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**Understanding the physiology of probiotic yeast cells under different  
formulation, processing and environmental conditions**

By

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## **ABSTRACT**

Probiotics are live microorganisms including yeast and bacteria used as food supplements to offer health benefits. Currently available dosage forms of yeast probiotic products include powder and gelatine capsules, which are not most appealing to consumers. A new dosage form of tablets containing probiotic yeast is preferred, based on a market research commissioned by Lesaffre International, France. The aim of the present work was to study the physiological states of yeast cells and the effects of different formulation, processing and environmental conditions on cell viability with an intention to develop a new dosage form of probiotic yeast. Three different yeast samples: wet yeast (WY) directly from fermentation, active dried yeast granules (ADY) produced by drying wet yeast cells in a drum dryer and extrusion through a screen, milled yeast granules (MY) were received from Lesaffre. The cell culturability and the physiological states of yeast cells in different formulation, processing and environmental conditions were experimentally characterised by colony plate counting (CPC) and flow cytometry (FC) respectively.

Cells in ADY and MY samples needed to be released from the granules before their culturability and physiological states could be characterised. Therefore, ADY was agitated in a homogeniser at different speeds. It was found that the number of cells released increased with the speed, but cells were vulnerable to the mechanical forces generated by homogenisation – when the speed was greater than 10000 rpm, which was considered to be the optimum speed for releasing yeast cells from the granules. The number of viable cells in WY sample was found to be higher than that in ADY, and the latter was greater than that in MY, which indicates the industrial drum drying and milling processes caused damage to the cells.

In order to choose an appropriate formulation for making the final dosage form, it is important to know whether yeast cells can survive in an acidic environment such as that in the stomach. Therefore, yeast cells from ADY were exposed to a simulated gastric and intestinal fluid of various pH (1 to 7) for two hours. It has been found that the fluid with pH greater than 2 had no significant effect on the cell viability, but the fluid with pH of 1 caused some damage to the cells although this pH is not typical in the stomach. A strong linear correlation between the number of viable cells obtained from FC and the number of culturable cells obtained from CPC for the different processing and environmental conditions and various homogenisation speed has been established which suggests that most of viable cells were culturable.

Wet yeast cells were freeze dried before they were mixed with excipients to be compacted into tablets. In general, the number of viable cells decreased with freeze drying time. At a freeze drying time of 24 h without addition of protectants, the water activity ( $a_w$ ) and number of viable cells in CFU were  $0.43 \pm 0.02$  and  $8.1 \times 10^9 \pm 1.5 \times 10^9$  CFU/g respectively. Tablets containing a mixture of the freeze dried yeast (FDY) and microcrystalline cellulose (MCC) (50/50 weight ratio) resulted in a low tensile strength of  $0.27 \pm 0.03$  MPa and a number of viable cells of  $8.6 \times 10^8 \pm 2.5 \times 10^8$  CFU/g. The storage stability result of FDY in the formulation after 6 months at 4°C has revealed a 1.6 log loss.

Wet yeast cells were encapsulated with pectin crosslinked with  $\text{CaCl}_2$  which formed calcium pectinate (CaP) beads in order to create a friendly microenvironment which can protect the cells during tableting and storage. The effects of pectin and  $\text{CaCl}_2$  concentrations on cell viability and encapsulation efficiency were examined. The formed CaP beads were freeze dried for 24 h in order to reduce their water activity to  $0.22 \pm 0.02$ . It was found that compacting a mixture of freeze dried beads with MCC (50/50 wt/wt) at a pressure of 90 MPa,



a tablet containing a number of viable cells of  $9.0 \times 10^8$  CFU/g and a tensile strength of  $1.09 \pm 0.03$  MPa was achieved, which is highly desirable.

The material properties of CaP beads were investigated by diametrical compression. Two types of material behaviours of CaP beads were found from ten tested beads. Three beads had no fracture and behaved elastically at small deformation with Young's modulus of  $312 \pm 28$  MPa. Seven beads showed fracture points and irreversibly deformed with Young's modulus of  $162 \pm 14$  MPa. Finally, encapsulated cells in CaP beads were proved to be protected more from damage during compaction and show more resistance to environmental stress during storage than non-encapsulated cells. The material properties of CaP beads have been used to explain their protective benefits to yeast cells during compaction and storage.

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## NOMENCLATURE

D	Diameter of tablet, mm
EE	Encapsulation efficiency %
F	Maximum force to break the tablet, newton
T	Thickness of tablet, mm
v/v	Volume/volume
wt/wt	Weight/weight
$\sigma_T$	Tensile strength, MPa

## ABBREVIATION

ADY	Active dried yeast
$a_w$	Water activity
BOX	Bis-oxonol
BP	British Pharmacopoeia
CFU	Colony forming unit
CPC	Colony plate counting
DCW	Dry cell weight
DM	Dry mass
FC	Flow cytometry
FDY	Freeze dried yeast
FL1	Green fluorescent (533/30 nm)
FL3	Red fluorescent (>670 nm)

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FL4	Red fluorescent (675/25 nm)
FSC	Forward side scatter
FSC-A	Forward side scatter (area)
FSC-H	Forward side scatter (height)
GI	Gastrointestinal
MCC	Microcrystalline cellulose
MgSt	Magnesium stearate
MY	Milled yeast
PBS	Phosphate buffer solution
PI	Propidium iodide
RH	Relative humidity
RI	Refractive index
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIP	Sample introduction probe
SIF	Simulated intestinal fluid
SSC	Side scatter
WY	Wet yeast
YME	Yeast malt extract agar

## CHAPTER 1

### INTRODUCTION

An essential part of human well-being includes good physical health. The digestive system is one of the most important systems in animals including humans involving food consumption and assimilation. Digestion begins in the mouth by chewing and swallowing. After that food mixed with digestive juices and is passed through the digestive tract i.e. the stomach, small intestine and large intestine (“the gut”). Various enzymes help break down large molecules of food to smaller molecules. However, digestion is complete at the small intestine stage. In the large intestine, the liquid residue is absorbed and no breaking down of food takes place.

In healthy individuals, the microflora in the intestinal gut remains relatively constant by maintaining a balance between pathogenic and non-pathogenic microorganisms. Once the intestinal gut microflora is disrupted, the host can be affected resulting in illness and discomfort. In recent years, many chronic digestive problems have been reported and caused to an unprecedented number of patients (Perrott, 1945, Mönnikes et al., 2001). Digestive disorders in the gut are affected by imbalances in both the internal and external environment and symptoms can become worse including heartburn, abdominal pain, diarrhoea and irritable bowel syndrome (IBS). All such disorders can be treated with drugs and antibiotics but the disadvantage is these treatments kill a number of beneficial as well as pathogenic bacteria. The integration of natural microorganisms known as probiotics and intestinal environment protection is a solution that increases the amount of active useful

microorganisms in the digestive system and maintains an adequate balance between beneficial and pathogenic bacteria.

## **1.1 BACKGROUND AND SIGNIFICANCE**

Probiotics have been reported to have a number of benefits including improving well-being (Dugas et al., 1999, Klaenhammer, 2000) and to play an important role in the modulation of the intestinal immune system (Marteau and Rambaud, 1993), prevention of tumour development (Parodi, 1999), and suppression of cancer (Aso et al., 1995, Cross, 2002). Recently, various bacterial probiotics have been produced for the market in many formulations such as chewable tablets, yoghurt, liquid drinks and powders (Yamaguishi et al., 2011, Czinn and Blanchard, 2009). However, bacteria are found to be susceptible in low pH such as the highly acidic conditions in the stomach so that many researches on other microorganisms have been undertaken. Yeast is one choice that is commonly thought of as an example of non-pathogenic eukaryotic microorganisms and has contributed to human welfare by maintaining and restoring the natural flora in the digestive system and therefore can be developed in order to pursue probiotic applications (Kumura et al., 2004, van der Aa Kühle et al., 2005).

With yeast probiotics, it is important to develop a formulation that is rigid and contains an adequate amount of active microorganisms to be delivered to the human colon to offer probiotic benefits. Currently, there are several dosage forms of yeast probiotics which are widely available in the commercial market (Lourens-Hattingh and Viljoen, 2001, Graff et al., 2008). However, the tablet dosage form is found to be easy to manufacture and administer whilst the oral route is also a convenient and safe way to administer such tablets.

Compaction pressure during tableting is a major challenge to overcome in probiotic tablet production because yeast cells are susceptible to such pressure and can easily be damaged. After these cells are exposed to such pressure, they can become injured and consequently die due to external stresses. It is thus essential to develop a method to protect yeast cells from compaction pressure and enhance cell survival during storage. A modified encapsulation method by extrusion could be applied to yeast cells by trapping them as core material with some biodegradable materials. However, so far there has been a lack of understanding of how the formulation, processing and environmental conditions associated with tableting, storage and passage via a gastrointestinal tract may affect physiology of probiotic yeast cells. Cell physiology can be investigated using flow cytometry, which will be extensively applied in this research.

## **1.2      OBJECTIVES**

The main objectives of this research were:

- To study the physiological states of yeast cells produced from various processing conditions i.e. drying and milling processes and also various environmental conditions i.e. low pH values in the GI tract (simulated in this work) using flow cytometry
- To develop a tablet dosage form of yeast probiotic containing an adequate number of viable cells with long shelf life and enhanced consumer acceptability

- To reduce the number of damaged yeast cells in tablets by encapsulating them with some biodegradable materials before compacting the resulting beads into tablet dosage form
- To investigate the mechanical properties of encapsulating beads and to understand how these beads behave under tablet compaction

### **1.3      *OUTLINES OF THESIS***

A brief summary of the chapters in this thesis is given below.

**Chapter 2** *Literature review*: Health benefits offered by probiotics and experimental techniques used to determine cell viability i.e. traditional techniques and flow cytometry are introduced. A literature survey includes the factors that may affect cell viability and the theory of flow cytometry which is used to study various physiological states of yeast cells.

**Chapter 3** *Materials and methods*: the equipment and chemicals used throughout this study are described. The methods to examine cell viability and bead characteristics are introduced.

**Chapter 4** *Comparison of probiotic yeast viability produced from various processing and environmental conditions*: viability of yeast cells produced from different processes is investigated by colony plate counting (CPC) and flow cytometry (FC). Some factors that have an impact on cell viability are described. The correlation between the number of culturable and viable cells in particular processing and environmental conditions is established.



**Chapter 5** *Freeze drying of wet yeast and compaction of them into tablet dosage form:* yeast cells were freeze dried at various conditions before mixing with other excipients and compacting into tablets at different ratios and compaction pressures. Tablet properties i.e. tensile strength and water activity, and cell tablet storage stability results are described.

**Chapter 6** *Encapsulation of yeast cells to improve their survival after tableting:* yeast cells were encapsulated with two different biodegradable materials in order to maintain cell viability inside encapsulating beads and to protect cells from damage by external stresses. Physical properties of encapsulating beads as well as cell viability in the beads are presented.

**Chapter 7** *Evaluation of calcium pectinate (CaP) bead properties and compaction of CaP beads into tablet form:* mechanical properties of calcium pectinate beads were measured by diametrical compression tests. This chapter shows the results of cell viability in tablets containing CaP beads and other excipients produced at different compaction pressures, as well as cell storage stability in these formulations after 3 and 6 months.

**Chapter 8** *Final conclusions and future work:* the overall conclusions and recommended future studies are presented.

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## CHAPTER 2

### LITERATURE REVIEW

#### **2.1 INTRODUCTION**

In this chapter, the benefits of probiotic yeasts in animal nutrition are introduced. The measurement of cell viability by various methods is discussed, as well as the use of such measurements in investigations of factors influencing cell viability. By considering a number of recent studies concerning probiotic bacteria and yeast, the use of flow cytometry (FC) is summarized, together with some fluorescent dyes in the investigation of many aspects of yeast physiology. An overview of encapsulation technique to protect microorganisms from harsh conditions is introduced. Based on the review, encapsulation of yeast cells has been applied to protect yeast cells from tablet compaction pressure in probiotic and food supplement application. In addition, the material properties of single calcium pectinate (CaP) beads determined by diametrical compression are considered.

#### **2.2 PROBIOTICS AND THEIR USE**

At the beginning of the 20<sup>th</sup> century the grandfather of modern probiotics, Elie Metchnikoff, demonstrated the health benefits of lactic acid bacteria in yoghurt that helped digestion and improved the immune system in human bodies. This was explained by the possibility of modifying harmful microbes to useful microorganisms in the large intestine. It has taken over a century for his hypothesis to be proved and the term of “probiotics” to be defined. In 1965,

Lilly and Stillwell first claimed probiotics as ‘substances secreted by one organism which stimulate the growth of another’ (Lilly and Stillwell, 1965). Nine years later, a different view from Parker (1974) introduced probiotics as ‘organisms and substances which contribute to intestinal microbial balance’.

The definition of probiotics which is now internationally accepted is “live microbial food supplements that beneficially affect the host by improving its intestinal microbial balance” (Fuller, 1989). More recently, probiotics have been known in prevention and treatment of infections, for example reduction of cholesterol, suppressing the growth of pathogens, stimulating the immune system and reduction in risk of cancer (Anderson and Gilliland, 1999, Agerholm-Larsen et al., 2000, Kailasapathy and Chin, 2000, Gotcheva et al., 2002, Nomoto, 2005, Nguyen et al., 2007, Jones and Jew, 2007).

### **2.2.1 Benefit of probiotics**

The selection of microbial strains for probiotics has been decided based on the amenability of the cells to industrial handling and their ability to remain viable until they reach the appropriate site in the body. However, there are differences in the composition of intestinal microflora in various host species, therefore this should be taken into account in selecting the strain of probiotic. There is some scientific evidence showing that particular species of bacteria such as *Bifidobacterium*, *Lactobacillus* and *Streptococcus* and particular strains of yeast such as *Saccharomyces* and *Torulopsis* have health benefits in human and animals (Tannock, 1995, Tannock, 1997, Berg, 1998). Beneficial effects of these bacteria include the regulation of the immune system, the balance of micro-flora and improvement of lactose digestion (Saad et al., 2013, De Vrese et al., 2001).

*Saccharomyces boulardii* and *Saccharomyces cerevisiae* are the most common yeast strains that have desirable properties used in probiotic products. They are resistant to antibiotics and reconstitute the intestinal microflora and thus, offer probiotic effects (Nousiainen and Setälä, 1993). Two strains of *Saccharomyces* isolated from infant faeces and Feta cheese have proved to have the ability to remove and assimilate cholesterol in the conditions found in the gastrointestinal (GI) tract (bile and low pH level of gastric juice) which is promising for their use as probiotics (Psomas et al., 2003). Moreover, some strains of dried yeast have been used for the prevention of vitamin B deficiency, the treatment of diarrhoea, lactose intolerance conditions, irritable bowel syndrome and those symptoms associated with *Clostridium difficile* colitis infection (Reynolds, 1982, Enache-Angoulvant and Hennequin, 2005, Marteau et al., 2001, McCullough et al., 1998).

### **2.2.2 Viability of microorganisms within the GI tract**

The adverse environment in the GI tract, especially the low pH in the stomach, is the main problem for microorganisms to remain viable during their passage through the intestine. The survival of probiotics in the GI tract mainly depends on the strain and formulation of the final product (Holzapfel et al., 1998, Schillinger, 1999). It has been reported by Conway et al. (1987) that four strains of lactic acid bacteria had low survival when they were freely suspended in gastric juice but had the ability to survive and adhere to endothelial cells enhanced by the addition of milk. Similar results were obtained by Charteris et al. (1998) in that lactobacilli and bifidobacteria are bile-salt resistant but are sensitive to stomach acidity. However, the addition of milk and mucin helped protect them from acidic conditions in the upper gastrointestinal transit.

Several studies have claimed that it is not necessary that some probiotic strains remain viable whilst still offering health effects such as immune modulation and lactose tolerance condition (Ouwehand and Salminen, 1998, Salminen et al., 1999), which may be called prebiotics. Viable and non-viable lactic acid bacteria showed a health effect in the treatment of candidiasis and in reducing the duration of diarrhoea (Ouwehand and Salminen, 1998). Understanding prebiotic effects although interesting is not considered in this work.

Moreover, it is thought that probiotic microorganisms have to be able to adhere to the intestinal endothelium and subsequently be capable of exerting probiotic effects (Tannock, 1995, Tannock, 1997, Fuller, 1992). However, the effectiveness of the probiotics is affected by the varying levels of indigenous and exogenous microflora found in the GI tract (Freter, 1992). Investigation of adhesion of probiotic cells on intestinal endothelium is out of the scope of this project.

## **2.3 CULTURABILITY AND VIABILITY**

### **2.3.1 Colony plate counting**

Reproductive growth is one proof of life which is associated with cell viability. The characteristics which indicate viable cells are the presence of structure, changeable genetic information and metabolism or functional activity. Colony plate counting is a classical technique to measure cell culturability, which is used to indicate cell viability. This technique has limitations particularly when the cells are stressed or injured. Lack of reproduction on agar does not prove that cells are dead. Likewise, lack of culturability may be accounted for by several factors which include cells being in a dormant state, prolonged nutrient limitation,

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or the culture plates not being incubated for long enough for the cells to adjust to the new environment and nutrient availability.

### **2.3.2 Viable and non-culturable cells**

One of the factors indicating whether microbial cells are dead or alive depends on their capability to reproduce themselves. It is possible to define cells with reproductive capability as 'viable' (Bogosian and Bourneuf, 2001). Concerning bacteria, the authors showed that once the energy source in the medium is exhausted, the bacteria will lose their cytoplasmic membrane potential. If the nutrients are restored, such dormant bacteria may regain metabolic activity, membrane potential, and the capacity to grow. Cells with no cytoplasmic membrane potential but retaining cytoplasmic membrane integrity have the potential to become healthy and metabolically active once suitable conditions are restored. Such cells are considered as dormant or viable but non-culturable (VBNC). VBNC recognises the potential of dormant cells with an intact cytoplasmic membrane but no membrane potential (MP) to become healthy once again and thus fully function and possibly reproduce.

The terms 'viability' and 'culturability' in microbiology have similar definitions, however the term viable but non-culturable has been applied to cells which failed to yield growth on solid media (Kell et al., 1998). It has been stated that VBNC is a survival strategy using the main control networks to regulate a genetically programmed sequence to enhance survival, hence these cells are capable of reversing this response and resuscitate to an actively metabolising state when the conditions for growth improve. The results in terms of public health studies indicate that these cells may not cause disease or be harmful.

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## **2.4 FLOW CYTOMETRY**

A unique and powerful technique, flow cytometry, consisting of the words ‘flow’, ‘cyto’ and ‘metry’, means the physical and/or chemical measurement across a wide range of single cells or biological/ nonbiological particles within a sample flowing in a liquid stream past a series of laser beam detectors. It allows quantitative investigation of whole cells and cellular compositions which have been marked by various fluorescent dyes, i.e. cell physiology. The advantage of flow cytometry is its ability to analyse both homogeneous and heterogeneous cell cultures within an enormously complex population of different cell types. This quantitative analysis technology describes the different characteristics of single cell populations as they flow in a liquid medium past an excitation light source of a laser beam with the capability of analysing up to 1,000 cells per second, providing real-time microbial analysis. Non-target cells are discriminated from target cells by using multi-fluorescence colour specific labelling to increase the information available (Vesey et al., 1994).

### **2.4.1 History and background**

The history of flow cytometry began in Stockholm in 1930 when Caspersson and his colleagues pioneered microspectrophotometric measurement and obtained the apparatus for cytology automation (Shapiro, 2003). Moldaven proposed the concept of cell counting by flowing fluid through a capillary tube using a photoelectronic sensor, but this instrument has not hitherto been successfully built (Shapiro, 2003). Albert Coons was successful in applying the fluorescent antibody technique to mark specific cellular proteins (Radcliff and Jaroszeski, 1998). A paper published by Gucker et al. (1947) accomplished the flow cytometric detection

of bacteria in aerosols using a laminar sheath stream of air. This work was done by analysing dust particles and airborne microbial spores used as biological agents.

Crosland-Taylor (1953) adapted the sheath flow principle and attempted to count red blood cells in fluid flowing through a narrow tube. However, the experiment was described as suffering from some problems and found to be difficult due to the fluid being easily blocked in the tube. A wider tube was tried in order to overcome these drawbacks by allowing more particles to travel side by side, but this led to errors in counting (Shapiro, 1988). Kamensky et al. (1965) performed an experiment to measure the nucleic acid content and light scattering of mammalian cells in a flow stream which can analyse 500-1000 cells per second. The applications of flow cytometry have been mainly used for the study of mammalian cells, particularly in the field of microbiology which is fully developed in the commercial market.

Initial flow cytometry was limited by the size of the microorganism which had to be smaller than leucocytes. Improvements in optics technology led to obtaining the better fluorescent stains and were applied successfully to microorganisms thus allowing flow cytometry to be further developed. Hutter and Eipel (1979) investigated DNA contents in *Saccharomyces cerevisiae* by staining with propidium iodide (PI) and then using flow cytometry to obtain accurate results. This technique is able to discriminate between live and dead cells on the basis of the altered light scattering behaviour and can be used in large populations.

### **2.4.2 Flow cytometry principles**

A cell suspension, prepared for analysis and introduced into a narrow tube, can be assessed by two types of physical phenomena: light scattering and fluorescence behaviour. A single



cell in suspension is hydrodynamically focussed through the flow cell, which forms a laminar flow across the surface in order to prevent mixing of sample and sheath fluid stream. Cell concentration is controlled at a low level of nearly zero to ensure that it contains a single cell. Some vibration within the flow cell takes place to make a uniform stream of the droplet through the flow cell (Davey and Kell, 1996). After cells of interest are fluoresced, the analysis of light scattering and fluorescence data are electronically assessed utilizing a computer and software following cell sorting procedure.

In the beginning of cell counter operation, an electronic counter was produced to count thousands of cells per second by which the sample passes through the small hole at constant flow rate. The measurement system detects the output which goes above the threshold level and then the cells are counted. Before the cell counter was available in the market, people used a haemocytometer to count the blood cells under a microscope; therefore the precision of cell counter became a problem.

### **2.4.3 Advantages and disadvantages**

The advantages of flow cytometry over the CPC technique (in section 2.3.1) are well described by Shapiro (1995) and Davey and Kell (1996). By using various mixtures of fluorescent dyes it is possible to study the physiological state of yeast cells beyond culturability in either homogeneous or heterogeneous populations. It is used to allow rapid data acquisition with direct methods for the detection of viable microorganisms in real time providing statistically accurate results.

Disadvantages of flow cytometry include the high cost involved in a laser based system and instrument servicing. Flow cytometry is also inappropriate for the analysis of filamentous

organisms, or cells that have a tendency to aggregate resulting in coincidental events. It is intended to use an existing FC to characterise physiology of probiotic yeast cells, which can be well dispersed in a suspending liquid.

## **2.5 DETERMINATION OF CELL VIABILITY BASED ON THE APPLICATION OF FLUORESCENT PROBES**

Fluorescent dyes function as viability probes to detect physiological metabolic changes of cells after a short period of exposure (Mason et al., 1994). When applied with flow cytometry, fluorescent dyes allow the detection of different applications such as metabolic activity, reproductive ability and membrane integrity in order to determine basic cell functions (Caron and Badley, 1995). The method characterizes cell populations based on light scattering and fluorescent dyes binding to the specific site of individual cells. Each of the fluorescing cells reacts by producing a light scattering signal and a fluorescent emission which varies depending on the specific properties of the individual cells. For example, propidium iodide (PI) and SYTO9 are used to measure membrane integrity which is achieved by binding to the DNA of the cell (Figure 2-1).

A broad range of reviewed articles on fluorescent stains have been reported, which have subsequently been applied in further microbiological analyses. Many of them have emerged for flow cytometric studies of eukaryotic cells such as yeast by distinguishing the properties and measuring the activities of cells in artificial or natural mixtures (Carter et al., 1993, Davey and Kell, 1996, Deere et al., 1998).

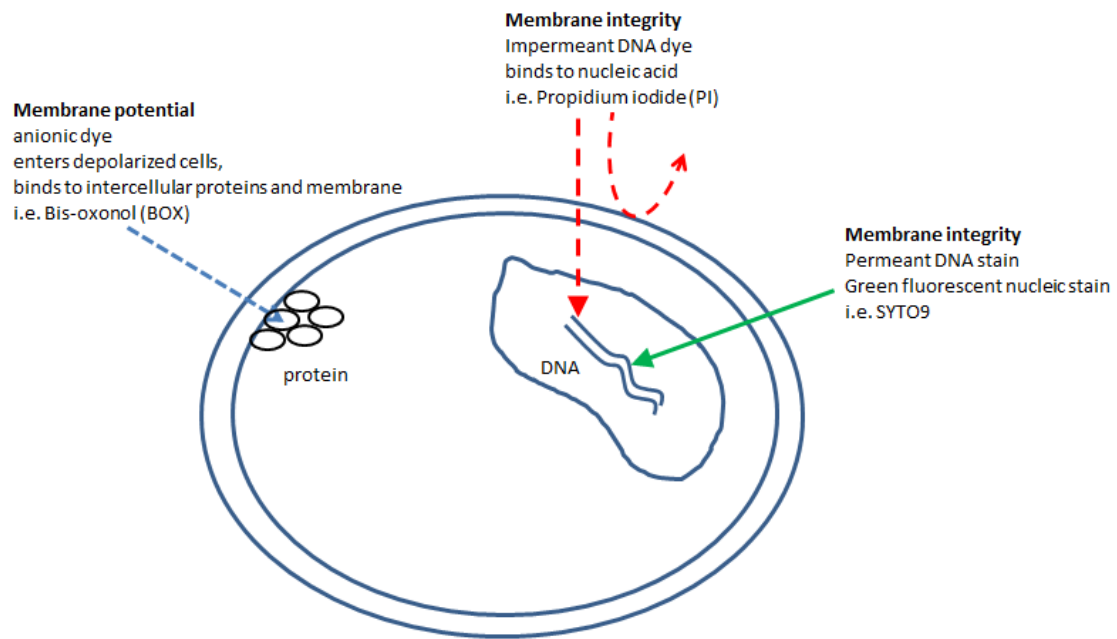


Figure 2-1 Schematic diagram showing application of fluorescent probes to investigate cellular viability and cell physiology

### 2.5.1 Cytoplasmic membrane potential

Across the cytoplasmic membrane the cell generates an electrochemical gradient referred to as the membrane potential. It determines if the cells have selective permeability and active transport of charged molecules through the cytoplasmic membrane. The presence of cytoplasmic membrane potential is one of the most important characteristics of viability. Assessment of viability can therefore be based on the presence or absence of an intact polarised cytoplasmic membrane and the transport mechanisms across it.

When exponentially growing cells are stressed by heat or energy starvation, they are particularly possessed by an active extrusion pump and become metabolically compromised before electrical depolarisation. At this stage, membrane potential is substantially decreased and the cells allow lipophilic dyes to pass through the cytoplasmic membrane easily and

accumulate due to the charges (Shapiro, 2005). Depolarisation can be measured with anionic probes known as Bis-(1,3-Dibutylbarbituric acid) Trimethine Oxonol (bis-oxonol, BOX, DiBAC<sub>4</sub>). This dye is allowed to access the permeabilised cytoplasmic membrane. When it is used together with PI, the culturability of the cells can be detected based on whether there is the presence of an intact fully energised cytoplasmic membrane (Davey and Kell, 1996, Hewitt and Nebe-Von-Caron, 2004).

### **2.5.2 Membrane integrity (impermeant dye)**

Dead cells with a permeabilised cytoplasmic membrane can be identified with propidium iodide (PI), which fluoresces red and binds to nucleic acid and is unable to cross an intact cytoplasmic membrane. PI concentration in an extracellular medium must be maintained in excess due to the fact that the binding of PI to the DNA is reversible (King, 2000). Dead cells are also classified as those without an intact cytoplasmic membrane. The membranes of dead or dying cells are permeabilised and unable to maintain the cytoplasmic membrane potential; hence the internal structures are freely exposed to the environment. Additionally, the indication of dead cells can be noticed from a reduction of ATP or energy in the cytoplasmic membrane. Therefore, another characteristic of dead cells is an increase of cell permeability of the cytoplasmic membrane.

### **2.5.3 Membrane integrity (permeant DNA stain)**

The permeant DNA stain, SYTO9, has been combined with PI in a commercially-available viability stain (*FungaLight*, Molecular Probes). This green fluorescent nucleic acid stain has the ability to penetrate and label all yeast cells with intact membranes and damaged

membranes. When used with the red fluorescent nucleic acid stain PI (see section 2.5.1), PI only penetrates to the DNA of those cells with damaged membranes where it displaces SYTO9, reducing the green fluorescence. Therefore, yeasts with intact cell membranes stain fluorescent green and those with damaged cell membranes stain fluorescent red. Zhang and Fang (2004) quantified the viability of *Saccharomyces cerevisiae* using flow cytometry by staining cells with SYTO9 and PI.

## **2.6 FACTORS INFLUENCING YEAST VIABILITY**

### **2.6.1 Processing conditions**

#### **2.6.1.1 High pressure homogenisation**

There are a number of reports on studying the effect of high pressure homogenisation on cell viability of gram positive and gram negative microorganisms (Lanciotti et al., 1994, Guerzoni et al., 1999, Vannini et al., 2004). The pressure from homogenisation influenced cell viability at different temperatures where cell viability of *Saccharomyces cerevisiae* decreased when a homogenisation pressure over 1000 bar was used at 20-30 °C (Guerzoni et al., 1999). Shear from high pressure homogenisation (Kleinig and Middelberg, 1998) and cavitation from ball valves have been identified as the primary mechanisms to cause cell disruption in yeast (Middelberg, 1995). Cell disruption is not of interest in this work, but the effects of mechanical forces generated in various processing steps including tableting on cell physiology is worth being investigated.

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### ***2.6.1.2 Drying process***

Microorganisms for various products such as food, pharmaceuticals and beverages are produced by fermentation in aqueous solution. Preservation by desiccation is therefore an important process used to prolong the stability of microorganism for long-term storage. The preservation of the suspension should contain high initial cell concentration as the majority of cells died during the drying process (Bozoğlu et al., 1987). Adverse temperatures, poor choice of protectant and rehydration methods are the causes of cell death in drying.

Labuza et al. (1972) investigated the effects of drying conditions in spray and drum drying on yeast cell viability. When cells were exposed very rapidly to high temperatures even with a short residence time, this caused small damage to cells (Labuza et al., 1970). These studies all demonstrate the drying temperature and time had a negative impact on cell viability.

### ***2.6.1.3 Milling process***

Interest in the reduction of the particle size of freeze dried probiotic products, especially by jet milling and ball milling, has been rising, mainly for preparing powder in small sizes for microencapsulation applications (Gèze et al., 1999, Hayakawa et al., 1993). Picot and Lacroix (2003) reported that reduction in particle size of freeze dried bacterial products caused additional damage to cells, and that cells were then more susceptible to other stresses.

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### 2.6.2 Environmental conditions

Several studies have reported an insufficient number of microorganisms in commercially available probiotic products (Kailasapathy and Chin, 2000, Varnam and Sutherland, 2001). Not only the processing conditions cause reduction of bacteria survival, but also environmental conditions i.e. highly acidic fluid in the stomach. The environmental conditions during storage can decrease product shelf life even with storage at low temperature (Lee and Salminen, 1995). Due to such environmental effects, probiotic organisms might be in some physiological state that the colony plate counting (CPC, section 2.3.1) cannot detect, but these cells might still be able to adhere to the intestine or target site and offer probiotic effects to the host (Kell et al., 1998).

However, little information regarding the effects of processing and environmental conditions on yeast cells is currently available in the literature. Therefore, one of the main objectives of this thesis is to investigate the survival of *S. cerevisiae* in different processes and a simulated gastric liquid with varying pH.

### 2.6.3 Residual moisture during storage

Regarding the effects of the drying process in section 2.6.1.2, it is important for probiotic products to contain the minimum possible level of moisture in order to extend product shelf life. However, the determination of water content or moisture content is not sufficient for reliably predicting microbial responses and chemical reactions in materials because its definition and measurement does not inform if the water in the product is bound or free. The molecules in bound water are firmly packed and the density is greater than that of free water

so bound water can only be frozen at low temperature. Therefore, the determination of water activity is a more accurate indication of how tightly water is bound within the material (Mathlouthi, 2001). Water activity ( $a_w$ ) is a parameter that affects product quality and safety especially in food and pharmaceutical products. It influences colour, odour, flavour, texture and shelf life of the products and is also related to the stability with respect to chemical and biochemical reaction rates and physical properties (Troller and Christian, 1978, Duckworth, 1975, Beuchat, 1981). In this thesis, the required water activity value ( $a_w$ ) of probiotic tablets should range between 0.1 and 0.3 in order to maintain probiotic storage stability (Dr Peter Jüsten, Lesaffre, France, Personal communication).

#### **2.6.4 Direct compression of probiotics into single tablets**

There are numerous routes by which drug substances can be administered. Oral dosage forms are the most widely used for the administration of small or organic drugs. They allow ease transportation, absorption to the large surface area of the small intestine and circulation in the body. Oral dosage forms include tablets, suspensions, capsules and solutions.

##### ***2.6.4.1 Advantages and disadvantages of the tablet dosage form***

In the pharmaceutical industries, oral dosage is the most natural and easiest method to administer drugs to adult patients. The advantages of tablets are easy to administer and transport, economical to produce, accurate to control the dose, able to contain more than one therapeutic ingredient and can have a pleasant appearance. However, the disadvantages of tablet dosage form include the complexity of the manufacturing process, physiological factors during the absorption of active ingredient from tablets i.e. gastric emptying rate and the tablet



size which is likely to be problematic for children (Turkoglu and Sakr, 2009, Banker et al., 2002). Overall, it is appealing to develop probiotic yeast in tablet dosage form if an adequate number of active cells can be incorporated into the tablet and their viability can be maintained.

#### **2.6.4.2 Direct compaction**

Direct compaction of powders into tablets has been widely used compared to the wet granulation process due to the elimination of some drying steps. Its advantages are lower labour costs, less processing stages and lower energy consumption (Jivraj et al., 2000, Rubinstein, 1988, Armstrong, 1997). The formulation of tablets prepared by direct compaction contains many excipients e.g. diluent, binder, lubricant and disintegrant. The mixing step is very important because of the differences in density of these substances and thus, segregation of the powder blend before compression is problematic. However, a solution to avoid segregation is to select the excipients having similar particle sizes.

Microcrystalline cellulose (MCC) has been commonly used as filler or binder because of its good binding property, low bulk density, high dilution potential and lubricating property (Reier and Shangraw, 1966, Bolhuis and Waard, 2011). Many studies have explained the good compactibility properties of MCC over other fillers (Bolhuis et al., 1979, Williams et al., 1997). During compaction, the pressure increased with punch displacement and the particles will rearrange themselves causing volume and porosity reduction as shown in Figure 2-2. After MCC undergoes stress, it deforms plastically, its molecules come in contact, and their physical and chemical interactions lead to formation of a rigid tablet (Bolhuis et al.,

1979). At low stress, MCC is slightly elastic (Aulton et al., 1974) but at high stress, plastic deformation can occur permanently (Reier and Shangraw, 1966).

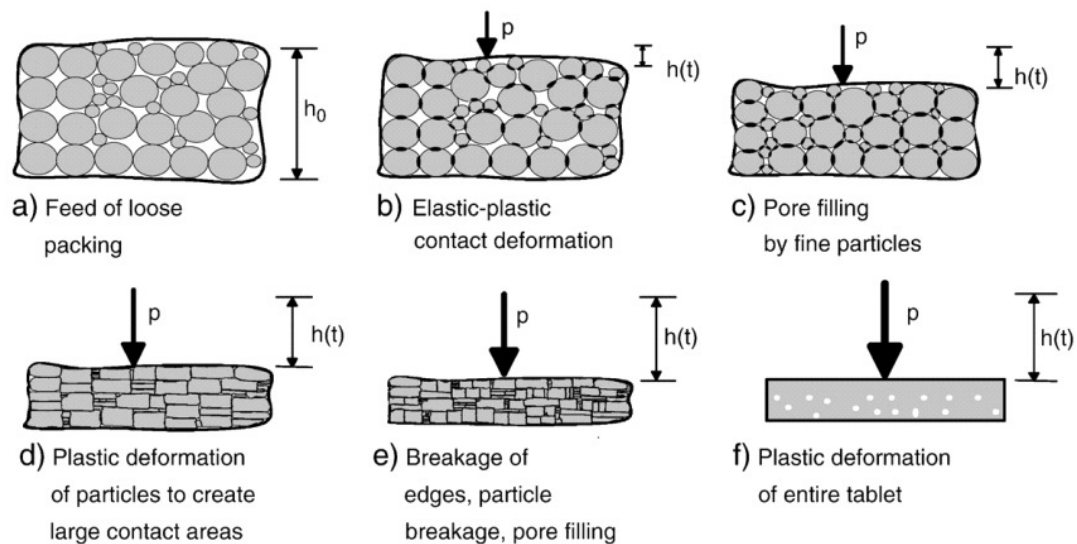


Figure 2-2 Process of deformation mechanism during compaction (Stasiak et al., 2010)

#### 2.6.4.3 Effect of compaction pressure on cell death

Several studies have shown the effects of compaction pressure on the reduction of the number of viable microorganisms (Chesworth et al., 1977, Plumpton et al., 1986b, Schiller et al., 1968). The results indicated that cell death may be caused by shear or heat during compression (Chesworth et al., 1977). Blair et al. (1991) and Yanagita et al. (1978) claimed that larger microorganisms were ruptured by shear rather than heat. In addition, the authors also reported that plastically deforming materials at low stress compaction caused greater cell rupture than fracturing materials. For compaction of *S. cerevisiae*, lethal effects were influenced by pressure (shear), particle size of dried yeast and heat sensitivity (Plumpton et al., 1986b, Plumpton et al., 1986a). Added to this, Fassihi and Parker (1977) demonstrated that cells were killed due to shear stress at a low compaction pressure but at high pressure cell

disruption was attributed to local heat. One of the aims in this study was to investigate the effect of compaction pressure on yeast cell viability using colony plate counting (CPC; section 2.3.1) and flow cytometry (section 2.4) by varying compaction pressures during tableting.

## **2.7 DRYING MICROORGANISMS**

Drying technologies have been applied to production of food and drug in bulk quantities, and also found to be the preferable preservation method (Morgan et al., 2006). Interest in drying methods has been shown in numerous industries including food and pharmaceuticals (Morgan et al., 2006). The dried form of microorganisms, produced via water removal presents many advantages in that it is light, compact and does not require subzero temperatures for storage and transport (Chan, 2002). There are numerous available drying methods, from which the ideal technique is selected based on the requirements of the sample in the final application.

### **2.7.1 Freeze drying**

Freeze drying involves the separation of water from a wet solid product, with the use of freezing and vacuum drying, resulting in the material in its anhydrous state (Franks, 1998). It involves heat and mass transport respectively. It can be scaled up for manufacturing (Tsinontides et al., 2004). Freeze drying is considered as the most convenient and successful method and widely used as a dehydration method for cell preservation (Chan, 2002, Abadias et al., 2001b) and enhances cell survival during storage in comparison with other drying methods (King and Su, 1994, Chávez and Ledebøer, 2007, Fu and Chen, 2011). Materials to

be dried are initially frozen (at temperatures between  $-10^{\circ}\text{C}$  and  $-30^{\circ}\text{C}$ ), followed by a sublimation of ice in a high vacuum (Chambers, 1949). Following the freeze-drying process, dried yeast cells generally remain unchanged morphologically and physiologically (Kirshop and Snell, 1984).

Freeze drying enables heat sensitive materials to be dried without decomposition and it results in a product which can be rehydrated more easily. The water activity in the product can be very low therefore the storage stability of freeze-dried material is seen to be good (Chambers, 1949). However, some loss in cell viability can occur in the freeze-drying method. One of the main causes of cell viability reduction is the freezing stage, specifically the freezing rate, if not correct, can be detrimental to cell viability. After freeze drying, the cell survival rate can be as low as 0.1% (Atkin et al., 1949, Abadias et al., 2001a, Morgan et al., 2006) but for *Saccharomyces*, the average cell viability obtained after freeze drying is 5% according to National Collection of Yeast Cultures (NCYC) (Kirshop and Snell, 1984). Poor performance of yeast survival may be attributed to the larger size of cells compared with bacteria (Kirshop and Snell, 1984). The damage from the freezing stage often takes the form of injury to the protoplasmic membrane, possibly as a result of intracellular ice formation or due to the production of high concentrations of solutes. Morgan et al. (2006) found that damage to the fragile cell membrane is caused by the formation of large ice crystals which cannot be repaired thus reducing cell viability. It is ideal to employ a higher freezing rate to enable smaller ice crystals to form in order to minimize cell damage. A further disadvantage of freeze dried encapsulated products is the open porous structure in the final products (Fyfe, 2011), the possibility of contamination and volume limitations within the freeze drying container. The variations in volume changing can have detrimental effects on the viability and appearance of freeze dried products.

Freeze drying has also been known to cause some adverse effects to proteins that appear to be denatured as well as causing damage to the cell membrane and cell wall. In this thesis, wet yeast was freeze dried as explained in section 3.10.1. In order to reduce these detrimental effects, numerous additives providing protection have been investigated in order to increase cell viability (Abadias et al., 2001a, Chan, 2002, Morgan et al., 2006).

### **2.7.2 Protective agents**

During drying, microorganisms are more susceptible to an increase of osmotic stress as the water activity begins to decrease (Morgan et al., 2006). Completion of freezing is dictated when more than 99% water is removed from the initial dilute solution, and so as a result, rapidly increasing the total concentration of solute (Franks, 1998). One way in which microorganisms respond to this stress is by counteracting the osmotic stress and maintaining osmotic balance between concentrated extracellular and the more dilute intracellular environment. The solutes in the protective media facilitate the cells by aiding to stabilize the proteins and cell membrane during osmotic stress conditions (Morgan et al., 2006). The components of the protective media have two main functions in preserving cell viability. A dry residue with a definite physical structure behaves as a support medium and receptor in the rehydration step. The other function is to protect microorganisms biochemically from damage (Abadias et al., 2001a).

There are a variety of simple and complex compounds that have been tested for their protective actions including polyols, saccharides or sugars, polymers, vitamins and minerals (Abadias et al., 2001a, Chan, 2002, Morgan et al., 2006). Coutinho et al. (1988) stated that an addition of 10% trehalose can maintain cell viability in yeast suspensions before drying.

Berny and Hennebert (1991) found that the viability of *Saccharomyces cerevisiae* increased from 30% to 98% by using an appropriate protecting medium containing 10% skimmed milk in the freeze-drying process. When selecting a suitable protective agent, it is important to note that the protection provided varies from strain to strain and can also be affected by the residual moisture content (Chan, 2002).

## **2.8 ENCAPSULATION**

Encapsulation is a method involving physicochemical and/or mechanical processes in protecting a core substance from adverse environments (Champagne and Kailasapathy, 2008). The core material is entrapped by a shell material, and capsule sizes range from nanometres to a few millimetres (Chen and Chen, 2007) and capsules can also be in different shapes (Shahidi and Han, 1993). In food and drug applications, the shell/coating materials are required to be biodegradable such as chitosan, alginate and pectin, which also does no harm to bioactive core components (yeast cells in this work) and the cells are maintained in a favourable environment whilst the capsule passes through the GI tract.

The main purpose of encapsulation of probiotic products is to protect the microorganisms from adverse environments and deliver adequate numbers of viable cells to the GI tract in order to offer probiotic benefits (Picot and Lacroix, 2004). For probiotic bacteria, there are various types of adverse environment i.e. high acid and bile salt conditions. For probiotic yeast in tablet dosage form, the compaction pressure is also likely to be an issue. Cell viability in the encapsulated matrix is dependent on various parameters such as the physicochemical properties of the matrix, initial concentration of cells, particle size and shell material (Chen and Chen, 2007). Kim et al. (2008) have reported the benefits of

microencapsulation of bacteria against acidic medium and enhancing the product storage stability. However, bacterial cells entrapped in a Ca-alginate matrix were not protected from acidic conditions due to the porosity of the matrix which allowed diffusion of acidic fluid to the cells (Sultana et al., 2000). Nevertheless, it is not known how yeast cells are susceptible to low pH liquid environment. The effects of suspending liquid with different pH on cell viability will be investigated. Moreover, the potential for increasing cell survival by encapsulating probiotic yeast with some biodegradable materials to provide favourable environments and thus to maintain cell survival rate during tableting and storage needs to be exploited.

### **2.8.1 Extrusion method**

Numerous encapsulation techniques have been developed and applied to microorganisms in food and drug applications. Extrusion is the most common and oldest encapsulation method involving in forcing the cell suspension through a small capillary or syringe into a crosslinking solution which hardens the coating material to trap the core material in a form of solid particle (Sara, 1995, King, 1995). This technique was first patented and developed by Swisher (1957) and later on applied in the manufacture of unstable and volatile flavour using carbohydrate matrices (Gunning et al., 1999, Reineccius, 1989) where shelf lives of up to 5 years were reported (Schultz et al., 1956). The active ingredient encapsulated by the extrusion method is surrounded by the coating material, but the disadvantage of this method is the resultant large particle size and defective structures i.e. thin wall and pores after processing (Westling et al., 1988, Kailasapathy, 2002, Wampler, 1992).

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## 2.8.2 Biopolymers for encapsulation of microorganisms

Some desirable characteristics of the carrier materials to encapsulate microorganism are: no activity or reaction with the cells, easy to handle and able to provide good protection of the active ingredients (Shahidi and Han, 1993). As the active ingredients in probiotic applications are microorganisms which are easily susceptible to damage in adverse environments, they require biocompatible and food-grade coating materials in order to maintain cell viability and exhibit appropriate physical properties for engineering processing leading to final use. The most suitable type of material for coating with the extrusion method is carbohydrate, water-soluble and water-insoluble polymers (Desai and Jin Park, 2005, Anal and Singh, 2007). Encapsulation in an alginate matrix is the most popular system for bacterial cells (Champagne et al., 1994, Jankowski et al., 1997). Chandramouli et al. (2004) and Sheu et al. (1993a) found that cell survival in calcium alginate beads increased with an increase of bead size and alginate concentration when the beads were exposed to acidic conditions. However, Hansen et al. (2002) claimed that alginate microspheres larger than 1 mm have an adverse effect on the quality and texture in food applications but small beads less than 100  $\mu\text{m}$  did not give protection to cells exposed to acidic conditions.

### 2.8.2.1 Shellac

Shellac is a biodegradable, renewable purified product of natural polymer resulting from the secretion of the insect *Kerria lacca* (*Coccoidea*) which mostly are found on trees in Asian countries i.e. India, Thailand and China (Cockeram and Levine, 1961, Chambliss, 1992). Shellac has been used as an enteric coating material in food and drug application due to its low water permeability and excellent barrier properties in gastric fluids (Sheorey et al., 1991,



Pearnchob et al., 2004). However, shellac has the disadvantages in low solubility and stability in neutral pH solutions and therefore, the slow dissolution of shellac in intestinal fluids is a major problem. Pearnchob et al. (2003b) have reported the benefits of shellac as a moisture barrier, taste masking and high stability coating in tablet dosage form. Tablets coated with shellac resulted in extended drug release which is potentially useful in pharmaceutical applications. Limmatvapirat et al. (2008) have developed shellac as an excipient in the tablet for oral controlled drug delivery. Recently, a novel method using shellac as an encapsulating material of carbamide peroxide for tooth whitening has been introduced (Xue and Zhang, 2008).

In addition to the applications mentioned above, the use of shellac with microorganisms (bacteria) in probiotic application is a promising application to protect the cells against acidic pH and achieve controlled release in the GI tract as discussed by Stummer et al. (2010a). However, there has been no work on encapsulation of yeast cells *Saccharomyces cerevisiae* with shellac.

#### **2.8.2.2 Pectin**

Pectin is an anionic heterogeneous and water soluble polysaccharide found in the cell walls of most plants. It is non-toxic substance and can totally degrade by colonic bacterial enzyme (Cummings et al., 1979). Pectin contains a linear chains of (1→4) linked D-galacturonic acid and can easily be cross-linked with calcium ions in mild conditions. The carboxyl groups of this acid are esterified with methanol, which are naturally presented as methyl esters. Low degree of esterification (DE <50%) pectin forms more rigid gels with a cross-linking between galacturonic acid and calcium ion (Rolin, 1993).

Many authors have reported the benefits of pectin as a drug delivery to the colon (Rubinstein et al., 1990, Rubinstein and Radai, 1991, Munjeri et al., 1997) and as a carrier for sustained drug release (Aydin and Akbug˘a, 1996, Srimornsak and Nanthanid, 1998a, Srimornsak and Nunthanid, 1999a). Pectin has been used as a carrier of protein (BSA) for oral delivery since it can react with calcium ions and form calcium pectinate gel beads (Srimornsak, 1998). The author also reported the effects of bead size, the amount of protein added, entrapment efficiency, drying condition and calcium concentration on the release of BSA in calcium pectinate gel beads (Srimornsak, 1998, Srimornsak, 1999).

Probiotic bacteria are found to be susceptible to pH in the GI tract, whilst a poor survival rate is also evident during product shelf life until point of consumption (Charteris et al., 1998, Conway et al., 1987, Holzapfel et al., 1998, Wang et al., 2004). Many studies have reported the improvement of cell viability by encapsulation of bacteria cells with some biodegradable and food-grade polymer such as alginate (Yoo et al., 1996, Jankowski et al., 1997, Sultana et al., 2000) and whey protein (Picot and Lacroix, 2004). More examples of probiotic bacteria encapsulated by the extrusion method are shown in Table 2-1. A few have reported encapsulation of yeast cells with alginate (Mahmoud et al., 1990, Cheong et al., 1993, Choi et al., 2007), chitosan (Raymond et al., 2004) and gellan gum (Tan et al., 2011). To the best of my knowledge, there has been no report concerning the encapsulation of yeast cells with pectin as a physical barrier in order to protect cells from severe environments, so that an adequate number of active cells can be released at appropriate sites and provide beneficial effects for the host. A major challenge of manufacturing probiotic yeasts in tablet dosage form is that yeast cells are exposed to compaction pressure so they might easily be damaged or become vulnerable to damage.

Table 2-1 Probiotic strains and encapsulation materials

Probiotic strain	Materials	Reference
<i>Bifidobacterium infantis</i>	Gellan/Xanthan gum	Sun and Griffiths (2000)
<i>Lactobacillus acidophilus</i> , <i>Bifidobacterium infantis</i>	Ca-alginate	Krasaekoopt et al. (2004)
<i>Lactobacillus acidophilus</i>	Alginate-chitosan	Urbanska et al. (2007)
<i>Lactobacillus casei</i>	Alginate/pectin	Sandoval-Castilla et al. (2010)
<i>Lactobacillus rhamnosus</i>	Whey protein	Reid et al. (2007)

## 2.9 MECHANICAL PROPERTY DETERMINATION BY DIAMETRICAL COMPRESSION

One of the important characteristics of excipients used for tablets made by compaction is to know their mechanical properties (e.g. brittle or ductile) in order to produce formulations that will result in rigid tablets. The investigation of mechanical properties of excipients in pharmaceutical applications by diametrical compression has been widely used to measure the strength of the materials (Fell and Newton, 1970, Stanley and Newton, 1977, Pitt et al., 1988). This technique has been used with brittle materials and large particles (>200  $\mu\text{m}$ ) exhibiting plastic deformation (Bonollo et al., 1994, Procopio et al., 2003). For small biological and non-biological particles (20-90  $\mu\text{m}$ ), a micromanipulation technique was developed for the determination by diametrical compression (Andrei D et al., 1996, Zhao and Zhang, 2004, Zhang et al., 1992).

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### 2.9.1 Hertz analysis

Hertz theory has been used to determine the mechanical behaviour of spherical particles (Hertz, 1882). Firstly, some assumptions to be used with this analysis are valid for (1) material having small deformations up to 30% at high compression speed (Andrei D et al., 1996), (2) materials are linear elastic and isotropic, (3) the contact interface between two solid materials is perfectly smooth. Later on, this analysis has been developed to use with various types of contact interfaces and material shapes (Stanley, 2001).

In a different study, Zhao and Zhang (2004) determined the mechanical properties of two biocompatible micro-particles using a micromanipulation technique by compressing at different speeds. Wang et al. (2005) compressed single calcium alginate microspheres with diameters ranging between 80-130  $\mu\text{m}$  using the same technique before mechanical behaviour for small deformation up to 30% was determined by an elastic model (Hertz, 1882).

### 2.9.2 Viscoelastic analysis

Viscoelastic characteristics of a material can be explained by some mechanisms that occur during relaxation. The process involves either the transport of water from inside the microsphere or changes in the polymer network. In the case of a dried microsphere, transport of water is negligible. Yan et al. (2009) evaluated the mechanical properties of agarose microspheres by diametrical compression using micromanipulation. The authors also presented a modified Hertz equation which was used to determine the viscoelastic material behaviour from the experimental force-displacement data. For a more detailed review of

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mechanical characterization of particles, beads and cells see Mercadé-Prieto and Zhang (2012).

Although a number of developed techniques used in studying compaction behaviours of spherical particles e.g. Kawakita (Kawakita and Lüdde, 1971) and Heckel (Heckel, 1961) have been reported, only few studies have reported the correlation between the mechanical properties of single particles of pharmaceutical excipients and their compaction behaviour (Yap et al., 2006, Yap et al., 2008). In a tablet containing various excipients, individual particles have different mechanical properties and this may affect their compactability. Therefore, it is essential to understand how the individual primary particles behave and deform under compaction.

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## **2.10 CONCLUSIONS**

The benefits of probiotic yeast in animal nutrition and a development of a yeast probiotic in tablet dosage form were introduced in this chapter. Two well-known methods to examine cell viability: colony plate counting (CPC) and flow cytometry (FC) were reviewed. The factors that influence cell viability i.e. drying, milling, pH and compaction pressure were summarised.

Therefore, the first objective in this study is to examine cell viability of yeast cells produced from various processing conditions by CPC and FC methods. Since yeast cells can be categorized into three physiological states after they are stained with propidium iodide (PI) and bis-oxonol (BOX), it is interesting to establish the correlation between the number of viable cells obtained from FC and the number of culturable cells obtained from CPC.

Next objectives in this study are to examine the effects of freeze drying on yeast cells and to make tablets containing freeze dried yeast and other excipients with sufficient tablet strength, the number of active cells and product shelf life.

The encapsulation technique offers some protection to core material to some extent. In this study, yeast cells are encapsulated with some biodegradable materials in order to protect yeast cells from tablet compaction pressure and to maintain cell viability during storage. Next objective is to minimize yeast cell damage during compaction with the help of encapsulation technique before producing tablets containing encapsulated yeast cells in beads and other excipients.

The beads with yeast cells embedded are subjected to pressure during tableting and therefore, the final objective is to understand the mechanical behaviour and deformation of these beads by diametrical compression tests.

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## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 INTRODUCTION

Yeast cells were provided by Lesaffre, France, which were mainly in two forms: embedded in dried granules and wet yeast cells directly from fermentation. In order to make rigid tablets with a sufficient number of viable cells, a series of experimental works have been conducted, including homogenisation of the yeast granules using different speeds to release cells from them, and exposure of yeast cells to artificial gastric/intestinal fluid of different pH to identify suitable formulations. Wet yeast cells were encapsulated into a matrix and then dried using different methods. Yeast granules or encapsulated and dried yeast cells were mixed with various industrial excipients, which were then compacted into tablets using different pressures. The tablets with yeast cells were then stored under ambient conditions to investigate their stability.

The cell viability of yeast in each form was determined by two main methods. One was a traditional method, colony plate counting (CPC) in which the number of culturable cells is counted after a certain period of incubation. The other method was flow cytometry (FC), which is more accurate and allows more detailed investigation of cell physiological state.

The main objectives of this study were: to use CPC and FC to determine the viability of probiotic yeast *S. cerevisiae* produced in various forms, to establish a correlation between the



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number of viable cells obtained from FC and CFU/g data from colony plate counting method, and to maximize the number of viable yeast cells in rigid tablets by producing them using minimum compaction pressure. Special attention has been paid to encapsulation of yeast cells in a biocompatible matrix to form beads, which can create a micro-friendly environment and provide certain protection to cells when they are compacted into tablets. It is hypothesized that the beads with cells should possess certain mechanical properties in order to protect the cells. Therefore, their mechanical properties have also been characterized.

Additionally, scanning electron microscopy (SEM), image analysis (QICPIC) and particle sizing (Mastersizer) were used to study the physical properties of the beads with yeast embedded. Other measurements include water activity ( $a_w$ ) of dried cells, dried beads, and excipients used for compaction and dry cell weight (DCW). The details of these methods are described below.

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## **3.2 COLONY PLATE COUNTING (CPC)**

### **3.2.1 Buffers and solutions**

TS buffer was prepared by dissolving 1 g of Bacto tryptone (BD Bioscience, UK) and 8.5 g of NaCl (Sigma Aldrich, UK) in 1 L distilled water. Oxytetracycline solution was prepared by stirring 1 g of oxytetracycline dihydrate (Sigma Aldrich, UK) in 100 mL of distilled water for 30 min before 2 mL of 0.2 M HCl solution was added and the mixer was stirred for another 30 min. The mixture was filtered by a sterile 0.25 µm membrane filter and kept at 4°C in a dark place.

Phosphate buffered saline solution was made by dissolving one PBS tablet (Sigma Aldrich, UK) in 200 mL distilled water, and autoclaved for 15 min at 121°C and 1 atm.

Aseptic technique was applied in all experiments to prevent contamination by other organisms, particularly bacteria. Distilled water was used in all experiment unless stated otherwise.

### **3.2.2 Yeast strain and samples**

*Saccharomyces cerevisiae* was originally received from Lesaffre International, France (Table 3-1) in the forms of (1) wet yeast (WY) i.e. yeast cake harvested from a fed batch fermentation then centrifuged and filtered using a rotary vacuum drum, (2) active dried yeast granules (ADY): yeast cake which was processed in a drum dryer at 40-42°C for total drying time of 10-20 h and (3) milled yeast (MY): ADY with the granule size reduced by a

comminutor before discharge through a 30 mesh ( $\approx 590 \mu\text{m}$ ) screen.  $d_{50}$  of MY is  $403 \mu\text{m}$ .

Before use, ADY and MY were kept at  $4^\circ\text{C}$  in vacuum packaging.

Table 3-1 Three different forms of yeast - ADY, MY and WY received from Lesaffre, France with description of process conditions and yeast characteristics used in this research.

Sample	Code	Process condition	Description
Wet yeast	WY	Fed batch fermentation/filtration	Suspension (27-32% DCW)
Active dried yeast	ADY	Drum dryer at $40\text{-}42^\circ\text{C}$ for 10-20 h	Spherical granules ( $\sim 92\%$ DCW), $d_{50} = 1252 \mu\text{m}$
Milled yeast	MY	Comminutor/30 mesh screen	Rod shapes ( $\sim 95\%$ DCW), $d_{50} = 403 \mu\text{m}$

DCW: dry cell weight

### 3.2.3 Cell viability determination

In this study, three different methods were used to determine cell viability. Colony plate counting (CPC) to determine colony forming units (CFU) has been used to evaluate the viability of yeast cells based on serial dilution and the number of colonies that can grow on an agar medium after a certain incubation time (Postgate, 1969). Cells stained by methylene blue and counted using haemocytometry is another method to determine the number of alive and dead cells based on a principle of the blue to penetrate the wall and membrane of dead cell (Richard, 1932). The third method which not only measures cell viability but also physiological state is flow cytometry as described in section 3.8.

