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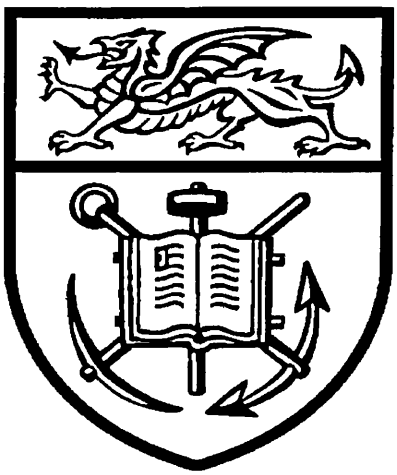
Zhao, Guoqun



Preservation and Utilization of Malolactic Fermenting Lactic Acid Bacteria

by

Guoqun Zhao BSc MSc

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Department of Chemical and Biological Process Engineering, University of Wales Swansea,
Singleton Park,
Swansea,
SA2 8PP

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Abstract

Preservation and Utilization of Malolactic Fermenting Lactic Acid Bacteria

Malolactic fermentation (MLF) is an important process in wine production. MLF results from metabolism of certain lactic acid bacteria (LAB) and involves the conversion of malate to lactate and CO_2. Except for deacidification, MLF can improve the quality and microbiological stability of wines. The aim of this project was to investigate the preservation and utilization of LAB with particular reference to MLF.

In order to measure the effect of various preservation methods and their productivities, an assay of cell vitality was developed. This measured the capacity to overcome and recover from freezing and freeze-drying. It was shown that this method was easily used and reliable.

The effect of cultural conditions on the cryotolerance and vitality of LAB was investigated, including: (1) the growth phase, (2) the growth temperature, (3) the medium pH, (4) the medium composition and (5) preincubation conditions. The optimal cultural conditions to obtain higher vitality after freezing varied with the species of LAB. When the pH of culture medium was controlled at pH 5 LAB attained the highest vitality after freezing. When cells of *L. plantarum* was preincubated in 5g/l yeast extract solution at 25°C for 1 hour, the viability of *L. plantarum* greatly increased, compared with those without preincubation.

The conditions of freeze-drying of LAB were investigated. It was found that 4% yeast extract was the most effective protectant for *L. plantarum* and *L. brevis* and 5% glutamate were the best protectant for *O. oeni*. When LAB was frozen quickly at -65°C, the vitality obtained after freeze-drying was higher than those frozen slowly at -20°C. Another factor to be considered important was ethanol tolerance when freeze-dried malolactic bacteria were used in wines. Among the suspension media tested, 5% glutamate and 10% sucrose were best for freeze-dried *L. brevis* and *O. oeni* respectively for high vitality in high ethanol concentrations.

These studies showed that there were no consistent underlying processes that could be easily identified and that preservation was a species specific, multifactorial process.

The MLF was then investigated further by studying the effect of wine components on batch MLF (*L. brevis* and *O. oeni*) using a defined synthetic wine. This uniquely allowed a systematic study of MLF in high alcohol environments. Alcohol tolerance was dependent on temperature and important fermentation intermediates such as citrate, pyruvate and malate. MLF was inhibited when glucose concentration increased from 2 g/l to 6 g/l. The inhibition to MLF of *O. oeni* caused by glucose was relieved when fructose was present. Nutritional status was also an important factor that affected MLF. When the synthetic wine did not contain added yeast extract, malic acid degradation of *L. brevis* and *O. oeni* was low (6.1% and 54.3% respectively).

Rapid and continuous MLF was achieved in a membrane bioreactor (MBR) with high cell densities of *O. oeni* (greater than 10^8CFU/ml). More than 95% degradation of malic acid in synthetic wine was obtained at 0.48 l/h of flow rate and 10.4 h residence time during 72 h continuous operation. High ethanol concentration of wine was the main factor that caused loss in malic acid degrading activity of *O. oeni* in MBR. The poor nutritional condition of wine was not the main factor causing loss in the stability of malic acid degrading activity of *O. oeni*. Shear stress had little influence on the malic acid degradation of *O. oeni* under the conditions investigated. Ethanol stress adaptation could improve the stability of malic acid degrading activity of *O. oeni* in MBR.

NOMENCLATURE

ADP	adenosine diphosphate
ATP	adenosin 5'-triphosphate
Aw	activity of water
$CaCl_2$	calcium chloride
CFU	colony-forming units
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
KH_2PO4	potassium dihydrogen phosphate
K_m	Michaelis constant
LAB	lactic acid bacteria
MBR	membrane bioreactor
$MnCl_2$	manganese chloride
MPN	most probable number
MLF	malolactic fermentation
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NaOH	sodium hydroxide
$(NH_4)_2SO_4$	ammonium sulphate
OD	optical density
OD_{660nm}	optical density at 660 nm
ODmax	maximum optical density at 660 nm
pH	$-\log_{10}$ proton concentration (activity), in mol^{-1}
VI	vitality index
SO_2	sulphur dioxide
YE	yeast extract
IUC	International Union of Chemistry
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})

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Chapter 1. Introduction

This investigation is concerned with preservation and utilization of lactic acid bacteria (LAB) with particular reference to malolactic fermentation (MLF). This study shows that preservation of LAB with high vitality by freeze-drying can be achieved. A rapid and continuous MLF in a membrane bioreactor has been designed and successfully operated.

Chapter 1 introduces and outlines the principal aims and objectives of this project. Background and literature summarizations help to understand bacterial responses to environmental stresses, freeze-drying process, principle and role of MLF and the possible solutions to the problems associated with MLF. Chapter 2 provides a detailed account of the methods and materials for assessment of cell vitality after freeze-drying, analysis of chemicals, the methods and equipment of freeze-drying and fermentation systems.

The experimental results of the project are documented in Chapter 3 to 7. Chapter 3 is concerned with study of a method to assess vitality. Chapter 4 investigates the effect of cultural conditions on the vitality and cryo-tolerance of *Lactobacillus plantarum, Lactobacillus brevis* and *Oenococcus oeni.* This focuses on the optimal cultural conditions to obtain cells of LAB with high vitality. Chapter 5 gives results of the vitality of freeze-dried LAB. This emphasizes the freeze-drying conditions such as suspension medium, freezing temperature, rehydration and storage of freeze-dried LAB in order to obtain high vitality. Chapter 6 investigates the ethanol tolerance of *Lactobacillus brevis* and *Oenococcus oeni.* Chapter 7 concerns the application of those bacteria to MLF alcohol environments. The effect of wine composition on MLF has been investigated in artificial wine (the synthetic wine). Rapid and continuous MLFs were carried out in a membrane bioreactor with a high cell density of *Oenococcus oeni.* The discussion and conclusions to this study are given in Chapter 8 together with suggestions for further work.

1.1 Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) are a group of non-motile, nonspore-forming, Gram-positive anaerobic bacteria that ferment hexoses to lactic acid (Konings *et al.*, 2000). They are normally cocci and rods. LAB occur in many genera. The most important genera of LAB are *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Weissella*, *Carnobacterium*, *Tetragenococcus*, and *Bifidobacterium*. Those most used in food industry are *Lactobacillus*, *Lactococcus* and *Streptococcus* (Michael and Holzapfel, 1997; Klein *et al.*, 1998). According to pathways by which hexose sugars are metabolised, LAB are divided into two groups, homo-fermentative and hetero-fermentative. Briefly, homofermenters such as *Pediococcus*, *Streptococcus*, *Lactococcus* and some *Lactobacilli* produce lactic acid as the major or sole end product of glucose fermentation. Heterofermenters such as *Weisella* and *Leuconostoc* and some *Lactobacilli* produce equimolar amounts of lactate, CO_2 and ethanol from glucose (Jay, 1978; Caplice and Fitzgerald, 1999).

LAB are generally mesophilic but can grow at temperatures as low as 5°C or as high as 45°C. Similarly, while the majority of strains grow at pH 4.0- 4.5, some are active at pH 9.6 and others at pH 3.0. Most LAB require a wide range of growth factors including amino acids, vitamins, fatty acids, purines, and pyrimidines for their growth and biological activities (Caplice and Fitzgerald, 1999; Kwon *et al.*, 2000; Fitzpatrick *et al.*, 2001).

1.2 The Utilization of LAB

LAB can ferment carbohydrates such as glucose and lactose, and produce lactic acid. Lactic acid is a commercially valuable product with applications in food, manufacturing and pharmaceutical industries (Hofvendahl and Hahn-Hligerdal, 1997). Apart from lactic acid, LAB also produce many other products. Bacteriocins from LAB are natural anti-microbial peptides or proteins with interesting potential applications in food preservation and health care (Konings *et al.*, 2000). Some LAB secrete polysaccharide polymers, improving the viscosity and the texture of fermented products (Welman and Maddox, 2003).

LAB have been widely used in food industries. For example, *L. plantarum* is often used in the production of fermented commodities such as sausage, cucumber pickles and silage (Fu and Mathews, 1999); *Oenococcus oeni* is implicated in the maturation of wine and cider; *L. lactis* is of great economic importance in the bulk production of cheese (Sanders *et al.*, 1999). *L. acidophilus* is used in yogurt production (Talwalkar and Kailasapathy, 2004). Usually LAB are used as starter cultures. In these processes, LAB have two major functions, namely (i) achievement of certain beneficial physico-chemical changes in the food ingredients, e.g., acidification, curdling and production of flavor compounds, and (ii) inhibition of the outgrowth of microbial pathogens and spoilage organisms (Karen and Van Impe, 2002). LAB have also been shown to have probiotic properties for both humans and domestic animals. However, not all LAB are useful, some of them are involved in food spoilage or may even be pathogens.

1.3 Preservation of LAB

The industrial uses of LAB as starter cultures for the food industry depends strongly on the preservation technologies employed. This means that LAB can be used rapidly and conveniently when required. Indeed, effective preservation technology determines the usefulness of the cultures. It is required to guarantee long-term delivery of stable cultures in terms of viability and functional activity (Carvalho *et al.*, 2004). Freezing and freeze-drying have commonly been used for this purpose.

1.3.1 Starter Cultures of LAB

A starter culture may be defined as a preparation or material containing large numbers of viable microorganisms, which may be added to accelerate fermentation processes (Holzapfel, 2002). LAB occupy a central role in these processes, and have a long and safe history of application and consumption in the production of fermented foods and beverages (Caplice and Fitzgerald, 1999).

The earliest production of fermented foods was based on spontaneous fermentation due to the development of the microflora naturally present in the raw material. The process

of fermentation cannot be controlled very well, and the quality of the end product was dependent on the microbial load and spectrum present in the raw material. The application of selected starter cultures to raw materials was a breakthrough in the processing of fermented foods. A typical starter facilitates improved control of a fermentation process and predictability of its products. In addition, starter cultures facilitate control over the initial phase of a fermentation process. Modern starter cultures are selected either as single or multiple strains, specifically for their adaptation to a substrate or raw material (Holzapfel, 2002). Recently, new starter cultures of LAB with an industrially important functionality have been developed. They can contribute to microbial safety or offer one or more organoleptic, technological, nutritional, or health advantages (Leroy and Vuyst, 2004).

The starter cultures of LAB can be preserved by many methods. Preservation of frozen cell cultures in liquid nitrogen is the best when compared with subculture, freezing, freeze-drying, and spray-drying (Urraza and Antoni, 1997). However, the high cost of liquid nitrogen prohibits its widespread use in the storage of frozen cultures. Freezing starter cultures at -20°C or -80°C is a simple and effective method. Many factories still use this method to preserve starter cultures. However, freezing preservation requires special shipping conditions such as dry ice packing, and has the risks of thawing during transportation or storage. Starter in dry form is a better alternative.

Dried starters can be produced by spray-drying, vacuum-drying and freeze-drying. As most of mesophilic LAB are very sensitive to higher temperature of airflow during spray-drying, only some thermoduric LAB could be spray-dried (Champagne *et al.*, 1991). The survival rates of vacuum-drying may be inferior to those of freeze-drying (Brennan *et al.*, 1983). Freeze-drying is one of the most effective methods for long-term preservation for many microorganisms. It is commonly used in the production of starter cultures for the food industry. The advantages of freeze-drying are: (i) protection from contamination or infestation during storage; (ii) long stability, most microbial strains can be preserved in lyophilized state for periods of up to 10 years (Chang and Elander, 1986). (iii) ease of distribution as the dried cultures can be transported without refrigeration. However, Freeze-drying is an expensive dehydration process because of the low drying rate, high capital and energy cost due to refrigeration and vacuum units, are directly dependent on the drying duration (Lombraña and Villarán, 1997). Moreover, Freeze-drying has some undesirable side

effects, such as membrane damage, denaturation of sensitive proteins and decreased viability for many cell types (Leslie *et al.*, 1995; Castro *et al.*, 1997; Abadias *et al.*, 2001a). Microbial survival is dependent on the strain of microorganisms, growth conditions, age of the cell cultures, nature of the suspending medium, and processing conditions (Bâati *et al.*, 2000).

1.3.2 Viability and Vitality

Freezing and freeze-drying are commonly used in the production of starter cultures of LAB. But LAB can be injured or killed during these processes. So it is necessary to evaluate the quality of starter cultures after freezing and freeze-drying.

Viability measurements are commonly applied to assess the quality of starter cultures. Traditionally, viability has been determined by the capacity of cells to multiply, often by viable count or plate counting. Each microorganism in a suspension after series dilution will form a single colony after incubation in a suitable medium under favourable conditions. After incubation, the number of colonies formed is counted to achieve an estimation of the number of viable microorganisms in the original suspension. However, not all cells will give rise to a colony because certain microorganisms have a tendency to clump or aggregate. When plated onto suitable culture media, a clump will give rise to only one colony, regardless of how many cells are in the clump. In addition, the microorganisms which are sublethally damaged, viable but non-culturable or dormant do not form colonies (Heckly, 1978; Parthuisot *et al.*, 2003). The counting medium used also influences the growth of microorganisms. For example, some injured cells are no longer able to grow on minimal media but are able to form colonies on complete media (Parthuisot, 2003). Therefore, viability is not an easily defined concept in absolute terms. Another disadvantage of plate counting is time consuming and tedious to perform. In addition, plate counting only provides information about the viability in a laboratory medium under specific growth conditions (Hornbak *et al.*, 2002).

With the recognition of different physiological states of microorganisms, new definitions of viability have been given. Ueckert *et al* (1995) found that assessments of cellular structure and functions were relevant indicators of cell viability. Hornbak *et al*

(2002) used the term viability to describe the culturability of cells and considered cytological methods such as direct fluorescence methods as indicators of activity or vitality rather than viability measurement.

Yeast vitality has been variously described as a measure of activity, fermentation performance, or the capacity to overcome and recover from physiological stresses. Yeast vitality methods usually involve the measurement of specific yeast cell components critical to fermentation activity, or the determination of a metabolic activity that may be related to fermentation performance (Guido *et al.*, 2004). Vitality methods enable a differentiated picture of various physiological states of microorganisms to be defined when the cells are subjected to different kinds of stresses (Ueckert *et al.*, 1995; Hewitt *et al.*, 1998; Hewitt *et al.*, 1999). For the user of starter cultures, vitality may be more important than viability because vitality is a better indicator than viability to indicate the quality of starter culture. For example, sublethal injured cells of LAB during freeze-drying can grow on the rich media used for plating but show reduced activity when added to milk (Champagne *et al.*, 1991).

1.4 Freeze-Drying

1.4.1 Principle of Freeze-Drying

At atmospheric pressure (approx. 1,000 mbar) water can have three physical states: solid, liquid, and gaseous. Below the triple-point (for pure water: 6.1 mbar at 0°C), only the solid and the gaseous states exist. The principle of freeze-drying /sublimation is based on this physical fact. The ice in the product is directly converted into water vapor without passing through the fluid state if the ambient partial water vapour pressure is lower than the saturated vapour pressure of the ice at its relevant temperature.

Figure 1.1 shows the phase diagram for the ice/water/vapour system. The curve A-B is termed the sublimation curve, at which the solid and vapour are in equilibrium. Similarly curve B-C is the melting curve and B-D is the evaporation curve, terminating at CP, the critical point. The point of intersection of the curves, TP, is called the triple

point, at which the three phases exist in equilibrium. It has a unique value for pressure and temperature, and is a reference point. It should now be apparent that for any pressure in a system below the triple point pressure, water can be changed from the solid to vapour phase without the liquid phase, if the temperature at which it is allowed to do this does not have any restrictions. In practice, the system pressure (relative vacuum) used is much lower than the triple point pressure. A freeze-dryer therefore maintains the conditions of pressure and temperature required to remove water from the product as a vapour.

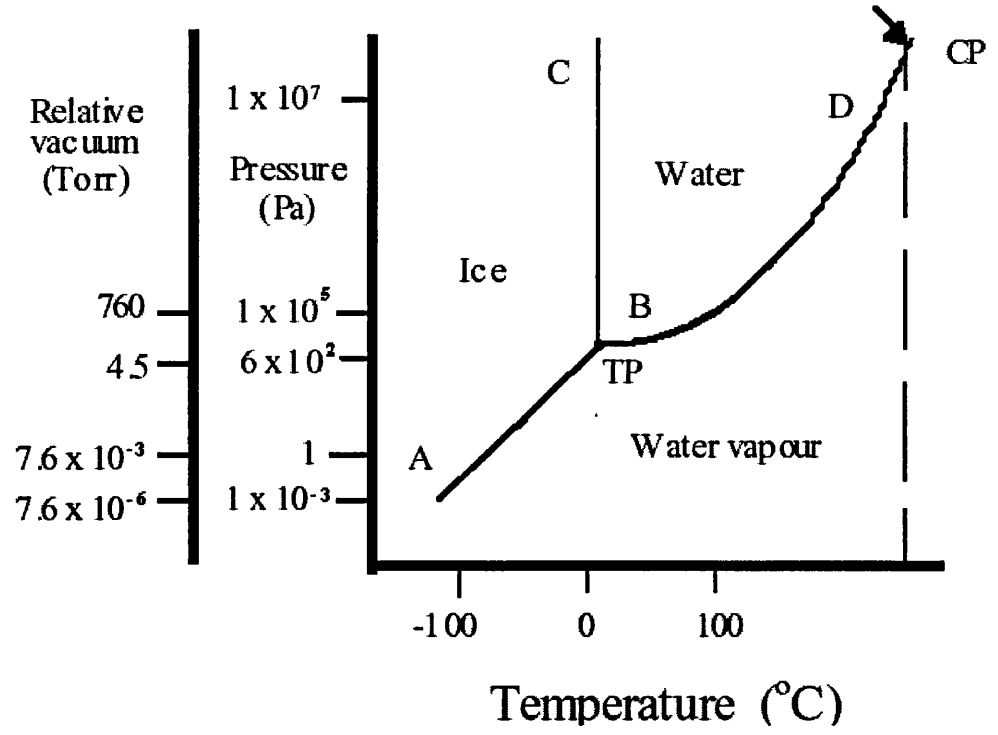


Figure 1.1 The typical phase diagram of water

1.4.2 Processes of Freeze-Drying

Freeze-drying, or lyophilization, is a process in which a product such as a microbial cell suspension is first frozen and then subjected to high vacuum (10^{-2} mbar). Under

these conditions frozen water will sublime and the product is dried. The principle of freeze-drying is shown in Figure 1.2. Freeze-drying process consists of two major steps: pre-freezing, and drying under vacuum. The drying step is further divided into two phases: primary and second drying.

