et al., 2004).

6.3.3.2 Effect of initial pH

In order to study the effect of initial pH on cell growth, *L. casei* 431 was cultivated at three initial pH values; 6.0, 6.5 and 7.0. The effect of initial pH was found insignificant (*P* value = 0.8) on *L. casei* 431 growth (log cfu ml^{-1}) as well as other properties such as OD, biomass and lactic acid. However, Bernardez et al. (2008) found that the viable cell count, biomass and lactic acid yield of *L. casei* CECT 4043 were maximum at initial pH of 7.0 and 6.5 and their values decreased significantly above and below these pH values, but the reported biomass production was much lower (1.7 g L^{-1} media after 312 h) . Even though the viable counts reported for *L. casei* CECT 4043 by Bernardez et al. (2008) were higher (1.7 X 10^{10} cfu ml^{-1}) than our counts, the fermentation time reported was much longer (312 h). Liew et al. (2005) studied effect of initial pH in the range 5.0 to 7.0 and found that optimized media at initial pH of 6.9 gave maximum growth of *L. rhamnosus*. Bevilacqua et al. (2008) reported significant effect of initial pH on the cell number and the biomass yield of *L. plantarum.* According to Poolman and Konings (1988), the amino acid or peptide transport, which is one of the growth-rate-determining steps, depends on the culture pH. For *L. lactis* and *Lactococcus cremoris* strains, the optimum pH value for amino acid transport varied between 6.0 and 6.5, decreasing rapidly at higher and lower pH values (Poolman and Konings, 1988). The fact that initial pH in our studies was not significant may be due to the only a narrow range of initial pH being studied (pH 6.0-7.0) and *L. casei* is known to grow well in this pH range.

6.3.3.3 Effect of glucose

Regarding the effect of carbohydrates, a slight positive effect of glucose on cell number of *L. rhamnosus* was reported by Liew et al. (2005) but glucose supplementation did not increase the total biomass yield of *Leuconostoc mesenteriodes* and *L. Curvatus* (Mataragas et al., 2004). Polak-Berecka et al. (2010) suggested that concentration of glucose + sodium pyruvate was more significant in increasing biomass yield of *L. rhamnosus* than glucose alone. Our study also showed that glucose had slight positive significant effect (*P* value = 0.03) on *L. casei* 431 viable cell count, but OD, biomass and lactic acid yield were unaffected (Table 6.3.2). However, the

residual glucose was higher when initial glucose concentrations were high, both in presence and absence of NaCl.

6.3.3.4 Effect of various nitrogen sources, vitamins and ammonium salts

The total % nitrogen of the growth medium at lowest levels of PB design experiments (Run 10) was 0.5 % and at highest levels (Run 13) was 1.4 %. Statistical analysis results showed that increasing meat extract and peptone concentration had a slight negative effect on cell growth of *L. casei* 431 whereas effect of yeast extract was not significant (*P* value =0.3) (Table 6.3.2). The results obtained in present study were however, in contradiction to the results reported in literature who reported positive effect of yeast extract on the cell number (Liew et al., 2005), the OD (Fung et al., 2008) and the biomass production (Bevilacqua et al., 2008; Liew et al., 2005; Mataragas et al., 2004; Polak-Berecka et al., 2010). Liew et al. (2005) found effect of yeast extract (YE) was strongest on the growth of *L. rhamnosus* and under optimum conditions for growth (pH = 6.9, vitamin solution=1.28%, glucose = 5.01% and YE = 6.0%) the viable count (9.35 log cfu ml^{-1}) was much higher than MRS media (8.81 log cfu ml^{-1}). Fung et al. (2008) showed that supplementing soy protein (containing 0.08% Nitrogen) with 1.7 % Nitrogen by a combination of 7.25 % meat extract, 4.7 % vegetable extract and 6.85% peptone, maximized growth (OD) of *L. acidophilus* in the soy whey medium. Polak-Berecka et al. (2010) observed that significant effect of nitrogen sources (yeast extract, meat extract, and peptone) and organic salts (ammonium citrate) on *L. rhamnosus* PEN biomass yield were very similar but the effect of vitamin supplementation was not important. Thus our obtained results were not in agreement with literature. PB design experiment however, only identifies the most important variables with their main effects, and in this study the effects of nitrogen sources were small. A better understanding of individual effects and interactions was sought through the factorial design and another PB experiment performed in the later section.

6.4 Central composite design (CCD) to optimize the growth medium composition

Based on the results of PB design, a full-factorial CCD was applied to test the influence of yeast extract and glucose on cell growth. Because increasing the meat extract and peptone concentration had slight negative effect on *L. casei* 431 cell growth in the previous PB design, only yeast extract was selected for further analysis at centre level. Yeast extract was supplemented at 15-30 g l^{-1} (0.15-0.3 % Nitrogen) in MRS media. The lower level of pH (6.0) was selected as the set point for CCD experiments. In-spite of the slight positive effect of glucose in the PB experiment, its levels were kept low (between 0 - 25 g l^{-1}) in the CCD

experiments due to high residual glucose in PB experiment trials where initial glucose concentration were high. All the analysis of growth media was performed after 20 h of fermentation.

6.4.1 Results and discussion

The experimental design and obtained results are shown in Appendix 2 with complete statistical analysis results in Appendix 8. Results showed that the viable cell count was similar in all the trials and there was a significant lack of fit of data. The maximum biomass obtained was 2.9 g kg^{-1} media higher which was higher when compared to PB trial at centre level (Run no. 10) where total % nitrogen was same indicating yeast extract had significant effect (*P* value <0.001) on biomass yield. The regression coefficient table for biomass is shown in Table 6.4.1 with R^2= 0.91 showing yeast extract was the only significant factor in biomass production. And its effect was highly positive in this case as opposed to being negative in PB design. These results thus suggested a positive effect of yeast extract on biomass, as reported by Bevilacqua et al. (2008) and Mataragas et al. (2004) which could be due to the fact that yeast extract contains growth factors and a relatively larger proportion of free amino acids and short peptides of two or three amino acids long than protein hydrolysates (Bevilacqua et al., 2008). However, contradictory effects of yeast extract on viable cell count have also been reported in literature. Bevilacqua et al. (2008) observed that viable cell count *L. plantarum* was not affected by the amounts of yeast extract, di-ammonium hydrogen citrate and carbohydrates, whereas initial pH was found to be important, the minimum cell count was recovered at pH 4 and pH > 8.0. Liew et al. (2005) reported that effect of yeast extract was strongest on viable cell count of *L. rhamnosus.*

The results of viable cell count compared to the amount of biomass produced for *L. casei* 431 in the same conditions of CCD experiment could suggest a partial uncoupling between the increase in cell number and the biomass production, as they were affected by the independent variables in a different way. The amount of biomass is related to the cell number and to other factors, e.g. the cell dimension. Environmental variables like the different nutrients content of the medium could act in a different way on the cell number and their dimension (and consequently on the amount of biomass) and the data of this experiment suggested that the effect of the nutrients could be more significant on the dimension rather than on the population number (Bevilacqua et al., 2008).

Table 6.4.1 – Regression coefficients table for *L. casei* 431 biomass yield (g kg^{1} media)

Variable	Coefficient estimate	Standard error	*T* value	*P* value
Constant	2.46000	0.05265	46.723	0.000
Glucose	0.05000	0.04162	1.201	0.269
Yeast extract	0.35178	0.04162	8.451	0.000
Glucose*Glucose	-0.04250	0.04464	-0.952	0.373
Yeast extract*Yeast extract	0.00750	0.04464	0.168	0.871
Glucose*Yeast extract	-0.05000	0.05887	-0.849	0.424

In the light of these conflicting results of CCD experiment which showed the viable cell count was same under all the conditions another PB design was used to evaluate the effects of concentration of yeast extract, meat extract, peptone, glucose and harvesting time on cell growth of *L. casei* 431.

6.5 PB design to identify the important nitrogen sources effecting *L. casei* 431 viable cell count

A PB experimental design (Appendix 3) was designed with 13 trials to study the effect of 5 variables (glucose, yeast extract, meat extract, peptone and harvesting time). Harvesting time selected was early (16h) and mid stationary phase (24h) based on growth curve obtained in section 6.2.

6.5.1 Results and Discussion

Results showed that the viable cell count varied between 9.2 and 9.5 log units for different trials (Table 6.5.1). Cell growth was similar in most of the trials (9.2 - 9.3 log units) except the trials in which both meat extract and yeast extract were present at high levels, which showed maximum viable cell count of 9.5 log units indicating concentrations of both yeast extract and meat extract were important for achieving high viable cell count of *L. casei* 431. The estimated factors effect table for viable cell count (Table 6.5.2) confirmed that the *L. casei* 431 growth was mainly influenced by positive increase in the concentration of yeast extract and meat extract; however, increasing glucose concentration had a significant negative effect on cell growth. Also all the variables studied except glucose showed positive influence on biomass

yield (Table 6.5.3) which was in agreement with Mataragas et al. (2004) who found increasing glucose did not affect biomass of *L. curvatus* and *Leuconostoc mesenteroides* up to an intermediate level after which decrease in biomass was observed. Bevilacqua et al. (2008) also reported positive effect of glucose on biomass up to a threshold value of 20-25 g l^{-1} for some strains of *L. plantarum* after which a decrease was observed.

Results obtained in this experiment showed that supplementing growth media with meat extract and yeast extract could increase *L. casei* 431 viable cell number by more than 2 fold (9.5 log units) when compared to MRS (9.2 log units) and biomass by more than 1.5 fold (3.1 - 3.4 g kg^{1} media) as compared to MRS media (1.9 g kg^{1} media). Increasing glucose concentration had negative effect on viable cell number and biomass yield of *L. casei* 431. But for OD and residual lactic acid, only glucose and yeast extract were significant (data not shown here). Harvesting time also had a slight positive effect on cell count. Complete statistical analysis results can be found in Appendix 9.

The theoretical conversion yield of glucose to lactic acid showed that in addition to glucose, *L. casei* can also use other growth factors like yeast extract, peptone, meat extract for its growth and acid production, as observed in Run No. 6, 12, 13 of this experiment, in which concentrations of final lactic acid and final glucose when added, their sum was more than initial glucose concentration which confirmed the observations made by Brinques et al. (2010).