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### **3.2.3.1      *Yeast suspensions***

The preparation of yeast suspensions was based on the protocol adapted from Lesaffre International, France (Appendix 1) by dissolving 1 g of yeast in 100 mL of TS buffer and homogenising at 10-000 rpm using a T-25 Ultra-Turrax homogeniser (IKA, UK) for 3 min. This is equivalent to a dilution of 100 fold (initial concentration  $\times 10^{-2}$ ).

### **3.2.3.2      *Agar plate preparation and cell enumeration***

Yeast malt extract agar (YME) was purchased from Sigma Aldrich, UK and an agar stock was prepared from 4.1 g of YME agar and 100 mL distilled water which were mixed together thoroughly, autoclaved for 15 min and then left until the temperature cooled to 48°C before 1 mL of oxytetracycline solution was added. The agar stock was poured into a Ø90 mm petri dish and solidified at room temperature for approximately 30 min. Each cell suspension after homogenisation was serially diluted and 100 µL of each dilution was spread onto the YME agar plates by a sterilized spreader (Figure 3-1). The plates were incubated at 25°C for 72 h and at appropriate dilution, plates resulting in 30 to 300 colonies were counted (Appendix 1). This experiment was conducted in triplicate and the results were expressed as mean  $\pm$  95% CI. A statistical method (t-test) was used to determine whether or not data were significantly different at a confidence interval 95%.

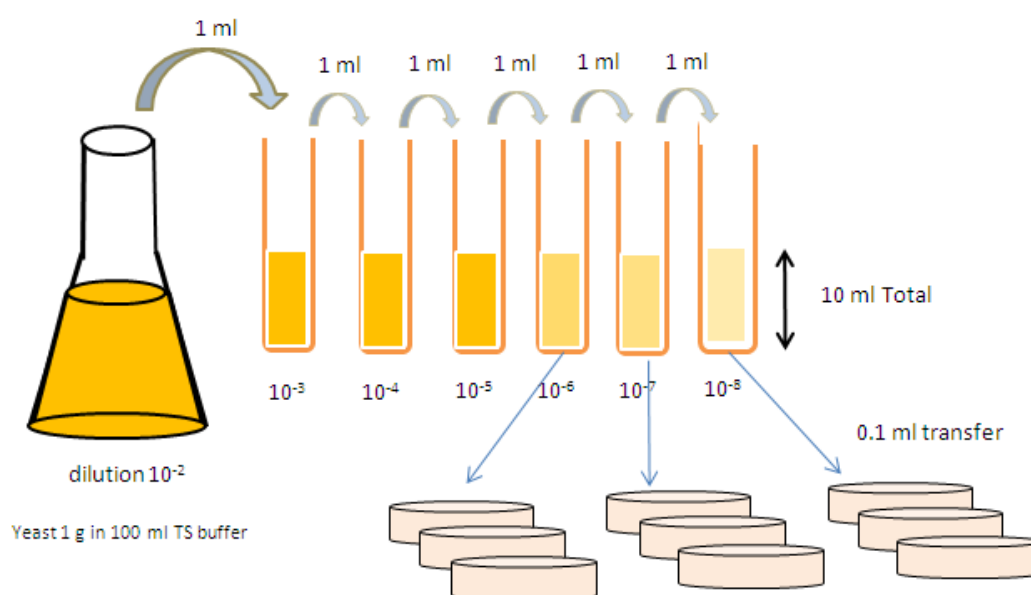


Figure 3-1 A schematic diagram showing how serial dilutions were made from a yeast sample (initial dilution 100 fold i.e.  $10^{-2}$ ). 1 mL of each sample was mixed into 9 mL of water and dilutions were made from  $10^{-3}$  to  $10^{-8}$ . 100  $\mu$ L of each dilution was plated onto YME agar and the plates incubated at 25°C for 72 h.

### 3.3 PARTICLE SIZE DISTRIBUTION

The size of yeast cells in ADY and MY suspension was determined by integrated light scattering using a particle size analyzer (Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK) as shown in Figure 3-2. The analyzer was fitted with a laser beam which measured the angular variation in intensity of light scattered when a dispersed sample passed through the beam. The scattering data were then analyzed to calculate the size of the particles based on a volume equivalent sphere model. According to this calculation, optical properties such as refractive index of the sample and dispersant are required. In the experiment, the refractive index (RI) of yeast cells was set at 1.53 and that for water as a dispersant was 1.33 (Smith et al., 2000).



Figure 3-2 Image of Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK)

### **3.4 DRY CELL WEIGHT**

Dry cell weight (DCW) means the weight of a sample after water removal by drying in an oven. A certain weight of given sample was placed on a glass plate at 80°C for at least two days or until dried mass was constant. Dried weight was calculated accordingly based on the difference in the weight before and after drying and expressed in g/L or % dry cell weight. Dried cell weight determination of each sample was carried out in duplicates and the average of dry cell weight was presented with standard errors.

### **3.5 Cell viability test based on staining by methylene blue and counting using haemocytometry**

A haemocytometer with methylene blue dye was used to determine yeast cell viability and the number of cells per mL in a suspension (Lehrer and Cline, 1969). The number of counted

cells in a given volume was converted to cell/mL by Eq. 3-1 and the percentage of cell viability was calculated by Eq. 3-2. Methylene blue was used to stain cells where dead cells with damaged cell walls allow methylene blue to penetrate into them resulting in a dark blue colour. In contrast, live or healthy cells remain unchanged. 0.9 mL of methylene blue staining solution (Sigma Aldrich, UK) was added to 0.1 mL of yeast suspension (of a known concentration), which was agitated for 1 min. One drop of this mixture after a 1000 fold dilution was placed onto the chamber covered with a cover slip and viewed under an optical microscope (Leica Microsystem Ltd, Milton Keynes, UK). Higher cell concentrations tend to create the blockage of light from the microscope. The counting chamber was thicker than a standard microscope slide and therefore it was necessary to use a 40x lens to give a clear image and avoid scratching the surface of the objective lens. At least 600 live and dead cells in 5 from 25 grids (see Figure 3-3) on 0.2 mm<sup>2</sup> of the haemocytometer surface were counted separately through the microscope and recorded, as described in Richard (1932).

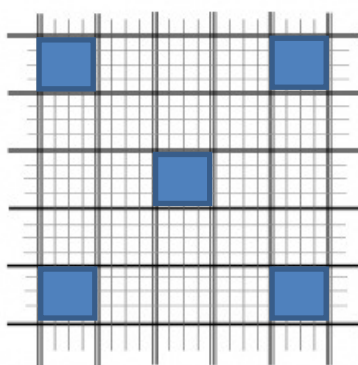


Figure 3-3 Haemocytometer showing 5 grids from 25 where live and dead cells were counted

$$\frac{\text{Number of cells}}{\text{suspension}} = \text{cells counted} \times 5 \times \text{dilution} \times 10^4 \quad (\text{Eq.3- 1})$$

---

$$\% \text{ cell viability} = \frac{\text{Live cell count}}{\text{Live cell count} + \text{Dead cell count}} \times 100\% \quad (\text{Eq.3- 2})$$

### **3.6 EFFECT OF HOMOGENISATION SPEED**

1g of ADY or MY was dispersed in 100 mL of PBS and homogenised using a T-25 Ultra-Turrax homogeniser (IKA, UK) at a speed between 6,000 and 15,000 rpm for 3 min prior to cell viability determination by CPC and FC analysis. Yeast suspension (initial dilution  $10^{-2}$ ) was serially diluted according to section 3.2.3.2 before plated onto YME agar and incubated for 72 h. For FC analysis according to section 3.8, yeast suspension at dilution  $10^{-4}$  was stained with PI & BOX and SYTO9 & PI respectively. CFU/g (based on dry basis) and the numbers of healthy, injured and dead cells were plotted against homogenisation speed with standard errors. Flow cytometric dot plots of two dual stains were also generated corresponding to different homogenisation speeds.

### **3.7 EFFECT OF ENVIRONMENTAL CONDITIONS ON CELL VIABILITY**

#### **3.7.1 Simulated gastric fluid preparation**

Acidic fluids of pH 1.2 and pH 2 were prepared by initially making up 0.2 M HCl and 0.2 M NaCl solution and then mixing these in different ratios depending on the required pH value (Table 3-2). Other acidic fluids were prepared by dissolving PBS tablets (pH 7) in distilled



water and then adjusting the pH by adding 0.2 M HCl. The pH of the solution was checked with a pH meter (Coming, pH meter 240, UK). All fluids were autoclaved for 15 min at 121°C and 1 atm before use.

Table 3-2 Preparation of simulated gastric fluid with different pH values  
(BP, Appendix XII B)

pH	0.2 M HCl (mL)	0.2 M NaCl (mL)
1.2	425	250
2	65	250

### 3.7.2 USP apparatus and in-vitro dissolution study

*In vitro* cell release and dissolution studies of tablets were performed using an apparatus-I USP rotating basket (Figure 3-4a), which is equipped with eight vessels, a temperature bath, shafts and a standard British Pharmacopoeia (BP, 1999) rotating cylindrical basket. The samples/tablets were placed in the cylindrical basket (40 mesh, welded seam, Fig. 3-4b) held with the motor shaft (Fig. 3-4C) and rotated in a cylindrical vessel containing the dissolution medium. This was carried out in a constant-temperature water bath at  $37 \pm 0.5$  °C for 2 h throughout each run. The rotation speed of the shaft was set at a constant speed of 100 rpm throughout the study.

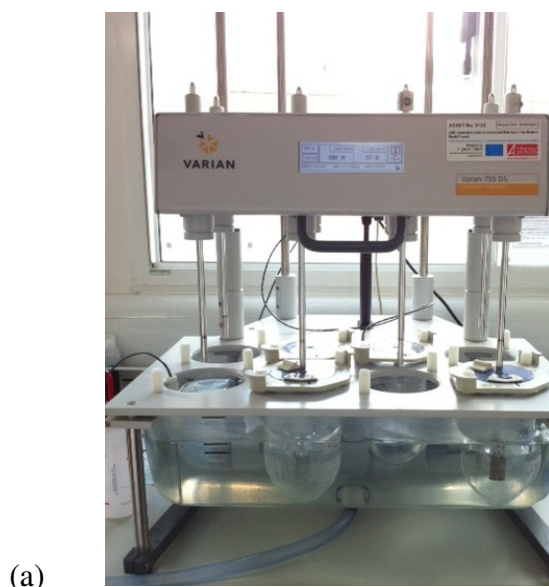


Figure 3-4 (a) USP dissolution apparatus containing eight vessels, equipped with a rotating shaft and operated in a constant temperature bath set at  $37 \pm 0.5$  °C (b) rotating cylindrical basket (40 mesh) which is held by (c) a motor shaft during operation

### 3.8 FLOW CYTOMETRY ANALYSIS

A BD Accuri C6 flow cytometer (MI, USA) (Figure 3-5) equipped with a 488 nm argon-ion laser for propidium iodide (PI) and bis-oxonol (BOX) excitation was used in this study. The red emission from PI (FL3) was collected with a 670 nm longpass filter. The green emission from BOX (FL1) was collected through a 533/30 nm band-pass filter. SYTO9, nucleic acid stain, emitting green fluorescence at 480/500 nm was combined with PI and used as a conventional direct-assay to measure viability based on membrane integrity. Yeast samples stained with single or dual dyes were passed through the flow cytometer and the data were collected. The acquired data of individual cell population combined with statistical data were plotted in histograms (frequency distribution) (Figure 3-6A) or dot plots (Figure 3-6B) which are the most common graphic representation.



Figure 3-5 Accuri C6 flow cytometer (Ann Arbor, MI, USA) connected with CFlow software

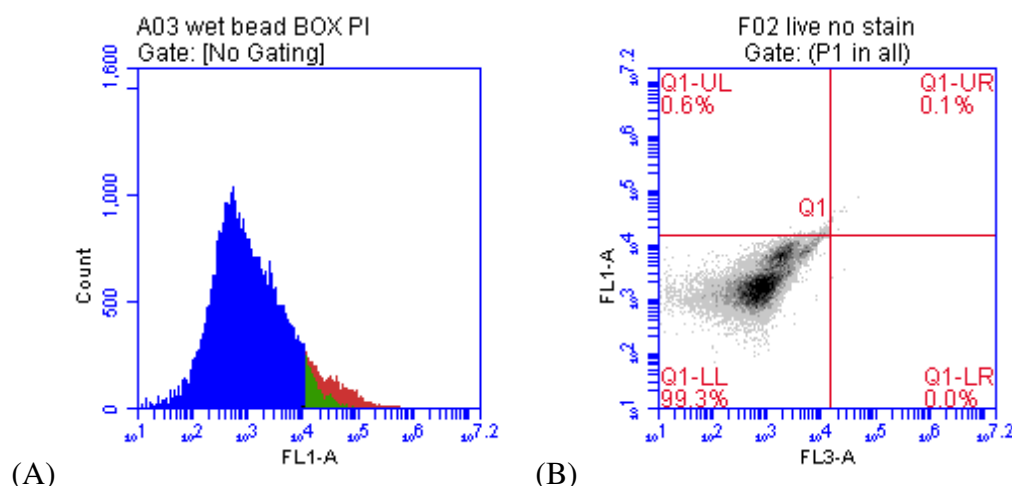


Figure 3-6 Flow cytometry data illustration (A) histogram (B) dot plot

### 3.8.1 Flow calibration and fluid calibration

Prior to flow cytometry experiments, it is necessary to validate the performance of the flow cytometer by using two default validation beads which are a 6 peak and 8 peak bead samples (Accuri C6, BD). These pre-diluted validation beads were stored at 4°C in the dark room and were used to analyze the fluorescence channels. The data of validation beads should look like the defined number of peaks as shown in Figure 3-7 (Accuri C6 user guide). Eight discernable peaks in FL1-H are shown for the validation of 8 peak beads and six discernable peaks beads in FL4-H are shown for the validation of 6 peak beads. If there was an indication of fewer peaks caused by an error of any of four fluorescence channels, the troubleshooting validation is needed (CFlow Accuri user guide).

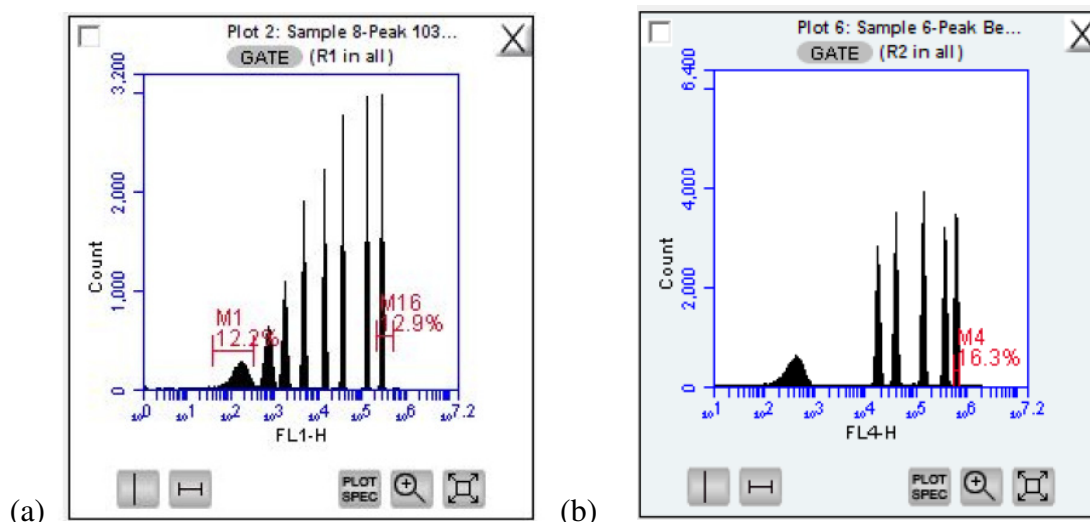


Figure 3-7 Validation data of 8 peak beads shown in FL1-H (a) and 6 peak beads shown in FL4-H (b)

### 3.8.2 Threshold setting and fluidics rate

Threshold is used to eliminate light scatter or fluorescent signals caused by debris or undesired events in cell samples. All the thresholds in BD Accuri C6 are set on the height signal for any given parameters. In this study, a primary threshold of 80,000 was set for yeast cells on FSC-H where given yeast cells can be collected and electronic noise in the system can be gated out (CFlow Accuri user guide). Any event not meeting the threshold criteria will not be acquired.

The software of the system (BD Accuri C6 software) can accommodate an upper limit of 10,000 events/sec where three different fluid speeds (low, medium and fast) can be adjusted. Fluidic rate setting which is recommended to acquire all samples is set at a low speed of 2,500 events/sec or less to ensure the best data resolution. If not stated, fast fluidic rate was used i.e. during performing flow cleaning or running decontaminant fluids. To analyze the

sample, gates can be drawn to the region where populations of interest are shown. The examples in Figure 3-8 show a gating of P1 and P2 population where cells of interest are. Cells presented in dot plots are selected based on the drawn gate and accordingly analyzed the physiological states.

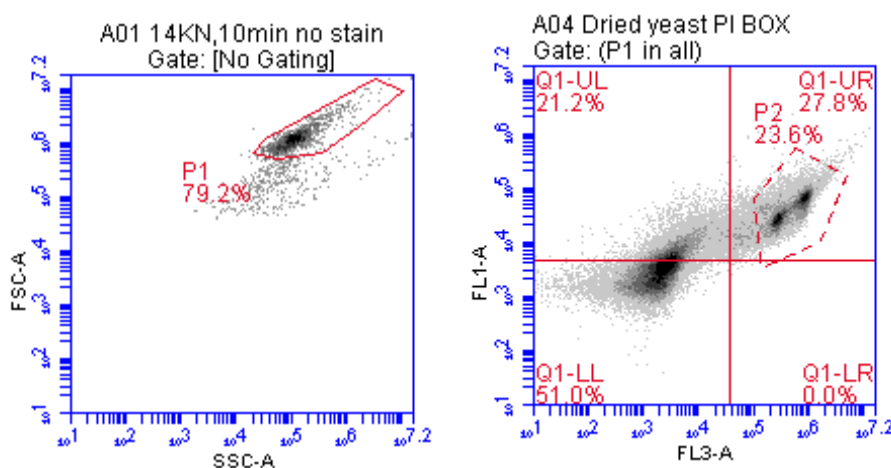


Figure 3-8 Flow cytometry data showing a gate of selected cell analysis within P1 and P2 population

### 3.8.3 Preparation of fluorescent dyes

PI and BOX were obtained from Molecular Probes, Europe BV, Leiden, Netherlands. PI at a working concentration of  $200 \mu\text{g mL}^{-1}$  made in distilled water and stored at  $4^\circ\text{C}$  was used to stain nucleic acid within the cells (Hewitt and Nebe-Von-Caron, 2001). For the case of the membrane potential dye, BOX, a working concentration of  $10 \mu\text{g mL}^{-1}$  was made from  $10 \text{ mg mL}^{-1}$  stock solution in DMSO and stored at  $-20^\circ\text{C}$ . For dual staining,  $20 \mu\text{L}$  PI and  $1 \mu\text{L}$  BOX were added into each sample, which was incubated for 5 min prior to flow cytometry analysis.

SYTO9, the green fluorescent nucleic acid stain, as a part of the LIVE/DEAD *Funga*Light yeast viability kit (Life Technologies Ltd, UK) has been used together with PI to determine yeast viability (Tanja et al., 2013, Zhang and Fang, 2004). The combination of 3.34 mM of SYTO9 and 20 mM PI were used together in a 1:1 mixture in 1 mL cell suspension. Samples were incubated at room temperature (37°C), protected from light for 15-30 min prior to flow cytometry analysis without cell washing. The data of the desired populations based on green and red fluorescent plot are shown in Figure 3-9.

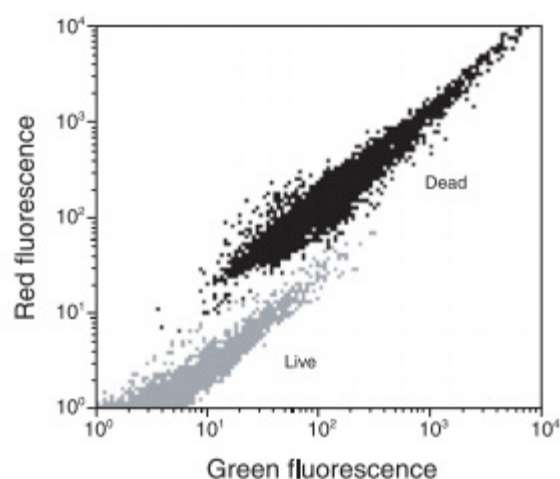


Figure 3-9 Yeast cell suspension containing a mixture of both live and dead cells stained with SYTO9 and PI and analyzed by FC

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### 3.8.4 Control studies

For flow cytometry, it is important to do control studies in order to discriminate between live and dead cells in population plots. 1 mL of yeast suspension as described in section 3.2.3.1 was used as a population of healthy cells. To prepare a dead cell population, 1 mL of healthy cell suspension was added in an Eppendorf tube (Sigma Aldrich, UK) and centrifuged at 5,000 rpm for 5 min. The supernatant was removed and 100% v/v ethanol was added to healthy cell pellet and left for at least 15 min to kill all cells present. After that, cells were washed and centrifuged with PBS solution twice before being re-suspended in PBS solution.

### 3.8.5 Sample analysis

Cell suspensions were serially diluted before staining or were used without staining with fluorescent dyes. 1 mL of sample at a known concentration was added in the polypropylene test tube and placed on a sample introduction probe (SIP, Figure 3-10). Data were collected and saved on an empty well in BD Accuri C6 software *collect* tab (Figure 3-11). In the BD Cflow plus software, the rate (events/  $\mu\text{L}$ ) was shown as default (Figure 3-11), in which the actual number of cells can be converted to a known concentration.





Figure 3-10 Sample introduction probe (SIP) and sample stage (Accuri C6 instrument manual)

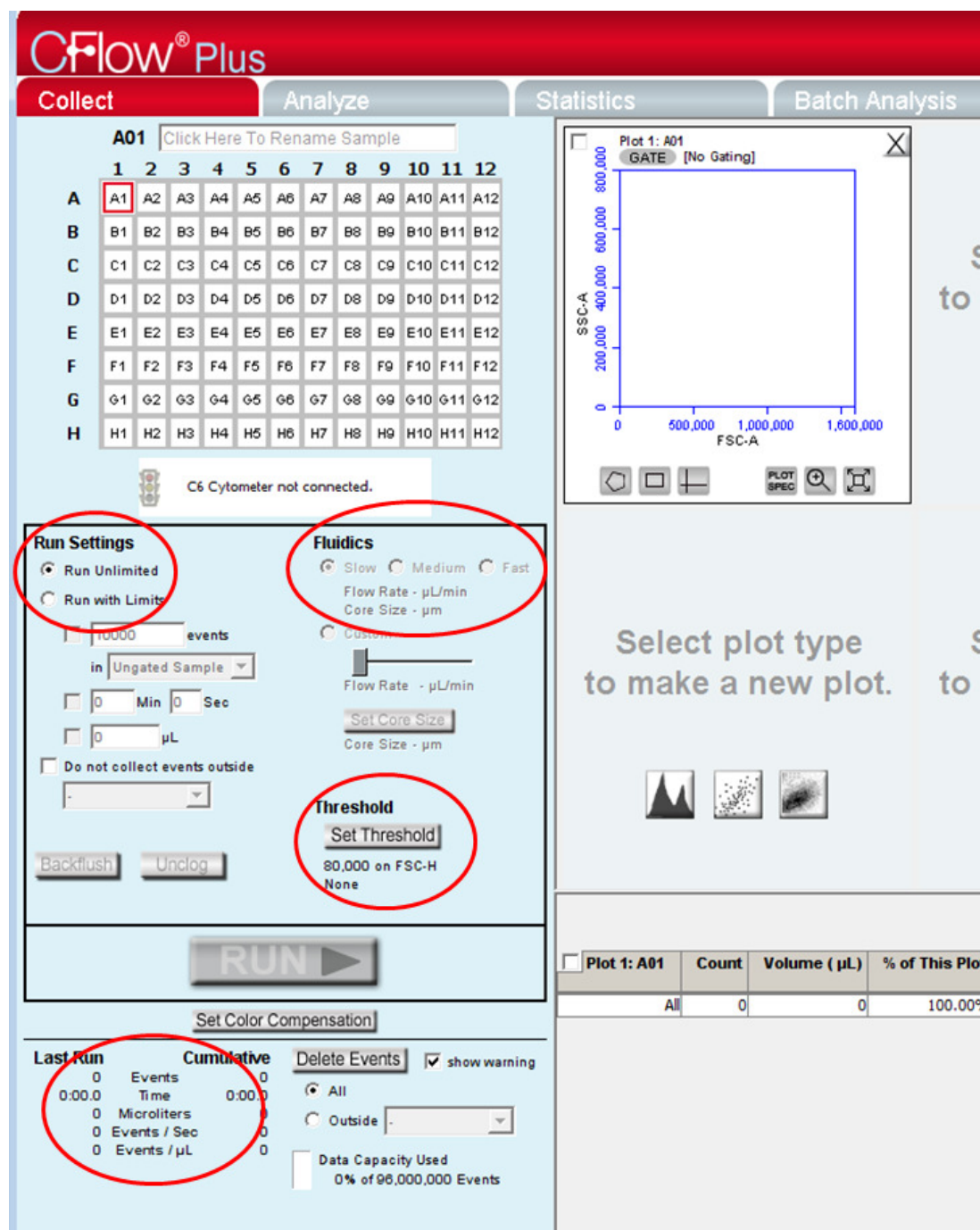


Figure 3-11 BD Accuri C6 software showing empty wells to save the data, fluidic rate, threshold and run setting

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### **3.9 CORRELATION BETWEEN CFU AND FC DATA**

In PI and BOX plots, cells which do not uptake PI are considered to be viable cells (healthy and injured cells). In order to determine the correlation between viable, healthy and injured cells and the number of culturable cells (CFU/g) obtained from CPC, the data in three different conditions: (1) three yeast samples (2) ADY suspensions homogenised with varying speed and (3) ADY suspensions exposed to a fluid of different pH were investigated. These data were fitted in a linear relationship by plotting the mean values with standard errors of two observed variables in a scatter plot and log scale. CFU/g is presented as the dependent variable on the ordinate and the number of viable, healthy or injured cells as the independent variable on the abscissa. Best fit straight lines and the corresponding linear equations were generated by Excel software. The correlation coefficient ( $R^2$ ) and slope were presented.

### **3.10 COMPACTION OF FREEZE DRIED YEAST INTO TABLET DOSAGE FORM**

#### **3.10.1 Freeze drying condition**

WY was placed in a freeze dryer (Edwards, EF03, Crawley, UK) for 24 h and at 0.6-0.7 mbar (Figure 3-12). The initial temperature of its refrigeration unit was switched on until it reached -50 °C and then the drying temperature was maintained at 25°C to minimize residual water content until the end of the process. In case of adding protective agent, 10% (w/v) of skimmed milk (Berny and Hennebert, 1991, Abadias et al., 2001a) was added to WY prior to freeze drying (Figure 3-13).

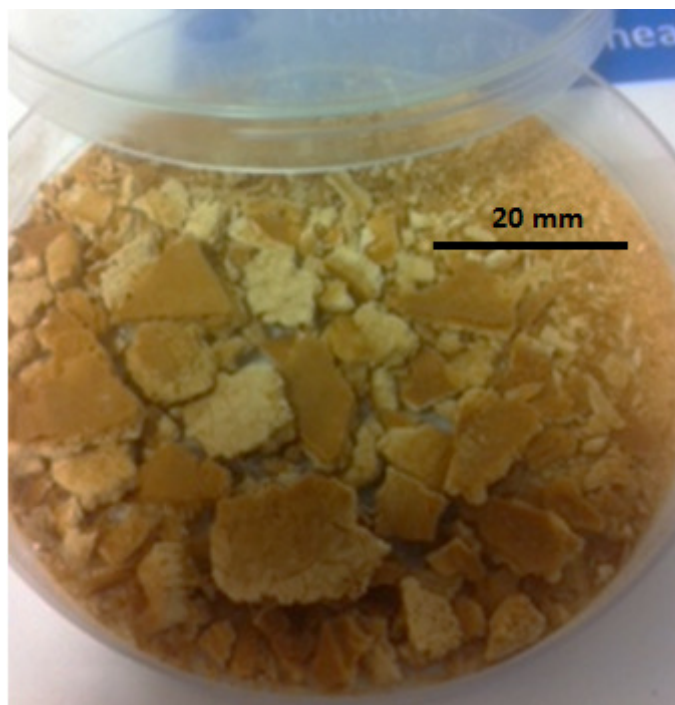


Figure 3-12 Freeze dried yeast (FDY) after freeze drying at 0.6 mbar for 24 h

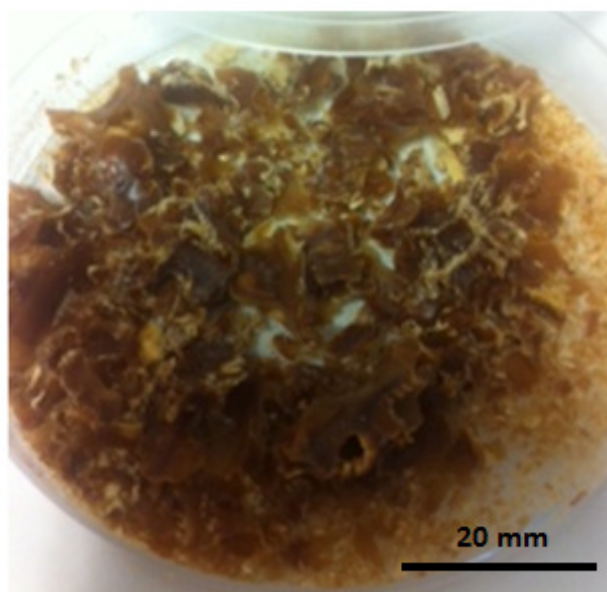


Figure 3-13 Freeze dried yeast with addition of 10% skimmed milk after freeze drying at 0.6 mbar for 24 h

### 3.10.2 Particle size distribution (QICPIC)

After 24 h freeze drying time, FDY (and MCC) was collected and milled by a food processor (Kenwood, FP260 series, UK) for 3 min. Particle size distribution was carried out using an image analyzer (QICPIC, Sympatec GmbH Inc., Clausthal-Zellerfeld, Germany) equipped with a dry dispersing unit GRADIS/L as shown in Figure 3-14. The minimum number of particles used to measure the mean diameter was 400 in order to give statistically representative results. Dried particles were fed into a high-speed dry disperser, which was connected to a Venturi tube. During the process, the dried beads were dispersed by the gravity disperser and centrifugal force caused by velocity gradients. The images of particles were captured by a high-speed camera and recorded by the image sensor therefore; the size and shape of particles were analyzed using Windox 5.0 software. The results of particle size

presented were based on triplicate experiments and expressed as mean  $\pm$  95% CI. The graph was plotted between density distribution and diameter (mm).

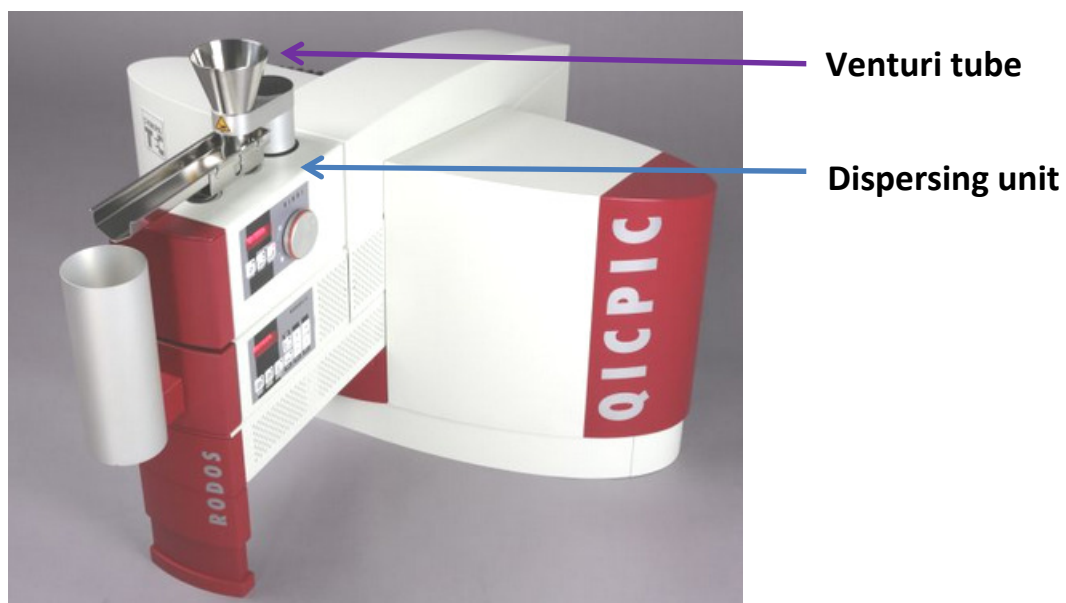


Figure 3-14 QICPIC particle size and shape analysis (Sympatec, GmbH, Germany)

### 3.10.3 Water activity

To determine water activity of FDY particles, a disposable cup was filled to not more than half full with them. A Pawkit water activity meter (Figure 3-15) was used by placing it on the level surface of the disposable cup at room temperature.



Figure 3-15 Portable water activity measurement system (Pawkit, Decagon Devices Inc, PA, USA)

### 3.10.4 Preparation of excipients

In this study, microcrystalline cellulose powder (MCC or Avicel Ph102, FMC Biopolymer, UK) as a widely used filler for direct compaction (Shangraw R and Demarest D, 1993) and magnesium stearate (Mg Stearate) (Sigma Aldrich, UK) as a lubricant (Armstrong, 1988) were introduced in all tablets. MCC was mixed with 1% w/w of MgSt using a double cone Gardner Laboratory Mixer (Kemutec Manufacturing Division, UK) for 2 min. MgSt has a lubricating property and helps preventing ingredients from sticking the die during compression process. The tablet total weight in this research was 1 g, which contained active ingredient (FDY), MCC and MgSt in particular ratios (Table 3-3).

Table 3-3 The amount of ingredients used in a one gram tablet

		Amount (g) in one tablet		
		90/10	80/20	50/50
Filler	MCC	0.09	0.19	0.49
Active ingredient	FDY/CaP beads	0.9	0.8	0.5
Lubricant	MgSt	0.01	0.01	0.01

### 3.10.5 Tableting by direct compaction

A Zwick/Roell Z030 universal testing machine (Herefordshire, UK) equipped with a 50 kN load was used for all compression studies (Figure 3-16a). A punch, 13 mm die and base plate manufactured from toughened stainless steel were used to make all tablets (Figure 3-16b). Compaction velocity was  $30 \text{ mm min}^{-1}$  and three tablets of each formulation were compressed. Aseptic technique and conditions by using Virkon (multi-purpose disinfectant) to eliminate other microorganisms were applied throughout the compression process.





Figure 3-16 (a) Zwick/ Roell Z030 universal testing machine for compressive and tensile strength of materials, force ranging 10 mN to 30 kN (b) tablet die with a diameter of 13 mm and base plate

### 3.10.6 Diametrical compression test for tablets

The tensile strength of tablets was determined by diametrical compression to their failure and typically it was evaluated not more than 24 h after compaction. A Zwick/Roell Z030 equipped with a 50 kN load was set up as shown in Figure 3-17. The diameter and thickness of each tablet were measured accurately by a digital caliper micrometer gauge (Mitutoyo, U.K). Compaction velocity was  $0.01 \text{ mm s}^{-1}$  and three tablets of each formulation were compressed. Single tablets were placed upright at the middle of a base plate and compressed vertically. The plunger was moved downward and stopped once the tablet failure was detected. The force required to break the tablet was recorded and tensile strength ( $\sigma_T$ ) was calculated by the following formula (Eq. 3-4):

$$\sigma_T = \frac{2F}{\pi dT} \quad (\text{Eq.3- 3})$$

Where;       $\sigma_T$       is tensile strength (MPa)  
                   $F$         is maximum force needed to break the tablet (N)  
                   $d$         is diameter of tablet (mm)  
                   $T$         is thickness of tablet (mm)

The experiments of measuring tablet tensile strength were performed in triplicate and the results are expressed as mean  $\pm$  95% CI.

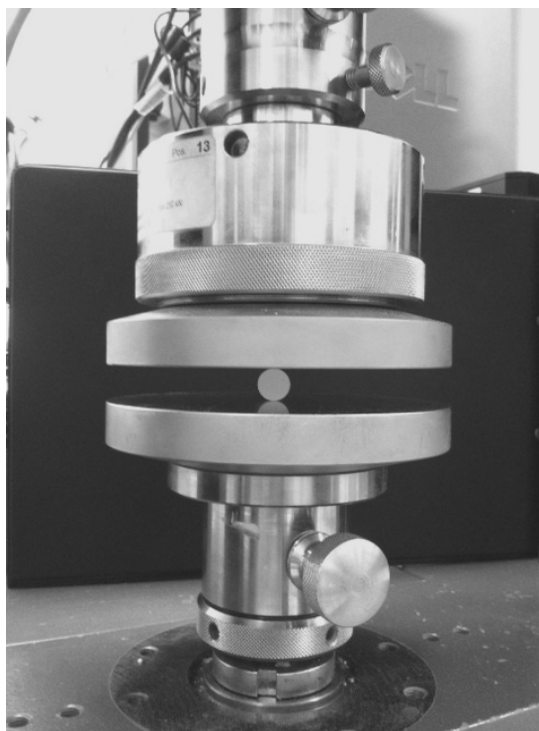


Figure 3-17 Zwick machine set-up equipped with a 50 kN load for measuring tablet tensile strength

After compression, it was evident that a tablet showed a crack towards the end on the compaction direction. There was also a flat part of the tablet towards the crack as shown in Figure 3-18. Tensile strength can be calculated as defined in Eq.3-4.

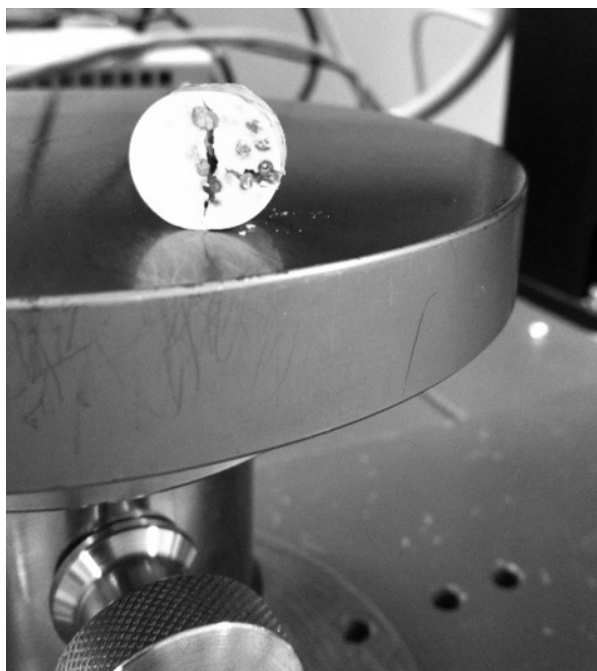


Figure 3-18 A tablet containing calcium pectinate bead and MCC after the diametrical compression test

### 3.10.7 Scanning electron microscope (SEM)

SEM visualization of materials in this research was kindly assisted by Mrs. Theresa Morris, School of Metallurgy and Materials, University of Birmingham. Some specimens of tablets containing FDY and MCC were cut with a steel blade through the center to examine the cross-sectional internal structure. Samples were coated with a 30 nm layer of gold and then observed at an accelerating voltage of 10 keV prior to analysis. Morphological characteristics and images of samples were obtained using a scanning electron microscope (Philips XL-30 SEM).

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### 3.10.8 Tablet storage stability test

Stability testing is used to establish a shelf life of a product before consumer handling. Tablets containing FDY and MCC in 50/50 (wt/wt) ratio, resulting from compaction at 75, 90 and 105 MPa, were placed in a plastic container and stored in a cool place at  $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , ( $69\%\text{RH} \pm 5\%\text{RH}$ ) for 3 and 6 months prior to storage stability test. These tablets were immersed in PBS buffer for 2 h using a USP apparatus (section 3.7.2) and homogenised at 10,000 rpm for 3 min prior to colony plate counting and FC analysis. The experiments were performed in triplicate and the results are expressed as mean  $\pm$  95% CI.

## 3.11 ENCAPSULATION OF YEAST CELLS WITH SHELLAC BY EXTRUSION

Shellac (Marcoat 125) was provided by SYNTAPHARM Harke Group, Germany which had been produced by a solvent extraction method (Buch *et al.*, 2009). This shellac solution with a dissolved solid content of 25% and pH  $\sim$ 7.5 was a clear, slightly amber and quite stable aqueous solution known as an ammonium salt. As it did not contain any preservatives, it could be diluted or mixed with any ingredients to the required concentration. Calcium chloride was acquired from Sigma-Aldrich (Dorset, UK) and of analytical grade. They were used as received without further purification.

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### 3.11.1 Preparation of core material and cross-linking solution

The core material was prepared by homogenizing 5 g of ADY in 100 mL of PBS for 3 min. The suspension was aseptically transferred into 50 mL centrifuge tubes and centrifuged at 3,000 rpm for 10 min. The supernatant was removed and yeast cell pellet was collected and used as the core material in the encapsulation process. The pellet was washed thoroughly with 40 mL sterilized TS buffered solution. Afterwards, the pellet was resuspended in 20 mL of ammonium shellac to produce a suspension with a concentration of 20% (w/v). This suspension in a 250 mL beaker was stirred for 30 min with an overhead stirrer (Eurostar, IKA, Germany) at a high speed of 1,500 rpm and left for 15 min to remove bubbles before further usage.

For preparing the cross-linking calcium chloride solution, 1.5 g of calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) was dissolved in 100 mL sterilized distilled water. During capsule formation, the cross-linking solution was stirred constantly to avoid the aggregation of the prepared capsules. When the core material in droplets came into contact with calcium chloride solution, there was diffusion of calcium ions to shellac molecules, resulting in formation of the beads. The encapsulation process using different concentrations of  $\text{CaCl}_2$  was to study the mechanical properties of beads and the effect of  $\text{CaCl}_2$  concentration on yeast cell survival in tableting.

### 3.11.2 Production of calcium shellac bead

The cell suspension containing cell pellet and shellac solution after stirring for 30 min was filled in a glass syringe and extruded through a Nisco Encapsulation unit (model Var J1, SPA-00195, Nisco, Zurich, Switzerland) with an inner diameter of 0.4 mm (Figure 3-19) by a syringe pump at a flow rate  $120 \text{ mL h}^{-1}$  before the droplets were dripped into a cross-linking solution bath for gelation. The distance between the nozzle and cross-linking solution was set at 2 cm. Calcium shellac beads containing cells prepared by this extrusion method, were formed instantly, which were allowed to immerse in  $\text{CaCl}_2$  for 15 min for hardening before being rinsed three times with sterilized distilled water.



Figure 3-19 A Nisco encapsulation unit model VAR J1, SPA-00195

### 3.12 ENCAPSULATION OF YEAST CELLS WITH PECTIN

GENU pectin type LM 101 (DM 36%) was generously supplied by Copenhagen Pectin, Denmark. These are low methylester pectin extracted from citrus peel which is suitable for pharmaceutical applications. The product has light colour and relatively fine particle size (100 mesh or 149  $\mu\text{m}$ ). Calcium chloride was acquired from Sigma-Aldrich (Dorset, UK) and used as supplied.

#### 3.12.1 Preparation of core material

Pectin powder was dispersed in distilled water to a concentration of 5% w/v unless a different concentration is stated in Table 3-4 in order to determine how the mechanical strength of calcium pectinate beads affects viability of yeast cells in tablets produced at different compaction pressures.

Table 3-4 The preparation of core material at different concentrations

Pectin concentration (%w/v)	Amount of pectin (g)	Water (mL)
3	1.5	50
4	2	50
5	2.5	50

For encapsulation of yeast cells with pectin, core material was prepared by 10 g of wet yeast (WY) mixed with 50 mL of each pectin concentration in a 250 mL beaker and stirred with an overhead stirrer (Eurostar, IKA, Germany) for 30 min at a high speed of 1,500 rpm, which was left for 15 min to remove bubbles before further usage.



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### 3.12.2 Production of calcium pectinate beads using a G23 needle

The suspension of pectin and wet yeast as described in section 3.12.1 was pumped through a glass syringe and extruded through a G23 needle (0.33 mm in diameter) using a syringe pump. Droplets containing yeast were dripped into a bath of calcium chloride cross-linking solution for gelation at room temperature. The distance between the dripping tip and the gelling bath was at 7 cm (Chan *et al.*, 2009). Magnetic stirring was used constantly in the cross-linking solution in order to avoid the aggregation of calcium pectinate beads. When the core droplets came into contact with the calcium ions, the beads were formed, which were further hardened in the same  $\text{CaCl}_2$  solution for 30 min. They were washed with distilled water twice before they were filtered and dried in a freeze dryer (Edwards, EF03, Crawley, UK) for 24 h.

### 3.12.3 Optimization of calcium chloride concentration

The concentrations of calcium chloride solution in the encapsulation process were varied to be 1%, 1.5%, 2% and 2.5% in order to determine whether calcium ion has an effect on viability of cells in entrapped beads by leaving them hardening in the solution for 30 min.

### 3.12.4 Freeze drying of calcium shellac/pectinate beads

Calcium shellac/pectinate beads prepared using the procedures described in section 3.11.2 and 3.12.2 were freeze-dried in the freeze dryer (Edwards, EF03, Crawley, UK) before they were compacted into tablets. The beads were placed on a petri dish and subsequently put in the drying chamber. The refrigeration unit was switched on until the temperature reached -50

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°C and the beads were dehydrated at 0.6-0.7 mbar for 24 h. The drying temperature was maintained at 25°C until the end of the process when the pressure was at 2.5-3.5 mbar.

### **3.12.5 Characterization of beads with encapsulated cells**

Cell encapsulation efficiency and the properties of calcium shellac and pectinate beads obtained from the encapsulation process described above i.e. water activity value, particle size distribution and internal structure were studied.

#### ***3.12.5.1 Particle size analysis***

The sizes of dried calcium shellac/pectinate beads were analyzed using an image analyzer (QICPIC, Sympatec GmbH Inc., Clausthal-Zellerfeld, Germany) as described in section 3.10.2. The minimum number of particles measured to determine the mean diameter was 400 beads in order to give statistically representative results.

#### ***3.12.5.2 Water activity***

To determine water activity of the dried calcium shellac/pectinate beads, a disposable cup was filled to not more than half full with them. A Pawkit water activity meter (Figure 3-15) was used by placing it on the level surface of the disposable cup at room temperature.

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### **3.12.5.3 Viability of cells entrapped in beads/tablets**

Calcium shellac or calcium pectinate beads with cells entrapped were homogenised in 100 mL of PBS solution at a homogenisation speed of 10,000 rpm using a T-25 Ultra-Turrax homogeniser (IKA, Germany) for various times to fully release cells from the beads. Cell viability by CPC and FC was performed as described in section 3.2 and 3.8 respectively.

0.5 g of calcium shellac beads was compacted into tablets using a Zwick/Roell Z030 universal testing machine (Herefordshire, UK) as described in section 3.10.5. These tablets were immersed in PBS solution using the USP apparatus (see section 3.7.2) and cell viability was performed by CPC and FC as described in section 3.2 and 3.8 respectively.

### **3.12.6 Optimizing calcium chloride concentration**

The effect of calcium chloride as a cross-linking molecule on cell viability was studied by varying its concentration at 1%, 1.5%, 2% and 2.5% (w/v). Cell viability in CaP beads made in each concentration was determined by CPC and FC together with PI and BOX and SYTO9 and PI respectively (see section 3.2 and 3.8). The numbers of healthy, injured and dead cells against  $\text{CaCl}_2$  concentration with standard errors were obtained from FC analysis after cell suspension being stained with fluorescent dyes.

### 3.12.7 Encapsulation efficiency (EE)

The encapsulation efficiency (EE) was calculated based on the initial number of yeast cells and those encapsulated in calcium pectinate beads (see Eq.3-5).

$$EE = \frac{\text{Total number of cells in CaP beads (OUTPUT)}}{\text{Total number of cells in WY (INPUT)}} \times 100\% \quad (\text{Eq.3- 4})$$

The number of cells in calcium pectinate beads was determined after they were freeze dried at 0.6 mbar for 24 h and it is assumed that there was no loss of cells during the drying process. Prior to determination of encapsulation efficiency, the beads with cells trapped must be completely dissolved before their total number can be counted. Firstly, 1 g of the beads was disintegrated in 100 mL PBS solution (pH 7) by using a USP apparatus operated at 37 °C and 100 rpm for two hours. 1 mL of pectinolytic enzyme was added in order to help degrade calcium pectinate beads. The use of the enzyme was to mimic the enzyme in human colon (Rubinstein et al., 1993, Ashford et al., 1994). After that, this suspension was homogenised for 3 min before the total number of cells was measured using flow cytometry.

Another measurement to determine encapsulation efficiency based on number of culturable cells (%) of the beads was calculated using Eq 3-6 which was based on one gram of bead and WY.

$$EE \text{ based on number of culturable cells} = \frac{\text{CFU in CaP beads (OUTPUT)}}{\text{CFU of WY (INPUT)}} \times 100\% \quad (\text{Eq.3- 5})$$

### **3.12.8 Varying pectin concentration**

The concentrations of pectin were varied at 3%, 4% and 5% in order to investigate if high concentration of pectin solution could offer more protection to encapsulated yeast cells. It had been found that no spherical calcium pectinate beads could be formed when pectin concentration lower than 3% was used. The suspensions of yeast cells mixed with pectin concentration higher than 5% were very turbid and it was difficult to form the beads through a 23G needle and a syringe pump.

### **3.12.9 Viability test under fluorescence microscope**

Cell suspensions of calcium shellac and pectinate beads were stained with SYTO9 and PI for 30 min, a part of LIVE/DEAD Funga*Light* yeast viability kit, before they were observed under a fluorescence microscope (Olympus, AX70, Tokyo, Japan) equipped with a camera (Olympus DP70). The images obtained from the fluorescence microscope was analysed to determine live cells stained in green and dead cells stained in red.

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### **3.13 EVALUATION OF CALCIUM PECTINATE (CAP) BEAD PROPERTIES AND COMPACTION OF CAP BEADS INTO TABLET FORM**

#### **3.13.1 Diametrical compression test for CaP beads**

In order to study the mechanical properties and estimate the Young's moduli of calcium pectinate beads, diametrical compression was performed using a 100 N load connected to Zwick/Roell machine as shown in Figure 3-20. At least ten calcium pectinate (CaP) beads were compressed. Firstly, one CaP bead was placed under a 100 N load. The maximum compression force used was set to be 95 N when the platen stopped compressing. The compression speed was set at 0.01 mm s<sup>-1</sup> with/without holding time while the load and displacement data were recorded. The force-sampling time data for compressing single calcium pectinate beads were examined by the data acquisition software testXpert V9.01 which had been installed in a computer directly connected to the Zwick machine. A typical force-displacement curve was plotted as shown in Figure 3-21.

Nominal rupture stress is one of the parameters used to determine the mechanical strength of the particle (Yap et al., 2008). It is defined as the ratio of the rupture force at failure to the initial cross sectional area of the microparticle.

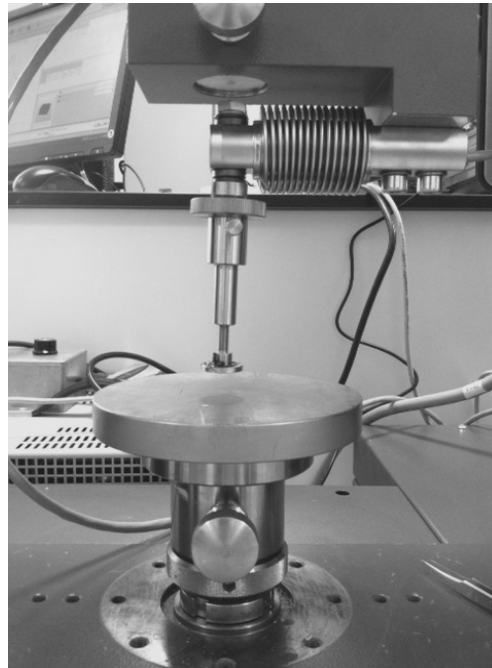


Figure 3-20 Zwick machine set-up with a 100 N load for measuring the mechanical properties of single calcium pectinate beads

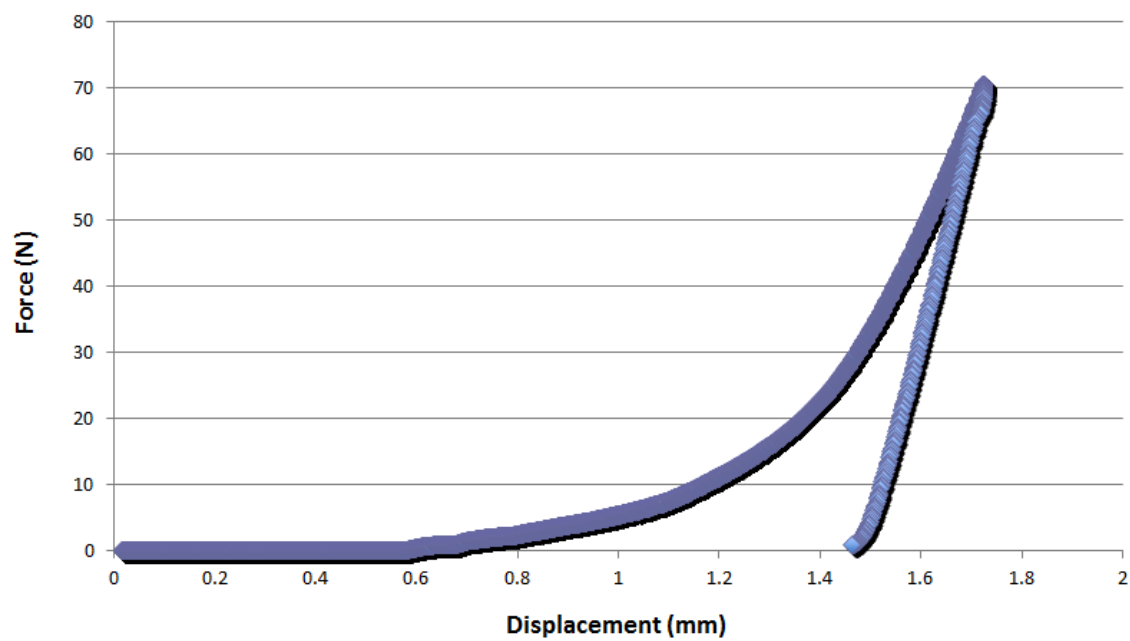


Figure 3-21 Typical load and unloading-displacement curve of a single CaP bead (2.3 mm in diameter) performed at a speed of 0.01 mm s<sup>-1</sup>

Young's modulus of single calcium pectinate beads for the case of small deformation was calculated by Hertz equation (Hertz, 1882) (Eq 3-7). This equation is valid for deformation of CaP beads up to 30% nominal deformation or 10% strain (Andrei D et al., 1996). A Poisson ratio ( $\nu$ ) of 0.5 was chosen as the material was presumed negligible water loss and might be close to incompressible material (Wang et al., 2005).

$$F = \frac{E \sqrt{2R}}{3(1 - \nu^2)} \cdot \Delta^{3/2} \quad (\text{Eq.3-6})$$

Where E = Young's modulus (MPa)

F = applied external force (N)

$\Delta$  = total displacement of the moving platen (mm)

R = radius of calcium pectinate bead (mm)

$\nu$  = Poisson's ratio

### 3.13.2 Force relaxation

In order to identify whether the beads were viscoelastic, single dried CaP beads were compressed by a 100 N load at a speed of  $0.01 \text{ mm s}^{-1}$  and then held at constant deformation for 3 min to allow force relaxation. Since there was no water loss under compression, any force relaxation during the holding can be considered to be due to their viscoelasticity.



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### 3.13.3 Compaction of CaP beads into tablet

Dried calcium pectinate beads (as described in section 3.12.4) were mixed with MCC and MgSt in different ratios as shown in Table 3-3. Every tablet weighed 1 g which contained 1% of MgSt to help prevent ingredients from sticking the die during compression process. Tablets were compacted using a Zwick/Roell machine (section 3.10.5) at a compaction velocity of  $30 \text{ mm min}^{-1}$ .

### 3.13.4 Storage stability test

Tablets containing CaP and MCC in 50/50 (wt/wt) ratios compacted at 75, 90 and 105 MPa were stored in a cool place at  $5 \pm 1^\circ\text{C}$ , ( $69\%\text{RH} \pm 5\%\text{RH}$ ) for 3 and 6 months. Tablets compacted at 90 MPa were also stored in a desiccator ( $45\% \text{ RH} \pm 3\%\text{RH}$ ) at  $5 \pm 1^\circ\text{C}$  in order to determine if relative humidity has an impact on cell viability. The average CFU/g tablet and FC data were plotted against compaction pressures. Flow cytometric dot plots of cells stained with PI and BOX and, SYTO9 and PI respectively were presented.

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### **3.14 CONCLUSION**

This chapter details the materials and methods used to investigate the viability and physiological states of probiotic yeast cells in a range of processes related to preparation of samples from fermentation, compacting dry cells with excipients into tablet dosage form and their storage including encapsulation of them in biocompatible matrixes with an aim to improve cell survival in tableting and storage. The following chapters will present the detailed results with associated discussions.

## CHAPTER 4

### COMPARISON OF PROBIOTIC YEAST VIABILITY PRODUCED FROM VARIOUS PROCESSING AND ENVIRONMENTAL CONDITIONS

#### 4.1 INTRODUCTION

Three different samples of yeast (as shown in Table 3-1) received from Lesaffre International (France) have been used to investigate cell viability by measurements techniques, i.e. colony plate counting (CPC) (Jayapal et al., 1991), haemocytometry with methylene blue (Richard, 1932) and flow cytometry (Pore, 1991, Wenisch et al., 1997). Working at the single cell level, flow cytometry (FC) combined with a variety of fluorescent dyes e.g. propidium iodide [PI] (Ramani and Chaturvedi, 2000) and bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC<sub>4</sub>(3) or BOX] (Dinsdale et al., 1995) has been used to assess microbial physiological states (Hewitt and Nebe-Von-Caron, 2001). PI is used to investigate DNA content and distinguish between live and dead cells. Dead cells with permeabilised cytoplasmic membrane or injured membrane can be stained with PI, which binds to nucleic acid but is unable to cross an intact cytoplasmic membrane. A membrane potential (MP), an electrical potential difference across the cytoplasmic membrane of a cell is required for energy production in the form of ATP during nutrient uptake. The lipophilic anionic dye known as BOX has been used to detect changes of MP by producing green fluorescence when the cytoplasmic membrane is depolarised or fully energised (Caron and Badley, 1995, Hewitt and Nebe-Von-Caron, 2004). The use of BOX combined with PI allows allocation of cells into three physiological states: (a) intact polarized cells without staining (b) injured cells with no membrane potential or

depolarized cytoplasmic membranes and (c) dead cells with permeabilised membranes (Davey and Kell, 1996, Hewitt and Nebe-Von-Caron, 2001). Another commercial viability kit, *FungaLight*, has previously been developed only for bacteria (Hope and Wilson, 2003, Lehtinen et al., 2003) and recently it has also been used to quantify yeast viability (Zhang and Fang, 2004, Tanja et al., 2013).