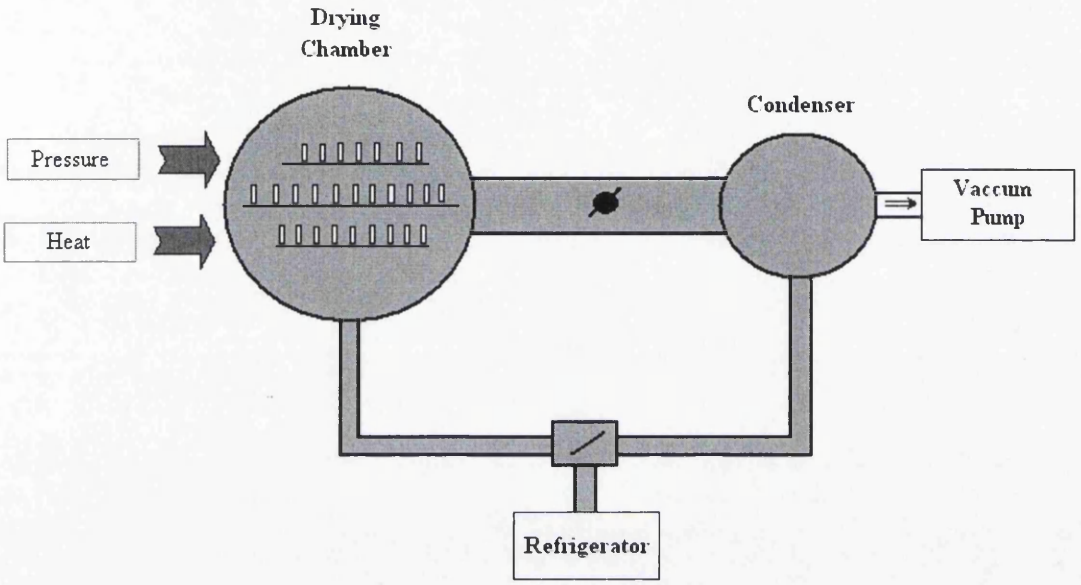


Figure 1.2 The schematic diagram of the principle of freeze-drying. The frozen product such as bacterial cells suspension is placed in the drying chamber. The refrigerator makes all the system, mainly drying chamber and condenser, cool down to -45~-50°C. The vacuum pump is used to lower the pressure of the environment around product and encourage the flow of water molecules from the product to the condenser. The condenser collects the moisture that leaves from frozen product. Under these conditions, freeze-drying starts, and the ice in the frozen product sublimes. As ice sublimes the temperature of product decreases and the rate of sublimation declines. Heat must be supplied to the product. The product should be freeze-dried at a temperature slightly lower than its eutectic or collapse temperature. The product is dried as ice sublimes.

Pre-Freezing:

Since freeze-drying requires a change in state from the solid phase to the gaseous phase (vapour), the product to be freeze-dried must first be adequately frozen. The majority of product consists primarily of water, the solvent, and the materials dissolved or suspended in the water, the solute. Many products that are to be freeze-dried form eutectics, which are mixtures of substances that freeze at lower temperatures than the

surrounding water. Products can be frozen in two ways, depending on the composition. When the aqueous suspension is cooled, changes occur in the solute concentrations of product matrix. As cooling proceeds, the water is separated from the solutes as it changes to ice, creating a more concentrated solution. These concentrated materials have a lower freezing temperature than water. Although the product may appear to be frozen because of all the ice present, in actuality it is not completely frozen until all of the solution is frozen. The mixture of various concentrations of solutes with the solvent constitutes the eutectic of the product suspension. Only when all of the eutectic mixture is frozen is the suspension properly frozen. This is called the eutectic temperature. It is very important to pre-freeze the product to below its eutectic temperature before beginning freeze-drying process. Small pockets of unfrozen materials remaining in the product expand and damage the structural integrity. Another type of frozen product is a suspension that undergoes glass formation during the freezing process. Instead of forming eutectics, the entire suspension becomes increasingly viscous as the temperature is lowered. Finally the product freezes at the glass transition point forming a vitreous solid. This type of product is extremely difficult to freeze-dry.

Primary Drying:

After pre-freezing the products, conditions must be established in which ice can be removed from the frozen products via sublimation, resulting in dry, structurally intact products. This requires very careful control of two parameters, temperature and pressure, involved in the freeze-drying system. The rate of sublimation of ice from frozen product depends upon the difference in vapor pressure that is related to temperature, it is necessary that the product temperature is warmer than the cold trap (ice collector) temperature (Figure 1.2). It is extremely important that the temperature at which products are freeze-dried is balanced between the temperature that maintains the frozen integrity of the products and the temperature that maximizes the vapor pressure of the products. This balance is key to optimal drying. The typical phase diagram shown in Figure 1.1 illustrates this point. Most products are frozen well below their eutectic or glass transition temperature, and then the temperature is raised to just

below the critical temperature (eutectic temperature) and they are subjected to a reduced pressure. At this point the freeze-drying process starts.

Some products such as aqueous sucrose solution can undergo structural changes during the drying process, resulting in a phenomenon known as collapse. Although the product is frozen below its eutectic temperature, warming during the freeze-drying process can affect the structure of frozen matrix at the drying front. This results in a collapse of the structural matrix. To prevent collapse of products containing sucrose, the product temperature must remain below a critical collapse temperature during primary drying. The collapse temperature for sucrose is -32°C. No matter what type of freeze-drying system is used, conditions must be created to encourage the free flow of water molecules from the product to the trap. Therefore, a vacuum pump is an essential component of a freeze-drying system, and is used to lower pressure of the environment around the product. The other essential component is a collecting system, which is a cold trap (generally around -50°C) used to collect the moisture that leaves the frozen product. The collector condenses out all condensable gases, i.e. the water molecules, and the vacuum pump removes all non-condensable gases.

As water sublimes, the product cools. Heat must be supplied to the product to encourage the removal of water in form of vapor from the frozen product. The heat supply has to be accurately controlled so that sublimation does not cease and the frozen product does not melt during the drying operation. The product should be freeze dried at a temperature slightly lower than its eutectic or collapse temperature since the colder the product, the longer the time required to complete primary drying. Usually the temperature of the product shelf is 20-40°C. During the primary drying stage it is usual to remove 98-99% of the contained moisture. Sublimation of the ice commences at the surface of product and the dry surface layer so formed remains static. As drying progresses the ice surface recedes gradually within the product, leaving a porous structure in the dried product, which like a 'cake'.

Secondary Drying:

After primary freeze-drying is complete, and all ice has sublimed, bound moisture is still present in the product. The product appears dry, but the residual moisture content may be as high as 7-8%. Continued drying is necessary at warmer temperature to

reduce the residual moisture content to optimum values, which is normally 0.5%. Secondary drying is normally continued at a product temperature higher than ambient but compatible with sensitivity of the product. The drying rate is affected by the thickness of material—the thinner the layer the higher the drying rate—and the nature of the product. The dry product usually retains its original shape and size and will have considerable porosity, resulting in subsequent ready solubility or dispersion in water (Williams-Gardner, 1971; Schelenz *et al.*, 1995). Usually drying requires 12-24 hrs under a vacuum of 25-50 μm of mercury, at a platen temperature of 20-40°C (Champagne *et al.*, 1991).

1.4.3 Freeze-drying of Bacteria

1.4.3.1 Effect of Freeze-Drying on Microbial Cells

Freeze-drying is a complex process and during such a treatment microbial cells are exposed to freezing and drying processes that subject cells to the stresses of high concentration of solutes, to extremes of pH, to low temperature, to the formation of ice crystals and to the removal of water from within the cell. The loss in viability and vitality of cultures can occur during freezing, drying or storage. Much of the work in this area has been done using organisms associated with dairy LAB.

Bacteria cells are sensitive to freezing, which usually leads to a slow rate of death. Indeed, freezing/thawing cycles are often used to kill bacteria cells (Panoff *et al.*, 1998). Water plays an important role in the maintenance of structure and functional integrity of biological membrane and bio-macromolecules. The removal of this water, by freezing or dehydration, often results in vast structural and functional alterations such as hydration-dependent phase changes and lateral phase separation of membrane components, intramembranous particle aggregation, loss of membrane permeability (Rudolph and Crowe, 1985). Brennan *et al.* (1986) reported that freeze-drying of *L. acidophilus* removes the bound water, destabilizing the structural integrity of macromolecules present in the cell wall and cell membrane. The ice formation and increase in salt concentrations within and around cells during freeze-drying might also cause cell membrane and cell wall damage, proteins and DNA denaturation (Jay, 1978; Panoff *et al.*, 1998; Wolfe and Bryant, 1999). Changes in intracellular pH and ionic

strength may increase the rates of detrimental chemical reactions (Efiuvwevwere *et al.*, 1999). The resistance of LAB to freezing has been related to fatty acid composition of cells (high levels of unsaturated fatty acids favour cryo-resistance) and to the nature of key proteins present within the membranes (Bozoglu *et al.*, 1987; Panoff *et al.*, 1998; Bâati *et al.*, 2000). It is found that up to 88% of *L. acidophilus* cells having survived freezing were sublethally injured and could not form colonies on MRS agar supplemented with 0.15% bile (Wright and Klaenhammer, 1981).

1.4.3.2 Bacteria Responses to Environmental Stresses

In nature and in industrial processing, LAB often encounter a number of stress conditions, such as low temperature, low pH, and low water activity that cause a loss of viability. However, microorganisms have developed some mechanisms to adapt to environmental stresses.

After the transfer of a bacterial cell population from the optimum temperature to a low temperature above 0°C, some bacterial strains develop an increased ability to survive at extremely low temperature, a phenomenon known as cryo-tolerance (Panoff *et al.*, 1998). This cryotolerance depends on the temperature and the duration of cold preincubation as well as the initial bacterial cell concentration. The bacterial cells will make some physiological and biochemical responses to cold shock: (I) synthesis of cold shock proteins (CSPs). For instance, the response of *Lactobacillus acidophilus* CRL 639 to cold-shock (freezing) is over-expression of six proteins (22.5–105.0 kDa) (Lorca and de Valdez, 1998). The cold acclimation proteins might act as antifreeze proteins or as anabolic enzymes which would be involved in the synthesis of putative antifreeze substances or even in maintaining some metabolic functions of the cells (Phadtare *et al.*, 1999); (II) modifications of lipid composition and membrane fluidity.

The physical properties of the cell membrane depend on the degree of saturation of the fatty acids. Cooling leads to desaturation of fatty acids, resulting in increased fluidity of the membrane, which is necessary for proper functioning at the lower temperature (Graumann and Marahiel, 1996; Kim *et al.*, 1998; Panoff *et al.*, 1998; Bâati *et al.*, 2000). These processes can be seen as mechanisms by which organisms protect themselves when exposed to cold temperature. During this adaptation, it is found that some bacteria change their morphological shapes. For example, *L. acidophilus* CRL

639 showed an important morphological transition from short rods to long and curled filaments when grown at 37°C and 25°C respectively (Lorca and de Valdez, 1998).

Bacterial cell envelopes are permeable to water. Therefore, an increase in the osmolarity of the growth medium would result in rapid efflux of water from the cytoplasm. To retain water in the cell and, thus, to maintain turgor pressure, bacteria have systems to accumulate compatible solutes that do not interfere with cell physiology. The rapidly accumulating compatible solutes prevent the loss of water and loss of turgor pressure. Such compatible solutes are either taken up from the environment or newly synthesized in the cytoplasm. The uptake mechanism is found in most LAB. Physiological studies on osmoregulation have revealed that the amino acids, glutamate and proline, and the quaternary ammonium compounds, glycine betaine, are the most important compatible solutes used by bacteria (Sanders *et al.*, 1999; Konings *et al.*, 2000).

LAB produce lactic acid during sugar fermentation. This implies that they are frequently confronted with acid and osmotic stresses. Lactic acid is a weak organic acid that is not charged at low pH and can easily cross the cell membrane. At cytoplasmic pH, it dissociates and, thus, poses a stronger stress to cells (Kashket, 1987). A number of mechanisms have been shown to confer acid resistance. The primary mechanism for control of intracellular pH is the F_0F_1 ATPase that translocates protons to the environment at the expense of ATP. Both the expression level and the activity of this protein complex are increased at low pH (Nannen and Hutkins, 1991).

Exhaustion of an essential nutrient limits growth of a culture that then enters the stationary growth phase. It is generally accepted that stationary phase is the most common state of bacterial cells in nature. Total cell proteins are degraded during exponential growth and in the first 30–90 min of stationary phase, but proteins are stably maintained afterwards. Bacterial cells react to starvation by increasing the level of 14 proteins, despite the lack of metabolic energy (Sanders *et al.*, 1999).

Subjection to a mild stress makes bacterial cells resistant to a lethal challenge with the same stress condition, as has been discussed above for a number of stress conditions. Moreover, pre-adaptation to one stress condition can render cells resistant to other stress imposing conditions.

1.4.4 Factors that affect freeze-dried bacterial survival

LAB represent the major group on which research on preservation of freeze-drying bacteria has been focused. Maximization of survival of LAB cultures during drying and subsequent storage for long periods is of vital importance, both technologically and economically. Bacterial cell survival throughout drying and storage is dependent on many factors, including growth conditions (Palmfeldt and Hahn-Hägerdal, 2000), growth medium (Carvalho *et al.*, 2003a), drying medium (Font de Valdez *et al.*, 1983a and 1983b; Leslie *et al.*, 1995; Abadias *et al.*, 2001a), initial concentration of microorganisms (Costa *et al.*, 2000) and rehydration conditions (Font de Valdez *et al.*, 1985a and 1985b; Abadias *et al.*, 2001b).

1.4.4.1 Cultural Conditions

The production of freeze-dried cultures requires that the cells must be prepared to withstand the stresses of freezing and drying. The culture conditions such as pH, temperature, and the medium composition will influence the survival of bacteria (Wright and Klaenhammer, 1983a and 1983b; Neidhardt, *et al.*, 1990; Palmfeldt and Hahn-Hägerdal, 2000; Carvalho *et al.*, 2003a).

Growth Medium

Specific compounds in growth media have been found to influence the survival rate of LAB after freeze-drying. Tomato juice media was supplemented with Tween 80 or oleic acid to improve the viability of *Lactococcus lactis* or *lactobacillus sp.* upon freeze drying (Goldberg and Eschar, 1977). Addition of Tween 80 to the growth medium improved the survival rate of *L. acidophilus* by a factor of ten (Chang and Elander, 1986). When *L. bulgaricus* and *L. acidophilus* were grown in a nutrient broth medium to which calcium was added (0.006 M), there was increased survival of the cells after freeze-drying (Wright and Klaenhammer, 1983a and 1983b). It was found that the addition of magnesium and calcium cations to the growth medium of *L. bulgaricus* promoted short bacilloid rod morphologies, as opposed to filamentous morphologies, ever under controlled conditions. The protective effect of calcium to

freezing was related to morphological characteristics of the bacterium, the short bacilloid rods being more resistant to freezing than filamentous forms (Wright and Klaenhammer, 1981 and 1983a). Calcium was ineffective, however, in preventing freeze injury, since the cells displayed decreased acid production in milk following -20°C storage (Wright and Klaenhammer, 1983b). Calcium has an important role in the structure of the cell wall, possibly influencing its contraction and expansion (Champagne *et al.*, 1991). Since freeze-drying causes damage to the cell wall (Brennan *et al.*, 1986), it is thus logical that minerals that contribute to its structure may have a positive effect on subsequent survival after freeze-drying.

Growth Phase

Growth phase of cells also influences survival after freeze-drying. Exhaustion of an essential nutrient limits growth of a culture that enters the stationary growth phase. It is generally accepted that stationary phase is the most common state of bacteria cells in nature (Sanders *et al.*, 1999). The cells harvested in stationary phase showed a higher resistance compared to those obtained in exponential phase (Thammavongs *et al.*, 2000). The survival rate of yeast during drying was influenced by the energy levels in the cells. Yeast cells in stationary phase showed an increase in ATP-level when they were dried, whereas with exponential phase cells this behaviour was not observed. The accumulated ATP can serve as an energy source during rehydration of the cells, which could result in a higher survival rate (Lievense and Riet, 1994).

Growth Conditions

Growth temperature affects the fatty acid composition of microorganisms, e.g. *E. coli,* growth at sub-optimum temperatures increases the proportion of unsaturated fatty acids in the cells (Marr and Ingraham, 1962). Thus, it may be that producing cultures at low temperature might increase their survival to the freezing process.

It was found that when *L. lactis* was frozen at –20°C for 24 h, the cell viability decreased drastically. But when they were cold shocked at 10°C for 2 h prior to

freezing, viability improved significantly, however, not for all LAB. When the period for cold shock was extended to 5 h, the viability increased even further for all tested strains (Sanders *et al.*, 1999). Survival of bacteria first grown at 30°C to stationary phase and subsequently incubated at 4°C was 30%, whereas 0.03% of the culture survived when held at 30°C throughout the experiment. Similarly, survival from a freezing-thawing cycle was better (95%) after preincubation at 8°C for 48 h than without such an adaptive treatment (75%). The resistance of *L. delbrueckii subsp. bulgaricus* to freezing can be increased by preincubation at suboptimal temperatures in the medium with a fermentable sugar (Urraza and Antoni, 1997). *L. lactis* subspecies *lactis* strain was shown to acquire freezing resistance by pre-adaptation at 10°C whereas strains of the subspecies *cremoris* are equally freezing sensitive with or without pre-adaptation (Woojin *et al.*, 1999).