Table 6.5.1 - PB design to study the effects of concentration of yeast extract, meat extract, peptone, glucose and harvesting time on the viable cell count

StdOrder[1]	Run Order[2]	Glucose (gl^{-1})	Yeast (gl^{-1})	Meat (gl^{-1})	Peptone (gl^{-1})	MRS (gl^{-1})	Harvesting Time (h)	Final pH	% Initial glucose	% Final glucose	% Final Lactic Acid	OD	Biomass (g kg^{1} media)	log cfu g^{1} media
1	1	20	0	16.7	0	55	16	4.25	3.81	1.97	1.54	7.03	2.3	9.25
11	2	0	20	0	0	55	16	4.26	2.06	0.24	1.71	7.93	2.8	9.32
6	3	20	20	16.7	0	55	24	4.1	3.67	1.33	2.24	9.18	3.2	9.41
8	4	0	0	16.7	40	55	24	4.28	1.76	0.00	1.81	7.24	3	9.42
2	5	20	20	0	40	55	16	4.33	3.68	1.65	1.90	7.97	3.1	9.26
7	6	0	20	16.7	40	55	16	4.4	1.66	0.00	1.82	7.75	3.4	9.5
13	7	10	10	8.35	20	55	20	4.13	2.88	0.56	2.23	8.04	3.3	9.37
12	8	0	0	0	0	55	16	4.19	2.05	0.60	1.34	6.15	1.9	9.19
4	9	20	0	16.7	40	55	16	4.31	3.67	1.66	1.83	7.22	3.2	9.2
5	10	20	20	0	40	55	24	4.15	3.5	1.11	2.26	9.18	3.5	9.29
10	11	20	0	0	0	55	24	3.99	3.94	2.17	1.74	7.51	2.3	9.23
3	12	0	20	16.7	0	55	24	4.21	1.82	0.00	1.93	8.26	3.1	9.58
9	13	0	0	0	40	55	24	4.19	1.83	0.54	1.82	7.18	2.7	9.32

[1]Standard order (StdOrder) is the order designed by Minitab

[2]Run Order is the order in which the experiment is conducted

Table 6.5.2 - Estimated Effects and Coefficients for *L. casei* 431 viable cell count (log cfu ml^{-1} media)

Variable	Effect	*P* value
Glucose	-0.11500	0.002
Yeast	0.12500	0.001
Meat	0.12500	0.001
Peptone	0.00167	0.942
Harvesting Time	0.08833	0.007

Table 6.5.3 - Estimated Effects and Coefficients for *L. casei* 431 biomass yield (g kg^{-1} media)

Variable	Effect	*P* value
Glucose	0.11667	0.251
Yeast	0.61667	0.001
Meat	0.31667	0.014
Peptone	0.55000	0.001
Harvesting Time	0.18333	0.093

6.6 Effect of % nitrogen present in growth media on viable cell count of *L. casei* 431

The results for the effect of yeast extract on *L. casei* 431 viable cell counts were contradictory. However, if the results of the above three experiments were combined and expressed in terms of effect of supplemented % nitrogen present in growth media on growth of *L. casei* 431 (Fig 6.6), the results appeared to show a distinct increase in *L. casei* 431 cell counts with increase of supplemented % nitrogen in the MRS media, even though R^2 was found to be low. In the literature, the effect of yeast extract on viable cell count and cell growth (OD) was significant only when amount of total % nitrogen was high (Fung et al., 2008; Liew et al., 2005) rather than low (Bevilacqua et al., 2008) under uncontrolled pH conditions. Further experiments need to be done to confirm this hypothesis.

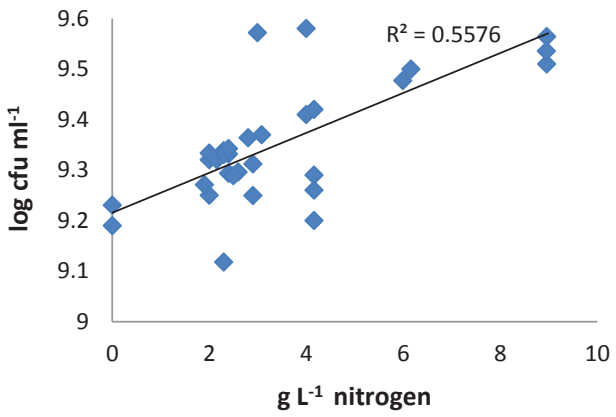


Fig 6.6 - Effect of supplemented nitrogen on viable cell count of *L. casei* 431

6.7 Conclusions

The following general conclusions may be drawn from the results discussed in this chapter:

i. Presence of salt (NaCl) (osmotic stress) during growth had a highly significant negative effect (*P* value <0.001) on viable cell count, OD, biomass yield of *L. casei* 431 whereas its effect on lactic acid yield was much less.

ii. Viable cell count of *L. casei* 431 was not affected by increasing both glucose and yeast extract concentrations upto the studied levels in MRS media during CCD experiment whereas yeast extract was found to have highly significant positive effect (*P* value <0.001) on biomass production.

iii. Supplementation of both meat and yeast extract in MRS media increased the viable cells of *L. casei* 431 by more than 2 fold and biomass by more than 1.5 fold whereas increasing glucose concentrations had a slightly negative effect (*P* value =0.002) on viable cell number.

iv. The biomass and OD often showed a response to glucose and nitrogen sources, even when there was no difference in viable counts, indicating the effect of the nutrients was more significant on the dimension or on growth of cells rather than on the population number.

v. Combining all the results of three experiments showed a distinct trend of increase in *L. casei* 431 cell counts with increase in supplemented % nitrogen.

CHAPTER 7

COMPARING STABILIZATION OF *L.CASEI* 431 HARVESTED FROM PH UNCONTROLLED GROWTH MEDIA DURING LABORATORY AND INDUSTRIAL SCALE PRESERVATION PROCESSES AND STORAGE

7.1 Introduction

In order to achieve long-term stability of probiotic cultures as well as the feasibility of scale-up of the process, the stabilization of *L. casei* 431 after laboratory scale and industrial scale processes and storage was investigated. On a laboratory scale, bacterial cells are usually harvested by a high speed Sorvall centrifuge. Sorvall centrifugation is a batch process but in pilot scale as well as in industries, the bacterial cells are usually concentrated using some type of continuous separator. Concentrated cells obtained after laboratory centrifugation (Sorvall) in the experiments performed, had around 23 % solids compared to an industrial separator (Fonterra) which yielded an average dry mass of 6.5 % for bacterial suspensions of *L. casei* 431. In order to simulate pilot/industrial conditions in the laboratory, supernatant obtained after Sorvall centrifugation was added back to the dense pellet, to adjust the solids to 6.5 %. This simulated concentrate with 6.5 % solids would thus still contain some metabolites. Also on a laboratory scale, collected cells are usually washed with peptone water; however, on industrial scale a washing step is not feasible. To determine the magnitude of this effect, the viability of powders made with washed and unwashed harvested cells was compared. Also the effect of harvesting time on the cell viability during drying and storage has been investigated.

7.2 Viability of *L. casei* during laboratory scale and pilot scale preservation processes and storage

A Plackett-Burman design (Appendix 4) in two blocks (washed and unwashed) was used to study the effect of (i) harvesting time (ii) concentrate solids (23 %, 6.5 % solids) on viability of *L. casei* 431 during drying and storage. Effect of block and other factors on residual moisture content of dried powder was also investigated. Two factors (harvesting time, concentrate solids) were varied over two levels. Harvesting time chosen was early (16 h) and late stationary phase (36 h) based on previous growth curve for *L. casei* 431 (Chapter 6, section 6.2).

L. casei 431 was grown in Durham bottles containing 1L MRS medium supplemented with 30 g l^{-1} yeast extract and 0.03 g l^{-1} $MnSO_4.H_20$. The bottles were incubated at 37 °C in an orbital shaker at 160 rpm. *L. casei* 431 cells were harvested/concentrated by centrifugation (8min at

10000 rpm, Sorvall) after required harvesting time. For Block 1 trials, concentrated cells were washed with peptone water and were centrifuged again before adding to WMP of a_w 0.3. For Block 2 trials, concentrated cells were directly added to WMP (a_w 0.3) without washing with the peptone water. Around 7.5 g of pellet was added to 100g WMP in all the trials. In case of trials with 6.5 % solids, around 19 g supernatant along with 7.5 g precipitate was added to 100 g powder. The powders were dried at 50˚C in FB drier for 30–120 min until a_w of dried powder was between 0.25 -0.3 and residual moisture content between 4.5–5.5 %. The dried powders were packed and heat sealed in aluminium pouches and stored at 25 ˚C and at accelerated storage temperature of 37 ˚C. Accelerated storage testing is a widely used method for the prediction of storage stability and quality, and for the estimation of shelf-life (Achour et al., 2001; King et al., 1998). The Arrhenius equation is the most common and generally valid assumption for the temperature-dependence of the deterioration rate. The accelerated shelf life testing (ASLT) method was used to predict shelf-life of FB dried *L. casei* 431 at 25 ˚C. The viability of *L. casei* 431 was monitored by enumerating the viable counts in dried powder for 4 weeks at 37 ˚C and 3 months at 25 ˚C. Viability of powders was checked in duplicate from 4 g powder taken after 15 and 30 days of storage at 37 ˚C and after 1 and 3months of storage at 25 ˚C. Rehydration conditions were held constant for all the samples.

7.3 Results and Discussion

All the powder viability data is presented in Table 7.3.1 where each result represents the average of three individual experiments. Results showed that mean drying losses of *L. casei* 431 in both blocks (washed and unwashed) were the same, but harvesting time and total solids of cells concentrate had a significant impact on cell viability after drying (Table 7.3.2). During storage although significant reductions in cells viability occurred in powders stored at 37 ˚C for 28 days, total decline in viability was highest in the powders made with low solids concentrate (4.0 - 4.2 log unit reduction) which was almost double that for the powders made with high solids concentrate obtained at early stationary phase, in which storage losses were minimal (2.1 - 2.2 log unit reduction). Powders at centre level conditions (15 % solids, 26 h harvesting time) had intermediate viability losses (2.9 log unit reduction). Moreover these storage losses were irrespective of whether powders were made with washed or unwashed cells. At the end of the 37 ˚C storage period, the highest count observed in powders made with concentrate containing 23 % and 6.5 % solids was obtained at early stationary phase (16h) i.e. 8.6 and 6.5 log cfu g^{-1} powder respectively which was around 1 – 1.3 log units higher than respective counts in powders made with cells at late stationary phase (36 h). Powders made with factors at centre level (15 % solids and 26 h harvesting time) had better storage stability (7.88 log cfu

g^{-1} powder) than both powders made with 6.5 % solids/ 16h harvesting time and 23 % solids/ 36 h harvesting time.

Table 7.3.1 – Results from PB design used for comparing powders made under laboratory and industrial processing

	Block	Total % solids	Harvesting Time (h)	Final a(w)	Final % Moisture	log cfu g^{-1} powder						% Moisture after 3 months
						Before drying	Day 0	Day 14 @ 37C	Day 28 @ 37C	1month @ 25C	3months @ 25C	
1	1	23	16	0.21	4.65	10.31	9.68	8.99	8.21	9.64	8.99	5.00
					(0.16)	(0.03)	(0.05)	(0.28)	(0.19)	(0.09)	(0.12)	(0.22)
2	1	6.5	16	0.29	5.64	9.96	9.15	7.32	5.97	8.57	7.5	5.86
					(0.25)	(0.25)	(0.40)	(0.89)	(0.59)	(0.31)	(0.05)	(0.81)
3	1	15	26	0.26	5.25	10.59	9.35	8.42	6.91	7.88	8.18	5.36
					(0.29)	(0.03)	(0.05)	(0.22)	(0.15)	(0.01)	(0.1)	(0.1)
4	1	23	36	0.26	5.16	9.59	8.96	7.36	6.66	7.94	7.34	5.52
					(0.36)	(0.01)	(0.15)	(0.12)	(0.05)	(0.18)	(0.15)	(0.43)
5	1	6.5	36	0.28	5.66	9.53	8.81	6.84	5.47	6.91	5.59	7.09
					(0.15)	(0.15)	(0.26)	(0.29)	(0.10)	(0.09)	(0.03)	(0.14)
6	2	23	16	0.2	4.49	10.77	10.15	9.52	8.60	9.69	9.31	4.72
					(0.16)	(0.09)	(0.04)	(0.01)	(0.29)	(0.07)	(0.03)	(0.25)
7	2	6.5	16	0.27	5.32	10.68	8.74	7.31	6.53	8.7	8.37	4.89
					(0.38)	(0.11)	(0.23)	(0.31)	(0.04)	(0.05)	(0.09)	(0.41)
8	2	15	26	0.21	4.62	10.39	9.11	8.53	7.88	9.33	8.46	4.65
					(0.09)	(0.08)	(0.07)	(0.03)	(0.03)	(0.01)	(0.05)	(0.12)
9	2	23	36	0.2	4.32	10.15	9.16	7.58	7.28	9.15	8.29	4.52
					(0.12)	(0.09)	(0.02)	(0.10)	(0.03)	(0.13)	(0.17)	(0.11)
10	2	6.5	36	0.28	5.64	9.75	7.57	6.16	5.53	7.46	6.01	5.77
					(0.05)	(0.19)	(0.08)	(0.07)	(0.03)	(0.01)	(0.09)	(0.41)

