Microencapsulation of probiotics (*Lactobacillus acidophilus* and *Lactobacillus rhamnosus*) in raspberry powder by spray drying: optimization and storage stability studies

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A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of

Master of Science

in

Bioresource Engineering

ABSTRACT

Non-dairy probiotic foods are becoming popular because they do not pose problems of lactose intolerance and high cholesterol content while they offer an alternative from traditional sources and for personal preferences. The storage of probiotic products requires to maintain viability as high as > 10^6-10^8 CFU/unit. To tackle these aspects, the microencapsulation of probiotics in a raspberry juice powder by spray drying was studied. A combination of probiotics (Lactobacillus acidophilus NRRL B-4495 and Lactobacillus rhamnosus NRRL B-442) was chosen to maintain high viability along with multiple health benefits. The chosen microencapsulating agent-maltodextrin's role as carbon source was also assessed (in an MRS recipe) for its prebiotic potential. High temperatures employed during spray drying are detrimental to the probiotics and can be circumvented by sub-lethal thermal shock (50°C for L. acidophilus and 52.5°C for L. rhamnosus) before spray drying. Optimization of the process through response surface method was performed. Inlet temperature (°C), total solids: maltodextrin ratio, and inlet feed rate (mL/min) were fixed as independent variables while % recovery, % survival and color (ΔE-total color change) were the dependent outputs. The optimized model with all the significant factors had an overall desirability of 0.91. Storage study of the raspberry encapsulated probiotic powder was performed in glass containers stored at room and refrigerated temperatures for 30 days. CFU/g, water activity, color (powder and rehydrated liquid) were analyzed throughout the storage period. Since the CFU numbers do not necessarily correlate with functionality, basic probiotic characteristic tests like acid and bile tolerance and antibiotic sensitivity assay were performed before spray drying and after 30 days storage. At the end of storage, the three best responses with respect to % viability retention at cold and room temperature were chosen for electron image analysis of the microspheres. The optimized model was obtained at the following conditions: inlet temperature of 100°C, Maltodextrin ratio of 1:1 and inlet feed rate of 40 mL/min.

***Keywords**: sub-lethal effect, microencapsulation, optimization*

RÉSUMÉ

Les aliments probiotiques non laitiers augmentent en popularité puisqu'ils offrent un choix de consommation pour les problèmes d'intolérance au lactose, les problèmes d'hypercholestérolémie et selon les habitudes ou préférences personnelles. La durée de conservation de ces produits probiotiques doit préserver leur viabilité à plus de 10^6 – 10^8 UFC/portion. À cet effet, le séchage par atomisation d'un jus de framboise et de probiotiques a été étudié. Un mélange de probiotiques (Lactobacillus acidophilus NRRL B-4495 et Lactobacillus rhamnosus NRRL B-442) a été choisi afin de produire une haute viabilité et un aliment santé. La maltodextrine a été choisie comme matière d'enrobage et son rôle à titre de source de carbone a été déterminé (dans un bouillon de type MRS) pour son potentiel prébiotique.

L'utilisation de températures élevées durant l'atomisation est nuisible aux probiotiques ce qui peut être atténué par un pré-traitement thermique modéré (50 °C pour L. acidophilus et 52.5 °C pour L. rhamnosus) et ce précédant l'atomisation. L'optimisation du procédé fut effectuée par réponse de surface (RSM). La température d'entrée (°C), le ratio solide : maltodextrine et le débit d'alimentation (mL/min) ont été choisis à titre de variables indépendantes, alors que le % de récupération, le % de survie et la couleur des échantillons (ΔE – changement de couleur) ont été choisis comme variables dépendantes.

Le modèle optimisé, tenant compte de tous les facteurs significatifs, avait une conformité de 0.91. La qualité de conservation de la poudre probiotique de framboise a été étudiée dans des contenant de verre maintenus réfrigérés ou à la température de la pièce pour 30 jours. Les mesures de UFC/g, d'activité de l'eau, et de couleur (poudre et liquide réhydraté) ont été analysées durant la période d'entreposage. La fonctionnalité des probiotiques a également été étudiée (résistance à l'acide, à la bile, sensibilité aux antibiotiques) avant l'atomisation et à la fin des 30 jours d'entreposage. À la fin de l'entreposage, les trois meilleures réponses, en termes de % de survie pour les échantillons réfrigérés et à la température de la pièce, ont été sélectionnés pour l'analyse, par microscopie électronique, des microsphères produites lors de

l'atomisation. L'optimisation du procédé obtenue par le modèle RSM propose une température d'entrée de 100°C, un ratio de maltodextrine de 1 :1 et un débit d'alimentation de 40 mL/min.

ACKNOWLEDGEMENTS

I sincerely thank my supervisor, Professor Valérie Orsat, for accepting me as a graduate student and letting me work on what I really dreamt of. I am grateful for her considerate discussions and vast assistance in preparing and editing all my work, direction and reassurance.

I extend my gratitude to Dr. GSV Raghavan, Dr. Mark Lefsrud, Dr. Martin Chénier (Department of Food Science) and Dr. Michael Ngadi for generously letting me use their labs for experiments. I would like to thank USDA for donating the lactobacilli cultures. A special thanks to Dr. Darwin Lyew for his patience and willingness in helping me to set up my initial microbiology experiments and data analysis. I thank Mr. Yvan Gariépy for the technical support on the use of various instruments. I also thank Ms. Kathy and Ms. Aghdas (Department of Microbiology and Immunology, McGill University) for donating antibiotic disks and their continuous suggestions on microbiological techniques.

I am indebted to the great help by my friend and lab mate Kiruba Krishnaswamy for the continuous encouragement and suggestions regarding the experimental protocols. I am also grateful to my friends and colleagues Simona Nemes, Ashutosh Singh and Ramesh Murugesan in the help they provided for experimental set up and data analysis. I thank Ms Susan Gregus, Ms Abida Subhan and Ms Trish Singleton for their support and help with the administrative work throughout my stay here.

My heartful thanks to my family, well wishers and friends (quite a long list!) who have always been morally supportive and encouraging throughout my stay at Macdonald Campus of McGill University.

I dedicate my thesis to my loving and supporting Family Sailaja (Mom), Prabhakar Reddy (Dad) and my little brother Koushik (Chikku)

CONTRIBUTION OF AUTHORS

The present research work was performed by the candidate and supervised by Dr. Valérie Orsat. The entire work was performed in Dr. Valérie Orsat's lab and Post Harvest Technology lab.

The authorship of the manuscript under preparation is presented below:

Microencapsulation of probiotics *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* in raspberry powder by spray drying: optimization and storage studies. Authors: Kartheek Anekella and Valérie Orsat.

The research was presented as a poster at the NABEC, Burlington, Vermont July 2011 meeting with the following authors: Kartheek Anekella, Darwin Lyew and Valérie Orsat

Dr. Darwin Lyew's was involved with his expertise in setting up the microbiology experiments and the protocols and he provided his valuable suggestions in the data analysis.

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NOMENCLATURE

A_w - Water Activity

CFU- Colony Forming Unit

DE- Dextrose Equivalent

FOS- Fructooligosaccharide

GRAS- Generally Regarded As Safe

H^+-ATPase- Hydrogen ion Adenosine Tri Phosphatase

Hsp- Heat Shock Protein

kV- Kilo Volts

IBS/D- Inflammatory Bowel Syndrome/Disease

Ig G, M, A- Immunoglobulin G, M, A

K_{max} - Maximum specific growth rate constant

LAB- Lactic Acid Bacteria

MD-Maltodextrin

MRS- de Man, Rogosa and Sharpe

OD- Optical Density

Pa- Pascals (SI unit)

PBS- Phosphate buffer saline

Rpm- revolutions per minute

PET- Polyethylene terphthalate

SEM- Scanning Electron Microscope

Tsl- Sub-lethal Temperature

Tg- Glass transition Temperature

USDA- United States Department of Agriculture

µL- microlitre

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INTRODUCTION

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1.1 Introduction

Versatility in consumption with added health factors in addition to nutrition and flavor are characteristics of a functional food. In addition to exorbitantly high-priced health care and medicines, the desire for better quality of life (Vasiljevic and Shah, 2008) encourages novel functional foods consumption with multiple health benefits apart from basic nutrition. The link between diet and health is growing stronger day by day. Healing an illness through particular food consumption to restore natural defense with fewer side effects than medicine is always appealing to all age groups (Reid, 2002). Hence research and development on functional food components like phytochemicals, probiotics, and omega fatty acids is presently an important focus of the food industry (Guarner and Schaafsma, 1998). Functional foods act beyond the normal nutrition.

The term "probiotics" was first used by Lilly and Stillwell in 1967, although this concept existed since ancient Greek times. Probiotics represent over 65% of the functional food market (Agrawal, 2005). More than 2000 probiotic products were launched in the year 2008 (Jankovic et al., 2010), and more than 1700 research articles were published with key word "probiotics" in 2010 till June 2011 (an average of 3.8 publications per day). Clearly the numbers are raising significantly due to the reported multiple health benefits and the increase in public awareness (Stanton et al., 2001). Though the health benefits are widely established scientifically, the technological hurdles and solutions in probiotic product formulation is yet to be firmly established. In US alone, the probiotics product sales were $764 million in 2005 and expected to be 1.1 billion by 2010 (Hibberd and Davidson, 2008). Probiotics are considered as "good buddies" to human health. Although historically, probiotics were products of the pharmaceutical industry, the current trend is moving towards the health food sector, making Hippocrates' statement *"Let food be your medicine"* true once again.

Lactic fermented foods represent over 20% of all fermented foods (Reddy et al., 2009) and the variety of available products has more than tripled in number. Consumer interest in probiotic products is rapidly increasing due to their multiple health benefits (Prado et al., 2008). Fermented drink consumption dates back to pre Egyptian era and their health benefits are

traditionally well known. The main goal of any food industry is to increase the versatility in consumption of different forms of food without losing its basic properties which are addressed in the current research.

1.1.1 Health Benefits of Probiotics

Probiotics are one of the prime gut microflora inherited by infants from their mother's vagina as well as breast feeding. It has been demonstrated that these microbiota protect the gut from enteric pathogens (Wang et al., 2010). Probiotic dosage may not be a cure but rather an addendum to the regular medicine prescribed to patients especially suffering from gastro-intestinal related disorders. Probiotics help in immune modulation, disease prevention and/or symptoms alleviation, for diarrhoea, lactose intolerance (by hydrolyzing lactose to glucose and passing it through the intestinal tract), allergies, colon cancer and atopic eczema (by stimulating the production of IgM and IgG as inflammatory response). Probiotics may also be useful for patients with high blood pressure, coronary diseases, intestinal inflammatory diseases like irritated bowel syndrome, constipation, disordered growth of intestinal bacteria, bladder and cervical cancer, upper respiratory tract and urinary tract infections (Holzapfel et al., 1998; Isolauri et al., 2001; Mattila-Sandholm et al., 2002). Probiotics exert an antimicrobial effect on pathogens like *Campylobacter jejuni, Clostridium difficile, Helicobacter pylori* by producing IgA and antimicrobial bacteriocins against these pathogens. They prevent disruption of cytoskeleton by pathogens and improve mucosal barrier function and electrolytes transport.

Probiotics have also recently been claimed to treat alcoholic cirrhosis (scarring of the liver and poor liver function as a result of chronic liver disease) by regulating interleukin secretion,. They also inhibit the cholesterol synthesizing enzymes which inhibit hydroxyl methyl glutaryl-CoA reductases and co-precipitate cholesterol with the de-conjugated bile salt via fecal route. Carcinogens are metabolized by specific binding and altering metabolic activities of intestinal microflora thus acting as antimutagenic and anticarcinogenic. Allergies during pregnancies, urogenital infections, pancreatitis, encephalopathy, Crohn's disease, ulcerative colitis, travelers' diarrhoea can be treated by probiotic dosage (Harish and Varghese, 2006).

Studies have demonstrated that strains of *L. acidophilus* and *L. rhamnosus* reduce the toxicity levels of ochratoxin A (a common food mycotoxin) from foods up to 50% (Piotrowska and Zakowska, 2005). Recent studies have shown that probiotics could have therapeutic effects on stress, anxiety and mood behavior (Jankovic et al., 2010).

However, the usage of probiotics for a long term and its effect on immune-regulatory system has not yet been assessed (Vasiljevic and Shah, 2008). Furthermore, there are no reports specifically dealing with the probiotic effects on health for under weight infants, immuno-compromised patients and patients with chronic inflammatory bowel syndrome. But all the above mentioned health benefits are strain specific and hence administered according to illness if consumed as a pharmaceutical product. Studies have proven that even dead probiotic (heat treated or UV killed) cells can stimulate an immune response although not comparable with all the health benefits of live cells, however this research is still at its initial phase (Kataria et al., 2009).

Use of berries with probiotics has been tested for uropathogenic and urogenital disorders. The proanthocyanins present in cranberry can modulate the immune system in conjunction with probiotics (Reid, 2002). This supports our premise of studying the combination of probiotics organisms with raspberry juice.

1.2 Probiotic foods

An interesting observation noted by Nobel laureate (1908) Metchnikoff, of Bulgarian peasants who lived for more than 87 years which was an unusually high life expectancy in 1900s was attributed to the large amount of fermented milk in their diet. As discussed earlier, probiotics are active against colon infections, gastro intestinal disorders and in the maintenance of healthy gut microflora (Gibson and Roberfroid, 1995). Formulating and enriching foods with probiotics would not only improve public health but also the diversity in food choices.

Traditional dairy probiotic foods are present widely like *Dahi* (*Lactobacillus* strains) in India, *Kindrimo, Nono* and *Warankasi* (types of cheese with *Lactococcus* and *Lactobacillus* strains) in Nigeria, and *Amasi* (fermented milk with *Lactococcus* species) in Zimbabwe (Ukeyima et al., 2010).

Though initially most probiotics were used as fermentation additives, the application development are now driving towards health additives. The food matrix can act as buffer during storage as well as in stomach transit until the probiotic is delivered to the intestinal tract (Ranadheera et al., 2010). The probiotic microorganisms present in food should survive in significant numbers of at least 10^6-10^8 CFU/g though the numbers vary from strain to strain (Boylston et al., 2004; Chávez and Ledeboer, 2007; Ishibashi and Shimamura, 1993; Kailasapathy and Rybka, 1997) and exert their effect even under extreme conditions. The dosage levels are still unclear partly because it is dependent on the diet and preferences of the individual. And not all probiotics exert their health benefits in a similar way. However, very few probiotic products in the current market declare the actual content of microorganisms present.

Maintenance of existing endogenous gut microflora with added benefits like prevention of various cancers, diarrhoea, allergy and other gastro intestinal disorders has lead to the huge commercialization of this health sector. Probiotics are considered GRAS (Generally Regarded As Safe) in foods, although it is strain specific. Probiotics are usually sold as capsules, powders and a combination of different species might have multiple advantages (Timmerman et al., 2004). In many cases, the functionality of probiotics is an issue due to the poor quality in the standards of preparation of probiotics foods and lack of clinical studies (Azcarate-Peril et al., 2009; Hamilton-Miller and Shah, 2002; Klaenhammer, 2000; Timmerman et al., 2004).

Probiotics in a food carrier must reach the gut effectively while they must withstand harsh acidic and bile environments and enzymes. Most probiotic foods in the current market are refrigerated dairy-based products (Burgain et al., 2011) while preparations of non-dairy foods are attracting a broader range of consumers with different preferences. Probiotic foods must be consumed regularly as the adverse conditions in the stomach may continuously deplete the number of live cells (Kearney et al., 2009). On the other side of the coin, probiotics

might have safety issues where the possible risks associated could be systemic infection or lethal metabolic activities, undue immune stimulation in immune-compromised patients and gene transfer. However the vast health benefits outnumber the safety issues for which there is only little evidence (Saarela et al., 2000).

1.2.1 Probiotic food market in Canada

Probiotics are limited to non-pathogenic microorganisms and are regulated by the Natural Health Products Directorate (NHPD). There are several food ingredients as well as tablet formulation widely used in Canada as probiotic supplements. Some probiotic food products produce in Canada include Probiostick (*Bifidobacterium Rosell-175* and *Lactobacillus Rosell-52*) by Institut Rosell, Oasis juice (*B. bifidus* and *L. acidophilus*) by Lassonde foods, YogActive (*L. acidophilus* beads with cereal) by Belgo and Bellas, Yoptimal immuni+ (*L. acidophilus* LA-5 and *B. lactis* BB-12) by Yoplait and Activia yogurt by Danone (*B. animalis* DN 117-001).

Unless Health Canada approves them as drugs their potential health claims are not validated. Most of the probiotic products have benefitting claims like structure/functional modification (role of a nutrient or ingredient intended to affect normal structure or functions of the human body), disease prevention (reducing the risk of developing a specific disease or abnormal physiological condition), illness treatment (refer to the diagnosis, cure, alleviation or prevention of a disease, disorder or abnormal physical state or symptoms). Different countries have different policies on probiotics depending on the awareness of the probiotics. For example most UK based probiotics food products publicize the coronary and other disease health benefits, whereas in Germany the claim is for general disease resistance.

1.2.2 Non-Dairy Probiotic Foods

Non-dairy probiotic foods offer an advantage for individuals with lactose intolerance and high cholesterol which is a major drawback of dairy-based probiotics (Prado et al., 2008). Traditional and economic reasons can also be accounted for the encouraged use of non-dairy probiotics. Dairy allergens are absent in fruits and vegetables and in addition they contain dietary fibers, phytonutrients and antioxidants which makes them acceptable by almost all age groups of the population (Luckow and Delahunty, 2004; Yoon et al., 2006). It is equally important for non-dairy formulations that they help in maintaining the desired viability and functionalities of probiotics.

Kanji is an ayurvedic fermented rice drink in India well known for its probiotic content (Kumar Reddy et al., 2007). *Boza is a* traditional non-dairy probiotic food in Bulgaria, Albania and Turkey which is a colloid suspension composed of wheat, rye, millet and saccharine. The chief microbes present in this drink are yeast (*Candida* sp.) and LAB (lactic acid bacteria which include *Lactobacillus* spp. and *Leuconostoc* spp. Etc,). *Kimchi,* like *Sauerkraut* (fermented cabbage, onion, carrots, etc.) is prepared with various *Lactobacilli* and is famous in Korea. *Bushera, Mahewu* and *Togwa* comprising of similar ingredients (sorghum, millet and maize porridge) are few examples of non-dairy fermented probiotic drinks widely popular in African countries. *Pozol*, a refreshing fermented drink made from maize flour balls in 1% lime solution, is popular in eastern Mexico. *Hardaliye* is a grape fermented chilled probiotic juice with several *Lactobacillus* sp. consumed since ancient period in Turkey (Angelov et al., 2006; Prado et al., 2008).

Oat-based probiotic (*L. plantarum B28*) drink has also been developed by Angelov et.al. (2006), with high beta-glucan content. Tomato, beet root and cabbage juice were successfully evaluated as potential probiotic carrier juices (Yoon et al., 2004). *Grainfields*- whole gram liquid, *Vita Biosa*- comprising of herbs and probiotics (Denmark), *Rela* and *Proviva* (Sweden), *Gefilus*

and *Biola* (Norway), are few of the commercially available non-dairy probiotic beverages in Europe.

Dry sausages (Muthukumarasamy and Holley, 2006), soy milk (Shimakawa et al., 2003), oat based bar (Ouwehand et al., 2004), chocolate and chocolate mousse (Maillard and Landuyt, 2008; Possemiers et al., 2010), table olives (Lavermicocca et al., 2005), cabbage juice (Yoon et al., 2006), starch, grape, orange juice, apple juice, tomato and banana juice (Tsen et al., 2007) are few non-dairy food products being worked on. Most of these non-dairy carriers were assessed for the probiotic survival in free and encapsulated states, and as expected the encapsulated probiotics had a better survival with minimal interference with the sensory and nutritional properties of the food. Due to the global boom in the probiotic food market, the International Probiotic Association (IPA) has suggested the usage of a "quality seal" to identify and qualify the right probiotic foods.

1.3 Canadian Regulations on Probiotic Foods (Guidance Document, Health Canada, 2009)

In the past there have been few products in Europe with fake and imaginative probiotic species like *Bifidobacterium digestivum, Digestivum essensis, Lactobacillus anti-caries* etc. Hence enforced and detailed regulations are critical in any country for successful probiotic product launch. Dose information is essential although higher counts do not necessarily correlate to better health claim (Saxelin, 2008).

A probiotic is categorized under Natural Health Product (NHP) by the Food and Drug Act and intended for therapeutic usage. Functional claims of probiotics must include or target:

- Scientifically proven physiological effects of the probiotics ("improves digestion", "promotes regularity", etc.). If the study is performed only on sick patients it may not be considered as scientifically supported;
- Maintaining and supporting limited body functions;

- If the manufacturing involves latest technology like microencapsulation, consultation with the Food Directorate is necessary prior to commercialization of the product if the technology meets the definition of "novel";
- The species should not possess the risk of transferable antibiotic resistance which might cause multi-drug resistance related diseases;
- The stability and viability of the probiotics serving size in the food must be present until consumption. Quality assurance dossiers should be maintained for all these products and ingredients.

1.3.1 Labelling "Probiotics"

- The claimed health effect should not be false, misleading or deceptive
- Should accompany a statement of proven health benefit
- Latin name (genus and species name) of all the bacteria present should be printed
- Level of probiotic content- CFU size should be given along with the dosage

1.4 Strain Selection

Probiotics broadly comprise species and sub species of LAB- *Lactobacillus, Bifidobacterium, Lactococcus, Streptococcus* and *Enterococcus* and non LAB-species and sub-species of *E. coli*, *Bacillus, Pediococcus* and yeast- *Saccharomyces sp*. Usually probiotics derived from human origin exert their full potential effects and since probiotics are GRAS they are non-pathogenic (Coeuret et al., 2004; Collins et al., 1998; Saarela et al., 2002). Strains from human origin have better chance of outcompeting in growth than the resident microflora. But as long as the probiotic strain is able to exert its health benefits, its origin is not a major concern. *Lactobacillus* species are generally aerotolerant or microaerophilic or obligate anaerobes, non-spore forming and Gram positive rods, devoid of cytochromes, catalase negative and strictly fementative (lactic acid as end product) (Stiles and Holzapfel, 1997; Vasiljevic and Shah, 2008).

1.4.1 *Lactobacillus acidophilus*

L. acidophilus is probably the most explored lactobacilli which are found in most probiotic foods in the market. It is naturally found in human gastrointestinal tract and vagina and microaerophilic. Strains of *L. acidophilus* are homofermentative (metabolic end product is only lactic acid) having S-layers which help in surface adhesion and hence exert their probiotic property at the same time as they compete with pathogens and eradicate them. *L. acidophilus* enhances phagocytosis, prevents kidney stones, secretes lysozomal enzymes and reactive oxygen species (ROS) that can help in treating Crohn's disease. Patients administered with this strain have experienced deconjugation of bile salts and hence reduced serum cholesterol by lipid metabolism to treat coronary heart diseases (Gilliland, 1981). *L. acidophilus* strain conducted peroxidation of linoleic acid and thus scavenged the free radicals (Lin and Yen, 1999). *Helicobacter pylori* and *Salmonella enterica* were eliminated due to extracellular proteins and cytoskeletal rearrangements by *L. acidophilus* (Coconnier et al., 1997; Lorca and de Valdez, 2001). Live probiotic *L. acidophilus* ATCC strains eliminated adhesion and invasion of *E.coli* (Resta-Lenert and Barrett, 2003). Acute diarrhoea was reduced in children fed with probiotic dosage compared to placebo group (Simakachorn et al., 2000).

1.4.2 *Lactobacillus rhamnosus*

L. rhamnosus is homofermentative and mostly used in yogurts as a natural preservative. It attaches to the epithelial cell wall lining of the stomach and encourages the growth of useful organisms. It can survive in extreme acidic and bile conditions. Eczema affected children fed with *L. rhamnosus* diets showed a significant decrease in gastro intestinal activity by reinforcing gut defense through immunomodulation and immunoregulation (Isolauri, 2001; Rosenfeldt et al., 2004). It is also proven very effective against *Clostridium difficile* associated diarrhea and traveller's diarrhea, suppressing the side effects of *H. pylori* antibiotics, alleviating symptoms of Crohn's disease, preventing dental caries, atopic infections, and increasing the non-specific antibody secreting cells against rotavirus-induced diarrhea (Jankovic et al., 2010; Vasiljevic and

Shah, 2008). *L. rhamnosus* can also interrupt the transportation of enterococcus in kidney-related infections. *L. rhamnosus* aids postmenopausal women in fighting against chronic urinary tract infections. Studies have proven that even dead *L. rhamnosus* was able to trigger an inflammatory response, but slightly lesser than live cells. This could be an advantage when there might be possible risks associated with the live cells, though a high dosage is recommended (Kataria et al., 2009). Elevation of serum concentration of interleukin-10 (a better immune response) was reported in children fed four weeks with *L. rhamnosus* strains and *L. acidophilus* (Rosenfeldt et al., 2004).

1.5 Potential Probiotic Characteristics

Initial assessment of basic probiotic characteristics might give an insight into their performance during harsh processing conditions (Ross et al., 2005). In order to be qualified as a potential probiotic species, the microbes should possess initial screening characters for (Kailasapathy and Chin, 2000; Ledeboer et al., 2006; Prado et al., 2008):

- Acid resistance
- Bile salt tolerance
- Antibiotic resistance
- Cholesterol assimilation
- Antimicrobial (due to bacteriocins) and antimutagenic activity
- Adherence to epithelial cell wall
- Immuno-modulation and stimulation etc.