FC is much more powerful microbiological technique than the traditional method of colony plate counting (CPC). It has the ability to discriminate between cells in a mixed population or heterogeneous suspension and can be used to resolve rapidly the physiological and metabolic state of single cells including those that are not culturable (Pore, 1991, Wenisch et al., 1997). Nevertheless, some characterization of cultures is best done by plate counting if one's interest is limited to only those cells that have the ability to grow on solid media (Caron and Badley, 1995). This method requires colonies to be visualized under a microscope after dilution plating of  $10^9$  to  $10^{12}$ , depending on the initial cell concentration (Jayapal et al., 1991). It assumes that the diluted sample is a homogeneous suspension of cells so that each colony grows from a single cell. However, the CPC method has always had drawbacks e.g. it is time-consuming and labour intensive because it involves solid media preparation, as the plates require incubation time and manual counting is often used.

In this work, the size distribution of yeast suspension homogenised at different speeds was studied and hence, the effects of homogenisation speed on cell viability were evaluated. The factors influencing yeast cell viability i.e. heat from drying process, size reduction by milling process, compaction pressure from tableting and various pH values in the GI tract (simulated) were also investigated. Preliminary results of tablet dissolution time were examined by

dissolving a tablet in a USP apparatus for two hours. This was to determine whether all active ingredients from the tablet can be released within the limit of disintegration test (B.P).

The aim of this study was to use both CPC and FC to determine the viability of probiotic *Saccharomyces cerevisiae* produced from various processing conditions and to study the effect of simulated GI tract conditions (pH values between 1.7 and 6.1) on such cells. It was also intended to correlate the number of viable cells obtained from flow cytometry and the number of cultural cells from CPC, and such a correlation may lead to the determination of how the stress level from either processing or environmental conditions affects yeast cell viability.

## **4.2 RESULTS AND DISCUSSION**

### **4.2.1 Particle size analysis**

As the yeast samples (ADY and MY) received from Lesaffre was in the solid granule form, it is necessary to disperse them in a buffer using homogenisation and hence, to optimize the homogenisation speed which can release the maximum amount of yeast cells but generate minimum damage to them. Particle size data of ADY and MY suspension in Figure 4-1 and Figure 4-2 show a sharp peak ranging from 3-8  $\mu\text{m}$  in size, which corresponds to the typical size of yeast cells (Jorgensen et al., 2002). It appears that after 3 min homogenisation, higher homogenisation speeds enhanced the number of cells released from the granules, as might be expected. The particle size distribution data of WY suspension (data not shown) show only one peak at all homogenisation speeds. This might be because WY received from fermentation did not contain other excipients. Viability of ADY cells released after applying different homogenisation speeds was determined (more detail in section 4.2.3.1) and it was found that in

this experiment, the maximum CFU/g and number of viable cells were found when a homogenisation speed of 10 000 rpm was used, hence this speed was applied in consequent experiments.

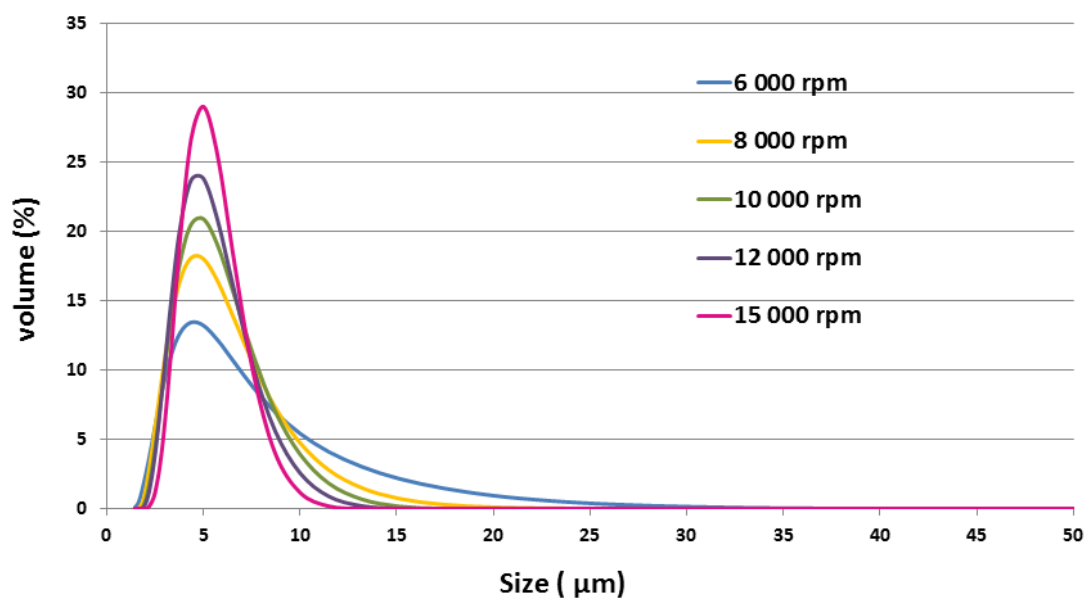


Figure 4-1 Particle size distribution of ADY suspension after being homogenised for 3 min using a T-25 Ultra-Turrax homogenizer at various speeds.

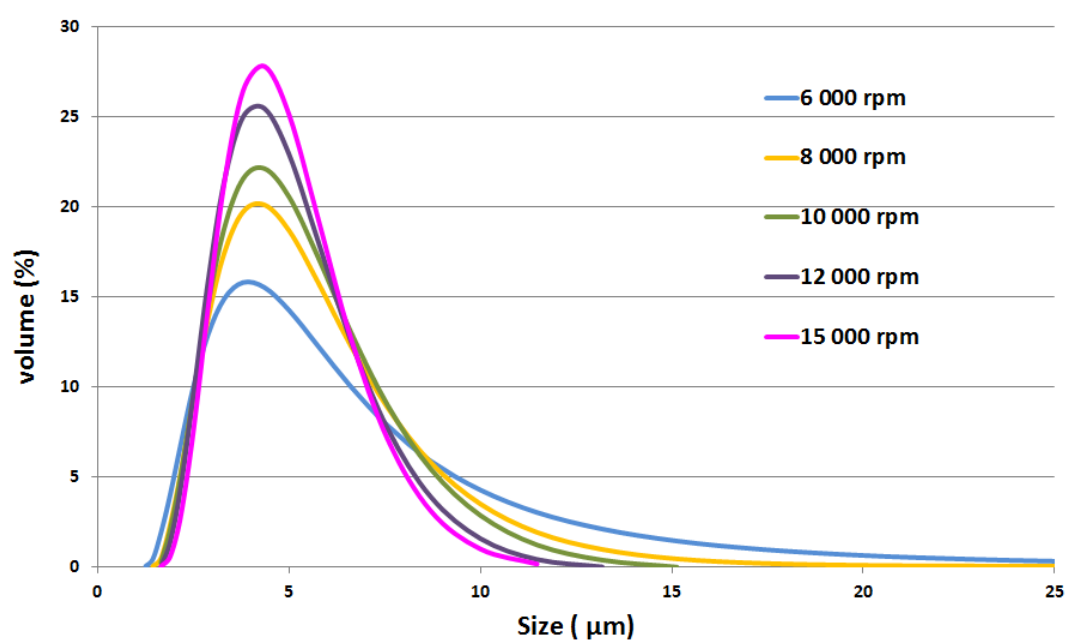


Figure 4-2 Particle size distribution of MY suspension after being homogenised for 3 min using a T-25 Ultra-Turrax homogenizer at various speeds.

### 4.2.2 Dry cell weight

Since CFU calculation was based on dry cell mass, dry cell weights of ADY, MY and WY were obtained as shown in Figure 4-3. MY sample had a mean DCW of  $95.6 \pm 0.4$  %, which contained the least amount of water among the three samples. WY harvested after fermentation contained the highest amount of water with an average of  $26.1 \pm 0.4$  %. These DCW values were used to calculate true values of CFU/g dry cell mass.

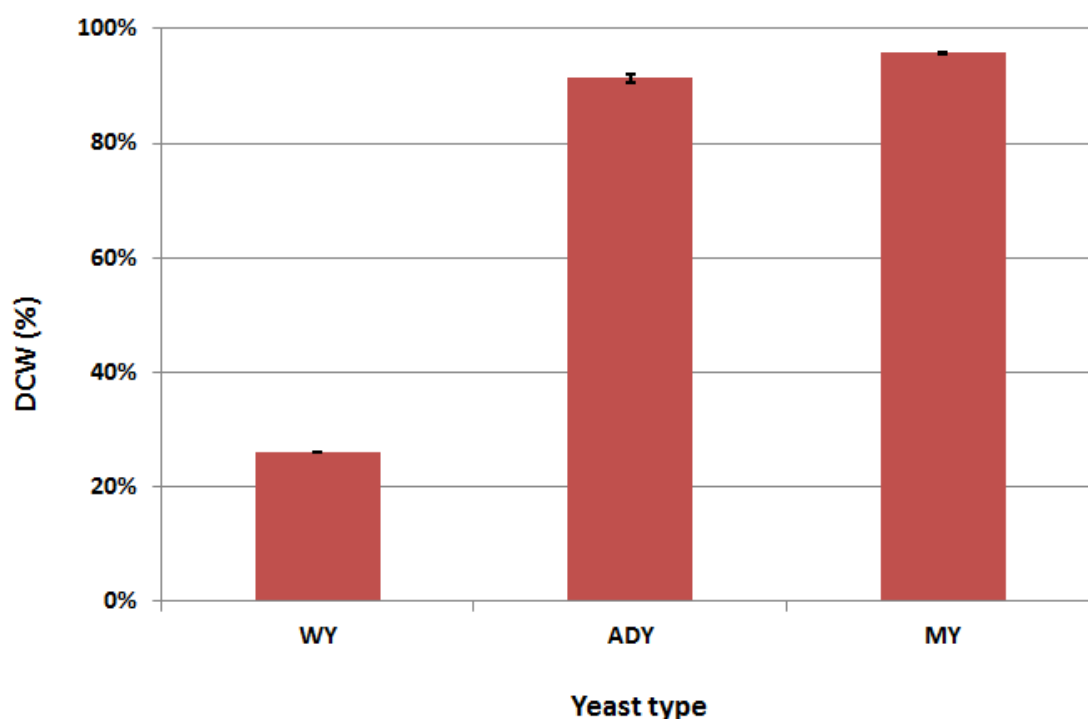


Figure 4-3 Percentage of dry cell weight comparison between ADY ( $91\% \pm 3\%$ ), MY ( $95.6\% \pm 0.4\%$ ) and WY ( $26.1\% \pm 0.4\%$ ) after being left in the oven for 48 h; results are obtained from two measurements ( $n=2$ ) and presented with standard errors

### 4.2.3 Viability determination

In order to determine whether processing conditions have an effect on cell viability, studies were conducted on ADY, MY and WY. Cell viability was determined by FC using dual staining of PI and BOX. Three physiological states of cells were detected as described in Table 4-1. The fluorescent emissions of BOX and PI represent green and red respectively. Moreover, two groups of dead cell population as shown in top right quadrant of PI and BOX staining (Figure 4-6; upper row) and in lower gating of SYTO9 and PI staining (Figure 4-6; lower row) were observed. Furthermore, when these populations of dead cells were sorted based on forward scatter/side scatter plots (FSC/SSC), the results can be explained by the presence of doublets which have two different sizes of dead cells (flow dot plots are not shown).

Table 4-1 Distinct physiological states of *S. cerevisiae* after stained with PI and BOX

Characteristics	Staining pattern	Quadrant
Healthy	BOX-, PI-	Lower left
Injured	BOX+, PI-	Upper left
Dead	BOX+, PI+	Upper right



### 4.2.3.1 Effect of homogenisation speed on yeast cell survival

Suspensions of ADY were prepared at different homogenisation speeds for 3 min. The homogenisation speed was varied between 6,000 to 15,000 rpm. Cell viability was determined by FC using dual staining of PI and BOX and SYTO9 and PI. At low homogenisation speeds (<10,000 rpm), it appears that a large number of dead cells at granule surfaces were released which are thought to be cells damaged by heat (40-42 °C) in the drying process (Lesaffre, France) as shown in Figure 4-4. It can be noticed that similar results from using the two dual stains were obtained at homogenisation speeds from 6000 to 15000 rpm (Figure 4-6). At high homogenisation speeds, it is presumed that cells were vulnerable due to mechanical forces generated in the homogeniser.

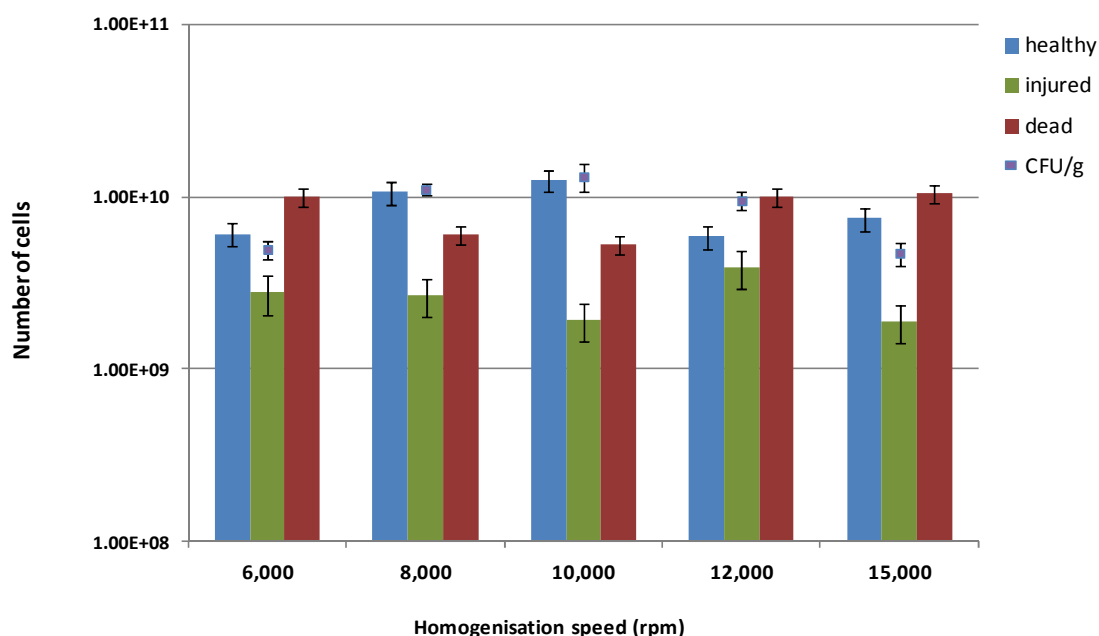


Figure 4-4 CFU/g and the number of healthy, injured and dead cells of yeast suspension (based on 1 g of ADY) homogenised at different speed for 3 min and stained with BOX and PI. Data are obtained from triplicate studies (n=3) with standard errors

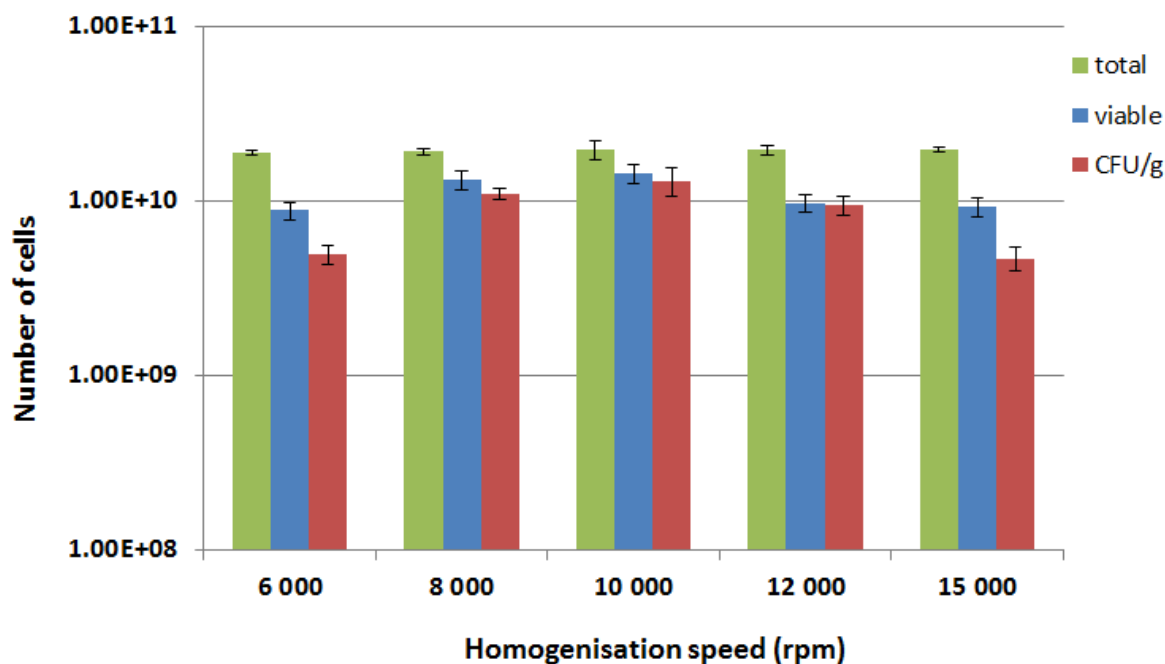


Figure 4-5 The number of total and viable cells measured by FC vs. CFU/g in CPC of ADY suspension at various homogenisation speeds

Based on the data finding in Figure 4-5, CFU decreased when yeast granules were homogenised from 10,000 rpm to 15,000 rpm. At a speed of 10,000 rpm, the maximum number of viable cells measured by FC was  $1.4 \times 10^{10} \pm 0.5 \times 10^{10}$  cells which corresponded to colonies of  $1.3 \times 10^{10} \pm 0.3 \times 10^{10}$  CFU/g. The colonies on the agar plates are thought to be the number of single cells that are able to grow at the particular condition i.e. enough nutrients in the medium. Some cells that are determined as viable but unable to be cultured on the solid medium are typically called viable but nonculturable (VBNC) cells (Hewitt and Nebe-Von-Caron, 2001, Oliver, 2005). However, the number of culturable cells or CFU/g always underestimates the number of viable cells present as CFU/g measures the capability to reproduce rather than a true measurement of viability. A correlation between the number of viable cells and CFU/g data at different homogenisation speeds is described in section 4.2.4.

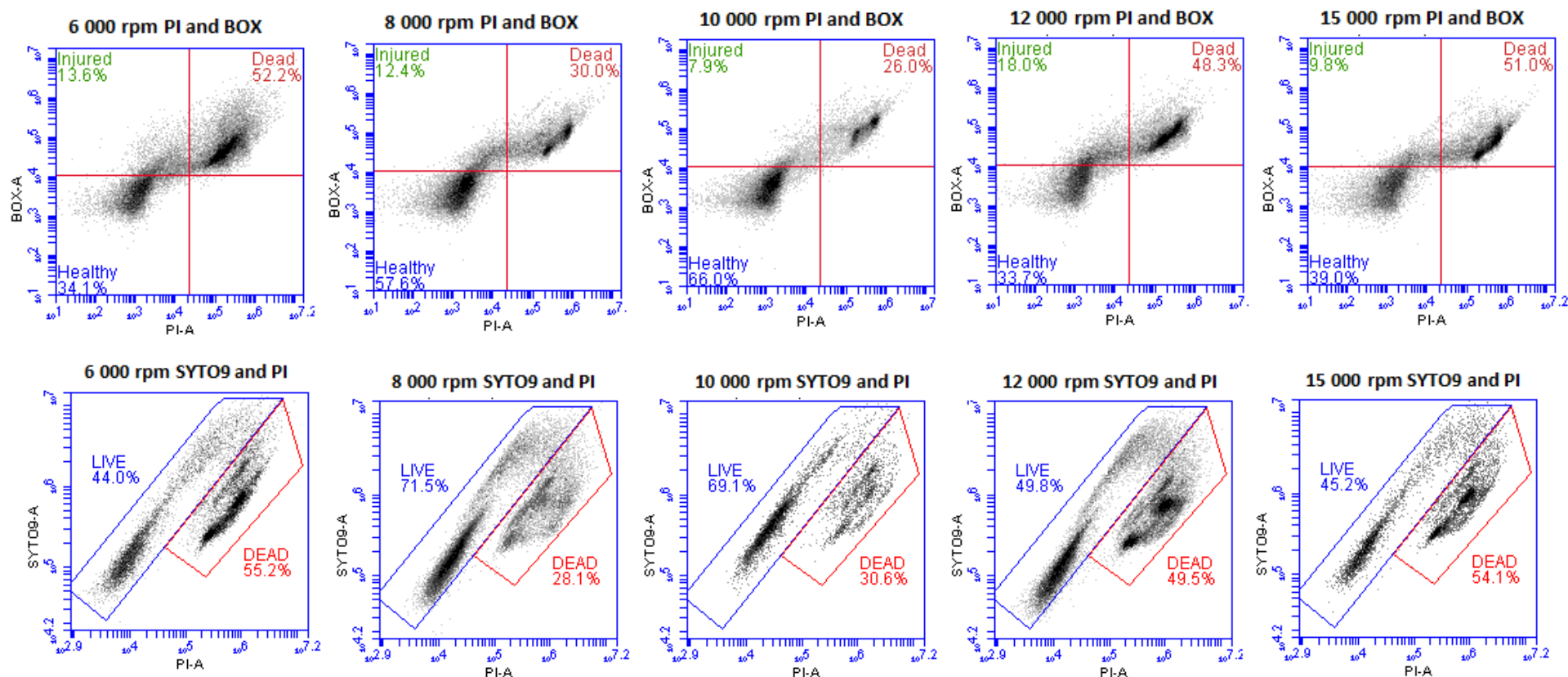


Figure 4-6 Flow cytometric density plots of ADY suspension homogenised at different speed, stained with BOX and PI (upper row) and with SYTO9 and PI (lower row). Green and red fluorescence are presented on the ordinates and abscissae axes respectively.

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#### 4.2.3.2 Viability of three yeast samples

Flow cytometric dot plots in Figure 4-7 present the physiological states of healthy, injured and dead cells after being stained with PI and BOX. It can be seen that WY showed the maximum percentage of healthy cells at  $98.4 \pm 0.2$  % among the three samples. Consistent data of the percentage of live and dead cells in SYTO9 and PI plots (Figure 4-8) can be observed. The definition of a live cell in this paper is a cell with an intact polarized cytoplasmic membrane as proposed by Caron and Badley (1995) including healthy and injured cells in the PI and BOX plots. The percentage of live cells in WY was approximately  $27.1 \pm 2.5\%$  and  $41.2 \pm 0.1\%$  more than that in ADY and MY respectively. Cell viability loss in ADY which was drum dried at 40-42°C for 10-20 h (Lesaffre, France) is thought to be due to the effect of adverse temperature during the drying process which can cause cell death. This is in agreement with Labuza et al. (1970) and Bozoğlu et al. (1987) who reported considerable viability loss in spray and drum drying at short residence time.

In ADY production, the granules were extruded through the wide extrusion screen resulting in a particle sizes between 500 and 2000  $\mu\text{m}$ . FC results of ADY from two dual stains show the average percentage of viability of  $71 \pm 2\%$  which is higher than 57% of MY ( $D_{50} \sim 400$   $\mu\text{m}$ ). This slight decrease in viability of MY suggests that the milling process had a small adverse effect on cell viability. The present findings seem to be consistent with other research which reported particle size reduction can cause additional damage to cells and these cells are then more susceptible to other stresses (Picot and Lacroix, 2003).

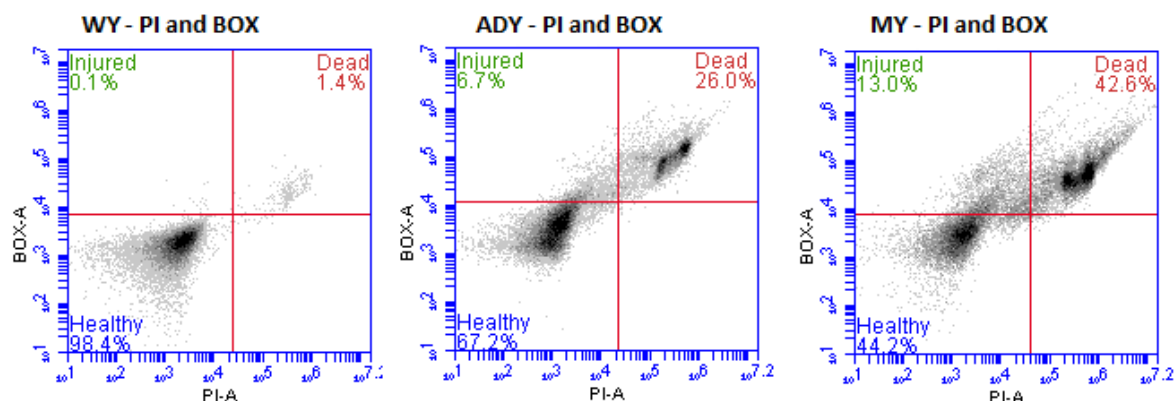


Figure 4-7 Flow cytometric dot plots of WY, ADY and MY suspensions discriminating healthy, injured and dead cells after being stained with PI and BOX

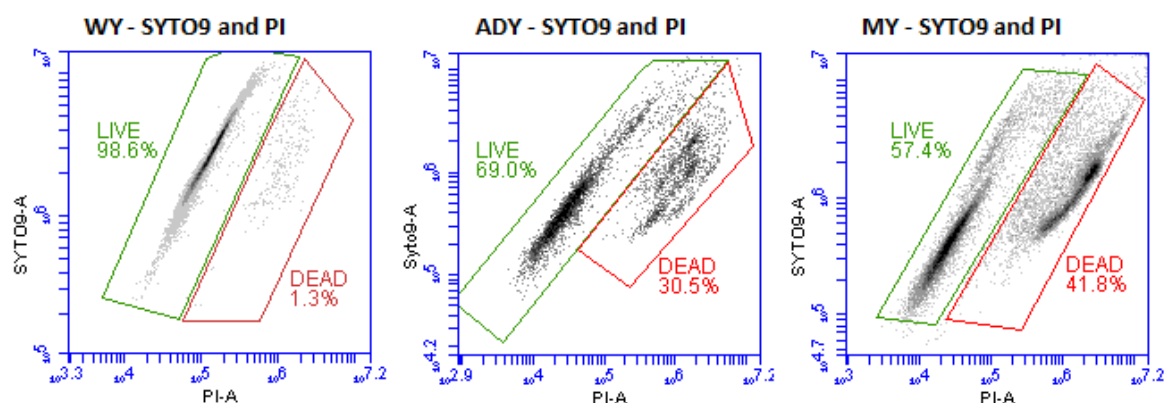


Figure 4-8 Flow cytometric dot plots of WY, ADY and MY suspensions discriminating live and dead cells after being stained with SYTO9 and PI

The numbers of healthy, injured and dead cells of each yeast sample after staining with PI and BOX are plotted with CFU/g in Figure 4-9, and all data are based on 1 g dry cell weight. It can be seen that WY contained the maximum number of healthy cells, which was more than ADY and MY by 1 and 1.5 log respectively. WY displayed the minimum number of dead cells/g DCW, which was significantly less than those in ADY and MY ( $p \leq 0.05$ ). Differences between means of CFU/g between three yeast samples were significant. In addition, CFU/g value was consistent with FC data where CFU/g of WY was almost one log higher than that of ADY. Relative correlation between the number of viable cells measured by FC and CFU data of various yeast samples is described in section 4.2.4.

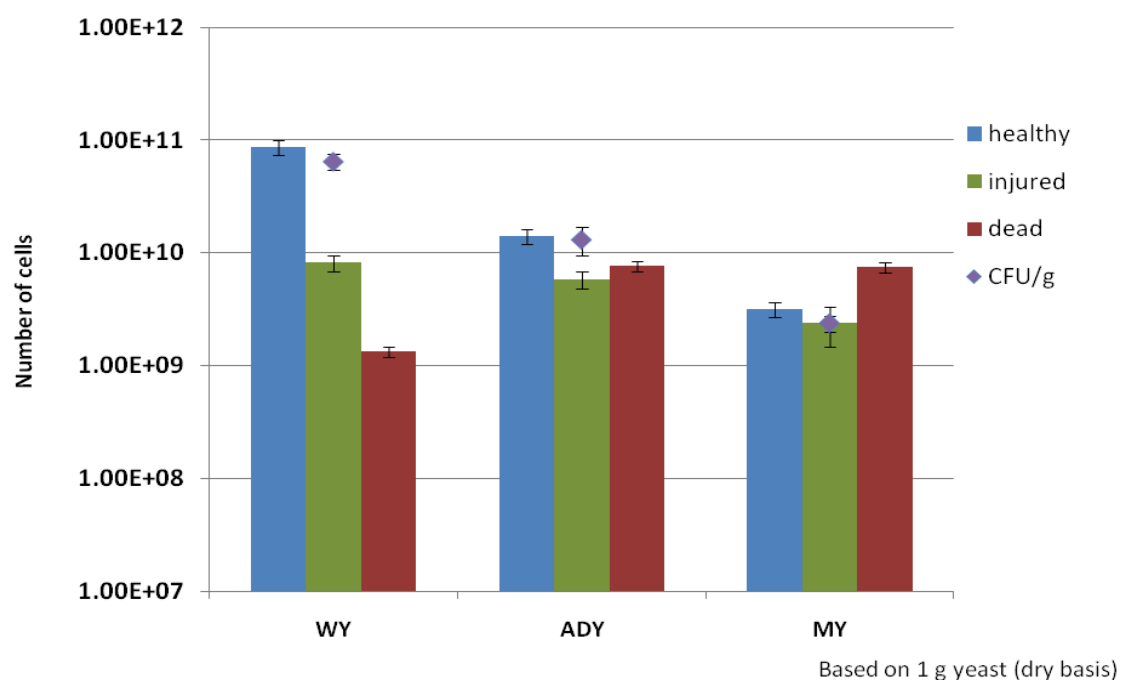


Figure 4-9 The number of healthy, injured and dead cells of three different samples: WY, ADY and MY after being stained with BOX and PI. Data shown are based on 1 g dry cell mass basis with standard errors.

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#### 4.2.3.3 Effects of pH on cell viability

In the GI tract, pH can vary between 1.7 and 6 due to food consumption and various enzymes (Blanquet et al., 2004). A study was conducted in order to investigate whether environmental condition of pH during the digestive system has an effect on cell viability. ADY suspensions with different pH were tested using a USP apparatus operated at 37°C and a speed of 100 rpm (as explained in section 3.7.2). Each sample was taken after two hours after the cells were exposed to a fluid with a given pH before cell viability was determined by CPC and FC. The numbers of healthy, injured and dead cells in different pH fluids are presented in Figure 4-10. The number of healthy cells dramatically decreased in strong acid whereas that injured cells slightly fluctuated. Figure 4-11 shows that the number of viable cells after exposure to fluids with different pH was all higher than  $10^9$  CFU/g except the number corresponding to the fluid of pH 1 in which the viable cell number dropped to approximately  $10^8$  CFU/g. This was consistent with FC data when cells were stained with PI and BOX. The loss of viability when cells were exposed to a simulated gastric fluid (SGF) of pH 1 might be caused by the high acidity and osmotic pressure of pH 1 fluid (Appendix 2). It was found that viability loss of *S. cerevisiae* after being exposed in hypertonic solution caused the reduction of cell volume, therefore cells were osmotically responsive (Morris et al., 1986) and cell membranes were subjected to cell shrinkage by mechanical constraints (Beney et al., 2000). Although yeast was found to be susceptible in pH 1 fluid, pH in the human stomach is rarely as low as 1 (typically it is pH 1.6) and tends to increase after food consumption, so the effect of pH on yeast cell viability is not significant in practice.

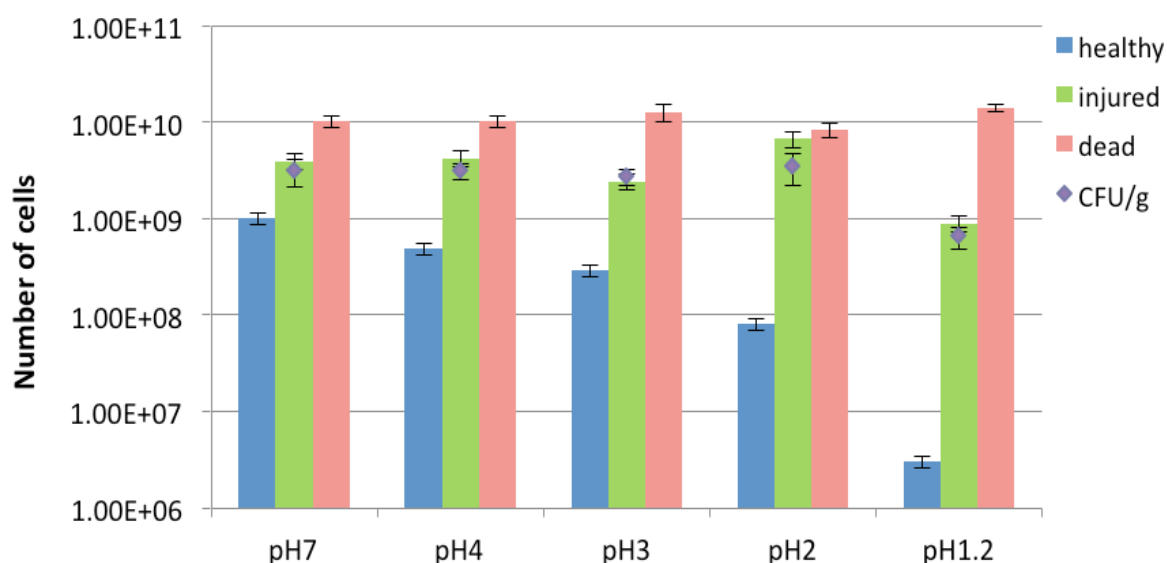


Figure 4-10 CFU/g and the number of healthy, injured and dead cells of yeast suspension (based on 1 g of ADY) homogenised at a speed of 10,000 rpm for 3 min and stained with BOX and PI. Data are obtained from triplicate measurements (n=3) with standard errors.

The number of viable cells as shown in Figure 4-11 is a summation of healthy and injured cells which are cells without PI staining. When comparing viable cells and culturable cells (CFU), it can be noticed that CFU/g values were always underestimated. This is because CFU/g measures the reproductive capability rather than a true measurement of viability.

Another cell viability measurement of yeast exposed to various pH fluids for 2 h by using a haemocytometer and methylene blue was made. The data also show that the number of alive cells in pH 1 fluid was significantly less compared to those exposed to other pH values (Figure 4-12), which is consistent with data obtained from CPC and FC.



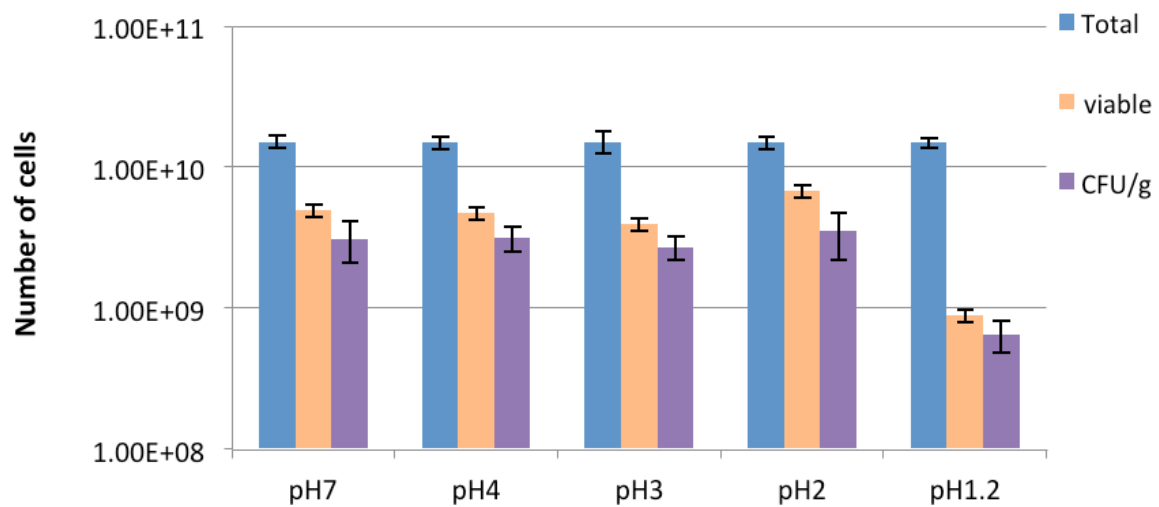


Figure 4-11 The number of total and viable cells of ADY measured by FC when they were stained with PI and BOX vs. CFU/g measured by CPC, after being exposed to a fluid of varying pH. Data shown are based on 1 g of ADY with stand errors.

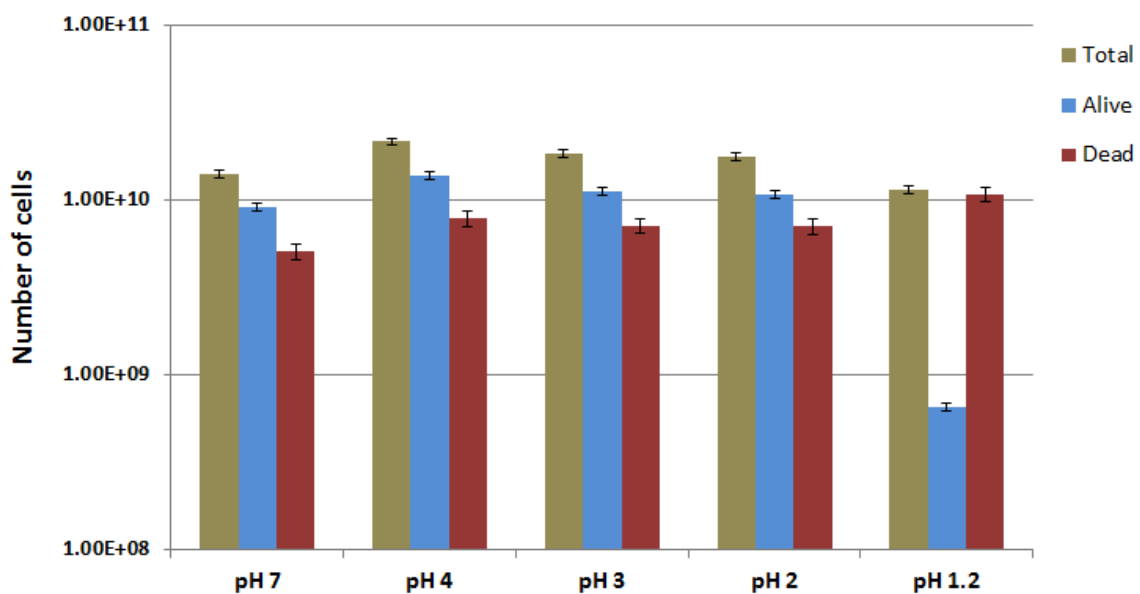


Figure 4-12 The number of total, alive and dead cells of ADY suspension after being exposed to various pH fluids for 2 h, stained with methylene blue and counted by a haemocytometer.

#### 4.2.3.4 Effect of compaction pressure on cell viability

In order to determine the effect of compaction pressure on cell viability, MY and MCC were mixed in a 50/50 (wt/wt) ratio (Table 3-3) and compacted into tablets using a compression pressure in a range of 75-135 MPa (see section 3.10.5). These tablets were dissolved in PBS for two hours using a USP apparatus (see section 3.7.2). The results of the number of healthy, injured and dead cells are shown in Figure 4-13 and compared with MY as a controlled experiment. Higher compaction pressure caused more dead cells and resulted in less number of healthy cells. It can be noticed that there was no significant difference in the number of cells when compaction pressures higher than 120 MPa were used.

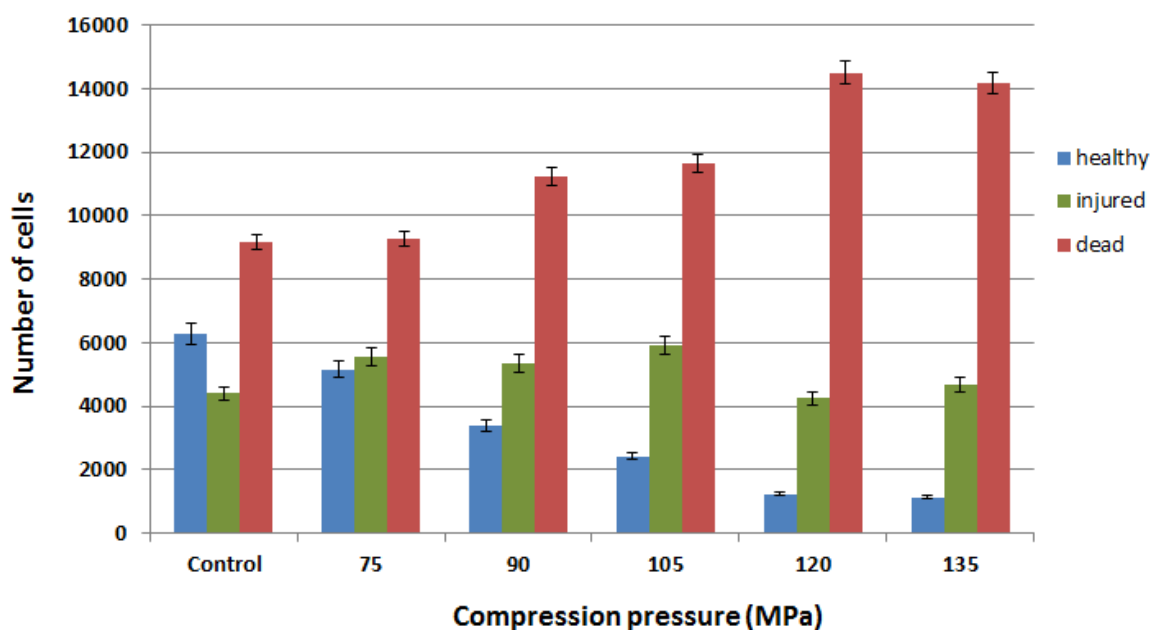


Figure 4-13 The number of healthy, injured and dead cells in tablets made by MY /MCC in 50/50 (wt/wt) ratio compacted at different compression pressure and exposed to PBS for 2 h. Data shown are based on 20,000 cells as measures by FC with standard errors.

Similar experiment to study the effect of compaction pressure on cell viability was performed with tablets made with ADY/MCC in 50/50 (wt/wt) ratio and compacted with a pressure ranging between 75 and 150 MPa. Cell viability in terms of CFU/g tablet is shown in Figure 4-14 in comparison with control experiment (before compaction) and a targeted CFU/g in one tablet. There was no significant difference in CFU/g when compaction pressures up to 105 MPa were used, however CFU/g dropped significantly when compaction pressures beyond 105 MPa were used. If the data in Figure 4-13 and Figure 4-14 are compared, the results are consistent where the optimized compaction pressure giving satisfactory CFU/g tablet was 105 MPa. Later on, however, it was confirmed by a fellow student that these formulations failed in tensile strength (Shriya Pancholi, Chemical Engineering, Birmingham, Personal communication).

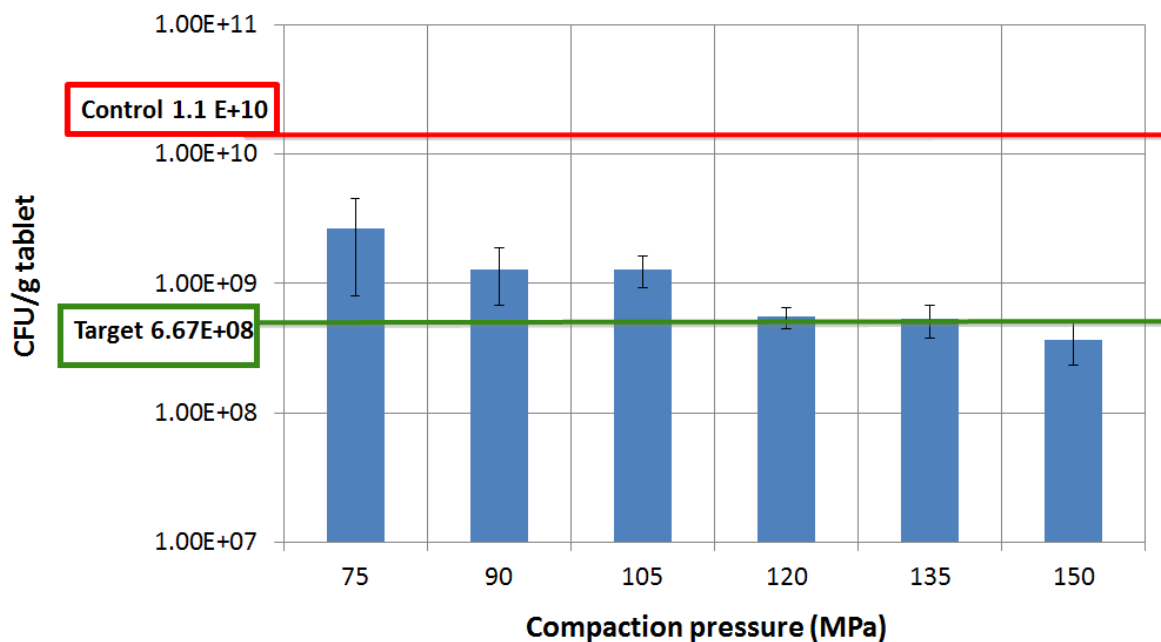


Figure 4-14 CFU/g tablet of tablets containing ADY/MCC in 50/50 (wt/wt) ratio and compacted at different compaction pressure. Error bars represent the 95% confidence limit from three measurements ( $n=3$ ). The green line demonstrates target CFU/g tablet (Lesaffre's specification) whereas the red line represents CFU/g ADY before compaction.

#### 4.2.3.5 Tablet dissolution time and the corresponding cell viability

The dissolution time of the produced tablets and the corresponding cell viability were studied by dissolving a tablet in PBS using a USP apparatus and the samples were collected every 20 min before being stained with PI and BOX. It was observed that a tablet started to disintegrate immediately after it was placed in a liquid medium. The results in Figure 4-15 show that all active ingredients from the tablet were released between 20 and 40 min. This did not comply within a limit of disintegration time of uncoated tablet (15 min) according to the B.P. (1998). The total numbers of cells were constant after 40 min of dissolution time.

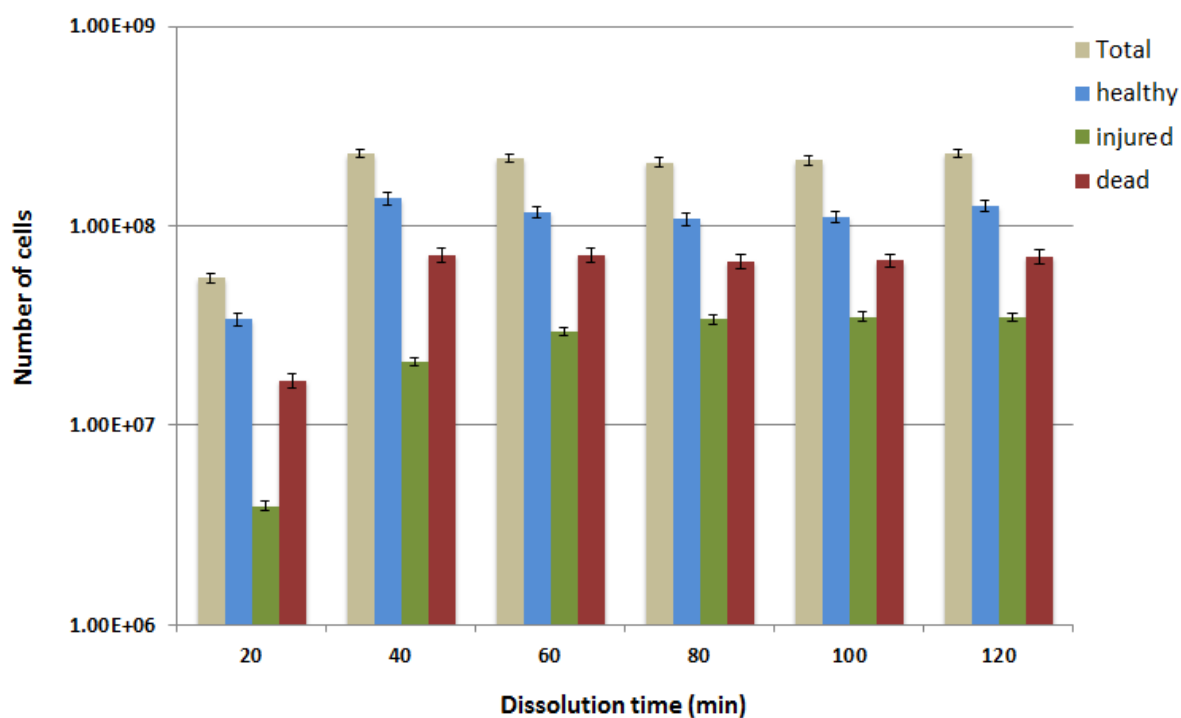


Figure 4-15 Number of total, healthy, injured and dead cells of a tablet containing MY/MCC in 50/50 (wt/wt) ratio and compacted at 105 MPa during dissolution studies by dissolving in PBS. Data are obtained from triplicate studies (n=3) with standard errors

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#### 4.2.4 Correlation between CFU and FC data

The number of healthy, injured and viable cells obtained from FC under different conditions (three yeast samples, ADY suspensions homogenised with varying speed and ADY suspensions exposed to a fluid of different pH) and the number of culturable cells obtained from CPC (see section 3.2.3.2) were compared. The numbers of healthy and injured cells are those which do not uptake PI by intracellular constituents, and their sum is defined as the total number of viable cells. In Figure 4-16 to 4-18, CFU/g (Table 4-2) is presented as the dependent variable on the ordinate and the number of injured, healthy or viable cells (Table 4-2) as the independent variable on the abscissa. Best fit straight lines and the corresponding linear equations were generated by Excel software.

It can be seen that CFU/g and the number of healthy cells had a strong linear correlation with an  $R^2$  of 0.76 where a poor correlation between CFU/g and the number of injured cells with an  $R^2$  of 0.39 was displayed. However, as shown in Figure 4-18, a correlation coefficient ( $R^2$ ) of 0.95 was found between CFU/g and the number of viable cells, indicating an excellent agreement. In addition, the slope of the linear equation in Figure 4-18 was 0.95 which suggests that most viable cells were culturable.

Table 4-2 The number of viable cells and CFU/g under different processing and environmental conditions (mean  $\pm$  CI 95%, n=3)

Condition		Healthy (H)	Injured (I)	Viable (V)	CFU/g
Three yeast samples	WY	$8.7 \times 10^{10} \pm 0.2 \times 10^{10}$	$8.1 \times 10^9 \pm 0.3 \times 10^9$	$9.5 \times 10^{10} \pm 0.2 \times 10^{10}$	$6 \times 10^{10} \pm 1 \times 10^{10}$
	ADY	$1.4 \times 10^{10} \pm 0.3 \times 10^{10}$	$6 \times 10^9 \pm 1 \times 10^9$	$2.0 \times 10^{10} \pm 0.3 \times 10^{10}$	$1.3 \times 10^{10} \pm 0.4 \times 10^{10}$
	MY	$3 \times 10^9 \pm 1 \times 10^9$	$2 \times 10^9 \pm 1 \times 10^9$	$5.6 \times 10^9 \pm 0.3 \times 10^9$	$2 \times 10^9 \pm 1 \times 10^9$
ADY suspensions with varying homogenisation speed (rpm)	6000	$6 \times 10^9 \pm 1 \times 10^9$	$3 \times 10^9 \pm 1 \times 10^9$	$9 \times 10^9 \pm 1 \times 10^9$	$5 \times 10^9 \pm 1 \times 10^9$
	8000	$1.1 \times 10^{10} \pm 0.3 \times 10^9$	$2.7 \times 10^9 \pm 0.4 \times 10^9$	$1.3 \times 10^{10} \pm 0.1 \times 10^{10}$	$1.1 \times 10^{10} \pm 0.1 \times 10^{10}$
	10000	$1 \times 10^{10} \pm 1 \times 10^{10}$	$2 \times 10^9 \pm 1 \times 10^9$	$1 \times 10^{10} \pm 1 \times 10^{10}$	$1.3 \times 10^{10} \pm 0.3 \times 10^{10}$
	12000	$5.9 \times 10^9 \pm 0.1 \times 10^9$	$3.9 \times 10^9 \pm 0.3 \times 10^9$	$9.8 \times 10^9 \pm 0.4 \times 10^9$	$1 \times 10^{10} \pm 1 \times 10^9$
	15000	$7 \times 10^9 \pm 1 \times 10^9$	$1.9 \times 10^9 \pm 0.2 \times 10^9$	$9.3 \times 10^9 \pm 0.3 \times 10^9$	$5 \times 10^{10} \pm 1 \times 10^9$
ADY suspensions exposed to a fluid of different pH	pH 7	$10.0 \times 10^8 \pm 0.2 \times 10^8$	$4 \times 10^9 \pm 1 \times 10^9$	$4.9 \times 10^9 \pm 0.2 \times 10^9$	$3 \times 10^9 \pm 1 \times 10^9$
	pH 4	$4.8 \times 10^8 \pm 0.1 \times 10^8$	$4 \times 10^9 \pm 1 \times 10^9$	$4.7 \times 10^9 \pm 0.3 \times 10^9$	$3 \times 10^9 \pm 1 \times 10^9$
	pH 3	$2.9 \times 10^8 \pm 0.3 \times 10^8$	$2.4 \times 10^9 \pm 0.4 \times 10^9$	$2.7 \times 10^9 \pm 0.1 \times 10^9$	$3 \times 10^9 \pm 1 \times 10^9$
	pH 2	$8 \times 10^7 \pm 1 \times 10^7$	$7 \times 10^9 \pm 1 \times 10^9$	$7 \times 10^9 \pm 1 \times 10^9$	$4 \times 10^9 \pm 2 \times 10^9$
	pH 1.2	$3 \times 10^6 \pm 1 \times 10^6$	$8.8 \times 10^8 \pm 0.3 \times 10^8$	$9 \times 10^8 \pm 1 \times 10^8$	$7 \times 10^8 \pm 1 \times 10^8$

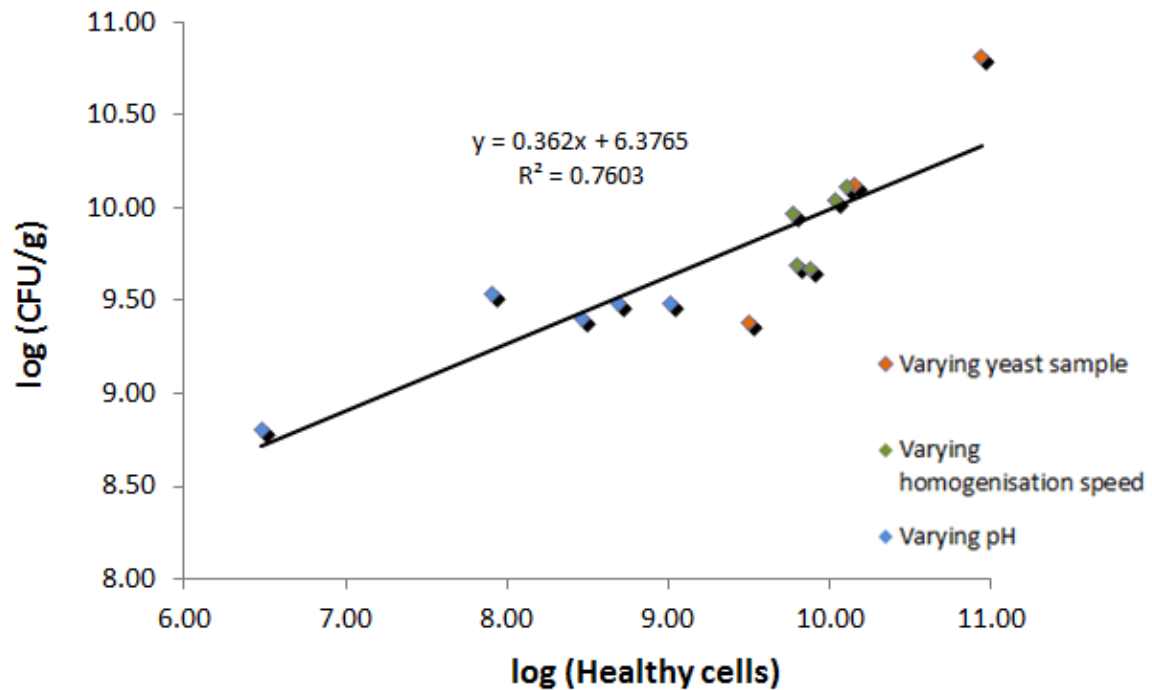


Figure 4-16 Correlation between CFU/g and number of healthy cells of yeast under three different conditions

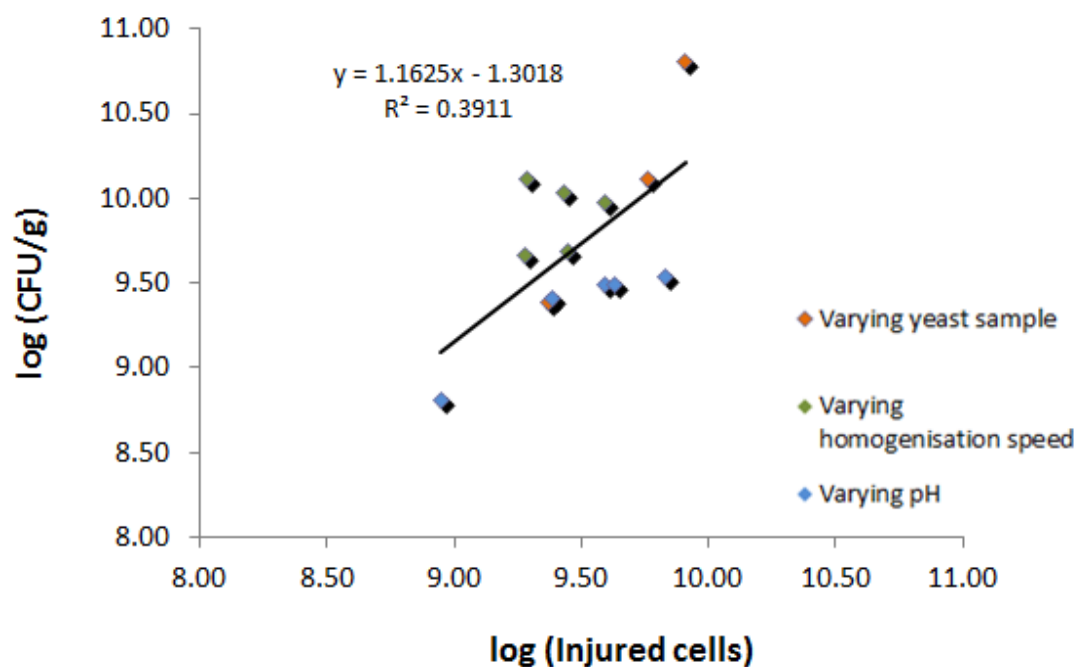


Figure 4-17 Correlation between CFU/g and number of injured cells of yeast under three different conditions

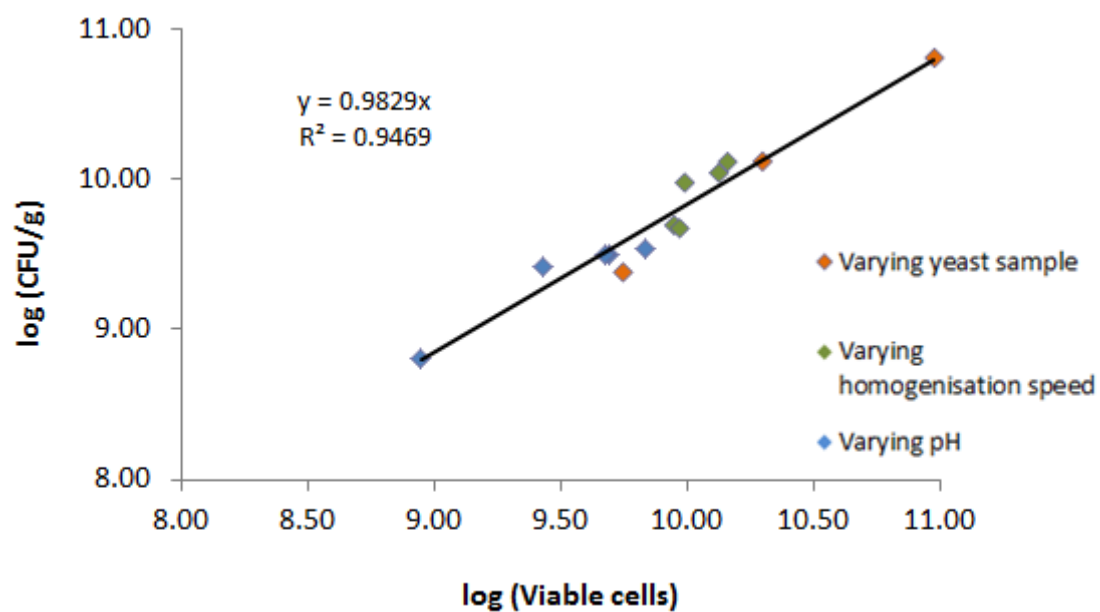


Figure 4-18 Correlation between CFU/g and number of viable (healthy plus injured) cells of yeast under three different conditions



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### **4.3 CONCLUSIONS**

This study has demonstrated the effects of processing and environmental conditions on yeast cell viability by using flow cytometry in conjunction with fluorescent dyes which has enabled an in-depth understanding of physiological state of individual cells. Cell viability of yeast cells in terms of culturability was determined using a traditional method of colony plate counting (CPC) and compared to flow cytometric data. Correlations between CFU/g from CPC and the number of healthy, injured and viable cells obtained from flow cytometry (in section 4.2.4) were sought. A strong correlation between CFU/g and the total number of viable cells determined by FC has been found ( $R^2 = 0.95$ ) and this suggests that most viable cells were culturable. Therefore, the correlation might be useful to estimate cell culturability from total viable cell number determined by FC for other processing and environmental conditions.