The pH must be controlled at two levels: during cell growth and in the suspension medium as freeze-drying of acid cultures is detrimental to survival. Low pH resulting from acid production is a growth-limiting factor. Growth of LAB under pH control not only results in higher biomass but also in increased survival. *L. reuteri* was cultivated at pH 5 and 6. The highest viability ... approximately 80% of the viability after freeze-drying ... was obtained for the cells from the pH 5 cultures harvested 2.5 h after entering stationary phase (Palmfeldt and Hahn-Hägerdal, 2000). It has been observed that pH 5 can trigger a stress response in LAB (O'Sullivan and Condon, 1997). Gilliand and Lara (1988) reported that *Lactococcus* cultivated at regulated pH 6 were more resistant to freezing at –17°C than those grown at non-regulated pH, and survival of LAB was improved by neutralisation of suspending medium or buffer.

1.4.4.2 Suspension Medium

Freeze-drying might cause cell membrane damage, proteins and DNA denaturation, and decrease viability. To prevent or reduce the loss of cell viability, protective substances such as skim milk, sucrose and glycerol are commonly added to samples before freezing or freeze-drying (Leslie *et al.*, 1995). The protective substances have two functions in preserving viability of freeze-dried cells. The first is to provide a dry residue with a definite physical structure acting as a support material and as a receptor

in rehydration, and the second is to protect the living cells biochemically against damage during freezing and/or drying (Abadias *et al.*, 2001a). A good suspending medium should provide cryo-protection to the cells during freeze-drying, be easily dried and provide a good matrix to allow stability and ease of rehydration.

Various groups of substances have been tested for their protective actions: (i) polyols, (ii) polysaccharides, (iii) disaccharides, (iv) amino acids, (v) proteins, (vi) minerals, (vii) salts of organic acids and (viii) vitamin-complex media. However, protection afforded by a given additive during these processes will vary with the species of microorganism. There are significantly different responses of various strains to different additives used. Protection afforded by a given additive during freeze-drying seems to vary from strain to strain (Font de Valdez *et al.*, 1983b; Costa *et al.*, 2000). For any given microorganism, there are only one or two suitable cryoprotective agents (Theunissen *et al.*, 1993).

Polyols

Glycerol is an effective cryoprotectant and widely used in frozen concentrates. It provides some protection in freeze-drying (Costa *et al.*, 2000). However, glycerol remains liquid at normal storage temperature (4°C), and may not be useful for freeze-drying. When the six polyols were added to skim milk and evaluated for their cryo-protection effects on 12 strains of *lactococci*, *lactobacilli*, and *Leuconostoc*, more than 80% survival was obtained with adonitol for 9 of the 12 strains tested. Dulcitol, mannitol, m-inositol and sorbitol were found to provide little or no protection when added to milk (Font de Valdez *et al.*, 1983a). Unfortunately the cost of adonitol limits its industrial use.

Polysaccharides

Polymers such as polyethylene glycol (PEG) and dextran accelerate drying. PEG in itself is not effective in protecting *L. bulgaricus*. But the addition of PEG to milk did increase survival to freeze drying by 10-20% with mesophilic *streptococci* (Font de

Valdez *et al.*, 1985b). Gum was found not having protection when it was added to milk medium. Pectin and dextran increase survival to freeze-drying for most *lactobacilli* and *lactococci* (Champagne *et al.*, 1991).

Disaccharides

Disaccharides such as trehalose, sucrose are most wildly used as cryo-protectants. Kilara *et al* (1976) found that lactose (7%) was a useful protectant of LAB obtaining viabilities of between 23% and 50% depending on the genera studied. Sucrose can also be used successfully. An increase in viability was observed when the concentration of protectant was increased from 1% to 10% (Abadias *et al.*, 2001a).

A few mechanisms have been proposed to explain the protection afforded by sugars or polyols. Damage to biological systems resulting from freeze-drying can be attributed to two primary causes: changes in the physical state of membrane lipids and changes in the structure of sensitive proteins. As water is removed from lipid bilayers of cell membrane the headgroups are brought closer together, resulting in an increase of van der waals interactions between the acyl chains. The increase in attraction between the acyl chains forces the dry bilayer into the gel phase at room temperature, which could lead to phase separation of membrane components. When the dry lipid is rehydrated it undergoes a phase transition from the gel back to liquid crystalline phase, during which leakage occurs (Leslie *et al.*, 1995).

Drying with trehalose or sucrose can prevent the damaging effects of drying and rehydration on membranes by maintaining the lipid in liquid crystalline phase at room temperature, even when dry. It has been proposed that sugars depress the phase transition in dry phospholipid by hydrogen bonding to the polar headgroups, an idea known as the water replacement hypothesis. In addition, trehalose and sucrose have been shown to preserve both structure and function of isolated proteins during drying, this ability to stabilize proteins results from disaccharides forming hydrogen bonds with the proteins when water is removed, thus preventing protein unfolding, aggregation and denaturation (Leslie *et al.*, 1995; Da Costa *et al.*, 1998; Costa *et al.*, 2000). Differences exhibited by sugars are connected with their water-bonding capacity and prevention of intracellular and extracellular ice crystal formation. Trehalose has less of a tendency to crystallize than sucrose and lactose (Costa *et al.*,

2000). It was suggested that the ability of sugars to stabilize phospholipid bilayers was dependent on the stereo-conformation of the hydroxyl groups (Lievense and Riet, 1994).

Amino acids and proteins

Amino acids such as sodium glutamate, aspartic acid and cysteine have been successfully used as cryoprotectants during freeze-drying while asparagine is not effective (Font de Valdez *et al.*, 1983a; Costa *et al.*, 2000). This protection by amino acids is thought to be the result of a reaction between the carboxyl groups of the microorganism's protein and the amino group of protectant, stabilizing the proteins structure. Cysteine, however, is thought to prevent the oxidation of SH group to intermediate SS bonds. It could also restore the SH groups from the intermolecular SS bonds (Champagne *et al.*, 1991). Skim milk powder (20%) is also commonly used as a cryoprotective agent (Arnold *et al.*, 1986). Peptone, yeast extract and protein hydrolysates increase survival of most strains when compared with water but are generally less effective than milk or sucrose. The same can be said of proteins such as gelatine or bovine serum albumin (Font de Valdez *et al.*, 1983a).

Other compounds

The salts of phosphate as well as acetate and citrate can be effective when compared to water but they are generally less effective than milk solids (Champagne *et al.*, 1991). *Lactobacillus bulgaricus* cultures retained better activity following freeze-drying when 0.01% Tween 80 was added to 10% skim milk prior to freeze-drying (Naghmoush *et al.*, 1978).

1.4.4.3 Freeze-Drying Conditions

Cooling rate of cells is also a critical factor in freeze-drying process. If cooling is slow enough, water will have time to flow out of the cell by osmosis, dehydrating the cell and thus avoiding freezing. If cooling is very fast, the cell does not loose water quickly

enough to maintain equilibrium, ice crystals eventually form intracellularly. Ice formation inside the cell may cause lethal damage. The optimum rate of freezing varies from one genus to another (Champagne *et al.*, 1991; Sanders *et al.*, 1999). There is little information about the effect of freezing temperature on cell viability. The final temperature after rapid freezing to –70°C or –196°C had no effect on the recovery of *chlamydias* after freeze-drying (Theunissen *et al.*, 1993).

Drying level will affect viability of bacteria not only after freeze-drying itself, but also upon storage. Over-drying cellular materials would remove all the water, causing degradation of proteins and exposure of hydrophilic sites to gas such as oxygen. Font de Valdez *et al* (1985b) reported that there were indeed high mortality rates when residual moisture dropped under 0.5%. Although under-drying of viral preparations left a water barrier that acted as a protectant against oxidation of macromolecules at the surface of particles, chemical reactions might occur when residual water was available, and under-dried cultures of *L. oenos* showed poor stability upon storage. The optimum residual moisture content varies with composition of the fluid in which organisms are dried, with the storage atmosphere, and probably with the species and physiological state of the organisms. The optimum available water (Aw) level for acceptable viability was determined to be 0.1 with LAB (Champagne *et al.*, 1991).

In order to obtain a suitable commercial product it is necessary to achieve a high density of viable dried cells. Some studies have shown that the initial bacteria load affects the survival rate during treatment. The initial bacterial load affects the survival ratio. With all strains tested by Kilara *et al* (1976), there was higher viability when the highest initial cell density was freeze-dried. The protective effect of high initial bacterial load is a function of the interaction between microorganisms through decreasing the exposed area of each cell to the environment and therefore preventing possible damage. An initial biomass that would be too concentrated (10^{12} cells/ml) would, however, be harmful because of an unbalanced osmotic pressure (Bozoglu *et al.*, 1987).

1.4.4.4 Rehydration of Freeze-Dried Bacteria

Rehydration is a critical step in the recovery of freeze-dried bacteria. Direct rehydration of *O. oeni* in wines for the purpose of MLF, however, can result in

mortality of 95% of the freeze-dried culture (Davis *et al.*, 1985). Freeze-dried bacteria suspensions usually contain dead cells and unharmed cells as well as those sublethally injured. The injured cells may repair and regain normal function if they are rehydated under appropriate conditions (Font de Valdez *et al.*, 1985a; Theunissen *et al.*, 1993).

The rehydration of freeze-dried cultures in liquid media is usually completed within a few seconds, so that the cells are subjected to very fast changes from dry or near-dry conditions to a mixture of hydrated colloids and aqueous solutions that make up the living cells. The complexity of the changes and the speed with which they occur make it extremely difficult to measure or control the events which take place (Font de Valdez *et al.*, 1985b).

The medium itself, its molarity and the rehydration conditions can significantly affect the rate of recovery (Costa *et al.*, 2000). Sinha *et al* (1982) researched the effect of different rehydrating media on the viability of freeze-dried *lactic Streptococci.* They found that the rehydration medium had a considerable effect on viability. In that case, rehydration media composed of sucrose (10%), dextrose (10%) and reconstituted skim milk (10%) substantially increased recovery. To a lesser extent, yeast extract (5%), peptone (10%), sodium glutamate and horse serum were also effective. Water alone is not a good suspension medium for freeze-dried cultures. A broth containing 1.5% peptone, 1% yeast extract, 1% tryptone, 1% glucose, 0.1% Tween 80 and 0.05% formic acid gave good results for *streptococci* and *Leuconostoc.* Minerals often gave good recovery rates but were strain dependent. Marked variations in their capacity to repair cellular damage after freeze-drying were observed among the species and strains under consideration (Font de Valdez *et al.*, 1985a). The viability of *Lactobacillus plantarum* was enhanced by immobilizing cells in calcium-alginate beads incorporating cryoprotectants (Kearney *et al.*, 1990).

Very little information is available on the effect of rehydration medium pH, but a value of 6.5 appears recommendable (Font de Valdez *et al.*, 1985a). Rehydration at 22°C permits better recovery of *lactic streptococci* than 5°C or 37°C. While higher temperatures (30-40°C) enabled better recovery of *Bifidobacterium longum* and *Lactobacillus acidophilus* (Champagne *et al.*, 1991). The volume of liquid used to rehydrate a freeze-dried culture will influence the survival of LAB. Adding large volumes of liquid give less recovery. This effect is attributed to a lethal osmotic shock.

A popular way is to rehydrate the culture back to the volume it had prior to being freeze-dried (Font de Valdez *et al.*, 1985a).

1.4.5 Storage of Freeze-Dried LAB

The storage conditions can affect the freeze-dried LAB stability. Freeze-dried LAB cultures are usually stored at temperatures below 5°C. it is generally believed that lower storage temperatures (-20°C to -70°C) are superior for long-term culture stability. If stored at -20°C, many freeze-dried cultures remain active for one year. Most cultures can be kept for 6 months at 4°C. Some cultures maintained their activities for three months at 22°C if stored under vacuum. Maintenance of vacuum in vials is the most critical factor during storage. It is generally recommended to store freeze-dried cultures under vacuum or under dried N_2 at low temperature (Arnold *et al.*, 1986; Castro *et al.*, 1995).

Phenomena observed during storage may differ from those noted immediately following freeze-drying. Many compounds offer protection to freeze-drying itself, but do not prevent viability losses during storage (Font de Valdez *et al.*, 1983b; Lievense and Riet, 1994; Champagne *et al.*, 1996). So it is necessary to conduct storage trials to evaluate a protective compound. An interesting approach to maintain high viability during storage is the microencapsulation of LAB (Kim *et al.*, 1988).

LAB are notorious for variability in terms of response to protective compounds for freeze-drying or cell recovery. There were significantly different responses of the various strains to different additives used at all storage temperatures. There was a significant interaction between strain and storage time at all incubation temperatures. Thus, the mortality rates during storage were strain-dependent (Champagne *et al.*, 1996).

1.4.6 Productivity of Freeze-Drying of LAB

Freeze-drying is an expensive dehydration process because of the low drying rate, high capital and energy cost due to refrigeration and vacuum units (Lombraña and Villarán,

1997). So it is necessary to maintain the productivity of freeze-dried of LAB as high as possible. The productivity is directly related to the cell vitality after freeze-drying. It has been known that freeze-drying can cause cell membrane and cell wall damage, proteins and DNA denaturation (Jay, 1978; Panoff *et al.*, 1998; Wolfe and Bryant, 1999), and loss in vitality. Low vitality means low productivity. The cell vitality of LAB after freeze-drying depends on many factors, which are summarized in Figure1.3. During the preparation of freeze-dried LAB as starter cultures, each step should be controlled very carefully in order to maximize the vitality and productivity of freeze-dried of LAB.

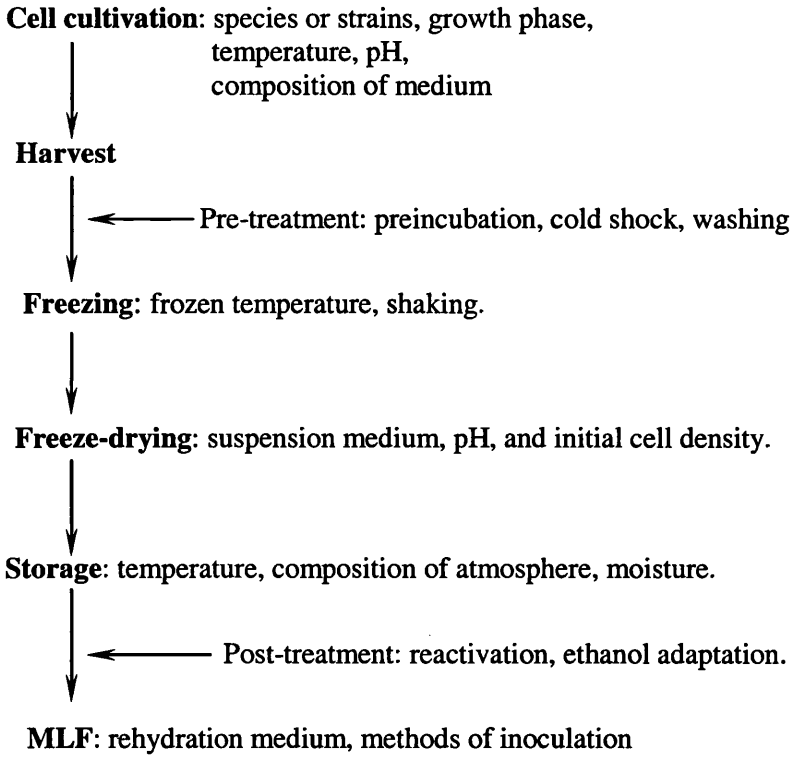


Figure 1.3 The process of freeze-dried starter cultures of malolactic bacteria and the factors that affect cell vitality.

1.5 Malolactic Fermentation

1.5.1 What is Malolactic Fermentation (MLF)

In many winemaking processes, two main fermentation processes are present: (a) alcoholic fermentation conducted by yeasts, and (b) malolactic fermentation (MLF).

MLF is the bioconversion of the malic acid in wines to lactic acid and CO_2. MLF results from the metabolism of certain LAB in wines. Because malic acid is a dicarboxylic acid and lactic acid is a monocarboxylic acid, MLF causes a decrease in acidity of the wine.

MLF was shown by Peynaud (1967) in the first published results on the lactic acid microflora of grape must and wines. After the works of Ribereau-Gayon *et al* (1975), MLF has been intensely studied world (Vivas *et al.*, 1997).

Apart from decreasing of the wine acidity, MLF can improve the flavor and the microbiological stability of wine (Davis *et al.*, 1985 and 1986; Maicas *et al.*, 1999a). MLF is an important process in production of wine, and is essential for most red wines and some white wines. MLF occurs naturally in red wines approximately 2-4 weeks after alcoholic fermentation has been completed (Wibowo *et al.*, 1988).

Most wines contain 0.55% to 0.85% titratable acid, and MLF is a major means by which the winemaker is able to reduce such acidity. Reduction in acidity by MLF can vary from 0.1% to 0.3%, and pH may rise by 0.1 to 0.3 of a unit. However, MLF is not favorable for all wines. Wines of high pH, such as those that are produced in the warmer viticultural regions of California, Australia, and South Africa, have a low acidity (0.5% to 0.65%). MLF could reduce the acid level too much, resulting in a flat, insipid wine and growth of spoilage bacteria (Davis *et al.*, 1985).

1.5.2 Malolactic Bacteria

When fermentation tanks are filled after crushing, the grape juice contains yeasts, LAB and acetic acid bacteria. Viable populations of 10^2 to 10^4 cells/ml of bacteria occur, varying mainly according to the conditions during the final days of ripening and harvest. Addition of sulphite to the grape must is the first step in vinification. It reduces the bacterial population. Yeasts, which are less sensitive to sulphite, grow rapidly initiating the alcohol fermentation. During this phase the conditions are very unfavourable for bacteria growth. After a short period of multiplication, interaction between yeasts and bacteria results in a decrease in the population. At the end of alcohol fermentation the numbers are usually about 10^2 to 10^3 LAB cells/ml.