Values in brackets represent SD

Table 7.3.2 - Main factor effects for PB design used for comparing powders made under laboratory and industrial processing conditions

		log cfu g^{-1} powder/main factor averages					
				37 °C		25 °C	
Factors	% Moisture	Before drying	Day 0	Day 14	Day 28	1month	3months
Block							
Block 1 (Washed)	5.3	10.0	9.2	7.8	6.6	8.5	7.5
Block 2 (Unwashed)	4.9	10.3	8.9	7.8	7.2	8.9	8.1
P value	<0.001	<0.001	<0.001	0.96	< 0.01	<0.001	<0.001
Total solids (%)							
6.5	5.6	10.0	8.6	6.9	5.9	7.9	6.9
23	4.7	10.2	9.5	8.4	7.7	9.1	8.5
P value	<0.001	0.18	<0.001	<0.001	<0.001	0.001	<0.001
Harvesting Time(h)							
16	5.0	10.4	9.4	8.3	7.3	9.2	8.5
36	5.2	9.8	8.6	7.4	6.2	7.9	6.8
P value	0.46	< 0.001	< 0.001	0.003	0.02	p< 0.001	< 0.001

Storage stability of powders stored at 25 ˚C and accelerated storage temperature of 37 ˚C were compared in order to predict shelf-life of FB dried *L. casei* 431 under ambient conditions. As expected the viability of the bacteria declined much more quickly at 37 °C than at 25 °C, but Student's t- test comparison of cell counts in powders stored at 37 ˚C for 14 days with powders stored at 25 ˚C for 3 months showed no significant difference in their mean viable cell counts (data not shown). Thus it appeared that the shelf-life of *L. casei* 431 was 6 times as long at 25 ˚C compared to 37 ˚C which was in agreement with Achour et al. (2001), who reported that the half-life of *Lactococcus lactis* was 4 to 5 times longer at 25 ˚C compared to 37 ˚C. It was further stated that this relationship holds well generally as long as the storage temperature is less than glass transition temperature (Achour et al., 2001). At the end of the 25 ˚C storage period, the highest counts of *L. casei* 431 were observed in powders made with unwashed cells

at early stationary phase (16 h) with their highest counts as 9.3 and 8.3 log units for concentrate containing 23 % and 6.5 % solids respectively.

The main factor effects investigated in PB design on moisture content and viable cell count in FB dried powders are presented in Table 7.3.2 and are discussed in detail below. The complete statistical analysis results are shown in Appendix 10.

7.3.1 Effect of harvesting time/ growth phase

The drying and storage viability of *L. casei* 431 during FB drying in different phases of growth were compared and optimal survival was observed in powders made with cells at early stationary phase (16 h) as compared to cells at late stationary phase (36 h). Mean viable counts of powders even before drying were significantly higher (*P* value <0.001) for cells harvested at 16 h than at 36 h (Table 7.3.2) even though the cell counts of their concentrate were similar (11.7-11.9 log cfu g^{-1}), indicating cells harvested at 36 h had higher mixing losses when homogenized with WMP of a_w 0.3. The cells with prolonged periods in stationary phase had also lower drying and storage stability. But no difference in storage stability was found for powders made with cells at early and mid (24 h) stationary phase (data not shown). Effect of harvesting time on residual moisture content in dried powders was negligible which was expected.

It has been generally accepted that cells harvested at the stationary phase during a batch culture showed the highest stress tolerance, compared to those harvested at the lag and exponential phases due to combined effect of starvation and acid stress (see section 2.6.2.1). In this regard, cells harvested from the early and mid-stationary phase have been widely used in all kinds of drying processes, e.g. spray drying (Corcoran et al., 2004; Teixeira et al., 1995), FB drying (Linders et al., 1997a, b, c; Mille et al., 2004; Prasad et al., 2003), vacuum drying (Tymczyszyn et al., 2007) as well as freeze drying (Costa et al., 2000). Thus the results of our experiments supported findings in the literature as in the present study it was observed that *L. casei* 431 cells harvested at early stationary phase (16 h) were more robust during drying and storage. However, a much longer time in stationary phase was found to be detrimental to cell stability. Drying and storage stability data of LAB harvested at late stationary phase (36h) under uncontrolled pH conditions was not found in the literature.

7.3.2 Effects of cell concentrate solids

Total solids of cell concentrate had a significant impact on *L. casei* 431 viability during drying and storage. Powders made with 23 % solids cell concentrate had significantly lower (*P* value <0.001) drying and storage losses than powders made with 6.5 % solids concentrate (Table

7.3.2). The total mean cell count of powders after drying and storage was 0.9 and 1.6-1.8 log units higher for powders made with high solids concentrate than powders made with low solids concentrate (Table 7.3.2). Another major influence of total solids was on the final moisture content of dried powders. The powder dried with cells concentrate of 23 % solids had significantly lower (*P* value <0.01) residual moisture content after drying and at the end of storage period (data not shown) than powders dried with concentrate of 6.5 % solids. Even though drying time for powders made with 6.5 % solids concentrate was almost 3–4 times longer than powders made with 23 % solids concentrate, the resulting water activity (data not shown) and moisture content in these dried powders was significantly higher (*P* value <0.01). The longer drying time (Santivarangkna et al., 2007) and the higher moisture content (Selmer-Olsen et al., 1999b; Strasser et al., 2009) of these powders could have resulted in higher drying and storage losses. The studies investigating effect of moisture content on storage stability of fluidized bed dried powders are very few. Selmer-Olsen et al. (1999b) reported decline in cell counts of *L. helveticus* entrapped in Ca-alginate in dark at 2-3 ˚C during storage was related to their high water content. The rehydration time for powders made with 6.5 % solids was also thrice that of powders made with higher solids. The longer desiccation time could have induced conformational changes in proteins which affected the powder rehydrability properties.

In general, literature on FB drying of LAB - especially on pilot/industrial scale as done in this study is limited (Santivarangkna et al., 2007). In the published data for LAB related to fluidized bed (Linders et al., 1997b; Mille et al., 2004; Prasad et al., 2002; Selmer-Olsen et al., 1999b; Strasser et al., 2009), cells were harvested from growth media by high speed centrifugation except in study performed by Strasser et al. (2009), who FB dried *L. plantarum* and *Enterococcus faecium* cells concentrated through a separator (to 7.3 % solids) in presence of various sugars by spraying concentrated liquid cell suspensions onto fluidized carrier material. The drying and storage losses (35 ˚C for 6 months) for *L. plantarum* in powders made by Strasser et al. (2009) were very high just like our results. The high decline in viability during drying and storage of powders made with 6.5 % solids could be due to presence of acidic metabolites present which could have caused acid stress on the cells.

7.3.3 Effect of block

Apparently block (washing) sometimes had a significant effect, but it was not consistent. The overall trend was for washing to have a negative effect on counts. Even though the mean viable count before drying was significantly lower (*P* value <0.001) in powders made with

washed cells (which was expected because a lot of cells are lost during washing step), their mean drying loss was lower. This could be because washing of harvested cells reduced metabolites present in them. The effect of washing was not significant for 14 days of storage at 37 ˚C, but with further increase in storage time, significantly higher (*P* value <0.001) viability losses were recorded in powders made with washed cells. In contrast effect of washing was significant during storage at 25 ˚C. However, it was found that the mean residual moisture content in the dried powders made with washed cells was significantly higher (5.3 %) than powders made with unwashed cells (4.9 %). The final moisture content in the dried powder could affect its storage stability. The differences in moisture content were due to the fact that powders made in block 2 (without washing) were dried for much longer time in order to reduce their water activity to around 0.2 and moisture content to less than 5.0 %.

7.3.4 Effect of storage

Cell injury and inactivation occur not only during processing, but also during storage of dried cultures. Our results showed that viability of *L. casei* 431 in powders manufactured at all the conditions decreased gradually during storage but this decrease in viability differed depending on processing conditions and the storage temperature. The factors which affect survivability of bacteria during storage and the mechanism of the loss of cell viability at elevated temperature is discussed in detail in Section 2.8. All the storage studies found in literature showed much greater losses in viability at temperatures above refrigeration conditions (Strasser et al., 2009; Teixeira et al., 1995). Cytoplasmic membrane damage and increase in membrane permeability has been reported for LAB during drying and subsequent storage (Lievense et al., 1992, 1994; Teixeira et al., 1995). Teixeira et al. (1995) observed that the ratio of unsaturated/saturated fatty acids in *L. bulgaricus* decreased during storage at 20 ˚C in air, evidence of lipid oxidation, but this decrease was significant only after 49 days of storage. Lipid oxidation could further result in DNA damage during storage thus affecting the viability.

Although viability was significantly affected by the factors investigated in this chapter, the highest viability of the powders also correlated well with their residual moisture contents after drying. The high moisture content combined with longer drying times could have resulted in higher loses in viability in powders made with low solids concentrate. Moisture content was found to further increase with increase in storage time (Table 7.3.1). This could be due to the fact that all the powders were stored in bulk (100 g) in aluminium pouches at both temperatures. So whenever the pack was opened during storage analysis at various intervals, powder came into contact with atmospheric air of high relative humidity and moisture

absorption took place. Champagne et al. (2011) suggested that situations where a commercial pack is open, a sample is taken and rest is kept for later use, a moisture absorption by the powder increases the a_w. Indeed, it has been shown that an increase in a_w from 0.1 to 0.3 of a milk based product will result in only a 2 % increase in moisture, but the stability during storage will be 10 times lower (Champagne et al., 2011). Also the gain in moisture would be higher during storage at temperature higher than refrigerated temperatures. Powders with a water activity value of approx. 0.2, equivalent to 4% moisture, usually maintain the best viability during storage at ambient conditions (Simpson et al., 2005). Thus reducing moisture content may also be important in achieving storage stability.

Very few studies have been done on storage stability of FB dried lactobacilli (see section 2.5.3). But studies on storage stability of spray-dried lactobacilli at various temperatures have been widely investigated. A poor viability for spray dried *L. paracasei* was reported following storage at various temperatures (4, 15, 30 ˚C) (Desmond et al., 2002). Teixeira et al. (1995) reported 4 log reduction of spray dried *L. delbrueckii* ssp. *bulgaricus* stored at 15 ˚C for 30 days and concluded that changes in the unsaturated/saturated fatty acids and survival of *L. bulgaricus* during storage were related. The stationary phase cultures of *L. rhamnosus* GG, *L. rhamnosus* E800 and *L. salivarius* suffered 1.45 - 2.95 log unit losses in viability during 37 ˚C for 8 weeks (Corcoran et al., 2004). Gardiner et al. (2002) reported 2 log unit decline in viability of *L. paracasei* NFBC 338 after 7 week storage at 30˚C, with no change in viability at 4 ˚C and 15 ˚C for same storage period. The spray drying conditions were identical for all these powders with similar final moisture content (< 4%), but storage and drying losses varied considerably. Thus the processing and storage losses can be strain specific. The cell membrane damage was associated with low viability for these spray dried powders. The reported survival values following storage for spray dried Lactobacilli were much lower than those presented in the current study for FB dried *L. casei* 431.

7.4 Conclusions

The following general conclusions may be drawn from results discussed in this chapter:

- Total solids in harvested cells had a significant effect (*P* value <0.001) on *L. casei* 431 viability during mixing and after drying and storage. Powders made with low solids cell concentrate (6.5 %) had almost double storage losses at 37 ˚C when compared to powders made with high solids cell concentrate (23 %). Even though drying time for powders made with 6.5 % solids was 3 - 4 times higher than powders made with 23% solids, the resulting water activity and moisture content in these powders was significantly higher than the

powders made with 23 % solids. Higher drying time and moisture content of dried powders could have caused higher cell death during drying as well as storage. Re-dispersibility of powders made with 6.5 % solids was also difficult.