High homogenisation speeds enhanced the release of yeast cells from ADY and MY granules (as explained in section 4.2.3.1). Moreover, homogenisation speed had an influence on cell viability of ADY where a speed of 10000 rpm produced the highest CFU/g. At a homogenisation speed of 15000 rpm, it is speculated that yeast cells became injured by the generated shear force and had lower culturability (lower CFU/g). WY obtained from fermentation contained a very high number of viable cells while drying process, milling process (section 4.2.3.2) and tableting of yeast with MCC using high compaction pressure (section 4.2.3.4) had negative effects on cell viability. It was also found that cell viability reduced when yeast was exposed to a simulated gastric fluid of pH 1 (section 4.2.3.3) which might be due to cell shrinkage in highly acidic and high osmotic pressure fluid. In the

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dissolution test, all the cells were fully released from the tablets containing ADY and MCC which disintegrated within 40 min after being exposed to the liquid medium (section 4.2.3.5).

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## CHAPTER 5

### **FREEZE DRYING OF WET YEAST CELLS AND COMPACTION OF THEM INTO TABLET DOSAGE FORM**

#### **5.1 INTRODUCTION**

In addition to processing and environmental conditions (in the GI tract), storage conditions can also have negative effects on probiotic microorganism survival. Using drying technology has been the preferred method for the preservation of microorganisms in many industrial applications enabling long term storage in bulk quantities. Freeze drying or lyophilisation (Costa et al., 2000, Miyamoto-Shinohara et al., 2000a) is one of the drying technologies used for the preservation of microorganisms. It works by freezing them and creating a vacuum to remove water by sublimation. Miyamoto-Shinohara et al. (2000a) have reported that yeast *Saccharomyces cerevisiae* cell viability only dropped by 10% after freeze drying and no further loss of viability occurred over the subsequent 10 years storage. It has been suggested that a high initial cell concentration should be used for freeze drying in order to maintain a relatively high number of viable cells afterwards (Bozoglu et al., 1987). In this study, therefore, wet yeast was used since it contains a higher number of viable cells per gram than active dry yeast (ADY) or milled yeast (MY) since the latter contains other ingredients. Although freeze drying is the preferred method for preserving microorganisms, during the freezing process large ice crystals can form leading to cell membrane and cell wall damage, and therefore a reduction in cell viability. It has been found that cryoprotectants in the

medium can reduce the damage, for example, Costa et al. (2000) have found that when non-fat skimmed milk was used as a protective medium when freeze drying, a higher cell recovery was achieved. Carvalho et al. (2004) reported that the survival of the freeze dried cells depended on both the protective medium and the strain of microorganism.

In the research described in this chapter, wet yeast was dehydrated using a freeze dryer with and without a protective medium -skimmed milk. The effect of drying time on the loss of viable cells and the resulting water activity of cell powders was determined. Freeze dried yeast (FDY, subjected to an optimal freeze drying time) was mixed with MCC in different ratios before being compacted into tablets (target  $\geq 6.67 \times 10^8$  CFU per 1 g tablet or,  $2 \times 10^9$  CFU/day and maximum 3 tablets per day, specified by Lesaffre, France) and the effects of formulation and compaction pressure on the tensile strength of the formed tablet and cell survival were evaluated. MCC was chosen to make tablets because tablets containing MCC had the higher tensile strength compared with those containing starch or HMPC (Wu et al., 2005). Moreover, the size distribution of MCC and FDY particles and the internal structure of the formed tablets were studied. The viabilities of cells in tablets containing FDY and MCC with different ratios were assessed by flow cytometry (FC) and colony plate counting (CPC) methods (Postgate, 1969). Two dual stains of PI and BOX (Hewitt and Nebe-Von-Caron, 2001) and SYTO9 and PI (Tanja et al., 2013, Zhang and Fang, 2004) were used together with FC to determine cell physiological states.

To examine their storage stability, cells in tablets made using a particular formulation 50/50 (wt/wt) were placed in a plastic bag and stored at 4 °C for 3 and 6 months. The storage time was partially limited by the duration of the study but the data collected may still be useful for indicating the storage stability of yeast in tablet to a certain extent. CFU and FC data of

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tablets containing FDY before and after storage were evaluated against the existing correlation established in section 4.2.4.

## **5.2 RESULTS AND DISCUSSION**

### **5.2.1 Particle size distribution**

Freeze dried yeast (FDY) (see section 3.10.1) was milled using a food processor (Kenwood, FP260 series, UK) for 3 min. The particle size distribution of FDY was determined by QICPIC (as described in section 3.10.2) and the result is shown in Figure 5-1. The number of FDY particles observed in three successive measurements ranged between 80,000 and 150,000 particles. The result shows a wide distribution with a mean size of  $0.85 \pm 0.1$  mm. The maximum particle size was 2.55 mm but it was thought that a longer milling time to cause further reduction in particle sizes could also cause some damage to cells.

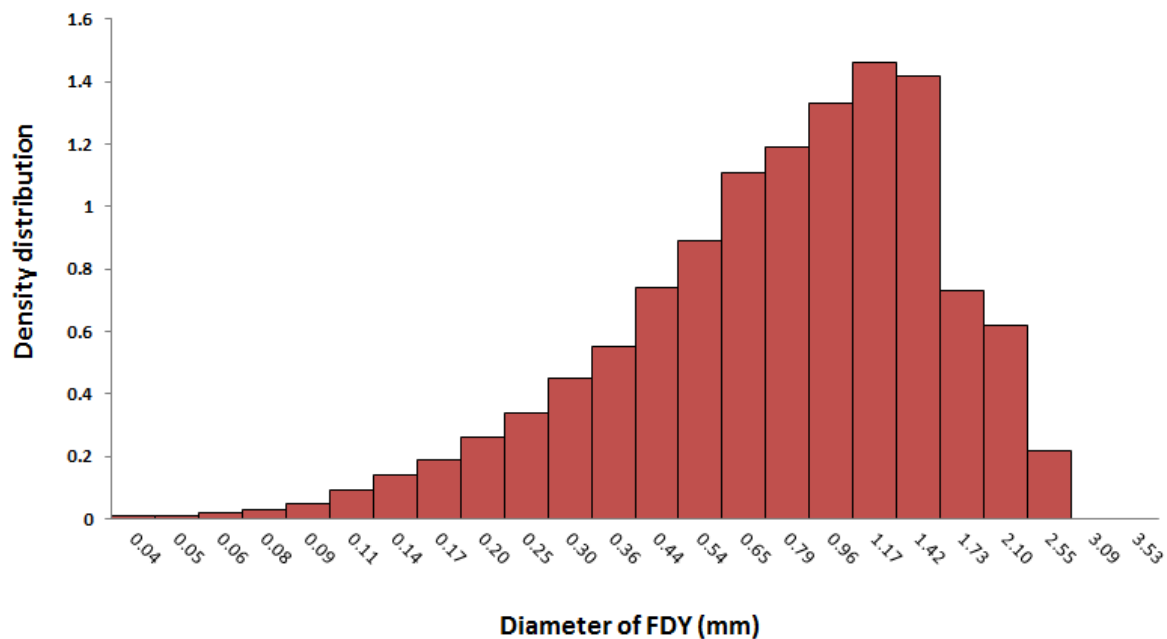


Figure 5-1 Particle size distribution measured by QICPIC of FDY after freeze drying at -50°C and 0.6-0.7 mbar for 24 h and milling for 3 min.

Particle size of MCC was also determined by QICPIC (as described in section 3.10.2) and the data are shown in Figure 5-2: MCC particles showed a moderately wide distribution with an average particle size of  $0.19 \pm 0.01$  mm which are approximately 5 times smaller than FDY particles. Three successive measurements were taken, as with FDY particles, and the data were generated based on 200,000 to 250,000 particles.

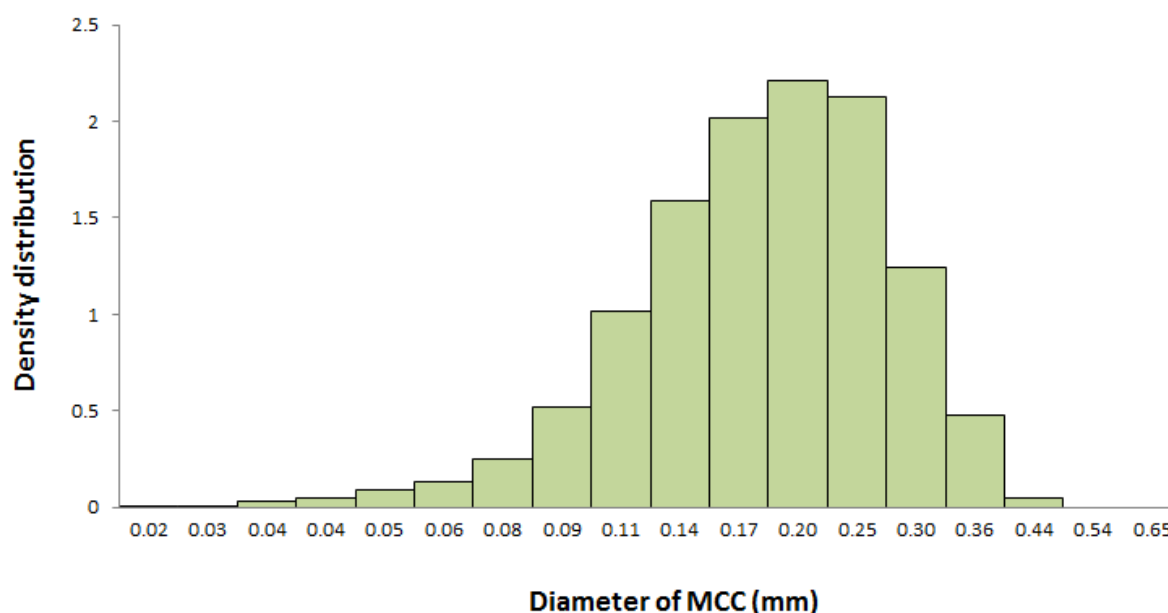


Figure 5-2 Particle size distribution of MCC measured by an image analyser (QICPIC).

### 5.2.2 Effect of freeze drying time on cell survival and water activity ( $a_w$ ) of remaining powders

To determine the effect of freeze drying on the water activity of remaining cell powders, which can affect cell long term stability during storage, the freeze drying time (at  $-50^{\circ}\text{C}$  and 0.6-0.7 mbar) was varied between 12 h and 48 h. Cell viabilities every 12 h were then measured by CPC (Figure 5-3) and FC (Figure 5-4).

WY containing  $6.5 \times 10^{10} \pm 1.1 \times 10^{10}$  CFU/g initially was used in this experiment. At 12 h of freeze drying time, CFU/g decreased to  $1.2 \times 10^{10} \pm 1.4 \times 10^9$  CFU/g FDY corresponding to 0.73 log loss and had  $a_w$  of  $0.64 \pm 0.02$ . It can clearly be seen that cell viability decreased with freeze drying time where 0.90, 1.02 and 1.29 log loss were observed at 24, 36 and 48 h of freeze drying time respectively. This indicates that a longer freeze drying time had a

deleterious effect on CFU/g. However, the  $a_w$  values at 36 and 48 h were constant at  $0.38 \pm 0.02$  but the extra drying caused further reduction in CFU/g FDY. Added to this,  $a_w$  of FDY obtained at a 24 h freeze drying time was  $0.43 \pm 0.02$  which was still higher than the value of 0.3 desired for long term storage stability (Dr Peter Jüsten, Lesaffre, France, Personal communication).

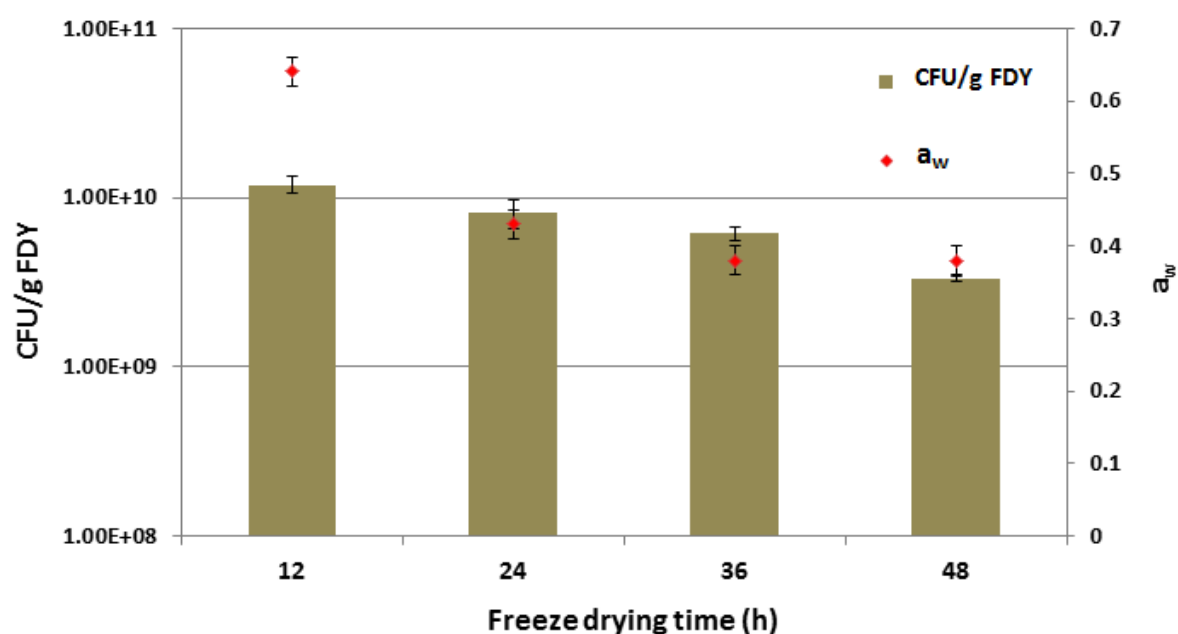


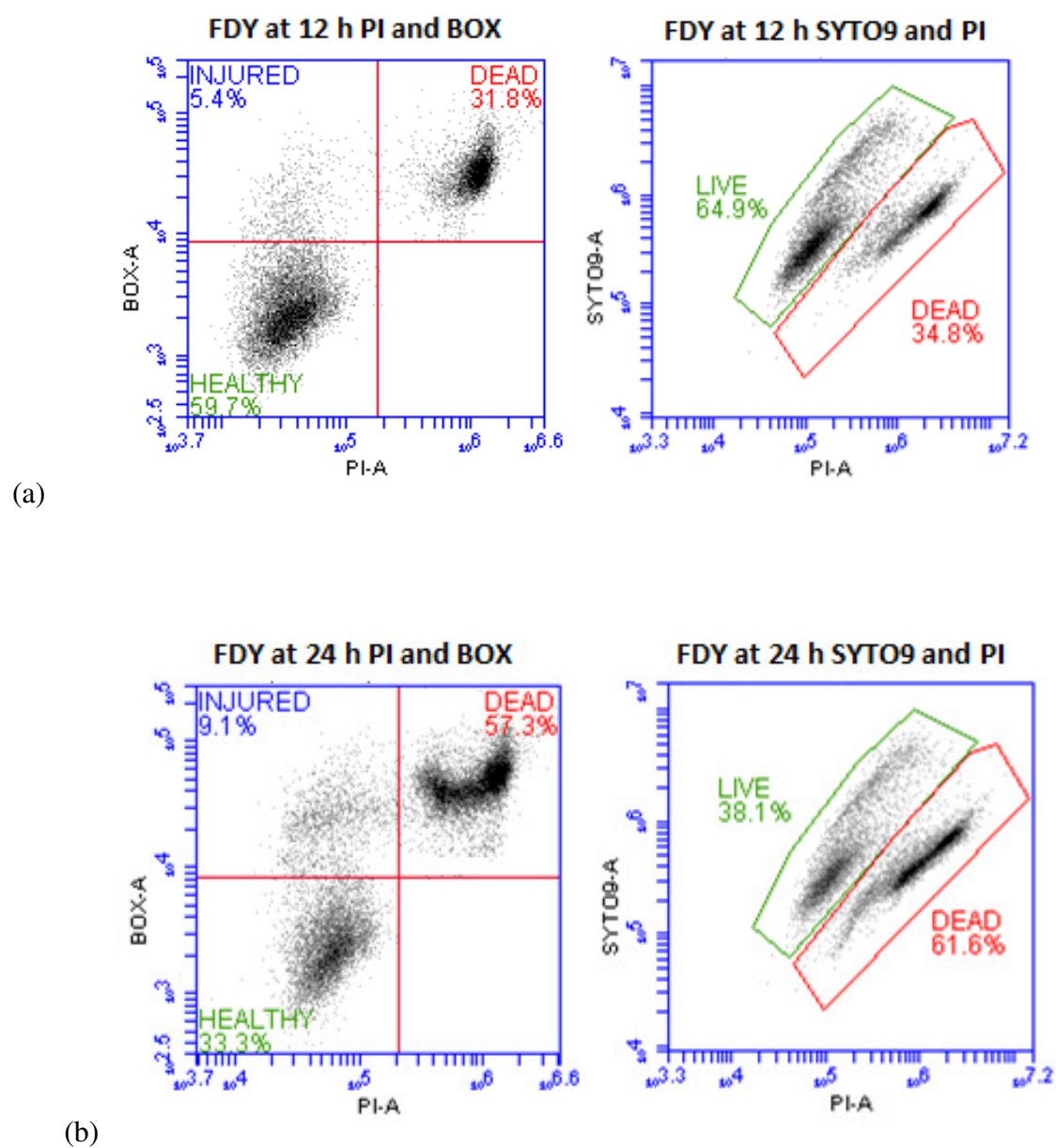
Figure 5-3 CFU/g FDY as measured by CPC and water activity ( $a_w$ ) at different freeze drying times (n=3)

Cell viability at different freeze drying times was further investigated by FC. Flow cytometric dot plots can be seen in Figure 5-4 and show various states of FDY suspensions with dual stains of BOX and PI and SYTO9 and PI at various freeze drying times. Clear discrimination of healthy, injured and dead cells was achieved by monitoring the extents to which yeast cells accumulated PI and BOX. The number of healthy cells (PI<sup>-</sup> and BOX<sup>-</sup>) showed a subtle fluctuation during exposure to freeze drying.



FC using SYTO9 and PI reagents (LIVE/DEAD *FungaLight*) cannot distinguish the overall health of cells, i.e., identification of the three physiological states – healthy, injured and dead – as PI and BOX staining can (Bunthof and Abee, 2002). In this study, the results of two dual stains were consistent in that the highest number of live cells and lowest number of dead cells were discovered at 12 h of freeze drying time. The number of dead cells detected in SYTO9 and PI plots appeared higher than those in PI and BOX plots. This is because the relative concentration of PI used in *FungaLight* (20 mM) was considerably higher than the one in PI and BOX staining ( $200 \mu\text{g mL}^{-1}$ ) (Soejima et al., 2009).

The numbers of healthy, injured and dead cells determined by FC employing fluorescent staining with PI and BOX are presented in Figure 5-5. A decreasing trend of healthy cells in conjunction with an increase of injured and dead cells with freeze drying time was observed. A clear reduction in the number of viable cells (corresponding to healthy and injured cells together) indicated loss of culturability of cells, which is consistent with a declining trend of CFU/g FDY. Viability reduction during freeze drying can likely be attributed to membrane permeabilisation resulting in an increase of dead cells which could be caused by dehydration during drying (Rault et al., 2007, Meng et al., 2008, Fu and Chen, 2011). Injured yeast cells with membrane potential loss might be manifested by an inability of such cells to be culturable i.e. able to form colonies on the solid medium. However, the cells may regain normal function under appropriate conditions e.g. adequate nutrients (Mazur, 1965, Mazur, 1966, Novo et al., 2000). Taking  $a_w$  and cell viability determined by CPC and FC into account, FDY obtained after 24 h freeze drying was selected for further experiments.



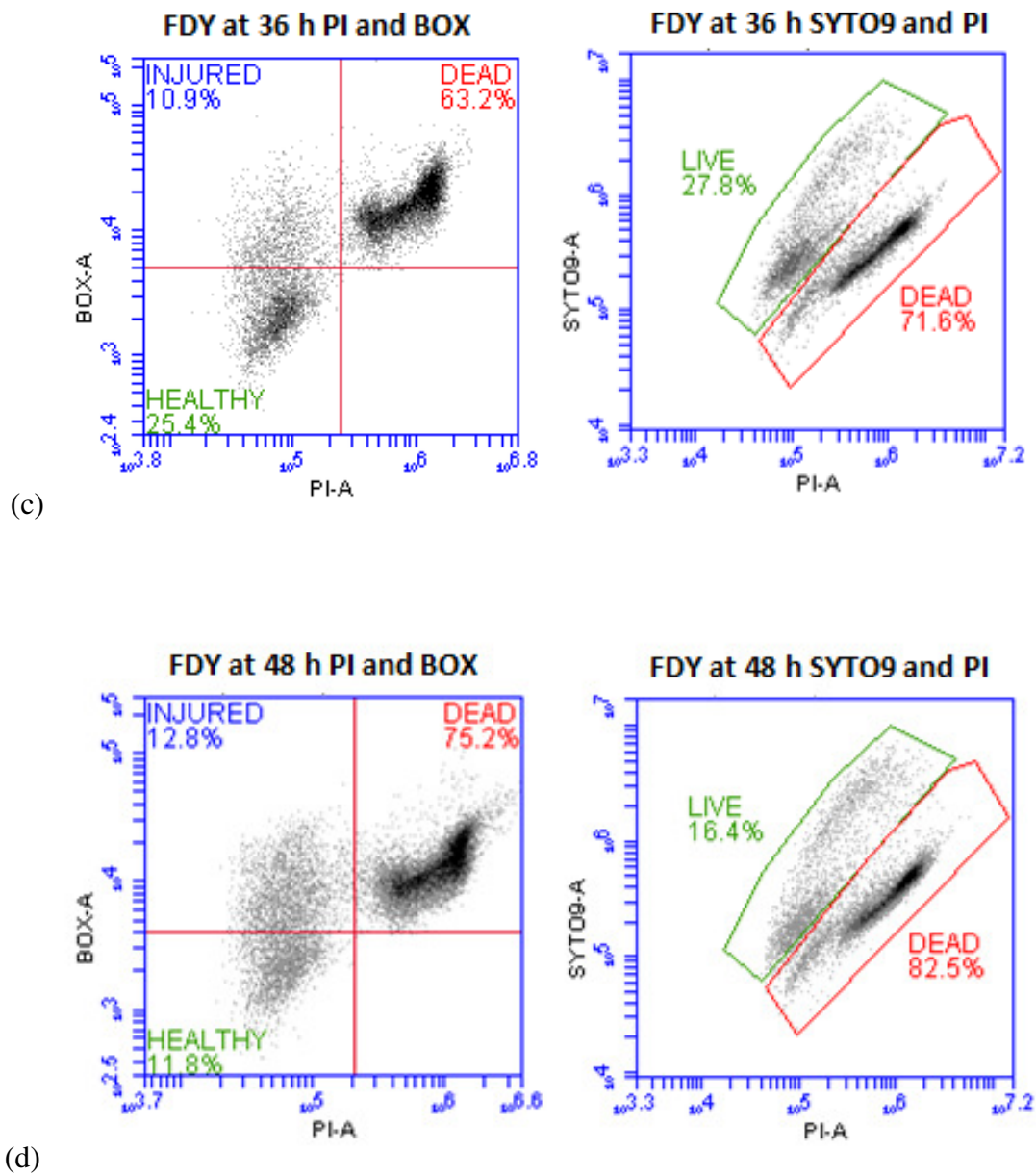


Figure 5-4 Flow cytometric density plots showing the percentage of healthy, injured and dead cells after staining with PI and BOX (left) and SYTO9 and PI (right) at various freeze drying times: (a) 12 h (b) 24 h (c) 36 h and (d) 48 h.

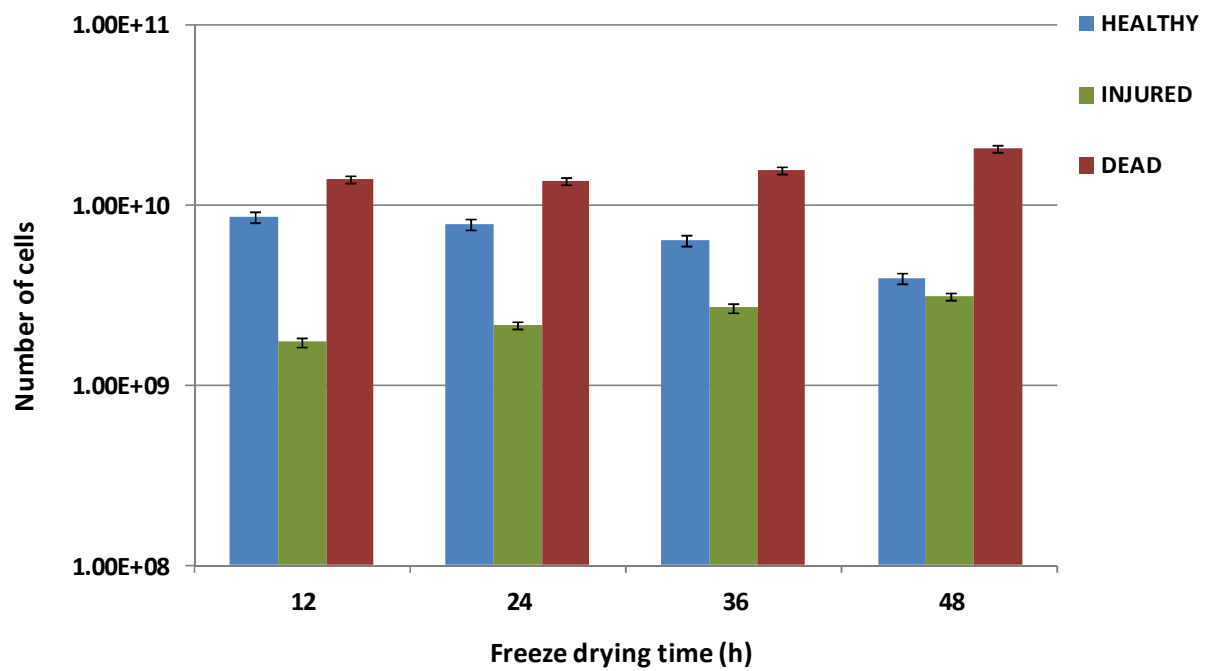


Figure 5-5 Number of healthy, injured and dead cells of freeze dried yeast (FDY) at various freeze drying times after being stained with PI and BOX and sorted by FC. Data shown are based on 1 g of FDY

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### 5.2.3 Freeze dried yeast with protective agent

Many papers have reported that the addition of cryoprotectant to microorganisms before freeze drying enhanced cell viability by protecting cell membranes and maintaining protein stability (Benaroudj et al., 2001, Leslie et al., 1995, Zhao and Zhang, 2005). It was found that *Lactococcus lactis* had more than a 60% survival rate after adding skimmed milk and sucrose into microorganisms before freeze drying (Berner and Viernstein, 2006). In addition to this, freeze drying *Candida sake* at -20°C was better than using liquid nitrogen since in the latter case the water was removed too fast and resulted in cell damage (Abadias et al., 2001a). In this work, addition of skimmed milk on protection of yeast during freeze drying was investigated.

In the experiments described in this section, 10% (w/v) of skimmed milk without any sugar was added to WY prior to freeze drying (see method section 3.10.1) (Blanquet et al., 2005). The flow cytometric plots of various physiological states of FDY with and without the addition of skimmed milk are shown in Figure 5-6. The addition of 10% skimmed milk suspended in WY gave a 5% increase in viability as observed in SYTO9 and PI plots and exhibited a 2% cell viability increase in PI and BOX plots.

During freeze drying, yeast cells are faced with increasing osmotic stress as the water activity begins to decrease (Morgan et al., 2006). A cryoprotectant is a substance that aids the protection of microorganisms from cell damage during the freeze drying process and helps maintain viability by reducing the osmotic pressure between the cells and the external environment (Capela et al., 2006, Kets et al., 1996), stabilizing the proteins and cell membrane during osmotic stress conditions (Morgan et al., 2006). Survival rates of *S.*

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*cerevisiae* after freeze drying were variable due to differences of yeast strain and drying conditions such as volume of suspension and initial cell concentration (Blanquet et al., 2005). Results in this study are in accordance with those of Blanquet et al. (2005) who reported the cell survival rate was improved by only 1% after 5% w/v of milk protein was added to yeast suspension prior to freeze drying, whereas higher survival rates of 13% were obtained when a mixture of milk protein and trehalose were added. Similar protective effects on cell viability were observed with another strain of yeast: *Candida sake* (Abadias et al., 2001a) showed an increase of 29% survival rate when trehalose and skimmed milk were used. Many reports claimed the addition of protective agents such as a mixture of sugar and skimmed milk was found to improve cell viability of lactic acid bacteria and yeast (Berner and Viernstein, 2006, Berny and Hennebert, 1991, Capela et al., 2006, Hsi-Chia et al., 2006). In this section, skimmed milk was added without the addition of carbohydrate (hence sugar was not available to form hydrogen bonds with the protein from denaturation when water was removed) so the influence on yeast viability was very marginal. Previous research found that cryoprotectant only helped some strains of probiotic to survive during freeze drying (Rybka and Kailasapathy, 1995) by acting on cell membrane integrity or protein structure (Carvalho et al., 2004). Subsequent experiments were therefore carried out without the addition of skimmed milk to reduce process complexity.

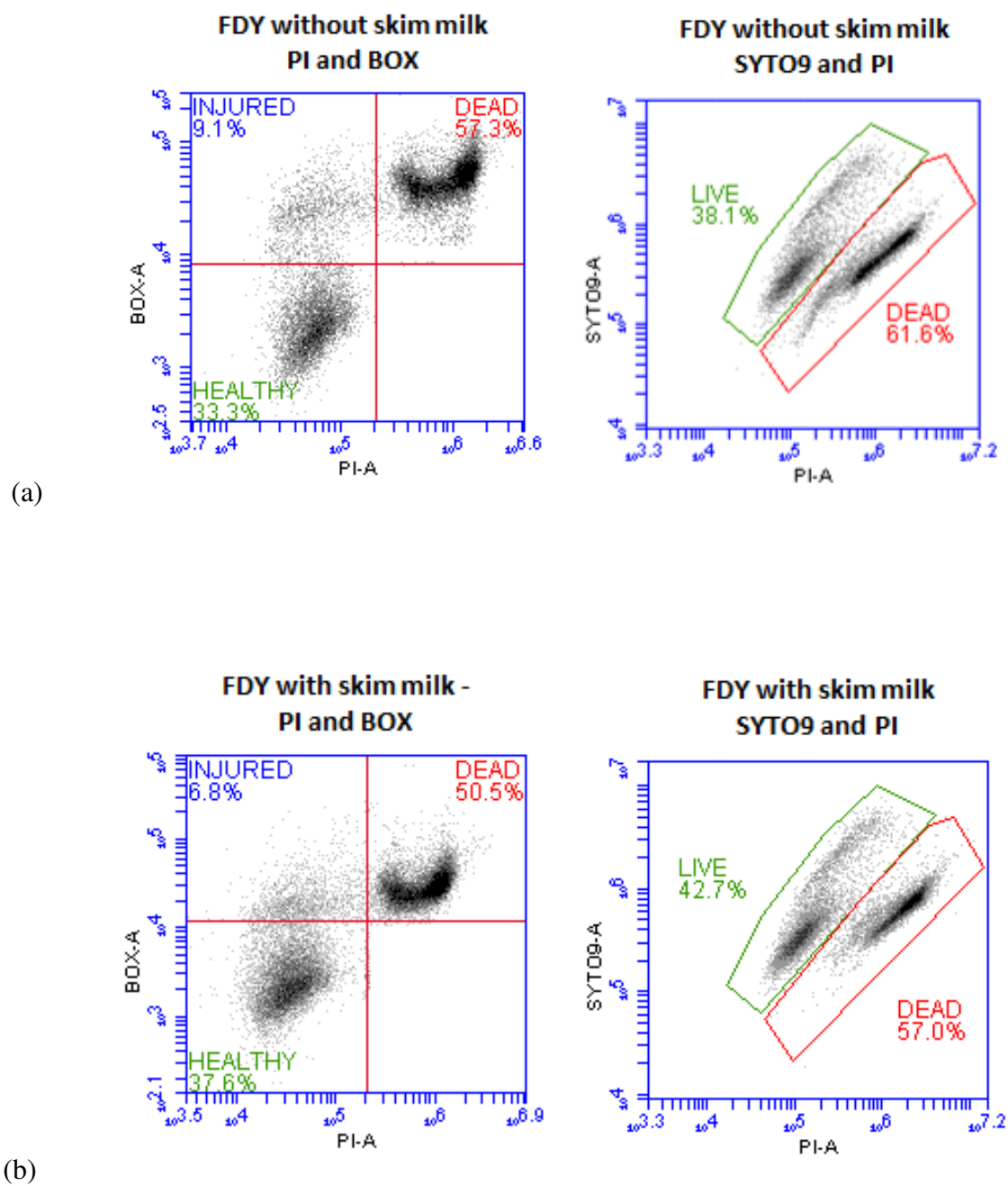


Figure 5-6 Flow cytometric plots showing various physiological states of (a) FDY without skimmed milk and (b) FDY with the addition of 10% skimmed milk, after being stained with PI and BOX (left) and SYTO9 and PI (right).

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## 5.2.4 COMPACTION OF FREEZE DRIED YEAST

### 5.2.4.1 *Tablet formation containing freeze dried yeast*

A preliminary experiment showed that FDY could not be compressed into rigid tablets, which can withstand handling, by direct compression with a pressure up to 105 MPa (data not shown). Therefore, FDY without the addition of skimmed milk and freeze dried for 24 h was mixed with MCC in ratios of 90/10 and 80/20 (wt/wt) (see Table 3-3) before compaction into tablets. The results of tablets made with FDY and MCC in 90/10 (wt/wt) ratio and compacted at 105 MPa are shown in Figure 5-7. These tablets were of cylindrical shape with a diameter of 13 mm and thickness of  $7.2 \pm 0.1$  mm. A similar thickness of tablet made with FDY and MCC in 80/20 (wt/wt) ratio at  $7.2 \pm 0.2$  mm was observed at the same compaction pressure. High thickness of the tablet could be caused by poor compressibility of FDY particles. In addition to this, due to the difference in FDY and MCC particle sizes, a clear segregation of MCC and FDY was observed on different sides of the tablet. This is thought to affect the mechanical strength of tablet, which was investigated further in the work described in section 5.2.4.3.



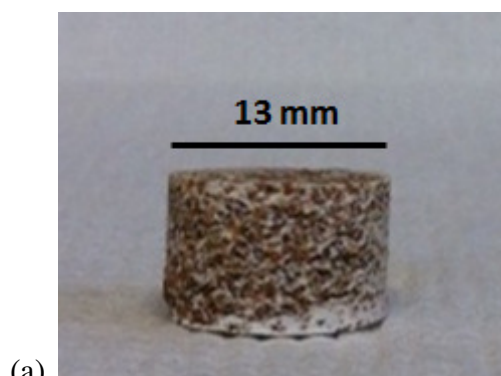
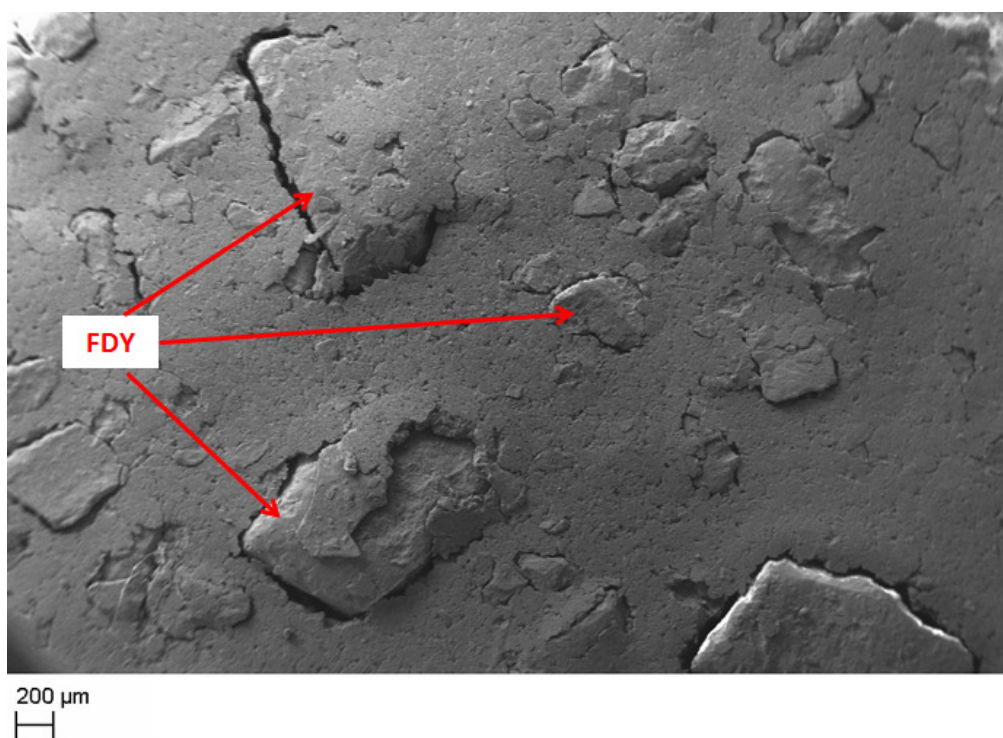


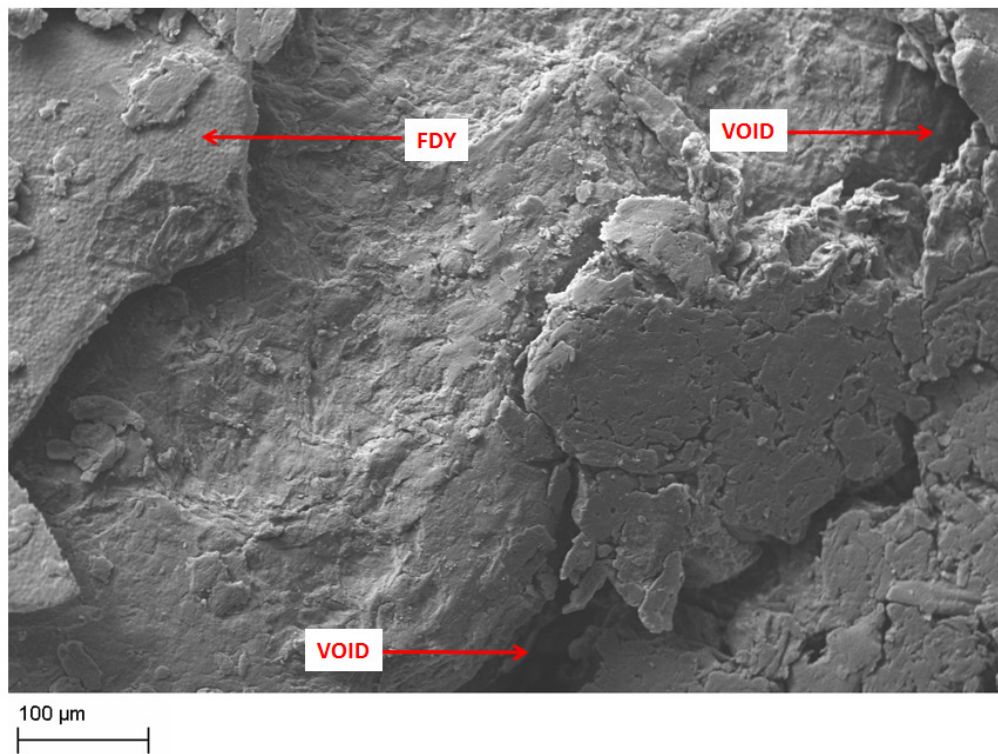
Figure 5-7 A tablet containing FDY and MCC in 90/10 (wt/wt) ratio after compaction at 105 MPa (a) side view (b) top view

### 5.2.4.2 SEM analysis

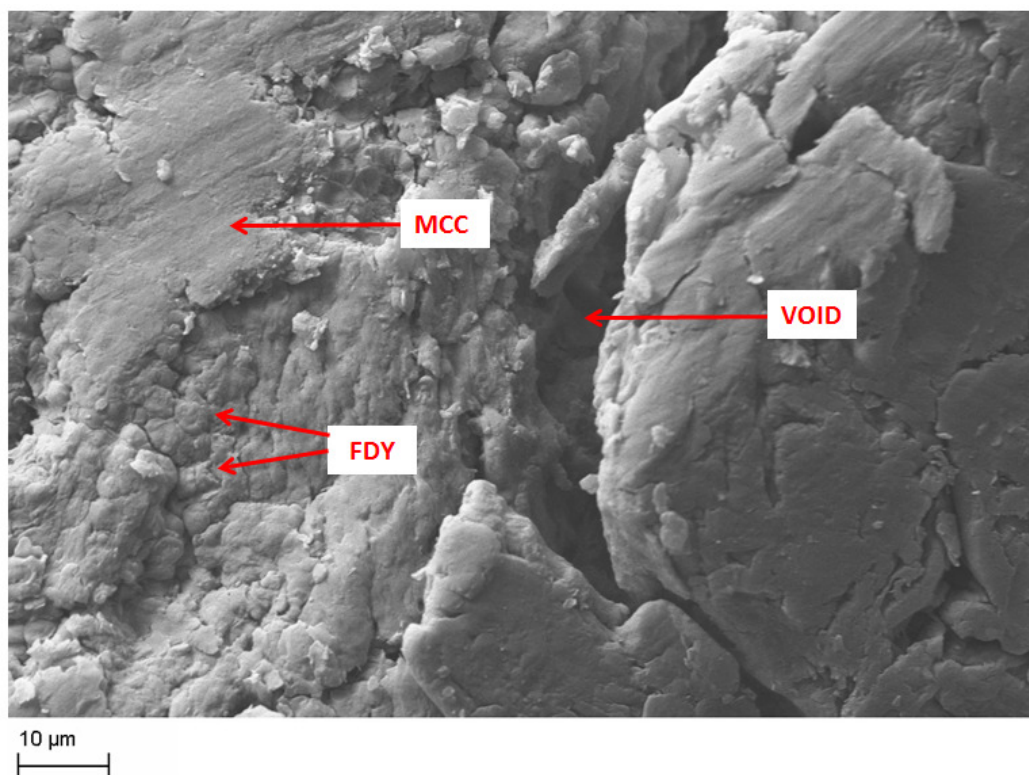
In order to see the arrangement of FDY and MCC particles, the tablet was cut across its cross-sectional area and a SEM analysis (see section 3.10.7) was carried out as illustrated in Figure 5-8. During tablet compaction, there was a deformation mechanism of powder excipients which resulted in the rearrangement of a tablet. SEM images highlight a difference in particle sizes between FDY and MCC, which might cause poor tablet compressibility. In addition, void spaces and high porosity of the tablet may adversely affect tensile strength (Wu et al., 2005) which will be explained more in the next section.



(a)



(b)



(c)

Figure 5-8 SEM images of a tablet containing FDY and MCC in 90/10 ratio compacted at 90 MPa showing some void spaces between FDY and MCC at different scales (a) 200 μm (b) 100 μm and (c) 10 μm

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#### ***5.2.4.3 Cell viability and tablet tensile strength at different compaction pressure***

Cell viability in tablets containing FDY and MCC in 90/10 and 80/20 (wt/wt) ratios was studied and the results are shown in Figure 5-9. It was found that a tablet compressed at higher compaction pressure was stronger than one compressed at lower pressure but resulted in lower cell viability. An increase in the amount of filler (MCC) by 0.1 g exerted a slightly positive effect on the tablet strength as MCC demonstrated its properties of high compressibility and plasticity. No significant change in cell viability between 90/10 and 80/20 formulations at the same compaction pressure was observed. This finding is not consistent with Klayraung et al. (2009) who claimed cells are better protected from stress factors when a tablet contains higher cell loading due to cell-cell interactions. Interestingly, cell viability of a formulation (80/20) compacted at 90 MPa was higher than that of a formulation (90/10) compacted at 105 MPa although the former contained less FDY. From this it can be concluded compaction pressure might have a more significant impact on cell survival compared to the amount of active ingredient.

Tensile strengths of the tablets containing FDY and MCC in 90/10 and 80/20 (wt/wt) ratios were also determined (Figure 5-9). It was found that tablets containing FDY/MCC in 90/10 ratio exhibited tensile strength of  $0.11 \pm 0.01$  MPa and  $0.24 \pm 0.02$  MPa when compacted at 90 and 105 MPa respectively. A similar increase of tensile strength from  $0.22 \pm 0.03$  MPa to  $0.29 \pm 0.01$  MPa was obtained from a formulation (80/20) when 0.1 g of MCC content was added. The results showed that tensile strength increased when compaction pressure and the amount of filler increased. Nevertheless, tensile strengths of these formulations were significantly less than 1 MPa (Lesaffre's specification).

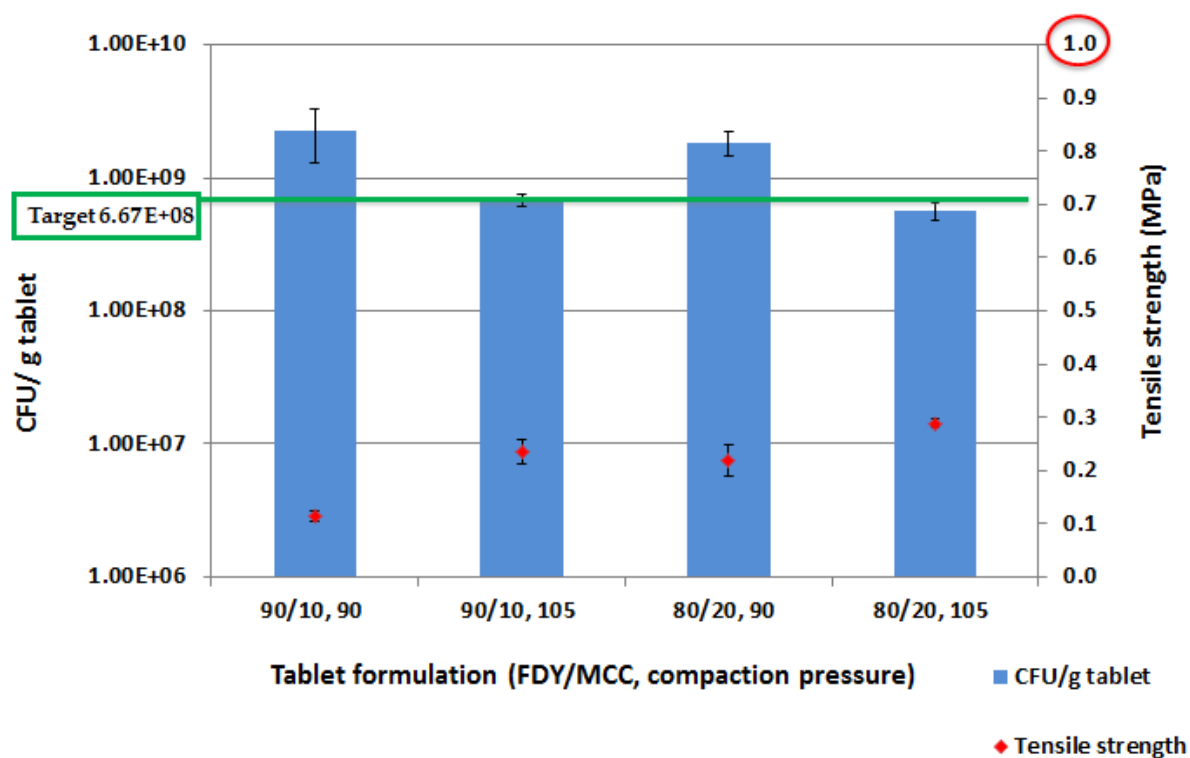


Figure 5-9 CFU/g tablet versus tensile strength of tablets containing FDY and MCC in 90/10 and 80/20 (wt/wt) ratio after compacted at 90 and 105 MPa. The green line demonstrates the target CFU/g tablet (Lesaffre). The red circle represents the target tablet tensile strength (Lesaffre)



#### ***5.2.4.4 Survival of cells in tablets made of different amounts of cells and excipient and their tensile strength***

The tablet formulations containing FDY and MCC in a ratio of 90/10 and 80/20 shown in the previous section appeared to be thick and brittle. It has been reported that increasing filler concentration can decrease porosity and pore size in a tablet, leading to higher tablet strength (Chan et al., 2011). Accordingly, decreasing the amount of FDY and increasing the amount of MCC was attempted in this study. The new formulation of FDY and MCC in 50/50 ratio was produced at 90 MPa, and typical tablets are shown in Figure 5-10. The mean thickness of this formulation based on measurements from three tablets was  $6.98 \pm 0.02$  mm which was slightly less compared to those made in 90/10 and 80/20 (wt/wt) ratio. This might be because a 50/50 ratio tablet had better tablet compressibility.

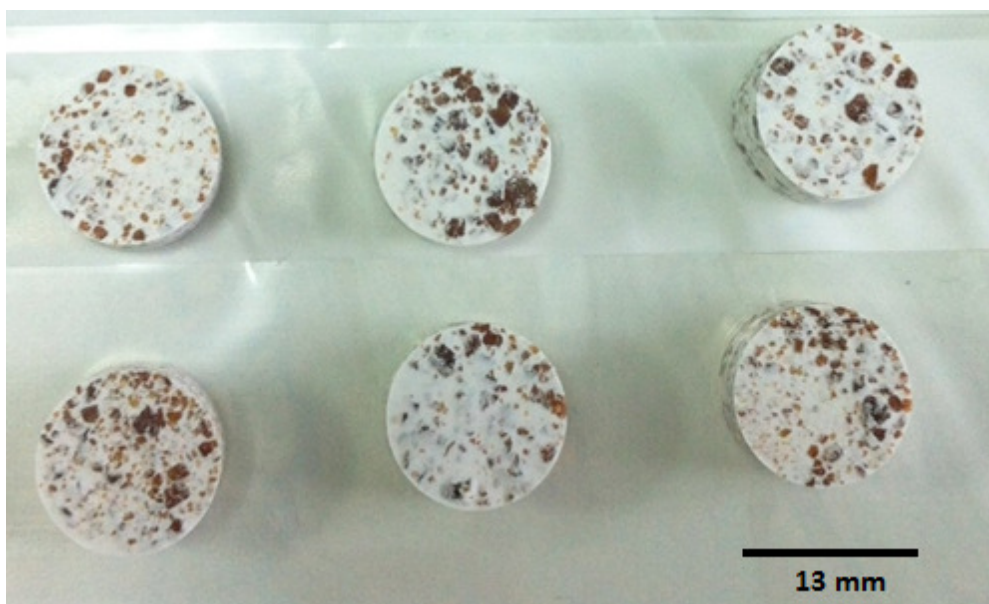


Figure 5-10 Tablets containing FDY and MCC in 50/50 (wt/wt) ratio and compacted at 90 MPa

Table 5-1 Cell survival and tensile strength of tablets containing FDY/MCC at different ratios and compaction pressures

Formulation	Compaction pressure (MPa)	CFU/(g tablet)		Tensile strength (MPa)	Log loss
		Before compaction	After compaction		
WY*		$6.5 \times 10^{10}$	$8.1 \times 10^9$		0.90
90/10	90	$7.29 \times 10^9$	$2.27 \times 10^9$	0.11	0.51
90/10	105	$7.29 \times 10^9$	$6.71 \times 10^8$	0.26	1.06
80/20	90	$6.48 \times 10^9$	$1.84 \times 10^9$	0.22	0.55
80/20	105	$6.48 \times 10^9$	$5.63 \times 10^8$	0.29	1.08
50/50	75	$4.05 \times 10^9$	$9.34 \times 10^8$	0.15	0.64
50/50	90	$4.05 \times 10^9$	$8.60 \times 10^8$	0.27	0.67
50/50	105	$4.05 \times 10^9$	$5.43 \times 10^8$	0.36	0.87

\*CFU/g was calculated based on WY before and after freeze drying for 24 h

Table 5-1 shows CFU/g tablet before and after compaction, tensile strength and log loss depending on the formulation and tablet compaction pressure. The new formulation of tablet containing FDY and MCC in 50/50 ratio compacted at 90 MPa had an average tensile strength of  $0.27 \pm 0.03$  MPa which was slightly higher than those made with 90/10 and 80/20 ratios (0.16 and 0.05 MPa respectively). This highlights that increasing filler content to 50% improve tablet tensile strength significantly but such tablets still did not meet the required specification in term of tensile strength. In addition, increasing the compaction pressure might produce significant deformation of MCC particles (Denny, 2002). Despite increasing the quantity of MCC and using a higher compaction pressure of 105 MPa, the tensile strength only increased slightly to  $0.36 \pm 0.03$  MPa (Table 5-1) which is still less than 1 MPa (Lesaffre's specification).

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The number of viable cells in tablets containing FDY and MCC in 50/50 ratio which were formed by compaction at different pressures was also considered (see Figure 5-11). To produce a 50/50 formulation, the amount of FDY was decreased by 44% from the 90/10 formulation and 37.5% from the 80/20 formulation. Therefore, less CFU/g tablet was to be expected in both cases due to less amount of FDY content. At 90 MPa, viable yeast cell numbers decreased by 0.51, 0.55 and 0.67 log after tablet compaction of 90/10, 80/20 and 50/50 ratio formulations. An increase of MCC offered stronger tablets but more excipient particles might interfere with cell surfaces resulting in cell destruction (Klayraung et al., 2009).

At a compaction pressure of 105 MPa, viable yeast cell numbers reduced by 1.06, 1.08 and 0.87 log after tablet compaction of 90/10, 80/20 and 50/50 ratio formulations which the losses at 105 MPa in all formulations were greater than those at 90 MPa. Loss of viable cell numbers has also been found by other authors to increase with compaction pressure (Plumpton et al., 1986b, Stadler and Viernstein, 2003). It should be noted that in all cases the final CFU/g tablet was lower than Lesaffre's specification which was  $6.67 \times 10^8$  CFU/g tablet.



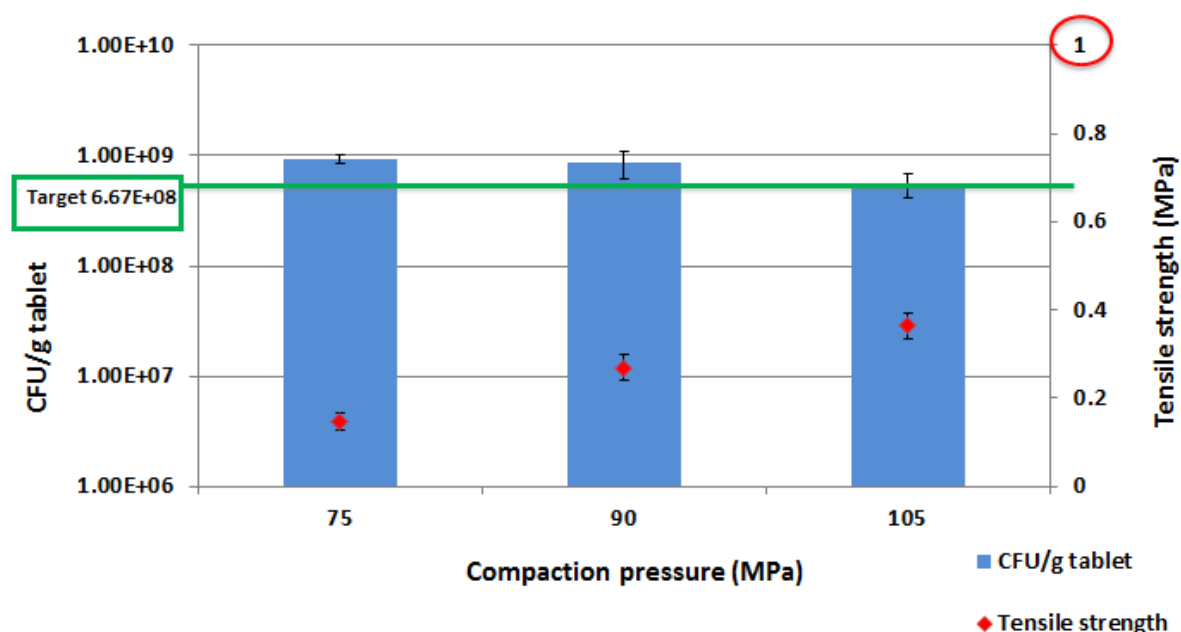


Figure 5-11 CFU/g tablet and tensile strength of tablets containing FDY and MCC in 50/50 (wt/wt) ratio after compaction at 75, 90 and 105 MPa. The green line demonstrates the target CFU/g tablet (Lesaffre). The red circle represents the target tablet tensile strength (Lesaffre)

The flow cytometric results of cell physiological states of yeast in tablets containing FDY/MCC in 50/50 (wt/wt) ratio compacted at different compaction pressures are presented in Figure 5-12. The results show significant changes in the number of healthy, injured and dead cells between tablets produced at different compaction pressures. When increasing compaction pressure during tableting, the gradual decrease in number of injured cells (i.e., those cells with diminished membrane potential) was observed as revealed by BOX staining. Bunthof and Abee (2002) define injured cells as “intact and metabolically active but not culturable”. Therefore, it is important to examine whether injured and/or healthy cells contributed to the number of viable cells in tablets containing FDY and if such cells were culturable, which will be presented in section 5.2.4.6.