Therefore, MLF normally follows alcohol fermentation if the general conditions are favourable (Lonvaud-Funel, 1995).

During alcoholic fermentation the bacterial population is also exposed to a natural selective process. The strains of LAB isolated from wines belong to the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*, although MLF is mainly driven by *Oenococcus oeni*, a species formerly known as *Leuconostoc oenos* (Dicks *et al.*, 1995). During wine fermentation, *Oenococcus oeni* has a higher capacity to degrade malic acid under such unfavourable conditions as low pH, high ethanol concentration and the presence of other compounds (for example, fatty acids) from the yeast metabolism or from technological aspects (for example, sulphur dioxide) (Lonvaud-funel, 1995; Vaillant *et al.*, 1995; Carreté *et al.*, 2002). It reaches a population of 10^6 cells/ml which is necessary to achieve significant malic acid degradation (Davis *et al.*, 1986; Lonvaud-Funel, 1995). During MLF, *O. oeni* ferments residual sugars, which are not fermented by yeasts. Glucose, fructose, xylose and arabinose are metabolised by *O. oeni* by heterofermentative pathway to produce lactic acid, acetic acid, ethanol and CO_2 as main products.

Malolactic bacteria are fastidious in nutritional requirement. Whatever strains are used, arginine, isoleucine, glutamic acid and tryptophan are essential for growth. Although some deficiencies (glycine, phenyalanine, proline or tyrosine) had no influence on the growth, they noticeably limited MLF (Fourcassie *et al.*, 1992).

1.5.3 Biochemistry of MLF

The term 'malolatic fermentation' was given to this step of winemaking by analogy to alcoholic fermentation long before the real mechanism was discovered. From a biochemical point of view, the conversion of malic acid to lactic acid is not a fermentation step, since it only consists in its simplest form of decarboxylation.

In the past, it was thought that the major pathway was that malic acid might first be decarboxylated to pyruvate with the reduction of NAD (malic enzyme activity), and then pyruvate reduced to lactic acid with reoxidation of NADH to NAD (lactate dehydrogenase activity). After further research it was found that a novel enzyme catalyses directly decarboxylation of malic acid to lactic acid without production of

pyruvate as an intermediary product (Radler, 1975; Ansanay *et al.*, 1993; Lonvaud-Funel, 1995; Versari *et al.*, 1999). This enzyme has been termed malolactic enzyme (L-malate: NAD carboxy lyase) (IUC number1.1.1.38). As shown in Figure 1.4, malic acid has two acidic groups that can release two protons while lactic acid contains only one proton that can be released. Note that the other terminal carbon in lactic acid has three hydrogen atoms. One of the "free protons" in the system has been fixed in the conversion of malic acid to lactic acid. Thus the acidity is decreased and the pH of wine is increased. The theoretical yield of this reaction is 0.67 g of lactic acid produced from 1 g of malic acid degraded (Fourcassie *et al.*, 1992).

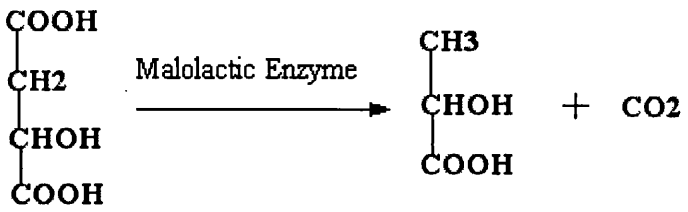


Figure 1.4 Malolactic fermentation

At the same time, the residual sugars (glucose, fructose, xylose and arabinose) are metabolized to produce lactic acid, acetic acid, ethanol and CO_2.

When research on MLF was first started, the most common result was that bacteria grew more easily in the media with added malic acid. Nevertheless, the malolactic reaction by itself was not thought to provide energy to the cell. The most usual comment was that in an acidic medium like wine, the decarboxylation of malic acid induced an increase in pH which was beneficial to bacterial growth. However, recent results have demonstrated that in an acidic medium, the malolactic reaction induces ATP production via a chemiosmotic mechanism. The transport of malic acid into the cell, its decarboxylation and efflux as lactic acid and the concomitant efflux of a proton create an energetic proton motive force. Thus, the transfer of malate and lactate through the membrane as the result of MLF generates energy, which explains the improvement of bacterial growth in such a medium (Figure1.5). The energy of the proton movement can then be captured on ATP (Lonvaud-Funel, 1995; Versari *et al.*, 1999).

Malolactic enzyme in *O. oeni* has been isolated and characterized. It is an interacellular enzyme that has a molecular weight of about 130 000, requires both NAD and $Mn^{2+}$and has optimum activity at pH 5.5~5.8 (Lonvaud-Funel and de Saad, 1982; Spettoli *et al.*, 1984). Malolactic enzyme is inducible. The induction is produced by L-malic acid only in the presence of glucose and amino acids. NAD^+ protects the enzyme against inactivation and its addition, after dissociation, restores the malolactic activity. NAD^+ is bound first to the protein, independently of malate concentration. Mn^{2+} acts as an allosteric activator. Malate is bound to the complex enzyme-NAD-Mn^{2+}. Oxamate, fructose 1,6-diphosphate and malonate act as non-competitive inhibitors, whereas citrate and tartrate produce competitive inhibitions (Strasser de Saad *et al.*, 1988; Lonvaud-Funel, 1995).

The pathway of MLF includes the uptake of malic acid, its decarboxylation to lactic acid and CO_2, and excretion of the end product. However, little is known about the mechanisms for transport of malic acid into cell or lactic acid out of cell. A specific permease is responsible for transport of malic acid into the cell (Gao and Fleet, 1994). Tourdot-Marechal *et al* (1993) reported that the cells of *O. oeni* could possess two uptake mechanisms for malic acid, active transport and passive diffusion. It appears that different species of malolactic bacteria possess different transport systems (Versari *et al.*, 1999).

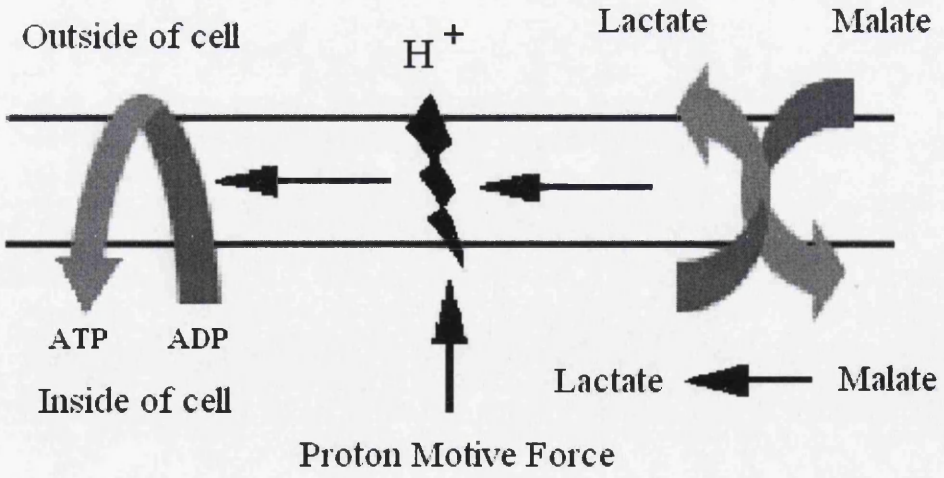


Figure 1.5 The schematic diagram of energy generation from MLF. The conversion of malate to lactate and accompanying "fixing" of a proton decreases the proton concentration of the cytoplasm upon efflux of lactate thereby creating a "proton motive force" across the membrane. The energy of the proton movement can then be captured on ATP.

1.5.4 Effect of MLF on Quality of Wines

The main effect of MLF is a decrease in total acidity resulting from the decarboxylation of malic acid to lactic acid. MLF induces a dramatic change in the organoleptic quality of wines, since, besides deacidification, the specific taste of malic acid disappears. However, MLF is a very complex process. It is now accepted that the role of MLF is more than just a deacidification process. During MLF malolactic bacteria can metabolise many other components of wine (see Figure 1.6), which give a positive or negative influence on the quality of wines.

The main wine sugars are glucose and fructose. Heterofermentative LAB convert glucose to lactic acid, ethanol and carbon dioxide whilst fructose is converted to mannitol, lactic acid and acetic acid. Sugar utilization can lead to wine spoilage under certain conditions, e.g. where there is more than 0.2% sugar in the wine and pH is above 3.8 (Tracy *et al.*, 1988; Pimentel and Silva, 1994).

Citric acid is one of the major organic acids in grape juice and wine, besides malic acid and tartaric acid. Citric acid can be transformed to lactate, acetate, diacetyl, acetoin and 2,3-butanediol (Martineau, 1995; Caplice, 1999). A small amount of citrate is converted to aspartate via oxaloacetate and aspartate aminotransferase. The most important oenological significance associated with citrate fermentation is the production of diacetyl (2,3-butanedione), an aroma compound with a buttery flavour note. In general, wines that have undergone MLF have higher concentration of diacetyl. But the final level of diacetyl in wine is affected by a number of factors, such as bacterial strain, wine type, sulfur dioxide and oxygen. Sensory threshold for diacetyl in wine was reported to be 2-3 mg/l. Wine with higher levels of diacetyl could be considered spoiled (Susan *et al.*, 1990).

Phenolic acids (mainly ferulic and p-coumaric acids) are natural constituents of grape juice and wine. These phenolic compounds can be decarboxylated microbially during fermentation into volatile phenols such as 4-ethylguaiacol and 4-ethylphenol. The volatile phenols can contribute to wine aroma positively or negatively, dependent on the concentration, due to their low detection thresholds and their distinct flavor. Whereas wine yeasts are, to a large extent, responsible for the decarboxylation of phenolic acids, wine LAB may also be able to do this. It has been observed that the

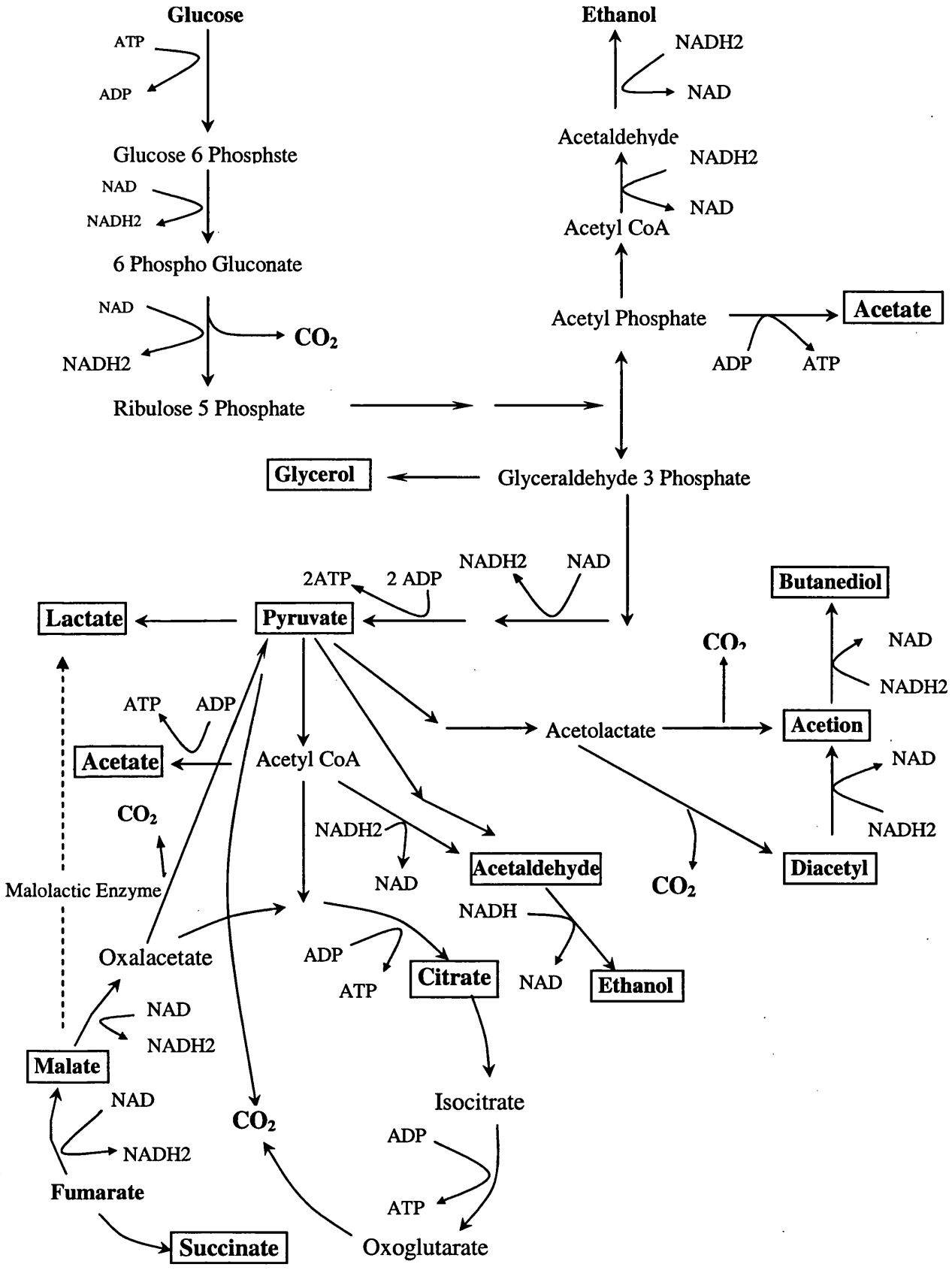


Figure 1.6 Summary of metabolic pathways of glucose, organic acids and metabolised products by wine LAB. The boxed compounds are important flavour compounds.

concentration of volatile phenols increased markedly during MLF, suggesting that wine LAB might be involved. This is verified by the catabolism of ferulic and p-coumaric acids by several wine lactobacilli and pediococci and the detection of corresponding volatile phenols (4-ethylguaiacol and 4-ethylphenol) (Liu, 2002). Tannins, anthocyans are also metabolised, which affect the taste and colour of wine (Lonvaud-Funel, 1995).

One of main reasons carrying out MLF is that wines that have undergone MLF are, in a microbiological sense, more stable than those that have not. In general, few winemakers will risk bottling wines before MLF has been completed, as they believed from experience, that wines without MLF may undergo this reaction in the bottle, as a consequence, the sediment, haze, and gassiness produced would be considered as spoilage. This may be explained not only by deprivation of nutrients, but also probably by synthesis of antibacterial compounds. However, many aspects still remain unclear and practical difficulties are often encountered (Davis *et al.*, 1985; Lonvaud-Funel, 1995).

MLF is not favorable for all wines. Grapes grown in warmer areas tend to be less acid and a further decrease in acidity by MLF may be deleterious for the sensory properties and biological stability of wine (Martineau and Henick-Kling, 1995; Versari *et al.*, 1999). An excess of acetic acid, the synthesis of glucane, biogenic amines and precursors of ethylcarbamate are undesirable (Lonvaud-Fune, 1999).

Benefits and disadvantages of MLF are dependent on viticultural region, grape variety, wine composition, winemaking techniques, and the style objectives of the winemaker (Davis *et al.*, 1985 and 1986). The process of MLF in wine is only partially understood and difficult to predict. Therefore, an improved knowledge of MLF is essential to control (stimulate or arrest) this process.

1.5.5 Factors Influencing MLF

MLF actually begins when the bacterial population reaches about 10^6 cells/ml. Therefore, from the end of alcoholic fermentation, when the level is 10^2-10^3 cells/ml to the beginning of MLF, a multiplication phase occurs (Lonvaud-Funel, 1995). Various physico-chemical factors (ethanol, SO_2, pH and temperature) are known to affect the

growth of bacteria in wine. Vaillant *et al* (1995) studied the effects of 11 physico-chemical parameters (pH, temperature, SO_2, ethanol, glycerol, citrate, malate, tartrate, fructose, glucose and pentoses) on malolactic activities of three *O. oeni* strains (commercial B1, and B16, and experimental 13A1) using a complex experimental design. Ethanol showed the greatest inhibitory effect, followed by pH and SO_2. The increase of malic acid concentration prolonged the duration of MLF. SO_2 was more effective against LAB at pH 3.4 than pH 3.8. Generally, the growth of malolactic bacteria is easer at relatively high pH (pH > 3.5), with an ethanol content less than 13% and at 19-20°C and it is nearly impossible at pH < 3.0, ethanol > 14% and below 17°C. However, these figures are not absolute since all these factors and other inhibiting or activating components of wine interact to determine the 'malofermentability' of wine (Lonvaud-Funel, 1995). Nutritional status of wines (Fourcassie *et al.*, 1992), fatty acids (Guilloux-Benatier *et al.*, 1998), phenolic compounds, lysozyme (Gerbaux *et al.*, 1997), nisin (Daeschel *et al.*, 1991), and bacteriophages (Arendt *et al.*, 1990; Arendt and Hammes, 1992) also affect the growth of malolactic bacteria in wines. Anaerobic condition has been observed to stimulate the growth of LAB in wines and thus the rate of MLF (Stamer, 1979).

Spontaneous MLF in wines is often unreliable. The main reasons may be nutrient availability and/or the presence of physico-chemical stress factors, such as low pH, high concentration of ethanol and SO_2. Optimisation of malolactic starter cultures for MLF induction as well as achievement of MLF in wines largely depends on understanding complex interaction between bacteria and the environment. More information about the mechanisms involved in adaptation to stress conditions and the possible interactions of each wine component on the structure, growth and activity of malolactic bacteria is still required.

The growth of malolactic bacteria in wine is important for success of MLF. But cell growth is not necessary to accomplish MLF (Lafon-Lafourcade, 1975; Fourcassie *et al.*, 1992; Maicas *et al.*, 2000). The rate of MLF in wine depends on bacterial cell density, specific malolactic activity and physiological state of the cells. Despite the fact that cells from the exponential phase of growth have the highest specific malolactic activity, these cells are not able to start MLF in wines because of their sensitivity to ethanol. On the contrary, inocula taken from the stationary phase are able to degrade malic acid (Granchi *et al.*, 1996). Malate is degraded more rapidly at 25°C

than at 32°C or 18°C. The rate of malate consumption is pH-dependent (Pimentel and Silva, 1994).