- Growth phase or harvesting time of cells also had a significant effect (*P* value <0.001) on cell viability. The prolonged periods in stationary phase resulted in reduced cell counts after drying and storage. Drying and storage counts of powders made with cells at early stationary phase (16 h) were around 1.2 times higher than powders made with cells at late stationary phase (36h).
- Powders made with factors at centre level (15 % solids and 26 h harvesting time) had intermediate storage stability and were better than both powders made with 6.5 % solids/ 16h harvesting time and 23 % solids/ 36 h harvesting time.
- Although storage losses for the powders made with washed cells were significantly higher (*P* value <0.001) than unwashed cells, the highest viability correlated well with the residual moisture content of the dried powders. The mean moisture content of powders made with washed cells was significantly higher (*P* value <0.01) than unwashed cells. Since the residual moisture content of the final product may have a considerable impact on survival rate after drying and during storage, it is conceivable that the unexpected effect of washing is at least partially due to indirect effects of different moisture contents after the drying process. Thus moisture content needs to be standardized in further studies.
- Students T- test comparison of *L. casei* 431 counts in powders stored at 37 ˚C for 15 days with powders stored at 25 ˚C for 3 months showed no significant difference (*P* value >0.01) in their mean viable cell counts (data not shown). Thus it appeared that the shelf life of *L. casei* 431 was 6 times as long at 25 ˚C compared to 37 ˚C.

CHAPTER 8

OPTIMIZING THE DRYING CONDITIONS TO GET PROBIOTIC POWDERS WITH LOWER RESIDUAL MOISTURE CONTENT AND HIGH STORAGE STABILITY UNDER LABORATORY AND INDUSTRIAL PROCESSING CONITIONS THROUGH RESPONSE SURFACE METHODOLOGY

8.1 Introduction

The aim of this chapter is to discuss the drying conditions for *L. casei* 431 in order to achieve a shelf-stable dried probiotic powder with low moisture content without huge cell death during laboratory and scale-up drying process. A dehumidifier added to the FB drier provided drier air with lowered relative humidity (33% RH) as compared to atmospheric air (55% RH).

Powders heat sealed and stored as bulk packaging in aluminium pouches in Chapter 7 showed an increase in the moisture content with increase in the storage time at both stored temperatures. So the dried powders (12 g) made in this chapter were individually placed in sealed polythene bags with outer layer of hermetically sealed aluminium pouches in order to avoid moisture penetration in the powders.

8.2 Effect of drying time and temperature of FB drier on *L. casei* 431 stability during laboratory scale preservation

A PB design (shown in Appendix 5) was used to determine the effect of FB drying time and drying temperature on viability of *L. casei* 431 during preservation under laboratory conditions. The two factors; inlet drying temperature and drying time were set at 50/60 ˚C and 15/30 minutes respectively. Inlet air temperatures set to 50 and 60 °C resulted in a maximal bed temperature of 42 and 47 ˚C respectively. Growth media composition and rehydration conditions were kept same as in Experiment 7. Harvesting of growth media was done after 16-17 h of fermentation by high speed centrifugation (Sorvall) to get a cell concentrate of 23 % solids. Around 7.5 g of washed (with peptone water) cell concentrate was mixed with 100g of WMP (a_w 0.3) before drying and dried powders were stored at 37 °C and 25 °C for 4 and 12 weeks respectively.

8.2.1 Results and discussion

Table 8.2.1 shows the viability data of powders dried under different conditions. The maximum count after drying was obtained in powders dried at 50 ˚C (9.7 log units). All the powders except the one dried at 50 ˚C for 15 min were relatively stable after 3 months of storage under ambient conditions but had 1-1.5 log unit reduction in counts after storage at 37 ˚C for 28 days. The highest count after end of storage period at 37 ˚C and 25 ˚C was obtained in powders dried at 50 ˚C for 30 min i.e. 8.73 and 9.63 log cfu g^{-1} powder respectively. All these powders dried with the aid of dehumidifier had moisture content between 4.0-4.2 % and water activity of ≤ 0.2 (data not shown) after drying. Moisture values remained constant after 3 months of storage under ambient conditions (data not shown). The lowered moisture content during storage thus could be an essential factor for stability of dried cells. The drying loss (< 10%) was much lower when compared with the results obtained by Mille et al. (2004) who reported 80 % survival of *L. plantarum* dried to final a_w of < 0.2 in FB drier for 30 min at temperature ≤ 35 ˚C. The mean storage loss (day 0-3months count) for *L. casei* 431 at 25 ˚C was negligible when compared with storage loss reported by Prasad et al. (2003) who observed 1.6 log unit reduction in heat shocked stationary phase *L. rhamnosus* cells after 14 weeks of storage at 30 ˚C.

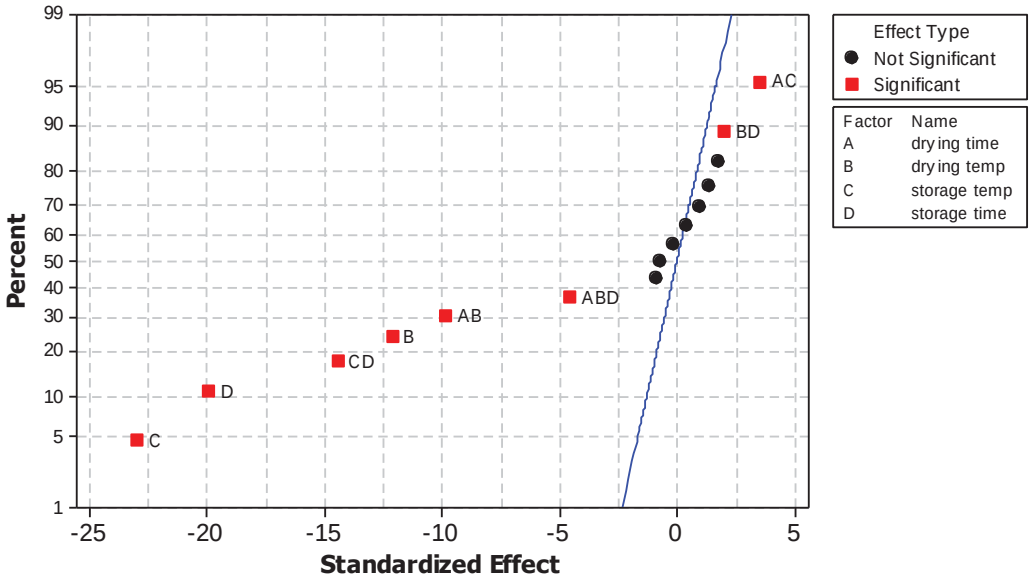
Table 8.2.1 – The viability data of powders dried for different time-temperature combinations in FB drier

Drying Time/Temp	Day 0 Moisture (%)	log cfu g^{-1} powder				
		Day 0	Day 14 @ 37 °C	Day 28 @ 37°C	Day 28 @ 25C	3 months @ 25°C
15min/50°C	4.19	9.73	9.37	8.13	9.65	9.29
	(0.08)	(0.12)	(0.01)	(0.01)	(0.13)	(0.1)
15min/60°C	4.15	9.29	9.12	8.16	9.42	9.45
	(0.08)	(0.04)	(0.05)	(0.15)	(0.01)	(0.01)
30min/50°C	4.04	9.75	9.50	8.73	9.74	9.63
	(0.12)	(0.06)	(0.02)	(0.08)	(0.05)	(0.02)
30min/60°C	4.14	9.05	9.02	8.04	9.13	8.96
	(0.04)	(0.02)	(0.01)	(0.08)	(0.04)	(0.28)

Values in brackets represent SD

All the viability results obtained from this experiment were plotted as a full factorial two-level design (2^4) with 3 replications, and response as *L. casei* 431 viable cell count. The factors studied were drying time (15min, 30min), drying temperature (50 ˚C, 60 ˚C), storage time (short, long) and storage temperature (25 ˚C, 37 ˚C). Short and long storage time at 25 ˚C represented 1 and 3 months and at 37 ˚C meant 14 and 28 days of storage. The normal probability plot of the effect estimates is presented in Figure 8.2.1.1. The plot was useful in comparing the magnitude of main effects and interaction effects. The significant effects were larger and farther from the straight line than non-significant ones, at an α level of 0.05. The main effect of all the factors except drying time and 2-way interaction between all the factors except drying time & storage temperature were large. Interaction effect of drying time & storage time was significant only at an α level of 0.10 (shown in Table 8.2.2).

Figure 8.2.1.1- Normal probability plots of effects (α=0.05) with *L. casei* 431 viable cell count as response



To confirm these results, a main effects plot (Figure 8.2.1.2) and interaction plot (Figure 8.2.1.3) were used. The main effect plot compared the overall grand mean of the factors in the form of reference line with the main effects, whereas interaction plot showed the interaction effect of two factors on the response and compared the relative strength of these effects. Table 8.2.2 summarizes the analysis of variance of this experiment with R^2=0.98. It was clear from Figure 8.2.1.2 that low drying temperature, low storage temperature and short storage

time gave higher viable counts than their counterparts. ANOVA results (Table 8.2.2) confirmed these results as the main effects of drying temperature, storage temperature and storage time were significant at the 0.05 α-level with their effect slightly negative whereas main effect of drying time was not significant for viable cell count. Effect of drying time was however found significant (*P*-value=0.05) for final moisture content but the difference was slight (data not shown). ANOVA results also showed that the 2-way interactions of drying time & drying temperature, storage time & storage temperature, drying time & storage temperature and drying temperature & storage time were significant at 0.05 α-level and with their effect slightly negative for first two combinations and slightly positive for last two combinations. The interaction plot (Figure 8.2.1.2) of drying time & drying temperature and drying time & storage temperature indicated that longer drying time (30 min) at high temperature of 60 ˚C resulted in lower cell count but longer (30min) drying time gave higher counts during storage at accelerated temperature of 37 ˚C. Higher counts during storage at 37 ˚C for powders dried could be due to the fact that drying for longer times resulted in slightly lower (mean) residual moisture content and water activity (data not shown) in dried powders. The plot of interaction between storage time & drying temperature and storage time & storage temperature indicated that decrease in cell count with increase in storage time was greater for powders dried at of 60 ˚C and stored at 37 ˚C. The interaction plot of drying time & storage time was significant at 0.10 α-level indicating slightly higher cell counts during storage for longer periods were achieved in powders dried for longer drying time (30min).