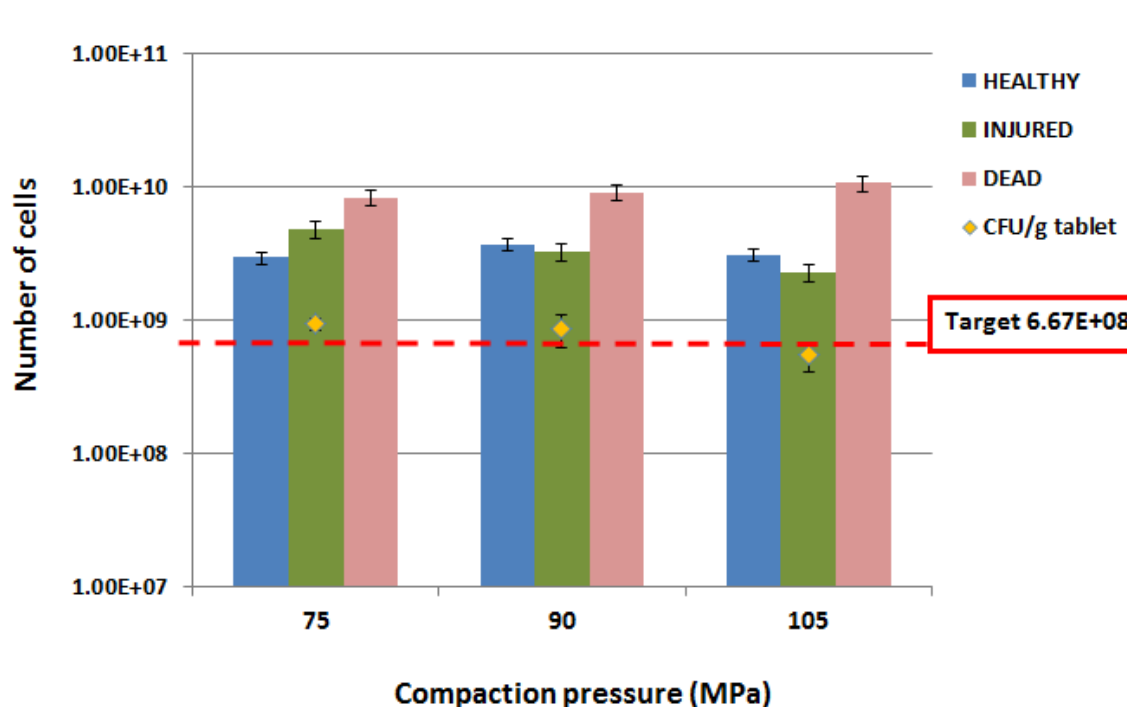


Figure 5-12 CFU and the number of healthy, injured and dead cells in 1g tablets containing FDY and MCC in 50/50 (wt/wt) ratio after compaction at 75, 90 and 105 MPa before storage. The red line demonstrates the minimum required CFU/g tablet (Lesaffre).

#### 5.2.4.5 Storage stability of yeast in tablets

Storage stability tests on yeast in tablets containing FDY/MCC in 50/50 (wt/wt) ratio compacted at 75, 90 and 105 MPa were also conducted. Three tablets were placed in a plastic bag and stored at  $4 \pm 1^\circ\text{C}$  ( $69\% \pm 5\%$  RH) for 6 months. These tablets at each compaction pressure were tested at 3 and 6 months. However, the tablets were not placed in a desiccator where the moisture level could be maintained. Therefore, it is conceivable that cells could have been affected by unknown fluctuations in moisture and light during storage.

Viable cell numbers for the 50/50 ratio tablets after 3 and 6 months storage were determined by the FC technique (Figure 5-13, Figure 5-14, Figure 5-15) and colony plate counting (Figure 5-16, Figure 5-17, Figure 5-18). Based on the PI and BOX stain plots, tablets before

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storage contained relatively high percentages of both healthy and injured cells. Such cells corresponded to the dense spread of live cells where healthy cells (gated in blue) and injured cells (gated in pink) are shown in the SYTO9 and PI stain plots. Therefore it is plausible that differing levels of SYTO9 penetration may depend on the health of individual cells. After three months storage, a high number of healthy cells appeared to have been injured, which might be due to changes in cell structure and function caused by exposure to stress factors such as moisture and oxygen (Miyamoto-Shinohara et al., 2000b). This is consistent with a loss of cell viability by membrane permeabilisation due to relative moisture content and light in the fridge which led to changes of proteins and nucleotides in cells functionality (Morgan et al., 2006, Santivarangkna et al., 2007). The total number of cells in these tablets determined by FC remained constant during 3 and 6 months storage (data not shown). Therefore, cell lysis can be considered to have been negligible.

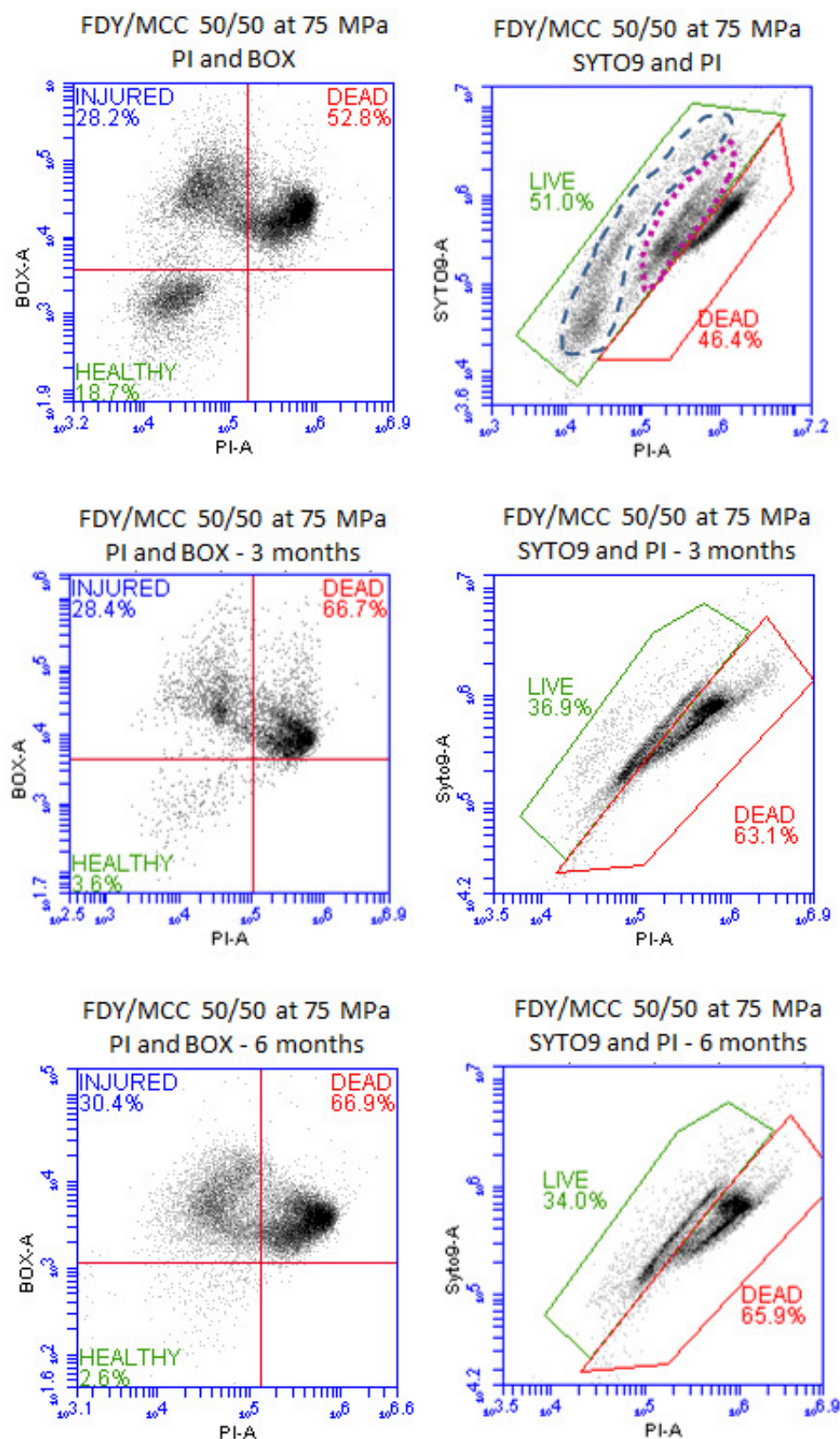


Figure 5-13 Flow cytometric dot plots showing various physiological states of yeast cells for tablets containing FDY and MCC in 50/50 (wt/wt) ratio compacted at 75 MPa (10 kN) after 3 and 6 months storage at 4°C and being stained with PI and BOX and SYTO9 and PI.

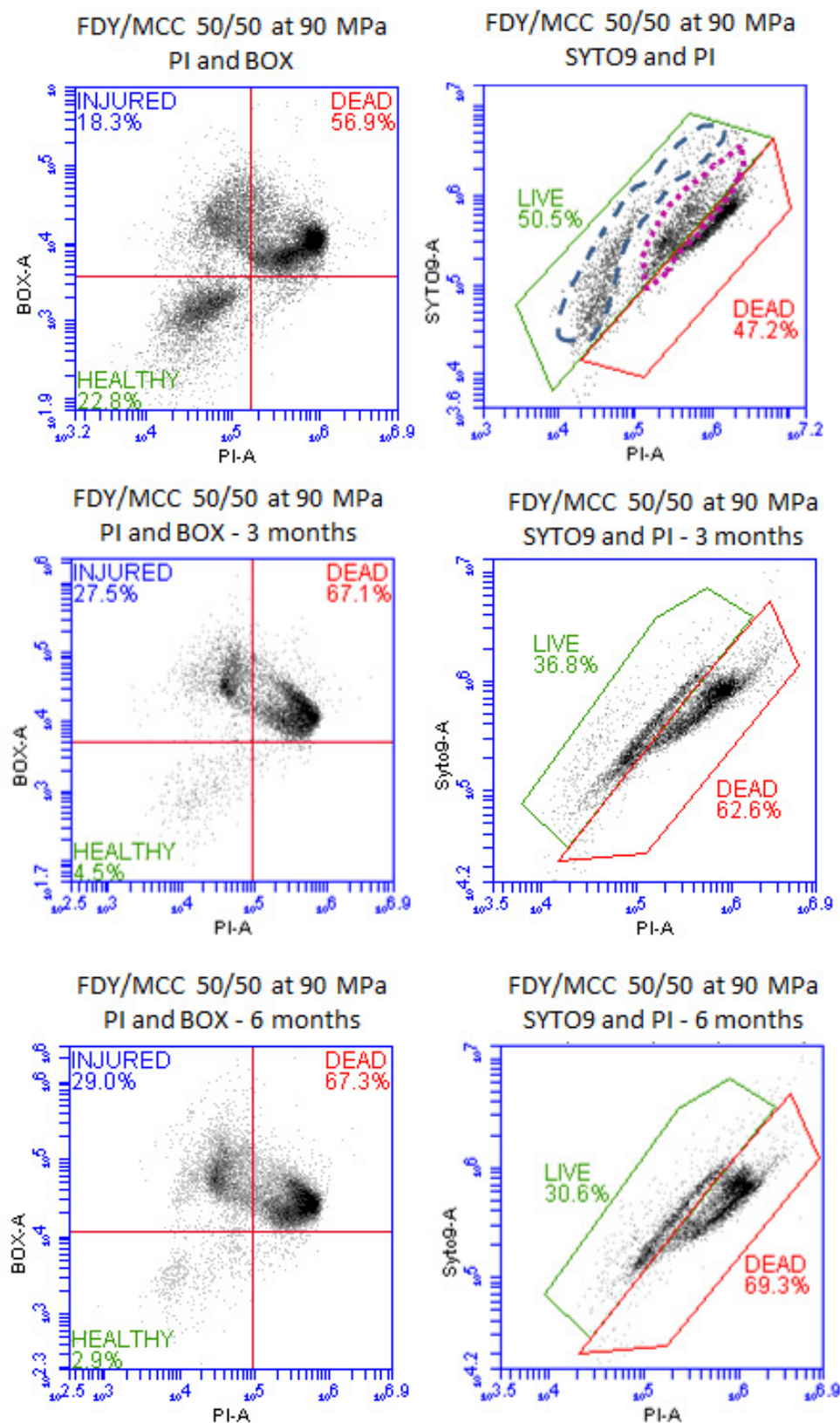


Figure 5-14 Flow cytometric dot plots showing various physiological states of yeast cells for tablets containing FDY and MCC in 50/50 wt/wt ratio compacted at 90 MPa (12 kN) after 3 and 6 months storage at 4°C and being stained with PI and BOX and SYTO9 and PI.



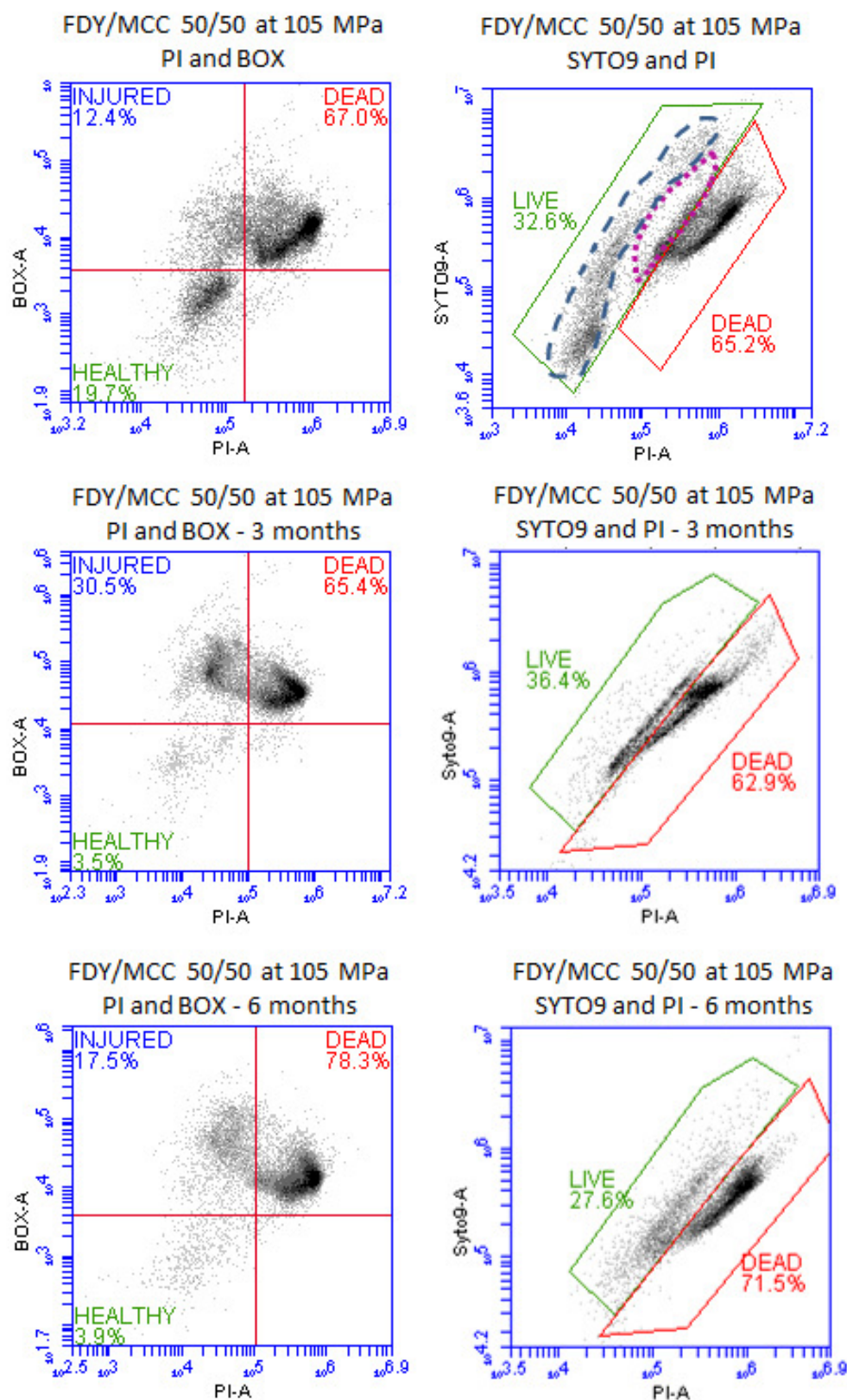


Figure 5-15 Flow cytometry dot plots showing various physiological states of yeast cells for tablets containing FDY and MCC in 50/50 wt/wt ratio compacted at 105 MPa (14 kN) after 3 and 6 months storage at 4°C and being stained with PI and BOX and SYTO9 and PI.

Figure 5-16 shows the number of healthy, injured and dead cells in tablets containing FDY and MCC in 50/50 ratio as well as CFU/g tablet after storage at  $4 \pm 1^\circ\text{C}$  ( $69\% \pm 5\%$  RH) for 3 months. After storage, the number of healthy cells significantly decreased and the number of dead cells increased while the number of injured cells slightly fluctuated with compaction pressure. It can be seen that the number of healthy cells alone was more than the CFU value but not all healthy cells were able to form colonies. The declined trend of such cells with compaction pressure is consistent with that before storage but demonstrates less culturability when exposed to the adverse effects of storage.

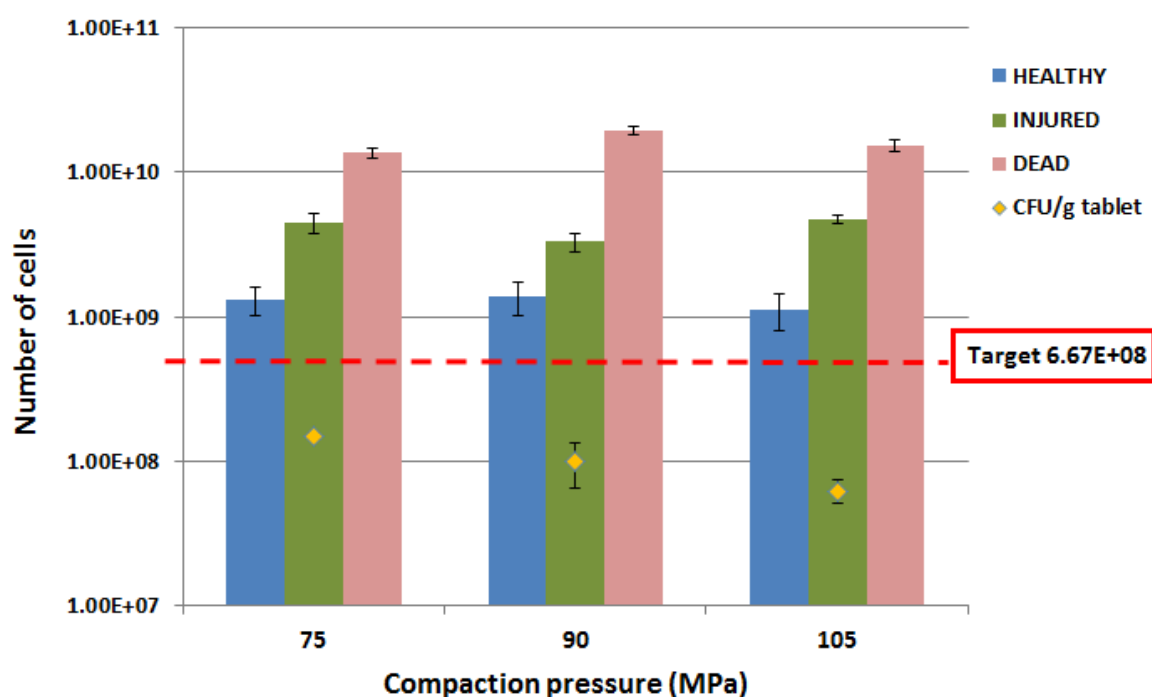


Figure 5-16 CFU and the number of healthy, injured and dead cells in 1g tablets containing FDY and MCC in 50/50 (wt/wt) ratio compacted at 75, 90 and 105 MPa after 3 months storage at  $4^\circ\text{C}$ . The red line demonstrates the minimum required CFU/g tablet (Lesaffre).

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The data of cell viabilities after being stored for 6 months are shown in Figure 5-17. There was a significant loss in CFU/g tablet ( $p < 0.05$ ) at all compaction pressures compared with tablets that had been stored for 3 months. There is also a decrease in the numbers of both healthy and injured cells (as more cells died) as storage time increased. Decreased stability of tablets containing FDY after storage could be due to the adverse effects of moisture, light and/or oxygen during storage. Since the tablets were not stored in a vacuum package they were exposed to oxygen in the plastic bag during storage and the loss of culturable cells at 4°C might be caused by the extent of oxygen induced cell injury following protein denaturation (Morgan et al., 2006, Ghandi et al., 2012). Besides exposure to oxygen, moisture level may also affect viability during storage especially since FDY with  $a_w$  of  $0.42 \pm 0.02$  was used to produce the tablets. The water activity value is thought to influence the phase transition of the cell membrane leading to cell deterioration and structural collapse (Kurtmann et al., 2009). Therefore, it is suggested tablets should be stored at a high level of desiccation or vacuum packaged to maintain high cell viabilities.



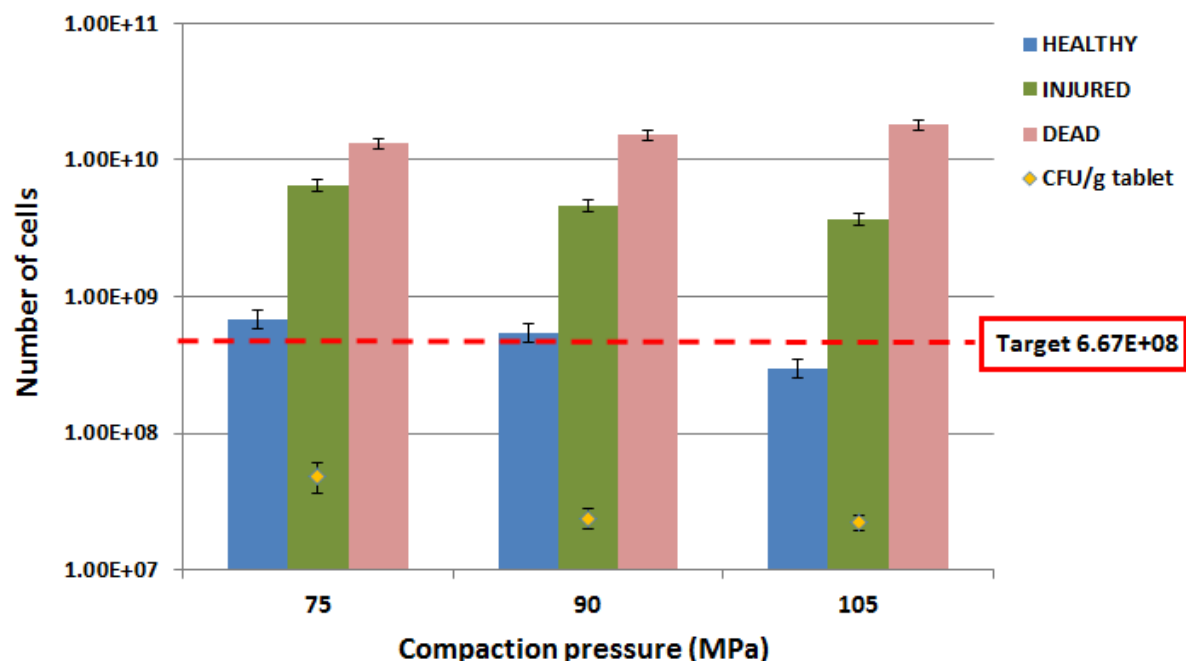


Figure 5-17 CFU and the number of healthy, injured and dead cells in 1g tablets containing FDY and MCC in 50/50 (wt/wt) ratio compacted at 75, 90 and 105 MPa after 6 months storage at 4°C. The red line demonstrates the minimum required CFU/g tablet (Lesaffre).

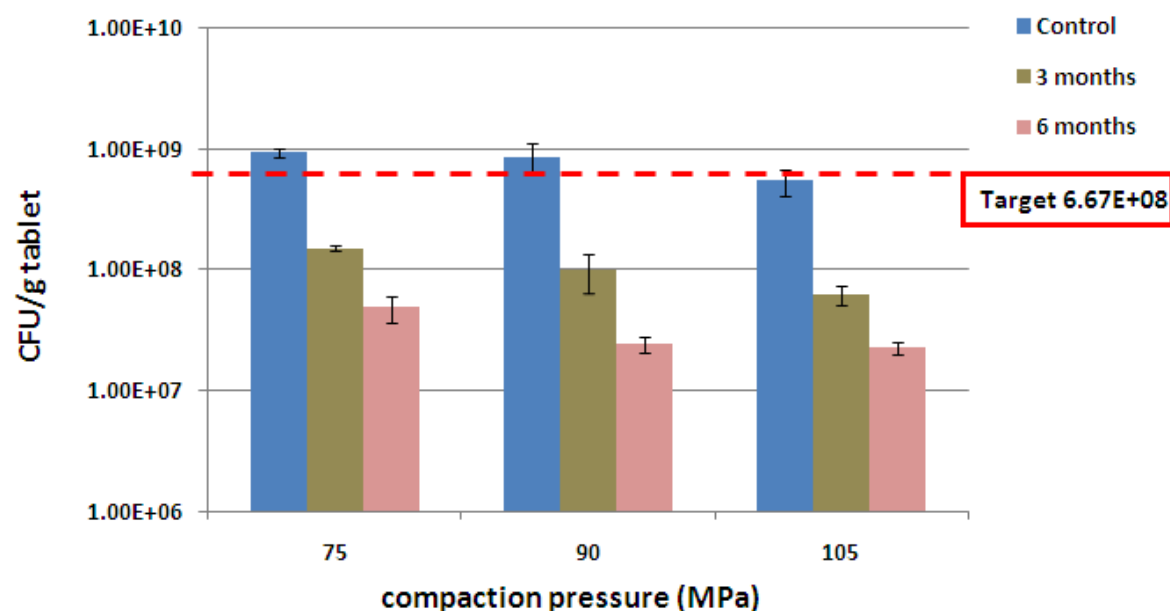


Figure 5-18 CFU in 1g tablets containing FDY and MCC in 50/50 (wt/wt) ratio compacted at 75, 90 and 105 MPa before and after 3 and 6 months storage at 4°C. The red line demonstrates the minimum required CFU/g tablet (Lesaffre).

Table 5-2 Cell viability loss in log scale during tablet compaction and after storage at 4°C over a 3 and 6 month period for tablets containing FDY/MCC in 50/50 ratio

Compaction pressure (MPa)	Cell viability log loss		
	After compaction	After storage for*	
		3 months	6 months
75	0.64	0.79	1.28
90	0.67	0.93	1.55
105	0.87	0.94	1.38

\* cell loss data after storage was calculated based on cell viability data after compaction

After 3 months storage at  $4 \pm 1^\circ\text{C}$  ( $69\% \pm 5\%$  RH), all tablets produced at three different compaction pressures had approximately 1 log loss of cell viability which reduced further after 6 months storage to 1.6 log loss (Figure 5-18). This finding is consistent with findings from Klayraung et al. (2009) who reported 1 log loss of freeze dried *Lactococcus lactis* after being stored for 6 months at  $30^\circ\text{C}$ . These results further support the observation of Brennan et al. (1983) and King and Su (1994) that cell viability loss rapidly decreased during the early stage of storage. When comparing cell viability loss during tablet compaction and storage, FDY had a higher survival rate during tablet compaction than those stored for 3 and 6 months. Table 5-2 shows a summary of losses of viable cell number during tablet compaction and storage (based on the CFU values). Storage at  $4^\circ\text{C}$  was found to have a greater impact on cell viability loss in comparison with cells that were subjected to tablet compaction pressure; this might be due to the duration and conditions of storage. Many factors i.e. moisture, light and oxygen during storage were detrimental to cells (FDY) in tablets, hence they had negative effects on cell survival (Porubcan and Sellars, 1979). There are very few studies on maintaining cell viability during storage but the available literature suggests use of high barrier bags that can control levels of oxygen, moisture, light and temperature better than low barrier bags (Costa et al., 2002). In a previous study ascorbic acid was found to protect *Lactobacillus delbrueckii* spp. *bulgaricus* cells which were stored at  $4^\circ\text{C}$  and then spray dried (Teixeira et al., 1995). The addition of an antioxidant such as ascorbic acid could be used to

diminish lipid oxidation in cell membranes, which typically occurs during storage and is responsible for cell death. In addition, there was more survival of microorganisms in fermented soymilk after being freeze dried than being spray dried. After 4 months storage in a vacuum package, cell survival at 4°C was higher than that at 25°C (Wang et al., 2004). Therefore, superior storage data at 4°C can be used to predict cell viability for products stored at 25°C according to an Arrhenius equation (Ishibashi et al., 1990).

#### ***5.2.4.6 Correlations of yeast cells in tablets containing FDY***

The correlations between CFU/g and healthy, injured and viable cells of tablets containing FDY and MCC in a 50/50 (wt/wt) ratio before storage and after being stored for 3 and 6 months were plotted with the correlation in section 4.2.4 as shown in Figure 5-19, Figure 5-20 and Figure 5-21 respectively. It can be seen that the storage data (outlined in red) were plotted in the lower positions in all figures which weakened the linear correlation coefficients shown in Figure 4-16 to 4-18. Therefore, it can be concluded that the correlation in section 4.2.4 cannot be applied to the storage stability data of tablets containing FDY. This might be due to some different stress factors affecting cell viability between storage and processing conditions and exposure to simulated fluids with different pH as presented in Figure 4-16 to 4-18.

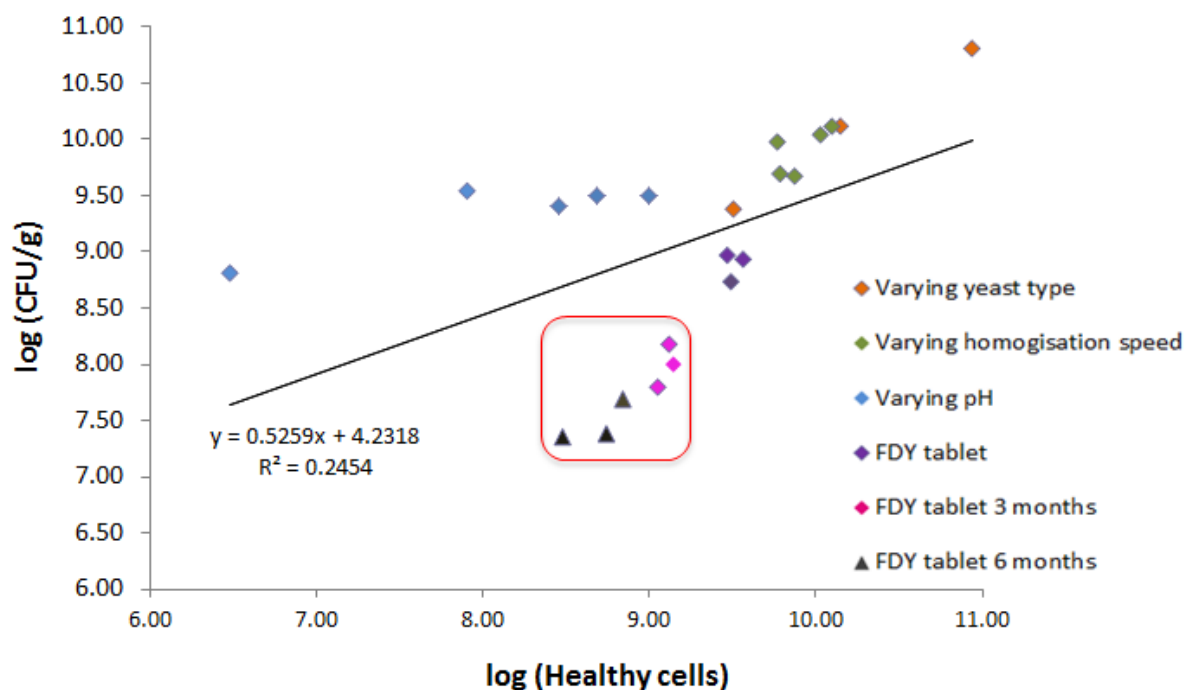


Figure 5-19 Correlation between CFU/g and number of healthy cells established in section 4.2.4 plotted with data of tablets containing FDY and MCC in 50/50 (wt/wt) ratio before and after storage (outlined in red). A new correlation line was weakened with  $R^2$  of 0.25.

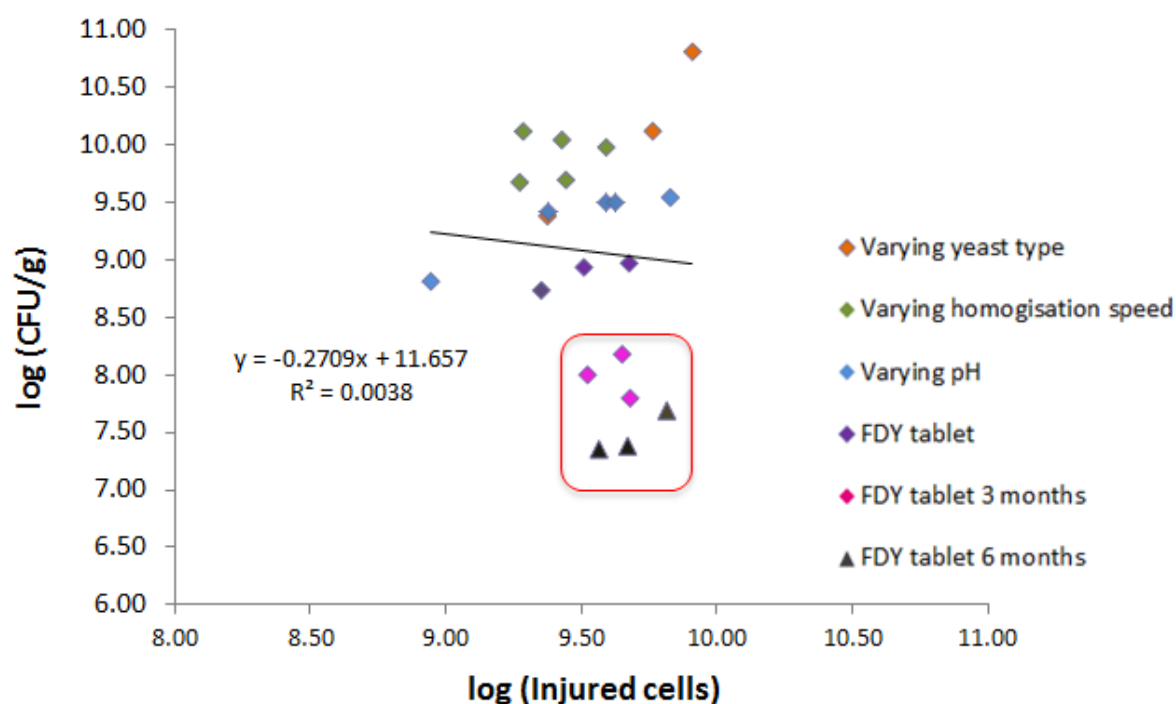


Figure 5-20 Correlation between CFU/g and number of injured cells established in section 4.2.4 plotted with data of tablets containing FDY and MCC in 50/50 (wt/wt) ratio before and after storage (outlined in red). A new correlation line was weakened with  $R^2$  of 0.004.

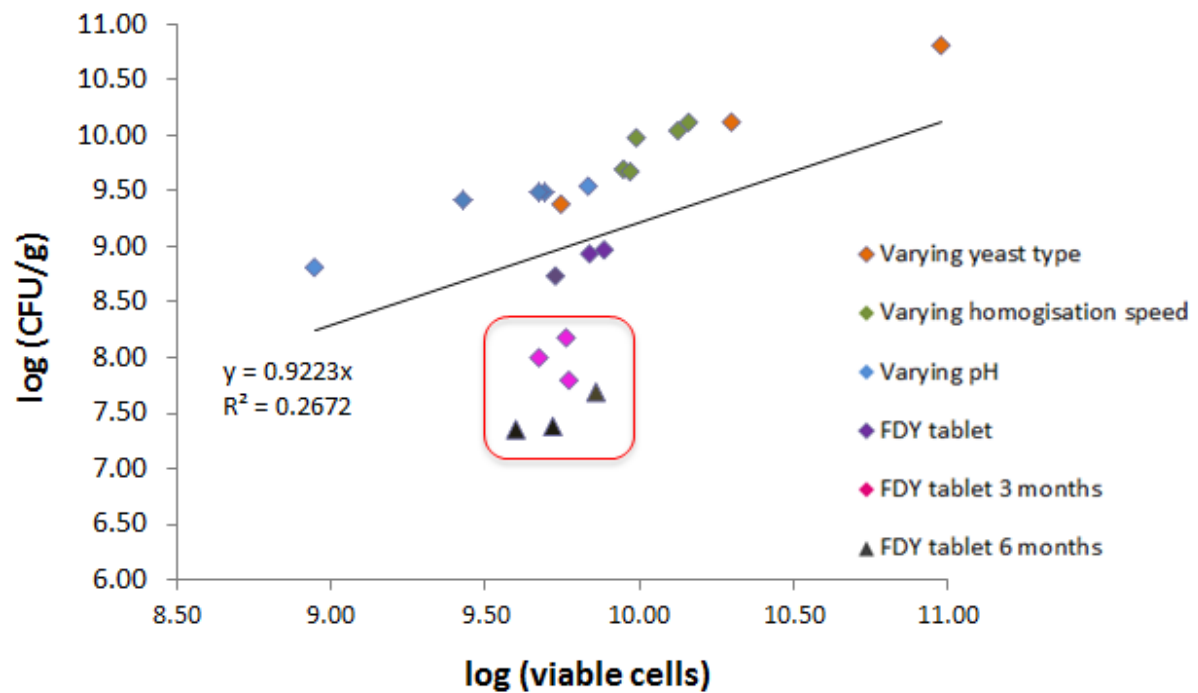


Figure 5-21 Correlation between CFU/g and number of viable cells established in section 4.2.4 plotted with data of tablets containing FDY and MCC in 50/50 (wt/wt) ratio before and after storage (outlined in red). A new correlation line was weakened with  $R^2$  of 0.23.

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### **5.3 CONCLUSIONS**

Flow cytometry has been shown to be a useful technique to determine the individual physiological state of yeast cells. Using a flow cytometric method in conjunction with PI, SYTO9 and BOX, it is possible to characterize cells into separate groups based on membrane potential and membrane integrity which is beyond culturability explored by the colony plate counting method.

The effects of freeze drying time on cell survival and water activity have been explored using FC and demonstrated in greater detail. The study in section 5.2.2 has shown a declining trend of viable yeast cells with increased freeze drying time which might be caused by dehydration. Such reduction of viable cells is attributed to membrane permeabilization resulting in PI uptake and consistent with a decline trend of CFU/g FDY. The addition of skimmed milk without a carbohydrate source (sugar) had a very small influence on the cell viability by which the number of viable cells increased by 5% in SYTO9 and PI plots and 2% in PI and BOX plots.

Compression of FDY and MCC into tablets was found to disrupt the cytoplasmic membrane of some cells by diminishing membrane potential as revealed by BOX staining. These tablets have shown high porosity from SEM analysis and resulted in low tensile strength (section 5.2.4.2). Increasing the amount of MCC in a tablet reduced segregation which slightly improved the tensile strength of the tablet (section 5.2.4.4). Attention is also drawn to the primary particle size of FDY and its properties which reflected poor binding properties within a tablet formulation.

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According to Lesaffre's CFU specification, two tablet formulations which contained FDY and MCC in 50/50 (wt/wt) ratio and compacted at 75 and 90 MPa can meet the minimum required CFU of  $6.67 \times 10^8$  CFU/g tablet. Nevertheless, these two formulations could not form rigid tablets having in low tensile strength (Figure 5-11).

The storage stability at 4°C of the formulation compacted at 90 MPa revealed approximately 1 and 1.6 log loss after 3 and 6 months storage respectively. Significant cell viability loss during storage could potentially be caused by tablet sensitivity due to moisture, oxygen, light and temperature. Therefore an additional cell protection by an encapsulation technique could potentially be applied to reduce cell viability loss during tableting and storage. The correlation between CFU/g and viable cells as explained in section 4.2.4 could not be used to estimate the culturability of cells which have been stored for 3 and 6 months. However, a strong correlation between healthy cells and CFU/g has proved that healthy cells were the main contribution to the number of viable cells but not all healthy cells were culturable.

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## CHAPTER 6

### ENCAPSULATION OF YEAST CELLS TO IMPROVE THEIR SURVIVAL AFTER TABLETTING

#### 6.1 INTRODUCTION

In the previous chapter, some formulations of tablets containing freeze dried yeast (FDY) and microcrystalline cellulose (MCC) exhibited an adequate number of viable cells at low compaction pressure for tableting, but they were vulnerable to high compaction pressure. The low compaction pressure resulted in tablets with low tensile strength, and it is presumed they could not stand further processing and handling. Moreover, during storage, FDY cells in tablets appeared to be vulnerable to moisture, oxygen and light resulting in lower CFU/g tablet. In addition, cell viability loss during storage for these tablets proved greater than the losses from compaction.

Stummer et al. (2010a) reported that dried bacteria cells coated with shellac were protected in acidic conditions and cell survival rates were maintained for four months after storage at 5°C. Since shellac has low permeability, it is suitable as a taste masking coating (Pearnchob et al., 2003b) or protective coating against moisture for water sensitive materials including microorganisms (Phan The et al., 2008). In addition, shellac is suitable as an enteric coating material to prevent some types of active ingredients from being released in acidic conditions when they pass through the stomach (Ravi et al., 2008a, Ravi et al., 2008b). Sandoval-Castilla et al. (2010) reported that *Lactobacillus casei* entrapped in pectin beads were protected after exposure to simulated gastric fluid and bile salts. Pectin is also used as a



carrier for oral drug delivery (Chung and Zhibing, 2003) and a carrier for sustained release (Sriamornsak and Nanthanid, 1998a, Sriamornsak and Nunthanid, 1999a). It is, therefore, hypothesized here that encapsulation of cells can protect them from being damaged by compaction to a certain extent and enhance their storage stability.

From many available encapsulation techniques, the extrusion method (droplet method) is the longest established and most common approach due to its low cost and mild operating conditions (King Alan, 1995, Sara, 1995). Previous works have shown viability of bacterial cells encapsulated in various beads produced by the extrusion method was up to 80-95% (Audet et al., 1988, Rao et al., 1989, Sheu et al., 1993b, Jankowski et al., 1997). This method involves forcing a mixture of cells and gelling liquid (e.g. sodium alginate, shellac or pectin) through a small nozzle or a syringe needle (Smidsrød and Skjåk-Bræk, 1990) to form a droplet, which drops to a hardening solution e.g. calcium chloride (Hansen et al., 2002, Lee and Heo, 2000). Despite the applications of shellac in probiotic products, the encapsulation of cells in shellac beads by extrusion method has not been reported so far.

The aim of the work described in this chapter was to encapsulate yeast cells in beads that maintain cell viability while protecting them from harsh environments. Several factors can influence the properties of the calcium pectinate (CaP) beads (and therefore cell viability) during the encapsulation process such as the concentration of cross-linking calcium chloride ( $\text{CaCl}_2$ ) solution to harden the encapsulated beads, the concentration of pectin as an encapsulating material and the freeze drying time. Entrapped yeast cells were surrounded by shellac and pectin in calcium crosslinked beads. The physical properties of calcium shellac and calcium pectinate beads obtained from the encapsulation process i.e. particle size, internal structure (Allan-Wojtas et al., 2008, Rosenberg et al., 1985) and water activity were

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investigated. The number of viable cells in these beads was measured by colony plate counting (CPC) (Postgate, 1969) and a flow cytometry technique together with staining by PI and BOX (Hewitt and Nebe-Von-Caron, 2001, Boswell et al., 1998) and SYTO9 and PI (Tanja et al., 2013, Zhang and Fang, 2004, Stocks, 2004). Consequently encapsulation efficiency (EE) of yeast cells in CaP beads at different calcium chloride concentrations was measured based on the number of viable cells obtained from flow cytometry. Together with a calculation of cell viability based on CFU (%), the conditions that maximized cell viability were used to determine a formulation for subsequent compaction experiments in this chapter.

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## **6.2 RESULTS AND DISCUSSION**

### **6.2.1 Encapsulation of yeast cells with shellac**

Encapsulation of yeast cells with shellac by an extrusion method using a Nisco encapsulation unit (model VAR J1, SPA-00195, Switzerland) was performed as described in section 3.11. The results obtained from measuring the properties (e.g., particle size; water activity and inner structure) of calcium shellac beads are introduced in this section.

#### ***6.2.1.1 Morphology of calcium shellac beads***

Shellac was provided in a form of ammonium shellac solution. When a mixture of ammonium shellac and yeast was dropped into calcium chloride solution, there was an exchange of ions with ammonium ions replaced by calcium ions resulting in solid calcium shellac particles. Calcium shellac beads obtained from the encapsulation process and before freeze drying had yellowish hard outer shells that were almost spherical. Some beads before freeze drying had distinct tails which was due to the collective effects of surface tension, viscosity of the mixture and the collecting distance between the dripping tip and the gelling bath where the minimum was about 2 cm (Chan et al., 2009). In this study, the distance between a nozzle and a cross-linking solution was set at the minimum of 2 cm. Potentially, the distance could be adjusted, which might be done to obtain spherically shaped beads. However, since these beads were to be compacted into tablets by direct compaction, it was presumed that the shape of the beads was not critical, assuming the shape would not have a significant effect on the rearrangement and deformation of beads and MCC during tableting.

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The particle sizes of prepared calcium shellac beads before freeze drying were in a range of 3.5 to 4.5 mm (Figure 6-1a) and after freeze drying for 24 h, the size slightly reduced to a range of 2-3 mm (Figure 6-1b). This might be due to the removal of liquid content from the beads during freeze drying and thus reduction of particle volume (Velings and Mestdagh, 1995). In addition, the size of calcium shellac beads was dependent on the encapsulation nozzle size and the co-axial air stream rate used to create droplets and consequently particles in the encapsulation process.

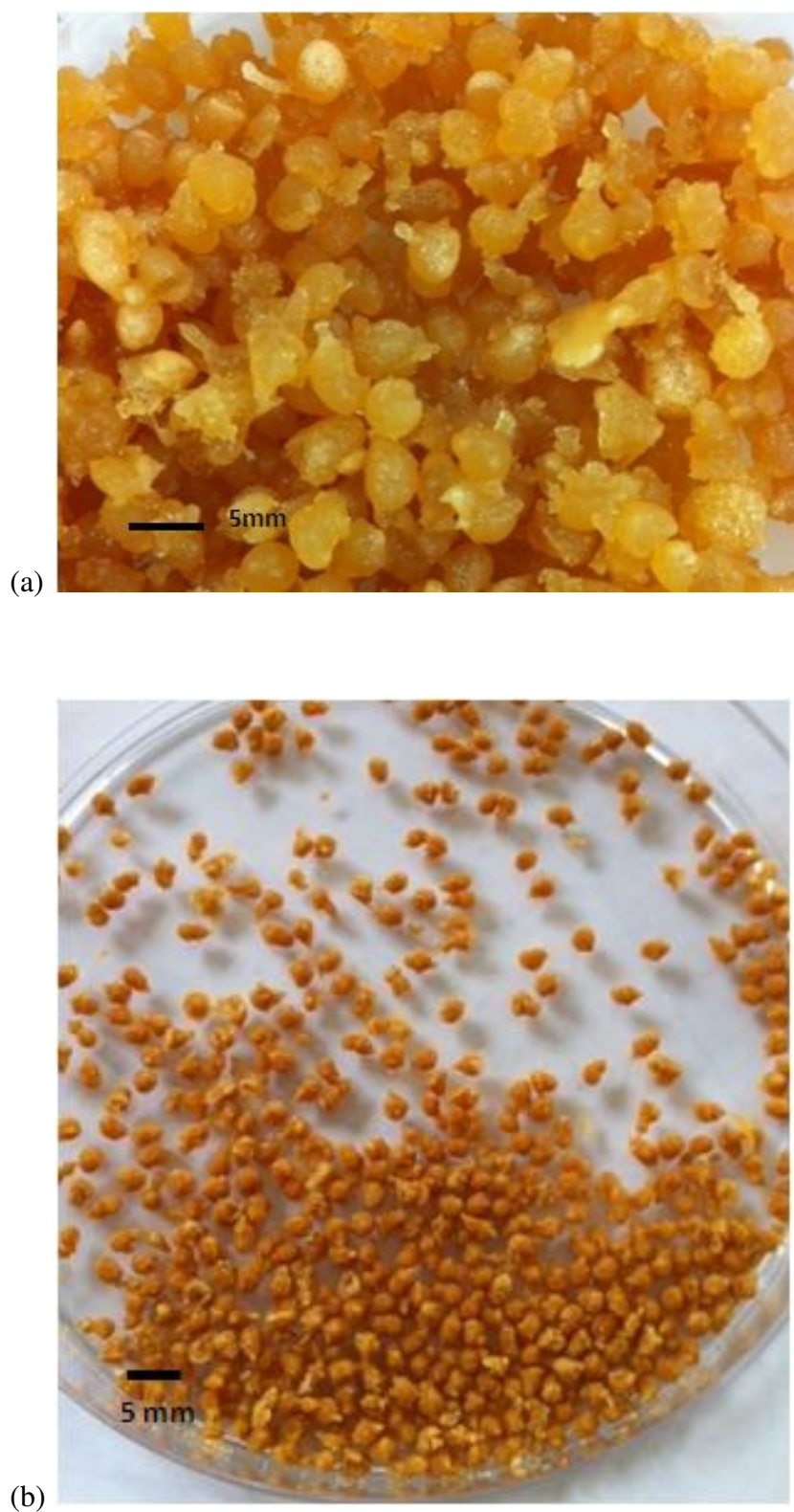


Figure 6-1 Calcium shellac beads containing yeast cells (a) before freeze drying (b) after freeze drying for 24 h

### 6.2.1.2 Particle size distribution

As the particle size of calcium shellac beads may affect tablet compactibility, the size distribution of calcium shellac beads after freeze drying for 24 h was measured by QICPIC image analysis as described in section 3.12.5.1. In this experiment, the number of particles counted in three measurements ranged between 6,000 and 8,000 particles. As shown in Figure 6-2, dried calcium shellac beads had a very wide size distribution but the peak was between 2.55 and 3.09 mm with the average particle size of  $2.9 \pm 0.1$  mm. This analysis indicated that the size of calcium shellac beads was significantly different to MCC particle size (mean size  $0.19 \pm 0.01$  mm; as described in Chapter 5).

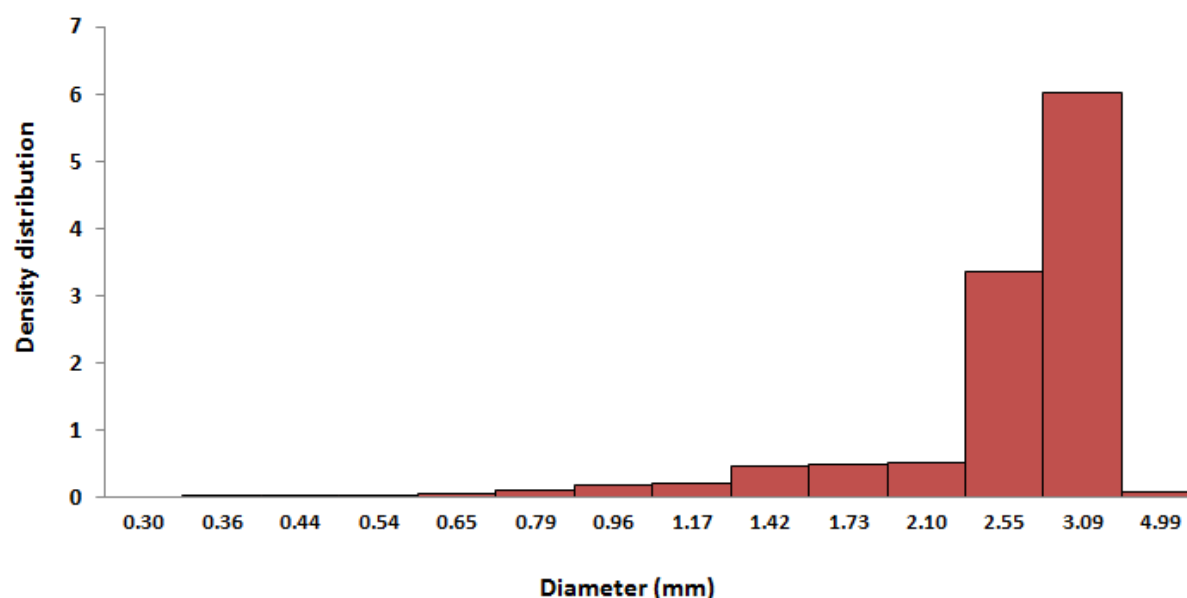


Figure 6-2 Particle size distribution of dried calcium shellac beads measured by an image analyser (QICPIC). The density distribution is defined by Eq 3-3.

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### 6.2.1.3 Water activity

The previous chapter describes how non-encapsulated cells of FDY were sensitive to humidity demonstrating the importance of ensuring calcium shellac beads have low water activity ( $a_w$ ). The results in Figure 6-3 show the water activity versus drying time of calcium shellac beads subjected to (1) air drying and (2) freeze drying. Calcium shellac beads before drying had a very high water activity at  $0.97 \pm 0.02$  because these solid particles were formed and immersed in an aqueous cross-linking solution for 15 min to allow ion exchange to take place (as described in section 3.11.2). After calcium shellac beads were dried for 12 h a significant drop of  $a_w$  was obtained to 0.55 and 0.47 for air drying and freeze drying respectively. Since water activity has an effect on proliferation of microorganisms and also product stability (Hahn-Hägerdal, 1986, Harris, 1981), the  $a_w$  value should be below 0.3 prior to storage (Dr Peter Jüsten, Lesaffre, France, Personal communication), thus improving the shelf life of probiotics in the tablet formulation. Hence, prolonging the drying time of calcium shellac beads was necessary.

The  $a_w$  of calcium shellac beads after freeze drying for 24 h was  $0.12 \pm 0.02$  whereas air drying for 24 h produced  $a_w$  of  $0.4 \pm 0.2$ . The former is desirable for long term storage stability (Dr Peter Jüsten, Lesaffre, France, Personal communication), whilst the latter may still be too high. No significant changes of  $a_w$  were observed after prolonging drying time up to 36 h. Therefore, freeze drying was preferable to air drying since a suitable  $a_w$  value could be achieved within 24 h.

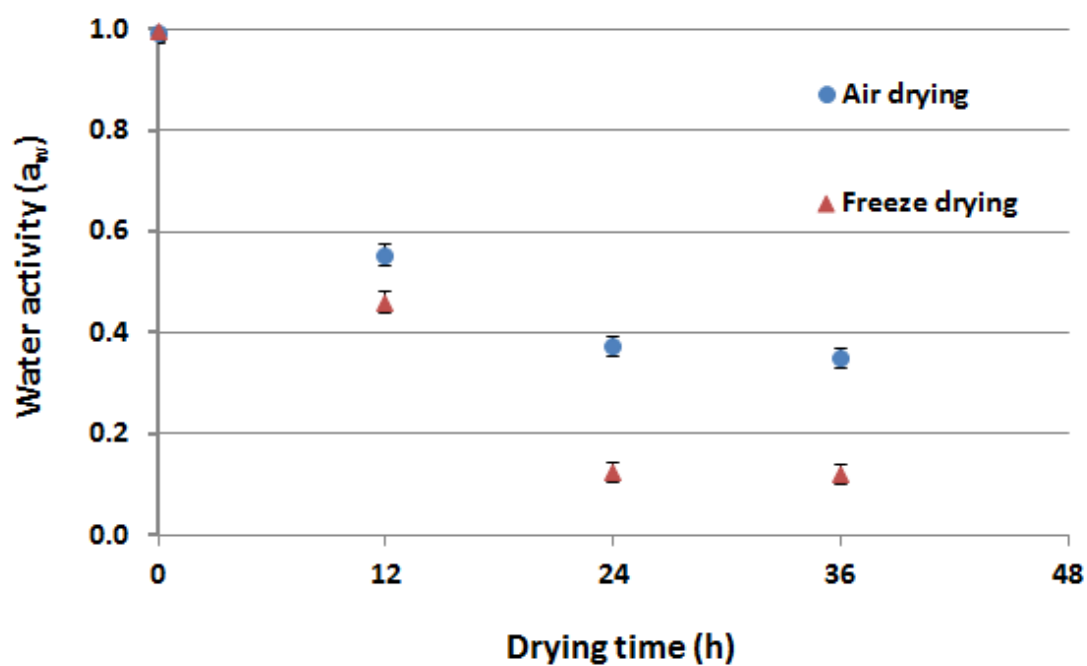
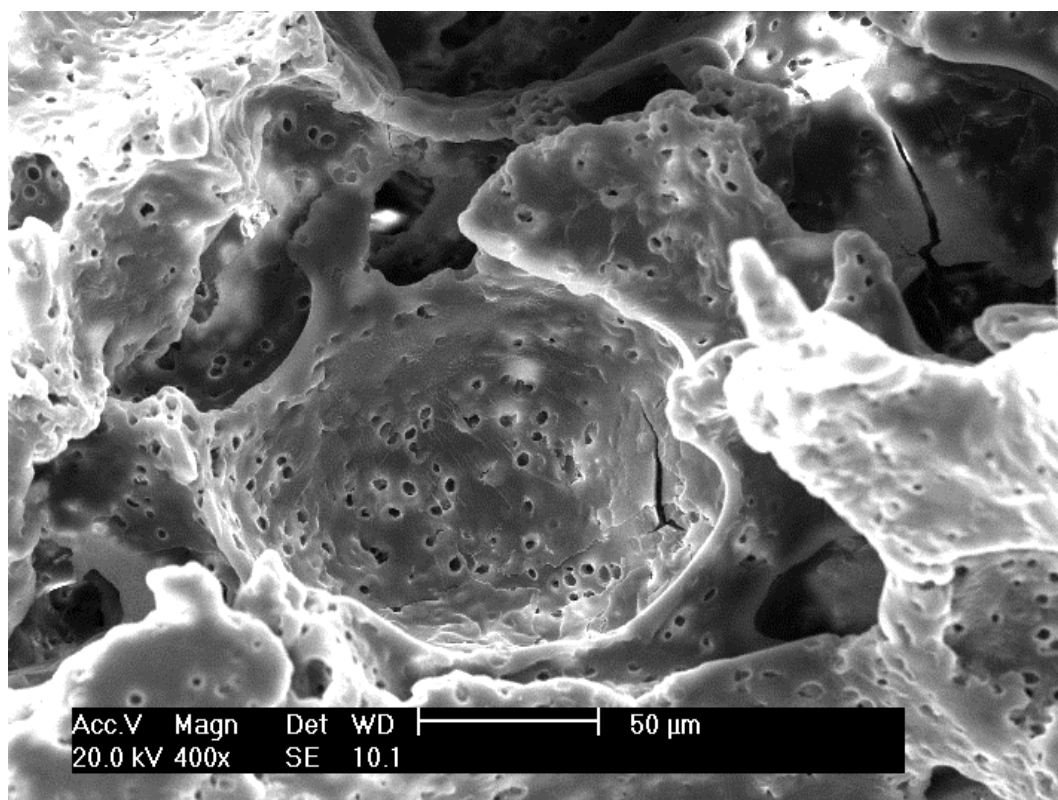


Figure 6-3 Water activity of calcium shellac beads that were either air dried or freeze dried. Data were measured every 12 h using a Pawkit water activity meter with standard errors (n=3).