Once administered, probiotics prevent the colonization potential of pathogens by producing antimicrobials or competing for the cell surface and mucin binding sites, hence the pathogens translocation is prevented from the colon to lungs. Certain lactobacilli participate in reducing oxidative damage and help in the prevention of cancer, atherosclerosis and other chronic diseases. Lactobacilli ferment the indigestible long chain carbohydrates and fibers to

short chain fatty acids which inhibit cholesterol synthesis in liver and relocate cholesterol from gut to liver (De Boever et al., 2000; Doncheva et al., 2002). These properties are strain specific and hence each strain should be checked individually to be qualified as probiotic (Verdenelli et al., 2009). Presently industries aim at formulating probiotic products not only with higher shelf life but also with high retention of the probiotic characteristics.

The probiotics act on host pathogens by reducing the pH in the gut by their fermentation end products (lactic acid, propionic acid), compete for food against pathogens or produce bacteriocins (Kailasapathy and Chin, 2000).

1.6 Combination of Probiotics

Klaenhammer (2000), described the issues associated with the potential probiotics health benefit as follows:

1. The variability of the stomach environment for which the probiotic is aimed for, might complicate the study of their health benefits;
2. Identification of the specific species with viability and activity might be confusing during clinical trials;
3. Single strain might induce multiple effects which may be difficult to study.

It was proven with various animal studies that the use of one or more strains/species of probiotics can have beneficial effect. Furthermore, since probiotics have multiple health benefits it is logical that a mosaic of probiotics could help in exerting all the necessary benefits (Famularo et al., 1999; Sanders, 1999). Using a cocktail of probiotics (rather than a monostrain) inhibited pathogens adhering to the intestinal mucus walls, however, the health benefit combinations are strain specific and each strain must be assessed individually (Collado et al., 2007). Multi-strain probiotic feeding had better performance in cell adherence as well as reduction in the mortality rate of chicken (Jin et al., 1996). Multi species probiotic dosage (*B. bifidum and L. acidophilus*) can prevent imbalance in microflora, antibiotic diarrhea and

dysbiosis (microbial imbalances) due to ceftriaxone antibiotic treatment for upper respiratory tract infection (Zoppi et al., 2001).

In a study conducted by Perdigon et al. (1990), multi strain (*L. acidophilus* and *L. casei*) fermented milk was fed to mice while *Salmonella* was injected into their liver and spleen and they survived for a longer time than the mice fed with a monostrain. *E. coli O157:H7* shedding in ruminants were reduced by probiotic dosage (*L. acidophilus* and *Ec. faecium*) in sheep (Lema et al., 2001). Though the exact mechanisms of multi strain/species beneficial effects are unknown, colonization resistance in the gut is a major reason. Since the probiotics must withstand acid, bile, pancreatic and other enzymes in the stomach, a combination of species may be helpful to ensure at least partial survival of the probiotics (Timmerman et al., 2004).

In summary the advantages of multi strain/species probiotic dosage include (Timmerman et al., 2004):

- Additive health benefits of several individual strains in a single dose particularly for users with several gastrointestinal ailments;
- Enhanced survival of at least one or more strain/species;
- Creation of probiotic niche and colonization against pathogenic microflora;
- Synergistic and symbiotic (exchange of metabolites, growth factors, production of extracellular polysaccharides) relations between the species;

Further investigation is required to check the possibility of antagonistic activity by production of peroxides or antimicrobial compounds like bacteriocins by the multi strain/species mixture (Harish and Varghese, 2006).

1.7 Prebiotics

Prebiotics are simply defined as non digestible foods that are essential for the viability or stimulation of growth of microflora in the gastro-intestinal system (Gibson and Roberfroid, 1995). Fructo/galacto oligosaccharides, inulin and maltooligosaccharides are the most widely

studied prebiotics (Kaur et al., 2002). Prebiotics promote the growth of probiotics in the colon by stimulating proliferation and host immunological response or probiotic activity. The effectiveness of prebiotics depends on the ability to influence the growth conditions of probiotics by selective utilization. Other gut microorganisms may also compete with these prebiotics (Huebner et al., 2007). There are currently probiotic products in the market like beverages, gums, chocolates, juices, infant foods etc which are fortified with prebiotics. Foods that naturally contain prebiotics are desirable, because they avoid the necessity of adding extra ingredients and thus making the product natural and reducing the cost of production.

Prebiotics have been shown to improve the growth rate and reduce the fermentation time of probiotics (Desai et al., 2004) and in the presence of certain prebiotic food components the probiotic properties can be enhanced (Saxelin et al., 1999). They can also inhibit pathogen multiplication conferring additional benefits to the host (De Souza Oliveira et al., 2009). Prebiotics, besides being selective to the probiotics, are broadly distinguished from some of the dietary fibers by the fact that prebiotics are hydrolyzed or absorbed in the upper intestinal tract. So when they reach the lower intestinal tract they serve as food for probiotics as well as the host (Gibson and Roberfroid, 1995). But this differentiation has taken a new turn to co-relate between the physiological role of the dietary fiber and the health benefits conferred suggesting new definitions by the American Dietetic Association and American Association of Cereal Chemists.

Since prebiotics and probiotics have a synergistic interaction, consuming both simultaneously might offer some advantage (Mattila-Sandholm et al., 2002). However, the combined effects of prebiotics and probiotics are highly strain- and substrate-specific (Huebner et al., 2007; Nagpal and Kaur, 2011). The combination of prebiotics and probiotics, collectively called ‘synbiotics’, gained a wide popularity among functional foods (Collins and Gibson, 1999).

In summary prebiotics should possess the following properties (Kolida and Gibson, 2007):

- resist host digestion and absorption in upper gastro-intestinal tract;

- selective substrate for microflora (specifically probiotics) in the gut;
- modify the gut to a healthier environment;
- induce multiple systemic and local health benefits.

1.7.1 Dietary Fibers

Dietary fibers, though ambiguous in defining, broadly encompass the edible and non-digestible food biopolymers present in various plant foods including but not limited to resistant and non resistant starches, cellulose, gums, lignin, fructo-oligosaccharides etc. In 2001, the American Association of Cereal Chemists defined dietary fibers as *". . . the edible parts of plant or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine . . . promotes beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or glucose attenuation..".*

It is recommended to take 20-35 g/day in adults and 5 g/day in children of dietary fibers through daily food intake. Dietary fibers usually should be present between 0.03-0.06 g per gram of solid foods and 0.015-0.03 g in liquid foods (De Souza Oliveira et al., 2009). Most dietary fibers are not digested in the stomach or upper intestinal tract which offers an advantage of slow digestion and acting as prebiotics to *Lactobacilli* (Marlett et al., 2002). Dietary fibers act as good adhering material for the probiotics and hence can help to deliver them live/metabolically active to the gut (Crittenden et al., 2001). Digestion of fiber rich foods in stomach is slow and thus increases the nutrient absorption intake. The slow processing also has benefits like relaxed laxation and prevention of flatulence, rate of glucose formation is slow which is highly advantageous for patients suffering from diabetes mellitus.

The soluble dietary fiber may increase the viscosity of the transit thus reducing the cholesterol reabsorption. And also the fermentation products of dietary fibers (acetic acid and propionic acid) may interfere with the metabolic cholesterol production in the stomach. Most of the soluble and a portion of insoluble fibers are digested by the colonic microflora in the

large intestine (Marlett et al., 2002). Inclusion of citrus fibers (from orange and lemon) as prebiotic sources had a beneficial effect on survival of probiotics with good sensory perception in yogurt (Sendra et al., 2008).

Since harsh processing steps like high temperature treatments, extrusion etc. alter the physico-chemical properties of fibers, there might be a significant change in their function after processing. The long chain fibers may break down into smaller molecules and sometimes sugars might undergo Maillard reaction if subjected to high temperatures. However, little research is done on the effects of processing on fibers and their functionality. The smaller fibers may be retained for a very short time not giving enough time for absorption and fermentation which was the case for rice bran milling (Mongeau, 2003).

1.7.2 Starch Derivatives

Starch is indigestible in the upper colon of the human body and hence it reaches the large intestine where it is metabolized by the intestinal flora by fermentation. It is degraded to short chain acids and thus beneficial to both the micro flora as well as the host (Gibson and Roberfroid, 1995). Resistant maltodextrin (hydrolyzed from starch) was proven to increase the bulkiness of feces and reduce the problems of constipation from altered gut microflora. Maltodextrin is metabolized by microflora to produce short chain fatty acids and lower the pH which destroys the pathogens (Fastinger et al., 2008).

Maltodextrin was added as a carrier during spray drying of probiotics which yielded significant number of live cells even after the imposed thermal stress retaining the probiotic properties like acid, bile tolerance, cholesterol assimilation etc (Reddy et al., 2009). Addition of corn starch at 2% concentration as a prebiotic increased the recovery of the *L. acidophilus* cells after spray drying, while a concentration of corn starch above 4% acted as a microencapsulating agent in combination with alginate (Sultana et al., 2000). Iyer and Kailasapathy (2005) have studied the effect of the inclusion of corn starch (1%) as a prebiotic and microencapsulating

carrier material under *invitro* acidic and bile conditions for *L. acidophilus* and proved its efficiency in improving its viability. Starch also acts on the surface of adherence to the probiotics during processing and transport into the gut making their synbiotic formulation robust and metabolically active (Mattila-Sandholm et al., 2002). The viability during storage was undoubtedly increased with the cells' coating with microencapsulation with starch prior to spray drying (O'Riordan et al., 2001). This concept can be extended in protection against high acidic and bile concentrations during the gastro-intestinal transit.

1.8 Raspberry

The physico-chemical properties of the food matrix in which the probiotics are delivered to the host also has a major effect on the absorption and exertion of a health benefit. The same probiotic strain may have varying health effects depending on the food matrix (Ranadheera et al., 2010). So it is extremely important that the food matrix be non toxic, uninterrupting the bioactivity of the probiotics and if possible should act as prebiotic. The synergistic (synbiotic) effect of prebiotics and probiotics should be well studied before being incorporated into the food. Raspberry has a high amount of dietary fibers with good absorption characteristics which could potentially serve as a carrier and microencapsulating agent as well as a prebiotic (Chiou and Langrish, 2007). Yogurt supplemented with fruit juice with high fiber content have shown higher survivability of probiotics (Kailasapathy et al., 2008). Fruits also attribute other nutrients like vitamins and minerals which are essential to probiotics as well. The ascorbic acid present in certain fruits have an oxygen scavenging effect which is also responsible for the higher survival of the probiotics as they create micro-aerobic conditions. (Dave and Shah, 1997).

Raspberries (*Rubus strigosus*) are perennial fruits and they are widely cultivated in most of the cold temperature climate countries. They have high nutritional values and they are consumed either in fresh or frozen form, where fresh is more common. The USA and Canada contributed around 65000 and 12000 metric tonnes respectively of raspberries for the year 2009 (David, 2010), those numbers being significantly higher than previous years. But at the

same time, frozen raspberry economic returns to the growers are rapidly decreasing (0.4 pounds per capita) for the reason that consumers are interested in buying “fresh” fruits (Cook, 2009). So there seems to be an opportunity to develop new fruit-based products with added nutritional benefits such as probiotics while at the same time, maintaining the nutrients and organoleptic quality of the raw fruit.

Raspberries, apart from their nutritional health benefits (Table 1.1) to human beings, have a high dietary fiber component that could potentially act as prebiotics to the *Lactobacilli*. The sugars present in raspberry can protect cells from thermal injuries and also maintain storage survival as several studies have shown that various sugar sources like trehalose, sucrose, etc, are thermoprotectants (Carvalho et al., 2004).

Table 1.1: The nutritional values of raspberries (per 100 g):

Nutrients	Value/100 g
Calories	52 Kcal (220 kJ)
Water	85.75 g
Total Lipids (Fats)	0.65 g
Total dietary fiber	6.5 g
Protein	1.2 g
Total sugars (Fructose+ Glucose+ Sucrose)	4.42 g
Potassium (K)	151 mg
Phosphorus (P)	29 mg
Magnesium (Mg)	22 mg
Calcium (Ca)	25 mg
Sodium (Na)	1 mg
Other trace elements (Mn, Cu, Zn, Se)	1.872 mg
Total Carotenoids	0.166 mg
Vitamins B1-B6	1.073 mg
Vitamin C, total ascorbic acid	26.2 mg
Vitamin E	3.39 mg
Vitamin A	33 IU
Vitamin K	0.007 mg

Source: USDA Nutrition Database, No: 09302

1.9 Production of Functional Food ingredients

In the production of food ingredients, process modifications may alter the probiotics' properties. So care must be taken to ensure that the food composition and process variables are optimized in such a way that there is a minimal effect on the probiotics and their health promoting properties (Jankovic et al., 2010).

1.9.1 Changes in formulation:

- Mono strain/species or multi strain/species? If mono strain, all health benefits have to be provided while maintaining high viability. If multi species are considered, is there any chance of antagonistic activity?
- Attention must be paid to concentration of nutrient media, pH, growth phase, stress etc, presence of anti-microbial compounds, peroxides and incompatible solutes.
- Consider various prebiotic sources, growth factors, etc.

1.9.2 Modification in processing and production:

- Production methods need to be selected carefully: spray/freeze/vacuum drying-dehydration
- Probiotics culture responses must be determined for high temperature, pressure stress, sub-lethal stress adaptation, alteration/ denaturation of metabolic enzymes etc.
- Conditions of lethality of microbes and destruction of critical functional components must be determined.
- How does processing affect yield and sensory properties?

1.9.3 Changes in shelf life and consumption:

- Determine appropriate storage container and storage conditions as- air, humidity, temperature and water activity.
- Maintain total viable count >10^6–10^8 CFU/unit. Sometimes cells might be alive but non-replicating or without metabolism.
- Establish the nutrient depletion during storage.
- Determine what might be the survival in the gastrointestinal tract (against gastric, bile, microbial antagonism) to ensure adequate activity for expected health benefits.

Several methods for improving the viability of the probiotics have been proposed with an effect/modification on the process or the organism itself. Inclusion of prebiotics, optimizing production operation, selection of a cocktail of probiotic organisms, modification (physically or genetically) of the probiotics prior to microencapsulating are some of the possible solutions to ensure the bioactivity of the probiotic products.

1.10 Microencapsulation

Microencapsulation with respect to a food application, involves the reversible capture of active bio-molecules in a stable core and delivering it safely to a given target. Functionality and bioavailability are the key factors driving the microencapsulation of food products. It is widely used to preserve and control flavor, color, texture, functional properties and to maintain the potential health benefits. Probiotics, minerals, vitamins, phytosterols, fatty acids, lycopene and antioxidants are some of the compounds which have been delivered through microencapsulation in recent years. All the three forms of matter can be entrapped using microencapsulation techniques. Microcapsules are commonly spherical in shape but can take any random form with an outer layer. Sometimes they may be double walled depending on the carriers added (Gardiner et al., 1999; Gharsallaoui et al., 2007; Kailasapathy, 2002; Madziva et al., 2005).

Early on, microencapsulation was mainly used to mask off-flavors of food ingredients and for conversion of liquids to solids. Encapsulation helps in the physical separation of sensitive viable cells from the external adverse environment thus improving the viability of cells (Sultana et al., 2000; Weinbreck et al., 2010). Protection through physical barrier is considered an easy and efficient way of protecting these sensitive micro organisms against adverse conditions (Krasaekoopt et al., 2003). These applications can be extended towards formulation of various food products and ingredients in the health food sector.

Several methods of microencapsulating probiotics include: spray drying, freeze drying, extrusion, coacervation, chemical methods using Ca-Alginate, k-carrageenan, gums (xanthan, arabic, etc.), starch, etc. All methods have their pros and cons. The substance to be microencapsulated is called the core material or bioactive and surrounding it is the wall material. The sphere dimensions vary depending on the physico-chemical interactions between the core and wall and also the technique of microencapsulation. It could be a simple sphere or an irregular sphere or multiple core spheres, etc. The size of the microspheres usually ranges between 0.2 to 5000 micrometers.

Challenges in bioavailability and viability of probiotic microorganisms inside the gut are of prime concern for the current health-food industry. The purpose of microencapsulation is not just a protection through physical barrier but also a controlled release of the functional probiotics passing through the stomach to effectively reach the intestines (Picot and Lacroix, 2004). Once the encapsulated, bioactive cores reach the targeted organs, it is ideal for the microencapsulated matrix to release them in a controlled fashion. Undesirable interactions between bioactives and external medium can be prevented when they are microencapsulated. The release of the product follows a predetermined kinetics when microencapsulated which can be modified depending on the system to be delivered (Ré, 1998).

In summary, the microencapsulation in food industry is employed to (Shahidi and Han, 1993):

- reduce the core reactivity to the environmental factors;

- decrease the transfer rate of the core material to the outside environment
- promote easier handling
- for masking the taste and for controlled release of core material

When probiotics are delivered to the host, they must retain viability and potential probiotic properties even after extensive processing techniques without altering food properties (flavor or textural changes) (Krasaekoopt et al., 2003; Rao et al., 1989). Microencapsulation technique is widely exploited to improve the shelf life and to retain probiotics health properties (Semyonov et al., 2010). Encapsulation of probiotics ensures active maintenance even under high water activity during storage (Crittenden et al., 2001; Weinbreck et al., 2010).

The stability and activity of microcapsules in gastro-intestinal system is dependent on several factors like pH of the core and the gut, particle size, chemicals present in the microencapsulating material and enzymes present in the gut. Several synthetic polymer microencapsulating agents were employed for an enhanced bioavailability of microorganisms but challenges still exist on survival against adverse and harsh conditions in gut. There were many successful attempts in microencapsulating probiotics in several media like Ca-alginate, k – carrageenan etc., but their microcapsule size may be a drawback for their incorporation into most powdered foods due to large size and undissolvability.

1.11 Spray Drying

Spray drying is one of the oldest methods of encapsulation since 1900s used initially for flavor capture (Gharsallaoui et al., 2007). It is a single step continuous processing operation. The process can produce purest and finest powders with high sterility reducing the post unit operation like grinding and conditioning (Menshutina et al., 2010). Spray dried powder particles are relatively small and uniform in size and shape. Spray drying allows a uniform dispersion of powder particle by diluting the bioactive core when a low amount is required. Spray dried

powders can be easily transported without any special requirements and they have a prolonged storage (Silva et al., 2005; Silva et al., 2002). Milk and coffee powders, dehydrated enzymes, fruit and vegetable powders, etc. are some of the spray dried food products currently available in the market.

It is a controlled process where the, fluid to be spray dried is passed through a very fine nozzle which comes in contact with hot air in co-current or counter current direction. The process of spray drying, illustrated in Figure 1.1, generally involves the following steps (Shahidi and Han, 1993)

1. Preparation of suspension/emulsion
2. Homogenization
3. Automated feed dispersion into the drying chamber through a fine nozzle
4. Dehydration of fine droplets (powder formation)
5. Collection of accumulated powder

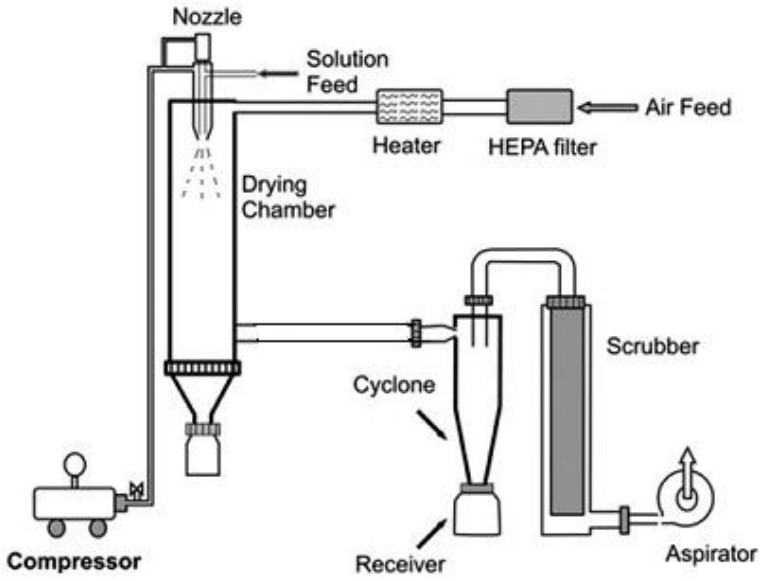

Figure 1.1: Schematic of a spray dryer (Devakate et al., 2009)

Spray drying allows the production of stable and functional compounds in native form (Koc et al., 2010). The principal driving force behind the powder formation is the temperature difference between the surface of the particle and the surrounding air, which is usually considered as wet bulb temperature of inlet air (Mani et al., 2002). The size of droplet should be small and viscosity of the liquid should be low for prevention of air in droplets (Lu and Walker, 2001). High viscosity of liquid to be spray dried increases its inlet time which in turn increases the power consumption. Rapid evaporation occurs mainly during co-current air drying when heat sensitive products are safely dried (Peighambardoust et al., 2011). However counter current air drying is more economical compared to co-current (Gharsallaoui et al., 2007).

Feed temperature has a direct impact on drying efficiency because it modifies the viscosity of the liquid. Drying rate of microsphere, final moisture content, water activity and ease of agglomeration are dependent upon the feed temperature. If feed temperature is very high, volatile and sensitive compounds might be lost before they get microencapsulated. Cracks on the microspheres and premature release and destruction of the ingredients were observed when high inlet temperatures were employed (Zakarian and King 1982). Nozzle obstruction due to high solid contents in the material to be spray dried is a common hurdle. Formation of large elongated particles has been observed when the viscosity of the liquid is too high (Doyle and Rosenberg, 1990). Also, retention of core substance in sphere is affected by emulsion properties and drying conditions. Moisture content, color and other sensory properties are greatly affected by outlet air temperature of spray dryer (Bielecka and Majkowska, 2000; Koc et al., 2010). Mathematical modeling of spray drying process is relatively difficult due to the combined and simultaneous effects of factors like droplet size, interaction between droplets and air, nozzle type, heat and mass exchange, agglomeration, etc. (Gharsallaoui et al., 2007) which are difficult to measure in a continuous operation.

1.12 Wall Materials

The encapsulation efficiency and the microsphere stability are greatly dependent on the encapsulating material known as wall material. Ideally the wall material should be water soluble since most spray drying suspensions are water based and possess good mechanical strength, compatibility with the core materials, emulsification properties and film forming and low viscous properties (Reineccius, 2004). Biopolymers, natural gums (acacia, k-carrageenan, alginates, etc), low molecular weight carbohydrates and proteins (whey protein, gelatin, etc.) are generally considered as good wall materials (Reineccius, 2004). This of course varies from strain to strain, however the carriers (like arabic gum, inulin, FOS, maltodextrin, polydextrose, skim milk powder, soy milk protein etc) in the suspension may have a significant effect on the viability (Ananta et al., 2005; Corcoran et al., 2004; Desmond et al., 2002; Espina and Packard, 1979; Lian et al., 2002; Santivarangkna et al., 2008a; Wang et al., 2004). Since these wall materials contain prebiotic sources, when mixed with probiotics the produced powders can be considered as synbiotics (Roberfroid, 1998).

1.12.1 Starch Derivatives

Starches, maltodextrins (hydrolyzed starch), corn syrup liquids are successfully used as microencapsulating agent owing to their low viscosity at high solid contents, good solubility and good water holding capacity. They are usually associated with other proteins or gums because of their low interfacial property. Incorporation of corn starch has been observed to successfully act as a prebiotic nutrient to the probiotics as well as to improve the microencapsulation efficiency of probiotics by providing a better physical barrier against the environment. Side chain chemical modification of the carbohydrates can be performed in order to improve the microencapsulation efficiency.

Studies performed by O'Riordan et al., (2001), proved that starch to cell ratios of 10:1 and 5:1 showed only 3 log cycles and 2 log cycles growth reduction respectively after a storage period of 5 days. Though the survival efficiency is relatively low (30%) compared to gums and

proteins the prebiotic factor of the starch makes them inevitable to use as adjuvants (Lian et al., 2002). Maltodextrins act as osmotically inactive bulking compounds and increase the cell space thus strengthening the glassy matrix (Chávez and Ledeboer, 2007).

Total solids content is proportional to the emulsion viscosity of the liquid to be spray dried. Higher viscosity results in rapid skin formation due to the reduced movements of particles. Internal mixing may delay the formation of semi permeable membrane due to low viscosity. But after a cut-off limit it may pose problems of longer drying times (and also film formation) and nozzle obstruction and larger surface particles which leads to uneven drying (Ré, 1998). Carbohydrates have somewhat of a gelling capacity which can prevent the solution from agglomeration and coalescence.

1.12.2 Maltodextrin

Maltodextrin is a white granular hygroscopic powder usually soluble in water. It is a polysaccharide of dextrose obtained by partial hydrolysis of starch. The number of dextrose units in maltodextrin is given as Dextrose Equivalent (DE). The DE value is usually between 4 and 20. Proteins (soy protein and whey protein) are often used as adjuvants along with maltodextrin at a defined ratio, to improve the microencapsulation of selected microorganisms. High DE maltodextrins help in preventing lipid oxidation by forming a strong barrier. Caking, crystallization and collapse was observed when low molecular weight carbohydrates were used because of their low glass transition temperature (Tg) which requires very low temperature for spray drying. Research suggests that spray drying process is unsuitable for sugar and acid rich foods due to their stickiness to the spray drying chamber caused by their low Tg (Chávez and Ledeboer, 2007).