Figure 8.2.1.2 – Main effect plot of factors studied with response as *L. casei* 431 cell count

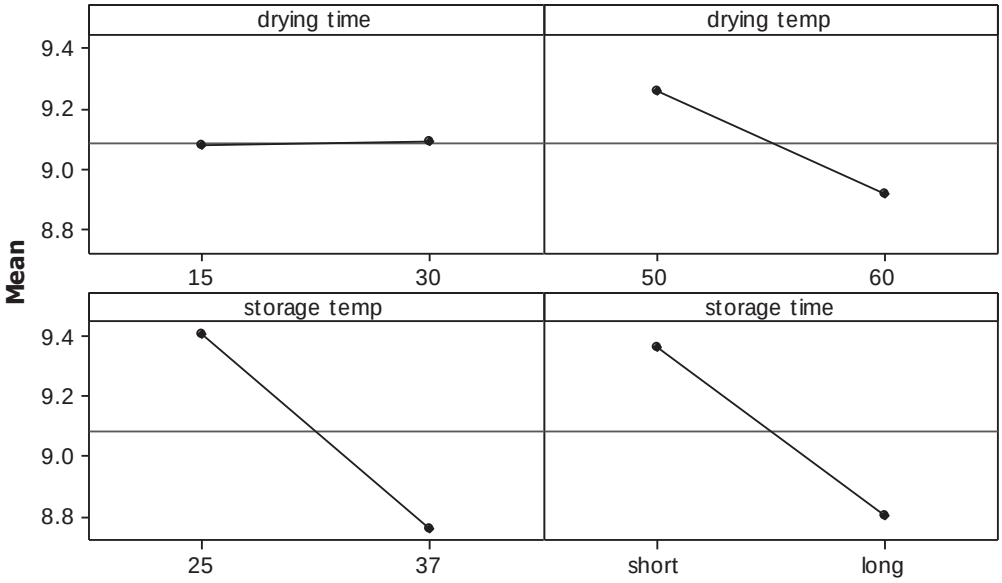


Figure 8.2.1.3 – Interaction plot of factors studied with response as *L. casei* 431 cell count

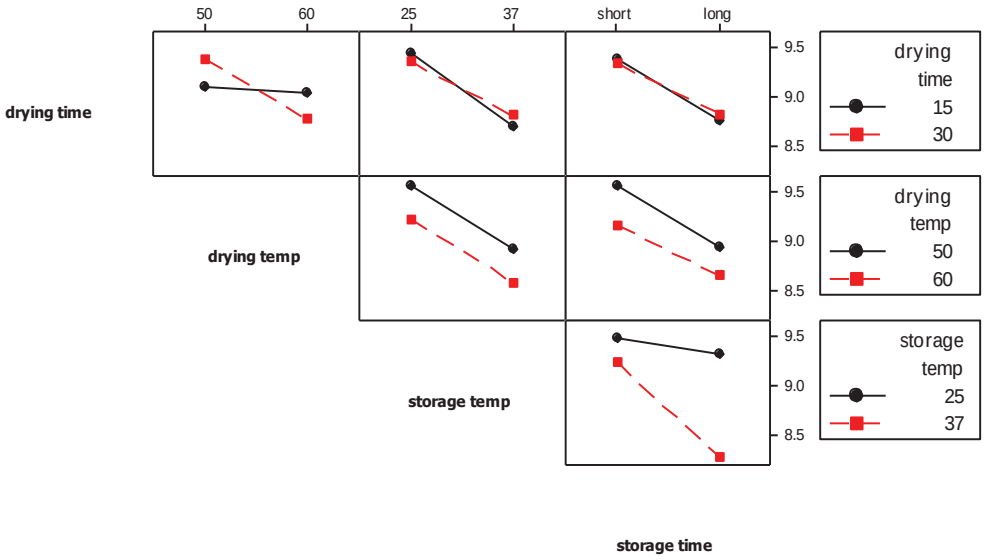


Table 8.2.2 Analysis of variance table for the experiment

Source of variation	*F* value	*P* value
Main Effects	267.24	0.000
drying time	0.20	0.660
drying temp	146.26	0.000
storage temp	526.49	0.000
storage time	396.00	0.000
2-Way Interactions	54.20	0.000
drying time*drying temp	96.95	0.000
drying time*storage temp	12.59	0.001
drying time*storage time	3.25	0.081
drying temp*storage temp	0.01	0.907
drying temp*storage time	4.16	0.050
storage temp*storage time	208.21	0.000
3-Way Interactions	6.00	0.001
drying time*drying temp*storage temp	0.90	0.351
drying time*drying temp*storage time	20.47	0.000
drying time*storage temp*storage time	1.85	0.183
drying temp*storage temp*storage time	0.79	0.382

Exposure of microorganisms to high temperatures should be as short as possible and correct choice of times and temperatures of dehydration are crucial to achieve the best FB drying conditions. Our results showed that bacterial cells inactivation was higher for powders dried at 60 ˚C than at 50 ˚C. Dehydration inactivation is the most important inactivation mechanism at drying temperature ≤ 55 ˚C but at high temperatures thermal inactivation becomes important (Lievense et al., 1992). Thus to avoid thermal inactivation superimposed on the dehydration inactivation, low drying temperatures are recommended. Also during storage powders with a_w of approximately 0.2, equivalent to 4 % moisture appeared to maintain the best viability (Simpson et al., 2005). Results from our experiments showed that drying time of 30 min at a temperature of 50 ˚C is preferable to achieve both desirable lower moisture content and high cell count. Moisture content and water activity of dried cells is very critical as discussed in section 2.8. So drying temperature of 50 ˚C was selected for further experiments performed. Also from the interaction plot of storage temperature & storage time it could be concluded that the mean viable count after 15 days of storage at 37 ˚C was similar to 3 months storage at 25 ˚C which was in accordance with the results obtained in Chapter 7. Thus shelf life of FB dried *L. casei* 431 cells at 25 ˚C could be considered as 6 times longer than at 37 ˚C.

8.3 Comparison of cell viability in powders dried in presence and absence of dehumidifier

Table 8.3 shows Students T-test comparison of powders made with 23 % solids concentrate and dried with and without dehumidifier in FB drier at 50 °C in terms of their drying time, final moisture content and viability losses during drying and storage. No difference in viability was found in powders after drying (Day 0) and during storage at 37 °C for 28 days but the viability of *L. casei* 431 in the powders dried in presence of dehumidifier, and stored under ambient conditions for 3 months was significantly lower than the powders dried in absence of dehumidifier. This could be due to fact that the powders dried with dehumidifier had significantly lower moisture content, even although their drying time was half (30min) that of the powders made without dehumidifier (60 min). Bayrock and Ingledew (1997) concluded that dehydration and not moist heat, dry heat, or oxidation, is the mechanism that leads to losses in yeast cell viability during fluidized bed drying at 40 °C. On the other hand Fu and Chen (2011) suggested that the relative humidity (RH) of drying air is important, as moist heat is more effective in inactivating microorganisms than the dry heat due to the fact that heat is conducted faster when the material is not dry. The reason that there was no difference in viability after drying in our experiments could be due to the fact that at 50 °C the increase in thermal conductivity of air that occurs with decrease in RH from 55% (in absence of

dehumidifier) to 33 % RH (in presence of dehumidifier) is negligible (Tsilingiris, 2008), but at air temperatures of around 100 °C the increase in thermal conductivity is significant. Thus effect of RH is only important at temperatures around and above 100 °C (Tsilingiris, 2008). It would be interesting to compare storage stability of these powders after longer periods of storage at 25 °C.

Table 8.3 – Students T-test comparison of powders FB dried (at 50 °C) in presence and absence of dehumidifier

Powders made	% Moisture at Day 0	log cfu g^{-1} powder		
		Day 0	Day 28 @ 37 °C	3 months @25 °C
Without dehumidifier	4.6	9.4	8.3	9.5
With dehumidifier	4.1	9.6	8.4	9.2
P value	0.007	0.30	0.27	<0.001

n=6

8.4 Effect of initial moisture content (before drying) and drying time at 50 °C on *L. casei* 431 stability during industrial preservation

The previous experiment showed that when a high solids (23 %) cell concentrate was used, the viability of the cells during storage was better if they had been dried at a lower temperature (50 °C) but drying time had little effect. The question remained whether this would also apply to wetter cell concentrates, such as would be obtained in an industrial situation. The powders made with cell concentrate containing 6.5 % solids (22.2 % initial moisture in powders) in Chapter 7 showed huge drying and storage losses at ambient storage temperature when compared to cell concentrate of 23 % solids (8.4 % initial moisture in powders). The powders at center level, with 14.7 % solids in cell concentrate (14.2 % initial moisture in powders), were better than powders made with 6.5 % solids concentrate in terms of their storage stability. It should be possible, with wetter cell concentrates to reduce the moisture in the powders for drying by reducing the ratio of concentrate to milk powders, although obviously there are limits to the dilution of the cell concentration in the powder. Thus a CCD (shown in Appendix 6) was used to standardize the drying conditions with two factors; initial moisture content of the powder before drying and drying time. Initial moisture content was varied in the range

between 8 and 14 %. A moisture level of 8 % before drying represented the conditions where 7.5 g of unwashed cells concentrate containing 23 % solids was mixed with 100 g WMP and moisture level of 14 % represented the conditions where 7 g of supernatant obtained after centrifugation was added along with 7.5 g of unwashed concentrate to 100 g WMP. Drying time was varied between 20 and 80 min. Longer drying times at 50 °C were used with the aim of achieving final water activity ≤ 0.2 and moisture content between 4.0-4.2 % in the dried powders.

8.4.1 Results and discussion

Table 8.4.1 shows experimental design with the obtained results. Results showed that initial counts after drying were much lower than results obtained in Experiment 8.2 under similar drying conditions, indicating huge drying loss which could be due to presence of metabolites in the powders, which was in agreement with the viability results obtained in Chapter 7 after drying under centre level conditions. The count after drying varied between 8.5 -9.3 log cfu g^{-1} powder with lowest count in powders with maximum (14 %) initial moisture and highest count in powders made with lowest (8 %) initial moisture. The moisture content was determined in vacuum oven due to the fact drying in conventional oven for moisture content determination showed occasionally slight increase in moisture content in powders made in experiment 8.2 even when powders were individually stored in aluminium pouches with inner layer of polyethylene. The high temperatures of conventional oven (105 °C) could lead to loss of organic matter in WMP which could affect the moisture results. Results from vacuum oven showed that moisture content was stable during 14 days of storage at 37 °C except in one trial (Run No. 7) which could be due to experimental error. Storage stability results of powders stored at 37 °C for 14 days showed that all the powders were stable during this storage period with no decline in viability except for the powders with 11 % initial solids with 20 min of drying time which showed nearly one log reduction in cell count.

Table 8.4.1 – CCD results to study effect of initial moisture (before drying) and drying time (at 50 °C) in FB drier on *L. casei* 431 stability during drying and storage

Std Order[1]	Run Order[2]	Drying time (min)	Initial moisture %	Day 0, % moisture	Day 0, a(w)	log cfu g^{-1}		Day 14, % moisture
						Day 0	Day 14 @ 37 °C	
12	1	50	11		0.2	9.26	9.3	3.0
13	2	50	11	3.3	0.2	9.26	9.28	3.3
2	3	71.2	8.9	3.1	0.17	9.27	9.29	3.1
4	4	71.2	13.1	3.1	0.17	9.14	9.29	3.0
10	5	50	11	3.2	0.2	9.27	9.32	3.2
6	6	80	11	3.0	0.17	9.19	9.2	3.0
8	7	50	14	3.3	0.2	8.68	8.61	3.8
3	8	28.8	13.1	3.5	0.2	8.45	8.71	3.6
9	9	50	11	3.0	0.18	9.26	9.29	2.9
1	10	28.8	8.9	3.2	0.21	9.59	9.46	3.4
7	11	50	8	3.1	0.17	9.48	9.46	3.2
5	12	20	11	3.6	0.17	9.09	8.2	3.7
11	13	50	11	3.3	0.2	9.25	9.25	3.2

[1]Standard order (STDOrder) is the order designed by Minitab

[2]Run Order is the order in which the experiment is conducted

Table 8.4.2 shows results from analysis of variance for day 0 count with R^2 as 0.86. Results showed that the effect of drying time and initial moisture was significant at 0.05 α-level with effect of drying time slightly positive and effect of initial moisture slightly negative. The *P* value of their interaction was also highly significant (*P* value <0.01). Table also showed small *P* value for square terms but because the square terms of factors studied were not important in the experiment, they are not further discussed. The contour plot (Figure 8.4.1) suggested that a relatively wide range of both initial moisture and drying times levels would result in acceptable values of 9.2-9.6, the optimum target cell count for day 0. However, analysis of variance results showed that the probability of lack of fit of the model was very high (*P* value =0.003), thus the model did not adequately describe the data. When lack of fit is detected, it implies that the

predictive knowledge of model is not statistically correct. This also means that the regression equation may not explain well the presence of several large residuals.