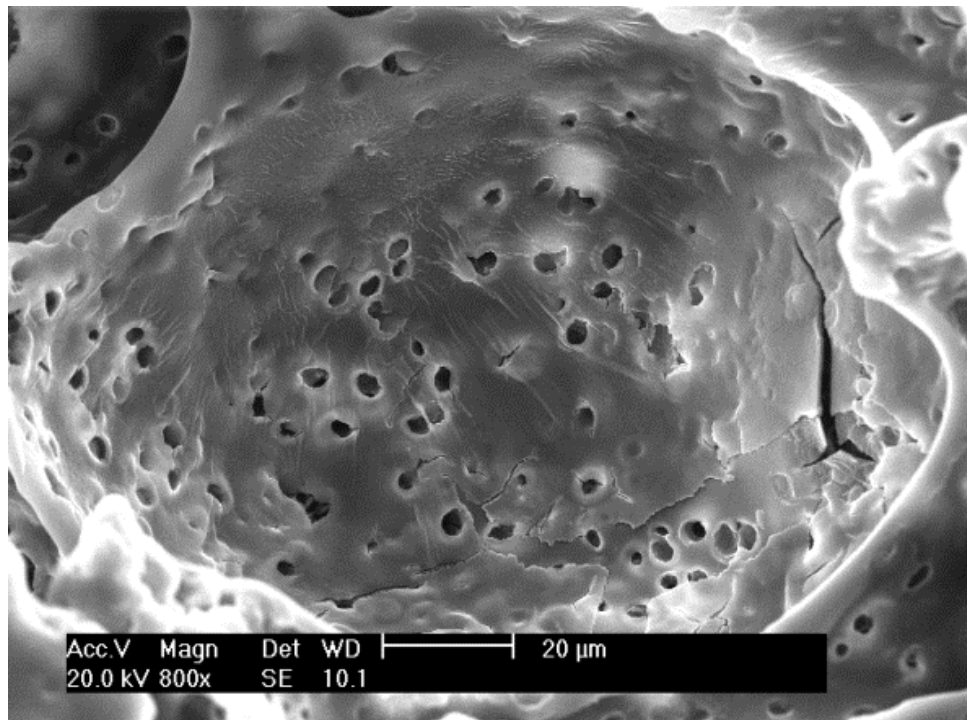


#### 6.2.1.4 SEM analysis

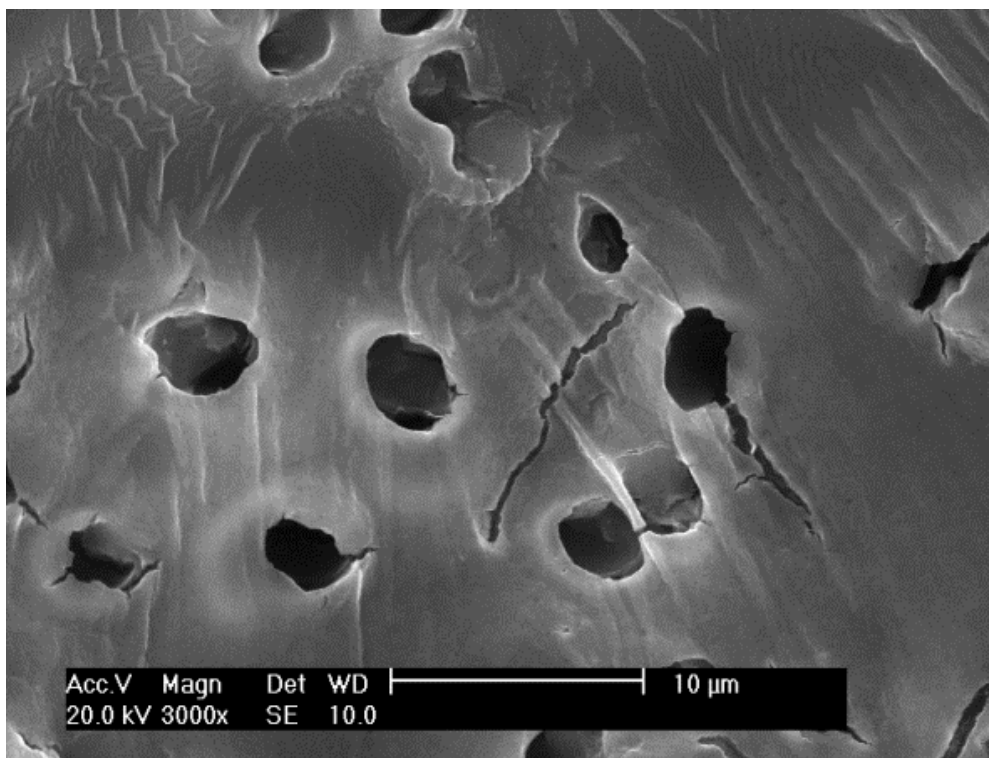
The morphology of calcium shellac beads was characterized by scanning electron microscopy. SEM images of cross sections of calcium shellac beads after freeze drying are presented in Figure 6-4. As can be seen, the beads had a macroporous and slightly condensed internal structure, which might be due to bubbles created during the mixing step (as described in section 3. 11.2). To diminish these bubbles, the mixture could have been left longer than 15 min before being extruded in the encapsulation unit. This finding is interesting since it may have an impact on the strength of tablets made with these beads. Moreover, it can be observed that embedded yeast cells were on the surface structure inside the microspheres.



(a)



(b)



(c)

Figure 6-4 SEM images of cross-sectional areas of dried calcium shellac beads at different magnifications (a) 400x (b) 800x and (c) 3000x

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### ***6.2.1.5 Viability of cells in shellac beads***

To determine whether homogenisation time had the effect of releasing yeast cells from calcium shellac beads, dried beads were homogenised at 10,000 rpm for 3 to 12 min prior to serial dilution (as described in section 3.12.5.3). The CFU/g beads at different homogenisation times together with active dried yeast (ADY) suspension homogenised for 3 min as a control are shown in Figure 6-5. It can be seen that longer homogenisation time resulted in higher CFU/g bead. However, it was observed that calcium shellac particles were very hard to break even by homogenisation. There was no significant difference in CFU/g bead when the beads were homogenised for 9 and 12 min. Despite this, it can be presumed that the total number of entrapped cells in the calcium shellac beads was not fully released by sufficient homogenisation or after 9 min homogenisation. “Shellac” actually comprises a mixture of shellac, water and ammoniumhydrogencarbonate, which has a pH that is more or less neutral. Therefore, it is likely that shellac itself has no effect on yeast cell viability. Approximately 1 log loss of CFU after 9 min homogenisation was observed when compared with the ADY control. This could be due to osmotic shock when the mixture of yeast suspension and shellac was dropped into the cross-linking solution ( $\text{CaCl}_2$ ). It also might be that not all yeast cells in calcium shellac beads were released after being homogenisation. Another possibility is that high concentration of 20% (w/v) of resinous shellac used might interfere with the transport of oxygen through the cell membrane (Stummer et al., 2010b).

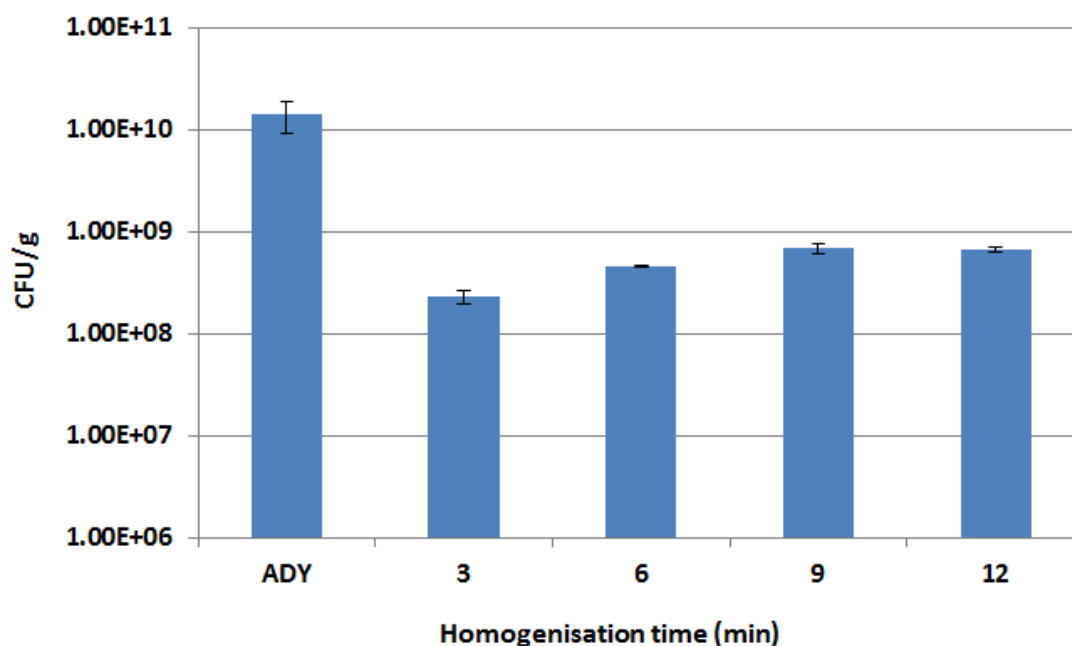


Figure 6-5 Viability of cells released from calcium shellac beads after being homogenised for various times compared with ADY suspension (controlled experiment),  $n=3$  with standard errors.

The viability of cells released from calcium shellac beads after being homogenised at 10 000 rpm for 3 min and stained with SYTO9 and PI was measured by fluorescent microscopy as described in section 3.12.9. It can be seen in Figure 6-6 that dead cells were clearly stained in red or dark orange and had spherical shapes. However, it was problematic to identify individual live cells, which appeared fluorescent green. The green particles appeared in distorted shapes where yeast cells might be embedded in non-breakable and insoluble shellac particles. This is thought to be due to an interaction of SYTO9 and some component in shellac which is highly variable depending on its origin and the way it has been refined (Buch et al., 2009). Therefore not only live cells, but also shellac particles were stained with green fluorescent SYTO9. This observation clearly indicates SYTO9 is not suitable for examining cell viability of encapsulated shellac beads.

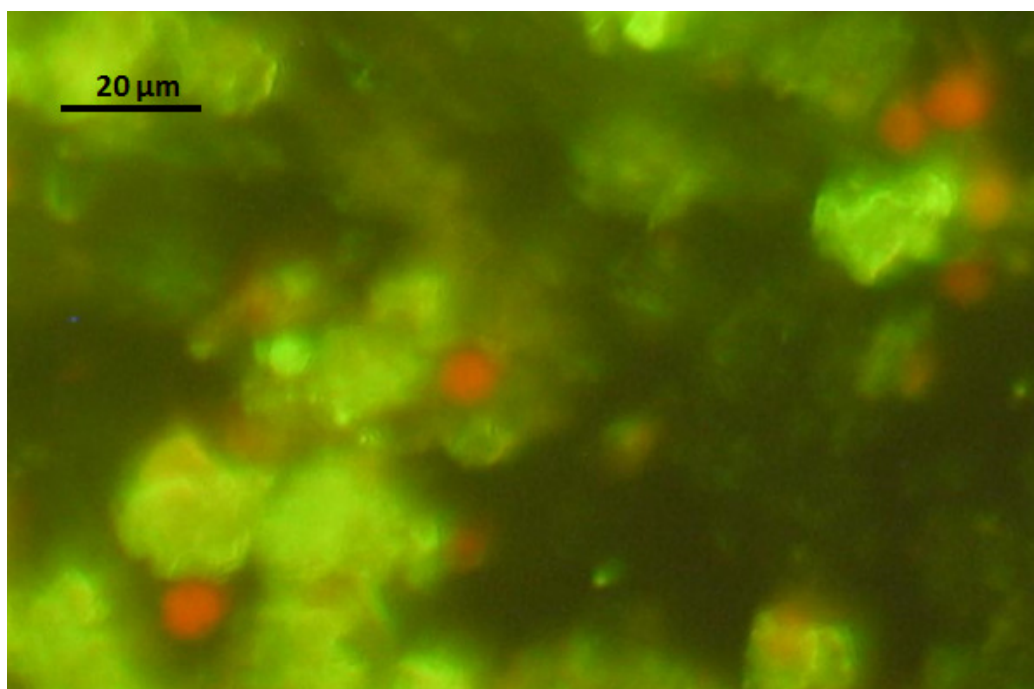


Figure 6-6 Fluorescent microscopy image of calcium shellac bead suspension after being homogenised at 10,000 rpm for 3 min and stained with SYTO9 and PI at room temperature and protected from light for 15 min

#### ***6.2.1.6 Dissolution study of tablets made with calcium shellac beads***

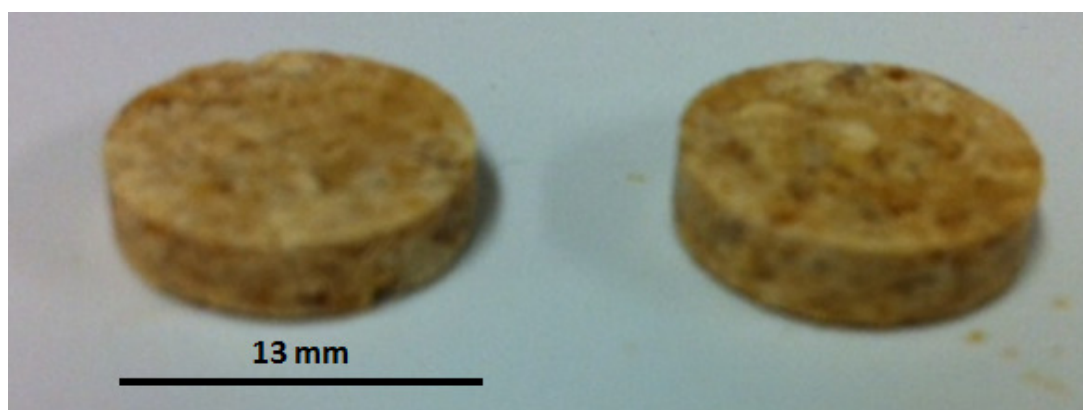
Due to the size and weight of calcium shellac beads, 1 g of calcium shellac beads was unable to fit in the die before direct compaction. Therefore, 0.5 g of calcium shellac beads without any filler were compacted into tablets as shown in Figure 6-7(a). It can be seen that calcium shellac beads have high compactibility, and did not require any binder, filler or lubricant for direct compaction. Based on Blanquet *et al.* (2004), they stated the physical transition time of ingested food from the stomach to the colon is 2-6 h. Figure 6-7(b) shows a tablet containing calcium shellac beads after being immersed in PBS (pH7.4) for 6 h. Although the tablet was immersed in neutral pH buffer for 6 h using the USP apparatus at a speed of 100 rpm operating at 37°C (as described in section 3.7.2), the tablets showed low solubility and poor

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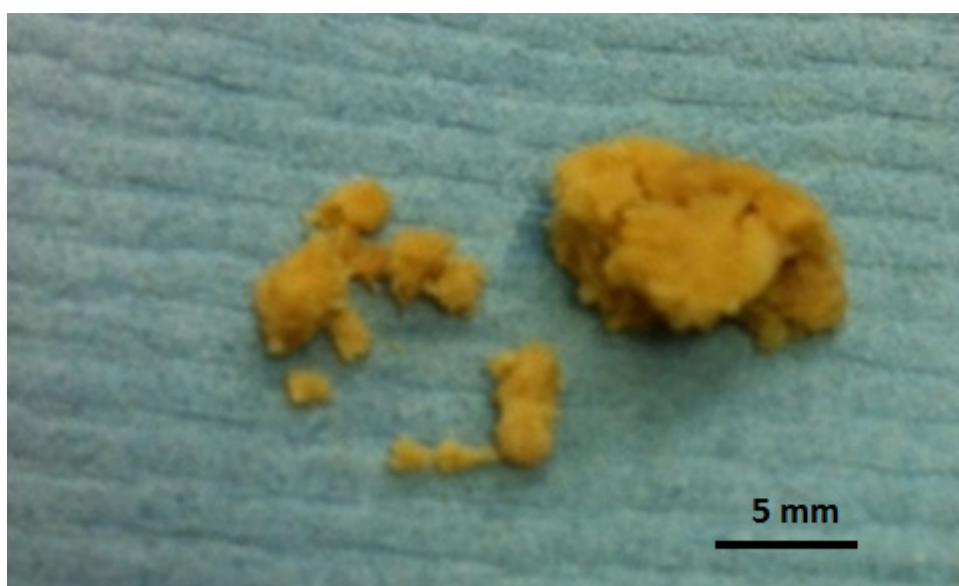
disintegration in simulated intestinal fluid. This slow dissolution of shellac in neutral pH buffer was also confirmed by Limmatvapirat *et al.* (2004) and Pearnchob *et al.* (2003a). As a result of poor dissolution of shellac, Pearnchob *et al.* (2004) improved the disintegration of shellac coated capsules in simulated intestinal fluid by adding some plasticizers which remained unchanged at low pH in simulated gastric fluids and rapidly disintegrated in simulated intestinal fluid. In this study, however, it was thought that the ratio of shellac to cells for encapsulation could alter the release rate of entrapped cells from calcium shellac beads (Limmatvapirat *et al.*, 2008). Using a low ratio of shellac to cells might overcome the problem of poor disintegration of calcium shellac beads by allowing more yeast cells to be released from the beads.

Since, it proved difficult to break calcium shellac beads in order to fully release entrapped cells prior to viability determination. Moreover, calcium shellac beads had poor solubility in a neutral pH buffer (i.e. simulated intestinal fluid (SIF)). Therefore, further investigation of the encapsulation process focused on the use of pectin only.





(a)



(b)

Figure 6-7 (a) A tablet containing 0.5 g of calcium shellac beads compacted at a compaction pressure of 105 MPa (c.a. compaction force 14 kN) and (b) Tablet after being immersed in PBS solution for 6 h which showed low dissolution and poor disintegration in neutral pH solution

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## 6.2.2 Encapsulation of yeast cells with pectin

Another biodegradable material selected to encapsulate yeast cells was pectin. The encapsulation of yeast cells with pectin was performed by the extrusion method using a G23 needle and a syringe pump according to section 3.12.2. The properties of calcium pectinate beads i.e. morphology, water activity and inner structure were examined.

### 6.2.2.1 Morphology of calcium pectinate beads

Calcium pectinate beads formed instantaneously after a mixture of cells and pectin was dropped to the gelling bath with calcium ions. The beads before freeze drying were white and fairly spherical shaped with a diameter of  $2.3 \pm 0.2$  mm as shown in Figure 6-8(a). It was claimed by Chan *et al.* (2009) that the shape of the beads produced by the extrusion-dripping method was principally influenced by process conditions and the liquid properties. None of the calcium pectinate beads appeared with a distinct tail as found in calcium shellac beads. It is likely that the egg shape of calcium pectinate bead might be due to (1) the concentration of pectin used and (2) the distance of 7 cm between the G23 needle and the gelling bath of calcium chloride solution. After freeze drying some beads became irregularly shaped and the colour changed to brown as shown in Figure 6-8(b). However, calcium pectinate beads produced from this encapsulation technique were generally spherical prior to tablet compaction.





(a)



(b)

Figure 6-8 Image of calcium pectinate beads with scale (a) before freeze drying and (b) after 24 h of freeze drying

### 6.2.2.2 Particle size distribution

Determination of the particle size distribution of calcium pectinate beads containing yeast cells after 24 h of freeze drying was performed using QICPIC image analysis (as described in section 3.12.5.1). The number of particles observed in three measurements ranged between 7000 and 9000 particles. As shown in Figure 6-9, the distribution is in a narrow range with the average particle size of  $2.1 \pm 0.3$  mm. Hansen et al. (2002) claimed that the particle size of encapsulated microspheres should be in the range of 100-1000  $\mu\text{m}$ . However, particle size larger than 1000  $\mu\text{m}$  obtained in this study did not affect the shape and quality of the CaP beads.

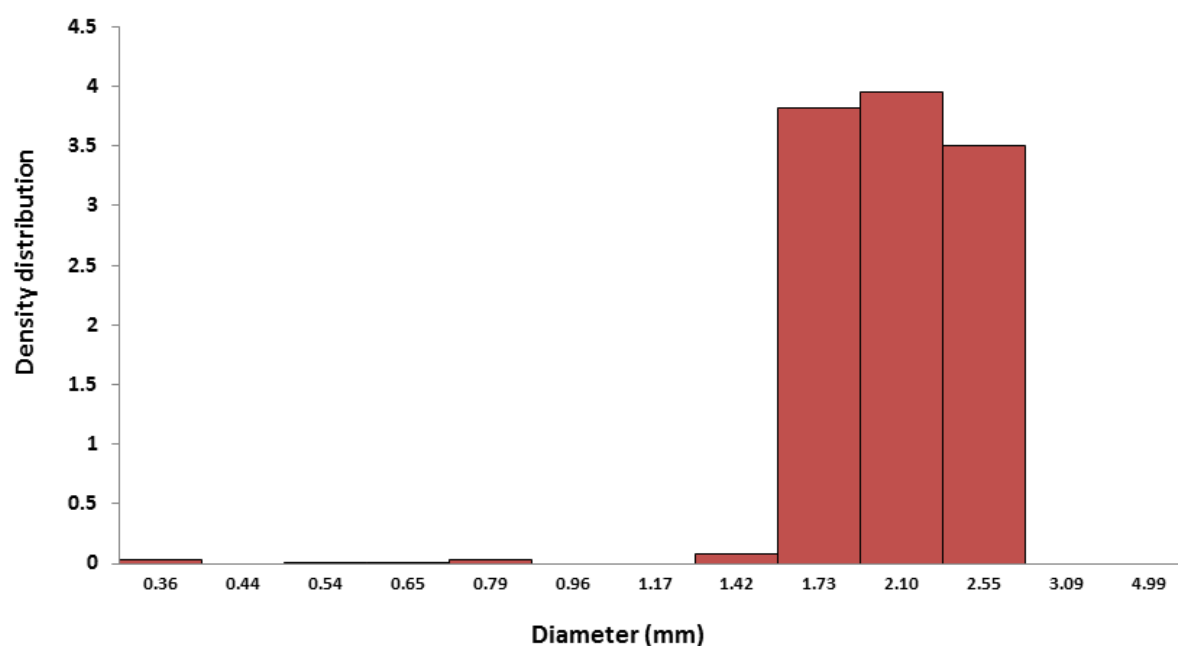


Figure 6-9 Particle size distribution of calcium pectinate beads after 24 h freeze drying measured by an image analyser (QICPIC). The density distribution is defined by Eq 3-3.

### 6.2.2.3 SEM analysis

The surface morphology and the inner structure of calcium pectinate (CaP) beads were examined using scanning microscopy electron (SEM) as described in section 3.10.7. An image of a calcium pectinate bead before freeze drying is shown in Figure 6-10, which was an egg shape and had smooth surface. The bead was cut through the centre by a blade to investigate a cross section (Figure 6-11). The SEM images showed that the internal structure of a calcium pectinate bead was porous and contained plenty of voids (Figure 6-11 c and d), which were probably caused by bubbles during the mixing step of the encapsulation process. A number of yeast cells embedded on the surface structure of microsphere can also be seen.

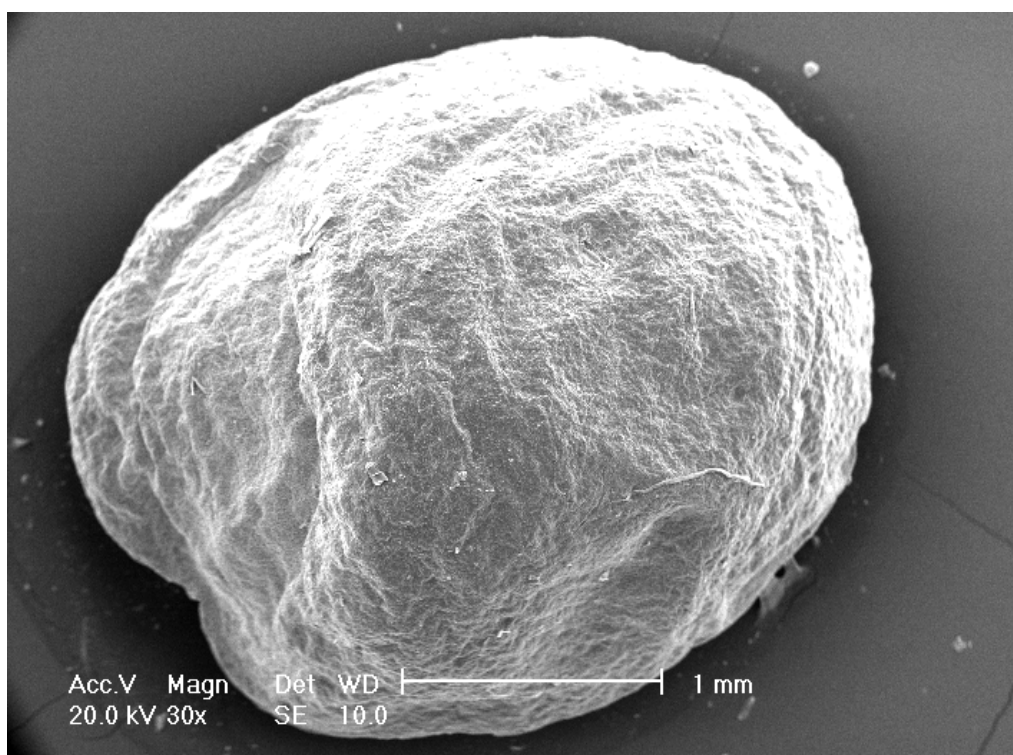
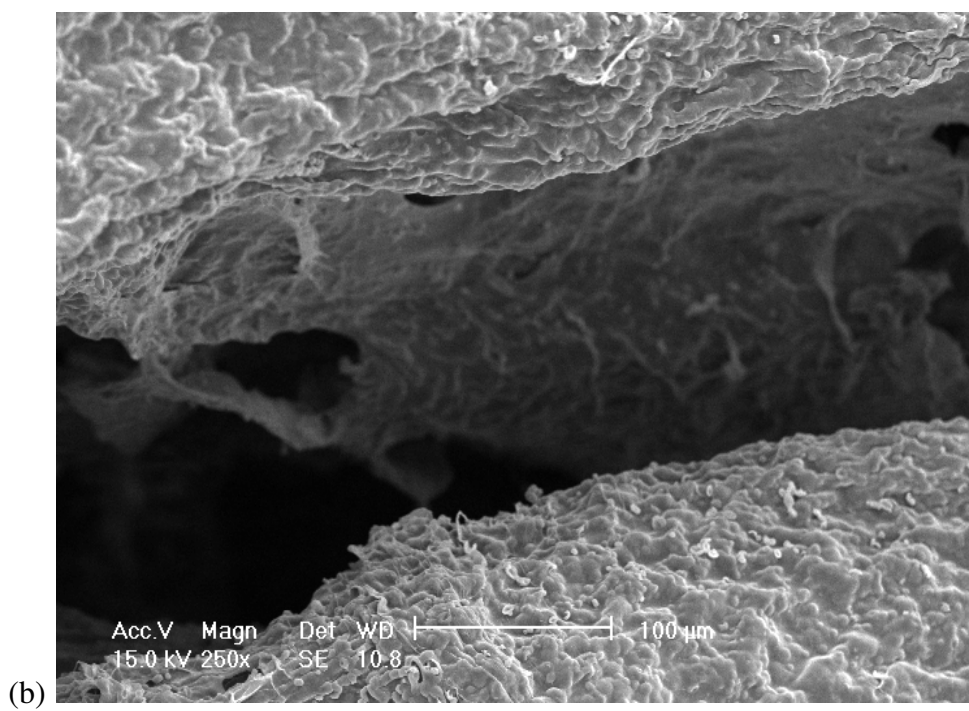
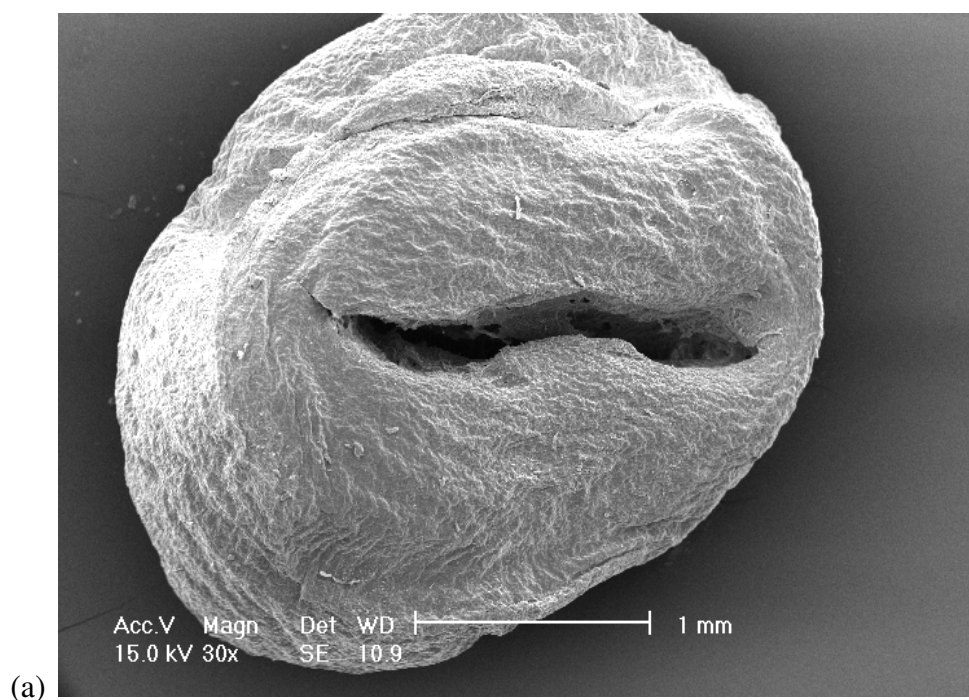


Figure 6-10 SEM image of a calcium pectinate bead before freeze drying





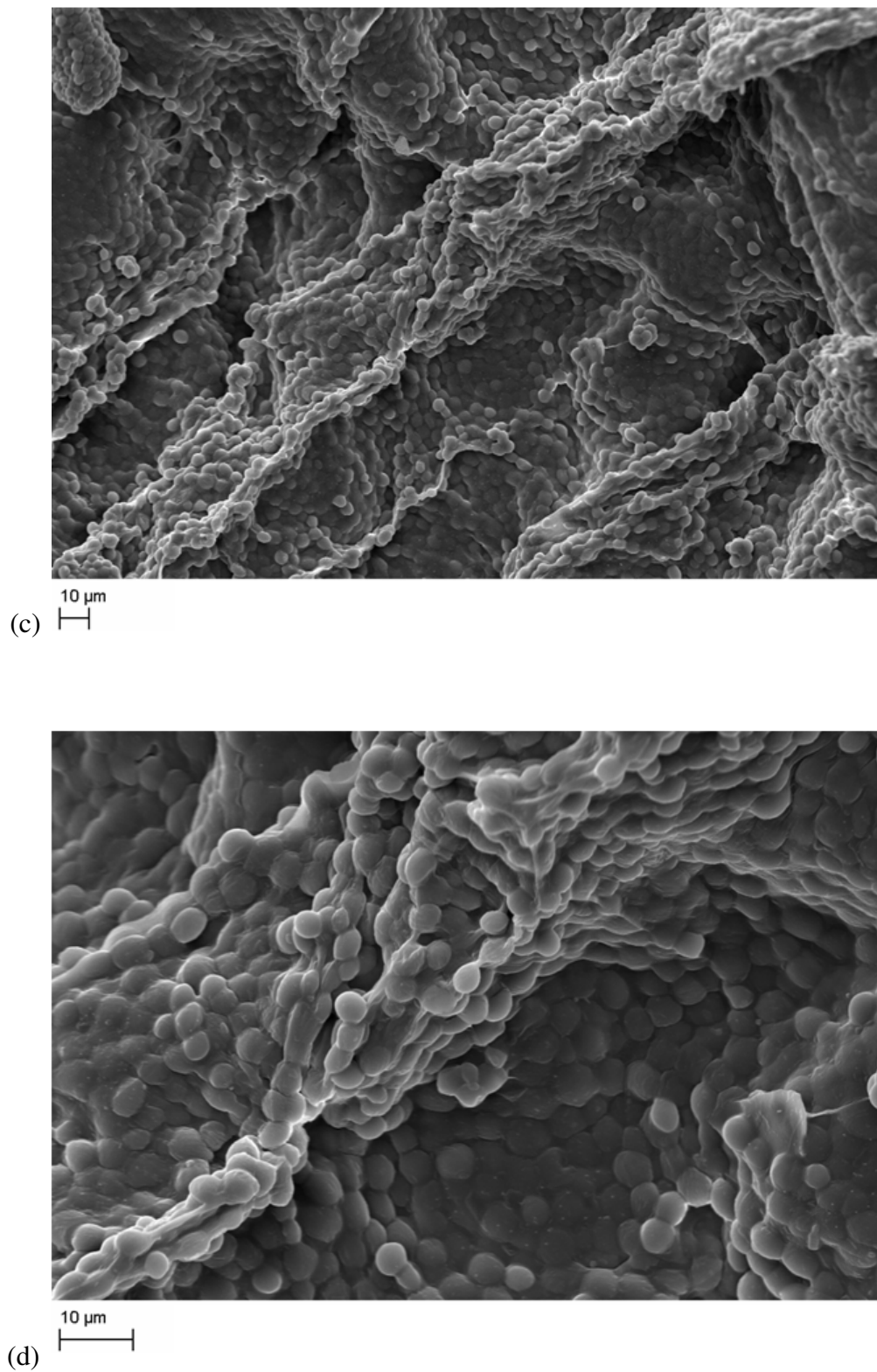
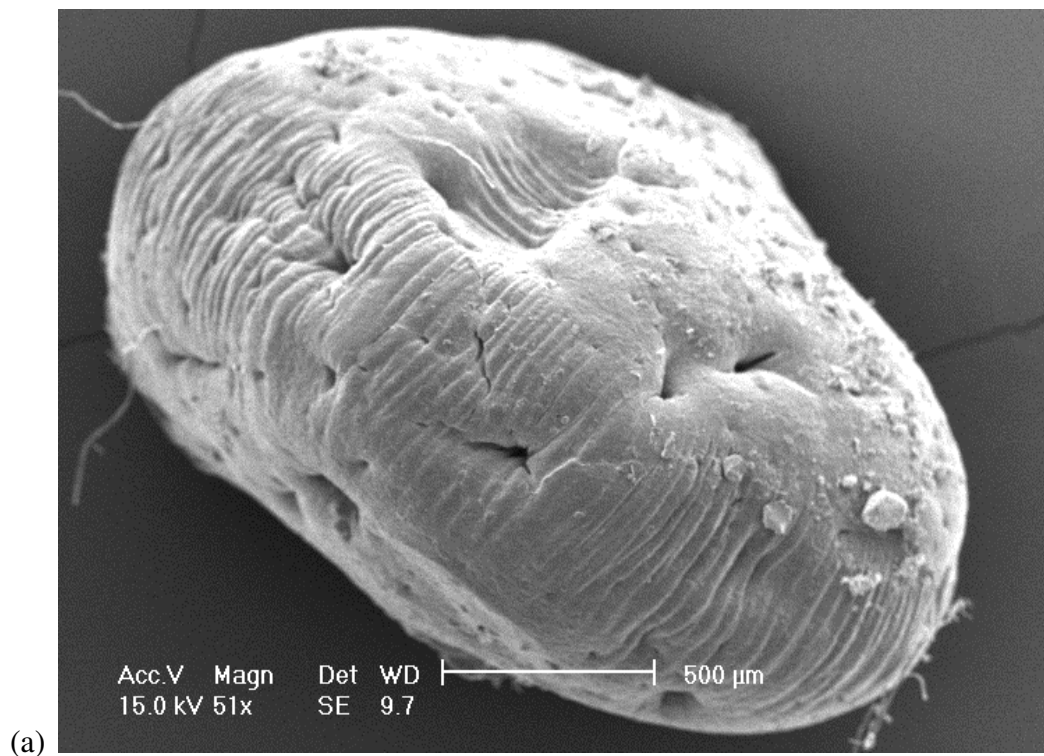


Figure 6-11 SEM images of a cross sectional area of a calcium pectinate bead before freeze drying at different scale (a) 1 mm (2) 100  $\mu\text{m}$  (3) 10  $\mu\text{m}$  (4) 10  $\mu\text{m}$

After 24 h of freeze drying, the bead size was smaller compared to those before freeze drying as a reduction of liquid content occurred with time. The structure of the dried bead was denser due to void spaces being removed during the freeze drying process. As shown in Figure 6-12(a), the bead had an irregular shape with a slightly rough surface consistent with the beads shown in Figure 6-8. With higher magnifications as shown in Figure 6-12(b) and (c), a large population of yeast can be seen on the surface. It can be noticed that the freeze drying time did not affect the shape and surface structure of the CaP bead.





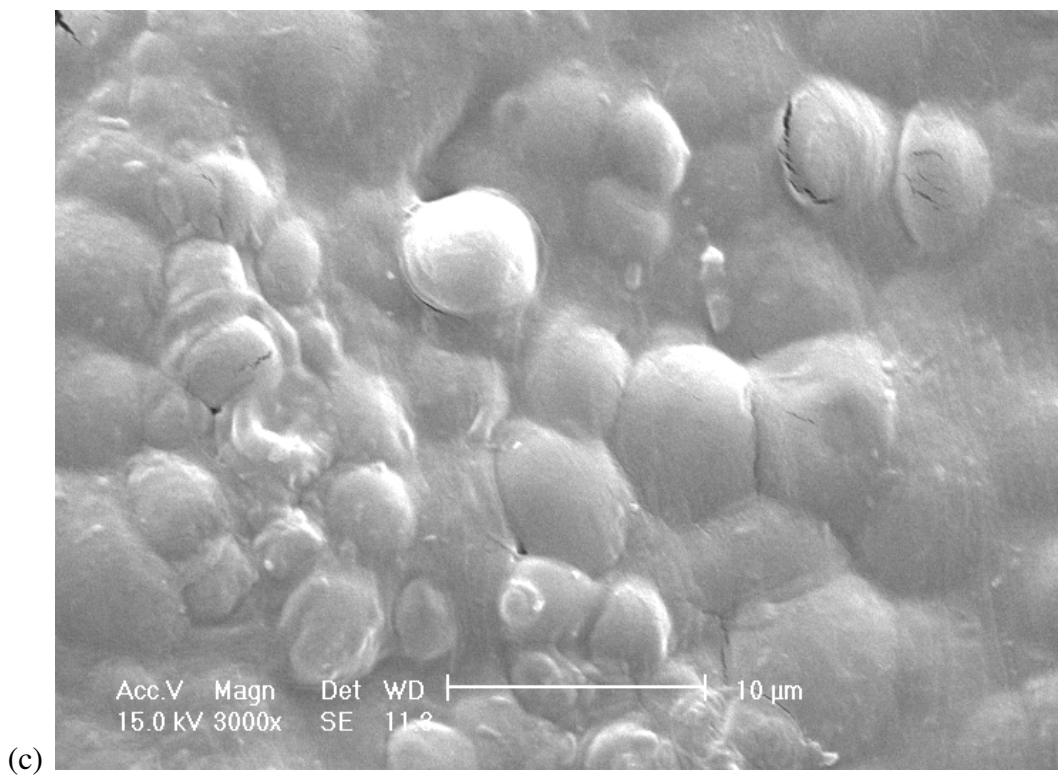
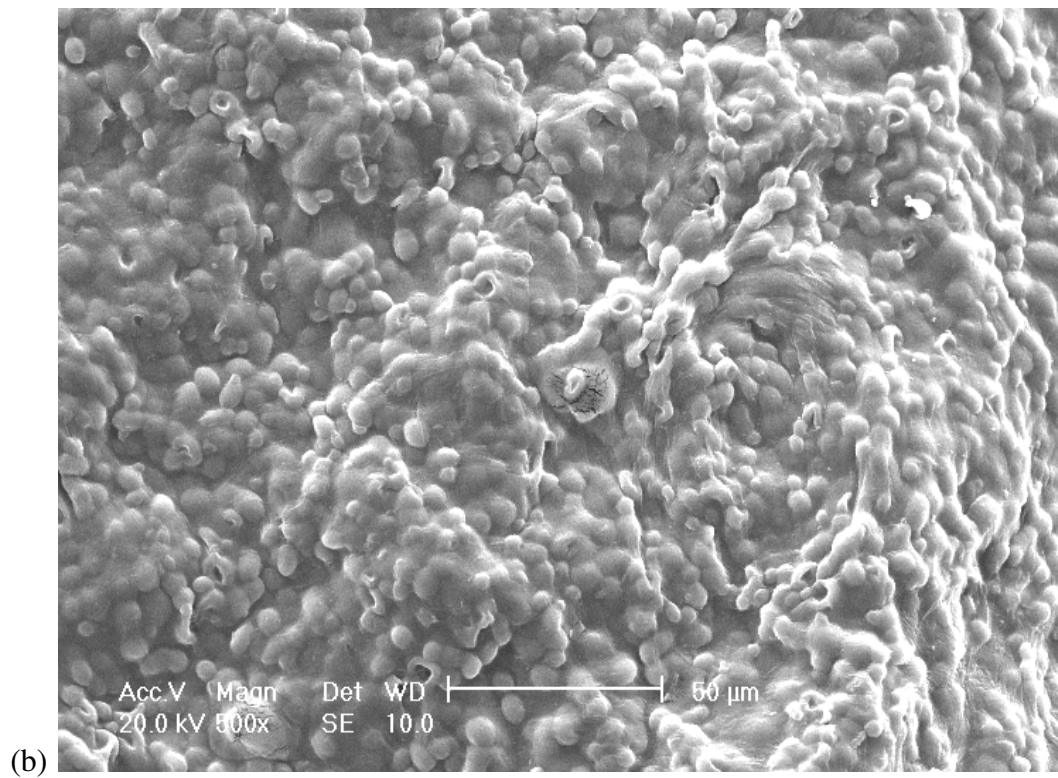


Figure 6-12 SEM images of a calcium pectinate bead after 24 h of freeze drying at different scale (1) 500  $\mu$ m (2) 50  $\mu$ m (3) 10  $\mu$ m

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#### 6.2.2.4 Water activity

Wet calcium pectinate beads obtained from the extrusion method before freeze drying ( $a_w$  0.97) were dried with (1) air drying and (2) freeze drying. The water activity of beads dried with these methods were measured every 12 h as shown in Figure 6-13. The water activity of the calcium pectinate beads after freeze drying dropped dramatically in the first 24 h to  $0.22 \pm 0.02$  which is lower (and more desirable) than the generally recommended range of between 0.25-0.3 for probiotic products (Chávez and Ledeboer, 2007, Manojlović et al., 2010, Viernstein et al., 2005). The values gradually reduced to 0.15 and 0.11 at 36 and 48 h freeze drying respectively. Pertinent to this, Viernstein et al. (2005) noted that oxidation of membrane lipids may occur thus reducing the cell viability of probiotic products if  $a_w$  is lower than 0.1.

On the other hand, water activities of calcium pectinate beads after air drying for up to 48 h were considerably higher than those from freeze drying. High water activity indicates the extent of free water in probiotic products and excess free water could allow microorganisms to be active thus shortening product shelf life. It is also reported that encapsulated cells in probiotic products are not stable when water activity is high which generally results in cell viability reduction (Mathlouthi, 2001, Weinbreck et al., 2010). Only one previous study from Crittender et al. (2006) reported high cell survival of encapsulated *Bifidobacterium infantis* in film-foaming protein-carbohydrate-oil emulsion after 5 weeks storage at 25°C and 50% relative humidity. Consequently, freeze drying was considered preferable for drying calcium pectinate beads containing yeast cells in subsequent experiments.



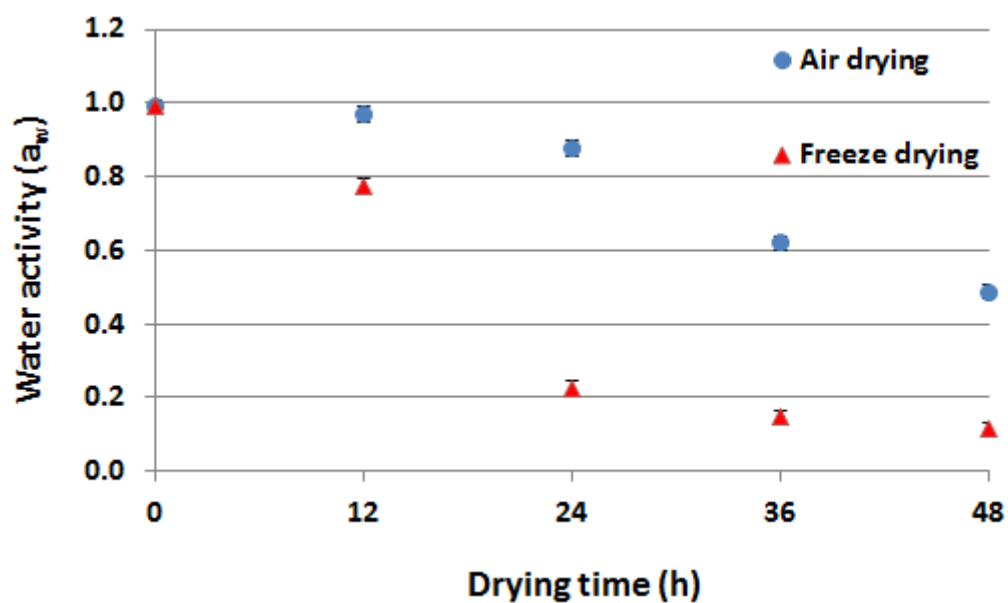


Figure 6-13 Water activity of calcium pectinate beads dried by two methods: air drying and freeze drying

### 6.2.2.5 Viability of CaP beads under fluorescence microscopy

Calcium pectinate beads (CaP) were immersed in PBS solution and stained with SYTO9 and PI before cell viability was examined under a fluorescence microscope (see section 3.12.9). The image in Figure 6-14 shows a high number of live cells stained in green and a few dead cells stained in red. It is clear that the components of SYTO9 had no interaction with pectin molecules. Therefore; flow cytometry together with SYTO9 and PI can be used to investigate viability of cells encapsulated in calcium pectinate beads and the data are comparable with BOX and PI stains.

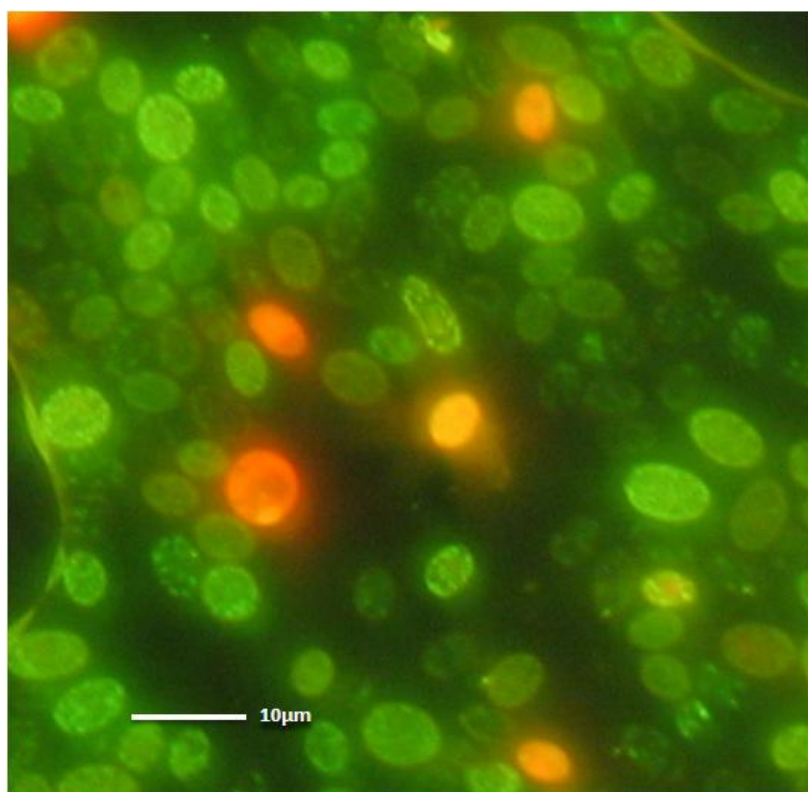


Figure 6-14 Fluorescent microscopic images of pectinate bead suspension after being stained with SYTO9 and PI (microscope magnification x40)

### 6.2.3 Optimizing calcium chloride concentration

Viability of cells in CaP beads made in different  $\text{CaCl}_2$  concentrations and freeze dried for 24 h was examined by CPC and FC.  $\text{CaCl}_2$  concentrations of 1%, 1.5%, 2% and 2.5% w/v were used in order to determine whether  $\text{CaCl}_2$  concentration had an impact on cell viability of encapsulated cells. The results in Figure 6-15 show that a maximum of  $1.7 \times 10^{10} \pm 3 \times 10^9$  CFU/g bead was observed in 1.5%  $\text{CaCl}_2$ . A similar CFU/g result was obtained at 1%  $\text{CaCl}_2$  but slightly less than that in 1.5%  $\text{CaCl}_2$ . At higher  $\text{CaCl}_2$  concentrations of 2% and 2.5%, cell viability dropped dramatically by 1.5 log and 1.4 log which corresponded to  $3.9 \times 10^8 \pm 3 \times 10^7$  CFU/g bead and  $4.6 \times 10^8 \pm 8 \times 10^7$  CFU/g bead respectively.

Flow cytometric dot plots showing various states of yeast cells encapsulated in CaP beads made in different  $\text{CaCl}_2$  concentrations are shown in Figure 6-16. The determination of cell viability by FC was done using two dual stains: PI and BOX and SYTO9 and PI. The flow cytometric results correlated well with CPC method through which the maximum cell viability was found to be at a  $\text{CaCl}_2$  concentration of 1.5%. For PI and BOX stain plots, three population groups (i.e., healthy, injured and dead cells) can be clearly seen. Using a  $\text{CaCl}_2$  concentration lower than 1.5% resulted in a higher number of viable cells (i.e., a higher number of healthy cells compared to injured cells). The percentage of live cells decreased with an increase of  $\text{CaCl}_2$ . This can be explained by the fact that calcium is one of the important ions in eukaryotic cell function, involved in metabolic processes and cell replication and that at higher concentrations, this had a negative impact on cell viability (Trofimova et al., 2010, Walker, 1994, Walker, 1999). Accordingly, it was reported that high osmotic pressure from high calcium concentrations could cause cell dehydration in fungi (Chardonnet et al., 1999). Moreover, Cao et al. (2012) have previously shown that high

calcium ion used as a cross-linking solution in a biofabrication process can damage the cell membrane by disturbing cell electrolyte and subsequently cell functions. However, there are many degrees of cell damage at which some cells can either repair themselves or recover from an injury state.

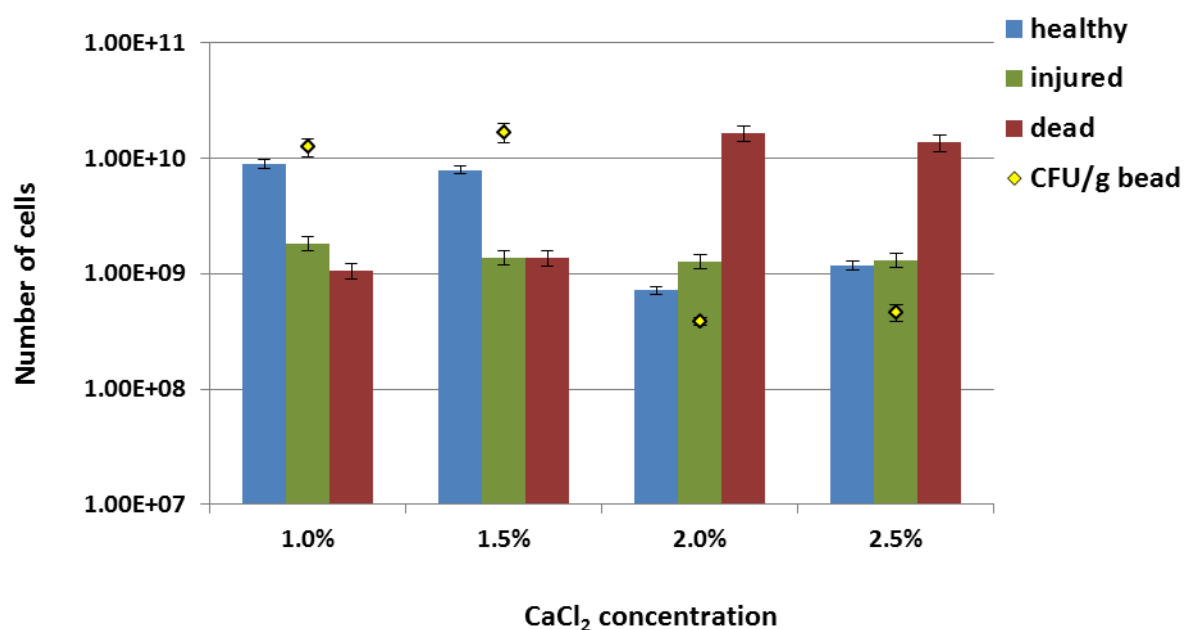
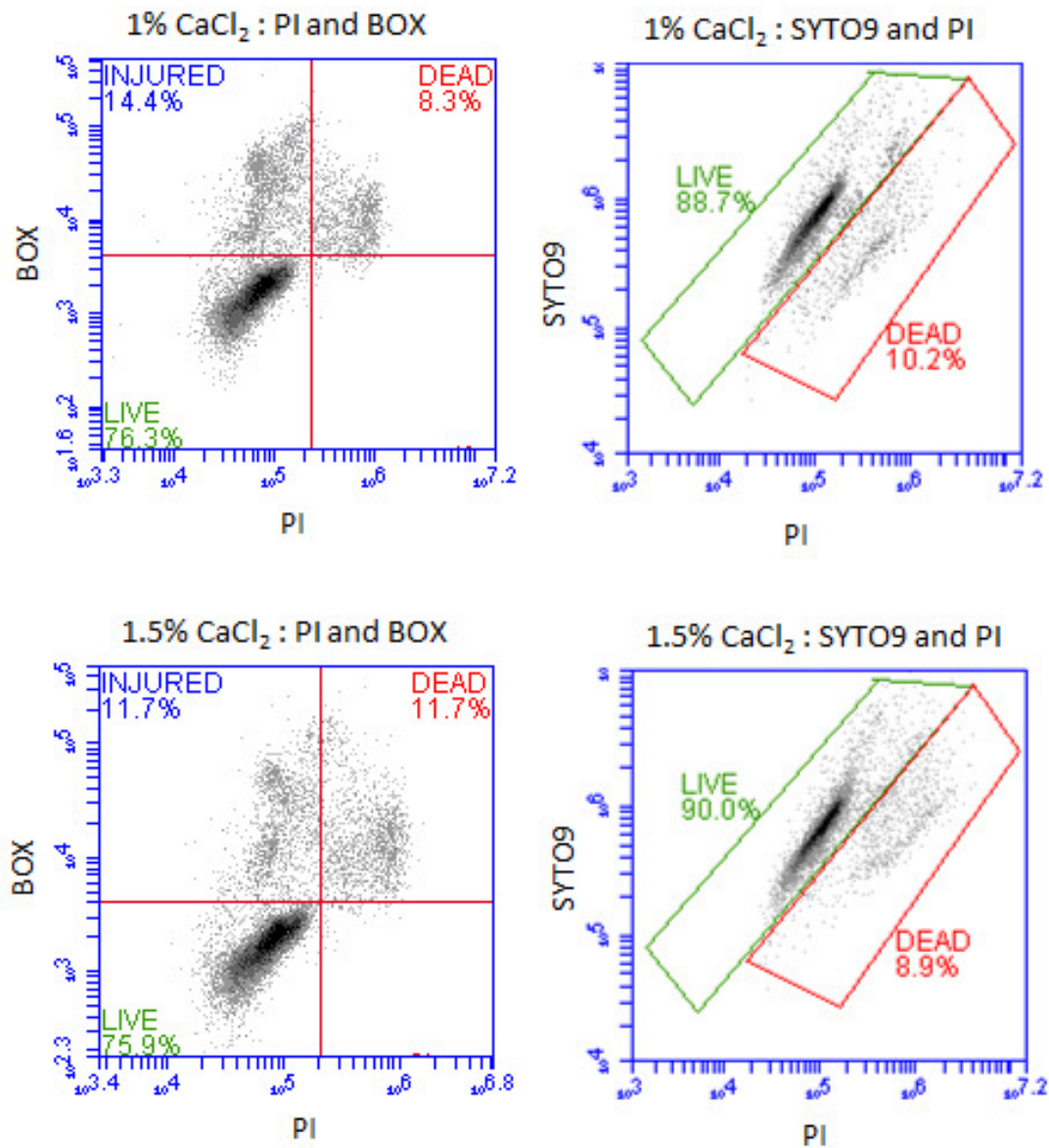


Figure 6-15 The number of healthy, injured and dead cells in 1 g of CaP beads made in different CaCl<sub>2</sub> concentrations measured by FC stained with PI and BOX with CFU/g measured by CPC

Pectinolytic enzyme - an enzyme used to simulate conditions found in the colon - considerably accelerated the release of cells from CaP beads and helped degrade pectin (Rubinstein et al., 1993, Ashford et al., 1994). It was previously reported that bovine serum albumin (BSA) encapsulated with pectin could maintain a controlled release in a pH 7.4 buffer for 5 h and BSA was released in the presence of pectinolytic enzyme afterwards (Sriamornsak, 1998). In this study, it was noticed that CaP beads degraded within two hours in the presence of the pectinolytic enzyme. Given this, it is likely that all the entrapped cells would be released in the colon. It is also worth noting this enzyme did not interact with the

fluorescent dyes used in this study (data not shown), which helps to avoid misleading data in the analysis.