Maltodextrins are metabolized by membrane bound glucosidases unlike simple sugars. Glucoamylase acts on the alpha 1,4 glucan link which is the non reducing end of maltodextrin. The end products have a high amount of organic acids (Yeo and Liong, 2010) lowering the pH

which is detrimental to the pathogens in the gut. Resistant starch is metabolized into butyric acid which was proven to have anti-carcinogenic effects in the colon by inhibiting the proliferation of tumor cells (Sharma et al., 2008). In addition, maltodextrin has a protective effect during the reconstitution of the probiotic powder before its usage (Muller et al., 2010).

1.12.3 Pectins

Pectins are water soluble colloidal carbohydrates (polysaccharides) naturally present in most fruit skins. They provide mechanical strength to the fruit. Pectins are soluble dietary fibers with natural emulsifying property when present in a suspension due to their high acetyl content. Pectin was also found to have microencapsulating property due to the protein residues present in the chain (Kailasapathy, 2002; Madziva et al., 2005). Sugar beet pectin was used to microencapsulate lipophilic food successfully and it was also observed that there was no interference in the spray drying(Drusch, 2007).

So, in summary the wall material should offer good emulsification and solubility, good rheological property, chemically non reactive to the core, and an ability to hold and protect the core during severe drying. Considering all the above factors and their manipulations, spray drying is a form of art rather than a science (Gharsallaoui et al., 2007).

1.13 Spray Drying of Probiotics

Spray drying of *Lactobacillus* cultures was first done in 1914 by Rogers but it was not adopted due to very low survival rate, difficulty in storage as wells as poor rehydration capacity (Porubcan and Sellers, 1975; Teixeira et al., 1995). Spray drying yogurt to preserve *Lactobacillus* and dairy starter cultures have been long investigated (Gardiner et al., 2000; Kim and Bhowmik, 1990; Metwally et al., 1989; Teixeira et al., 1994). Dried and stable probiotic cultures are usually prepared by spray drying or freeze drying which are the most common processing techniques (To and Etzel, 1997) though spray dried samples had a better stability and storage life than

freeze dried samples prepared under same composition of suspension and conditions of storage (Ying et al., 2010). Spray drying processing cost is approximately six times lower than that of freeze drying (measured as drying of water per liter). Yet there are difficulties like low survival rates of the probiotics during spray drying, poor rehydration properties of the resulting powders etc (Porubcan and Sellars, 1979). The final application of the food determines the type of microencapsulation and wall material to be used.

Moreover, dried and concentrated probiotic powder allows the ease of incorporation into several foods, transport and handling. Fermented rice drink *lao-chao* was successfully spray dried with *L. acidophilus* and *B. longum* strains and the higher the temperature, the better was the microspheres' uniformity with a high cell count (Su et al., 2007). Although there is a considerable amount of scientific data available on spray drying, it is impossible to compare them effectively as the species, culture conditions, suspension mixture, encapsulating agents, dehydration method, storage life vary in all cases (Zamora et al., 2006).

During convective drying, dehydration and heat inactivation are the two proposed mechanisms for cell death where the boundary operating parameters are not firmly established yet (Peighambardoust et al., 2011). The dehydration inactivation mode is different from the thermal inactivation of microbes. Cell wall damage is the main mechanism during dehydration inactivation (Lievense et al., 1994) whereas multiple critical components are involved during thermal inactivation. Loss of lesser critical components of the cell does not necessarily cause death. The identification of the critical components which are responsible for thermal injury resistance is difficult as multiple factors like DNA, ribosomes, proteins all act simultaneously (Gould, 1989).

Spray drying at lower outlet temperatures gave a better survival rate in studies performed using different microencapsulating materials like cellulose acetate phthalate, starch and its derivatives (maltodextrin), acacia gum, etc. (Corcoran et al., 2004; Fávaro-Trindade and Grosso, 2002; Reddy et al., 2009). Trehalose-MSG (monosodium glutamate) supplemented

medium also proved to be a good encapsulating material for preventing cell damage of probiotics (*L. rhamnosus*) and also ensuring longer storage (Sunny-Roberts and Knorr, 2009).

Spray drying outlet temperature is mainly responsible for the inactivation of the viable cells which is dependent on inlet temperatures, air flow and feed rate, suspension composition and nozzle droplet size (Santivarangkna et al., 2008b). High viability was achieved at lower outlet temperatures during spray drying (Ananta et al., 2005; Desmond et al., 2002; Meng et al., 2008) and high temperatures reduced the viability of the lactobacilli irrespective of cell load (Fu and Etzel, 1995). Most losses of volatiles take place in the early stage of drying before the dry outer crust is formed. This can be avoided by inclusion of thermo-protectants (Reineccius, 2004).

The temperature time profiles can be divided into two periods (Peighambardoust et al., 2011; Ré, 1998) - During the **constant rate drying period,** there is a rapid evaporation at the surface while it is still wet. Since temperature will not exceed the wet bulb temperature, the thermal inactivation is not seen, the evaporative cooling is higher where the cells are subjected to high temperatures for a very short time and hence the viability is dependent mainly on outlet temperature. The moisture lost is proportional to the heat gained. During the **falling rate period,** the surface dries out and the water activity of the microcapsule is reduced. The particle surface is dry, the temperature equilibrates to that of inlet and a maximal thermal inactivation is thus seen here. But if the water concentration is low at this stage the heat resistance is higher. Increasing the solid content reduces water content which again prevents thermal inactivation during spray drying and subsequent storage life. Therefore, the extent of the thermal inactivation depends on outlet temperature, residence time, feed rate and configuration of the dryer. Hence, during the falling rate period, the residence time represents the time for producing optimal recovery of the dry viable cells with minimal increase in temperature.

Denaturation or melting of DNA is the common cause for cell death at higher temperatures above 90^{o}C (Teixeira et al., 1997). Spray drying in conjunction with vacuum

drying has been employed by Ledeboer et al. (2007), making use of lower process temperature (45°C) for the production of a probiotic powder resulting in a higher survival of the probiotics. An outlet temperature of 70°C gave a maximum yield of 97% where as an outlet temperature of 120°C gave 0% survival in spray drying studies of *L. paracasei* NFBC 338 (Gardiner et al., 2000).

Inclusion of thermo-protectants and microencapsulating agents like maltodextrin and starch has obviously showed better resistance towards high temperatures. When fresh cultured cells and spray dried cells were grown in the presence of 5% NaCl, spray dried culture showed a reduction in viability. This reduced viability in presence of NaCl is accounted to extensive cell membrane damage and the stress during spray drying. But few properties like bacteriocin production, cell wall adherence, acid and bile tolerance (to an extent) were unaffected even after being subjected to high temperatures (Gardiner et al., 2000). The physical and genetic characteristics of the probiotics must be retained during production, storage and following ingestion. They should not have any adverse effects on sensorial properties of the food carrier. Multi faceted approach could be an alternative where efficiency of entire process is dealt in all possible ways (Weinbreck et al., 2010).

1.14 Sub-lethal Temperature Shock Treatment- Causes and Effects

Due to process hurdles like extreme temperatures or pressure, even the most robust probiotics are sometimes ruled out (Mattila-Sandholm et al., 2002). Exposure of bacteria to a temperature slightly above the optimal growth temperature induces tolerance and adaptation strategies during subsequent stress events (Boutibonnes et al., 1992; Gahan et al., 1996; Kim et al., 2001; Silva et al., 2005). It is possible to induce a sub-lethal effect on microorganisms which adapts them to adverse conditions during drying, storage and other processes (Broadbent and Lin, 1999). Different sub-lethal stresses studied so far include thermal, acid, salt, osmotic, high pressure, peroxides, UV, etc (Ananta and Knorr, 2004; Sanders et al., 1999). Usually a temperature rise 10°C above its optimal growth temperature leads to shock (Teixeira et al., 1994).

Most commonly studied stress adaptation is thermal stress. During the stationary phase, cells develop resistance mechanisms against adverse conditions caused by nutrient depletion and carbon source starvation. In studies conducted by Teixeira et al.(1997), the mixture was inoculated with the probiotic culture before spray drying and incubated for 30 min with constant stirring for the microbial adaptation. More than 50% survival was seen when *L. rhamnosus GG* was spray dried during the stationary phase of its growth (Corcoran et al., 2004).

The molecular mechanisms responsible for the induction have been investigated by various scientists, where heat shock proteins (Whitaker and Batt, 1991) like Gro ES (Silva et al., 2005), Gro EL and DNAk (Gouesbet et al., 2001; Péter and Reichart, 2001) were involved as chaperones during the stress adaptation. GroESL protein was characterized in *L. paracasei NFBC 338* which acts as chaperone during thermal stress in several ways like mRNA stability and, cytoplasmic protein folding, etc. With a prior over production of this protein, the survival during spray drying and freeze drying was increased, though it did not improve storage life (Corcoran et al., 2006). Studies revealed that there are no new proteins synthesized during damage repair after drying probably due to the loss of proteins during drying. The stress resistance proteins are produced mainly during the sub-lethal exposure prior to drying (Teixeira et al., 1994).

Physiological changes might be adapted by microbial cell to increase its resistance towards harsh environment (Prasad et al., 2003). Stress conditions provoke the substrate depletion as some pathways may be hindered during starvation irrespective of the concentration of the actual substrate present in extra cellular medium. This may lead to changes in cellular physiology by diminishing size or formation of spores (*Lactobacilli* are non-sporing though). However cell membrane seems to play a vital role in any type of stress (acid, bile, osmotic, etc) though characterizing the membrane proteins of individual strains is technologically unfeasible. But there is only limited evidence proving the physiological response of the microbial cells towards stress in *Lactobacillus*. Sub-lethal temperature is usually around 53°C for *L. acidophilus* while the lethal temperature is 60°C.

The stress mechanism can be induced in two ways - where the specific sub-lethal stress is induced for a similar later lethal treatment while the second type is a broader stress treatment i.e., cross protection induced by one stress to the other (Desmond et al., 2001; Kim et al., 2001). Cross resistance might also be seen where one stress induces resistance towards other stresses (salt, osmotic, high pressure, etc). Heat treatments gave the best viability after sub-lethal stress followed by salt, peroxides, and bile, maintaining high viability after spray drying in *L. paracasei* (Desmond et al., 2001).

However, spray drying during the exponential phase also gave significantly high recovery (83%) of probiotic cells (Zamora et al., 2006). Thermo tolerance in *L. bulgaricus* was induced effectively in the log phase of the growth cycle at 52°C for 20 min at constant agitation while in the latter growth, cells in the stationary phase were more thermotolerant than log phase bacteria (Teixeira et al., 1994). During storage in their dried form, following stress adaptation, mid log phase stressed cells showed better survival up to 14 weeks (Prasad et al., 2003). Studies show that acid resistance genes cross reacted with heat shock proteins which could be a possible explanation of cross tolerance (Lorca and de Valdez, 2001). Additionally, there were only few regulons to be induced for stress adaptation unlike the log phase regulons proving that resistance might be inherent during the stationary phase (Kim et al., 2001).

Heating at a temperature of 64°C and lower resulted in damage to the cytoplasmic membrane while above 65°C it caused a permanent damage (due to denaturation) to cell wall and cytoplasmic proteins. Increase in tolerance of *L. bulgaricus* towards other stress inductions like antibiotic resistance, high salt and pH concentration was observed when subjected to sub-lethal effect of heat stress below 64°C (Teixeira et al., 1997). Heating menstrum also has an effect on thermo-tolerance induction where a complex media is believed to give better adaptation to the bacterial cells rather than a simple media due to the presence of proteins. In order to assess the effect of food as a heating medium it is essential to determine the food as a menstrum during sub-lethal stress and also allowing enough time for the organisms to recover (Dabbah et al., 1969).

Thermal sub-lethal treatment can increase the survival rate of *Lactobacilli* remarkably (between 16-18 folds depending on the adaptation media) during and following spray drying (Desmond et al., 2001; Gardiner et al., 2002). Homology comparison of the protein modeling among the similar type of bacteria reveals the underlying mechanisms unique to each species (Sanders et al., 1999). Other possible factors leading to death during spray drying could be the damage of cell membranes, denaturation of DNA and ribosomes (Teixeira et al., 1997), and loss of essential minerals like Mg from the damaged cell membrane (Desmond et al., 2001). It was also observed that non controlled pH grown bacteria had a better survival rate (1 log cycle growth reduction) during spray drying than pH controlled bacteria (2 log cycles growth reduction). Heat shock proteins like GroEL and Hsp70 were expressed and seen on the western blot of protein gel. The physiological response to this stress is not yet studied though (Silva et al., 2005).

1.15 Spray Drying of Fruit Juices

Fruit juice powders are easy to store, handle and transport, offering stable natural aroma for a longer time and versatile in use. Fruit powders with moisture content less than 4% can be used to make toffees, flavor toppings, instant-mix drink powders, etc (Mani et al., 2002). Fruit juices have a low glass transition temperature due to the low molecular weight of the sugars present which increases the problem of stickiness during processing and handling.

Thermoplasticity and hygroscopicity (ability to absorb moisture from high relative humidity surrounding) of fruit juice might pose problems during the spray drying causing them to adhere to the chamber wall due to their stickiness, clogging and caking (Chegini and Ghobadian, 2005). Glass transition temperature (T_g) refers to the transformation temperature for transition from liquid to glass occurring during rapid cooling. T_g is usually lower than the melting temperature of the substance (Kingery et al., 2006). Inclusion of additives, like maltodextrin increases T_g and hence reduces problems of stickiness and agglomeration by

increasing the operating temperature. Mani et al. (2002), proved that inclusion of maltodextrin in fruit juice in a ratio of 45:55 greatly improved the resultant powder physical properties.

1.15.1 Recovery

Recovery is one of the main indicators of a successful spray drying process. Most solid material is lost during spray drying due to stickiness of the drying material to the walls of the drying chamber. Stickiness may lead to clogging which ceases the drying process which then requires a long time for clean up. Product recovery is dependent on several factors like the viscosity of the liquid to be spray dried, solid content (dissolved and suspended solids), additives (maltodextrins, soy proteins, starches, etc) added. Juice stickiness can be reduced by either increasing the drying temperature or by adding anti-caking/non sticking additives. But the usage of additives might increase the cost of the process.

The chemical nature of the additive and ratio in which it is added are considered before spray drying. The bulk density of the powder is inversely related to inlet temperature and maltodextrin ratio (it reduces the thermoplastic particles sticking and also it is a skin forming material containing air bubbles and thus decreasing the bulk density). Over all, higher inlet temperature, higher maltodextrin concentration with lower DE gave a higher rehydration, low hygroscopicity and hence low caking of powders from pulps like orange and tomato (Goula and Adamopoulos, 2010).

1.15.2 Cell Recovery after Spray Drying

The media used for resuscitation also plays a major role in activating the thermally injured spray dried probiotics (De Valdez et al., 1985). The physico-chemical properties of the rehydration media (pH, solutes in the media, temperature etc) as well as the conditions of rehydration also affect the viability and resuscitation of the injured encapsulated cells. Slow rehydration and higher temperatures were preferred for a better viability (Mille et al., 2004;

Teixeira et al., 1995). Increasing the rehydration media temperature increases the viability after spray drying linearly until a certain temperature. Injured cells normally have an extended lag phase and hence their growth cycle is longer than the regular ones (Boza et al., 2004; Ray et al., 1971; Teixeira et al., 1997) although this is strain dependent.

1.16 Particle Size

The smaller and more uniform the spray dried particles are, the better are their stability and uniformity in a food product application. Reduced droplet size has also been shown to reduce the thermal inactivation. Electron microscopy is usually employed to study the obtained microcapsules. Spray dried capsules are smaller than freeze dried particles and a better survival during storage is expected. Increasing the wall material ratio results in smoother particles due to reduced exposure to high temperature. Particle size and shape are dependent on the moisture content of the powder and the operating drying temperature. 40% maltodextrin solution was spray dried at different temperatures and observed using SEM. Small particles (12 μm) were obtained at lower temperatures while bigger particles (> 32 μm) were obtained at medium and higher temperatures (Fu and Etzel, 1995).

1.17 Response Surface Method (RSM)

RSM is a versatile statistical method employed to optimize multivariate processes with an experimental design. The number of replicates can be greatly reduced using this method yet giving a statistically significant model taking all possible variables into account. Optimization of any process can be performed taking the dependent and independent variables into account.

1.18 Storage and Shelf life

Upholding the probiotic characteristics throughout the product shelf life is essential for the product's marketable success (Espina and Packard, 1979). Probiotics are very sensitive to

environmental stresses like heat, oxygen, humidity, etc., and hence special protection is needed to maintain viability as high as 10^6-10^8 CFU/mL.

Packaging materials play a significant role in maintaining stable viable counts of the cells. Different authors have suggested different packaging materials like glass, metal pouch etc. Several factors like oxygen permeability, temperature, light, humidity, etc, need to be taken into account and are strain dependent. Due to the absence of electron transport chain during storage and catalase enzyme, the free oxygen from atmosphere is converted to peroxides during storage which is detrimental to the probiotic. An increase in relative humidity can lead to problems like caking and agglomeration. Glass bottle storage of spray dried microencapsulated (spray dried) probiotics had a high shelf life (>6 log CFU/g) of more than 40 days at low temperatures (4^oC) (Hsiao et al., 2004). Inclusion of desiccants can improve storage at 25^oC (at least 25 days) irrespective of the coating material used, while survival in glass bottles was higher than in PET bottles maintained under same conditions (Hsiao et al., 2004).

1.18.1 Color

Color of the raspberry powder is also important as the redness of the powder indicates the amount of phytochemicals and antioxidants present. Also, good color is the foremost quality parameter for consumer acceptance.

1.18.2 Water activity (A_w)

Water activity is the measure of “unbound” “free” “available” water present in any cells or a substance required to maintain or participate in metabolic activity and survival of living cells. Water activity is the ratio of vapor pressure of water in equilibrium with a food to the saturation pressure of water at a given temperature. It measures how the water is bound to a food, structurally as well as functionally. Multiplying A_w by 100 gives the % relative humidity of the atmosphere in equilibrium with the food. However the relation between moisture content

(%) and A_w is non linear (follows sorption isotherms) and is specific to each food. It is an essential parameter for determining the microbial activity and safety especially for a prolonged storage. Usually a low A_w is desired to reduce the unwanted chemical and biological reactions in the food. It can be used as well to predict the potential microorganisms that might contaminate the food.

Autocatalytic lipid oxidation and non enzymatic browning, enzymatic reaction, starch retrogradation, degradation of enzymes and vitamins are influenced by A_w. There is usually no microbial proliferation seen between the range of 0.2 to 0.5. A_w between 0.2-0.6 was suggested for stable maintenance of probiotics in spray dried milk powders (Corcoran et al., 2006). Although A_w >0.5 is detrimental to the cell viability due to lipid peroxidation and outgrowth of unwanted microorganisms. The textural and sensorial properties are also affected by water activity in many foods (Fontana, 1998). It is essentially the bound water on the cell membranes, which affects the death rate of cell during drying as it stabilizes the proteins and cell membranes (Santivarangkna et al., 2008b). Moisture content and water activity have to be carefully monitored to ensure quality during long term storage (Chávez and Ledeboer, 2007).

1.19 Consumer Interest

Consumer expectations and acceptance of novel health food must be studied before it is being launched. The novel food has to relish and be healthy at the same time. The successful launch of any health foods should also have an educational campaign explaining the benefits of the food (Cruz et al., 2009). One particular case study where orange juice was fortified with probiotics, gave off flavors to the juice and it was not initially accepted by the consumers. But given the health benefits , consumers were ready to accept minor deviations from the regular flavor (Luckow and Delahunty, 2004). However the off flavors can be easily masked by mixing other tropical juices. There is a growing trend in non-dairy probiotic food formulation as well as their acceptability as long as the health benefits are clearly stated.

There were several case studies where the probiotic claims were falsified or misnamed (Hamilton-Miller and Shah, 2002; Temmerman et al., 2001). In a study conducted by Coeuet et.al., (2004) on 10 selected European probiotic products for human consumption, it was surprising to find that five products were mislabeled and four out of them had none of the probiotics that were claimed. Similar studies in Taiwan on eight commercial probiotic products indicated that liquid foods are a better carrier of probiotics than solid foods, with two of the tested solid foods having no viable counts of probiotics (Lin et al., 2006).

Although probiotic claimed species (*E. faecium*) may not be non pathogenic, they may potentially raise a health risk as their antibiotic resistance levels are not same. So there is a pressing need to standardize the current regulations on probiotic claims of a food product. And it is also the responsibility of manufacturers to declare the species present and if possible the potential health benefits and risks. Labelling guidelines must be set which should state the strain name, state of the organisms, number and time of expiry (Weese, 2003) which is rarely seen on current probiotic products. Regulation and standardization should be made stricter than present to achieve this for consumer safety.

1.20 Objectives of the Current Research

- ✓ Compare dextrose and maltodextrin's ability as a carbon source for the growth of *L. acidophilus* and *L. rhamnosus*
- ✓ Determine sub-lethal shock temperature of both the lactobacilli in broth media as well as in raspberry juice
- ✓ Develop non-dairy raspberry probiotic powder by spray drying and optimize the process conditions
- ✓ Perform storage analysis (CFU/unit and A_w) of the obtained powder in glass containers at room as well as refrigerated temperature storage.
- ✓ Perform electron image analysis of the spray dried raspberry powders and determine the quality changes at the end of 30 days of storage.

Chapter 2

MATERIALS

AND

METHODS

2. Materials and Methods

All the initial screening tests and characteristics of the species were assessed individually unless otherwise mentioned.

2.1 Culture

After a detailed literature review on health benefits of probiotics, two species widely used in commercial probiotic foods were chosen. *Lactobacillus rhamnosus* NRRL B-4495 and *Lactobacillus acidophilus* NRRL B-442 were obtained from the USDA's Agricultural Research Services Culture Collection. Dried pellets were reconstituted in 50 mL MRS (deMan, Rogosa and Sharpe medium, Difco™) broth and grown over night (14-16 hours) in an incubator shaker (Model G24, New Brunswick Scientific, NJ, USA) at 110 rpm and 37°C. This was subcultured and grown overnight again. The culture thus obtained after the second sub-culture was used for further experiments. A small part of the culture was stored in sterile 30% glycerol at -80°C (-86C ULT Freezer Model 5698, Thermo Forma®, USA) for later usage. The composition of the MRS medium (Difco™) is presented in Table 2.1.

Table 2.1: MRS broth recipe (per liter)

Ingredient	Quantity
Proteose Peptone No. 3	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
Dextrose	20.0 g
Polysorbate 80	1.0 g
Ammonium Citrate	2.0 g
Sodium Acetate	5.0 g
Magnesium Sulfate	0.1 g
Manganese Sulfate	0.05 g
Dipotassium Phosphate	2.0 g

A similar recipe as shown in Table 2.1 was adapted to prepare MD-MRS medium where the dextrose was replaced by maltodextrin (MD) (Oxoid™). MRS agar plates were prepared by adding bacteriological agar to a final concentration of 1 g/L.

2.2 Determination of Growth Curve

A volume of seed cultures grown overnight in MRS was added to fresh sterile MRS broth to obtain an initial optical density at 600 nanometers (OD_{600nm} is defined as the absorbance of an optical element for a given wavelength λ per unit distance) ranging between 0.08 to 0.1 measured by a spectrophotometer (Ultraspec1000, Amersham Pharmacia Biotech, NJ, USA). The culture was incubated on a shaker at 110 rpm and 37°C for 24 hours. The OD and cell density (Log CFU/mL) were measured every 3 hours. A sample of the culture was serially diluted and 0.1 mL of the dilutions were plated in triplicate onto MRS agar. The plates were incubated at 37°C for 36-72 hours in a gravity convection incubator (Model 4EG, Precision Scientific, USA). The number of colony forming units (CFUs) was counted. Prepared graphs represent the average of triplicate Petri plates for each of three trials. The log CFU/mL at any given dilution was calculated using the formula in Eq 2.1

Log (CFU/mL) = Log {(Number of colonies*Dilution factor)/0.1} ---------- Eq 2.1

2.3 Dry Biomass Estimation

The increase in biomass over the growth cycle period provides information on kinetics of the growth in relation to substrate utilization. Fresh sterile MRS was inoculated with a seed culture to obtain an initial OD_{600} ranging between 0.08-0.1. A 5 mL sample of this culture was taken every 3 hours and centrifuged in a pre-weighed centrifuge at 4500 rpm for 6 to 7 min. The supernatant was decanted and stored for substrate estimation. The weight of dry biomass was measured as the weight difference between the tube and the pellet after drying at 103±2°C in hot air oven (Precision model 6528, Thermoelectron corporation®, USA) for over 12 hours.

(Harris and Kell, 1985; Li and Mira de Orduña, 2010; Major and Bull, 1985). Prepared graphs represent the average of triplicate Petri plates for each of three trials.