Analysis of variance of day 14 cell count (Appendix 12) also showed that drying time and initial moisture were significant (*P* value <0.05) but their interactions were insignificant (*P* value >0.1). Also the lack of fit of model was highly significant (*P* value <0.001). Overall, the results showed that cell viabilities were maintained for all moisture contents, provided sufficient drying time was allowed.

Table 8.4.2 – Analysis of variance table for the CCD experiment designed to study the effect of drying time and initial solids on the viable cell count at day 0

Source	*F* value	*P* value
Regression	165.36	0.000
Linear	288.36	0.000
Drying time	25.02	0.002
Initial moisture	551.70	0.000
Square	27.43	0.000
Drying time*Drying time	18.77	0.003
Initial moisture*Initial moisture	42.53	0.000
Interaction	195.19	0.000
Drying time*Initial moisture	195.19	0.000
Residual Error		
Lack-of-Fit	59.6	0.001

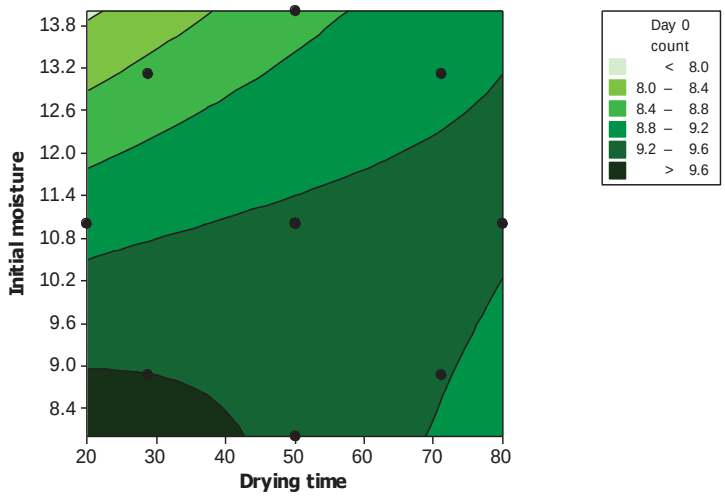


Figure 8.4.1 – Response contour plot with day 0 viable cell count as response

8.5 Conclusions

The following general conclusions may be drawn from the results discussed in this chapter:

- Optimization of drying conditions i.e. drying time and drying temperature resulted in relatively stable counts in powders after 3 months of storage at ambient temperature under laboratory processing conditions. The highest count after end of storage period at 37 ˚C and 25 ˚C was obtained in powders dried at 50 ˚C for 30 min i.e. 8.73 and 9.63 log cfu g^{-1} powder respectively. Also from the interaction plot of storage temperature & storage time it could be concluded that the mean viable count after 15 days of storage at 37 ˚C was similar to 3 months storage at 25 ˚C.
- Powders dried with the aid of dehumidifier had significantly lower (*P* value = 0.007) moisture content than the powders dried in its absence, even when their drying time was half (30min) that of the powders made without dehumidifier (60 min). The viable count after 3 months of storage at 25 ˚C was higher in powders dried in the presence of dehumidifier but no difference in storage counts in powders stored at 37 ˚C under both conditions was observed.
- Optimization of drying time and initial moisture of powder before drying lead to relatively stable powders after 2 weeks of storage at 37 ˚C under industrial processing conditions.
- The moisture content results from vacuum oven drying showed that moisture of dried powders stored in sealed polythene bags with outer layer of hermetically sealed aluminium pouches was stable with increase in storage time.

CHAPTER 9

OVERALL DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

9.1 Overall discussion

Probiotics are commonly found in chilled short shelf-life foods, such as fermented milk drinks and yoghurts, but consumer demand for healthy foods in a variety of formats is increasing. This is why there is a growing interest in including viable probiotics in dried foods with a long-term ambient shelf-life, and in sufficient numbers (10^8 CFU per serving or 10^6 to 10^8 CFU per gram of food) to provide a health benefit. A cost-efficient process (from growth to storage) would further help in expanding the market and promoting the usage of microorganisms for wider range of applications that could benefit consumers.

Following this line, the purpose of this research was to maximizing viability of *L. casei* 431 during fermentation, fluidized bed drying and ambient storage. This was achieved by investigating various parameters (Table 9.1.1) at various stages from growth to storage with the objective of finding best combination for maximizing the cell viability during laboratory and scale-up preservation of bacteria.

Table 9.1.1- Various parameters studied at each processing step to maximize *L. casei* 431l viability during drying and ambient storage

	Processing step	Parameters studied
1	Fermentation	Media composition, pre stress by acid and osmotic (NaCl) stress
2	Harvesting	Harvesting Time/growth phase (starvation stress) and harvesting method (laboratory and industrial scale)
3	Mixing	Mixing technique, carrier water activity
4	Drying	Drying time and temperature, initial solids/moisture before drying
5	Storage	Storage temperature

Large amount of information was available discussing the effects of various growth medium components and growth conditions on viable cell count and other growth properties such as OD and biomass of various lactobacilli strains. But most of these studies were performed

under pH controlled conditions in a bioreactor. Many of these studies included comparison of batch, fed-batch and continuous bioreactor in terms of cell growth. But very few studies have investigated increasing cell growth properties of lactobacilli under uncontrolled pH conditions and moreover the focus of these studies was to increase cell biomass or OD rather than viable cell count. Furthermore bulk of the attention has been given to the preservation of lactobacilli cells either through freeze drying or spray drying with very few studies describing the drying and storage stability of cells dried using a fluidized bed (FB). Also, in these studies, poor stability of FB dried lactobacilli during drying as well storage at temperatures above refrigeration temperatures has been reported. Maintenance of probiotic viability is not only important during drying but also during the shelf-life of powder. This is the main determinant of commercial success of a product.

9.2 Conclusions

A probiotic strain *Lactobacillus paracasei* subsp. *paracasei L. casei* 431 used in this study was found to grow well in MRS medium. During uncontrolled pH fermentation, pH of the growth medium dropped to ~4.0 over a period of 10-14 h and thereby preventing further multiplication of bacterial cells. The maximum cell density obtained in MRS media under these conditions was 9.3 log cfu ml^{-1} media. Supplementing MRS with glucose or nitrogen extract did not increase viable cell density significantly. However, concentration of nitrogen source (in the form of yeast extract) was found highly significant for *L. casei* 431 biomass suggesting a partial uncoupling between the increase in cell number and the biomass production, as they were affected by the independent variables in a different way. When pH during fermentation was controlled at 6.5 in a bioreactor, maximum cell density still remained 9.3-9.5 log cfu ml^{-1}. Yet again glucose supplementation did not affect the cell count probably due to substrate inhibition. However, fed-batch technique did increase the viable count to above 10 log cfu ml^{-1}.

For a commercial product, FB drying was chosen as an economical alternative to freeze drying. FB drying needs a carrier for bacterial cells and so whole milk powder (WMP) was chosen as a carrier for this work, but other carrier might offer more protection. During drying in a fluidized bed, pH controlled cells were found to suffer huge losses during drying and subsequent storage compared to pH uncontrolled cells. The reason for this loss was not related to osmotic shock caused during mixing of cells (wet) with WMP as the water activity of the WMP showed little effect on viability and the loss was also unrelated to presence of lactose crystals. The most probable reason could be due to the build up of resistance through starvation and acid stress in cells grown in uncontrolled pH conditions. The drying tests showed that the initial moisture content of harvested cells was a critical factor – if too high powder is difficult to dry

and drying and storage losses are high. Also, drying temperature should not be too high but drying time should be sufficient enough to dry powders to desirable final moisture content (~4 %) required for long term shelf-stability of dried bacteria.

For dried powders containing bacteria, the level of moisture appeared to be a critical factor. But, final moisture content highly depends on other factors such as drying temperature, drying time, initial moisture content before drying, harvesting techniques and type of protective carriers used. Thus, optimization of all these parameters in combination of final moisture content in dried powders is essential for stability of probiotic bacteria under ambient conditions. Furthermore, storage environment and type of packaging are important for long shelf-life for probiotics. An efficient packaging which could provide barrier to oxygen, light, heat and moisture would be beneficial. It is important to not overlook the necessity of inducing desiccation tolerance during the growth of the micro-organisms, and not just relying on protective agents within the drying matrix. It has clearly been demonstrated that cells need to be conditioned for desiccation tolerance, e.g. stationary phase cells in acidic growth conditions. Thus, each step ranging from growth to storage of cells could strongly influence viability of the cultures.

Some generic conclusions can be drawn from this study which may be useful in maximizing cell survival during drying and ambient storage:

- Growth media should be high in nitrogen content.
- Cell growth should be under uncontrolled pH conditions (acid stress).
- Harvesting of cells should be done during early to mid-stationary phase (starvation and acid stress).
- Total solids in harvested cells should be high.
- Mixing of cells with the protective carrier should be slow.
- Initial moisture content of the mix before drying should be low
- Maximum inlet drying air temperature of FB drier should not exceed 50 ˚C
- Relative humidity of drying air should be low
- Drying time should be aimed to get final water activity of ≤ 0.2 and moisture content close to 4 % in dried culture
- Packaging should be air tight and temperature of storage should not exceed 25 ˚C in order to ensure high viability of a commercial probiotic and starter culture.

9.3 Recommendations

Prestressing bacteria in the growth medium with osmotic shock (e.g. by addition of sodium chloride), cold shock and heat shock, as well presence of compatible solutes (e.g. betaine and carnitine) in the growth medium have been reported to improve survivability of LAB at high temperatures and during drying, and these conditions thus need to be further investigated. At the same time, it will be interesting to investigate the occurrence of accumulated compatible solutes, and induced specific stress proteins and chaperons (such as DnaK and GroEL). Stress responses in bacteria under above mentioned conditions and their role in preserving cell viability during preservation process could be very useful. Some other aspects that also need to be further examined include: effects of neutralizing media pH before harvesting, choice of protective agents that could protect both during dehydration and storage and packaging under vacuum or inert gas, with the intention to further improve storage stability of bacteria. The main challenge in making FB drying a wide spread methodology for microorganisms preservation is due to the difficulty of defining standardized conditions that would allow the comparison of results obtained in different laboratories. The reason of this difficulty is that the times and temperatures for the dehydration processes are related with the drying conditions such as relative humidity of drying air, weight or volume of the sample, initial water activity/moisture content, type of protectant etc. Therefore, to make results comparable, it becomes necessary to refer the experimental conditions, to a parameter that is independent to these experimental conditions, for example, the moisture content of the sample after dehydration at a given condition. A standard method of determination of moisture content is also required.