1.5.6 Induction and Control of MLF

Increased recognition of the influence of MLF on wine quality has led winemakers to seek better control over the occurrence and outcome of MLF. The following methods can be used to induce MLF: 1) selection of conditions to courage the growth of natural malolactic flora; 2) induction of MLF in wines by inoculation with wines that have already undergone MLF; 3) induction of MLF by inoculation with a LAB starter culture; 4) passage of the wine over supports of immobilized malolactic bacteria or membrane bioreactor charged with high cell densities of malolactic bacteria (Davis *et al.*, 1985; Lonvaud-Funel, 1995)

It may be desirable in some low acid wines to completely inhibit MLF to prevent deacidification. Inhibition is achieved through selection of conditions that restrict or halt bacterial growth. Such conditions include: maintenance of wine pH less than 3.2; alcohol concentration in excess of 14%; low storage temperature; adjustment of total SO_2 concentration to greater than 50 mg/l; early racking and clarification; and sterile filtration (Davis *et al.*, 1985).

1.6 Technology of MLF Systems

1.6.1 Batch MLF

Batch MLF is a traditional approach to carry out MLF. At the end of the alcoholic fermentation yeasts are dying and malolactic bacteria (mainly *O. oeni*) present in the wine start growing. When the bacterial population reaches about 10^6 cells/ml malic acid transformation becomes significant. It will take weeks or months to complete MLF. This natural process can be stimulated by: low total SO_2 (not greater than 40 to 50 mg/l) (Van Wyk, 1976); maintenance of wine at a temperature of approximately 16°C to 25°C (Beelman and Gallander, 1979, Van der Westhuizen and Loose, 1981); extended contact time with yeast lees to encourage autolytic release of nutrients for

bacterial growth and to increase the CO_2 content of the wine (Rankine, 1972; Van Wyk, 1976); long skin contact time, resulting in the extraction of factors stimulatory to the growth of LAB (Beelman and Gallander, 1979); and low alcohol levels (Kunkee, 1967). This spontaneous MLF is convenient and inexpensive, but it is slow and unreliable.

In order to better control the timing and speed of MLF, and the effect of MLF on wine aroma and flavour, winemakers use malolactic starter cultures to induce MLF (Davis *et al.*, 1985; Lonvaud-Funel, 1995). Although this technology increases the prospect of a rapid, reliable MLF, failures can still occur because the fermentation depends on the batch growth of bacterial cells introduced into wine. The conditions of cells at the time of introduction and the properties of wine will affect the growth and success of the reaction (Gao and Fleet, 1994). Another disadvantage of batch MLF is the relatively low productivity.

1.6.2 Fed-batch MLF

When MLF is completed in a tank, the wine is then filtered and the malolactic bacteria are retained in the tank. New wine is pumped in the tank to carry out MLF. Such procedure can be repeated many times, which is called fed-batch MLF. This approach for MLF also depends on the bacterial growth and the productivity is still low, although its productivity is higher than traditional batch MLF. Due to utilization of wild malolactic bacteria, the quality of wine cannot be predicted and controlled.

1.6.3 Continuous MLF

In recent years, immobilized cell technology has been developed to carry out continuous MLF. Malolactic bacteria are immobilized in beads of poly-acrylamide, alginate, carrageenan or "LentiKats". The immobilized cells are filled into column reactors, and wines pass continuously through the beads (Gestrelius, 1982; Spettoli *et al.*, 1982; Rossi and Clementi, 1984; Kosseva *et al.*, 1998; Durieux *et al.*, 2000). In this technology, the cells act as a biocatalyst, and because of their high concentrations, rapidly convert malic acid to lactic acid. It has the advantage of separating the malic acid bioconversion step and the cell propagation steps. The propagation of *O. oeni* can

be accomplished in more convenient conditions in a medium which allows a rapid growth rate in a separate bioreactor before encapsulation and used as a biocatalyst in the adverse conditions presented in wine (Durieux *et al.*, 2000). Other advantages of this technology include: continuous operation, greater tolerance to ethanol, better control over the timing and extent of acidification. Commercial application of these bioreactors in wine industry has not occurred: two main limitations appear to be instability of the reactors in retaining their malic acid degrading activity and slow rates of reaction. For cells immobilized in beads, the reaction kinetics are limited by the need for diffusion of substrate (malic acid) and product (lactic acid) into and out of the beads (Gao and Fleet, 1994 and 1995). Immobilized malolactic enzyme could be a solution for MLF. But it has not been successful, probably because the enzyme loses activity at wine pH (Davis *et al.*, 1985).

1.6.4 Membrane Bioreactor with High Biomass Systems

Membrane Bioreactor (MBR)

Integration of properties between synthetic membranes and biological catalysts, such as cells or enzymes, forms the basis of an important technology called Membrane Bioreaction (Belfort, 1989). Combining various functions of membranes and biocatalysts can generate quite a number of MBRs. The membrane has a micro-porous structure which can perform various degrees of separation. The cell retaining property of membranes and selective removal of inhibitory byproducts makes high cell density culture possible and better utilizes enzyme catalytic activity, which leads to higher productivity than using traditional bioreactors. MBR can be run continuously while maintaining high biocatalyst activity (Chang and Furusaki, 1991).

Membranes are usually made of polymers, but ceramics and stainless steel are also used. Membranes are traditionally divided into: microfiltration (0.1-10 μm), ultrafiltration (5 nm-0.1 μm) and nanofiltration (< 2 nm) types according to the pore size. The membrane itself is neither strong enough to build a membrane device alone nor has it enough surface area for mass transfer. Thus it is packed into a module in flat, spiral, tubular and hollow fiber configurations. MBR can be of various designs, which depend on the bioreactions or fermentations (Chang and Furusaki, 1991). Membrane

separations (microfiltration, ultrafiltration and nanofiltration) are driven by the pressure difference across the membrane.

Continuous MLF in MBR

Lafon-Lafourcade *et al* (1975) showed that resting cells of *O. oeni* at high density (10^6-10^7 CFU/ml) could bring about rapid degradation of malic acid to lactic acid in wines. Gao and Fleet (1994) found that cell suspensions of 10^{10} CFU /ml could degrade 90-95% of the malic acid in wine within 0.5 h. Under the condition of high cell density, there is no requirement for cell growth, and the mass of bacteria acts as a crude whole cell-enzyme preparation. MLF can be carried out very quickly.

With a view to obtain continuous, rapid MLF, using MBR charged with high densities of the cells is the best choice. The membrane separates the cells from the wine, while product (wine after MLF) is constantly removed. In this MBR, the cells at high cell densities can rapidly convert malic acid to lactic acid without the need for cell growth. In a MBR, bacterial cells are in direct contact with the process fluid (wine) whereas they are immobilized in beads in column reactors. The former therefore offers significant mass transfer and reaction rate advantages over the latter. Such bioreactor systems offer many advantages over batch fermentation, including very rapid and predictable reactions and use in a continuous mode (Gao and Fleet, 1995; Maicas, 2001). According to this concept, Fleet and Costello (1992) developed a MBR, charged with a high density of *O. oeni*, for the rapid and continuous MLF in wines. Gao and Fleet (1995) described an efficient MBR system used for MLF involving a high cell density. A reactor (300 ml volume) containing 10^{10} CFU/ml of *O. oeni* and operating at a flow rate of 6 ml/min for periods up to 56 h gave greater than 95% degradation of malic acid in a range of red and white wines. They also found that malic acid degrading activity of the three strains of *O. oeni* declined rapidly after 56 h operation, and the wines processed through MBR exhibited a slight off-flavour described as 'over-cooked' or 'burnt'.

In addition to cell density, the rate of malic acid degradation in a MBR is likely to be determined by environmental factors that affect the activity of malolactic enzyme which catalyses the reaction, and transport of substrate (malic acid) into the cell and of product (lactic acid) out of the cell (Gao and Fleet, 1994). Other limitations of recycle

bioreactors may include possible shear stress on the cells entering filtration unit and potential difficulties in scale-up due to filtration system (Maicas, 2001). Although the application of MBR in MLF has many advantages and has potential in wine production, more research is required.

1.7 Aims of This Study

The work in this dissertation is focused on the research and development of freeze-drying of LAB and their application on MLF. Three species of LAB are used in this work: *Lactobacillus plantarum*, *Lactobacillus brevis* and *Oenococcus oeni*. *Lactobacillus plantarum* is for the production of silage and represents a well-worked organism for comparison. *Lactobacillus brevis* and *Oenococcus oeni* are malolactic bacteria for MLF. The work is based on understanding of the bacterial physiological responses to environmental stresses associated with freezing and freeze-drying, the bacterial metabolism of wine components during MLF and biochemical engineering problems associated with them. The aims of thesis study are: (1) to develop an optimum LAB freeze-drying technology, and assess these in comparison to other LAB, (2) to carry out MLF by the malolactic bacteria in order to assess the importance of preservation on MLF systems, (3) to carry out MLF in a MBR. In order to achieve this aim, the research objectives are:

1. Investigation of culture conditions enhancing cryotolerance of the LAB
2. Investigation of factors influencing viability and vitality of the LAB after freeze-drying
3. Develop an optimum freeze-drying technology of the LAB with particular reference to MLF.
4. Investigation of growth properties of malolactic bacteria in wine
5. Investigation of factors affecting malolactic reaction of the malolactic bacteria
6. Construction and use of a suitable MBR for MLF

7. Investigation on the stability of malic acid degrading activity of the malolactic bacteria in a MBR.

The research objectives are very broad, but they are concerned with the technical development of freeze-drying and MLF as well as advancement of scientific knowledge of bacterial physiology and MLF. One of the most important topics is enhancing cryotolerance of these important LAB. Although a great deal of work has been done with dairy LAB, there are only a few reports of malolactic bacteria. It is the first investigation in which pre-incubation in yeast extract can improve cryotolerance of *Lactobacillus plantarum*. Although many studies have been carried out on MLF of wine, the stability of malic acid degrading activity of *Oenococcus onei* is the first investigation of prolonged use of this LAB in a MBR.

These studies will allow the identification of the important parameters and limitation affecting industrial scale-up. The studies preformed here will be used as guidelines for the development of successful industrial freeze-drying of LAB and MLF of wines.

Chapter 2. Materials and Methods

2.1 Introduction

This chapter describes the materials and methods used in the experimental works. Experimental works were carried out within the Centre for Complex Fluids Processing, School of Engineering, University of Wales Swansea. Microbial strains, media, experimental equipments used in this work are described. Chemicals used were purchased either from Fisher Scientific, Sigma Chemical Company (USA), or Oxoid Company (UK).

2.2 Microbial Strains and Media

2.2.1 Microbial Strains

In this work, *Lactobacillus plantarum* supplied from Interprise Company, *Lactobacillus brevis* offered by Bulmer Company and *Oenococcus oeni* 11648 from UKNCC (the United Kingdom National Culture Collection) were used.

2.2.2 Growth Media

The medium (GY) used for cultivation of LAB was composed of: glucose 5 g/l, (fructose 5 g/l for *O. oeni*), yeast extract (YE) 5 g/l, $(NH_4)_2SO_4$ 2 g/l, KH_2PO_4 2 g/l, resazurin solution (0.1% w/v) 1 ml/l and trace element solution (see Table 2.1) 20 ml/l, and pH of the medium was 6.0.

For large-scale experiments carried out in Chapter 6, a high strength medium (HGY) was used. The composition was: glucose 15 g/l, fructose 15 g/l, yeast extract 20 g/l, $(NH_4)_2SO_4$ 4 g/l, KH_2PO_4 4 g/l, resazurin solution (0.1% w/v) 1 ml/l and trace element solution (see Table 2.1) 20 ml/l.

Table 2.1 Composition of the trace element solution

Components	Concentration (g/l)
$MgCl_2 \cdot 6H_2O$	5.00
$MnCl_2 \cdot 4H_2O$	0.47
$FeCl_2 \cdot 6H_2O$	0.35
$ZnCl_2$	0.2
$CaCl_2$	0.1
$CoCl_2 \cdot 6H_2O$	0.02
$CuCl_2 \cdot 2H_2O$	0.02
$NaMoO_4$	0.01
$Na_2B_4O_7$	0.01

2.3 The Cell Cultivation of LAB

2.3.1 Anaerobic Broth Cultivation

Anaerobic broth cultivations were carried out in pressure tubes (25 ml, Bellco Glass Inc., Vineland, NJ, U.S.A) or serum bottles (50-100 ml, Wheaton Scientific Co., Milliville, NJ, U.S.A). Once prepared, the medium was boiled to expel dissolved oxygen. After boiling and some cooling, 10 ml aliquots of the medium were added to test tubes, via a 10 ml syringe attached to a piece of rubber tubing with a three way valve, from the flask containing the boiled medium. The system was flushed with O_2-free nitrogen. The headspace on top of the medium was replaced by O_2-free nitrogen. Resazurin, a redox dye, was added to the medium to indicate whether or not the broth had become fully anaerobic. Resazurin went colourless when oxygen was absent after autoclaving, and if oxygen was present, the medium would take on a pink/red appearance. After the headspace was filled with O_2-free nitrogen, the test tubes or bottles were sealed with rubber stoppers and aluminium caps. The aluminium cap

prevented the rubber stopper from being pushed out by the high pressures during autoclaving process. Once the test tubes or bottles had been prepared, they were placed in the autoclave and autoclaved at 121°C for 10 minutes.

For the experiments carried out in test tubes or serum bottles, inoculations were carried out by injecting some cell cultures from about 48 h old inoculum culture, using a sterile needle and syringe. Aseptic techniques were undertaken at all times, with inoculation being carried out in the sterile airflow cabinet. The tops of the rubber seal of test tubes or serum bottles were flamed to kill microbes that may have been present in the surface.

After introduction, the test tubes or serum bottles normally were incubated in an incubator or the constant temperature room. The cell growth was monitored by direct measurement of the optical density (see below).

2.3.2 Large-Scale Cultivation

In order to inoculate 20 L fermentors, a series of scaled-up cultivations were required. Each cultivation was kept for three days in a constant temperature room at 25°C before it was then used as inoculum for next sized vessel.

All vessels were anaerobic. The 100 ml serum bottles were sterilized, introduced and incubated. The larger vessels (500ml, 5L and 20L) were sealed with rubber stoppers. The stopper for these vessels contained receiving ports (stainless steel tubes) to allow degassing, introducing, and sampling. Each port was attached to butyl rubber tubing (impermeable to oxygen) and sealed with fibres or pinched off with clamps. The 5 L fermentor had an outlet port near the base, which could be connected with the introducing port of the 20 L fermentor.

The 20 L fermentor (Figure 2.1) had an extra port in the rubber stopper, which was used for sampling. The fermentor had an inlet port near the base, through which, after autoclaving and directly before inoculation, O_2-free nitrogen gas could be passed into the vessel to deoxygenate the system. A gas filter (0.2 μm, hydrophobic Fluoropore membrane, Millipore Corporation, U.S.A) ensured that the sterile medium in the vessel

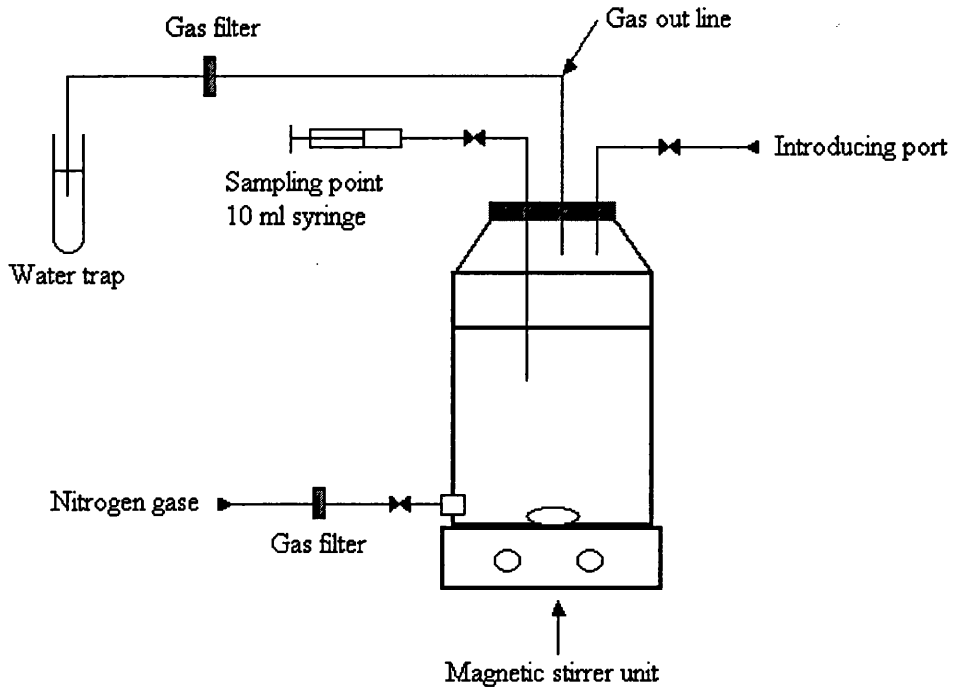


Figure 2.1 Schematic diagram of 20 L cultivation system for the growth of LAB

was maintained sterile when O_2-free nitrogen gas was bubbled through the liquor. The gas was allowed to escape through the outlet port and a water trap.

Bacterial cultures (100 ml) were grown in serum bottle, a sample (50 ml) of such a culture was drawn into a sterile syringe and injected into a anaerobic glass vessel (500 ml). This was incubated and the contents then were placed in the 5 L vessel. This 5 L vessel was designed not to be pressurized, and so a water trap was added with the outlet, in order to stop oxygen travelling up through the tubing and back into the headspace of the glass vessel. Air filters were placed on the nitrogen inlet lines to maintain sterility.

The outlet port near the base of 5 L fermentor was connected with the introducing port on the top of 20 L fermentor. A peristaltic pump was used to speed up the inoculum transfer. Approximately 3 litre of inoculum from the 5 L fermentor was added to 20 L fermentor. Flaming of the ports was necessary to ensure sterility.