The specific growth rate constant was determined to calculate the generation doubling time from the following equation Eq 2.2.

$$K = [\log(X_{t1}) - \log(X_{t2})] / 0.301 * (t2 - t1) \quad \text{Eq 2.2}$$

K= number of populations doubling in an hour (growth rate constant)

X_{t1}/ X_{t2}= number of cells/mL at times t1/t2

During any phase, the doubling time or generation time is the time required for the cell biomass to double in number as represented in Eq 2.3.

$$Tg = 1/K \quad \text{Eq 2.3}$$

2.4 Determination of Substrate Concentration (Anthrone method)

The anthrone method for quantification of sugars/carbohydrates is widely used for its sensitivity even at a very low sugar concentration. In the presence of strong acids and high temperature, carbohydrates are dehydrated to hydroxymethyl furfural which reacts with anthrone reagent to form a blue-green colored complex (Graham, 1963). The intensity of the color is directly proportional to the carbohydrate content which is measured by a spectrophotometer at 620 nm. Anthrone reagent (Sigma Aldrich™, USA) was prepared by dissolving 200 mg of anthrone in 100 mL of 95% sulfuric acid. A 1 mL volume of the supernatant obtained from the biomass estimation was diluted 10 times and 4 mL anthrone reagent was added. The mixture was heated in a boiling water bath (100±2°C) for 6 to 7 min until a stable green color was obtained after which the mixture was cooled immediately in an ice bath for 2 min. The maximum absorbance at OD_{620} was recorded. The concentration of pure dextrose/maltodextrin was estimated from the measured OD_{620} using the standard curve

obtained using a known concentration (0.1 g/L) of dextrose/maltodextrin. OD_{620} was plotted on the X-axis and concentration of substrate on the Y-axis.

2.5 Characterization of Probiotic Cultures

Preliminary probiotic biological properties such as acid and bile tolerance and antibiotic sensitivity were tested before and after spray drying to ensure that these characteristics were not lost as a result of the induced stress due to high temperatures of spray drying.

2.5.1 Acid Tolerance Assay

A five mL sample of cultures was taken during the late log phase and centrifuged at 3500 rpm for 1 to 2 min and this step was repeated again with neutral phosphate buffer saline (pH 7.2, PBS). The supernatant was decanted and the pellet was resuspended in phosphate buffer saline (PBS) at either pH 1.5 or 3 separately (pH adjusted by Acumet®AB15 basic, Fisher Scientific, USA). The buffer was added until the turbidity of the solution reached MacFarland standard of 0.452 @ OD_{600} (with water as blank). One hundred µL of this solution was inoculated on MRS agar at 0^{th}, 2^{nd} and 4^{th} hour of incubation without any dilution. Both the cultures were tested individually at both the pH values. CFUs were enumerated after 36-72 hours of incubation at 37°C using same formula presented in Eq 2.1. Prepared graphs represent the average of three trials.

2.5.2 Bile Tolerance Assay

Five mL of cultures were taken at the late log phase and centrifuged at 3500 rpm for 1 to 2 min and this step was repeated again with neutral PBS (pH 7.2). The supernatant was decanted and the pellet was resuspended in 10 mL of 0.3% Oxgall (Difco™, USA) prepared in MRS. One hundred µL of this mixture was spread on MRS agar at 0^{th}, 2^{nd} and 4^{th} hour without any dilution. CFUs were enumerated after 36-72 hours of incubation at 37°C using Eq 2.2. Prepared graphs represent the average of three trials.

2.5.3 Antibiotic Sensitivity Test

Antibiotic disks diffusion method was used to assess the antibiotic sensitivity of the *Lactobacilli*. The MRS agar plate was spread with 100 µL active late log phase culture and antibiotic disks were placed on the agar with sterile forceps. To diversify the selection of different classes of antibiotics, six different broad spectrum standard antibiotics were used. These antibiotics are commonly used to treat various infections in humans. Ampicillin (10 µg), chloramphenicol (30 µg), penicillin G (10 units), streptomycin (10 µg) (Oxoid™, England), sulfamethoxazole-trimethoprim (25 µg), tetracycline (30 µg) (BBL™, USA). The diameters of zones of inhibitions were measured and compared with previous data from literature to classify them as resistant, intermediate or sensitive (Tang et al., 2007 and NCCLS- National Committee for Clinical Laboratory Standards M11-A7).

2.6 Preparation of Raspberry Juice

Raspberry juice was prepared by blending (with Magic Bullet™, Canada) thawed previously frozen raspberries purchased from the local super market. The extracted pulp had a solid content of 13 to 14 °Brix measured by a portable refractometer (Fischer Scientific®, USA). The seeds and skin were sieved out using a fine metal sieve filter. The °Brix unit was adjusted to 11 (total solid concentration 0.1 g/L) as otherwise the pure extract was too viscous to be spray dried.

The dry weight (non volatile solids) was determined by measuring the weight difference after oven drying (103±2°C) 1 mL of raspberry juice.

2.7 Sub-lethal Temperature (T_{sl}) Treatment

A sub-lethal temperature treatment was selected to subject the microbes to thermal stress before spray drying. Since the sub-lethal temperature of any given microorganism is strain-specific, each strain was tested individually. Five mL of late log phase *Lactobacillus* cultures (incubated at 37°C, on a rotary shaker at 110 rpm for 12- to 14 hours) was subjected to different sub-lethal temperatures (45°C, 50°C, 52°C, 55°C) in a hot water bath (Isotemp® Fischer Scientific, USA). Test tubes containing either MRS or raspberry juice (with inserted

thermometers) were used as thermostats to regulate the temperature. One mL of sample was collected every five minutes and transferred into 9 ml of sterile MRD (maximum recovery diluents; 1 g/L- peptone, 8.5 g/L NaCl) until 15 min. CFU analysis was performed from each of these samples at regular intervals (0, 5, 10, 15 min). T_{sl} was determined from the curves in the graph where the temperature was retaining the highest number of cells after 15 min of thermal stress exposure. Prepared graphs represent the average of triplicate Petri plates for each of three trials.

2.8 Response Surface Design

Process optimization for the spray drying of probiotics in raspberry juice was performed using response surface method (RSM). Initial trials were performed by varying the different conditions for the spray drying of probiotics in raspberry juice such as inlet temperature, maltodextrin to juice solid content ratio, and the inlet feed rate as found in the literature. Spray drying trials on fruit juice with probiotic cells without heat shock treatment had lower viability than heat shock treated cells under similar conditions (Corcoran et al., 2004; Desmond et al., 2001; O'Riordan et al., 2001; Teixeira et al., 1995). After analyzing the effects of different variables on the probiotics viability, powder color and powder recovery, the maximum and minimum values of the each factor were adjusted for heat shock treated probiotic mixture in the raspberry juice. Inlet temperature (°C), total solids in juice to maltodextrin ratio, feed rate (mL/min) were the independent variables and % recovery, % survival and color (ΔE) were the dependent variables. A central composite design- uniform precision model by JMP-8® (SAS Institute, NC, USA) generated the 20 responses shown in Table 2.2.

2.9 Spray Drying

Mixture of probiotics culture, raspberry juice and additives were homogenized for 1 to 2 min using a magnetic stirrer just before spray drying. Spray drying of raspberry juice with maltodextrin as an additive (wall material) at different ratios and mixture of lactobacilli was performed- using a Buchi™ B-290 mini spray dryer. The spray dryer was allowed to reach uniform process temperature for 15-20 min prior to the spray drying. The aspiration was maintained 100% and cyclone air flow rate at 30 m^3/h. The optimization was performed by

response surface method (RSM) with inlet temperature, wall material ratio and inlet feed rate as independent variables and CFU following spray drying, change in CFU over 30 days, recovery and color as the dependent variables.

Table 2.2: Trials generated for response surface design by JMP-8®

Response	Pattern	Inlet Temp (°C)	Maltodextrin ratio	Inlet Feed Rate (mL/min)
R1	0,0,0	115	1.5	50
R2	0,0,0	115	1.5	50
R3	0,0,-1	115	1.5	40
R4	+1,−1,−1	130	1	40
R5	-1,+1,+1	100	2	60
R6	0,0,0	115	1.5	50
R7	−1,−1,+1	100	1	60
R8	0,+1,0	115	2	50
R9	0,0,0	115	1.5	50
R10	−1,+1,−1	100	2	40
R11	−1,−1,−1	100	1	40
R12	-1,0,0	100	1.5	50
R13	+1,0,0	130	1.5	50
R14	0,0,0	115	1.5	50
R15	+1,+1,−1	130	2	40
R16	0,-1,0	115	1	50
R17	+1,+1,+1	130	2	60
R18	+1,−1,+1	130	1	50
R19	0,0,+1	115	1.5	60
R20	0,0,0	115	1.5	50

The experimental data thus obtained were analyzed by SAS 9.2 TS2M2 and the graphs were generated by JMP-8 with same results obtained in both softwares.

The raspberry juice was adjusted to 11 °Brix by appropriate dilution with sterile distilled water because with a total solid content above this limit, the spray drying nozzle clogs up. The °Brix increased when maltodextrin and the probiotic cultures were added. °Brix was between 18- and 19 in a 1:1 suspension, 21- and 22 in a 1:1.5 suspension and 24- and 25 in a 1:2 suspension. A 50 mL volume (i.e 5 g of total raspberry solids) of raspberry juice was used for each trial. Homogenization of this pulp and maltodextrin was done using a magnetic stirrer. A 5 mL volume of each probiotic cultures (late exponential phase; grown over night; approximately 9.5 log CFU/mL) were centrifuged at 3500 rpm for 1- to 2 min and diluted with 1 mL of sterile distilled water homogenized by stirring just before spray drying.

Following spray drying, the raspberry powder thus obtained was stored in small glass jars with screw caps. The RSM trials were done in duplicate to obtain two samples under same condition. One sample was stored at room temperature (23±2°C) in the dark and the other at refrigeration temperature (4±2°C).

2.10 Moisture Content

The initial moisture content of the powder samples was determined on the 0^{th} day by drying 1 g of spray dried raspberry powder in aluminium dishes for more than 24 hours at 103±2°C. The moisture content was calculated as the weight difference between dried and undried samples.

2.11 Storage Studies

Powder samples were stored at 4°C and at 23°C for 30 day shelf life study. The number of CFU, color and water activity of the samples were measured every 10 days or 15 days (depending on the amount of recovered powder) for 30 days.

2.11.1 CFU/unit

The number of colonies was determined during the storage life of 30 days at every 10 days or 15 days intervals. A one gram sample of the powder was diluted in 10 mL of MRD and serially diluted. The powder was resuscitated in the MRD for 20 min. A one hundred µL sample of this suspension was spread on MRS agar and after incubating at 37°C for 48 to 96 hours, the CFUs were counted.

2.11.2 Water Activity (A_w)

Water activity is essential to predict microbial behavior and product caking during storage. The A_w of each raspberry powder sample was measured by a water activity meter, Model-3TE, AQUA® labs) throughout the storage (30 days) (stored at room and cold temperature) at every 10 or 15 days intervals.

2.11.3 Color

The predicted end product usage of the obtained raspberry powder is mostly as a rehydrated drink. Therefore, the powder as well as the rehydrated powder (1 gram in 10 ml of MRD) was assessed throughout the storage period by chromameter (Model- CR300, Konica-Minolta®, USA). The measurement was done in small glass tube filled with the powder/liquid fully covering the base diameter of the instrument. The glass tube fitted perfectly the diameter of the chromameter's testing window.

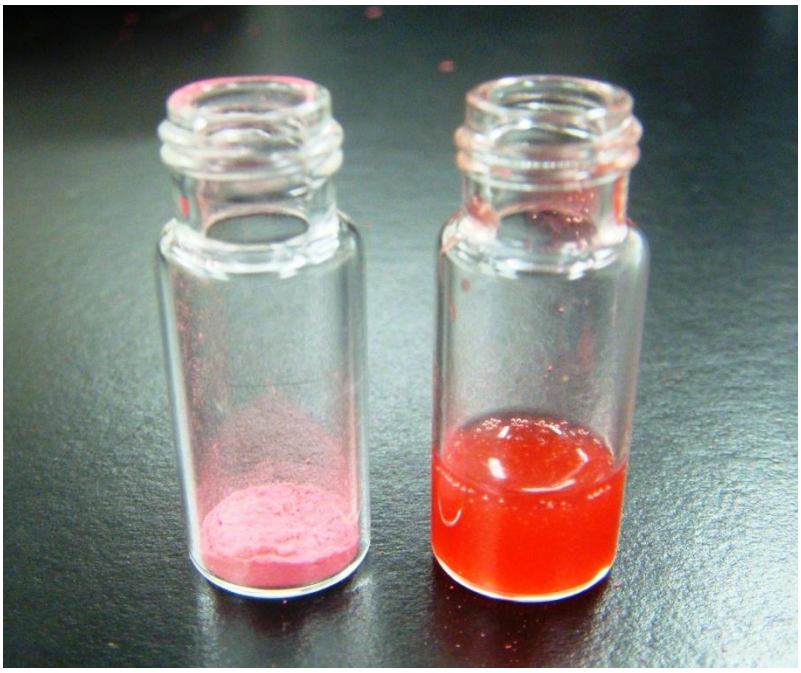

Fig 2.1: Glass tube with raspberry powder and rehydrated liquid

This glass tube with sample (Fig 2.1) was covered with a black material to prevent unwanted interference from ambient light and scattering of light from the source. L^* (light/dark), a^* (a^+- redness/green- a^-) and b^* (b^+- yellowness/blueness- b^-) values were recorded for each sample. From the obtained L^*, a^* and b^* values, the total change in colour (ΔE) was calculated using the following equation Eq 2.4:

$$\Delta E = \sqrt{(Lo - L*)^2 + (ao - a*)^2 + (bo - b*)^2} \quad \text{---------- Eq 2.4}$$

Note: L_o, a_o, b_o denotes the value of plain maltodextrin (for powder) and MRD (for rehydrated liquid) used as standard reference to compare the colour change of the extract.

2.12 Particle Size

Size of the microcapsules was measured for the best three samples in terms of CFU and recovery after 0^{th} day and 30 days to compare the changes in the particulate dimensions and surface properties. Vapor pressure scanning electron microscope (VPSEM, Hitachi® S-3400N,

Japan) was employed to estimate the size of the microcapsules. Images with 1500x and 250x magnifications were captured under vacuum pressure at 10 Pa and 25 kV.

Chapter 3

RESULTS

AND

DISCUSSION

3. Results and Discussion

The growth characteristics and the substrate kinetics for each bacterial species were measured and preliminary probiotic tests were done. The sub-lethal temperature-time profile was also characterized for both *Lactobacilli* species. Spray drying process was optimized using response surface design. Storage studies and microsphere imaging studies were analyzed.

3.1 Growth Curve

By understanding the growth phases, the sub-lethal stress conditions and other parameters like doubling time, antibiotic resistance, etc., can be assessed. Microbial survival can be monitored and controlled especially for industrial scale applications.

3.1.2 *Lactobacillus acidophilus*

The relation between OD_{600} and log CFU/mL helps us to understand the growth characteristics. Increase in OD represents the increase in cell number over time and hence an increase in log CFU/mL. In *L. acidophilus*, this relation between OD_{600} and log CFU/mL is modelled by the equation y= 0.74x+ 7.81 obtained from the graph presented in Figure 3.1. This relation was used to estimate the concentration of live cells of *L. acidophilus* in MD-MRS (Maltodextrin-MRS) from OD_{600} values obtained under the conditions presented in Figure 3.2.

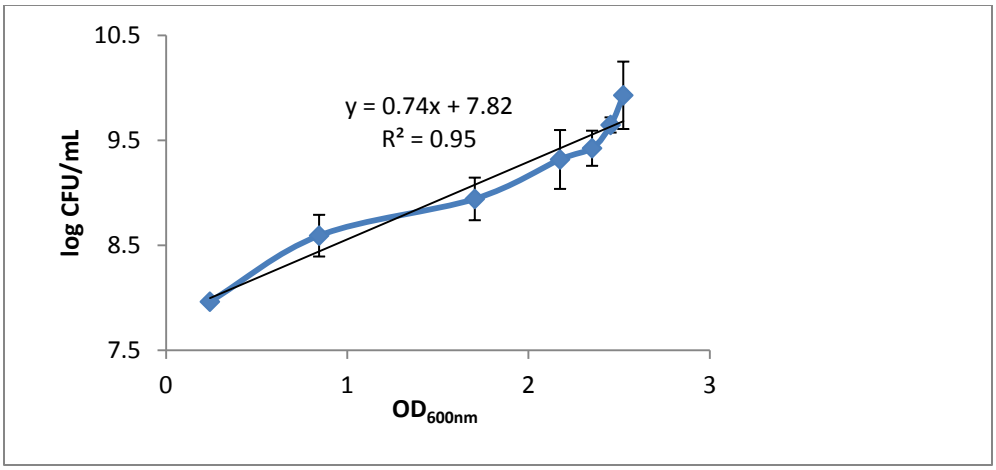

Figure 3.1 : Relationship between OD_{600} and bacterial cell concentration of *L. acidophilus* in MRS broth (37°C, 110 rpm, 24 hours)

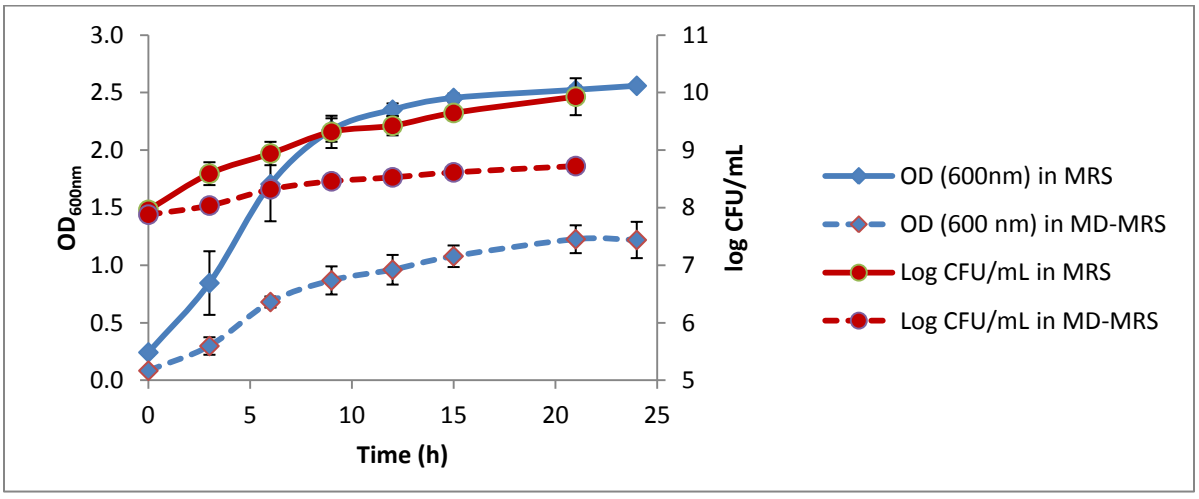

Figure 3.2: OD_{600} and log CFU of *L. acidophilus* over time in MRS and MD-MRS (37°C, 110 rpm)

From Figure 3.2, the growth cycle (with respect to OD_{600}) of *L. acidophilus* in MRS broth followed an exponential phase between 0-12 h and a stationary phase starting from 13 h. Under similar conditions in MD-MRS the growth cycle from OD measurements, although had an exponential phase till 6^{th} hour but had a longer stationary growth from 7^{th} hour showing minimal increase in OD_{600}. Although the growth curve with respect to log CFU/ml does not show different phases, the log CFU/mL in MRS reached from 7.96 to 9.71 and 7.88 to 8.72 in MD-MRS by the end of 24 hours. Hence it can be concluded that, under the given conditions, dextrose as a carbon source supports the growth of *L. acidophilus* better than maltodextrin. This analysis is

useful since the *Lactobacilli* in late exponential-stationary phase are required for thermal shock treatment and spray drying.

Both tested media are significantly different ($P<0.05$) from each other with respect to the growth rate of microorganisms. The maximum specific growth rate constant (K_{max}) and generation/doubling time (T_d) in MRS are 0.697 and 1.43 min; while K_{max} was 0.313 and T_d was 3.2 min in MD-MRS. Clearly the growth rate is better in MRS medium with dextrose as the carbon sugar. From the above values and graphs it can be concluded that maltodextrin also supported the growth of *L. acidophilus* but not as well as dextrose as the carbon source at similar concentrations.

3.1.2 *Lactobacillus rhamnosus*

Similar results were observed for the growth of *L. rhamnosus* on the two media under the same conditions (Figures 3.3 and 3.4). The relation between OD_{600nm} and log CFU/mL is modelled by the equation y= 1.91x+ 7.23 which was used to calculate log CFU/mL in MD-MRS medium from measured OD_{600} values.

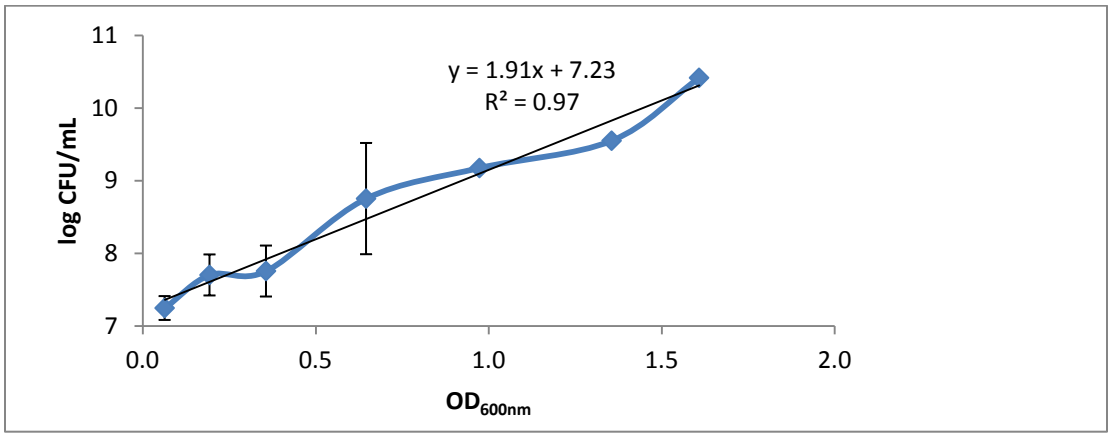

Figure 3.3: Relationship between OD_{600nm} and bacterial cell concentration of *L. rhamnosus* in MRS broth (37°C, 110 rpm, 24 hours)

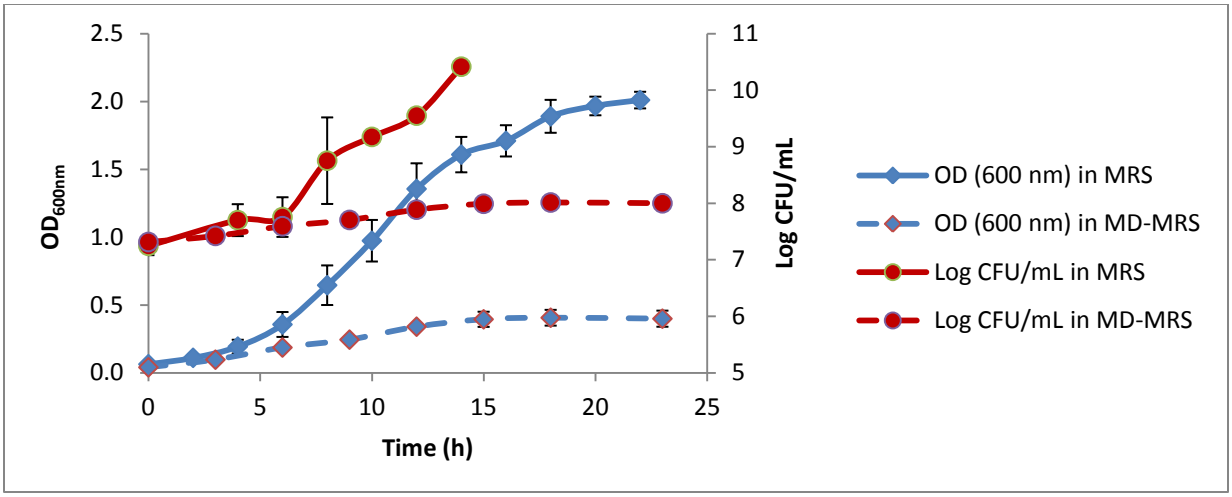

Figure 3.4: OD_{600nm} and Log CFU of *L. rhamnosus* over time in MRS and MD-MRS (37°C, 110 rpm)

The growth cycle of *L. rhamnosus* in MRS was monitored by measuring OD_{600}. An initial lag phase was observed until 5 h followed by an exponential phase from 6th-and 16th h; stationary phase started at 17th h. Under similar conditions in MD-MRS the growth cycle measured in terms of OD followed a steady and slow growth. The growth curve in MRS medium with respect to log CFU/mL also had a lag phase till the 5th hour followed by an exponential phase, however there was a steady minimal/no growth in MD-MRS. The number of cells, log CFU/mL in MRS reached from 7.25 to 10.41 by 15th hour and MD-MRS only from 7.31 to 8 even by the end of 24th hour. Hence it can be concluded that dextrose as a carbon source for *L. rhamnosus* was a better growth support than maltodextrin.

Both tested media are significantly different ($P<0.05$) from each other with respect to the growth microorganism. The maximum specific growth rate constant (K_{max}) and generation time (T_d) are 1.657 and 0.6 min respectively in MRS and 0.202 and 4.95 min respectively in MD-MRS. Again, the growth rate and live cell concentration were higher with dextrose as the carbon source and growth in maltodextrin was lower than *L. acidophilus* in MD-MRS indicating clearly that dextrose was utilized better as a carbon source than maltodextrin. Although the growth was not very high with maltodextrin it could potentially act as a prebiotic as well as a microencapsulating agent during spray drying.

3.2 Substrate Utilization Kinetics

Substrate utilization kinetics reflects the effectiveness of a medium to support the growth of a given microorganism. Understanding the substrate utilization pattern of a microorganism is also helpful to assess their growth behavior and survival in the gut which is their final destination (Verdenelli et al., 2009). The growth media in which the microbes are grown also have an effect on survival during spray drying and subsequent storage. Inclusion of different sugars (lactose, mannose, dextrose, etc.,) during growth has been shown to have varying effects on survival during thermal treatment and subsequent freeze drying although this was strain-dependent (Carvalho et al., 2004).