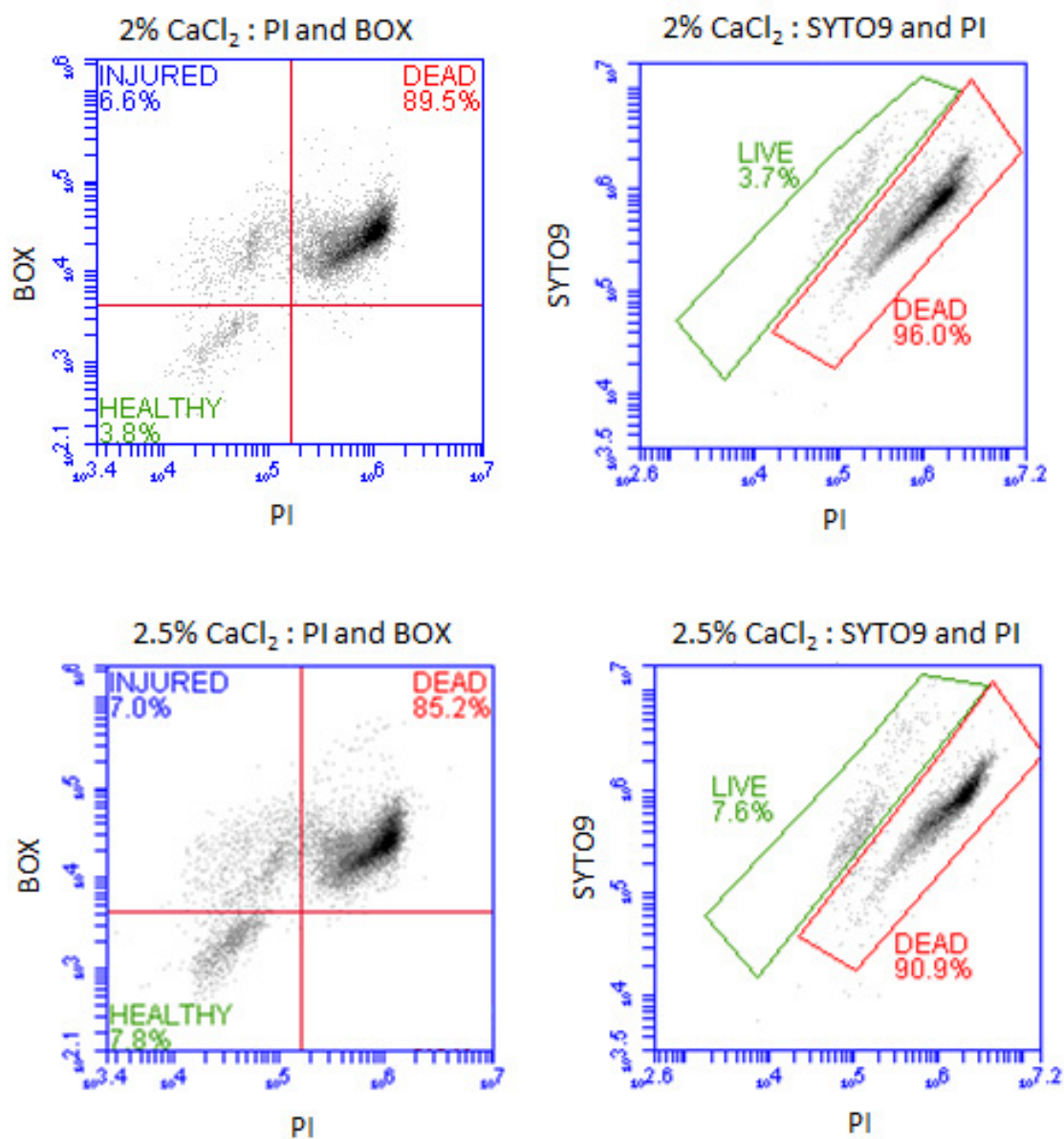


Figure 6-16 Flow cytometric dot plots showing various states of yeast cells in CaP beads made of CaCl<sub>2</sub> concentration between 1 and 2.5% (w/v) and stained with PI and BOX (left) and SYTO9 and PI (right)

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### 6.2.4 Encapsulation efficiency (EE)

Encapsulation efficiency (EE) and encapsulation efficiency based on number of culturable cell (%) of CaP beads produced in different  $\text{CaCl}_2$  concentrations were measured (as described in section 3.12.6 and 3.12.7) as shown in Figure 6-17. Encapsulation efficiency was calculated based on the total number of cells entrapped in the beads before and after encapsulation whereby such cells could be either live or dead. It has been demonstrated that more than 60% of yeast cells were encapsulated into calcium pectinate beads depending on the concentration of calcium ion presented in the solution when ion exchange occurred. The relatively high EE of 93% at a  $\text{CaCl}_2$  concentration of 1% showed good efficiency of cell entrapment and 76% of entrapped cells were culturable. However, highest encapsulation efficiency based on culturable cells at 99% was observed at a  $\text{CaCl}_2$  concentration of 1.5%, suggesting that a high number of entrapped cells were able to grow on the agar and they had great culturability. Encapsulation efficiency based on number of culturable cells per gram of CaP beads, produced using 2% and 2.5%  $\text{CaCl}_2$ , were very low which suggests that most entrapped cells at a higher calcium chloride concentration were not active and unable to form colonies onto the agar plates. In this study, cell loss during encapsulation by extrusion was very low due to the gentle method used whilst the high EE reflects the high number of cells that can be recovered after encapsulation.

The encapsulation of yeast cells with pectin in calcium chloride concentrations of 1% and 1.5% w/v proved to be effective as they showed relatively high EE and encapsulation efficiency based on number of culturable cells (%). The EE decreased with the increase of calcium chloride concentration, which might result from cell cytolysis (or cell bursting) in high concentrations of  $\text{CaCl}_2$  or the imbalance of osmotic pressure. As mentioned before,

calcium ion can have an impact on cell function so it is not surprising that high calcium ion at 2% and 2.5% w/v resulted in low encapsulation efficiency based on culturable cells. Cell shrinkage during bead formation in the gelation process might occur and also be affected by cross-linking time. This is in agreement with Sriamornsak (1999) that encapsulation efficiency of CaP beads with lower concentrations of calcium ion and a cross-linking time of 20 min was greater than using higher concentrations. Yeast cells exposed to calcium chloride solution for a long time might burst due to an osmotic imbalance, thus leading to cytolysis and increasing the number of dead cells. Such dead cells slightly affected EE presumably due to cell lysis and drastically affected encapsulation efficiency based on number of culturable cells (%).

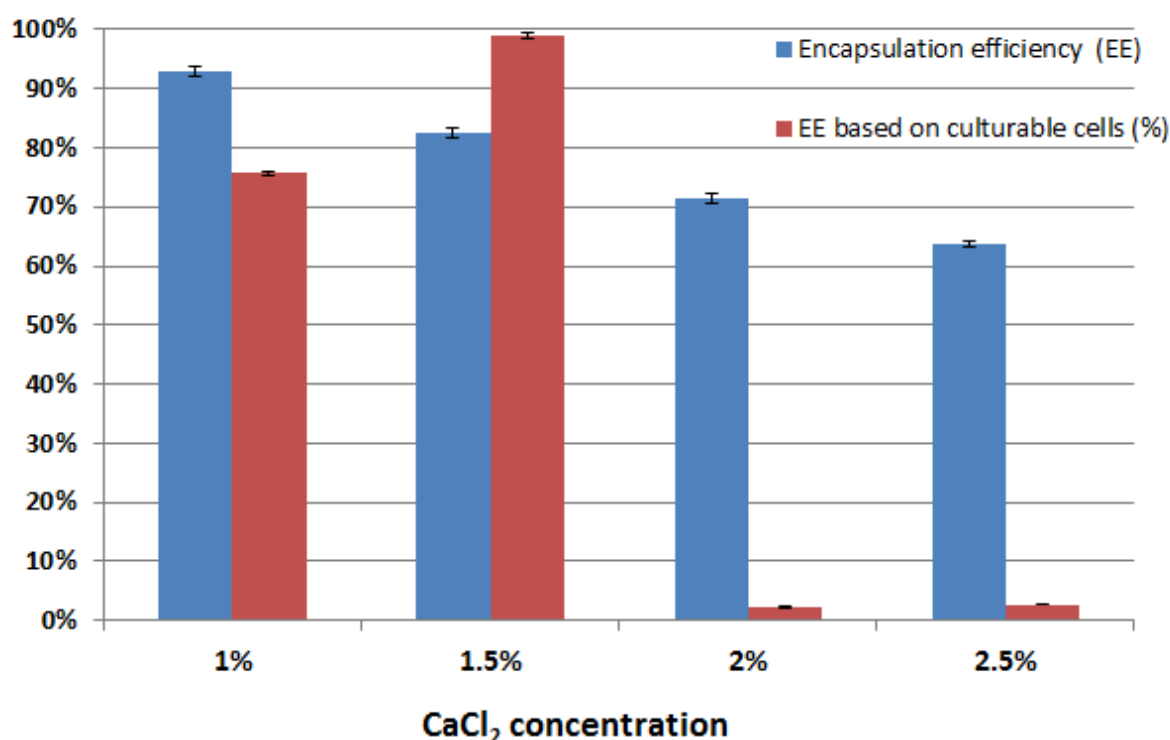


Figure 6-17 Encapsulation efficiency (EE) and EE based on number of culturable cells (%) of CaP beads prepared using different CaCl<sub>2</sub> concentrations



### 6.2.5 Effect of freeze drying time on viability and $a_w$ of calcium pectinate beads

Calcium pectinate (CaP) beads made with 5% (w/v) pectin and 1.5% (w/v)  $\text{CaCl}_2$  cross-linking solution were used in this experiment. The impact of freeze drying time on encapsulated cells in respect of  $a_w$  and cell viability is summarized in Table 6-1. It can be seen that after 12 h freeze drying time, CaP beads contained  $1.8 \times 10^{10} \pm 3.7 \times 10^9$  CFU/g bead and had  $a_w$  of  $0.34 \pm 0.02$ , which exceeded the long term storage stability of 0.3 (Dr Peter Jüsten, Lesaffre, France, Personal communication). Therefore, it is necessary to prolong the freeze drying time accordingly. After 24 h of freeze drying, CaP beads containing  $1.7 \times 10^{10} \pm 4.3 \times 10^9$  CFU/g bead and  $a_w$  of  $0.27 \pm 0.02$ . CFU/g bead further reduced to  $1.9 \times 10^9 \pm 6.8 \times 10^8$  over 36 h and  $1.4 \times 10^9 \pm 1.2 \times 10^8$  over 48 h of freeze drying whereas the  $a_w$  decreased to  $0.15 \pm 0.02$  and  $0.13 \pm 0.02$  respectively. It can be seen that the prolonging drying time significantly affected water activity, which is consistent with those reported in literature (Hahn-Hägerdal, 1986, Harris, 1981, Vesterlund et al., 2012).

Table 6-1 Cell survival and  $a_w$  of entrapped *S. cerevisiae* in CaP beads at different freeze drying times

Freeze drying time (h)	CFU/g CaP	$a_w$
12	$1.8 \times 10^{10} \pm 3.7 \times 10^9$	$0.34 \pm 0.02$
24	$1.7 \times 10^{10} \pm 4.3 \times 10^9$	$0.27 \pm 0.02$
36	$1.9 \times 10^9 \pm 6.8 \times 10^8$	$0.15 \pm 0.02$
48	$1.4 \times 10^9 \pm 1.2 \times 10^8$	$0.13 \pm 0.02$

Flow cytometric results for CaP bead suspensions after being stained with PI and BOX are shown in Figure 6-18. It can be noticed that at 12 h freeze drying time the number of healthy cells was higher than injured cells. Contrarily, a freeze drying time between 24 and 48 h resulted in a higher number of injured cells than healthy cells. This indicates that removal of water during freeze drying (low  $a_w$ ) could change the physiological state of cells causing more cell injury and cell death and thus affecting the number of culturable cells, i.e. CFU/g bead. While freeze drying is considered a method that preserves cell viability, cell losses usually occur during the freezing process prior to drying (Uzunova-Doneva and Donev, 2000). During the freeze drying process, ice crystal structures form on the surface of cells resulting in fragile cell membranes.

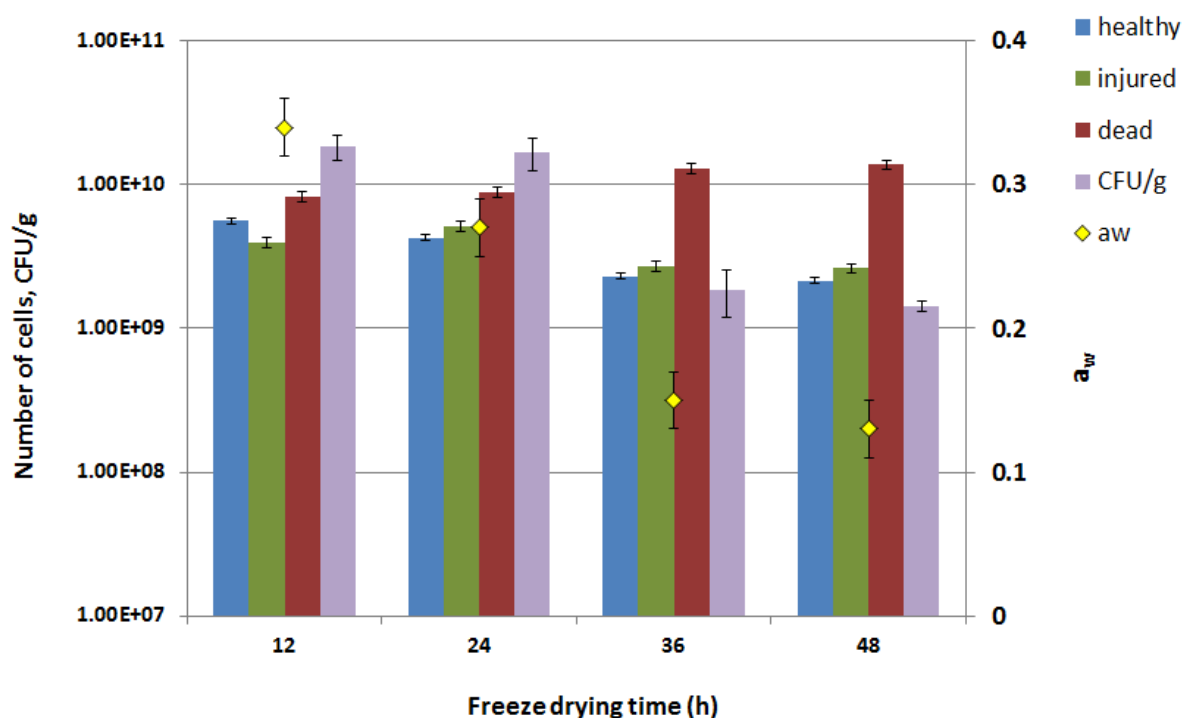
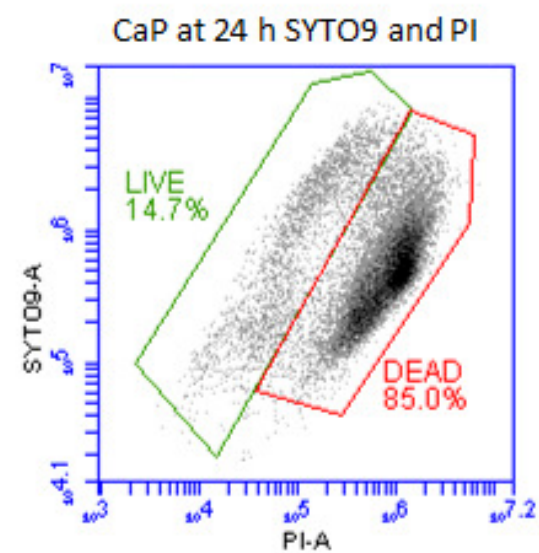
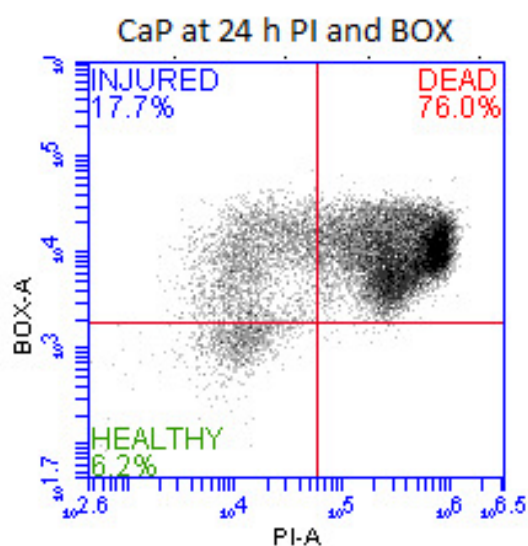
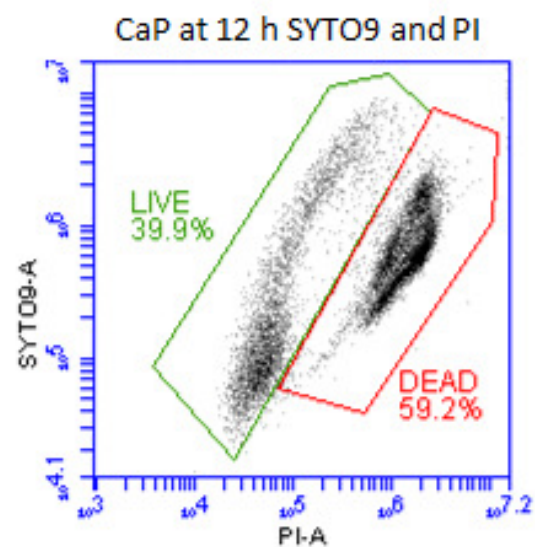
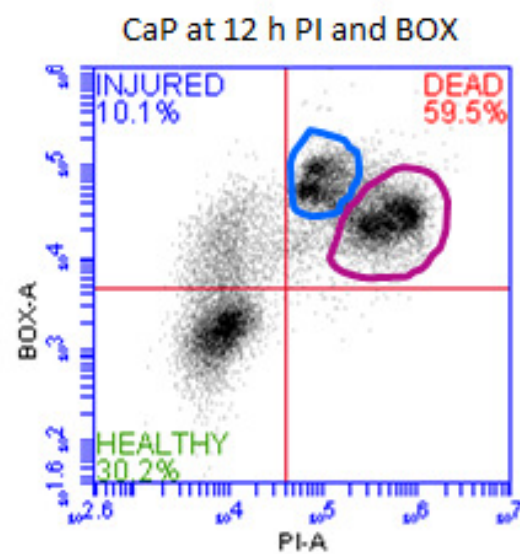


Figure 6-18 Number of healthy, injured and dead cells obtained from FC, CFU/g bead of CaP beads and water activity versus freeze drying times.

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Flow cytometric dot plots showing various physiological states of cells encapsulated in CaP beads at different freeze drying times are presented in Figure 6-19. For PI and BOX stain plots, it can be clearly seen that a population of healthy cells at 12 h freeze drying time became injured when prolonging freeze drying time to 24 h. It is possible that the change of cell physiological state occurred when large ice crystal structures formed onto cells resulting in fragile cell membranes as previously mentioned. However, when prolonging time from 24 h up to 48 h, some injured cells became dead whereas the number of healthy cells remained almost the same. The results of SYTO9 and PI stain plots were also consistent with those stained with PI and BOX. Interestingly, two groups of dead cells were observed within the population after 12 h freeze drying which might correspond to debris having different sizes (marked in blue and purple in PI and BOX plot). This debris might occur during the encapsulation process and smaller size was thought to be due to cell shrinkage in  $\text{CaCl}_2$  solution due to an osmotic imbalance. When CFU/g and  $a_w$  values are taken into account with FC data, it was decided to dry CaP beads for 24 h which resulted in desirable CFU/g bead and  $a_w$  and these beads were used in all subsequent experiments.



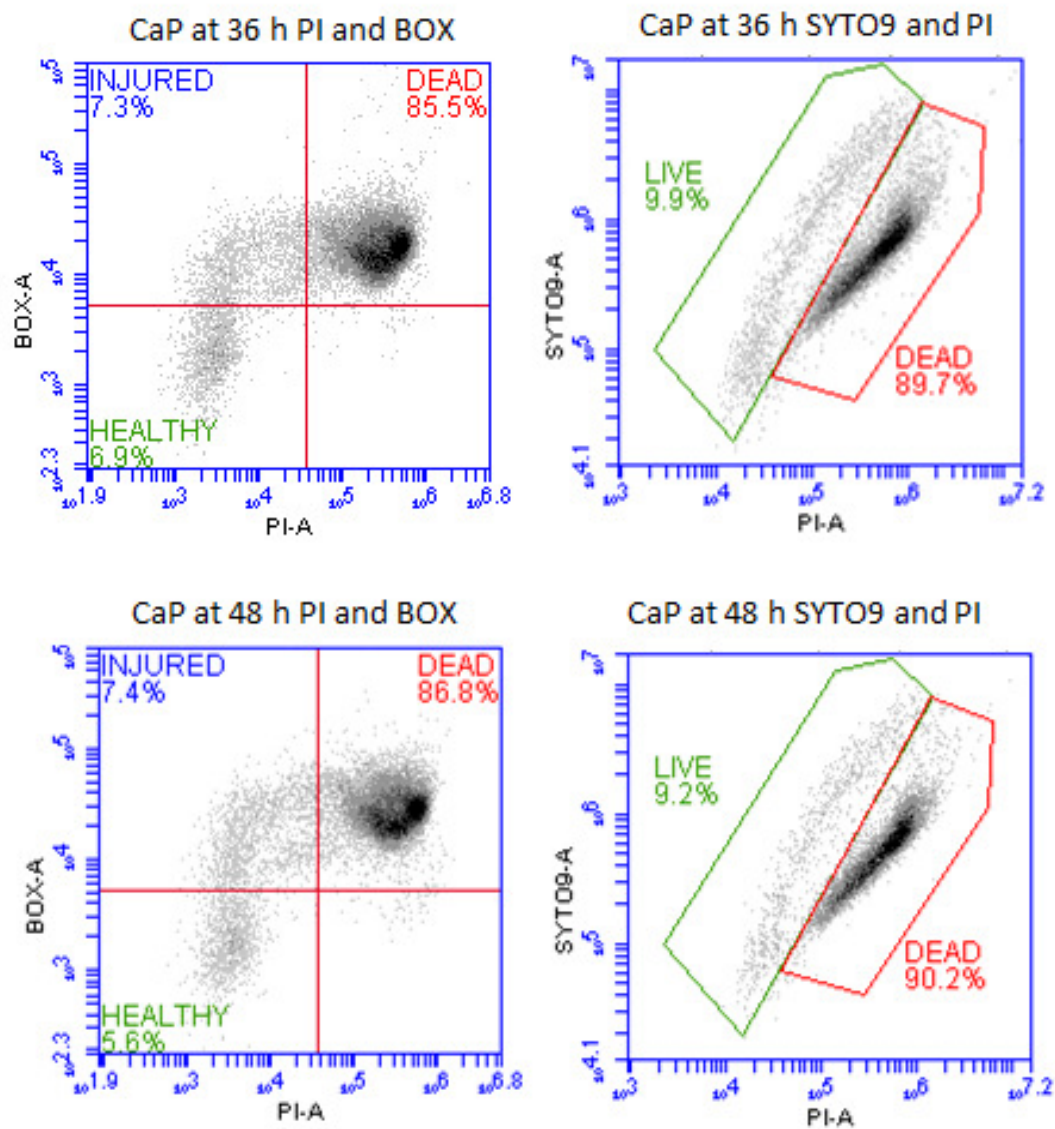


Figure 6-19 Flow cytometric dot plots showing various physiological states of yeast cells in CaP beads at various freeze drying times up to 48 h, which were stained with PI and BOX (left) and SYTO9 and PI (right)

### 6.2.6 Varying pectin concentration

Calcium pectinate beads were produced with different pectin concentrations (3%, 4% and 5% w/v) and freeze dried for 24 h. Pectin concentration had no influence on the appearance of the beads (images not shown). However, it was observed that calcium pectinate beads did not have a spherical shape in the cross-linking solution when pectin concentration lower than 3% (w/v) was used. Figure 6-20 shows an image of dried calcium pectinate particles made using a pectin concentration of 2% (w/v), which are flat. This might be due to low amounts of negatively charged carboxyl groups of pectin that cannot completely cross-link even with an excess of divalent calcium ions during the gelation process.

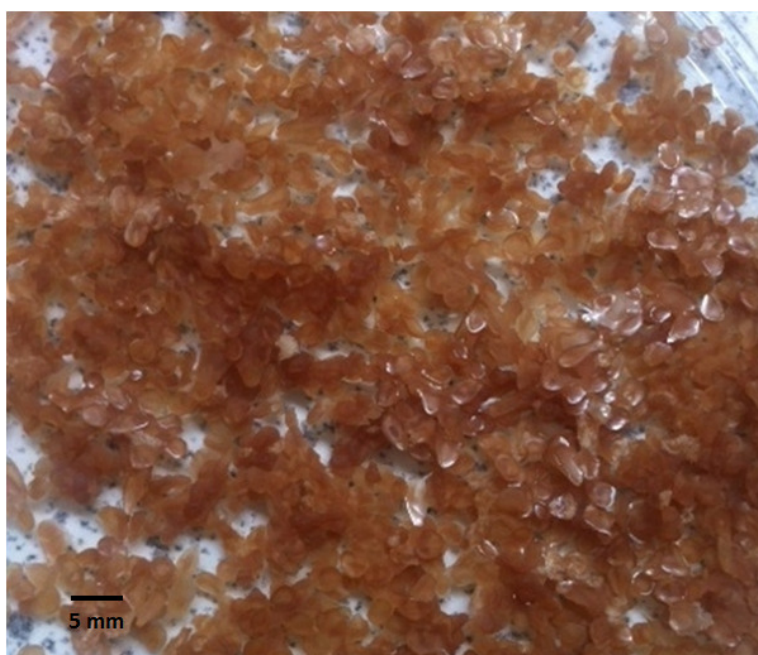


Figure 6-20 An optical image of calcium pectinate particles made of 2% pectin concentration and freeze dried for 24 h, scale bar 5 mm

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Further investigation of cell viability for beads made of 3%, 4% and 5% pectin was undertaken. The beads were immersed in PBS solution for 2 h and cell viability in these suspensions was examined. No significant difference in cell viability across the three different concentrations was found (data not shown). The beads containing cells were then mixed with MCC in 50/50 (wt/wt) ratio and compacted into tablets at 90 MPa. Tensile strength and cell viability for tablets containing these beads made from different pectin concentrations were measured by CPC and FC together with PI and BOX and the results are presented in Figure 6-21.

The pectin concentrations of 3% and 4% did not appear to have an impact on CFU/g tablet but with 5% (w/v) pectin, encapsulated cells were more protected from compaction pressure resulting in an increased CFU/g tablet. Tablet tensile strength increased with an increase of pectin concentration. Using 5% (w/v) pectin, a desirable tensile strength of  $1.1 \pm 0.1$  MPa was obtained (meeting Lesaffre's specification). It was also found that a mixture of WY and 6% (w/v) pectin was too viscous and consequently was not suitable for extrusion through a G23 needle. This highlights that an optimal amount of pectin can provide good protection to yeast cells from compaction pressure. Therefore, 5% (w/v) pectin was considered as a suitable concentration to make CaP beads for further development of the probiotic tablet.



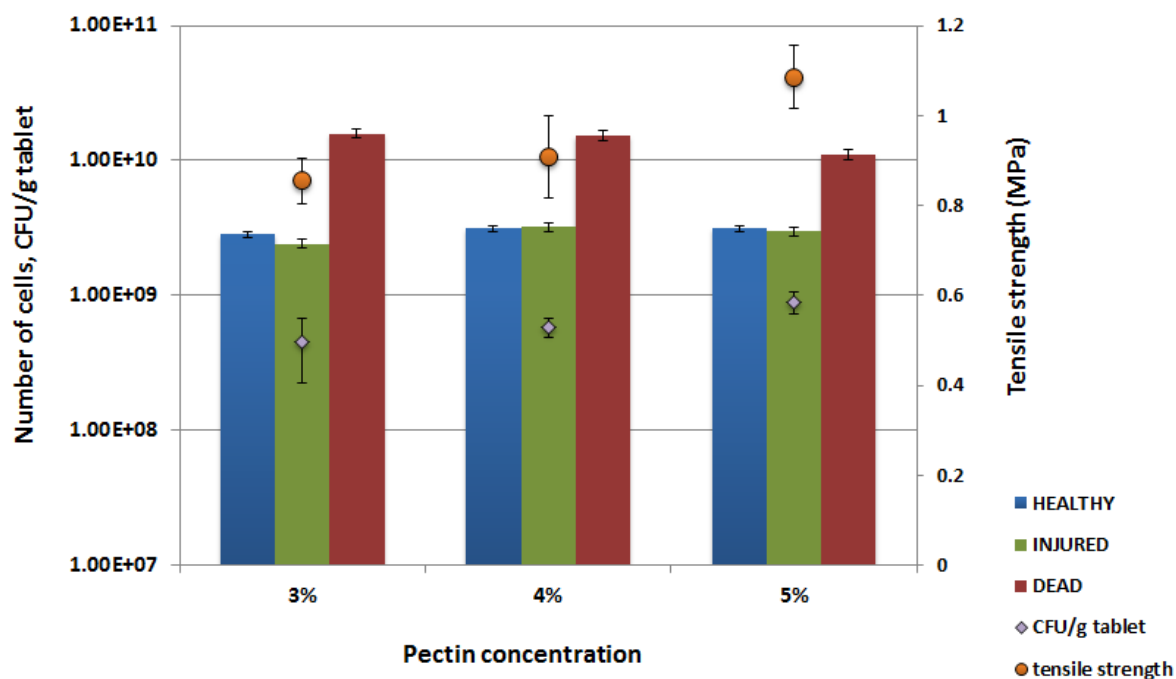


Figure 6-21 CFU/g tablet versus tablet tensile strength and the number of healthy, injured and dead cells in 1 g of calcium pectinate beads made using pectin concentration of 3%, 4% and 5% mixed with MCC in 50/50 (wt/wt) ratio before compacting at 90 MPa

Flow cytometric dot plots showing cell physiological states in tablets containing CaP beads made using different pectin concentrations and MCC in 50/50 (wt/wt) ratio are shown in Figure 6-22. In PI and BOX stain plots the percentage of injured cells was relatively low when 3% pectin concentration was used. However, the percentage of healthy cells remained constant at all pectin concentrations. SYTO9 and PI stain plots show no significant difference of live and dead cells between pectin concentrations of 4% and 5%. Therefore, flow cytometric data of two dual stains were consistent with CFU/g results and it is possible that the number of culturable cells (CFU/g) was related to the number of injured cells to some extent. Moreover, at low pectin concentration, encapsulated yeast cells were not well protected from compaction pressure resulting in a high number of dead cells. Moreover, tablets containing these beads were not rigid enough as shown by the low tensile strength.



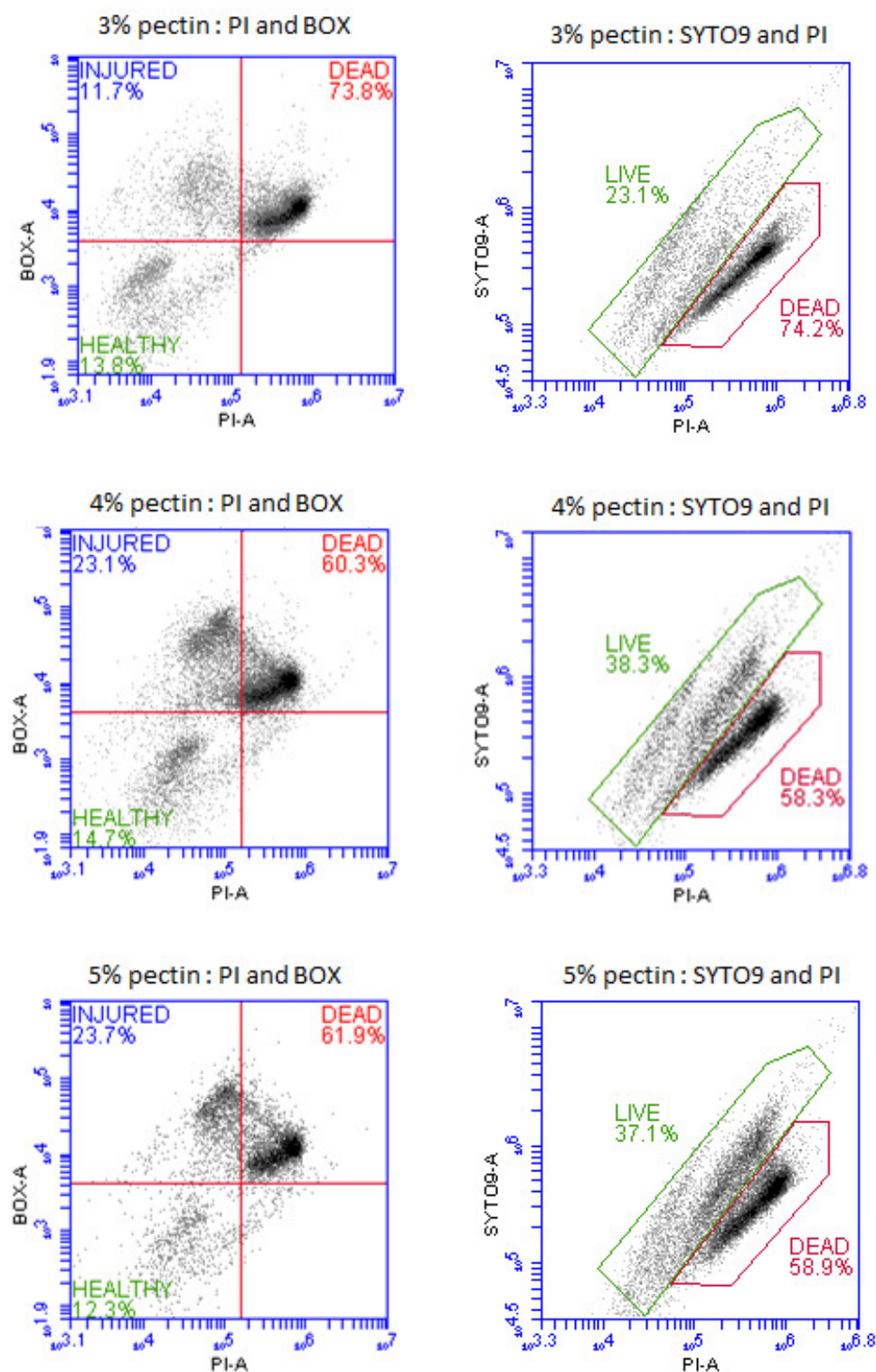


Figure 6-22 Flow cytometric dot plots showing various physiological states of tablets containing calcium pectinate beads made using different pectin concentrations and MCC in 50/50 (wt/wt) ratio before compaction at 90 MPa (c.a. 12 kN): PI and BOX (left) and SYTO9 and PI (right)

### 6.3 CONCLUSIONS

Yeast cells were successfully encapsulated with shellac and pectin cross-linked with calcium ions in order to protect them from tablet compaction pressure and from harsh environments during storage. The physical properties of calcium shellac and pectinate beads characterized by particle size distribution, SEM and water activity were explained. It was found that calcium shellac beads after 24 h freeze drying resulted in desirable water activity ( $a_w$ -0.12±0.02) as shown in Figure 6-3. Calcium shellac beads showed good compactibility during the tableting process because they were compacted to tablets without any filler. However, they did show low solubility and poor disintegration after they were immersed in neutral pH of SIF for 6 h as shown in Figure 6-7b. This finding highlights a strong interaction between shellac molecules and calcium ions in the cross-linking solution. Notwithstanding, the study also highlights a problem in the interaction between shellac and SYTO9, which makes it difficult to determine the viability of entrapped cells in calcium shellac beads (Figure 6-6). Therefore, yeast cells encapsulated with shellac might have limitations in probiotic applications.

The results have demonstrated that freeze drying is a more effective and efficient method to remove water from CaP beads than air drying. Encapsulated yeast cells with 5% (w/v) pectin in 1.5% (w/v) calcium chloride solution after freeze drying for 24 h appeared to provide the best cell survival rate and desirable water activity ( $a_w$ -0.27±0.02). This combination created spherical beads with narrow particle size distribution that offered high encapsulation efficiency (99%) and high encapsulation efficiency based on number of culturable cells (65%) as explained in section 6.2.4. Freeze drying time did not affect the shape of CaP beads but did affect viability of encapsulated cells. Prolonging freeze drying times resulted in

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decreasing of  $a_w$  and hence, a population of healthy cells became injured and consequently died resulting in low CFU. Using pectin concentration less than 3% (w/v), spherical beads cannot be formed and thus, cells were not well protected from compaction pressure. High calcium chloride concentration might cause cell bursting due to an osmotic imbalance, thus leading to an increase in the number of dead cells.

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## CHAPTER 7

### EVALUATION OF CALCIUM PECTINATE (CAP) BEAD PROPERTIES AND COMPACTION OF CAP BEADS INTO TABLET FORM

#### 7.1 INTRODUCTION

Results on calcium pectinate (CaP) beads made with 5% (w/v) pectin and crosslinked with 1.5% (w/v) calcium chloride solution presented in Chapter 6 demonstrated that the cells encapsulated in CaP beads were protected to some extent from tablet compaction pressure. Moreover, they had a low water activity ( $a_w \sim 0.27 \pm 0.02$ ) after 24 h freeze drying, which may inhibit bacterial growth during storage and be desirable for long term storage stability. In order to understand the mechanism of the cell protection during compaction of CaP beads with MCC, it is essential to characterize and understand the mechanical properties of these beads.

The diametrical compression test has been widely used to examine the material properties of pharmaceutical dosage forms (Fell and Newton, 1970, Stanley and Newton, 1977, Pitt et al., 1988, Stanley, 2001). It measures the compressive force between two flat platens by allowing the load-applying machine to compress the material up to the point of failure (Kristensen et al., 1985, Bonollo et al., 1994, Soltesz et al., 1995). This method can be used to evaluate the mechanical behaviour of large particles ( $>200 \mu\text{m}$ ) of brittle materials, which exhibit plastic deformation before fracture (Bonollo et al., 1994, Procopio et al., 2003). For smaller particles

than 200  $\mu\text{m}$  e.g. calcium alginate microspheres with a diameter between 80 and 130  $\mu\text{m}$ , Wang et al. (2005) used a micromanipulation technique to characterise their mechanical properties. Typical mechanical testing includes compression of single particles to a given deformation and then holding, compression of single particles at different speeds and loading and unloading in order to determine their elastic, viscoelastic or elastic-plastic properties.

The aim of the work described in this chapter was to evaluate the material properties of CaP beads by diametrical compression testing in order to understand why they can protect cells during tableting. In addition, CaP beads made with 5% pectin and crosslinked with 1.5%  $\text{CaCl}_2$  were mixed with MCC in 50/50 ratio before compacted at different pressures. A storage stability test was undertaken to estimate the long-term shelf life of these tablets. Cell viabilities for the tablets before and after storage at 4°C were measured by colony plate counting (CPC) (Postgate, 1969) and flow cytometry (FC) together with some fluorescent dyes as described in method section 3.2 and 3.8 respectively (Boswell et al., 1998, Hewitt and Nebe-Von-Caron, 2001). The factors that affected tablet formulation and cell viabilities including water activity (Costa et al., 2002) and tablet tensile strength, were investigated.

## **7.2 RESULTS AND DISCUSSION**

### **7.2.1 Diametrical compression test**

Single CaP beads made with 5% (w/v) pectin and 1.5% (w/v)  $\text{CaCl}_2$  crosslinking solution were compressed with a 100 N flat platen load at a speed of 0.01  $\text{mm s}^{-1}$  using a Zwick/Roell machine (as described in section 3.13.1). At least ten beads were compressed at this velocity to obtain statistically significant results. The data of compressive force and displacement

recorded from a Zwick machine were plotted for typical examples in Figure 7-1 and Figure 7-2. Within the ten beads, two kinds of material behaviours were observed. Figure 7-1 shows a fracture event at a force of 60 N suggesting the point at which the structure of a CaP bead was ruptured. After this point, the bead was irreversibly deformed and this was observed in 7 out of 10 beads tested. Typical images of the fractured CaP beads after being compressed with a 100 N load cell are shown in Figure 7-2. The second material behaviour is shown in Figure 7-3 where no fracture event was observed during compression. The unloading curve shows the bead was plastically deformed since the force drops to zero before the displacement drops to zero.

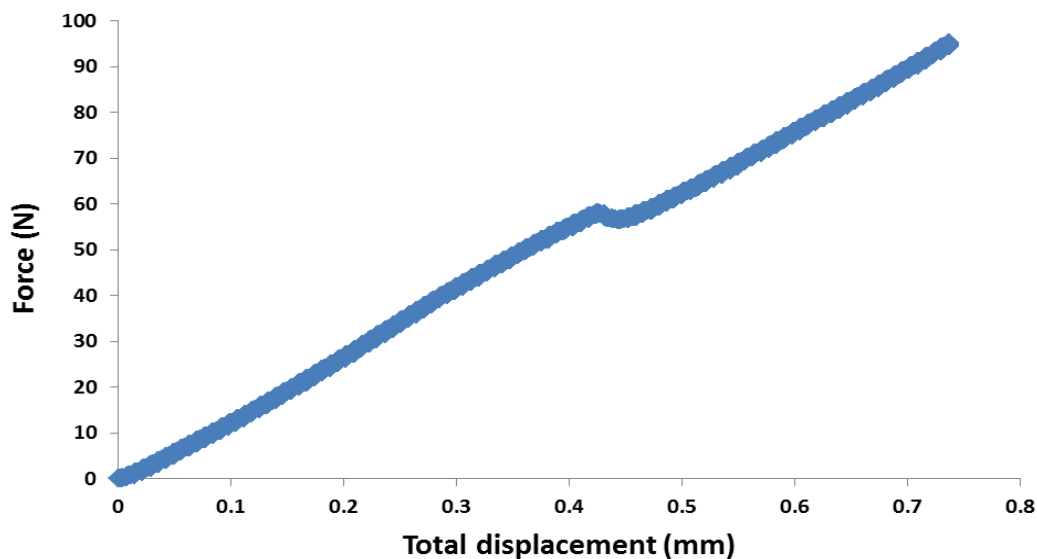


Figure 7-1 Force-displacement data of a single calcium pectinate bead (1.8 mm initial diameter) compressed with a 100 N load at a compression speed of  $0.01 \text{ mm s}^{-1}$



Figure 7-2 Typical optical image of CaP bead fractured by a diametrical compression test after being compressed with a 100 N load cell

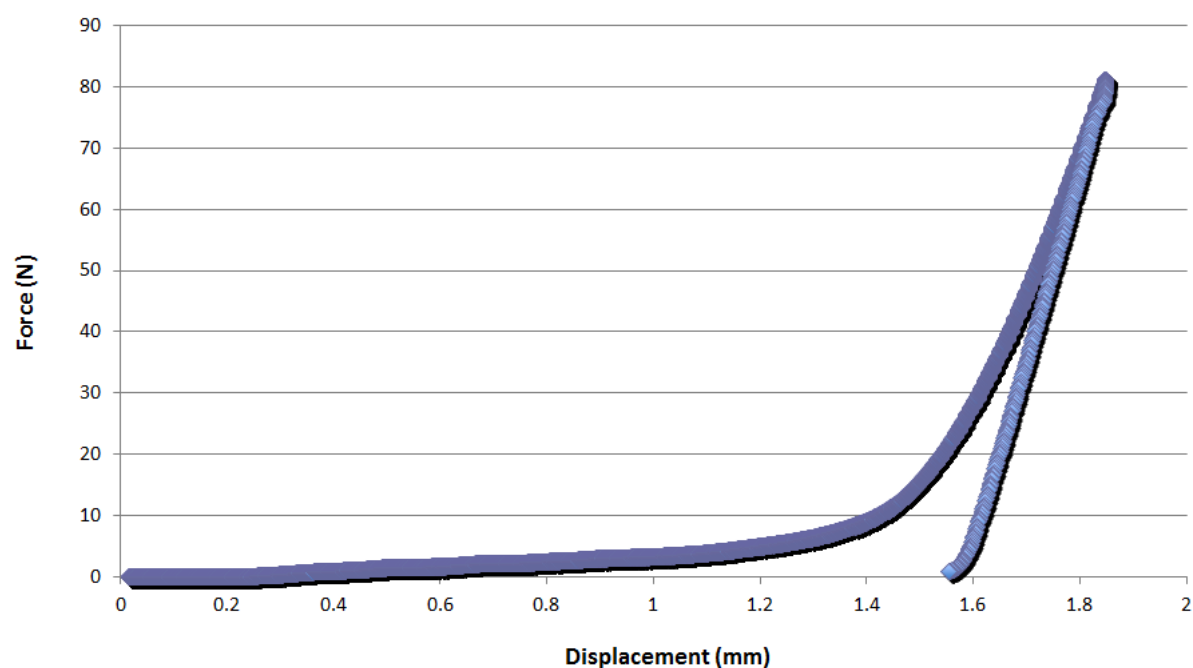


Figure 7-3 Force-displacement curve showing loading and unloading data of a single CaP bead (2.3 mm in diameter) at a compression speed of  $0.01 \text{ mm s}^{-1}$

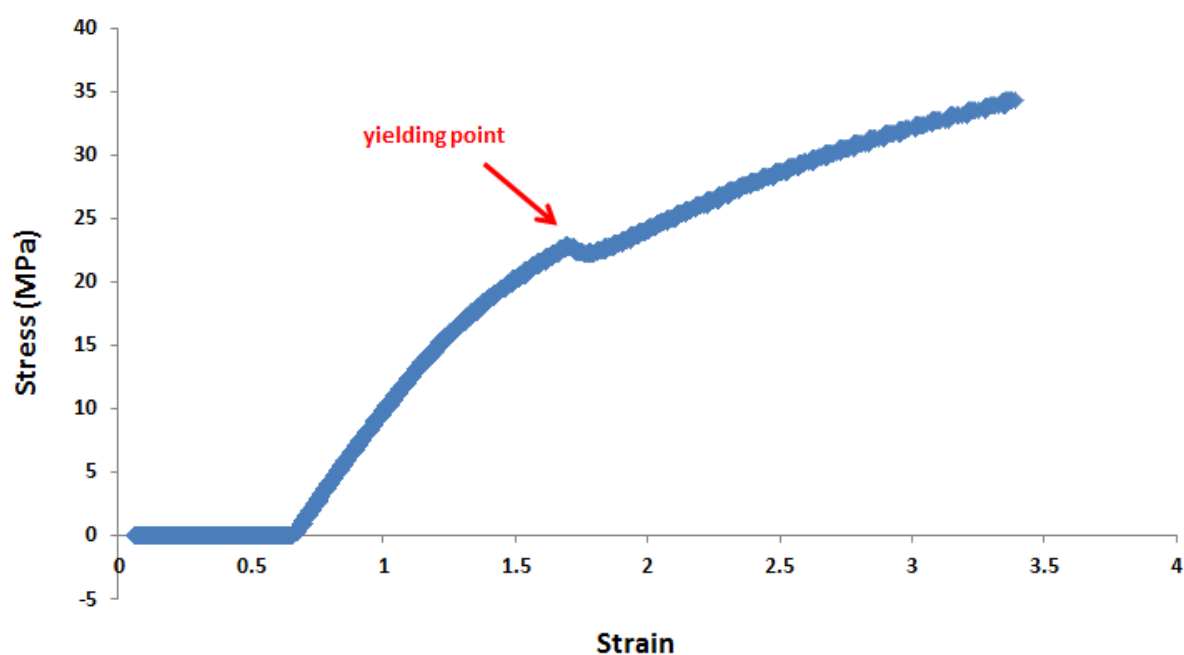


Figure 7-4 Stress-strain curve showing yielding point of a single CaP bead (1.8 mm in diameter)

### 7.2.2 Force relaxation

Typical force relaxation data for compression of a single CaP bead made with 5% pectin and 1.5%  $\text{CaCl}_2$  crosslinking solution having a diameter of 1.8 mm with no rupture event is shown in Figure 7-5. The bead was compressed to a deformation when the force reached 90 N and this deformation was sustained for three minutes (as described in section 3.13.2). The force imposed on a CaP bead increased with displacement (deformation) and gradually decreased while the bead was held. It was thought that the force relaxation could be due to viscoelasticity of pectin or water loss causing a volume decrease. In this study, however, dried CaP beads were compressed and thus water loss was considered negligible and this assumption unlikely to be true. Therefore, force relaxation was probably caused by viscoelastic properties of CaP beads.

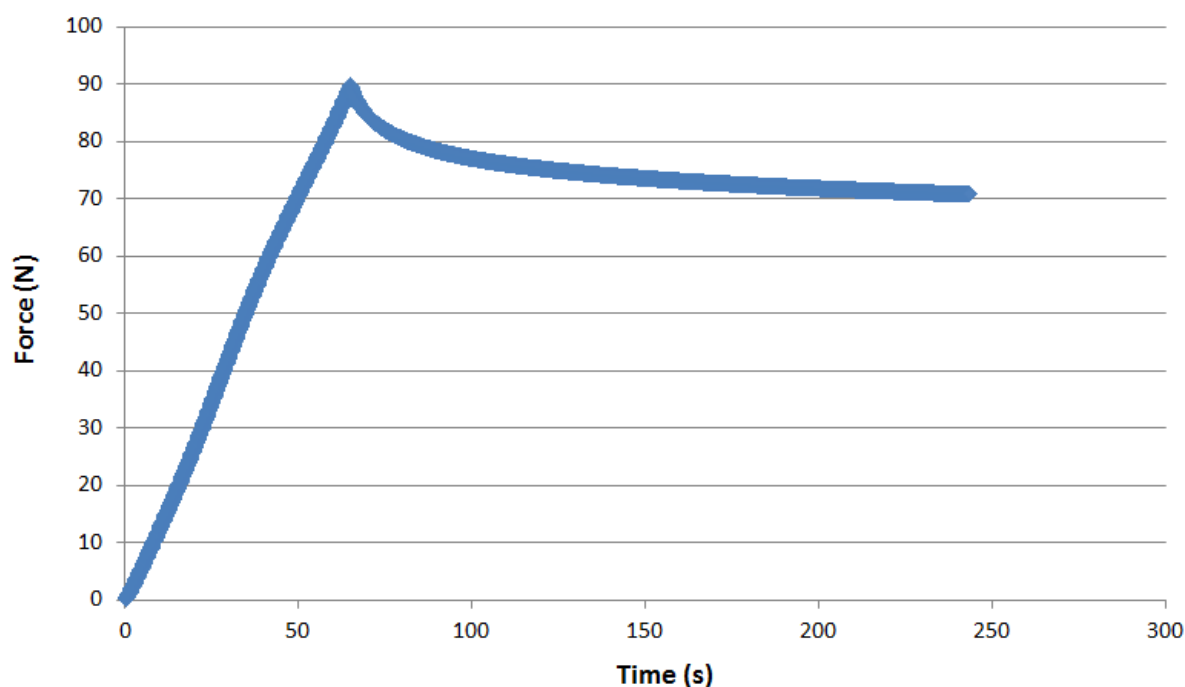


Figure 7-5 Typical force relaxation data for compression of a single CaP bead (1.8 mm in diameter) at a compression speed of  $0.01 \text{ mm s}^{-1}$ , and then holding



### 7.2.3 Mechanical property of CaP beads

Although the beads were viscoelastic, as shown in Figure 7-5, the maximum force relaxation is about 20% of the peak force. Therefore, Hertz analysis was applied to fit the force versus displacement data, as adopted by Andrei D et al. (1996) and Wang et al. (2005). In section 7.2.2, it was assumed that dried CaP beads had no changes in volume during compression, following Yap et al. (2008). Therefore, a Poisson's ratio ( $\nu$ ) of 0.5 was used for the incompressible material of CaP beads. This value was supported by Wang et al. (2005) who worked on the mechanical behaviour of alginate microspheres. Using this assumption and Hertz equation (as explained in Eq.3-7), a summary of the Young's modulus values and breakage behaviours of CaP beads is shown in Table 7-1. The mean Young's modulus value for the three beads which did not show clear breakage under compression is  $312 \pm 28$  MPa.

The mean Young's modulus value for the other seven CaP beads which showed breakage under compression is  $162 \pm 14$  MPa. The average of nominal rupture stress of these beads was  $14 \pm 3$  MPa which was less than the compaction pressure used in tableting (ranging between 75 and 105 MPa). The above results indicate that when the beads were compressed under high pressures, some might break. In a tablet, however, dried CaP beads were surrounded by many excipient particles of MCC. After compaction, the shape of the beads was slightly changed and small breaks appeared as shown in Figure 7-6. The image also shows some voids which might help to protect the cells inside the beads. Thus, it is likely that encapsulated cells in CaP beads were protected from compaction pressure and some cells within the beads remained intact to some extent. This was confirmed by a minor log loss of cell viability after compaction pressure was applied to the beads (Figure 7-8).

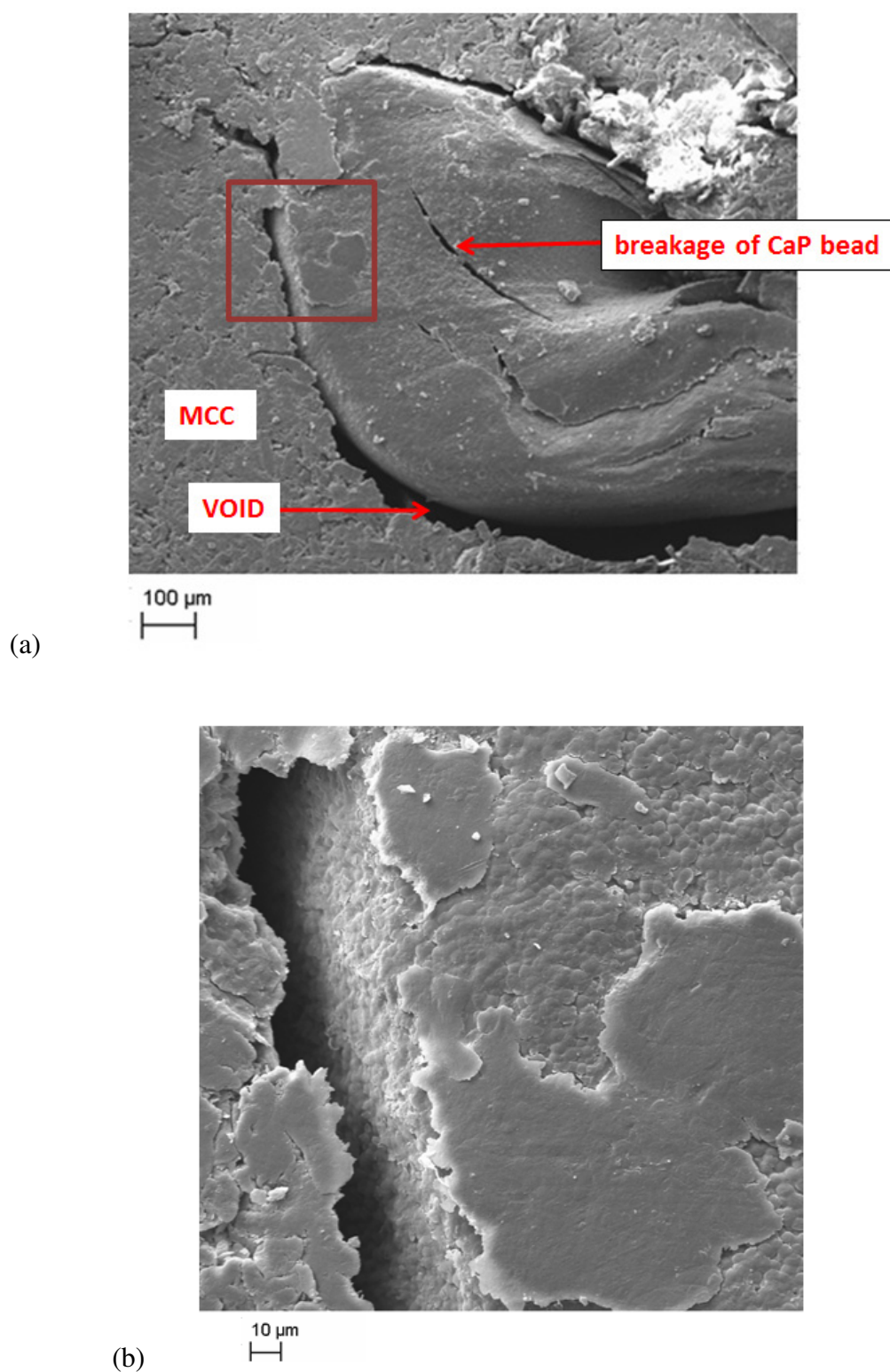


Figure 7-6 SEM image of a tablet containing CaP beads and MCC compacted at 90 MPa showing some breakage and void between CaP bead and MCC at different scale (a) 100  $\mu\text{m}$  (b) 10  $\mu\text{m}$

Table 7-1 Summary of the Young's modulus values and breakage behavior of CaP beads (ten tested beads)

Number of beads	Average particle size (mm)	Young's modulus (MPa)	Characteristics
7	$2.0 \pm 0.2$	$162 \pm 14$	Breakage point observed after pressure was applied
3	$2.2 \pm 0.1$	$312 \pm 28$	No breakage point

#### 7.2.4 Compaction of CaP beads made in 1.5% $\text{CaCl}_2$ into tablets

##### 7.2.4.1 *Formulation of tablets containing CaP beads made in 1.5% $\text{CaCl}_2$*

Preliminary results in Figure 6-21 show that CaP beads made of a 5% pectin concentration crosslinked with 1.5%  $\text{CaCl}_2$  and compacted at 90 MPa could produce a tablet with desirable tensile strength. Therefore, it would be interesting to produce tablets at different compaction pressures (75, 90 and 105 MPa) to determine whether which compaction pressure could produce the best tablet formulation in term of both tensile strength and an adequate number of culturable cells. A formulation containing CaP beads (made in 1.5%  $\text{CaCl}_2$ ) and MCC in 50/50 ratio compacted into a tablet at 90 MPa is shown in Figure 7-7. The appearance of the tablets showed slight segregation but none of the CaP beads broke off.