Once the inoculation had been completed, the connecting tube between the introducing ports of the two vessels was clamped shut and the end connected to the 5 L vessel was detached and flamed.

During the 20 L cultivation of LAB, samples were taken for biomass measurement. Samples were taken through the sampling port. A sterile syringe was used to draw up a sample out of the 20 L vessel. The connecting tube was clamped and the syringe containing the sample was removed for analysis. For subsequent samples, a sterilized syringe along with a section of rubber tubing was attached to the sampling port. After the tubing clamp was released, a 10 ml sample was taken. As the sampling tube was well below the surface of fermentation broth, and the sampling port was only open to atmosphere for a very short time period, the assumption was that a negligible amount of oxygen had been allowed into the vessel.

After introduction, the 20 L fermentors were incubated at 25°C in the constant temperature room and, cell growth was monitored.

2.3.3 pH-controlled Cell Cultivation

In order to examine the effect of pH of cultivation on cell vitality, pH-controlled cultivations of LAB were carried out in a reactor system (Figure 2.2 and Figure 2.3). The working volume was 2 litres. The glass electrode (5) was connected with a pH control meter (12) (Pye Model 290 pH meter, Pye Unicam Ltd, Cambridge). The pH control meter controlled a peristaltic pump (11) that could pump alkaline solution into the reactor. The flask (10) containing 1M NaOH was connected to the reactor with a rubber tube. In this way pH of the medium was automatically controlled within ±0.2 unit. Reactor temperature was maintained by a stainless steel coil (6) that was connected to a water bath. The temperature of water bath (14) was maintained by a thermostatically controlled electrical heater. Reactor temperature was controlled by pumping water from the water bath to the coil. The water bath temperature was set according to the experimental requirement. The magnetic stirrer unit (2) drove a magnetic follower (3) which rotated in the medium and allowed mixing of the fermentation medium to give a uniform pH. *L. plantarum*, *L. brevis* and *O. oeni* are all anaerobic bacteria. To obtain anaerobic cultural conditions, the reactor was connected

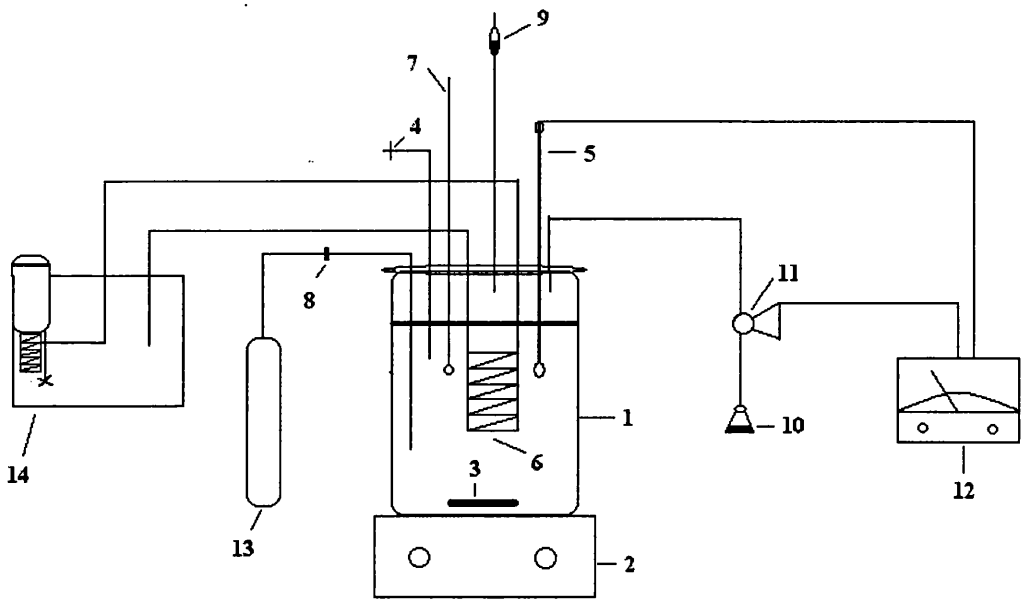


Figure 2.2 The schematic diagram of the pH-controlled cell cultural apparatus

1 — 2 L reactor
2 — Magnetic stirrer unit
3 — Magnetic follower
4 — Sampling port
5 — Glass electrode
6 — Stainless steel coil
7 — Thermometer
8 — Gas filter
9 — Water trap
10 — Alkaline solution
11 — Peristaltic pump
12 — pH control meter
13 — O_2-free N_2 gas cylinder
14 — Water bath

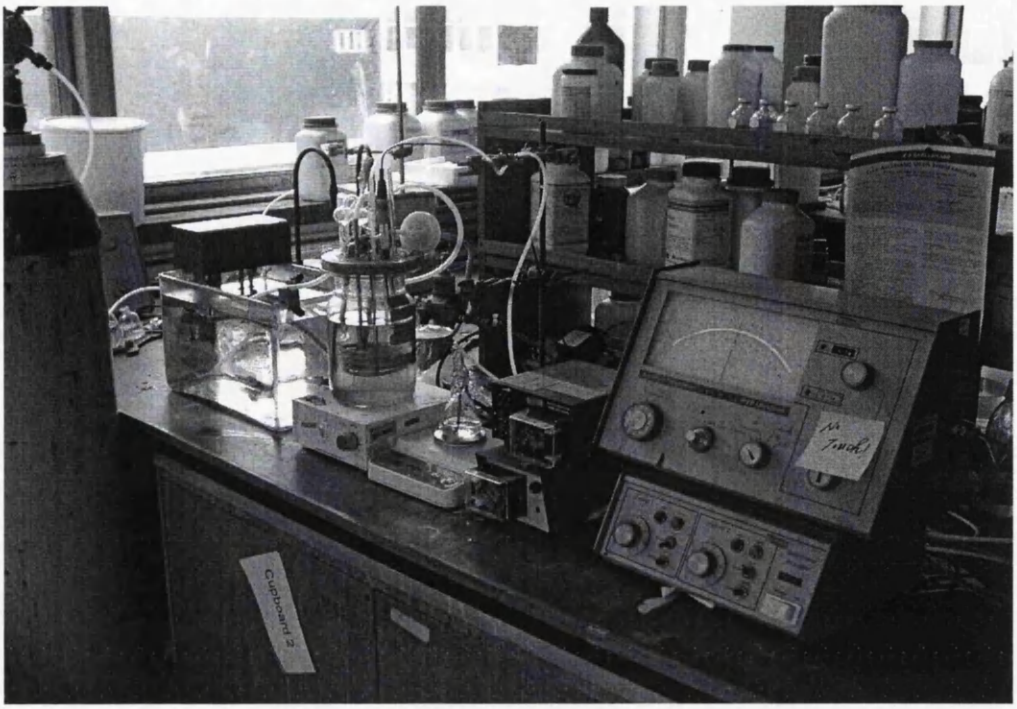


Figure 2.3 The photograph of the 2 L pH controlled cell cultural system.

to an O_2-free N_2 cylinder (13). N_2 was bubbled continuously through the medium and the headspace of medium was replaced with O_2-free N_2. The N_2 was introduced via an in-line filter (8) to ensure that sterility was maintained. The gas passed through the reactor and escaped through a water trap (9) that prevented air from entering the system. There was a sampling port (4) on the top of the reactor.

The medium used was the GY type. According to the experiment, final pH of the medium was adjusted with 1 M NaOH or HCl. The cover was fixed tightly to the glass reactor by three metal clamps, and there was a rubber gasket between the cover and the glass reactor to ensure that the cover was well sealed. Before autoclaving, the sampling port, the ends of gas filter and water trap were wrapped with aluminium foil. The reactor was autoclaved at 121°C for 15 minutes.

Fresh cultures of LAB, 40 ml (for *L. plantarum*) or 100 ml (for *L. brevis* and *O. oeni*), were introduced into the reactor. The cultures were incubated at 25°C. The pH was maintained at set value (4-7, depending on the experiments). The cell growth was monitored by measuring the OD of the cultures at 660 nm. This was done by sampling the cultures via the sampling port (4) (Figure 2.2).

2.4 Malolactic fermentation in Membrane Bioreactor

It has been known that traditional MLF is slow and unreliable. Rapid and continuous MLF in MBR was investigated. In this work, a synthetic wine was used: yeast extract, 4 g/l; glycerol, 2 g/l; glucose, 2 g/l; fructose 1 g/l; malic acid, 2 g/l; ethanol 10% (v/v) and trace elements solution, 20 ml/l, pH 4.0.

2.4.1 Construction of Membrane Bioreactor

A MBR was constructed at the Centre for Complex Fluids Processing, School of Engineering, University of Wales Swansea and is illustrated in the photograph in Figure 2.4.

2.4.1.1 Bioreactor Vessel

The bioreactor vessel was constructed from glass with a stainless sheet top plate and was operated under anaerobic conditions. The fermentation vessel employed was 5 litres operational volume, being 760 mm depth and 120 mm diameter. The vessel was sterilized by autoclaving at 121°C for 15 minutes. The top of glass vessel was sealed with a stainless steel plate using a rubber gasket. The top plate provided ports for feed, recycled process fluid, sterile nitrogen in and out, and sampling for microbiological and chemical analysis. The sterile nitrogen pipe was long enough to reach the surface of vessel contents so that the slow bubbling gas could be observed, giving positive proof of gaseous addition. The inlet for the feed was set above the process fluid level, again to ensure that the addition could be observed. The inlet pipe for recycled fluid had a 90° bend at its end to force the fluid onto the side of the glass vessel, causing vortices to occur, so as to ensure good mixing of the fresh feed and recycle. Finally, the outlet gas line passed out of the top of reactor vessel and through a sterile water gas trap to prevent diffusion of air containing oxygen, from atmosphere, back into the MBR. The volume of fluid within MBR was controlled by a level control, which is described later in this chapter.

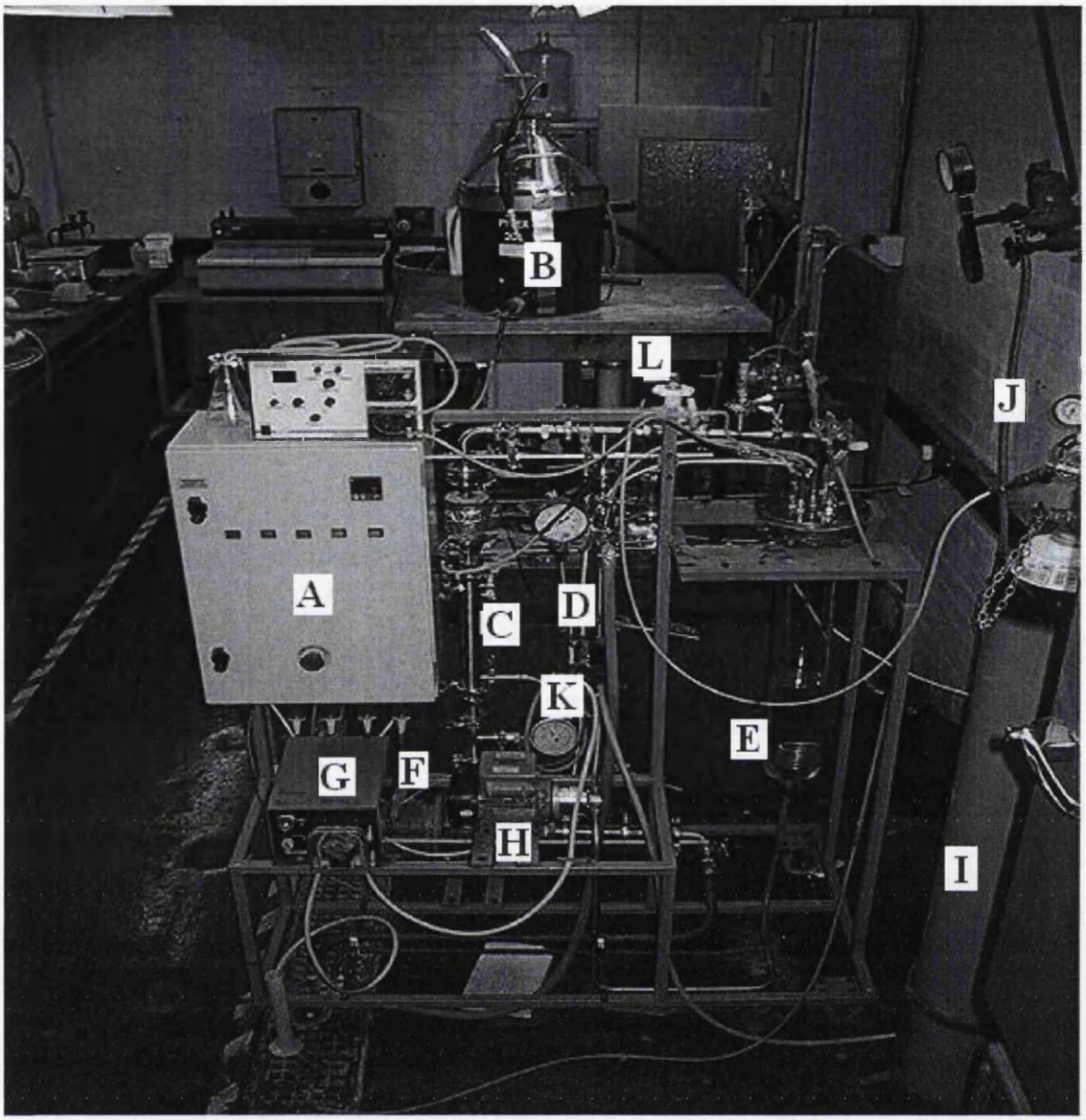


Figure 2.4 photograph of experimental MBR. A schematic diagram can be found in Figure 2.5 below. The operation of this bioreactor is also detailed in Section 2.4.3

The letters on Figure 2.4 are:

A): electrical control box.

B): the wine container

C): ceramic membrane

D): heat exchanger

E): fermentation vessel

F): pump (1)

G): pump (3)

H): pump (2)

I): free-O_2 N_2 gas cylinder

J): steam pipe

K): pressure gauge

L): diagram valve

2.4.1.2 Pumps

Pump (1) was a large centrifuge pump (Brook Hansen from Michael Smith Engineers Ltd.), type ED 80BD, 0.37KW, 2820r/min, which was situated on the base of frame. Pump (1) provided large flow rate required, ~100 litre/min, when the contents of bioreactor vessel were passed through the membrane. The large flow rate was required to prevent fouling of membrane by scouring action of process fluid.

Pump (2) was a gear pump (Brook Hansen from Michael Smith Engineers Ltd.), type PD63SFH, 012KW, 2870 r/min, capable of providing a flow rate of ~ 3 l/min at 3 bar. Pump (2) was smaller and situated on the shelf, the pump pressurised the MBR system. Pump (2) circulated process fluid around MBR. When pump (1) was not in operation, the process fluids pass from the base of glass bioreaction vessel through pump (2) into the stainless steel pipe work, through the heat exchanger and back into the top of the glass bioreaction vessel.

Pump (3) was a peristaltic pump (Watson Marlow Ltd), model 505Du. Pump (3) controlled the rate at which product was removed from the bioreactor system through the membrane and hence also controlled the rate at which the feed was added to the bioreactor system via the level control. The second purpose of operation of pump (3), was to allow the procedure of back-flushing membrane to take place, by reversing the action direction of pump (3) to reverse flow of products back through membrane and into the bioreactor system. This reverse flow was believed to unblock the pores of ceramic membrane and so reduce fouling. Pump (1) and pump (2) were both stainless steel pumps. These stainless pumps enabled the entire MBR system to be sterilized in situ or disassembled, further reducing the chances of contamination.

2.4.1.3 Membrane Unit

The membrane was ceramic of surface area 0.13 m^2, and pore size 0.2 μm. The stainless steel membrane housing was 340 mm long. The ceramic membrane had a flow diameter of 1.9 mm, maximum operating temperature of 130°C, minimum pH 2, a maximum pH 13, and flow velocity 1m/sec, or flow rate 786l/hr. The membrane was used to retain LAB within the liquid phase in the maturation process equipment. The ceramic membrane was encased within a stainless steel sheath, and the cells, being too

large to pass through the membrane, were retained inside the membrane. The process fluid (the synthetic wine) was able to pass through the membrane to the outside surface where it was removed through the outlet pipe. This permeate was fully clarified, and so a further downstream filtering process was not required. The membrane filtration operated was in a cross flow mode to minimize membrane fouling, and was able to withstand high pressures and back-flushing, because the membrane was constructed of ceramic material. Back-flushing was used to clean the membrane.

2.4.1.4 Heat Exchanger Unit

Heat generation in the system was appreciable because of the two pumps. Studies showed that the operation of both pumps without temperature control raised the temperature of process fluid to about 50°C. The metabolising cells also produced heat energy, (heat produced by metabolism was likely to be negligible at this scale), nevertheless the system could not maintain itself at constant temperature by simple interaction with its surroundings. To maintain the system at an optimum temperature for LAB, the broth had to be cooled by a heat exchanger. The heat exchanger was a single pass hollow tube exchanger, dimensions of outer shell are 435 mm by 50 mm, and the inside diameter of inner tube is 22 mm. Flow of the coolant was controlled by a solenoid valve linked to a thermocouple so as to ensure that temperature of the process fluid within MBR was kept constant. The process fluid passed through the inner tube and the coolant through the outer shell.

2.4.1.5 Piping

All rigid piping was constructed from 25.4 mm stainless steel, inside diameter 22 mm, with the exception of the pipes linking pump (2) to the main steel loop and the base of glass reaction vessel, which was 12.7 mm stainless steel. All of the piping was of food grade quality to allow for sterilization and cleaning. The stainless steel pipe sections were connected to the valves, pumps and themselves by clamp fittings making it easy to change the design of the pipe work to suit alternative operational configurations. Flexible pipes were constructed of butyl rubber (a compound that is not permeable to gases and hence will not allow atmospheric oxygen to enter the process).

2.4.1.6 Valves

Valves were required in the MBR system to regulate the flow of process fluid around it, and for operation during sterilization and cleaning. All valves were of hygienic food grade standard, and were not permeable to the outside atmosphere, (ensuring sterility), and were able to withstand direct steam sterilization of system.