3.2.1 Dextrose and Maltodextrin Utilization

Maltodextrin can act as a prebiotic as well as a microencapsulating agent during subsequent spray drying. Studies have demonstrated that there are clusters of genes responsible for the maltodextrin metabolism in *L. acidophilus* which produce maltose and then follow the glycolysis pathway to produce lactic acid as an end product (Nakai et al., 2009). Since *Lactobacilli* produce glycosyl hydrolase, they can metabolize maltodextrin to glucose units. Butyrate may be the end product which reduces the pH of the culture medium (Olano-Martin et al., 2000).

Figure 3.5 presents the change in substrate during the growth of *L. acidophilus*, and shows that at the end of the growth cycle for *L. acidophilus* under similar culture conditions for the two growth media, the residual concentration of dextrose (6.8 g/L) was lower than for maltodextrin (11.1 g/L) showing that dextrose is better metabolized by the bacteria than maltodextrin. However the dry biomass at the end of the growth cycle of *L. acidophilus* in MD-MRS (3.6 g/L) was higher than that of MRS (2.4 g/L). Indeed, after the centrifugation and before drying, there was a certain amount of insoluble maltodextrin present as residue in the pellet as a white mass in *L. acidophilus* cultures. This white pellet was not found for the same cultures grown in MRS medium. So the true weight might have been influenced by the

presence of insoluble maltodextrin which explains the higher trend of dry biomass in MD-MRS. In Figure 3.6, for *L. rhamnosus* for the residual concentration of dextrose (5.5 g/L) was lower than for maltodextrin (13.8 g/L) showing that dextrose is better metabolized by the *L. rhamnosus* than maltodextrin. Correspondingly the dry biomass in presence of MRS (2.7 g/L) was greater than MD-MRS (2 g/L).

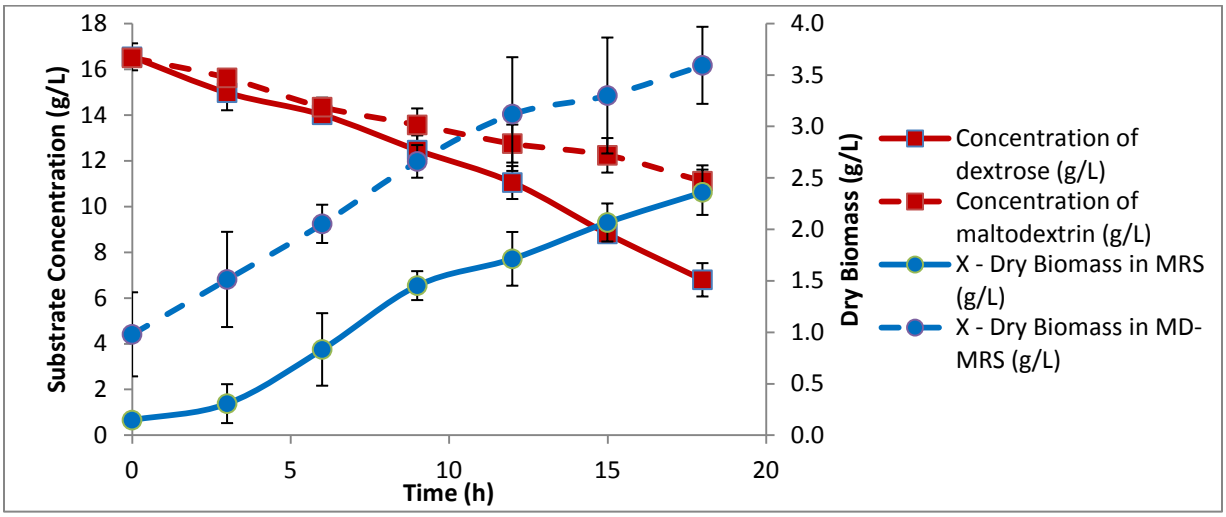

Figure 3.5: Changes in dry biomass and substrate over time for *L. acidophilus*

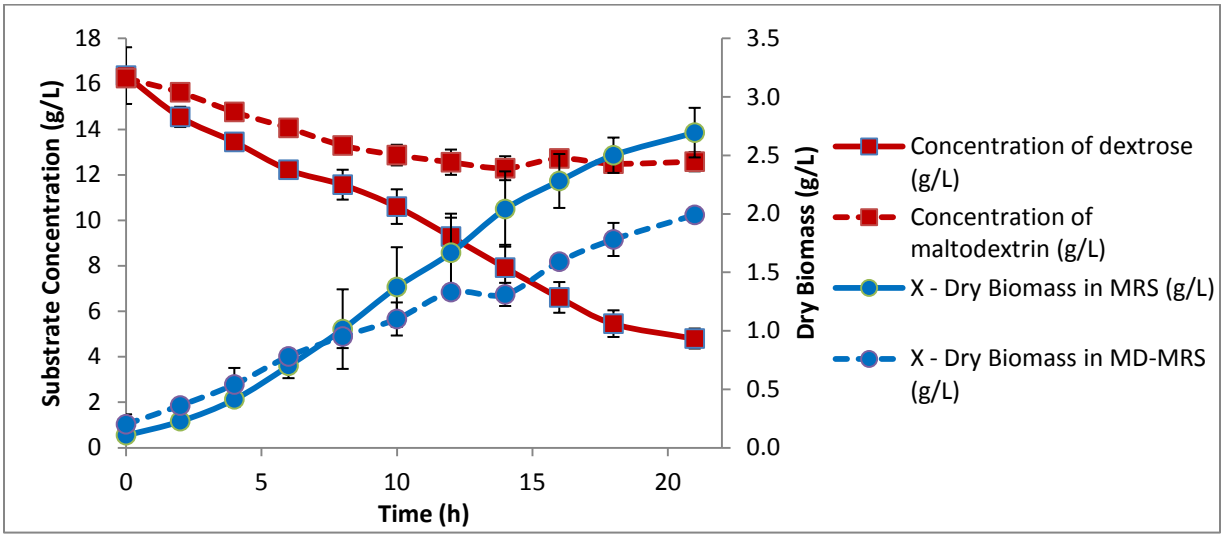

Figure 3.6: Changes in dry biomass and substrate over time for *L. rhamnosus*

3.3 Acid Tolerance Assay

The basic probiotic properties tests that were done in this study were- tolerance to acid and bile and sensitivity to antibiotics on each species separately. The pH of stomach, due to gastric juice is usually between 2 to 3 and during fasting it decreases to as low as 1.5 (Ross et al., 2005). Most fresh meal diets around the world comprise many acidic foods such as fruits, vegetables and fermented dairy products. So after ingestion into the gastro intestinal tract the probiotics must be able to withstand the high acidic environment yet still perform the function for which they are consumed. Both the cultures were tested individually at both pH values.

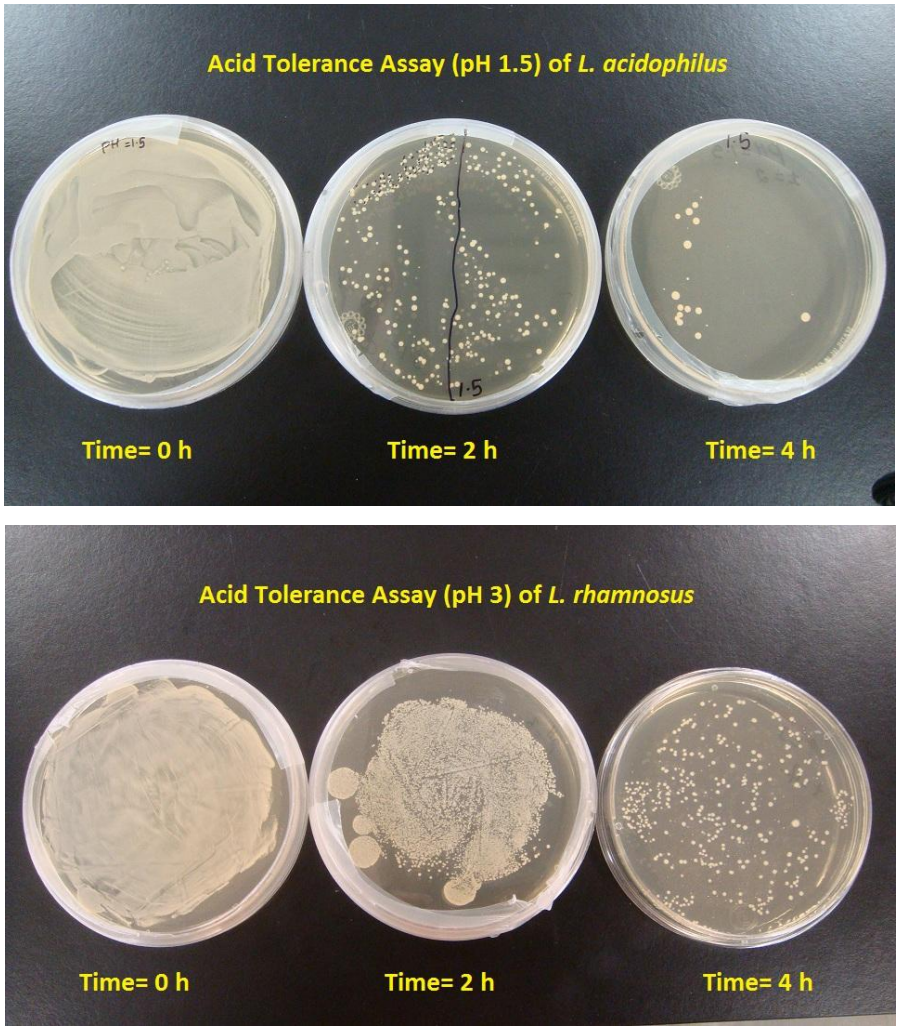

Figure 3.7: Acid tolerance of *L. acidophilus* and *L. rhamnosus* at different pH levels

Both species tested showed acid tolerance at pH 1.5 although the bacterial density was reduced by the end of 4 hours (Figure 3.7). At pH 3, incubation of both species produced a dense lawn growth on MRS agar plates indicating their resistance to acid. Since similar growth pattern was observed for all the tests, images of plates only for pH 1.5 for *L. acidophilus* and pH 3 for *L. rhamnosus* are shown in Figure 3.7.

Acid tolerance is also supposed to be mediated by membrane H^+-ATPases although there may be other uncharacterized proteins involved (Lorca and de Valdez, 2001). Acids passively diffuse through the cell membrane to enter the cytoplasm and dissociate into protons. This affects the transmembrane pH and proton motive force. It may also reduce the activity of enzymes and denature proteins and DNA (Van de Guchte et al., 2002).

3.4 Bile Tolerance Assay

The dense lawn culture growth on MRS agar plates, as seen in Figure 3.8, indicated that both cultures were strongly resistant to the bile salt Oxgall (0.3%) even after 4 hours of exposure.

Bile salts act as detergents and antimicrobial agents and disassemble the biological membranes. However *Lactobacilli* and *Bifidobacteria* are able to metabolize bile salts into amino acids and steroid derivatives by hydrolysis. This notably reduces the bile's solubility at low pH as well as its detergent property and hence permits a better survival. But the actual physiological response, regulatory pathways and molecular mechanisms are still obscure (Van de Guchte et al., 2002).

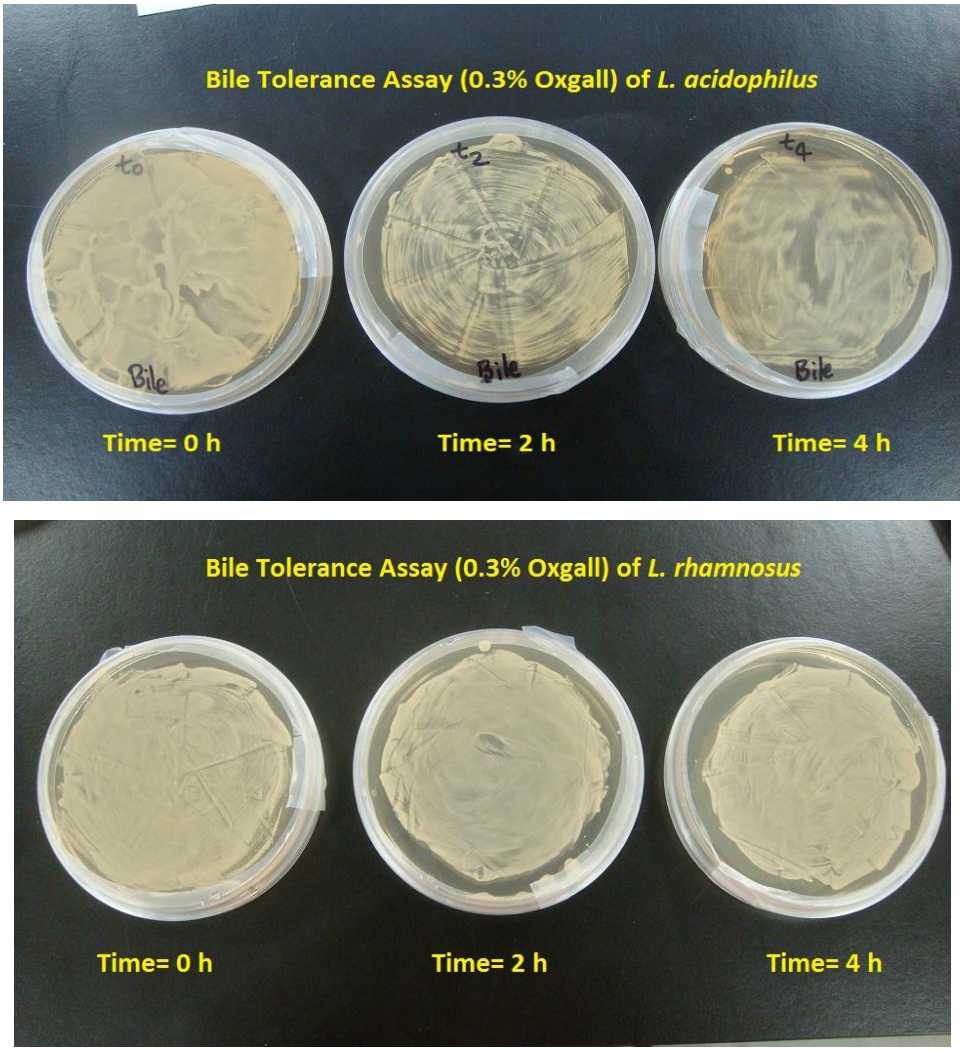

Figure 3.8: Bile tolerance (0.3% Oxgall) of *L. acidophilus* and *L. rhamnosus*

3.5 Antibiotic Sensitivity Assay

Human large intestine contains more than 10^{11} bacterial cells as native microflora with more than 500 different species of bacteria (mostly facultative anaerobes like *Enterobacteria, Coliforms, Lactobacillus,* etc.) (Mitsuoka, 1992; Tannock et al., 2000). When patients are under an antibiotic treatment there is a reduction in the microflora numbers. So it is critical that the probiotics are able to withstand these antibiotics for their sustained growth. Antibiotic resistance genes may be acquired due to continuous exposure to antibiotics especially in

irritable bowel syndrome (IBS) patients under long term antibiotic therapy. Hence the *Lactobacilli* were tested and compared for their survival against selected common broad spectrum antibiotics before and after spray drying (Figure 3.9 and Table 3.1).

Table 3.1: Antibiotic sensitivity profiles of *L. acidophilus* and *L. rhamnosus*

Antibiotic	Diameter of Inhibition zone (mm)		Characterization	
	L. acidophilus	*L. rhamnosus*	*L. acidophilus*	*L. rhamnosus*
Ampicillin	31	15	S	I
Chloramphenicol	19	18	S	S
Penicillin G	31.5	20	S	S
Streptomycin	12.5	0	I	R
Sulfamethoxazole-Trimethoprim	0	0	R	R
Tetracycline	27	25	S	S

*** S- Sensitive, I- Intermediate, R- Resistant**

From the data presented in Table 3.1 *L. acidophilus* is more sensitive than *L. rhamnosus* to some antibiotics (such as streptomycin and ampicillin). And similarly *L. acidophilus* was more sensitive to penicillin G than *L. rhamnosus*. Both species were sensitive to tetracycline and were resistant to sulfamethoxazole-trimethoprim. These tests check the resistance of lactobacilli against various antibiotics, which would be useful for their survival in the case of consumption by patients who are under antibiotic therapy. Since it is well known that the antibiotic resistance genes may be transferred to the host microbes it is essential to study this property. It has been well documented that there is a DNA, ribosome and enzyme melt-down/denaturation when the microbes are subjected to high temperature and thus it is expected that the antibiotic resistance might be altered as well (Teixeira et al., 1994 and 1997). Hence the antibiotic

sensitivity profile before and after spray drying were compared as the antibiotic resistance coding genes may be affected during spray drying. The molecular physiology involved in changing the antibiotic profile due to heat was not assessed as it is beyond the scope of the current study.

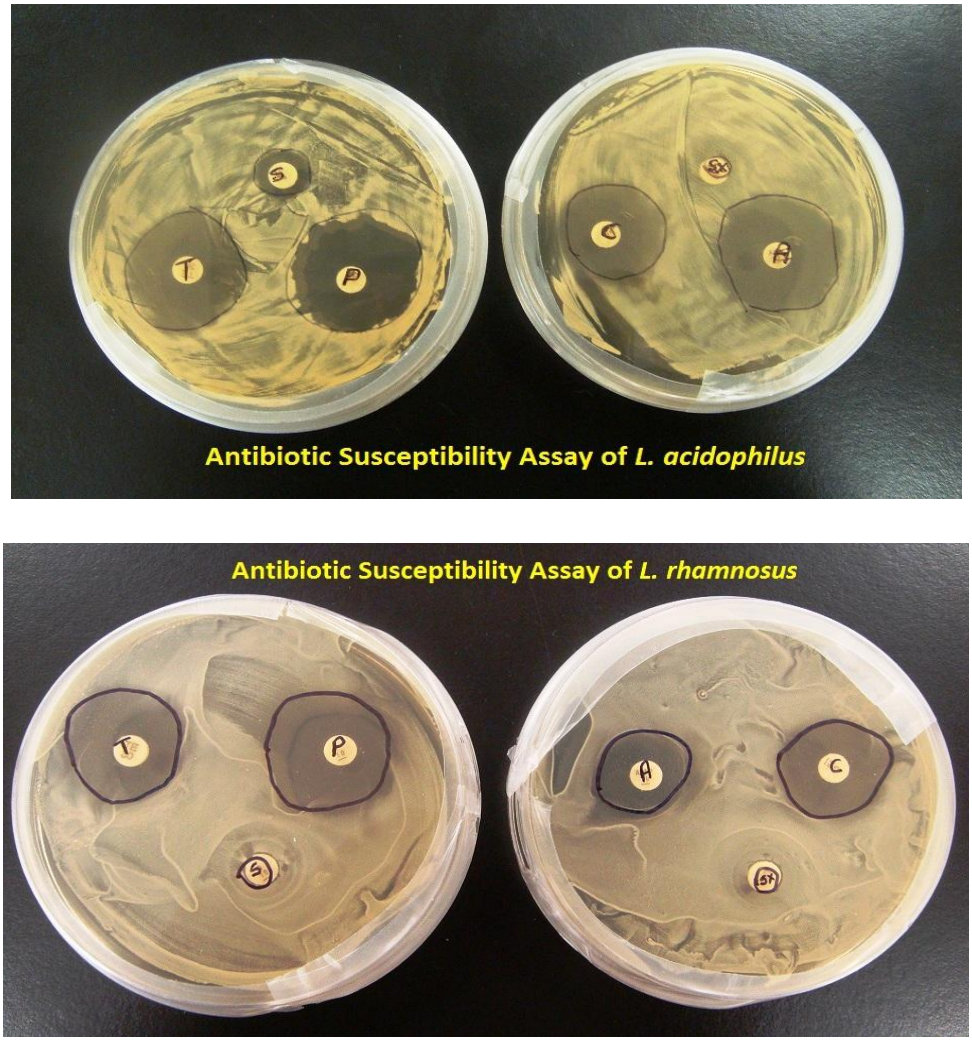

Figure 3.9: Zones of inhibition of *L. acidophilus* and *L. rhamnosus* by antibiotics (T- Tetracycline; S- Streptomycin; P- Penicillin G; SX- Sulfamethoxazole Trimethoprim; A- Ampicillin; C- Chloramphenicol

3.6 Sub-lethal Temperature Treatment

Exposing probiotics to sub-lethal thermal shock increases the subsequent tolerance to near lethal thermal stresses. In the current study, two different media were used in the assessment: MRS and raspberry juice. Once the microorganisms are heat shocked, the culture must be added to the juice before spray drying. A fruit juice juice was selected, such as raspberry juice because it obviates the extra steps of centrifugation and purification of the cultures before spray drying which was done in the case of MRS.

Cultures in their late exponential phase were chosen for sub-lethal exposure as they are still actively growing and about to reach carbon depletion. Hence there is a possibility of the culture being resistant either due to active growth and carbon depletion during the same phase of the growth cycle. Previous studies indicate that cells in the exponential phase are more easily adapted than in the stationary phase proving that age of cells also has a pronounced effect on the induction of thermo-tolerance (Corcoran et al., 2004 and 2006). Exposure to sub-lethal stress during the exponential phase was demonstrated to yield more resistant cells than during the stationary phase with *L. rhamnosus* (Prasad et al., 2003). Stationary cells however already have a few activated mechanisms for stress tolerance due to the onset of carbon depletion (Kim et al., 2001 and Teixeira et al., 1994). So if the cells are subjected at their late exponential phase/early stationary phase they are expected to have an added advantage with the above mentioned factors. The molecular physiology of proteins involved in this stress tolerance could be a potential future perspective of this study.

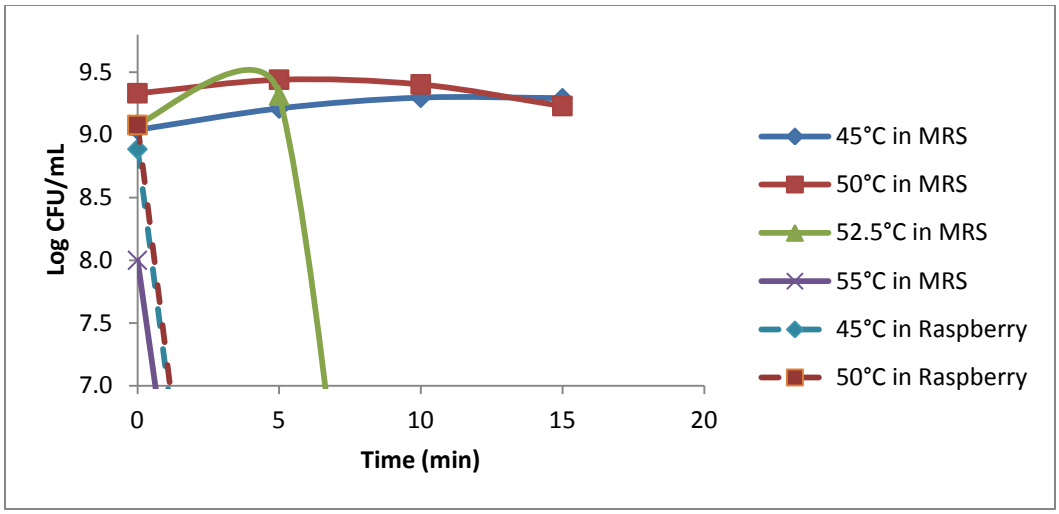

Figure 3.10: Sub-lethal temperature-time assessment of *L. acidophilus*

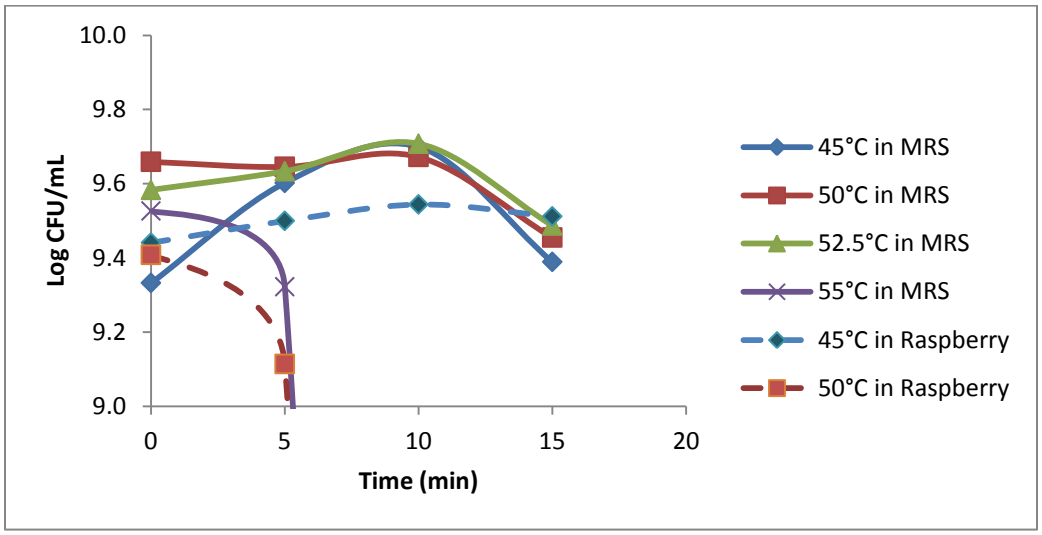

Figure 3.11: Sub-lethal temperature-time assessment of *L. rhamnosus*

From Figures 3.10 and 3.11, it can be seen that the viability of the probiotics was maintained up to 50°C for *L. acidophilus* and 52.5°C for *L. rhamnosus* for 10-12 min and hence they are considered as their sub-lethal temperatures. Raspberry did not help in any survival of both the *Lactobacilli* as the cell count decreased to zero within 5 min even at 45°C. MRS acted as a better heating medium than raspberry because of the more complex nutrients present in MRS medium than the plain raspberry juice which has mostly sugars and fibers. It is assumed

that the proteins present in the complex media may contribute to the stability of the intracellular proteins of the *Lactobacilli* during thermal shock.