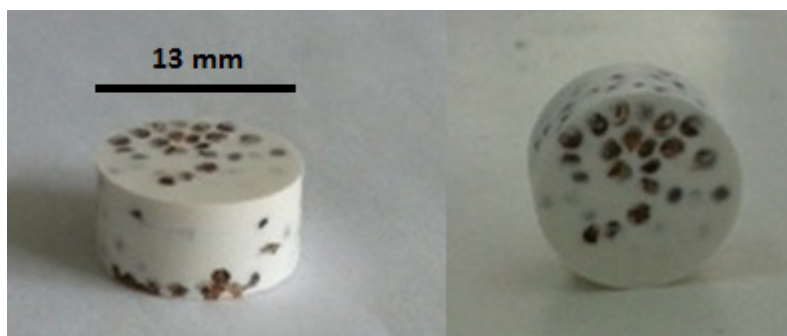


Figure 7-7 Tablets containing CaP beads made in 1.5%  $\text{CaCl}_2$  mixed with MCC in 50/50 (wt/wt) ratio compacted at 90 MPa (left: side view, right: front view)

A summary of cell survival and tensile strength of tablets containing CaP beads made in 1.5%  $\text{CaCl}_2$  and MCC compacted in 50/50 ratio at different compaction pressure is shown in Table 7-2. Tensile strength increased with the compaction pressure and it is also related to the porosity and relative density of the tablet (Wu et al., 2005). The authors also stated that tensile strength is independent of tablet diameter because they found similar tensile strengths for tablets where the diameter ranged between 8 mm and 13 mm.

Table 7-2 Cell survival and loss in log scale of tablets containing CaP beads (1.5%  $\text{CaCl}_2$ ) and MCC in 50/50 ratio compacted at different pressures

Formulation	Compaction pressure (MPa)	CFU/g tablet		Tensile strength (MPa)	Log loss
		Before compaction	After compaction		
CaP beads*	-		$2 \times 10^{10} \pm 8 \times 10^9$		
50/50	75	$8 \times 10^9 \pm 8 \times 10^8$	$9 \times 10^8 \pm 2 \times 10^8$	$0.74 \pm 0.07$	0.97
50/50	90	$8 \times 10^9 \pm 8 \times 10^8$	$9 \times 10^8 \pm 2 \times 10^8$	$1.09 \pm 0.03$	0.97
50/50	105	$8 \times 10^9 \pm 8 \times 10^8$	$5 \times 10^8 \pm 5 \times 10^7$	$1.37 \pm 0.06$	1.21

\*CFU/g was calculated based on CFU after 24 h freeze drying

Cell viabilities and tensile strength of the tablets in the 50/50 formulations are shown in Figure 7-8. The formulation compacted at 90 MPa had a tensile strength of  $1.09 \pm 0.03$  MPa which is a satisfactory value for a probiotic tablet with 13 mm diameter. In addition, cell viability of this formulation which contained  $9 \times 10^8 \pm 2 \times 10^8$  CFU/g tablet exceeded the minimum probiotic requirement. Consequently, this formulation produces an adequate number of culturable cells that can be released and adhere in the intestine thus exerting probiotic effects. Therefore, these findings in terms of minimum CFU/g tablet and tensile strength could potentially be useful in industrial applications if the cells can survive long term storage.

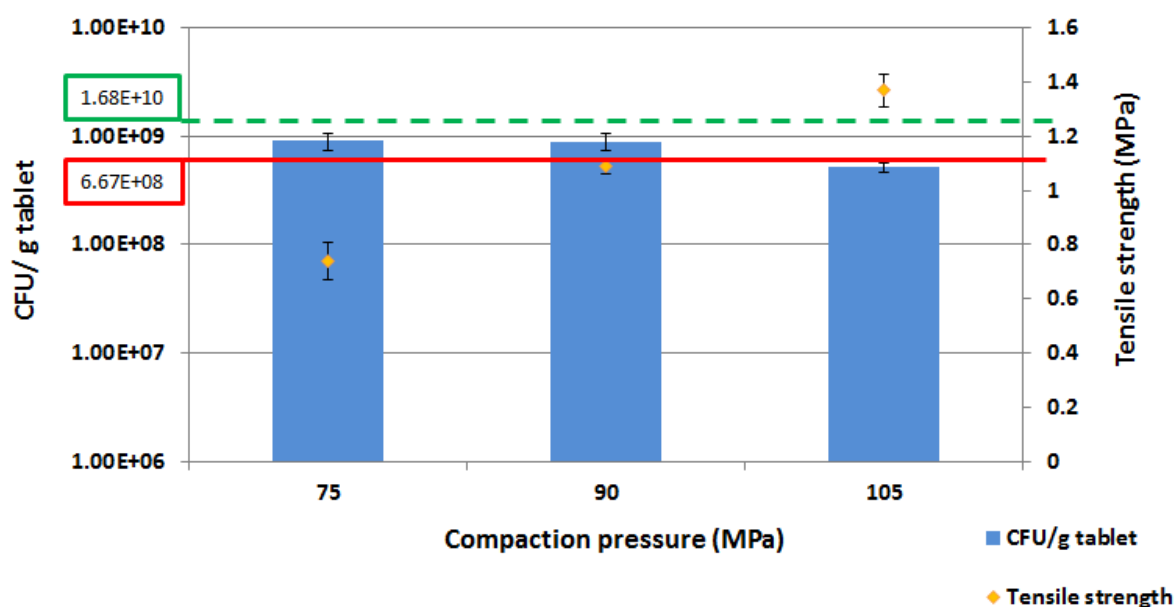


Figure 7-8 CFU/g tablet and tensile strength of tablets containing CaP beads made in 1.5%  $\text{CaCl}_2$  mixed with MCC in 50/50 (wt/wt) ratio and compacted at 75, 90 and 105 MPa. The red line demonstrates target CFU/g tablet (Lesaffre's specification) whereas the green line represents CFU/g of CaP beads before compaction.

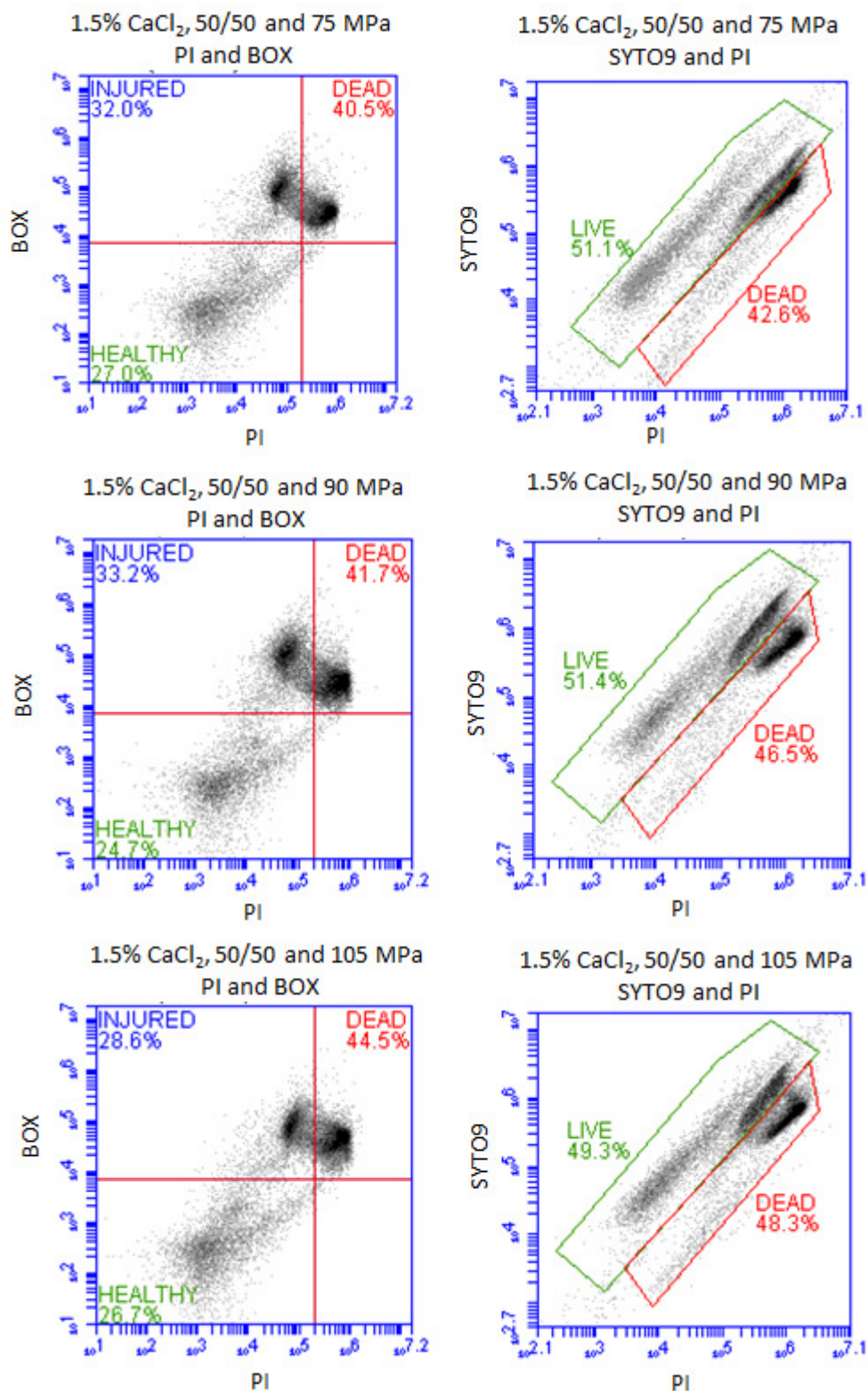


Figure 7-9 Flow cytometric dot plots showing the various cell physiological states of tablets made with CaP bead mixed with MCC in 50/50 (wt/wt) ratio and compacted at 75 MPa, 90 MPa and 105 MPa

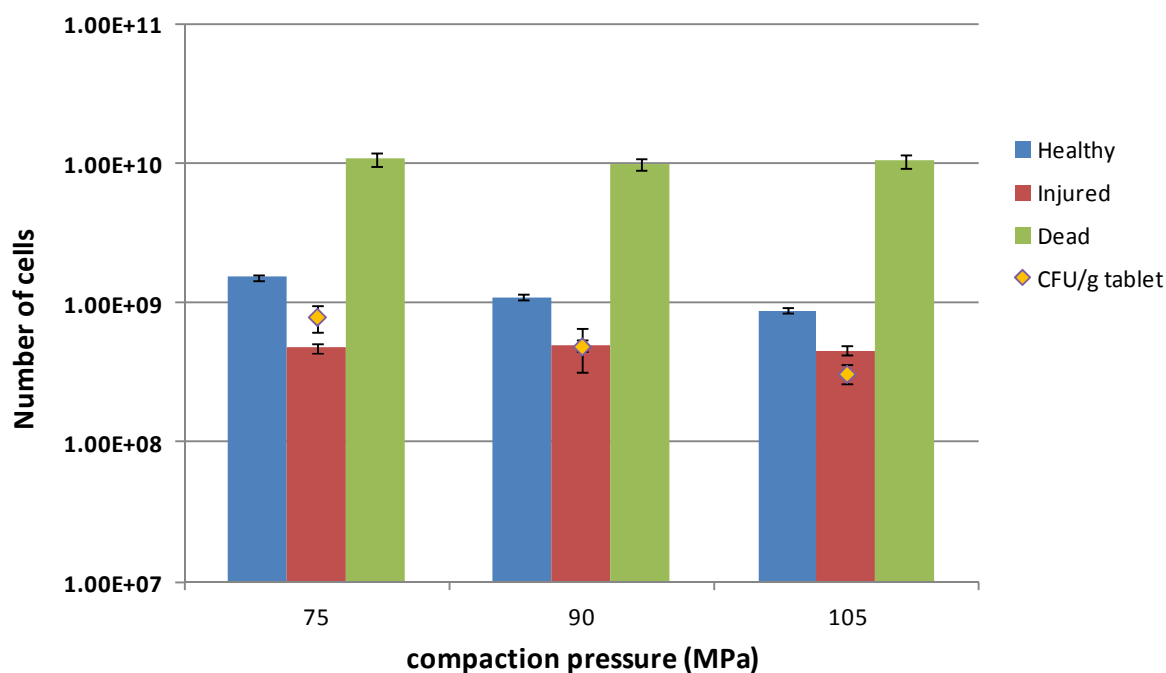


Figure 7-10 The number of healthy, injured and dead cells and CFU/g tablet versus pressure to compact CaP beads made in 1.5%  $\text{CaCl}_2$  and mixed with MCC in 50/50 (wt/wt) ratio

Flow cytometric results showing various states of tablets containing CaP beads made in 1.5% (w/v)  $\text{CaCl}_2$  and mixed with MCC compacted at 75, 90 and 105 MPa are presented in Figure 7-9. A slight decrease in the percentage of live cells in SYTO9 and PI plots was observed. The number of healthy, injured and dead cells and CFU/g tablet based on one tablet is shown in Figure 7-10. The CFU decreased with compaction pressure which might explain culturability of cells after being subjected to the stress of compaction pressure.



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#### 7.2.4.2 *Storage stability of an optimized formulation*

The tablets in 50/50 formulation compacted at 75, 90 and 105 MPa were stored at 4°C and the storage stability test was performed after 3 and 6 months (as described in section 3.13.4). Flow cytometric results showing various physiological states of cells after being stained with PI and BOX and SYTO9 and PI are shown in Figure 7-11, Figure 7-12 and Figure 7-13. The initial water activity ( $a_w$ ) value of tablets before storage was  $0.34 \pm 0.02$ . After 3 months storage, a significant decrease in the number of healthy cells was observed at all compaction pressures. As a result, there was an increase of dead cells as their cytoplasmic membranes became permeabilised and consequently stained by PI. This might be caused by changes of cell structure and function resulting from external stresses i.e. oxygen and moisture in the storage place as the tablets were not stored in a desiccator (Kurtmann et al., 2009, Costa et al., 2002).

Between 3 and 6 months, there were only slight changes in CFU/g tablet as shown in Figure 7-14. The external stresses in the storage did not have significant impact on cell viability in tablets between 3 to 6 months and this might imply that encapsulated cells inside CaP beads were resistant from these stresses to some extent. Figure 7-15 shows the number of cells in each state from FC (i.e., healthy, injured, dead) of tablets containing CaP beads and MCC in 50/50 (wt/wt) ratio at a compaction pressure of 90 MPa and the results correlated well with CFU data. It can be seen that the increase of dead cells in the first three months was greater than the following three months. During storage, the number of viable cells in the tablets remained constant but they had lower culturability after storage for longer.



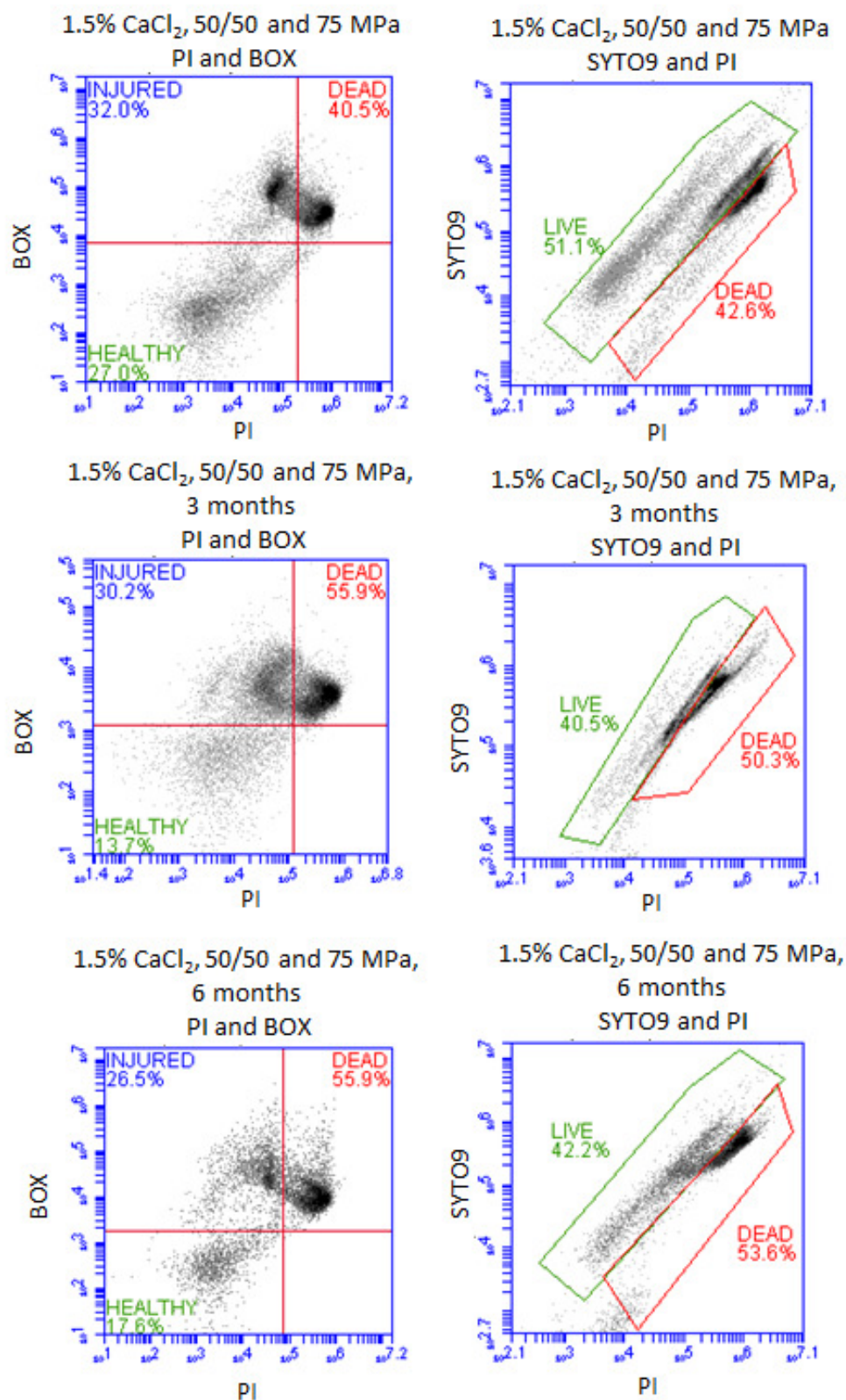


Figure 7-11 Flow cytometry dot plots showing various physiological states of yeast cells stained with PI and BOX or SYTO9 and PI for tablets containing CaP beads and MCC in 50/50 wt/wt ratio compacted at 75 MPa (c.a. 10 kN) after 3 and 6 months storage at 4°C

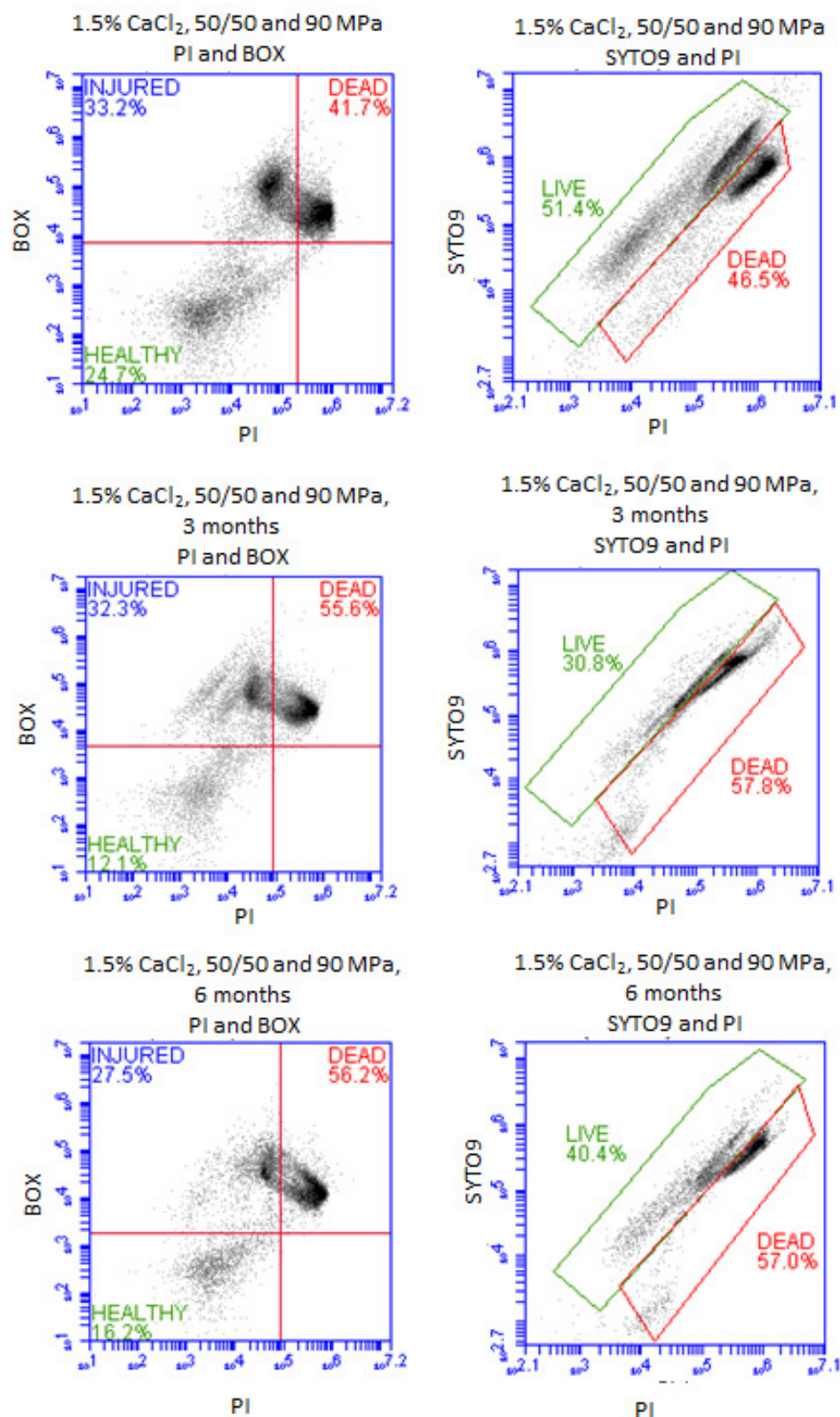


Figure 7-12 Flow cytometry dot plots showing various physiological states of yeast cells stained with PI and BOX or SYTO9 and PI for tablets containing CaP beads and MCC in 50/50 wt/wt ratio compacted at 90 MPa (c.a.12 kN) after 3 and 6 months storage at 4°C

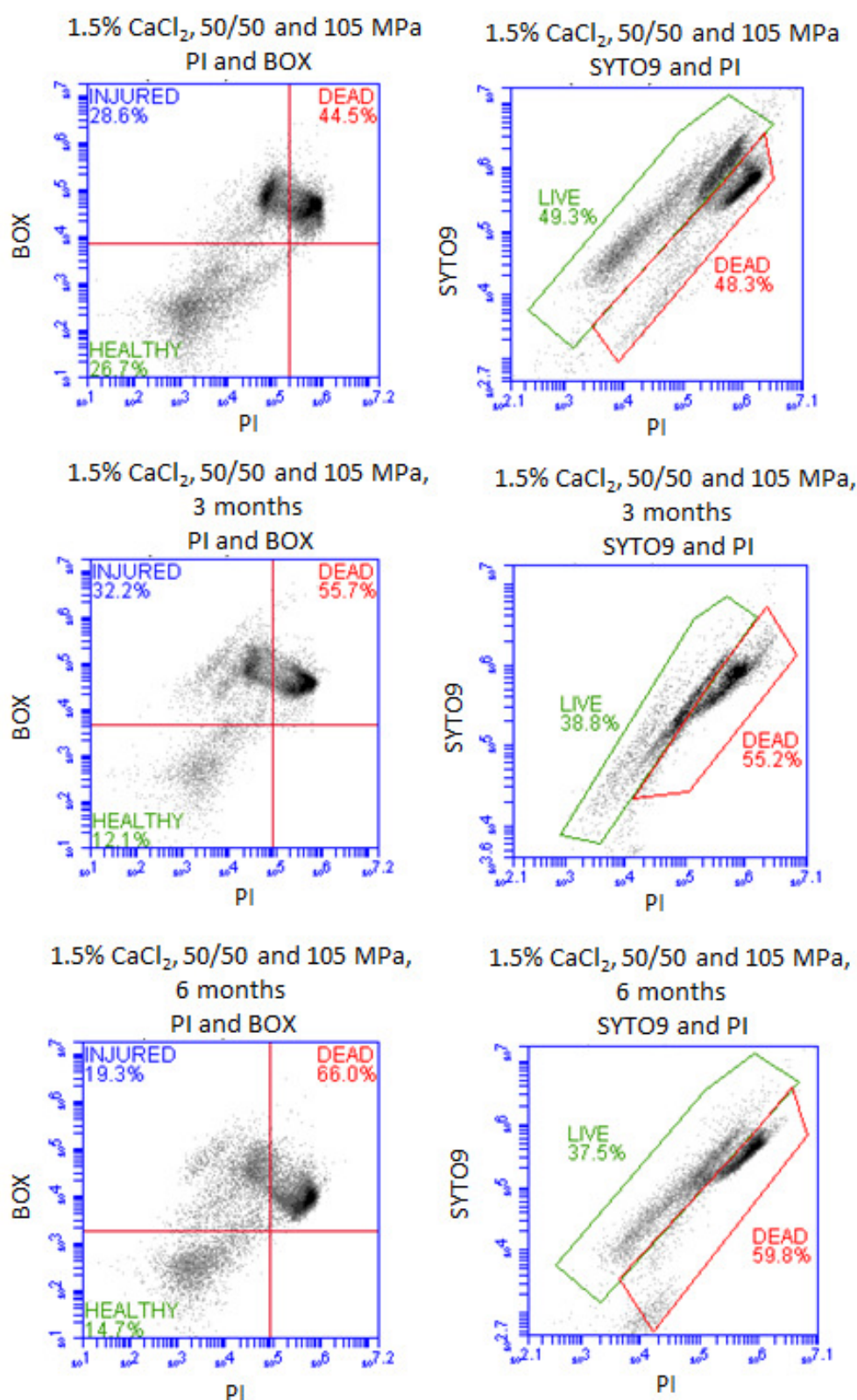


Figure 7-13 Flow cytometry dot plots showing various physiological states of yeast cells stained with PI and BOX or SYTO9 and PI for tablets containing CaP beads and MCC in 50/50 wt/wt ratio compacted at 105 MPa (c.a.14 kN) after 3 and 6 months storage at 4°C

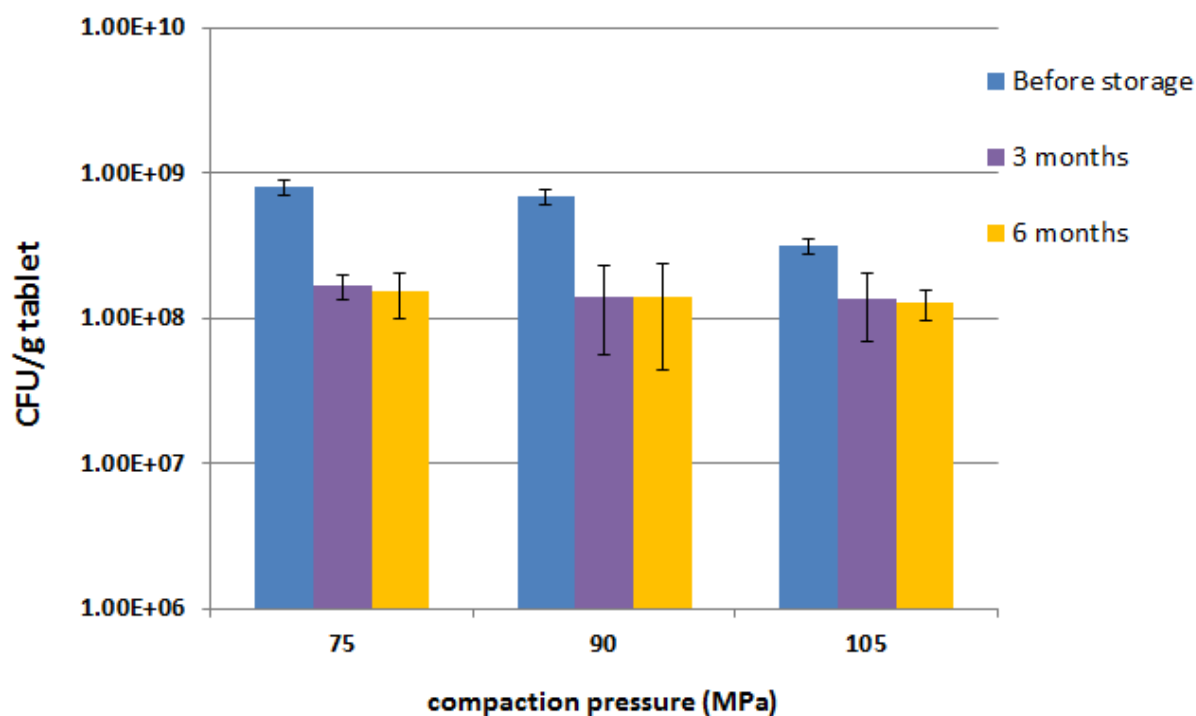


Figure 7-14 CFU/g tablet of tablets containing CaP beads made in 1.5%  $\text{CaCl}_2$  mixed with MCC in 50/50 (wt/wt) ratio and compacted at 75, 90 and 105 MPa before and after 3 and 6 months storage at 4°C

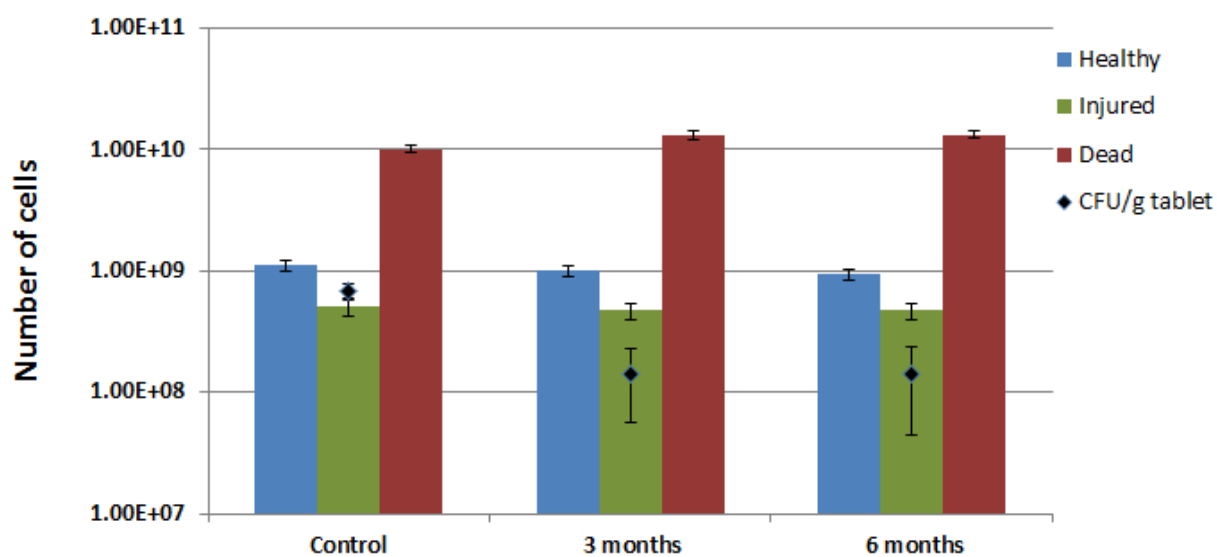


Figure 7-15 Number of healthy, injured and dead cells and CFU/g tablet of tablets containing CaP beads made in 1.5%  $\text{CaCl}_2$  mixed with MCC in 50/50 (wt/wt) ratio and compacted at 90 MPa after 3 and 6 months storage



The data of CaP beads before and after storage were plotted with the data in section 4.2.4 as shown in Figure 7-16, Figure 7-17 and Figure 7-18. It can be seen that the data of CaP beads before storage (symbol: red triangle) are in the same trend with a correlation in section 4.2.4. However, the number of viable, injured and healthy cells of storage data for 3 and 6 months (symbol: yellow triangle, outlined in red) could not correlate well with existing data and were plotted separately in the lower half of the figure and thus, it could weaken  $R^2$  of linear line. Therefore, it is suggested that the correlation found in section 4.2.4 could not be used to estimate cell culturability from total viable cell number determined by FC for storage stability data.

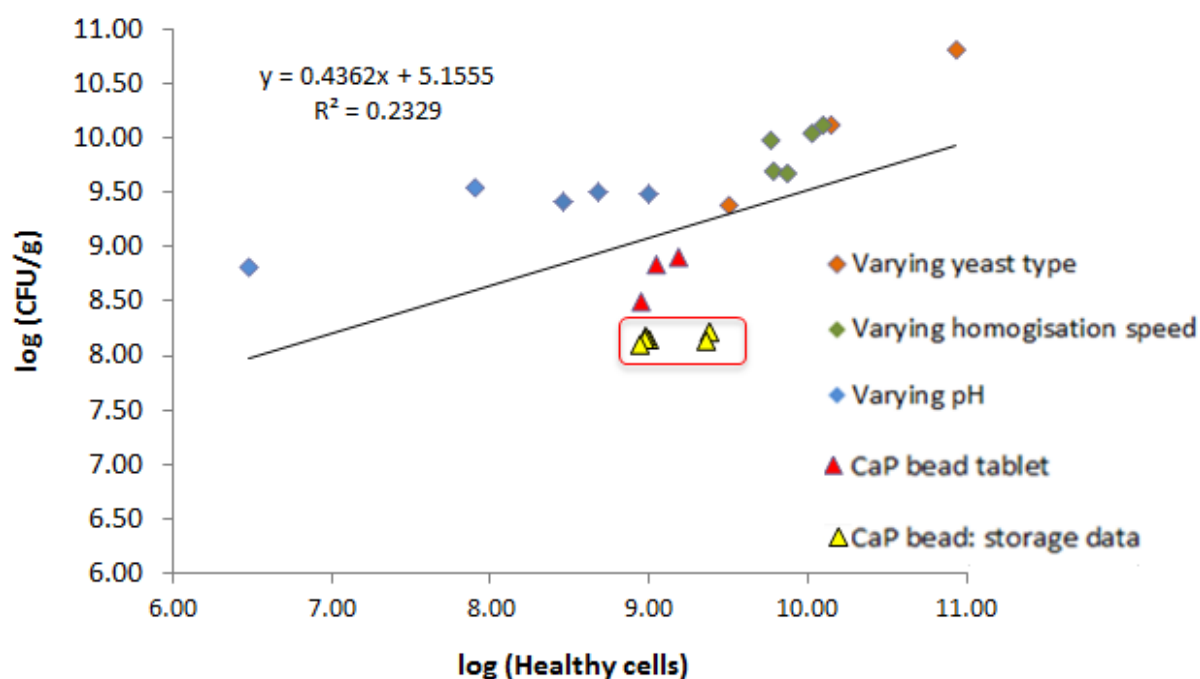


Figure 7-16 Correlation between the number of healthy cells and CFU/g established in section 4.2.4 plotted with the storage data (3 and 6 months, outlined in red) of tablets containing CaP beads and MCC in 50/50 (wt/wt) ratio compacted at 90 MPa

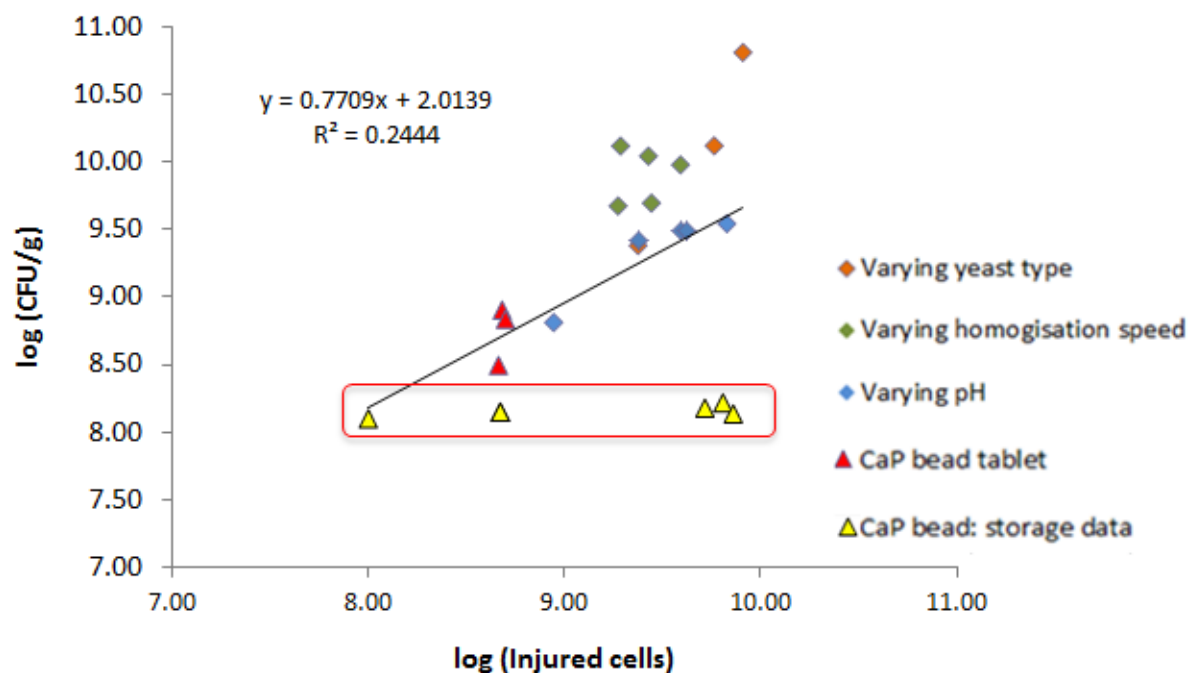


Figure 7-17 Correlation between the number of injured cells and CFU/g established in section 4.2.4 plotted with the storage data (3 and 6 months, outlined in red) of tablets containing CaP beads and MCC in 50/50 (wt/wt) ratio compacted at 90 MPa

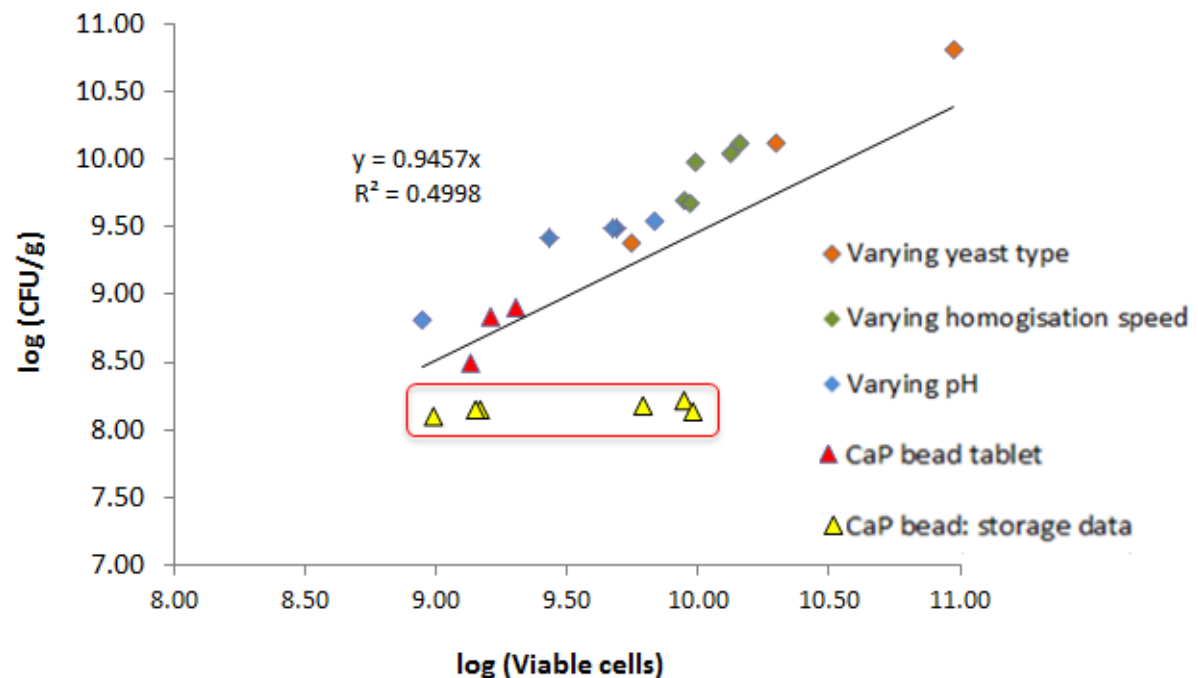


Figure 7-18 Correlation between the number of viable cells and CFU/g established in section 4.2.4 plotted with the storage data (3 and 6 months, outlined in red) of tablets containing CaP beads and MCC in 50/50 (wt/wt) ratio compacted at 90 MPa

### 7.3 CONCLUSIONS

The deformation behaviour of calcium pectinate beads made of 5% pectin and crosslinked in 1.5%  $\text{CaCl}_2$  has been investigated using a diametrical compression test. The investigation revealed two material behaviours of CaP beads. Three beads out of ten behaved elastically at small deformation and had no fracture events and therefore, force relaxation (Figure 7-5) could be due to viscoelasticity of pectin. Seven beads showed fracture points and irreversibly deformed when compressed to large deformation. Using the Hertz equation and a Poisson's ratio of 0.5 for incompressible material, the average Young's modulus of seven beads was  $162 \pm 14$  MPa. Another three beads demonstrated viscoelastic property and Young's modulus value of  $312 \pm 28$  MPa (Table 7-1).

Cells exposed to compression pressure resulted in a decrease in the number of healthy cells and caused more cell death by taking up more PI in FC analysis. Such decreased viable cells had lower culturability but were still viable. Additionally, when such injured cells were exposed to additional stresses during storage, their cytoplasmic membranes became permeabilised and thus resulted in cell death and PI up-take (Figure 7-11 to 7-13). Overall, cells encapsulated in CaP beads were resistant to external stresses in the storage. The encapsulation of yeast cells has created a favourable micro-environment, which protects the cells from being damaged by compaction to a certain extent and prolonged their storage stability.

The correlations between the number of viable cells and CFU/g data as explained in section 4.2.4 could be used to estimate the culturability of cells in tablets containing CaP beads and MCC before storage. However, these correlations could not be applied to the data of tablets

stored for 3 and 6 months, which implies there were different death mechanisms involved (Figure 7-16 to 7-18).



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## CHAPTER 8

### FINAL CONCLUSIONS AND FUTURE WORK

#### 8.1 FINAL CONCLUSIONS

The objective of this study was to gain an understanding of the physiological state of probiotic yeast cells when they were incorporated in tablet dosage form produced by compaction. The factors (i.e. formulation, processing and environmental conditions) that affected cell viability were studied by colony plate counting (CPC) based on culturability (CFU/g) and by flow cytometry (FC) based on the applications of fluorescent probes.

FC offered rapid data analysis of physiological states of microorganisms when used together with various fluorescent dyes. Cells stained with propidium iodide (PI) and Bis-oxonol (BOX) were classified into three different physiological states i.e. healthy cells with intact cytoplasmic membrane (PI<sup>-</sup>, BOX<sup>-</sup>), injured cells with no cytoplasmic membrane potential (PI<sup>+</sup>, BOX<sup>-</sup>) and dead cells with permeabilised cytoplasmic membrane (PI<sup>+</sup>, BOX<sup>+</sup>). Viable cells are defined as the summation of healthy and injured cells. The number of culturable cells was presented as CFU/g which was calculated based on the number of cell colony growing on agar plates.

The study on yeast cell viability in simulated gastric and intestinal fluids with different pH values (pH 1 to 7) has shown that yeast cells (*S. cerevisiae*) maintained their viability in the fluid for pH greater than 2. Since the pH value in the gastric fluid during food digestion is rarely below 2, therefore the adverse effect of pH on cell viability at pH 1 was disregarded in

respect of formulation of a probiotic dosage form. This implies that probiotic yeast in tablet dosage form does not require enteric coatings. Active dried yeast (ADY) as received from Lesaffre in spherical granules ( $d_{50} = 1252 \mu\text{m}$ ) resulted from being processed in a drum dryer at a relative low temperature between 40 and 42°C for 10-20 h. The FC results have shown that the drying process, in which 70% of residual moisture content was removed from yeast cells, caused the cell viability reduction by approximately 30%. Milled yeast (MY,  $d_{50} = 403 \mu\text{m}$ ) as received was produced from crushing of ADY with a comminutor before discharged through a 30 mesh screen to offer smaller particle sizes and thus, increased compactibility with other excipients for tableting. This process was found to have an additional adverse effect on cell viability by around 10%. All the dry yeast samples were suspended in PBS solution and then mixed by a laboratory homogeniser for 3 min prior to subsequent experiments. An optimized speed of the laboratory homogeniser used to prepare ADY suspension was found to be in the range between 8 000 to 12 000 rpm because at these speeds the maximum number of viable and culturable cells based on CFU/g data were released from the yeast granules. Moreover, active dry yeast (ADY) or milled yeast (MY) mixed with MCC were compacted into tablets using pressures ranging from 75 to 105 MPa but as the compaction pressure was increased, more dead cells and fewer healthy cells were found. In dissolution tests, the tablets produced using a Zwick machine disintegrated within 40 min after being exposed to a dissolution medium of PBS at 37°C. Therefore, if a tablet was ingested, the total number of cells in the tablet might be released in the GI tract. From FC and CFU data under the different conditions, a linear correlation between healthy and viable cells against CFU/g was applied. By considering the regression coefficient ( $R^2$ ), CFU/g and the number of healthy cells determined by FC showed a reasonably good linear relationship (Figure 4-16), whilst the correlation between CFU/g and number of injured cells was poorer. Therefore, healthy cells mainly contributed to those cells which were culturable, although

there could be more than one cell forming a single colony on agar plates. The slope of the regression line in Figure 4-18 which was less than 1 suggests that CFU/g data was typically underestimated in comparison with the number of viable cells detected by FC. This correlation might be useful to estimate cell culturability from the number of viable cells for other similar environmental and processing conditions.

A freeze drying method was utilized to preserve yeast viability in order to produce probiotic tablets with a long shelf life. An attempt with an additional protectant of skimmed milk to WY was carried out but marginal improvement on yeast cell viability was observed. One important challenge for freeze dried yeast (FDY) is to obtain the desirable water activity ( $a_w < 0.3$ ) in which prolonging freeze drying time was found to reduce water content but cause a reduction in cell viability. The flow cytometric results showed a declining trend of healthy cells in conjunction with an increase of injured and dead cells with freeze drying time. This reduction in cell viability might have been caused by a decrease in cell size due to dehydration, low temperature or absence of liquid in freeze drying process (Rault et al., 2007, Meng et al., 2008, Fu and Chen, 2011). These flow cytometric results consistently show a clear trend of declining CFU with freeze drying time. Those injured cells with membrane potential loss might be manifested by an inability to be able to form colonies on a solid medium, however they may restore and regain normal function under appropriate conditions or with enough nutrients (Mazur, 1965, Mazur, 1966, Novo et al., 2000). Even so, FDY particles after 24 h was mixed with microcrystalline cellulose (MCC) and compacted into tablets at different ratios and compaction pressures. However, the tablets containing FDY and MCC had a limitation in tablet compactibility which could be caused by a significant difference in particle size of FDY ( $d_{50} = 0.85$  mm) and MCC ( $d_{50} = 0.19$  mm) and thus resulted in low tensile strength. Another attempt was to improve tablet tensile strength by

increasing the amount of MCC as to enhance binding property in a tablet. Nevertheless, all formulations of tablets containing FDY and MCC did not meet the probiotic tablet requirements in term of CFU/g tablet ( $\geq 6.67 \times 10^8$  CFU/g tablet) and tablet tensile strength ( $\geq 1$  MPa, Lesaffre's specification, France). Overall, the reductions of cell viability were due to freeze drying time, tablet compaction pressure and stresses during storage to a certain extent. The numbers of healthy, injured and dead cells and CFU in tablets containing FDY and MCC before being storage were plotted in Figure 5-19 to 5-21 with the correlation explained in section 4.2.4 and they showed a good linear correlation. Nevertheless, this correlation could not be applied to the storage data of tablets as the correlation coefficient ( $R^2$ ) of linear line was weakened. The hypothesis for the storage data due to some external stresses requires further analysis.

Encapsulation of yeast cells with some biodegradable materials (shellac and pectin) was investigated in order to provide a favourable micro-environment to cells, which is resistant to compaction pressure. WY containing high initial numbers of viable cells was selected for all experiments in the encapsulation process. First attempt on using shellac for encapsulating yeast cells with an extrusion method was made. Calcium shellac beads ( $d_{50} = 2.9$  mm) containing yeast cells had desirable water activity value ( $a_w \sim 0.12$ ) after 24 h freeze drying and offered good tablet compactibility without any binders. These beads had a macroporous internal structure caused by bubbles created during the mixing step. Nevertheless, such beads showed a low solubility and disintegration rate after exposure to simulated intestinal fluid (SIF) for 6 h and therefore calcium shellac beads were proven to have limitations in encapsulation of probiotic yeast. Second attempt on using pectin as encapsulating material was made. Yeast cells were successfully encapsulated with pectin crosslinked with  $\text{CaCl}_2$ , which formed calcium pectinate beads ( $d_{50} = 2.05$  mm). The effects of crosslinking solution

concentration, pectin concentration and freeze drying time on cell viability were investigated. During the dissolution test, a pectinolytic enzyme mimicking conditions in the colon was added to the dissolution media. WY mixed with 5% pectin, dripped in a 1.5%  $\text{CaCl}_2$  crosslinking solution and freeze-dried for 24 h offered high encapsulation efficiency of 82%, encapsulation efficiency based on culturable cells of 65% and desirable water activity value of  $0.27 \pm 0.2$ . These beads were also mixed with MCC and compacted into tablets, which gave a desirable number of viable cells.

Measurement of the mechanical properties of CaP beads by diametrical compression testing showed two material behaviours of CaP beads based on the analysis of ten tested beads. Seven beads showed a breakage point and three beads had no breakage point after diametrical compression. The internal structure of such beads contained pectin and some free volume. When the beads were compacted into tablets, MCC as an excipient could protect the beads from breakage. Therefore, this explained good tablet compactibility and resulted in a rigid tablet. A best achievable tablet formulation with desirable CFU/g and tensile strength contained an equal amount of CaP beads and MCC at 50/50 (wt/wt) ratio, which was compacted at 90 MPa. Cells entrapped inside the beads benefited from greater protection against external stresses than non-encapsulated cells (or FDY) by 0.77 log after 6 months storage. Overall the encapsulation of yeast cells has two significant benefits; firstly it reduced the amount of cells being damaged by tablet compaction, secondly it helped to prolong the product storage stability. The relationship between the number of viable, injured and dead cells and CFU of tablets containing CaP beads and MCC before storage was fitted with Figure 4-16 to 4-18 as explained in section 4.2.4. It has been found this correlation could be useful to predict cell culturability of cells after encapsulation. However, this correlation could not be applied to storage data (as shown in Figure 7-16 to 7-18) as it resulted in poor linear

correlation coefficient which might be due to other external stresses affecting cell viability during storage i.e. oxygen or moisture.

## **8.2 FUTURE WORK**

In this study, yeast cells were successfully encapsulated in calcium pectinate beads which may be useful to make probiotic tablet. However, more work is required to understand the protective mechanisms and improve the formulation developed thus far.

Since adding skimmed milk to WY before freeze drying only improved the cell survival marginally, so other protective agents i.e. 10% trehalose could be added together with skimmed milk into WY before freeze drying in order to help maintain cell viability (Coutinho et al., 1988). Freeze drying at a high rate could be conducted to enable smaller ice crystals to form in order to minimize cell damage since large ice crystals can make cell membrane fragile and thus cause a reduction in cell viability (Morgan et al., 2006).

A G23 standard needle was used to encapsulate yeast cells with pectin solution and the obtained beads after freeze drying appeared very big with an average particle size of  $2.1 \pm 0.3$  mm. Smaller beads with bigger surface area could enhance tablet compactibility and reduce the segregation of the tablets when mixing with other excipients. To obtain such beads, needles with smaller diameters (e.g. G24 or G25 standard needles) could be used. Moreover, if a Nisco encapsulation unit (model VAR J1) is used to generate droplets, air flow rate could be adjusted. Additionally, pectin concentration should be optimised. Therefore the viscosity of suspension would not be problematic to form the beads.

Storage stability studies could be carried out in a closure system with desiccator to examine whether the tablets are sensitive to moisture. Moreover, probiotic tablets could be stored under the accelerated condition ( $40 \pm 2^{\circ}\text{C}$  and  $75\%\text{RH} \pm 5\%\text{RH}$ ) for 6 months to evaluate their thermal stability (Health and Services, 2003). For long term studies, the tablets should be stored at  $20\text{-}25^{\circ}\text{C}$  and  $60\%\text{RH}$  for a period of at least 12 months and the testing frequency should be every 3 months (Health and Services, 2003).

FC can simultaneously measure the physiological state of cells and detect whether the cells are damaged due to either compaction pressure or other stresses during storage. However, this technique can only measure the sample in liquid form or suspension. Therefore, a technique using fluorescent dyes and confocal microscope by adjusting light at the wavelength of interest could be developed in order to gain better understanding and explore cell behaviours. This method could possibly measure how many cells are killed in solid form or tablet.

Kawakita and Heckel equations have been validated in pharmaceutical powder compaction where they relate to material physical properties e.g. porosity and density. The primary particle data of CaP beads and MCC could be fitted into these equations and indicate the changes in the compaction mechanism. Since the relaxation force of some CaP beads might be due to viscoelastic property of pectin, modified Young's modulus using viscoelastic model could be used to study viscoelastic behaviour. Moreover, adhesion force between cells and excipient particles using AFM could be measured to get a fundamental understanding of the interaction between materials.

The final product tests such as friability of uncoated tablet, uniformity of mass (weight) and resistance to crushing could be conducted through batch samples to conform to the tablet specification. The appearances of the tablets i.e. taste and colour could be adjusted to meet the needs of the consumer. If the *in vitro* studies are successful, the next step in *in vivo* whilst a scaled-up process including tablets for animal use could also be carried out to establish the effectiveness of probiotic tablets, and to enhance consumer confidence.



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## APPENDIX 1

Serial dilution protocol from Lesaffre, France

### IV. MATERIALS AND EQUIPMENT

The media uses in this method (3.2) are commercially available and are to be prepared and sterilized according to the manufacturer's instructions. See also Appendix 1 for the formula of individual media.

#### 3.1 Material

- Sterile spreader
- Light microscope
- 1 ml sterile graduate transfer pipets (filled in with cotton)
- Tubes (20mL)
- Ø 90 mm
- Magnetic stirplate
- 1 L flask
- Sterile flasks (100mL) with "magnetic bar"
- Incubator capable of maintaining 25°C
- Water baths at 100°C and 48°C (to temper agar)
- Precision weighing balance  $\pm 0.001g$

**NOTE:** It is the responsibility of each laboratory to ensure that the temperatures of the incubators or water baths are maintained at the recommended temperatures. Where 35°C is recommended, the incubator may be 35  $\pm$  1°C. Similarly, lower temperatures of 30 or 25°C may be  $\pm$  1°C. However, where higher temperatures are recommended, such as 43 or 45.5°C, it is imperative that the incubators or water baths be maintained within 0.5°C due to potential lethality of higher temperatures on the microorganism being isolated.

### 5.2 Preparation and dilutions of the sample

Weigh with accuracy 1 g of sample in a sterile flask.  
Add 100 mL of TS buffer solution pre-warmed to 37°C.

The suspension is homogenized using an UltraTurax homogenizer (10000 to 12000 rpm for 3 min)

Homogenize the suspension before using it and prepare succeeding decimal dilutions as required (in distilled water pre-warmed to 37°C), using a separate sterile pipette for making each transfer

### 5.3 Plating and incubation

Yeast that may have been stressed should be enumerated by a surface spread plate technique rather than with pour plates. This technique provides maximal exposure of the cells to oxygen and avoids heat stress from molten agar. Pour plates may be used for all yeasts at the lab's discretion and if validated that the counts are not significantly different between spread plates and pour plates (Beuchat, L.R., Nail, B.V., Brackett, R.E., Fox, T.L.. "Comparison of Pour and Spread Plating for Enumeration of Fungi in Foods." 08/20/1990. 31: 27 - 30, 1992).

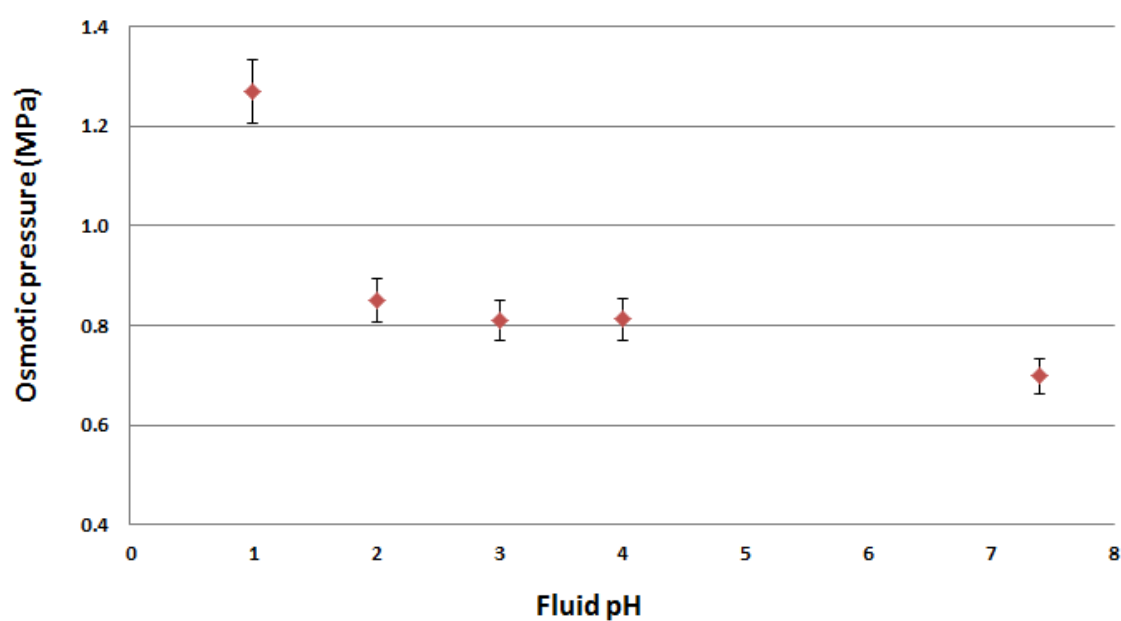
Transfer to the surface of the plate (with 1mL sterile pipet), 0.1mL or 0.5mL of the required dilutions to appropriate Petri plates. Gently spread the liquid culture onto the surface of the agar by moving the spreader in a circular manner while rotating the plate. This will ensure an even distribution of yeast.

Incubate plates in a reversed position to 25°C for 3 days.

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## APPENDIX 2

Osmotic pressure (MPa) of different pH fluids was measured by a vapour pressure osmometer (Wescor 5500 XRS, Ontario, Canada). Error bars represent the range from the mean of triplicate measurements (n=3).



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