- **Diaphragm valve**: one Teflon seal diaphragm valve was situated on the top of stainless steel loop, see the photo, on the recycle return line to the glass reaction vessel. This valve controlled the pressure over membrane, and hence the flow of process fluid permeation through membrane. When fully open, process fluid from the membrane flowed back into either the glass vessel or back down through the heat exchanger, the pressure across membrane was at a minimum. As the valve was closed, less process fluid was recycled back to the glass reaction vessel directing more process fluid down through the heat exchanger and back around the stainless steel loop, increasing the pressure across membrane thereby forcing a larger volume of process fluid through membrane.
- **Butterfly valves**: one butterfly valve was situated as drain valve at the base of stainless steel loop. This valve allowed for removal of concentrated LAB cells at the end of each operation and waste at the end of cleaning, or steam condensate from MBR during sterilization when opened. Steam was added to the equipment for on line sterilization, when opened the butterfly valve allowed the steam condensate to be removed from MBR to drain.

  Two additional butterfly valves were situated above the heat exchanger and the ceramic membrane housing respectively. These valves allowed complete steam sterilization of the system by regulating the routes that steam took around the stainless steel pipe work of MBR.
- **Solenoid valve**: the flow of water coolant into the heat exchanger was controlled using a solenoid valve. When the thermocouple measured that the temperature of MBR stainless steel housing has risen above a pre-set temperature, the solenoid valve would open, allowing cooling water into the heat exchanger to cool the process fluid. When the temperature measured by the thermocouple dropped below a second pre-set temperature, the solenoid valve would close to stop the flow of cold water into the heat exchanger.

- **Pinch valve**: a pinch valve controlled the flow feed into MBR system. The feed line was a flexible tube, which passed through the pinch valve. The valve was controlled by the level control. When the bottom electrode was uncovered the pinch valve would open, allowing the feed to enter the glass bioreaction vessel until the top electrode was touched by the feed, thus maintaining the volume of process fluid in MBR within the pre-set constraints.

2.4.1.7 Sampling Port

Samples of the bioreactor contents were taken for microbiological and chemical analysis, through a sampling port installed in the flexible hose between the top of glass bioreactor vessel and the stainless steel piping. The port was constructed from a plastic 'Y' piece, with a rubber bung being placed on the dead end, through which a sterile needle was placed to remove a sample of the process fluid from within the bioreactor system.

2.4.1.8 Frame

The support frame was constructed from 20 mm box section steel, cut to size and welded. The frame stood 1.54 m high and was 1.2 m long and 0.71 m wide. The frame was painted to avoid corrosion.

2.4.1.9 Instrumentations

- **Pressure gauge**: the trans-membrane pressure was read from a pressure gauge situated at the base of ceramic membrane on the outlet of pump 1.
- **Level control**: a system of electronic level control was added, so that the MBR could be run continuously without 24 h observation by the operator. The level control consisted of three electrodes, which were situated in the glass bioreaction vessel, and a pinch valve through which the feed tube passed. The first electrode was the earth, and was not insulated, reaching down to ~ 20 cm from the base of bioreactor vessel. The other two electrodes were both insulated and controlled the level of process fluid in the bioreactor. One electrode was about 10 cm longer than the other, and when level of the process fluid in the bioreactor dropped below this electrode, the pinch valve was

opened. This valve then allowed the feed to enter the glass bioreactor vessel, until the level reached the top electrode, the pinch valve then shut, cutting off the flow of feed into the bioreactor. The level in the system then dropped at the rate at which pump (3) was removing product through the membrane. So pump (3) controlled the feed rate.

2.4.2 Sterilization of MBR

Before each experimental operation, the stainless steel sections of MBR system were steam sterilized, and the glass bioreaction vessel was autoclaved. First the glass vessel and top steel plate were detached from the stainless steel pipe work of MBR. All the openings and flexible pipe ends were plugged with cotton wool, covered in aluminium foil and then the glass vessel was placed in the autoclave and sterilized at 121°C for 15 min.

A steam line was attached to the top of stainless steel pipe work. Steam, at less than 10 psi, was passed through the system by three separate routes, each for 30 minutes. It was important to allow steam through the steam line for 5 minutes prior to attaching the line to MBR, to remove dirt and rust from the steam line, which may damage the MBR membrane. The first route used for in-situ sterilization was from the top of stainless steel line down through the heat exchanger and to drain. Opening the bottom butterfly valve to the drain and butterfly valve above the heat exchanger and closing the butterfly valve to the membrane achieved this, seeing Figure2.5. The second route was from the top of stainless steel line down through the membrane and out to drain. Closing the butterfly valve above the heat exchanger and opening the butterfly valve above the membrane housing carried this flushing out. The final route allowed steam to pass through either the membrane or heat exchanger, and out through pump (2) to drain. This flushing was achieved by closing the bottom ball valve to the drain, and opening butterfly valves above both the heat exchanger and the membrane housing.

Once steam sterilization had been completed, the autoclaved glass vessel was replaced and the flexible tubing was reattached. The MBR was then sterile and ready for loading with LAB.

2.4.3 Cleaning of the MBR System

At the end of each operation, the membrane and bioreactor vessel were cleaned. The system was first drained and the MBR rinsed with warm water, approximately 50°C, until all the equipment was observed to be clean and the water going to drain from the membrane and stainless steel pipe work was clean. The bioreactor drain was then closed and the MBR was filled with 0.1 M NaOH solution. The bioreactor was then operated for approximately ½ hrs with this cleaning solution as a closed loop system, with the output from the membrane being recycled into the glass vessel. Using pump (3), the membrane was also back-flushed. This was achieved by collecting a volume of filtrate through the membrane, reducing the pressure across the membrane by fully opening the diaphragm valve and reversing the flow of fluid with pump (3), so that the filtrate was pumped back through the membrane into the bioreactor system, unclogging the membrane pores.

2.4.4 Principle of Rapid and Continuous MLF in MBR

The MBR was charged with a high cell concentration of LAB. The high cell concentration of LAB was used to carry out the bioconversion of malic acid to lactic acid, at an order of magnitude quicker than would be observed in traditional maturation vessels with very much lower concentration of LAB. The basic design of MBR system is given in the schematic diagram of the process of Figure 2.5.

2.4.5 Rapid and Continuous MLF in MBR

In order to operate MBR with high concentration LAB for rapid and continuous MLF, a large quantity of LAB, 40 L or more, were grown upon HGY medium. The cell cultures were added to the glass bioreactor vessel through the pinch valve and stainless steel top plate. Gravity was used to push the cell culture into the bioreactor system. As the culture liquid was filtered more cell cultures were automatically added up to operating volume of 5 litres in the MBR. The pore size of ceramic membrane was 0.2 μm, which allowed the cultural medium to pass the membrane but not the LAB,

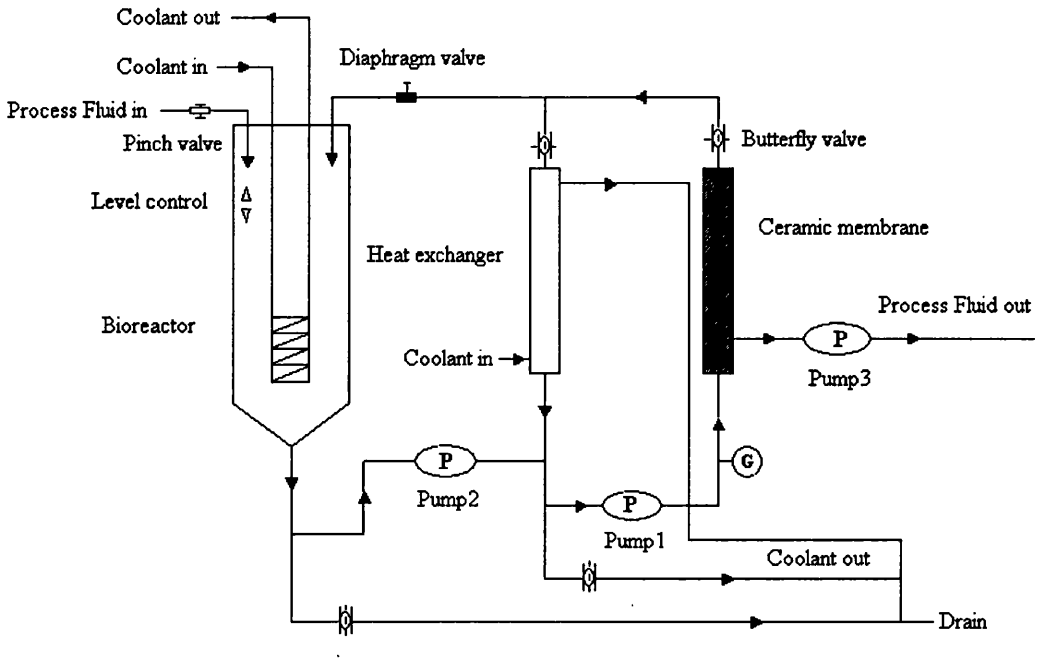


Figure 2.5 The membrane bioreactor system used

therefore retaining the cells within the MBR system. The level control was used to regulate the flow of cell culture into the MBR to prevent the system from overfilling or emptying. A more detailed description of the level control operation has been given in Section 2.4.1.9.

Once loaded with LAB, the synthetic wine was added through the pinch valve and stainless steel top plate, being driven by gravity. The rate at which the feed was added to the bioreactor system was governed by pump (3), a peristaltic pump situated in the outlet of membrane unit (see Figure 2.5). Pump (3) therefore determined the residence time of process fluid within the MBR. Once the synthetic wine entered the glass vessel, and came in contact with high concentration of LAB, MLF started. The synthetic wine was drawn down through the glass vessel and into the stainless steel piping by pump (2). Once in the piping system, pump (1) pumped the mixture of bacteria and the synthetic wine at a high velocity up through the ceramic membrane.

This high velocity was designed to create a scouring effect on surface of the membrane filter unit, which reduced fouling that would have occurred.

Some of the synthetic wine, the permeate, then passed out through the membrane at the flow rate governed by pump (3). The rest of synthetic wine, plus LAB, were retained and passed up through the membrane and into the top of MBR piping (see Figure 2.5). The synthetic wine then split into two paths. Some of the fluid was returned through the diaphragm valve situated on the top stainless steel pipe to the glass vessel, while the remainder passed down through the heat exchanger and back around the stainless steel loop. The diaphragm valve governed the system pressure that determined the permeate flux through membrane. The more the diaphragm valve was closed, the greater the pressure across membrane was. The heat exchanger was added to the MBR system to counteract the heat generated by operation of the two pumps and metabolism of LAB.

Samples of the permeate (the synthetic wine undergoing MLF) were taken regularly and analysed by HPLC (Prostar 210/215, Varian).

2.5 Freezing of LAB to Investigate Cryotolerance

In order to investigate the effect of culture conditions on the cryo-tolerance of LAB, after cultivation or after a variety of treatments, the cell cultures were taken and introduced into 10 ml of GY media. The inoculum concentration was controlled carefully by means of the spectrophotometer. The samples were frozen in a freezer at -20°C without shaking and kept at -20°C for about 48 hours.

2.6 Freeze-Drying of LAB

As already stated in the introduction, preservation by freeze-drying may be affected by many variables. The following sections details the fundaments associated with freeze-drying preservation of LAB.

2.6.1 Treatments Before Freezing

Serum bottles were washed with a detergent, then rinsed in tap water and finally in distilled water. The serum bottles were plugged with rubber stoppers and sterilized at 121°C for 15 minutes. The sterile serum bottles were stored at room temperature. Before use the bottles were labelled.

The cells of LAB were cultured in GY media at 25°C. When the cells had grown to stationary phase, the cultures were concentrated 3 times by centrifuging. The concentrated cultures were placed in sterile serum bottles and mixed with the suspension media which depended on the experiments.

2.6.2 Freezing for Freeze-Drying

The samples above were put into a freezer (-20°C) and left a static state overnight. The 10 ml of cell suspension gained about 1 cm thick layer in the serum bottle. The bottles were placed on stainless steel trays. These trays were filled with distilled water about 1 cm, and then frozen at a freezer (-20°C) for overnight. The ice in the bottom of the tray provides uniform heat distribution in the freeze dryer (see below).

2.6.3 Freeze-Drying

Freeze-Dryer

Freeze-drying was carried out in an Edwards freeze dryer (Figure 2.6 and Figure 2.7). The freeze dryer consisted mainly of drying chamber with shelves, condenser, refrigeration system, vacuum pump and the temperature control meter.

- **Drying Chamber:** there are 4 shelves in the drying chamber. The samples that will be freeze-dried are placed on the shelves. There are heating bases on the shelves, which are connected with a temperature control meter. The shelf temperature can be controlled electrically using the heating base. There are two rubber gaskets at the end of chamber cylinder to make the drying chamber seal

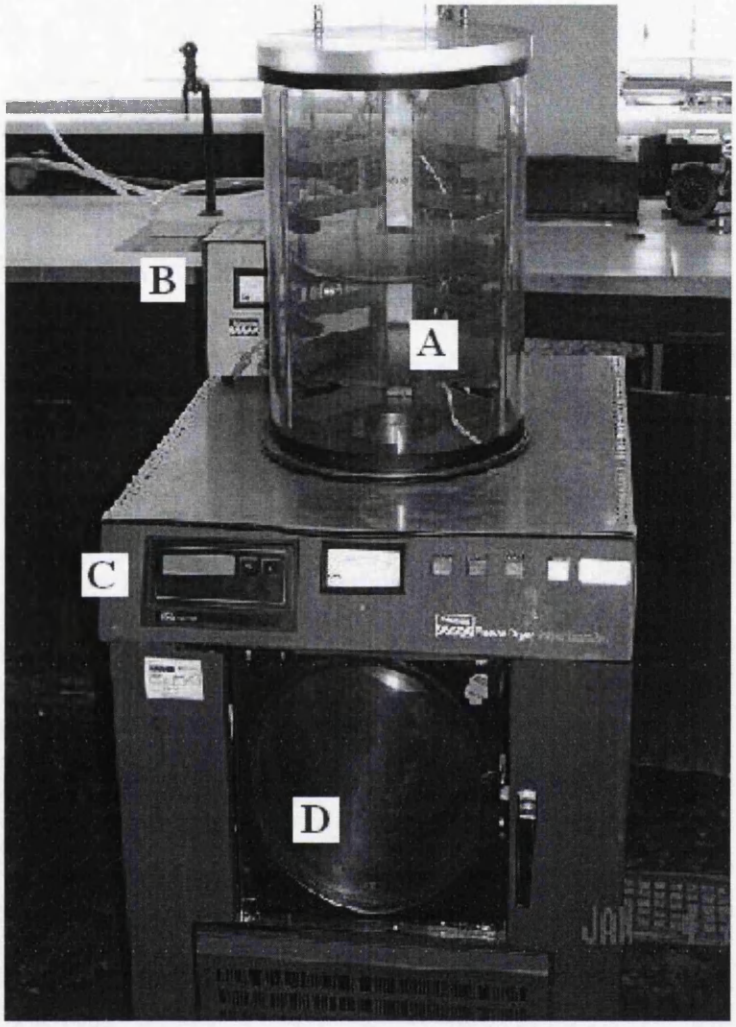


Figure 2.6 Photograph of the freeze-dryer. A schematic diagram can be found in Figure 2.7 below.

The letters on Figure 2.6 are:

A): drying chamber and the cylinder

B): temperature control meter

C): indicator of vacuum

D): condenser

tightly. When the lid is put over the chamber cylinder, a vacuum can be formed in the drying chamber if the vacuum pump is turned on.

- **Condenser:** The condenser is cooled by the refrigerator down about -50°C and traps the water vapour from the drying chamber during freeze-drying. The cooled water forms a layer of ice on the surface of condenser.

- **Refrigeration system:** This supplies refrigerant to the shelves and condenser, and reduce their temperatures.

- **Vacuum pump:** This reduces pressure in the drying chamber and condenser to facilitate the drying process. This is the most sensitive part of this system. Before freeze-drying, the drain valve was opened to ensure that excess water in the bottom of condenser chamber was removed.

Operation of Freeze-Dryer

The freeze-drying system is shown in Figure 2.7. In order to run freeze-drying, the door of condenser and drain valve of the freeze dryer were closed, the refrigerator was turned on. The condenser (cold trap) was cooled down to -45 ~ -50°C (about 20 to 30 minutes). The frozen serum bottles of samples on the stainless steel trays were placed into the drying chamber. The chamber cylinder was placed on the freeze dryer. In order to control the temperature of samples, a thermocouple was placed in one extra frozen sample. Once this was done, the lid was replaced on the chamber cylinder, and the wires were connected to the temperature control meter. The temperature in the control meter was set to 28°C. All the actions above were performed very quickly to prevent the frozen samples from melting. Once sealed, vacuum was applied. The pressure dropped rapidly. Water vapour of the samples was removed so as to freeze the samples by evaporative cooling and freezing, and then maintain the water-vapour pressure below the triple-point pressure. Once freeze-drying had started (0.6-2.5 mbar), the samples were left about 24 h and the final sample temperature about 20°C. After this the freeze-drying process was stopped and the vacuum was removed.