The physiological response with respect to the heat stress at the molecular level has not been firmly established in probiotics. The sub-lethal stress induces protective mechanisms such as an alteration or reprogramming of the metabolic pathways to adjust to the new environment (Teixeira et al., 1994), thus increasing their survival during subsequent harsh treatment and viability during storage (O'Riordan et al., 2001; Selmer-Olsen et al., 1999; Shah and Ravula, 2000). Over expression of conserved heat shock proteins like GroEL, GroESL, DnaK, etc., aided the intracellular proteins of the *Lactobacilli* during spray drying at higher outlet temperatures (95-100°C) by protecting their cellular components and other macromolecules during drying. The translation of proteins proceeds faster than usual during the response to heat shock stress (Abee and Wouters, 1999). The storage stability can also be improved by thermal adaptation due to the over production of stress proteins (Corcoran et al., 2006). However it should be noted that all these mechanisms are strain-dependent and hence cannot be generalized for all *Lactobacilli*. Preliminary experiments (results not shown) on spray drying of heat shock treated and untreated cells also showed similar viability results where heat shock treated cells had a higher survival than untreated under same conditions.

Thermotolerance alone may not be the only criterion to judge the best performance during spray drying as the cells also undergo various other stresses like osmotic shock, accumulation of toxic compounds/metal ions and cell membrane damage which cannot be induced by sub-lethal treatment (Sunny-Roberts and Knorr, 2009). Hence the knowledge on age and history of the cells and medium in which heating of the cells occurred are essential for a successful thermal sub-lethal treatment (Teixeira et al., 1994).

3.7 Spray Drying

After the preliminary experiments, three maximum and minimum points of each independent variables, inlet temperature (°C), feed rate (mL/min) and juice solids: maltodextrin

ratio were chosen. The effect of sub-lethal stress on overnight cultures mixed with raspberry juice was assessed by spray drying under same conditions with and without stress exposure. Sub-lethal temperature exposure improved the survival rate during spray drying during the preliminary trial studies (data not shown). Cultures were exposed to T_{sl} of 50°C for *L. acidophilus* and 52.5°C for *L. rhamnosus* for 10-12 min.

The optimization of the three dependent variables, % recovery, % survival and color change (ΔE) (Table 3.2) was performed individually as well as in combination and by eliminating the insignificant factors in the process equation. Viability retention (in terms of log CFU/g), color of powder and rehydrated liquid, and A_w were measured throughout the storage study and discussed in greater detail in a later section (Table 3.7). Among the 20 different trials performed, the best three trials with higher shelf life and recovery were chosen for further analysis consisting of electron imaging studies and probiotic characteristics assays (acid and bile tolerance, antibiotic susceptibility assay) which were performed on these three trials after 0 and 30 days.

Table 3.2: Spray drying responses with the outputs for each dependent variables

Response	Inlet Temp (°C)	Maltodextrin Ratio	Inlet Feed Rate (mL/min)	Outlet Temperature (°C)	% Recovery	% Survival	Color (ΔE)
R1	115	1.5	50	82-86	25.4	71.14	54.252
R2	115	1.5	50	80-85	32.9	84.44	54.094
R3	115	1.5	40	81-86	36	68.24	52.766
R4	130	1	40	92-97	55	58.80	56.719
R5	100	2	60	69-74	25.6	78.97	52.187
R6	115	1.5	50	80-85	35.2	69.10	52.149
R7	100	1	60	67-72	28	80.26	56.623
R8	115	2	50	81-85	31.1	64.38	52.689
R9	115	1.5	50	81-86	35.2	71.46	52.062
R10	100	2	40	71-76	32.1	82.62	55.443
R11	100	1	40	68-74	47.1	84.33	56.394
R12	100	1.5	50	71-76	30.75	79.08	53.313
R13	130	1.5	50	91-95	36.8	53.65	53.413
R14	115	1.5	50	83-88	27.6	67.70	54.337
R15	130	2	40	91-96	38	53.65	51.558
R16	115	1	50	79-83	41.5	69.74	57.082
R17	130	2	60	88-92	32.5	68.03	53.238
R18	130	1	50	90-94	35.5	56.87	58.018
R19	115	1.5	60	77-83	24.65	70.39	53.165
R20	115	1.5	50	80-85	27.6	67.70	54.337

Although there was no operating control on the outlet temperatures, an increase in the feed rate gave a lower range of outlet temperatures for the same inlet temperatures. This is explained by the fact that the faster flow rate results in a denser powder deposition which effectively cools down the outlet chamber. The factors and effects on the dependent variables [% recovery, % survival and color (ΔE)] with respect to outlet temperature (°C) are considered separately.

3.8 Raspberry Juice as a Carrier

The raspberry juice encapsulated probiotic powder is a synbiotic product combining prebiotic fibers (from juice) with maltodextrin and probiotics- *L. acidophilus* and *L. rhamnosus.* The sugars present in the raspberry may contribute to the survival during drying and storage since sugars act as thermoprotectants during spray drying (Carvalho et al., 2003) by replacing water molecules and stabilizing proteins (by forming hydrogen bonds) and phospholipid bilayer residues of the cell membrane (Rokka and Rantamäki, 2010b; Santivarangkna et al., 2008). Steric hindrance of large sugars might prevent them from interacting with proteins (Ananta et al., 2005; Crowe and Crowe, 1986). The presence of sugars can also aid during gastric survival and high acid conditions in the gut by enhancing the survival during storage by maintaining the energy status of the cell and thus enabling proton exclusion (Charalampopoulos et al., 2003; Corcoran et al., 2005).

Like for most other berries raspberry juice contains an appreciable amount of pectins extracted from the skin and it can be assumed that they partially contribute to emulsification of the probiotic suspension. Studies have proven that the functional properties of pectin were not affected by spray drying (Gharsallaoui et al., 2007). Pectins can also act as prebiotics if the probiotic species contain enzymes for galacturonate metabolism to break the carbon bonds (Yeo and Liong, 2010). Smaller fiber chains of the food component might still retain certain characteristic activities which are essential to remain functional as dietary fiber as well as a prebiotic.

Raspberries naturally have a high content of ascorbic acid, which is expected to enhance the storage of the probiotic powder. Inclusion of ascorbic acid as an antioxidant additive during spray drying of *Lactobacillus* cultures was reported to have both pros and cons. At room temperature it might act as a strong antioxidant and produce hydroxyl radicals which deteriorate biological molecules by oxidation. But at lower temperatures, they have a regular anti-oxidant property, protecting the cells (Teixeira et al., 1995a).

3.9 Process Optimization

Optimization is essential to scale up any lab scale process. In the current study, inlet temperature (°C), total solids: maltodextrin ratio and inlet feed rate (mL/min) were optimized with respect to % recovery, % survival and color (ΔE) as dependent variables. SAS 9.2 and JMP-8 were used to optimize the process and generate response surface plots.

3.9.1 Product % Recovery

The recovery varied between 25% and 55% depending on the maltodextrin ratio and feed rate. The walls of the spray drier were layered completely with the raspberry solids by the end of spray drying due to the stickiness of the solutions which lead to major losses in product recovery. An interesting observation from the data and graphs is that the temperature had a minimal role in recovery. The response plot for %recovery with respect to total solids: maltodextrin ratio and feed flow rate is presented in Figure 3.12. The grid on the top indicates the overall optimum condition with respect to maximum % recovery (48.79 %) at input conditions of 100°C inlet temperature, a maltodextrin: total solids in juice ratio of 1:1 and an inlet feed rate of 40 mL/min.

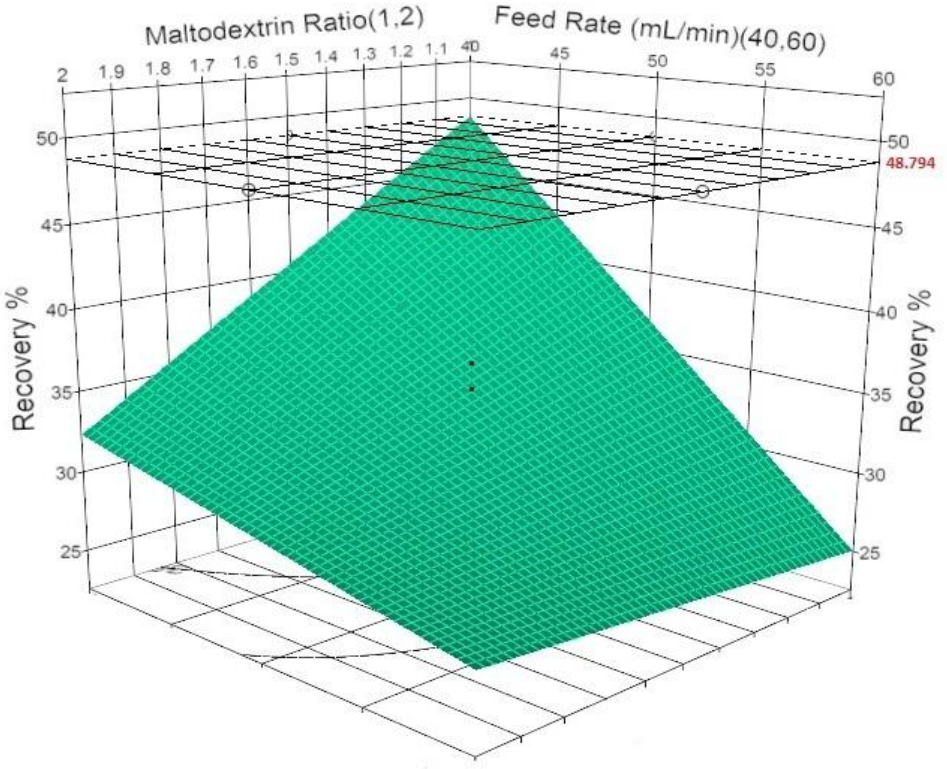

Figure 3.12: Response plot of % recovery with respect to maltodextrin ratio and feed rate (mL/min)

From the response plot in Figure 3.12, maltodextrin ratio and feed rate had significant effects on % recovery of produced powder from raspberry juice. Both variables inversely affected the recovery. As the maltodextrin ratio increased from 1 to 2 the recovery dropped to 33%. Similarly when the feed rate increased upto 60 mL/min the recovery dropped to as low as 25%. Reducing the maltodextrin ratio can increase the recovery however it may affect the survival of the probiotic due to reduced encapsulation efficiency. Reducing the feed flow rate could be an alternative approach to increase product recovery since it had minimal effect on probiotic survival as discussed below. But the possible disadvantage with reduced feed rate is the increase in residence time within the drying chamber which exposes the probiotics to longer thermal stress.

The optimized equation (Eq 3.1) follows a two factor linear form. From the analysis temperature did not have a significant role in product recovery. Ratio of maltodextrin and feed rate were the significant factors in the optimized predictive model. The R^2 value of the master model was 0.86 whereas the predictive model had an R^2 value of 0.71 (Table 3.3). The ANOVA table and effect estimates for % recovery of the master and predicted model are presented in appendix Table 1 and Table 2.

Table 3.3: Fit statistics for % recovery surface plot

	Master Model	Predictive Model
Mean	33.925	33.925
R-square	85.78%	70.72%
Adj. R-square	72.99%	65.23%

The optimized coded equation (-1,1) of predictive model

% RECOVERY = 33.331 - 3.593*MALTODEXTRIN RATIO - 7.236*FEED RATE +

4.63*MALTODEXTRIN RATIO*FEED RATE ---------- (Equation 3.1)

Low dextrose equivalent additives have been demonstrated to increase the product recovery by reducing the stickiness of orange juice and similarly higher Dextrose Equivalent (DE) value increased moisture content of the final product (Goula and Adamopoulos, 2010). In the study conducted by Phongpipatpong et al. (2008), on longan juice spray drying with maltodextrin as an additive, maltodextrin had a positive impact on the recovery of spray dried powder by reducing product stickiness.

3.9.2 % Survival

Prediction of microbial survival is essential to optimize any potential industrial process. High viability is required in the final probiotic product. The surface response plot for % survival with respect to varying temperature (°C) and feed flow rate (mL/min) is presented in Figure 3.13. The grid on top indicates the optimized conditions with respect to maximum % survival (81.17%) at the input conditions of100°C inlet temperature, a maltodextrin: total solids in juice ratio of 1:1 and an inlet feed rate of 40 mL/min.

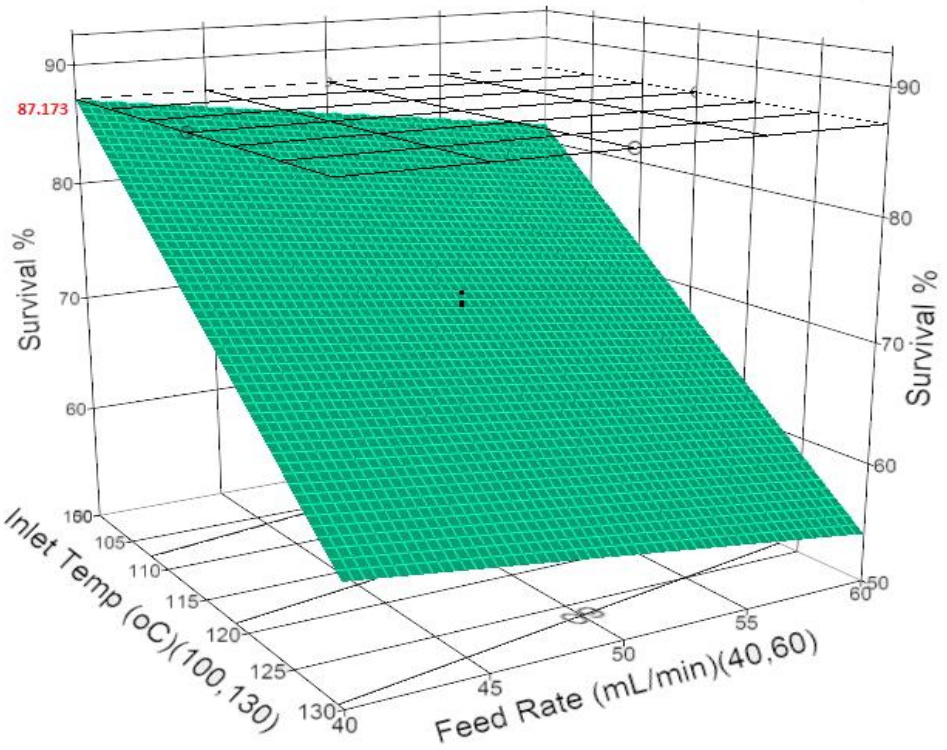

Figure 3.13: Response plot for % survival with inlet temperature (°C) and feed rate (mL/min)

From the response plot presented in Figure 3.13, the processing temperature had a major effect on the probiotics' survival as expected. The survival dropped down to almost 55% (9.5 to 5 Log CFU/mL) when the inlet temperature was raised to 130°C. The feed rate did not have a very significant role comparatively. It was also noted that the outlet temperature varied

with the inlet feed rate. From Table 3.2 it is seen that under same inlet temperature conditions for a high inlet feed rate, the outlet temperature was lower with an increased survival rate. This indicates that the survival is mostly dependent on outlet temperature. Increases in outlet temperatures is the major factor affecting the cell survival, more importantly than inlet temperature as expected from previous studies (Boza et al., 2004). In general outlet temperatures greater than 85°-90°C are lethal for probiotics (Corcoran et al., 2004; Gardiner et al., 2000; Zamora et al., 2006) but sub-lethal temperature pretreatment enabled cells to survive in that range with cell death occurring only after 92°C outlet temperature in our current study. Heat shock proteins produced during sub-lethal stress aid the probiotics during subsequent stress. They usually assist during the refolding of denatured proteins or removal of denatured proteins before they cause death (Kim et al., 2001).

However, the outlet temperature of a spray dryer is difficult to predict, control or fix for any given set of operating conditions. The effect of temperature on survival of the probiotics is also strain dependent as there are varying results from strain to strain (Silva et al., 2002). Though it is expected that higher temperatures reduce the viability of the probiotics, this inactivation is rather the combination of temperature and time (Kim and Bhowmik, 1990; To and Etzel, 1997).

In reported research, maltodextrin offered good adherence to the probiotics during drying, storage and also gastric transit (Mattila-Sandholm et al., 2002). Other studies have proven that probiotics encapsulated in starch were able to exert their health benefits and stress tolerance in the gut (Ding and Shah, 2007; Krasaekoopt et al., 2004). The survival is attributed to the strong adherence to the carrier, which protects cells from high acidic and bile conditions (Crittenden et al., 2001). Overall, maltodextrin is confirmed to serve as a good encapsulating matrix as well as a moderate prebiotic for high survival of probiotics (Cortes-Arminio et al., 2010).

The molecular nature of heat damage (above 90°C) is not clearly known but denaturation of critical proteins, DNA and ribosomes are few vital events (Teixeira et al., 1997). The cell membrane heat damage is one of the most susceptible target during spray drying. High temperatures during spray drying cause the cellular pores to leak the intracellular substances from the cell (Corcoran et al., 2004; Gardiner et al., 2000). Loss of metabolic activity might also be observed due to the denaturation of proteins (Meng et al., 2008). Critical components like ribosomes, DNA/RNA and their related enzymes may be lost which account for the loss of viability. The glycolytic enzymes production which is responsible for higher survival during and after spray drying, are also reduced due to the thermal stress (Prasad et al., 2003). Another mechanism which causes thermal cell death is the fact that Mg^{+2} ions ooze out of the cells during thermal stress and these ions are necessary for ribosome stability (Abee and Wouters, 1999).

The % survival optimized model equation (Eq 3.2) follows a two factor linear form where temperature plays the most important role. The R^2 value of the master model was 0.96 whereas the optimized predictive model had an R^2 value of 0.91 (Table 3.4). The ANOVA table and effect estimates for % survival of master and predicted model are presented in appendix Table 3 and Table 4.

Table 3.4: Fit statistics for % survival

	Master Model	Predictive Model
Mean	68.075	68.075
R-square	95.76%	91.25%
Adj. R-square	91.52%	87.89%

The optimized coded (-1, 1) equation of the predictive model for the probiotic survival is presented in equation 3.2

% SURVIVAL = 67.933 - 8.98*INLET TEMP - 2.681*MALTODEXTRIN RATIO + 3.124*FEED RATE + 4.863*INLET TEMP*MALTODEXTRIN RATIO + 5.840*MALTODEXTRIN RATIO*FEED RATE ---------- (Equation 3.2)

The optimization equation suggests that survival is a function of processing conditions as well as the chemical nature of the suspension. These results are in agreement with some of the previous studies where conditions of spray drying as well as composition of suspension had equal effect on probiotic survival and during storage as well (Chávez and Ledeboer, 2007; Cortes-Arminio et.al., 2010)

In another study, higher viability was seen when the spray pressure was reduced causing reduction in shear stress on the microorganisms (Lievense et al., 1994). Other alternatives to enhance survival as well as storage life of probiotics include the alteration of strain dependent parameters like growth pattern and design of culture (nutrient requirements); and encapsulation modifications (carrier materials, additives, glass transition, etc) to improve efficiency and barrier properties (Chávez and Ledeboer, 2007; Mattila-Sandholm et al., 2002; Weinbreck et al., 2010).

3.9.3 Color

Color is the foremost sensory perception for any successful launch of a novel food. Hence it was chosen as one of the dependent factor to be optimized. The color change is analyzed in the current study as the color differed from the standard control (white color). There was no significant loss of color at the end of the 30 day storage indicating color stability. As discussed earlier the produced raspberry powder is primarily consumed as a rehydrated liquid so the color of the rehydrated liquid was also assessed. The grid on top of the response plot for color, presented in Figure 3.14, indicates the optimized conditions with respect to maximum color (ΔE 57.210) at conditions of 100°C inlet temperature, maltodextrin: total solids in juice ratio of 1:1 and inlet feed rate of 40 mL/min.

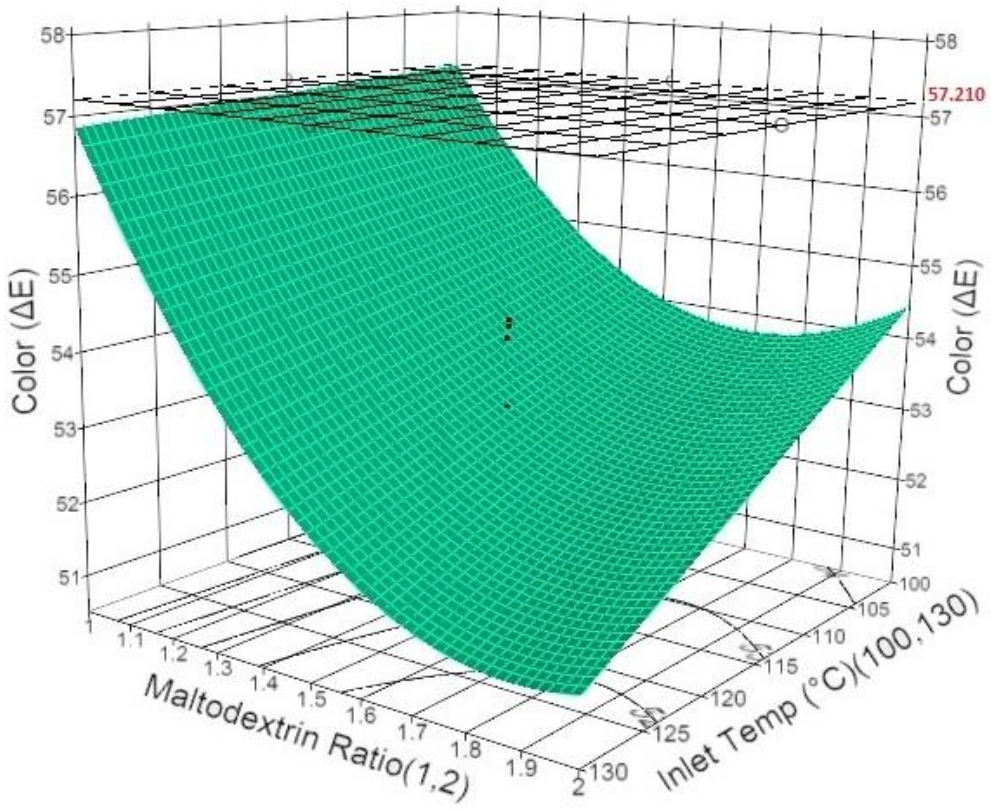

Figure 3.14: Response plot of powder color with maltodextrin ratio and inlet temperature (°C) as independent variables.

Figure 3.14 presents the response plot of powder color showing that the maltodextrin ratio had a major effect on color (ΔE) as it reduced (reaching towards white or increase in whiteness) with an increasing concentration of maltodextrin. On the other side, the rehydrated liquid (data not shown), produced from the powder for all the three MD ratios had similar bright red color visually though the ΔE values varied slightly. In the case where consumption of the raspberry powder is via rehydration then maltodextrin ratio can be increased further as the rehydrated liquid is more or less bright red in color irrespective of the MD concentration. Increasing maltodextrin ratio also maximizes recovery of powder and microbial survival. However, higher concentration of carriers leads to bigger spray dried particles and increased times of drying and exposure to high temperatures which may reduce the viability. Maillard

browning of sugars may take place during extended spray drying which contributes to the color of the powder.

The optimized model equation for the powder color (Eq 3.3) follows a quadratic form with maltodextrin ratio as a major factor. As discussed earlier, increasing the additives concentration reduced the color to pale pink. The R^2 value of the master model was 0.91 whereas the optimized predictive model had an R^2 value of 0.87 (Table 3.5). The ANOVA table and effect estimates for % color of master and predicted models are presented in appendix Table 5 and Table 6.

Table 3.5: Fit statistics for powder color

	Master Model	Predictive Model
Mean	54.184	54.184
R-square	90.97%	86.97%
Adj. R-square	81.93%	80.46%

The optimized predictive model (-1,1) for powder color is presented in Equation 3.3

COLOR = 53.283 - 0.005*INLET TEMP - 2.068*MALTODEXTRIN RATIO + 0.114*FEED RATE - 0.689*INLET TEMP*MALTODEXTRIN RATIO + 0.849*INLET TEMP*FEED RATE + 1.808*MALTODEXTRIN RATIO*MALTODEXTRIN RATIO

---------- (Equation 3.3)

3.10 Optimization of all Parameters

Overall parameter optimization is essential for scale up of any lab process to industrial scale. The three independent variables were optimized simultaneously to obtain the condition at which there is maximum % recovery, % survival and best color. Optimized conditions were determined at 100°C inlet temperature, juice solids: maltodextrin ratio of 1:1 and feed flow rate of 40 mL/min, where the maximum output dependent variables were obtained with 48.79 % recovery, 87.17 % survival and 57.21 color change (ΔE) as presented in Table 3.6.