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APPENDICES

Appendix 1 - Placket-Burman (PB) design matrix for increasing viable cell count of *L. casei 431* during uncontrolled pH fermentation

Random Order	Run Order	Glucose (10-30 g l^{-1})	Yeast extract (10-40 g l^{-1})	Meat extract (8.3-33 g l^{-1})	Peptone (18.5-74 g l^{-1})	Vitamin solution (0.5-1.5 g l^{-1})	Ammonium citrate (0.5-2 g l^{-1})	Salt (0-30 g l^{-1})	Initial pH (6.0-7.0)	Dummy 1	Dummy 2
10	1	1	1	-1	1	1	-1	1	-1	1	1
5	2	-1	-1	-1	1	1	1	-1	1	-1	-1
6	3	1	1	1	-1	1	1	-1	1	-1	-1
13	4	-1	1	1	-1	1	-1	-1	-1	0	0
2	5	1	-1	1	1	-1	1	-1	-1	1	1
11	6	-1	-1	1	1	1	-1	1	1	-1	1
1	7	1	-1	-1	-1	1	1	1	-1	1	-1
4	8	0	0	0	0	0	0	0	0	-1	1
12	9	1	1	-1	1	-1	-1	-1	1	-1	-1
3	10	-1	-1	-1	-1	-1	-1	-1	-1	1	1
9	11	1	-1	1	-1	-1	-1	1	1	1	-1
7	12	-1	1	-1	-1	-1	1	1	1	1	-1
8	13	-1	1	1	1	-1	1	1	-1	-1	1

Appendix 2 - Central composite design (CCD) matrix for optimizing glucose and yeast extract concentrations in growth media during uncontrolled pH fermentation

		Variables/ Levels		Actual levels							
Random Order	Run Order	Glucose	Yeast extract	Glucose ($g\ l^{-1}$)	Yeast extract ($g\ l^{-1}$)	Final OD	Initial Glucose (%)	Final Glucose (%)	Initial Lactic Acid (%)	Biomass ($g\ kg^{1}$ media)	log cfu g^{1} media
11	1	0	0	12.50	15.00	7.9	2.98	0.97	0.01	2.4	9.33
5	2	-1.41421	0	0.00	15.00	7.3	1.89	0.12	0.01	2.3	9.34
2	3	1	-1	21.34	4.39	7.6	3.82	2.03	0.01	2.3	9.12
4	4	1	1	21.34	25.61	8.2	3.61	1.51	0.01	2.9	9.25
3	5	-1	1	3.66	25.61	7.9	2.2	0.08	0.01	2.8	9.36
10	6	0	0	12.50	15.00	7.5	2.99	0.89	0.01	2.4	9.29
13	7	0	0	12.50	15.00	8.1	3	0.96	0.01	2.6	9.30
9	8	0	0	12.50	15.00	7.9	2.93	0.83	0.01	2.5	9.29
7	9	0	-1.41421	12.50	0.00	6.5	3.07	1.34	0.01	1.9	9.27
6	10	1.41421	0	25.00	15.00	7.8	4.05	2.15	0.01	2.3	9.33
12	11	0	0	12.50	15.00	7.8	2.97	1.08	0.01	2.4	9.34
1	12	-1	-1	3.66	4.39	7.8	2.27	0.35	0.01	2	9.33
8	13	0	1.41421	12.50	30.00	8.6	2.82	0.65	0.01	2.9	9.31

Appendix 3 - PB design matrix to study the effect of concentration of yeast extract, meat extract, peptone, glucose and harvesting time on *L. casei* 431 cell count

Random Order	Run Order	Glucose (0-20 g l^{-1})	Yeast extract (0-20 g l^{-1})	Meat extract (0-16.7 g l^{-1})	Peptone (0-40 g l^{-1})	Harvesting Time (16-36 h)
1	1	1	-1	1	-1	-1
11	2	-1	1	-1	-1	-1
6	3	1	1	1	-1	1
8	4	-1	-1	1	1	1
2	5	1	1	-1	1	-1
7	6	-1	1	1	1	-1
13	7	0	0	0	0	0
12	8	-1	-1	-1	-1	-1
4	9	1	-1	1	1	-1
5	10	1	1	-1	1	1
10	11	1	-1	-1	-1	1
3	12	-1	1	1	-1	1
9	13	-1	-1	-1	1	1

Appendix 4 - PB design matrix for comparing powders made under laboratory and industrial processing conditions

Random Order	Run Order	Blocks (1: washed cells 2: unwashed cells)	Harvesting Time (16-36 h)	Concentrate solids (6.5-23 %)
7	1	1	-1	1
10	2	1	1	-1
2	3	1	1	1
4	4	1	1	-1
12	5	1	-1	-1
6	6	1	1	1
3	7	1	-1	1
13	8	1	0	0
8	9	1	-1	-1
1	10	1	1	-1
9	11	1	-1	-1
11	12	1	-1	1
5	13	1	1	1
25	14	2	-1	-1
18	15	2	1	1
21	16	2	-1	-1
24	17	2	-1	1
20	18	2	-1	1
15	19	2	1	1
16	20	2	-1	1
17	21	2	1	-1
23	22	2	1	-1
19	23	2	1	1
14	24	2	1	-1
26	25	2	0	0
22	26	2	-1	-1

Appendix 5 - PB design matrix for powders made at drying times-temperature combinations in Fluidized Bed drier

Random Order	Run Order	Drying time	Drying temp	Drying tin (min)	Drying temp (˚C)
10	1	1	-1	30	50
9	2	-1	-1	15	50
7	3	-1	1	15	60
3	4	-1	1	15	60
12	5	-1	-1	15	50
6	6	1	1	30	60
8	7	-1	-1	15	50
2	8	1	1	30	60
4	9	1	-1	30	50
1	10	1	-1	30	50
5	11	1	1	30	60
11	12	-1	1	15	60

Appendix 6 - PB design matrix for powders made with cells of different initial moisture content and dried for various times

		Variables/ Levels		Actual levels	
Random Order	Run Order	Drying time	Initial moisture	Drying time (min)	Initial moisture (%)
12	1	0	0	50	11
13	2	0	0	50	11
2	3	1	-1	71.2	8.9
4	4	1	1	71.2	13.1
10	5	0	0	50	11
6	6	1.414214	0	80	11
8	7	0	1.414214	50	14
3	8	-1	1	28.8	13.1
9	9	0	0	50	11
1	10	-1	-1	28.8	8.9
7	11	0	-1.41421	50	8
5	12	-1.41421	0	20	11
11	13	0	0	50	11

Appendix 7 - Minitab results for PB design experiment used for increasing *L. casei* 431 cells count during uncontrolled pH fermentation

Analysis of Variance for viable cell count (Log cfu ml^{-1} media)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	8	5.45410	5.45410	0.68176	124.21	0.001
Glucose	1	0.08333	0.08333	0.08333	15.18	0.030
Yeast Extract	1	0.00853	0.00853	0.00853	1.55	0.301
Meat Extract	1	0.05333	0.05333	0.05333	9.72	0.053
Peptone	1	0.05070	0.05070	0.05070	9.24	0.056
Vitanmin soln	1	0.00213	0.00213	0.00213	0.39	0.577
Ammonium citrate	1	0.00213	0.00213	0.00213	0.39	0.577
Salt	1	5.25363	5.25363	5.25363	957.14	0.000
Initial pH	1	0.00030	0.00030	0.00030	0.05	0.830
Curvature	1	0.01600	0.01600	0.01600	2.92	0.186
Residual Error	3	0.01647	0.01647	0.00549		
Total	12	5.48657				

Analysis of Variance for Biomass (g kg^{-1} media)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	8	23.9000	23.9000	2.9875	11.96	0.033
Glucose	1	0.9075	0.9075	0.9075	3.63	0.153
Yeast Extract	1	0.0675	0.0675	0.0675	0.27	0.639
Meat Extract	1	0.3008	0.3008	0.3008	1.20	0.353
Peptone	1	0.0008	0.0008	0.0008	0.00	0.958
Vitanmin soln	1	1.0208	1.0208	1.0208	4.09	0.136
Ammonium citrate	1	0.4408	0.4408	0.4408	1.77	0.276
Salt	1	20.0208	20.0208	20.0208	80.17	0.003
Initial pH	1	1.1408	1.1408	1.1408	4.57	0.122
Curvature	1	1.7031	1.7031	1.7031	6.82	0.080
Residual Error	3	0.7492	0.7492	0.2497		
Total	12	26.3523				

Analysis of Variance for OD

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	8	140.454	142.626	17.828	8.07	0.115
Glucose	1	4.773	0.188	0.188	0.08	0.798
Yeast Extract	1	1.803	0.200	0.200	0.09	0.792
Meat Extract	1	2.305	0.186	0.186	0.08	0.799
Peptone	1	8.977	0.364	0.364	0.16	0.724
Vitanmin soln	1	1.835	1.170	1.170	0.53	0.543
Ammonium citrate	1	1.583	1.827	1.827	0.83	0.459
Salt	1	118.217	120.597	120.597	54.57	0.018
Initial pH	1	0.961	1.468	1.468	0.66	0.501
Curvature	1	8.783	8.783	8.783	3.97	0.184
Residual Error	2	4.420	4.420	2.210		
Total	11	153.657				

Analysis of Variance for Lactic acid (%)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	8	4.68350	4.68350	0.58544	1.58	0.385
Glucose	1	0.58080	0.58080	0.58080	1.57	0.299
Yeast Extract	1	0.26403	0.26403	0.26403	0.71	0.460
Meat Extract	1	0.00053	0.00053	0.00053	0.00	0.972
Peptone	1	0.02083	0.02083	0.02083	0.06	0.828
Vitanmin soln	1	0.17521	0.17521	0.17521	0.47	0.541
Ammonium citrate	1	0.25521	0.25521	0.25521	0.69	0.467
Salt	1	3.05021	3.05021	3.05021	8.25	0.064
Initial pH	1	0.33668	0.33668	0.33668	0.91	0.410
Curvature	1	0.22314	0.22314	0.22314	0.60	0.494
Residual Error	3	1.10897	1.10897	0.36966		
Total	12	6.01561				

Appendix 8 - Minitab results for CCD design experiment used for optimizing glucose and yeast extract concentrations in growth media during uncontrolled pH fermentation

Analysis of Variance for viable cell count (Log cfu ml^{-1} media)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	0.013036	0.013036	0.002607	0.99	0.487
Linear	2	0.005745	0.005745	0.002873	1.09	0.388
Glucose	1	0.002313	0.002313	0.002313	0.88	0.380
Yeast extract	1	0.003432	0.003432	0.003432	1.30	0.292
Square	2	0.002363	0.002363	0.001181	0.45	0.656
Glucose*Glucose	1	0.000878	0.001184	0.001184	0.45	0.525
Yeast extract*Yeast extract	1	0.001485	0.001485	0.001485	0.56	0.478
Interaction	1	0.004928	0.004928	0.004928	1.87	0.214
Glucose*Yeast extract	1	0.004928	0.004928	0.004928	1.87	0.214
Residual Error	7	0.018479	0.018479	0.002640		
Lack-of-Fit	3	0.016665	0.016665	0.005555	12.25	0.017
Pure Error	4	0.001814	0.001814	0.000454		
Total	12	0.031515				

Analysis of Variance for Biomass (g kg^{-1} media)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	1.03374	1.03374	0.206749	14.92	0.001
Linear	2	1.00997	1.00997	0.504987	36.43	0.000
Glucose	1	0.02000	0.02000	0.020000	1.44	0.269
Yeast extract	1	0.98997	0.98997	0.989975	71.42	0.000
Square	2	0.01377	0.01377	0.006885	0.50	0.628
Glucose*Glucose	1	0.01338	0.01257	0.012565	0.91	0.373
Yeast extract*Yeast extract	1	0.00039	0.00039	0.000391	0.03	0.871
Interaction	1	0.01000	0.01000	0.010000	0.72	0.424
Glucose*Yeast extract	1	0.01000	0.01000	0.010000	0.72	0.424
Residual Error	7	0.09703	0.09703	0.013861		
Lack-of-Fit	3	0.06503	0.06503	0.021675	2.71	0.180
Pure Error	4	0.03200	0.03200	0.008000		
Total	12	1.13077				

Analysis of Variance for OD

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	1.94890	1.94890	0.38978	2.57	0.125
Linear	2	1.78777	1.78777	0.89388	5.89	0.032
Glucose	1	0.08941	0.08941	0.08941	0.59	0.468
Yeast extract	1	1.69836	1.69836	1.69836	11.19	0.012
Square	2	0.06399	0.06399	0.03200	0.21	0.815
Glucose*Glucose	1	0.03245	0.04071	0.04071	0.27	0.620
Yeast extract*Yeast extract	1	0.03154	0.03154	0.03154	0.21	0.662
Interaction	1	0.09714	0.09714	0.09714	0.64	0.450
Glucose*Yeast extract	1	0.09714	0.09714	0.09714	0.64	0.450
Residual Error	7	1.06248	1.06248	0.15178		
Lack-of-Fit	3	0.87162	0.87162	0.29054	6.09	0.057
Pure Error	4	0.19086	0.19086	0.04771		
Total	12	3.01137				