Table 3.6 Summary of optimized variable values

Factor	Setting
Inlet Temperature (°C)	100
Maltodextrin ratio	1
Feed rate (mL/min)	40

Responses:

Response	Est. Value
Recovery %	48.794 [43.074, 54.515]
Survival %	87.173 [81.217, 93.128]
Color (ΔE)	57.210 [55.690, 58.731]

Desirability

Overall
91.15%

The overall desirability of the model was 91.15% which is quite high for a biological system on which there is minimal process control. These conditions were also further confirmed during the storage study. To the best of our knowledge there are no previous studies which optimized the microencapsulation of probiotics in fruit powder by spray drying.

3.11 Fruit Powders' Shelf Life

Fruit powders, such as raspberry powder are processed forms of the actual fruits and are versatile in consumption and usage. When adequately processed, they retain most of the natural benefits present in the original fresh fruit with the versatility of a longer storage life. They can be used as an additive or formulation for any nutritional or pharmaceutical product.

Fruit powders are added to dairy products such as ice cream, milk shakes, desserts, flavored yogurt etc. They can also be used in bakery and confectionary products, baby foods, snacks, salads, jellies, instant mixes, custards, puddings, ketchups and seasonings.

Inclusion of probiotics may give sensory concerns of having dairy or medicinal flavours due to the lactic acid end product (Luckow et al., 2005), but numerous non-dairy probiotic products have been released into the market which demonstrated the growing consumer acceptance. However very few studies have correlated the quality attributes, and storage conditions with the functionality of their contained probiotics (Vinderola et al., 2011).

The shelf life of the probiotics in our spray dried raspberry powder was reduced with an increase in storage temperature as presented in Table 3.7. Storage at lower temperature ensured longer shelf life and higher cell count retention at the end of 30 days which is in agreement with previous research (Corcoran et al., 2004). The presence of compatible solutes like sugars and small peptides also contribute in the maintenance of enzyme activity and cellular protection after drying. Lipid oxidation of the cells walls during storage and subsequent permanent damage is considered as the main cause for low shelf life of spray dried cultures (Teixeira et al., 1996); Meng et al., 2008).

The % survival of 0 presented in Table 3.7 indicates that those samples had a shelf life less than 30 days but greater than 15 days. Samples with ND were not determined at that point since they had a shelf life of less than one week even when stored under cold temperature. All of the ND samples were processed at the high inlet temperature of 130°C which is the obvious reason for very low survival and shelf life. The highlighted responses R3, R10 and R11 are the best three responses with respect to viability and they were further analyzed.

Table 3.7: Storage studies of spray dried raspberry probiotic powder

Response	% Survival (Day 0)	% Survival Day 30 (RT)	% Survival Day 30 (CT)	Aw-Day 0	Aw Day 30 (RT)	Aw Day 30 (CT)
R1	71.14	0		0.127	0.301	ND
R2	84.44		94.0	0.19		0.303
R3	68.24	86.2	91.4	0.154±0.001	0.312	0.3
R4	58.80		ND	0.259		ND
R5	78.97	0	0	0.27±0.002	0.311	0.323
R6	69.10		94.9	0.193		0.303
R7	80.26	66.8	81.3	0.172±0.003	0.323	0.266
R8	64.38	0	93.3	0.153±0.015	0.303	0.292
R9	71.46	0		0.193	0.319	
R10	82.62	76.6	98.4	0.172±0.009	0.338	0.307
R11	84.33	73.0	88.8	0.113±0.001	0.289	0.303
R12	79.08	0	96.6	0.124±0.013	0.259	0.256
R13	53.65		ND	0.099		ND
R14	67.70	0		0.133	0.317	
R15	53.65		ND	0.147		ND
R16	69.74	0	0	0.119±0.007	0.277	0.284
R17	68.03		ND	0.154		ND
R18	56.87		ND	0.199		ND
R19	70.39	0	0	0.15±0.01	0.321	0.265
R20	67.70	0		0.133	0.317	

RT- Room temperature (23±2°C), CT- Cold temperature (4±2°C), ND- Not determined

Survival during spray drying does not necessarily correlate or ensure survival during storage. Indeed, a series of optimal combinations of conditions are necessary to ensure survival during drying as well as during storage (Ying et al., 2010). Cell survival is the main criterion considered in the evaluation of the storage of probiotic foods but not the only criterion. Frequently, the reduction in cell viability is evident during storage but is not necessarily detected immediately after spray drying unlike what is experienced in freeze drying (Zamora et al., 2006). Due to the stress involved during spray drying, the cells tend to lose viability over the period of storage and hence it is essential to check that the required standard counts (> 10^6-10^8CFU/g) are maintained throughout the shelf life of the product (Ishibashi et al., 1985).

The viability of probiotic cells was assessed in this study for 30 days (Figure 3.15). Obviously, cold storage had higher retention (almost 98%) of cells at the end of 30 days. Low temperature processing caused less damage to cells and hence a higher shelf life can be correlated to the minimal processing stress. The rehydration property is inversely related to the Dextrose Equivalent (DE) value of the maltodextrin because a lower DE additive produces powder with lower moisture content (less sticky) thus ensuring faster rehydration (Goula and Adamopoulos, 2010). This property is particularly useful in cell re-activation in spray dried powder after thermal stress. The room temperature storage shelf life was also as high as 72-86%, though not comparable with refrigerated storage. Microencapsulation in a stable adhering matrix (raspberry+ maltodextrin) contributed to an adequate shelf life.

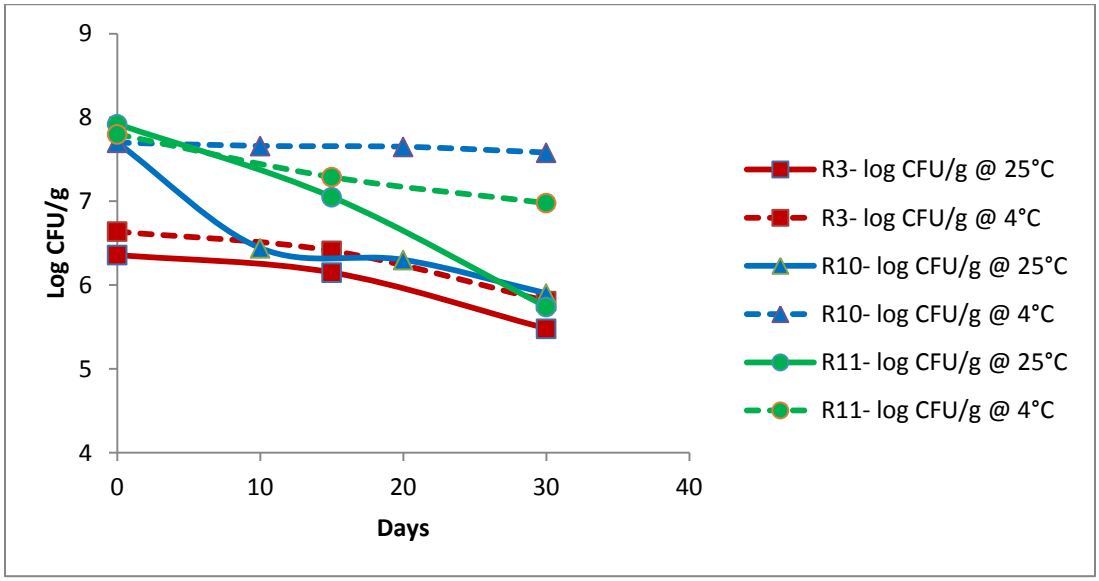

Figure 3.15: Viability of probiotics in raspberry powder (resuscitated in MRD) during the storage study for the three best responses (R3, R10 and R11)

The best three trials with highest viability at room and cold storage temperatures were determined as R3, R10 and R11. As expected the probiotic cell survival decreased during the storage (Figure 3.15). The spray dried samples stored at refrigeration temperature maintained higher viability than at room temperature. R10 samples retained highest viability indicating that a higher concentration of maltodextrin improved shelf life of the raspberry spray dried

probiotic powder. But R10 samples had a major drop in survival after 15 days probably due to the increased A_w which is discussed further below. R11 and R10 had same viability at room temperature storage.

From Table 3.7, R3 had lower cell number immediately after spray drying compared to the other two trials R10 and R11 but the % viability retention during storage at room temperature was higher. This suggests that spray drying at higher temperature may have higher % viability retention during room temperature storage following the high temperature stress. So in order to reduce the refrigeration storage costs spray drying at higher temperature with 1:1.5 total solids: maltodextrin ratio and a feed rate of 40 mL/min is suggested from these experiments. However, the best three trials suggest lower feed rate and lower temperature during spray drying for a better survival at the onset, and the microencapsulating wall material ratio can be varied depending on its chemical nature.

Survival stability of probiotics in spray dried raspberry powder is inversely related to temperature down to 4^{o}C. But cold storage might be a major limitation for commercializing production, storage and transport of probiotic products economically (Riveros et al., 2009; Simpson et al., 2005; Zamora et al., 2006). Furthermore, stability and maintenance of probiotic activity is higher in the presence of encapsulated additives and also with prior sub-lethal thermal stress conditioning (Chávez and Ledeboer, 2007; Corcoran et al., 2004; Desmond et al., 2002; Gardiner et al., 2002; Hansen et al., 2002; Kabeir et al., 2009; Krasaekoopt et al., 2004; Teixeira et al., 1995b; Wang et al., 2004)

3.12 Probiotic Property Testing of the Spray Dried Powder

Since the probiotics do not multiply when they are formulated in the powder, maintenance of stability is an issue. There have been reported discrepancies in the functionality of probiotics under *in vivo* and *in vitro* conditions after spray drying due to loss of vital components. It was also proven that though the viability of the probiotics is absent in the later drying stages, they were still able to exert, to some limited extent, their health benefits of carcinogen binding and immune modulation (Mattila-Sandholm et al., 2002).

The gastrointestinal tract is acidic with presence of bile salts which inhibit most gram positive microorganisms. Acid and bile tolerance was tested and present in the spray dried cells, indicating that the thermal stress had negligible effect on these intrinsic properties. Acid and bile resistance tests and antibiotic sensitivity tests were performed on the best three samples following 30 days of storage. From Figures 3.16 and 3.17, the acid resistance was slightly reduced because there was no growth at pH 1.5 even at initial 0^{th} hour exposure. However both the species were resistant at pH 3 which is still highly acidic. Bile resistance however remained high. The inclusion of maltodextrin has been shown to preserve the *Lactobacilli'* probiotic properties such as acid and bile tolerance and cholesterol assimilation even after spray drying (Reddy et al., 2009).

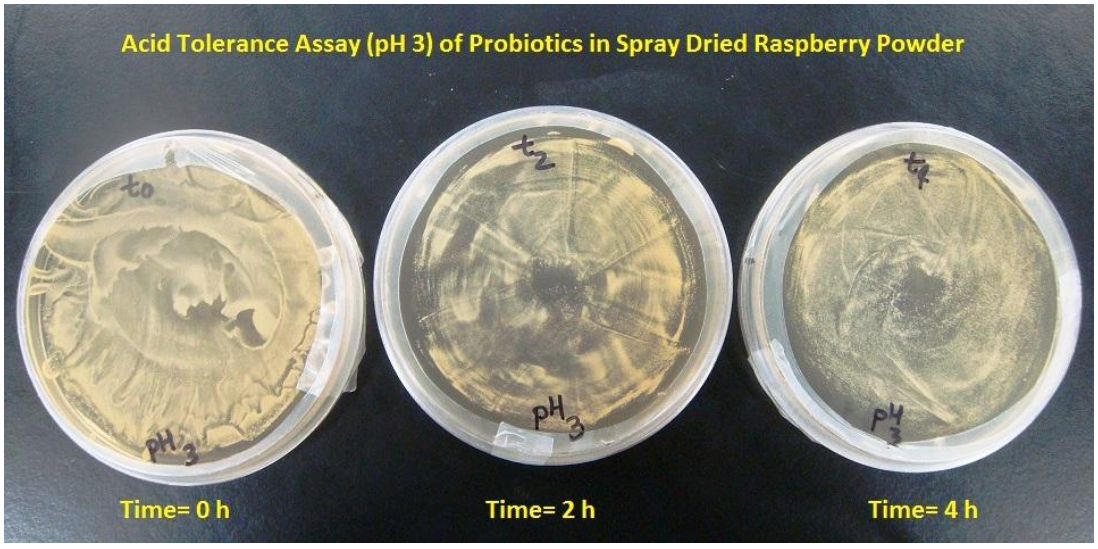

Figure 3.16: Acid Tolerance Assay (pH 3) of Probiotics in spray dried raspberry powder

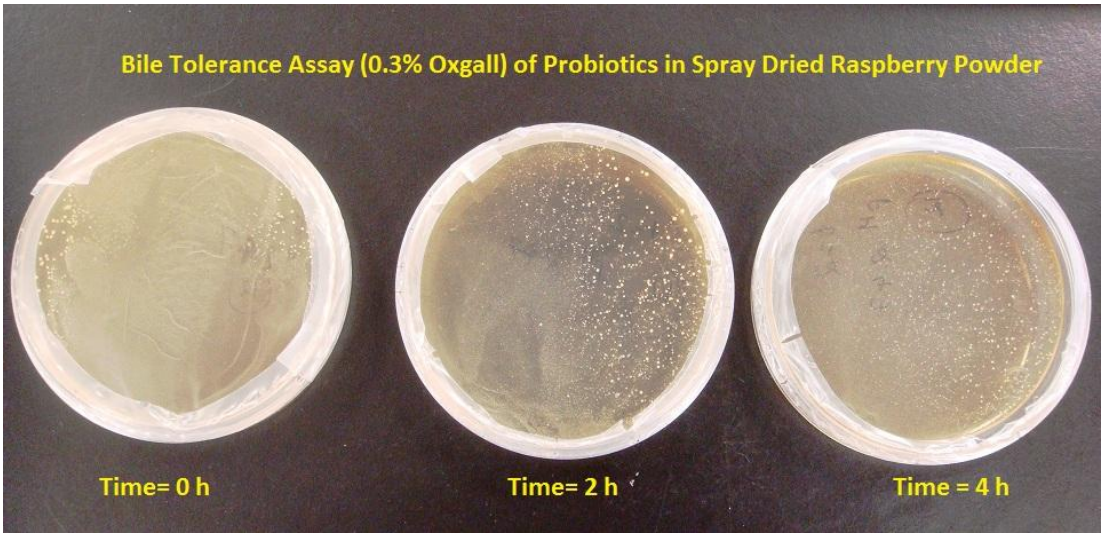

Figure 3.17: Bile Tolerance Assay (0.3% Oxgall) of Probiotics in spray dried raspberry powder

Antibiotic susceptibility profiles however changed and are presented in Table 3.8 with the sensitivity tests presented in Figure 3.18:

Table 3.8 Antibiotic sensitivity profile of the probiotics in spray dried raspberry powder

Antibiotics	Zone of Inhibition (mm)	Characteristic*
Ampicillin	27	S
Chloramphenicol	25	S
Penicillin G	0	R
Streptomycin	20	I
Sulfamethoxazole- Trimehtoprim	32	S
Tetracycline	27	S

***S- Sensitive, I- Intermediate, R- Resistant**

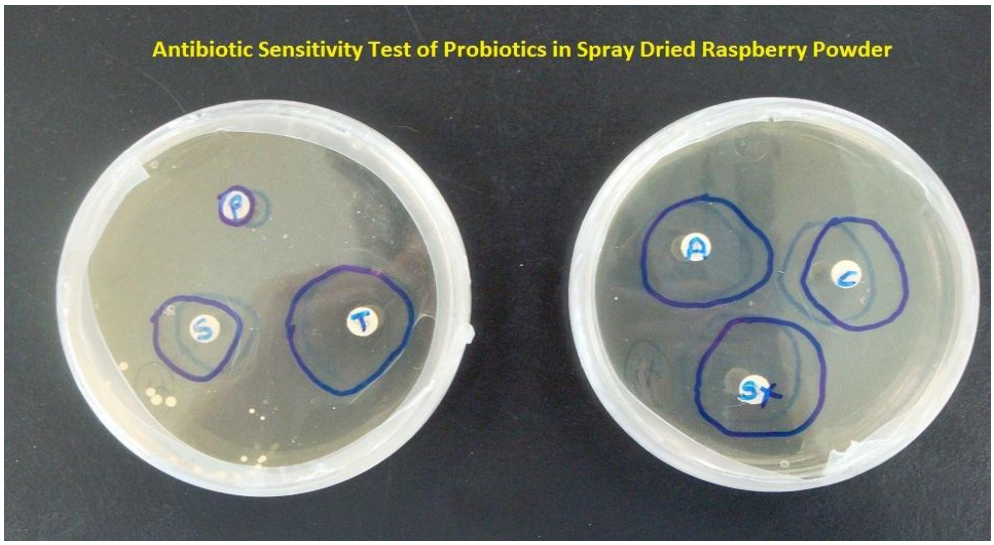

Figure 3.18: Zones of inhibitions of *L. acidophilus* and *L. rhamnosus against* antibiotics (T- Tetracycline; S- Streptomycin; P- Penicillin G; SXT- Sulfamethoxazole Trimethoprim; A- Ampicillin; C- Chloramphenicol

The species have become resistant after spray drying to sulfamethoxazole-trimethoprim. The reason for developing the resistance has not been established but can be hypothesized to be caused by a thermally induced gene mutation. Similar reasons can be accounted for the increased sensitivity towards antibiotics chloramphenicol and penicillin G. However no strong conclusion can be made superficially without molecular characterization of the genes involved which is a future prospect for research. Thus microencapsulation of *Lactobacilli* through spray drying offered protection against high acid, bile, gastric enzymes, temperature treatments which are also in good agreement with reported results from literature (Ding and Shah, 2007; Fávaro-Trindade and Grosso, 2002; Reddy et al., 2009).

The resuscitation conditions of the spray dried powders also have an effect on the restoration of viability and activity after spray drying. Particle size, wet ability of the constituents, pH and osmolarity, rehydration temperature, etc., affect the activity. But inability of growth on plates cannot be concluded solely as loss of probiotic activity. Probiotics may exert their health benefits even though they do not replicate metabolically (Rokka and Rantamäki, 2010). There is no universal resuscitation media and it varies from strain to strain (Muller et al., 2010). It is not possible to imitate the domestic consumption style if rehydration media is fixed because ideally any food must be versatile in consumption.

3.13 Storage in Glass

In the current study the spray dried probiotics raspberry powder was stored in glass bottles (Table 3.7). Storage in glass bottles is oxygen impermeable, non-toxic, safe and recyclable. It does not give any off flavors to the product like plastics sometimes do. Many studies proved that spray dried probiotics have a stable storage of at least 30 days under favorable conditions in an appropriate packaging material (Fu and Etzel, 1995; Gardiner et al., 2000; Kearney et al., 2009; Simpson et al., 2005; Wang et al., 2004).

Oxygen permeability might increase the death rate of the probiotics during storage for which reason storage in glass containers is recommended (Shah, 2000). High viability of microencapsulated bifidobacteria at the end of a storage life of 40 days was observed in glass bottles (Hsiao et al., 2004). There are still no commercial lactic acid cultures that are stable for a long period at room temperatures

3.14 Water Activity (A_w)

Residual water is essential, in spray dried probiotics, to maintain the protein conformation for enzyme activity, cell wall-lipid membrane structural stability, ribosomes etc. (Peighambardoust et al., 2011). Residual moisture content of the spray dried powder is dependent on the cell suspension media, carrier, additives and spray drying conditions (Wang et al., 2004). Reducing water content below a certain minimum has proved detrimental as the voids on the particles open up allowing oxidative degradation of lipids and proteins in the cell (Fu and Etzel, 1995). Water activity and presence of oxygen are factors which affect the viability of probiotics during storage (Anal and Singh, 2007; Weinbreck et al., 2010).

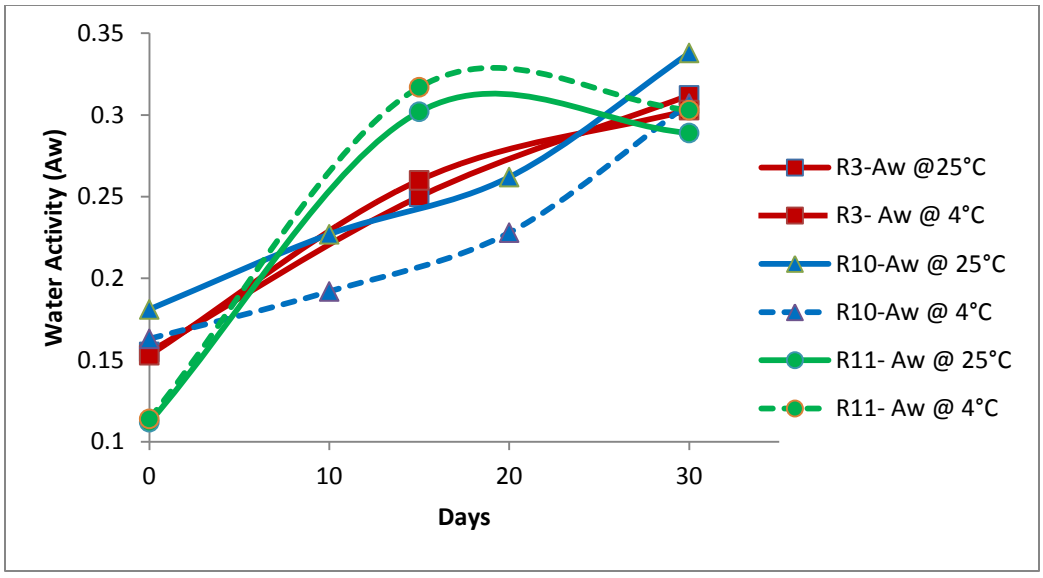

Figure 3.19: Water activity distribution of the raspberry probiotics powder during the storage of the best three responses (R3, R10 and R11)

A_w increased during storage time and reached around 0.305 for the best three responses (Figure 3.19). The R11 sample in particular showed a slight decrease in A_w from day 15 to 30 probably due to the high concentration of maltodextrin. Viability in cold storage for R11 was almost near R10 and higher than R3. At the end of 30 days at room temperature, the R3 and R10 had higher A_w than their corresponding cold stored samples. But R11 showed higher A_w at cold storage temperature than at room temperature probably due to the absorbed ambient moisture from the refrigerator from improper sealing.

A_w was higher (0.155-0.175) on day 0 for powders prepared at lower temperatures compared to (0.099-0.115) obtained at higher temperatures as the higher temperature drying process forces more of the free water out of the sample. Relative humidity in storage and moisture content of the samples significantly affected the A_w. There was no proper correlation between A_w and survival during storage because there was a survival even when A_w was between 0.245 and 0.338. It was reported that stable A_w<0.3 (moisture content of less than 5%) is essential for a good survival of the probiotics during storage (Chávez and Ledeboer, 2007; Teixeira et al., 1995b). Ideally A_w should be between 0.11 to 0.23 (moisture content of 4-5 %)

for most *Lactobacillus* species (Koc et al., 2010; Kumar and Mishra, 2004). Increased A_w encourages a faster death rate of the probiotics during storage as it encourages other microorganisms and fungi to grow as well as undesirable chemical reactions (Chávez and Ledeboer, 2007; Teixeira et al., 1995b; Wang et al., 2004; Ying et al., 2010b).

Smaller carbohydrates replace sugar molecules during low water activities and thus help in the survival of *Lactobacilli* by stabilizing the cell wall's functional integrity, thus preventing dehydration inactivation (Linders et al., 1997). The addition of maltodextrin to the spray dried powder also reduced the A_w thus increasing the shelf life as well as the rehydration capacity of the produced spray dried powder.

3.14.1 % Moisture Content

The relation between Aw and % moisture content is quite complex and specific to each food. % moisture contents (dry basis) of the best three responses were analyzed. R3 had a moisture content of 8%, R10- 7 % and R11- 12%. Although the temperature employed in R3 was higher the % moisture content is same as for R10 indicating that high maltodextrin concentration had similar effect on lowering moisture content as high temperature spray drying. It is expected that % moisture content would increase with an increase in maltodextrin concentration because the water molecules are unable to escape through the large maltodextrin molecules during the process. However R11 with a very low maltodextrin concentration had very high moisture content. The low temperature employed during the processing of this sample can be accounted for the high moisture content. It may be concluded that maltodextrin did not play a significant role in moisture content. Residual moisture content of 4% is ideal for the long storage of spray dried powders. The final moisture content of the powder was significantly reduced as the outlet temperature of the spray dryer increased as expected (Ananta et al., 2005).

3.15 Particle Size

Particle size can play an important role in the activity and applications of probiotics. The shape of the microcapsule could reveal information on surface porosity, and hence release kinetics of the core material, flow properties of the powder, etc. (Ré, 1998). The encapsulated probiotic capsule size is important as it also affects the textural and sensorial property of the subsequent food application (Burgain et al., 2011). Once encapsulated, the rehydration properties and potential application of spray dried powder are dependent on the capsule size. The mouth feel of the powder is greatly affected and it can be improved if the microcapsules are very small.

Figure 3.20 presents the SEM micrographs of the R3 raspberry powder with 1.5 ratio of maltodextrin which gave slightly large microcapsules compared to the other two MD ratios. During the storage period, the sphere dimensions did not vary much under cold storage conditions, however, there was slight agglomeration seen, due to the high relative humidity, for the samples stored under ambient room conditions.

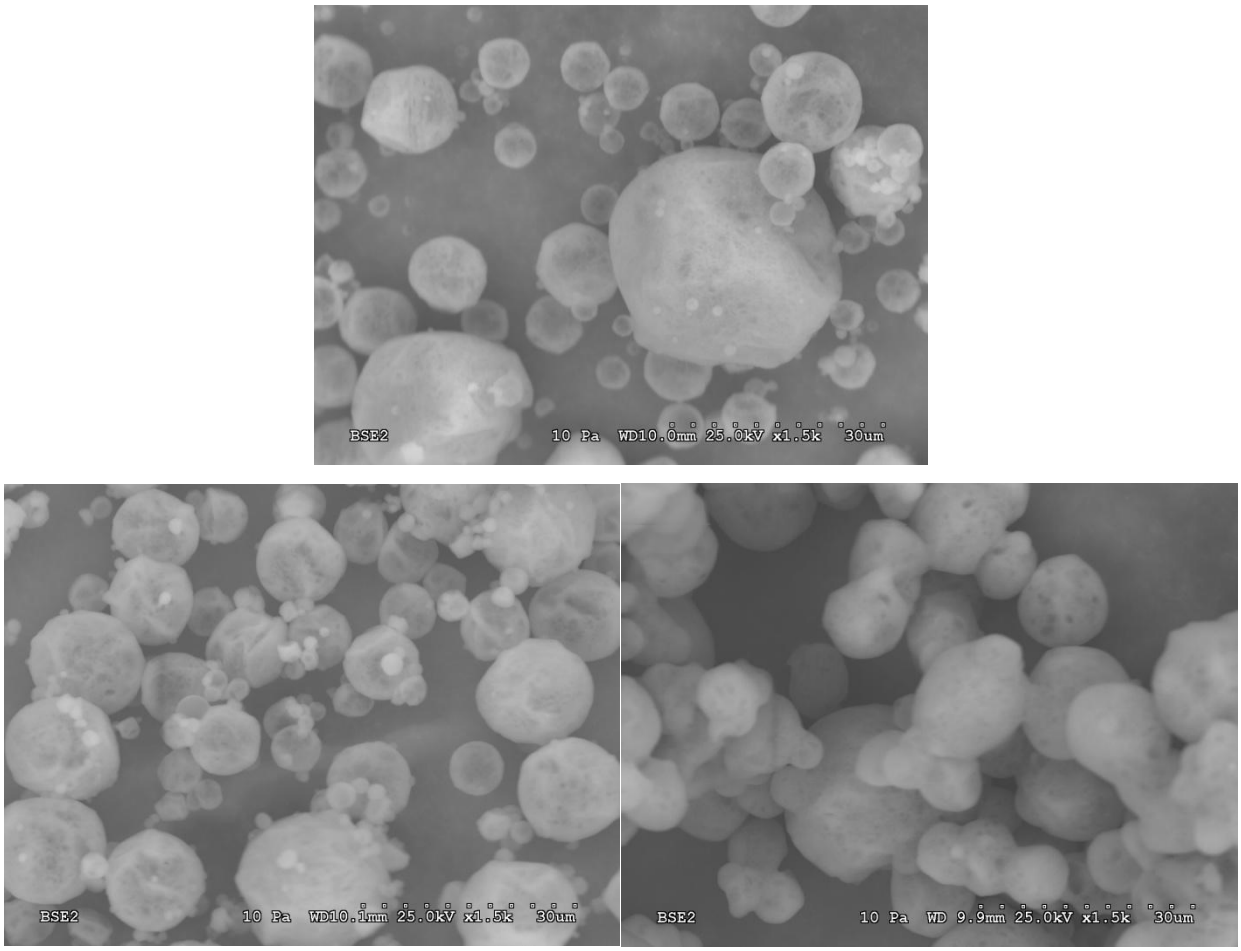

Figure 3.20: SEM micrographs at 1500X of raspberry powder from sample R3- at top centre at day 0; bottom left- 30 days of cold storage; bottom right- 30 days of room storage

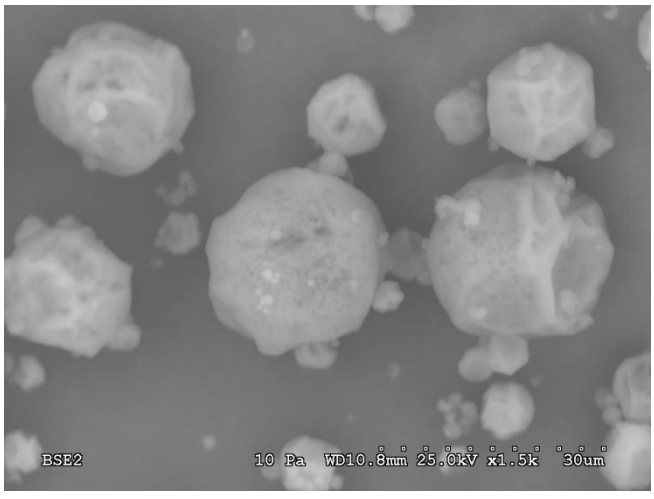

Figure 3.21: SEM micrographs of raspberry powder from sample R10 on day 0

The microspheres from the R10 samples (Figure 3.21) are slightly bigger with indentations (uneven on surface) than the other two responses R3 and R11 due to the higher concentration of maltodextrin. Increasing the additive concentration leads to the production of larger microspheres. The microsphere dimensions remained unchanged at the end of 30 days under cold storage conditions, however, there was agglomeration due to increased moisture content during room temperature storage. The viability remained as high as 98% during cold storage.

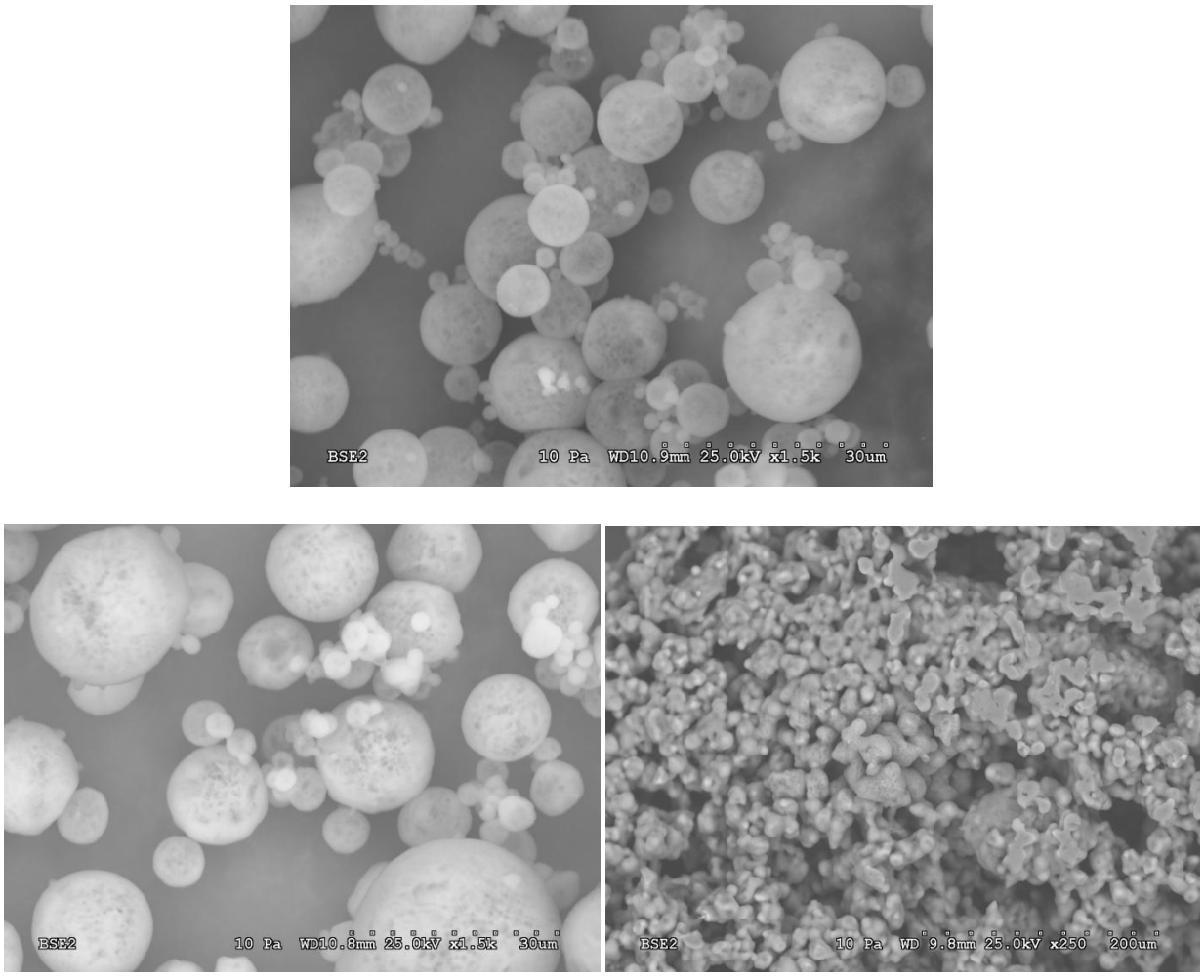

Figure 3.22: SEM micrographs of raspberry powder from sample R11- top centre at day 0; bottom left at 30 days of cold storage; bottom right- at 30 days of room storage

The microspheres were small and perfectly spherical in shape for R11 (Figure 3.22) probably due to the low concentration of maltodextrin since higher concentration of solids in

the suspension leads to larger microspheres. Again, the sphere dimensions did not vary much during cold storage but a greater degree of agglomeration was seen following room temperature storage with increased moisture content and reduced viability.

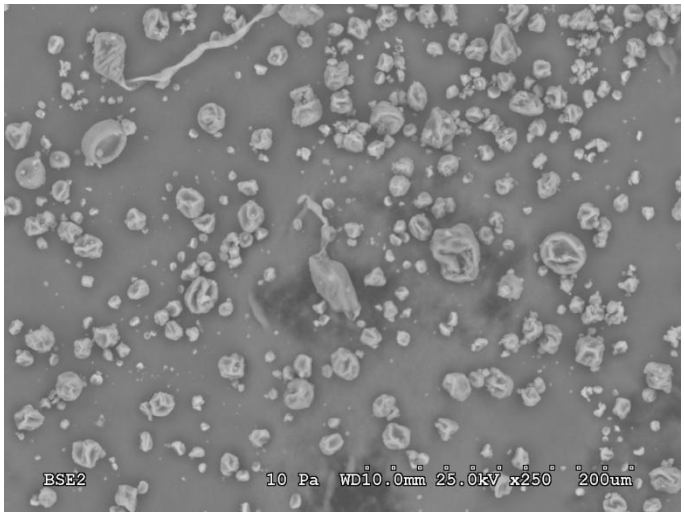

Figure 3.23: SEM micrographs of raspberry powder from sample R13

As observed in Figure 3.23, the microspheres for sample R13 were completely irregular in shape with numerous indentations. Poor microencapsulation and high temperature during spray drying for sample R13 appear to be the reasons for the drastic reduction in viability. Thick compact and irregular crusted particles were observed at low temperature drying and smooth and broken particles were observed at higher temperature drying as reported by others (Alamilla-Beltrán et al., 2005). The particle surface smoothness in our current study was however mainly dependent on the maltodextrin ratio. Lower concentration gave a more uniform size of the spheres. The rigid surfaces of microcapsules (due to surface alteration) provide a better barrier against water or any other physical or chemical deterioration, and hence an increased survival during storage is expected (Ying et al., 2010a).

It was shown that drying time increases as a function of the square of particle size which of course increases the thermal inactivation of *Lactobacilli*. But on the contrary larges particles had vacuoles which prevented cells and enzymes from inactivation. Thus the survival is a balance between vacuole size and dimensions of the sphere. More concentrated feed produces

larger spheres (higher surface area to volume ratio) and thus longer drying time which could be the possible explanation for lower survival of *Lactobacilli*. The longer drying time can be overcome by increasing the drying temperature (Boza et al., 2004).

3.16 Summary of Findings

- ✓ Dextrose was a better carbon source than maltodextrin in support of the growth of the *Lactobacilli* (*L. acidophilus* and *L. rhamnosus*) chosen in the current study.
- ✓ There was a minimal change of the preliminary probiotic characteristics (acid, bile tolerance and antibiotic sensitivity) when assessed before and after spray drying.
- ✓ MRS medium acted as a better heating medium than raspberry juice during the sub-lethal heat shock pre-treatment. Microorganisms were able to withstand upto 50°C (for *L. acidophilus*) and 52.5°C (for *L. rhamnosus*) in MRS as heating medium whereas both the microorganisms were killed at 45C in raspberry juice as the heating medium.
- ✓ Optimization of the spray drying process was performed using response surface design using the software JMP (SAS 9) with inlet temperature, feed flow rate, juice solids: maltodextrin ratio as independent variables and % recovery, % survival and color as dependent variables.
- ✓ Raspberry juice solid content to maltodextrin ratio and inlet feed rate have a major effect on recovery and the optimization equation followed a linear two factor model. The R^2 of the master model was 0.85 and predicted model was 0.71.
- ✓ Inlet temperature had a profound effect on % survival during spray drying. Although in the two factor linear optimization equation, the other independent variables inlet feed rate and maltodextrin ratio and also their products were significant. The R^2 of the master model was 0.96 and predicted model was 0.91.
- ✓ Maltodextrin ratio had a major effect on color of the raspberry powder. The optimization equation followed a quadratic form with an R^2 of 0.91 for master model and 0.87 for predicted model.

- ✓ Storage study at room and refrigerated temperatures was performed on the raspberry probiotic powder stored in glass bottles.
- ✓ Storability was higher under cold temperature storage than room temperature for raspberry probiotic powder produced under same conditions.
- ✓ Water activity increased during storage while the cell survival decreased.
- ✓ SEM imaging revealed the shape and size of microcapsules which are dependent mostly on the concentration of the added maltodextrin. The particle size varied with concentration of total solids present in the juice although the distribution did not have any relation. However the surface indentations were higher as the maltodextrin ratio increased with 1:1 concentration particles having the smoothest surface on Day 0.
- ✓ Particle agglomeration was observed (revealed under SEM) in some samples due to higher moisture and relative humidity in room storage which related to the lower stability observed at room temperature.

Chapter 4

FUTURE PERSPECTIVES

4. Future Perspectives

- Multi disciplinary approach involving immunologists, microbiologists, geneticists, bioprocess engineers, toxicologists, nutritionists, regulatory authorities is essential to enhance the quality and safety of the probiotic products being released (O'Brien et al., 1999).

- Health claims must be defendable and must be scientifically substantiated. Randomized and controlled placebo *in vivo* trials can explore more functional benefits and hence wider applications.

- Screening and selection of robust strains (with respect to physiological stress) over sensitive ones and tools to assess the fitness of the resistant strains must be developed for more versatile industrial application of these probiotics (Van de Guchte et al., 2002).

- Transfer of antibiotic resistance among probiotic species or the intestinal microflora, unwanted inflammatory response, virulence factors, sepsis in infants and immune-compromised patients are few risks which need to be addressed for safety purposes (Kataria et al., 2009; Vankerckhoven et al., 2008).

- Health benefits must be validated in the presence of food matrix dosage rather than simply with isolated pure culture.

- Since knowledge of entire genome sequence of most probiotic *Lactobacilli* is being explored by some, physiology modification or adaptation of strain-specific alteration like recombinations, insertions/deletions at molecular level could pioneer healthier functional properties. Stress resistance mechanisms during processing as well as gastric transit can be enhanced with genetic engineering techniques (Ross et al., 2005).

- The compatibility and stabilities between several bioactives when held together in the same microcapsule should be investigated, thereby widening the range of functional components that can be encapsulated.

- Microencapsulating agents' interaction between the protein-carbohydrate-probiotics formulation should be studied to ensure minimal toxicity and better bioavailability (Anal and Singh, 2007).

- Scale up of novel hybrid drying technologies with artificial intelligence, enhanced recovery (product and microbes) and energy efficiency needs to be developed (Chou and Chua, 2001).

- Rapid detection tools and bioassays are required to assess the functionality during storage.

- Further assessment is necessary on consumer acceptance rate, knowledge on the health benefits, safety and efficacy of the probiotics food as a whole which otherwise may lead to a product failure.

Probiotic foods are no more "complementary" or "alternative" medicine due to the widespread knowledge of their health benefits and the growing consumer acceptance. According to Daniel O'Sullivan, an expert in the field of probiotics, *"At best, your intestinal health is greatly improved and the immune system is strengthened with the probiotics. At worst, there are no adverse effects and you get some nutrients in the bargain."* (Chicago tribune; Condor, 1999)

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APPENDIX

The following tables represent the ANOVA and effect estimates for the three dependent variables: % recovery, % survival and color change (ΔE).

ITEMP= INLET TEMPERATURE (°C)

MRATIO= JUICE SOLID CONTENT: MALTODEXTRIN RATIO

FRATE= FEED RATE (mL/min)

Table 1: ANOVA for % RECOVERY

	Master Model					Predictive Model			
Source	**DF**	**SS**	**MS**	**F**	**Pr > F**	**DF**	**SS**	**MS**	**F**	**Pr > F**
ITEMP	1	43.7813	43.7813	2.8580	0.1218					
MRATIO	1	113.029	113.029	7.3786	0.0217	1	125.2911	125.2911	6.3536	0.0227
FRATE	1	357.4392	357.4392	23.3338	0.0007	1	453.7239	453.7239	23.0088	0.0002
ITEMP*ITEMP	1	3.2901	3.2901	0.2147	0.6530					
ITEMP*MRATIO	1	9.2460	9.2460	0.6035	0.4552					
ITEMP*FRATE	1	3.7401	3.7401	0.2441	0.6319					
MRATIO*MRATIO	1	37.4104	37.4104	2.4421	0.1492					
MRATIO*FRATE	1	100.1924	100.1924	6.5406	0.0285	1	143.6849	143.6849	7.286413	0.0158
FRATE*FRATE	1	0.0007	0.000788	0.00005	0.9944					
Model	9	924.4379	102.7153	6.705331	0.0032	3	762.1095	254.0365	12.8824	0.0002
(Linear)	3	695.5391	231.8464	15.13	0.0005					
(Quadratic)	3	126.9606	42.3202	2.7626	0.0974					
(Cross Product)	3	101.9382	33.9794	2.2182	0.1488					
Error	10	153.1846	15.3184			16	315.513	19.71956		
(Lack of fit)	5	60.5495	12.1099	0.6536	0.6739	5	99.5132	19.9026	1.0135	0.4546
(Pure Error)	5	92.635	18.527			11	215.9997	19.6363		
Total	19	1077.622				19	1077.622			

Table 2: Effect Estimates for % RECOVERY

	Master Model				Predictive Model			
Term	**Estimate**	**Std Err**	**t**	**Pr > |t|**	**Estimate**	**Std Err**	**t**	**Pr > |t|**
ITEMP	2.2331	1.3209	1.6905	0.1218				
MRATIO	-3.5881	1.3209	-2.7163	0.0217	-3.5929	1.4254	-2.5206	0.0227
FRATE	-6.9063	1.429	-4.8305	0.0007	-7.2395	1.5092	-4.7967	0.0002
ITEMP*ITEMP	1.0644	2.2969	0.4634	0.6530				
ITEMP*MRATIO	1.1647	1.4992	0.7769	0.4552				
ITEMP*FRATE	-0.8142	1.6478	-0.4941	0.6319				
MRATIO*MRATIO	3.5894	2.2969	1.5627	0.1492				
MRATIO*FRATE	4.2142	1.6478	2.5574	0.0285	4.6307	1.7155	2.6993	.0158
FRATE*FRATE	0.0166	2.3226	0.0071	0.9944				

Table 3: ANOVA for % SURVIVAL

	Master Model				Predictive Model				
Source	**DF**	**SS**	**MS**	**F**	**Pr > F**	**DF**	**SS**	**MS**	**F**	**Pr > F**
ITEMP	1	779.023	779.023	110.2365	<.0001	1	780.8711	780.8711	77.32866	<.0001
MRATIO	1	43.9433	43.9433	6.218244	0.0342	1	69.6283	69.6283	6.8952	0.0210
FRATE	1	42.626	42.6260	6.0318	0.0364	1	81.3797	81.3797	8.0589	0.0140
ITEMP*ITEMP	1	12.0278	12.0278	1.7020	0.2244					
ITEMP*MRATIO	1	199.8679	199.8679	28.2825	0.0005	1	181.8001	181.8001	18.0034	0.0010
ITEMP*FRATE	1	26.1006	26.1006.	3.6934	0.0868					
MRATIO*MRATIO	1	5.23694	5.2369	0.7410	0.4117					
MRATIO*FRATE	1	257.9487	257.9487	36.5013	0.0002	1	218.3966	218.3966	21.6275	0.0005
FRATE*FRATE	1	1.0822	1.0822	0.1531	0.7047					
Model	9	1437.247	159.6941	22.5976	<.0001	5	1369.573	273.9146	27.1254	<.0001
(Linear)	3	1023.96	341.32	48.2988	<.0001					
(Quadratic)	3	43.218	14.4060	2.0385	0.1790					
(Cross Prod)	3	370.0682	123.3561	17.4556	0.0004					
Error	9	63.6015	7.0668			13	131.2751	10.098		
(Lack of fit)	5	49.7046	9.9409	2.86134	0.1652	9	117.3782	13.0420	3.7539	0.1077
(Pure Error)	4	13.8968	3.47422			4	13.8968	3.47422		
Total	18	1500.848				18	1500.848			

Table 4: Effect Estimates for % SURVIVAL

	Master Model				Predictive Model			
Term	**Estimate**	**Std Err**	**t**	**Pr > |t|**	**Estimate**	**Std Err**	**t**	**Pr > |t|**
ITEMP	-9.4229	0.8974	-10.4994	<.0001	-8.979605	1.021144	-8.79367	<.0001
MRATIO	-2.2380	0.8974	-2.493	0.0342	-2.681395	1.021144	-2.62587	0.0210
FRATE	2.3856	0.9713	2.4559	0.0364	3.1245319	1.100642	2.838826	0.0140
ITEMP*ITEMP	-2.0432	1.5661	-1.304	0.2244				
ITEMP*MRATIO	5.4174	1.0186	5.3181	0.0005	4.8632567	1.146171	4.243045	0.0010
ITEMP*FRATE	-2.1516	1.1195	-1.9218	0.0868				
MRATIO*MRATIO	-1.3482	1.5661	-0.8608	0.4117				
MRATIO*FRATE	6.7641	1.1195	6.0416	0.0002	5.8405852	1.255894	4.650542	0.0005
FRATE*FRATE	-0.6198	1.5839	-0.3913	0.7047				

Table 5: ANOVA for COLOR (ΔE)

	Master Model				Predictive Model				
Source	**DF**	**SS**	**MS**	**F**	**Pr > F**	**DF**	**SS**	**MS**	**F**	**Pr > F**
ITEMP	1	0.0797	0.0797	0.1151	0.7422	1	0.0002	0.0002	0.0003	0.9859
MRATIO	1	41.2699	41.2699	59.558	<.0001	1	41.4139	41.4139	55.262	<.0001
FRATE	1	0.5147	0.5147	0.7428	0.4111	1	0.1076	0.1076	0.1436	0.7113
ITEMP*ITEMP	1	0.1728	0.1728	0.2494	0.6294					
ITEMP*MRATIO	1	4.5255	4.5255	6.5309	0.0309	1	3.6542	3.6542	4.8762	0.0474
ITEMP*FRATE	1	6.0343	6.0343	8.7084	0.0162	1	4.5743	4.5743	6.1038	0.0295
MRATIO*MRATIO	1	9.0002	9.0002	12.9886	0.0057	1	15.2453	15.2453	20.3430	0.0007
MRATIO*FRATE	1	2.5446	2.5446	3.6722	0.0876					
FRATE*FRATE	1	2.864E-6	2.864E-6	4.133E-6	0.9984					
Model	9	62.8019	6.9779	10.0702	0.0010	6	60.0453	10.007	13.3539	0.0001
(Linear)	3	39.2916	13.097	18.901	0.0003					
(Quadratic)	3	14.6396	4.8798	7.0423	0.0098					
(Cross Prod)	3	8.87062	2.9568	4.2672	0.0392					
Error	9	6.23637	0.6929			12	8.9929	0.7494		
(Lack of fit)	5	0.7978	0.1595	0.1173	0.9814	8	3.5544	0.4443	0.3267	0.9166
(Pure Error)	4	5.4385	1.3596			4	5.4385	1.3596		
Total	18	69.0383				18	69.03832			

Table 6: **Effect Estimates for COLOR (ΔE)**

	Master Model				Predictive Model			
Term	**Estimate**	**Std Err**	**t**	**Pr > |t|**	**Estimate**	**Std Err**	**t**	**Pr > |t|**
ITEMP	0.0953	0.2810	0.3393	0.7422	-0.0050	0.2782	-0.0180	0.9859
MRATIO	-2.1688	0.2810	-7.7174	<.0001	-2.0684	0.2782	-7.4338	<.0001
FRATE	0.2621	0.3041	0.8618	0.4111	0.1141	0.3010	0.3790	0.7113
ITEMP*ITEMP	0.2449	0.4904	0.4994	0.6294				
ITEMP*MRATIO	-0.8151	0.3189	-2.555	0.0309	-0.6897	0.3123	-2.2082	0.0474
ITEMP*FRATE	1.0345	0.3505	2.9510	0.0162	0.8495	0.3438	2.4706	0.0295
MRATIO*MRATIO	1.7674	0.4904	3.6039	0.0057	1.8080	0.4008	4.5103	0.0007
MRATIO*FRATE	-0.6718	0.3505	-1.9163	0.0876				
FRATE*FRATE	-0.0010	0.4959	-0.0020	0.9984				