Analysis of Variance for Final Lactic Acid (%)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	0.090887	0.090887	0.018177	2.22	0.164
Linear	2	0.089149	0.089149	0.044575	5.44	0.038
Glucose	1	0.005644	0.005644	0.005644	0.69	0.434
Yeast extract	1	0.083506	0.083506	0.083506	10.19	0.015
Square	2	0.000981	0.000981	0.000490	0.06	0.942
Glucose*Glucose	1	0.000583	0.000704	0.000704	0.09	0.778
Yeast extract*Yeast extract	1	0.000398	0.000398	0.000398	0.05	0.832
Interaction	1	0.000756	0.000756	0.000756	0.09	0.770
Glucose*Yeast extract	1	0.000756	0.000756	0.000756	0.09	0.770
Residual Error	7	0.057371	0.057371	0.008196		
Lack-of-Fit	3	0.029541	0.029541	0.009847	1.42	0.362
Pure Error	4	0.027830	0.027830	0.006957		
Total	12	0.148258				

Appendix 9 - Minitab results for PB design experiment used to study the effect of concentration of yeast extract, meat extract, peptone, glucose and harvesting time on *L. casei* 431 cell count

Analysis of Variance for viable cell count (Log cfu ml^{-1} media)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	5	0.156842	0.156842	0.0313683	21.76	0.001
Glucose	1	0.039675	0.039675	0.0396750	27.52	0.002
Yeast	1	0.046875	0.046875	0.0468750	32.51	0.001
Meat	1	0.046875	0.046875	0.0468750	32.51	0.001
Peptone	1	0.000008	0.000008	0.0000083	0.01	0.942
Harvesting Time	1	0.023408	0.023408	0.0234083	16.24	0.007
Curvature	1	0.001416	0.001416	0.0014160	0.98	0.360
Residual Error	6	0.008650	0.008650	0.0014417		
Total	12	0.166908				

Analysis of Variance for Biomass (g kg^{-1} media)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	5	2.49083	2.49083	0.49817	19.71	0.001
Glucose	1	0.04083	0.04083	0.04083	1.62	0.251
Yeast	1	1.14083	1.14083	1.14083	45.13	0.001
Meat	1	0.30083	0.30083	0.30083	11.90	0.014
Peptone	1	0.90750	0.90750	0.90750	35.90	0.001
Harvesting Time	1	0.10083	0.10083	0.10083	3.99	0.093
Curvature	1	0.16673	0.16673	0.16673	6.60	0.042
Residual Error	6	0.15167	0.15167	0.02528		
Total	12	2.80923				

Analysis of Variance for OD

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	5	8.06209	8.06209	1.61242	28.64	0.000
Glucose	1	1.05811	1.05811	1.05811	18.80	0.005
Yeast	1	5.24041	5.24041	5.24041	93.09	0.000
Meat	1	0.04856	0.04856	0.04856	0.86	0.389
Peptone	1	0.02001	0.02001	0.02001	0.36	0.573
Harvesting Time	1	1.69501	1.69501	1.69501	30.11	0.002
Curvature	1	0.09469	0.09469	0.09469	1.68	0.242
Residual Error	6	0.33776	0.33776	0.05629		
Total	12	8.49454				

Analysis of Variance for Lactic Acid (%)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	5	0.68446	0.68446	0.136892	21.08	0.001
Glucose	1	0.09630	0.09630	0.096302	14.83	0.008
Yeast	1	0.26850	0.26850	0.268502	41.35	0.001
Meat	1	0.01300	0.01300	0.013002	2.00	0.207
Peptone	1	0.07285	0.07285	0.072852	11.22	0.015
Harvesting Time	1	0.23380	0.23380	0.233802	36.00	0.001
Curvature	1	0.14738	0.14738	0.147385	22.70	0.003
Residual Error	6	0.03896	0.03896	0.006494		
Total	12	0.87081				

Appendix 10 - Minitab results for PB design experiment used to compare powders made under laboratory and industrial scale process

Analysis of Variance for viable cell count (Log cfu ml^{-1} media) before drying

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	1	1.2394	1.2394	1.23941	35.93	0.000
Main Effects	2	3.0151	3.0151	1.50754	43.70	0.000
Harvesting Time	1	2.7068	2.7068	2.70682	78.47	0.000
Dry matter in Precipitate	1	0.3083	0.3083	0.30827	8.94	0.007
Curvature	1	0.2942	0.2942	0.29416	8.53	0.008
Residual Error	21	0.7244	0.7244	0.03450		
Lack of Fit	5	0.4222	0.4222	0.08443	4.47	0.010
Pure Error	16	0.3023	0.3023	0.01889		
Total	25	5.2731				

Analysis of Variance for % moisture at day 0

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	1	0.83762	0.83762	0.83762	12.01	0.002
Main Effects	2	5.09928	5.09928	2.54964	36.57	0.000
Harvesting Time	1	0.17912	0.17912	0.17912	2.57	0.124
Dry matter in Precipitate	1	4.92016	4.92016	4.92016	70.57	0.000
Curvature	1	0.05511	0.05511	0.05511	0.79	0.384
Residual Error	21	1.46407	1.46407	0.06972		
Lack of Fit	5	0.61810	0.61810	0.12362	2.34	0.090
Pure Error	16	0.84597	0.84597	0.05287		
Total	25	7.45608				

Analysis of Variance for viable cell count (Log cfu ml^{-1} media) at Day 0

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	1	0.3947	0.39467	0.39467	2.58	0.123
Main Effects	2	8.9666	8.96655	4.48328	29.36	0.000
Harvesting Time	1	3.8882	3.88815	3.88815	25.46	0.000
Dry matter in Precipitate	1	5.0784	5.07840	5.07840	33.26	0.000
Curvature	1	0.0770	0.07696	0.07696	0.50	0.486
Residual Error	21	3.2066	3.20664	0.15270		
Lack of Fit	5	2.5905	2.59051	0.51810	13.45	0.000
Pure Error	16	0.6161	0.61613	0.03851		
Total	25	12.6448				

Analysis of Variance for viable cell count (Log cfu ml^{-1} media) at Day 14 at 37 ˚C

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	1	0.0030	0.0030	0.0030	0.01	0.909
Main Effects	2	22.7565	22.7565	11.3783	50.39	0.000
Harvesting Time	1	10.1270	10.1270	10.1270	44.85	0.000
Dry matter in Precipitate	1	12.6295	12.6295	12.6295	55.93	0.000
Curvature	1	1.3091	1.3091	1.3091	5.80	0.025
Residual Error	21	4.7417	4.7417	0.2258		
Lack of Fit	5	2.5920	2.5920	0.5184	3.86	0.017
Pure Error	16	2.1497	2.1497	0.1344		
Total	25	28.8103				

Analysis of Variance for viable cell count (Log cfu ml^{-1} media) at Day 28 at 37C

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	1	1.3328	1.3328	1.3328	13.28	0.002
Main Effects	2	26.8289	26.8289	13.4144	133.68	0.000
Harvesting Time	1	7.1723	7.1723	7.1723	71.47	0.000
Dry matter in Precipitate	1	19.6566	19.6566	19.6566	195.88	0.000
Curvature	1	0.7002	0.7002	0.7002	6.98	0.015
Residual Error	21	2.1073	2.1073	0.1003		
Lack of Fit	5	1.1311	1.1311	0.2262	3.71	0.020
Pure Error	16	0.9762	0.9762	0.0610		
Total	25	30.9692				

Analysis of Variance for viable for cell count (Log cfu ml^{-1} media) after 1 month at 25 ˚C

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	1	1.2628	1.2628	1.26280	11.71	0.003
Main Effects	2	18.5270	18.5270	9.26348	85.89	0.000
Harvesting Time	1	9.9588	9.9588	9.95882	92.34	0.000
Concentrte solids	1	8.5682	8.5682	8.56815	79.45	0.000
Curvature	1	1.3549	1.3549	1.35485	12.56	0.002
Residual Error	21	2.2648	2.2648	0.10785		
Lack of Fit	5	1.5803	1.5803	0.31607	7.39	0.001
Pure Error	16	0.6845	0.6845	0.04278		
Total	25	23.4094				

Analysis of Variance for viable cell count (Log cfu ml^{-1} media) after 3 months at 25 ˚C

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	1	2.4492	2.4492	2.4492	31.87	0.000
Main Effects	2	33.6784	33.6784	16.8392	219.08	0.000
Harvesting Time	1	18.0614	18.0614	18.0614	234.98	0.000
Dry matter in Precipitate	1	15.6171	15.6171	15.6171	203.18	0.000
Curvature	1	0.7700	0.7700	0.7700	10.02	0.005
Residual Error	21	1.6141	1.6141	0.0769		
Lack of Fit	5	1.4433	1.4433	0.2887	27.04	0.000
Pure Error	16	0.1708	0.1708	0.0107		
Total	25	38.5118				

Analysis of Variance for % Moisture at 3 months

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	1	4.6538	4.6538	4.65385	25.92	0.000
Main Effects	2	6.7270	6.7270	3.36352	18.73	0.000
Harvesting Time	1	2.1420	2.1420	2.14204	11.93	0.002
Dry matter in Precipitate	1	4.5850	4.5850	4.58500	25.53	0.000
Curvature	1	0.3487	0.3487	0.34867	1.94	0.178
Residual Error	21	3.7711	3.7711	0.17957		
Lack of Fit	5	2.4462	2.4462	0.48924	5.91	0.003
Pure Error	16	1.3249	1.3249	0.08280		
Total	25	15.5006				

Appendix 11 - Minitab results for PB design experiment used to compare powders made under different drying time-temperature combinations

Analysis of Variance for % Moisture content at day 0

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	2	0.053633	0.0536333	0.0268167	2.48	0.139
Drying time	1	0.053333	0.0533333	0.0533333	4.93	0.054
Drying temp	1	0.000300	0.0003000	0.0003000	0.03	0.871
Residual Error	9	0.097333	0.0973333	0.0108148		
Lack of Fit	1	0.004800	0.0048000	0.0048000	0.41	0.537
Pure Error	8	0.092533	0.0925333	0.0115667		
Total	11	0.150967				

Analysis of Variance for % Moisture content after 3 months

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	2	0.07908	0.07908	0.03954	3.19	0.090
Drying time	1	0.05201	0.05201	0.05201	4.20	0.071
Drying temp	1	0.02707	0.02707	0.02707	2.18	0.174
Residual Error	9	0.11154	0.11154	0.01239		
Lack of Fit	1	0.01021	0.01021	0.01021	0.81	0.396
Pure Error	8	0.10133	0.10133	0.01267		
Total	11	0.19062				

Appendix 12 - Minitab results for CCD experiment used to study effect of initial moisture (before drying) and drying time (at 50 °C) in FB drier on *L. Casei* 431 stability during drying and storage

Analysis of Variance for viable cell count (Log cfu ml^{-1} media) at Day 14 at 37 ˚C

Source	F	P
Regression	5.28	0.025
Linear	8.70	0.013
Drying time	8.11	0.025
Initial moisture	9.29	0.019
Square	3.13	0.107
Drying time*Drying time	6.21	0.041
Initial moisture*Initial moisture	0.29	0.605
Interaction	2.74	0.142
Drying time*Initial moisture	2.74	0.142
Residual Error		
Lack-of-Fit	177.31	0.000