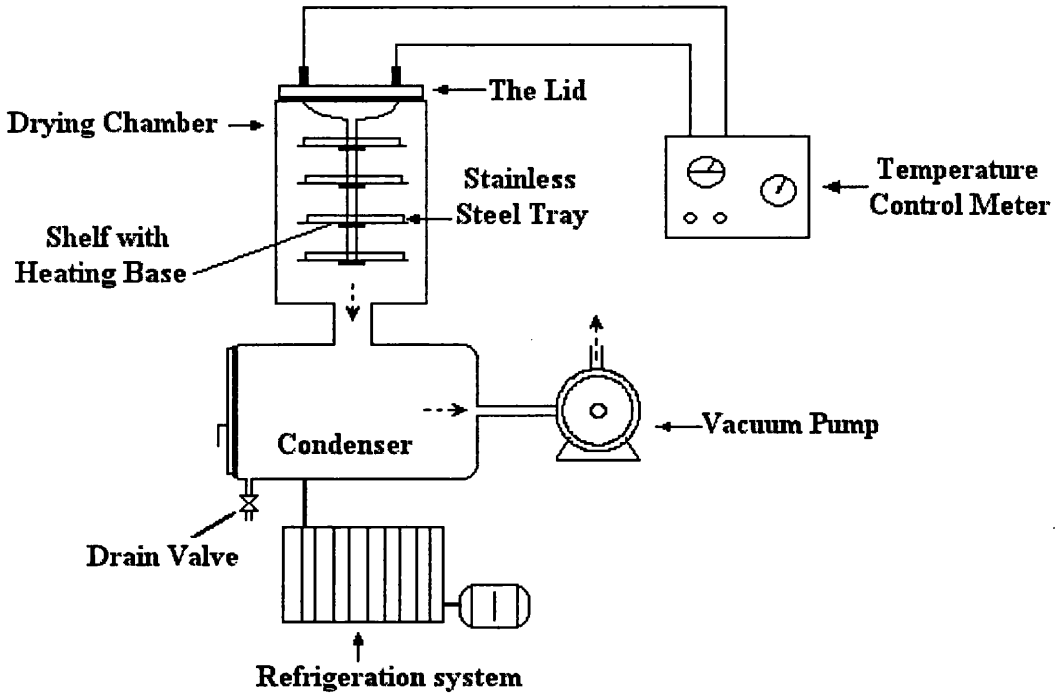


Figure 2.7 Schematic diagram of the freeze-drying system

2.6.4 Storage of Freeze-Dried LAB Bacteria

When freeze-drying was completed, the bottles of samples were sealed immediately with rubber stoppers and aluminium caps under vacuum made by a vacuum pump, and stored in a fridge at 4°C for 3 months.

2.7 Analysis

2.7.1 Measurement of Cell Growth

Cell growth was observed by regularly measuring the optical density (OD) of the cell culture in a spectrophotometer (Unicam 8625, UV/VIS spectrophotometer) at 660 nm. This was used as a measurement of growth and is proportional to biomass

concentration. Provided the measurement of the concentration was kept within the range of Beer-Lambert law.

2.7.2 Assessment of Viability and Vitality

2.7.2.1 Assessment of Viability by Plate Counting

GY medium agar was prepared by dissolving agar (10 g) in 500 ml of the liquid GY medium. The GY medium agar was autoclaved at 121°C for 10 minutes, and then cooled down about 50°C. It was gently poured into sterile petri dishes in a sterile airflow cabinet, ensuring aseptic conditions at all times, and allowed to harden on a flat surface with the lids resting on top. These agar plates were ready to be used for assessment of cell viability.

The cell suspension sample was serially diluted in autoclaved distilled water. 0.1 ml of the dilutions were transferred onto the surfaces of the agar plates and spread over the entire surface of the medium using a sterile glass spreader. The plates were put in an inverted position in an anaerobic jar (Oxoid). The jar was pumped to a -0.8 bar vacuum and filled with O_2-free N_2 to obtain anaerobic conditions. The plates were incubated at 37°C for 48 hours, and the colony forming units (CFU/ml) were counted. The cell viability was calculated as CFU/ml after freezing divided by CFU/ml before freezing.

2.7.2.2 Assessment of Vitality by Intercomparing Growth Curves

A method to assess cell vitality by intercomparing growth curves was developed, and it was demonstrated that this method was feasible and reliable (see Chapter 3 for the details). The procedures of the method were as follow:

1. The bacterial cells were grown up according to the methods described in Chapter 2, Section 2.3.1.
2. After cultivation, some cells, as inocula, were taken from the cell cultures and added into 10 ml GY medium. The amount added was carefully controlled

(measured by the optical densities) to give equal cell concentration. These cultures were then frozen without shaking at -20°C for about 48 hours.

3. After freezing the cultures were thawed for about 2 hours at room temperature (about 25°C).

   For freeze-dried LAB, the samples were rehydrated to their original volume (10 ml) at room temperature with GY media or other solutions, which depended on the experiments. Some cells as inocula were taken from the cell suspensions and added into 10 ml GY medium. The amount added was also carefully controlled to give equal cell concentrations.

4. These cultures were cultivated at 37C° (*L. plantarum*) or 25°C (*L. brevis* and *O. oeni*). The cell growth was monitored by measuring the OD values of samples in the spectrophotometer at 660 nm.

5. The growth curves were plotted by the OD values of cell cultures obtained. The growth curves of the samples were analysed by the software offered by Dr. R.W Lovitt, and then the vitality indexes of the samples were calculated (for detail, see Section 3.4.2, Chapter 3).

2.7.3 Calculation of Specific Growth Rate of LAB

In batch culture, after inoculation there is a period during which no growth appears to take place. This period is referred to as the lag phase and may be considered as a time of adaptation. Following a period during which the growth rate of cells gradually increases, the cells grow at a constant, maximum rate and this period is known as the log, or exponential phase. The exponential phase may be described by the equation:

$$\ln x_t = \ln x_0 + \mu t$$

Where x_0 is the original biomass concentration, x_t is the biomass concentration after the time interval, t hours, μ is the specific growth rate, in hours^{-1}. Thus, a plot of the natural logarithm of biomass concentrations against time yield a straight line, the slope of which equals μ.

According to the theory above, the specific growth rates of LAB were calculated: The fresh cultures of LAB were introduced into test tubes that contained 10 ml autoclaved medium. The cultures were incubated, and the cell growth was monitored by measuring OD values of samples in the spectrophotometer. The growth curves were plotted using OD values in natural logs against incubation time. The specific growth rate (μ) was determined from linear regression of the linear section of the growth curve using microsoft of Excel.

2.7.4 Determination of Malic Acid Concentration

The malic acid concentrations in the samples were determined by high performance liquid chromatography (HPLC) (Prostar 210/215, Varian), equipped with an electrochemical detector (ED40, Dionex). In the HPLC system two pumps were used together to proportion the solvent into a mixer. An Ionpac® AS11-HC analytical column (4×250 mm, P/N 052960) was used under the following conditions: column temperature, 35°C; mobile phase, mixed 0.5 mM and 100 mM sodium hydroxide; flow rate, 1.1 ml/min; volume injection, 20 μL. An optimised sodium hydroxide gradient elution (see Appendix-1.1) was used. A guard column (4×50 mm, AG11-HC, Ionpac®) was placed prior to the analytical column to prevent sample contaminants from eluting to the analytical column. An anion trap column (9×24 mm, ATC-1, Ionpac®) was installed in front of the guard column. The trap column contained high capacity, low efficiency anion exchange resin that striped trace anionic contaminants out of the eluent, and prevented them from reaching the guard and analytical columns. Solvents and reagents were HPLC grade. Analytical grade malic acid (without further purification) was used as standard. Quantification based on the peak area was performed using Interactive Graphics software (version 5.51, star, Varian). The calibration curve is shown in Appendix-1.2. The samples, permeated through the ceramic microfiltration membrane, were diluted 1:20 with deionised water before analysis in the HPLC.

Malic acid degradation (%) was expressed as the malic acid concentration after MLF divided by the malic acid concentration before MLF.

2.8 Reproducibility

All results (cell viability, vitality and malic acid levels) presented in this thesis were the mean of two or three independent replicate assays. Once methods were standardised, the variations were normally less than ±5% and are shown with the data.

Chapter 3. Study of Viability and Vitality

3.1 Introduction

LAB are widely used as starter cultures in the production of most fermented foods, such as cheeses, wines and yoghurt (Fu and Mathews, 1999; Sanders *et al.*, 1999; Talwalkar and Kailaspathy, 2004). The reproduction and metabolic activities of LAB are crucial in these fermentations. At the same time, the conditions of fermentation processes affect viability and activity of the bacteria (Bunthof *et al.*, 2000). Also, production of LAB starters and storage of the fermented products impose environmental stresses on bacterial cells. These so-called stress conditions include freezing, drying, low pH and high salt concentrations (Caplice and Fitzgerald, 1999; Bâati *et al.*, 2000).

Viability measurements are commonly applied to assess quality of starter cultures. Traditionally, viability has been determined by plate counting. However, plate-counting methods are time consuming and tedious to perform. Plate counting is not accurate because the microorganisms which are sublethally damaged, viable but non-culturable or dormant do not form colonies (Heckly, 1978; Parthuisot *et al.*, 2003). In addition, plate counting only provides information about the culturability in a laboratory medium under specific growth conditions (Hornbak *et al.*, 2002).

With the recognition of different physiological states of microorganisms, new definitions of viability have been given. Ueckert *et al* (1995) found that assessments of cellular structure and functions were relevant indicators of cell viability. Hornbak *et al* (2002) used the term viability to describe the culturability of cells and considered cytological methods such as direct fluorescence methods as indicators of activity or vitality rather than viability measurements.

Vitality has been variously described as a measure of activity, fermentation performance, or the capacity to overcome and recover from physiological stress (Guido *et al.*, 2004). Vitality methods enable a differentiated picture of various physiological states of microorganisms to be defined when cells are subjected to different kind of stresses (Ueckert *et al.*, 1995; Hewitt *et al.*, 1998; Hewitt *et al.*, 1999).

In this chapter, a simple and reliable method for measuring vitality was developed and compared with the plating count method.

3.2 Definition of Viability and Vitality

Defining cell death and cell viability is philosophically and experimentally difficult (Joux and Lebaron, 2000). In this work, according to Breeuwer and Abee (2000) and Rechinger and Siegumfeldt (2002), cell viability was defined as the ability to reproduce and form colonies in a suitable medium under favourable conditions, and was measured as CFU by plating serial dilutions and counting colonies. According to Guido *et al* (2004), cell vitality was described as the capacity to overcome and recover from the stress conditions such as freezing and freeze-drying. In this chapter an attempt to measure vitality by comparing the cell growth curves after different treatments was made. A measure of vitality in the form vitality index (VI) was determined.

3.3 Theory on Assessment of Viability and Vitality

3.3.1 The Batch Growth of Microbial Cells

When microbial cells are introduced into a batch reactor containing fresh culture medium and their increase in concentration is monitored, several distinct phases of growth can be observed. After introduction there is a period during which little growth takes place. This period is referred to as the lag phase (Figure 3.1), which is of variable duration. This is then followed by the exponential growth phase or logarithmic phase, where cell number increases exponentially. Following this is a short phase of declining growth, then the stationary phase and the death phase. The phases with a typical growth curve are illustrated in Figure 3.1.

The lag phase results from several factors. When cells are placed in fresh medium, intracellular levels of cofactors (e.g. vitamins), amino acids and ions (e.g.Mg^{2+}, Ca^{2+} etc) may be transported across the cell membrane and thus their concentration may decrease appreciably. If intermediates in metabolic pathways are required for enzyme

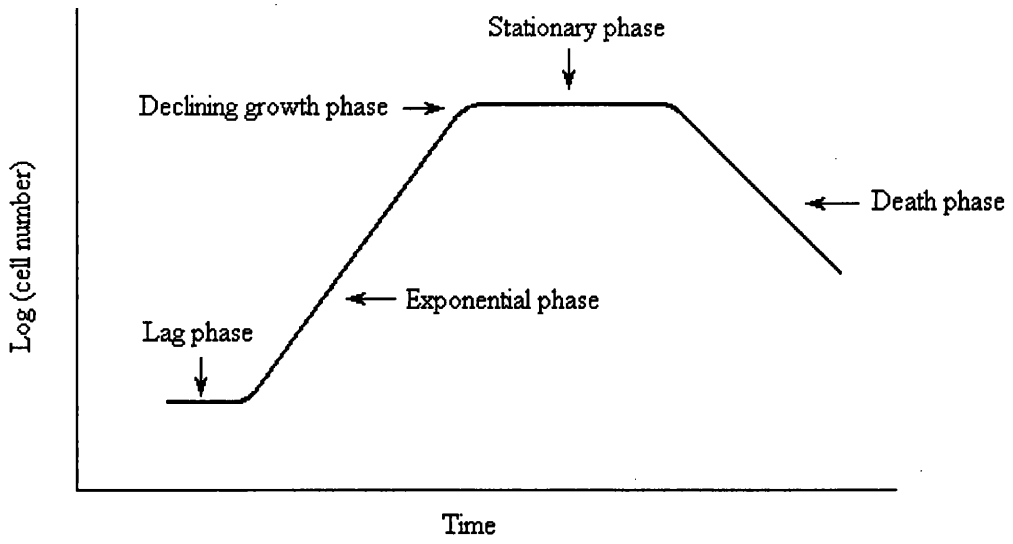


Figure 3.1 Typical growth characteristics of a microorganism in batch reactor.

activity, the dilution upon introduction may reduce the rate at which various pathways operate. Cells must then metabolise the available carbon source to replenish the intracellular pools prior to initiating cell division. Similarly, if the inoculum is grown in a medium containing a different carbon source from that of the new medium, new enzyme may need to be induced to catabolize the new substrate and this will also contribute to a lag. Cells taken from the exponential phase and used as inocula generally show a shorter lag phase than those taken from late stationary phase of the culture. These exponentially-derived inocula will have adequate concentrations of intermediates and will not suffer from the dilution effect. If an inoculum is placed in a rich medium, one containing many amino acids and other complex carbon and nitrogen sources, a shorter lag phase results as the intermediates of metabolism are already provided (Blanch and Clark, 1997).

After the lag phase, cells begin to grow and divide at their maximum growth rate. The culture is in the exponential or log phase of growth. (The number of microbial cells during exponential growth is often recorded in scientific notation to represent exponential changes conveniently.) Microbes continue to multiply at a logarithmic rate until one of two factors terminates the log phase; either (1) the supply of an essential nutrient is exhausted or (2) toxic products from microbial metabolism accumulate to

inhibitory levels. Fewer and fewer cells divide (declining growth), and culture begins the transition into the stationary phase.

During the stationary phase, there is not net increase in population size because the number of cells being produced is equivalent to the number of cells that are dying. More important, the microbes gradually stop multiplying and switch to the lowest levels of metabolism and energy expenditure that allow them to remain viable for long periods of time (Blanch and Clark, 1997).

As the stationary phase continues, the conditions continue to deteriorate, many microbes undergo changes that affect their structural integrity (e.g. sporulation) and the culture enters the death phase. There are many reasons for death of the cells, due to either accumulation of toxic metabolites, or the activity of lytic enzymes. Death, like growth, usually occurs logarithmically, i.e. single order reaction.

3.3.2 Mathematics of the Cell Growth

For analysis of growth a mathematics description is required. The lag phase is very difficult to describe mathematically as its causes are multifactor, and so models of these processes are normally avoided. Following a period during which the growth rate of the cells gradually increases, the cell growth enters the exponential phase, and the cells grow exponentially. The growth in exponential phase can be described by the equation:

$$\frac{dX}{dt} = \mu X \qquad (1)$$

Where X is the concentration of microbial biomass, t is time, in hours; μ is the specific growth rate of the cells, in hours^{-1}. On integration, equation (1) gives:

$$X_t = X_0 e^{\mu t} \qquad (2)$$

Where X_0 is the original biomass concentration, X_t is the biomass concentration after the time interval, *t* hours, and e is the base of the natural logarithm.

On taking natural logarithms, equation (2) becomes:

$$\ln X_t = \ln X_0 + \mu t \qquad (3)$$

Thus, a plot of the natural logarithm of biomass concentration against time should yield a straight line, the slope of which should equal to μ and the intercept of which should equal to $\ln X_0$. During the exponential phase nutrients are in excess and the organism is growing at a constant, maximum specific growth rate, μ_{max}. So $\mu = \mu_{max}$.

Time required for the cell numbers or dry weight to double, the doubling time t_d, is related to the specific growth rate by

$$t_d = \frac{\ln 2}{\mu} \qquad (4)$$

Following the exponential phase, the rate of exponential growth decreases because the concentration of substrates in the medium decreases and toxic metabolites accumulate. The growth enters the declining growth phase. The declining growth phase can be described by the equation:

$$\frac{dX}{dt} = \mu X \qquad (5)$$

But the value of μ is affected by the concentration of the substrate and products in the medium, and $\mu < \mu_{max}$. For example, the Monod equation:

$$\mu = \frac{\mu_{max} \times S}{K_m + S} \qquad (6)$$

Where S is the concentration of growth-limiting substrate, K_m is the substrate constant and has the same dimensions as substrate concentration.

During the stationary phase, there is no net increase in population size because the number of cells being produced is equivalent to the number of cells that are dying. The stationary phase can be described by the equation:

$$\frac{dX}{dt} = (\mu - k_d) X = 0 \qquad (7)$$

Where k_d is the death rate constant.

Following the stationary phase is the death phase. It is thought that cell lysis occurs and the population decreases. The rate of decline is also exponential, and can be represented by the equation:

$$\frac{dX}{dt} = -k_d X \qquad (8)$$

3.3.3 Principle of Assessment of Viability

The enumeration of microorganisms by viable count is based on the assumption that each microoraganism in a suspension will give rise to a single colony after incubation in a suitable medium under favourable conditions. After incubation, the number of colonies formed is counted to arrive at an estimation of the number of microorganisms in the original suspension. The viable count is considered to be a minimum count because the number of colonies on the plate represents only those microorganisms that can multiply under the condition that have been established. Not all cells will give rise to a colony because certain microorganisms have a tendency to clump or aggregate. When plated onto suitable culture medium, a clump will give rise to only one colony, regardless of how many cells are in the clump. For this reason, the results obtained by viable plate counts are expressed as colony forming units rather than cells/ml. Ideally, only plates containing 30-300 colonies are counted because this will enhance the accuracy of the count (Colomé *et al.*, 1986). This is the typical and traditional method to assess viability. Further elaborations and a feasible method to enumerate viable bacteria in samples is most-probable-number (MPN) counts. This method has been well described and reviewed elsewhere (Russek and Colwell, 1983; Arias *et al.*, 1996 Aurelio *et al.*, 1999).

3.3.4 Principle of Assessment of Vitality

If a new culture is inoculated with a mixture of living and dead cells, such as the cell cultures in stationary phase, the cell growth measurement of live bacteria will give different answers from determination of total bacterial mass. If cell growth is monitored by measuring turbidity of the culture, the figure obtained will include the contribution of dead organisms. The results will show a false lag in the resumption of growth. If the cell growth is measured by a viable count, the result shows that the living cells have very short or no lag phase (Figure 3.2).

Generally, under the same cultural conditions such as cell concentration, medium composition, pH, and temperature, the more viable cells present in a medium, the faster the cell cultures will grow, and the shorter the lag of the cell growth. Therefore,

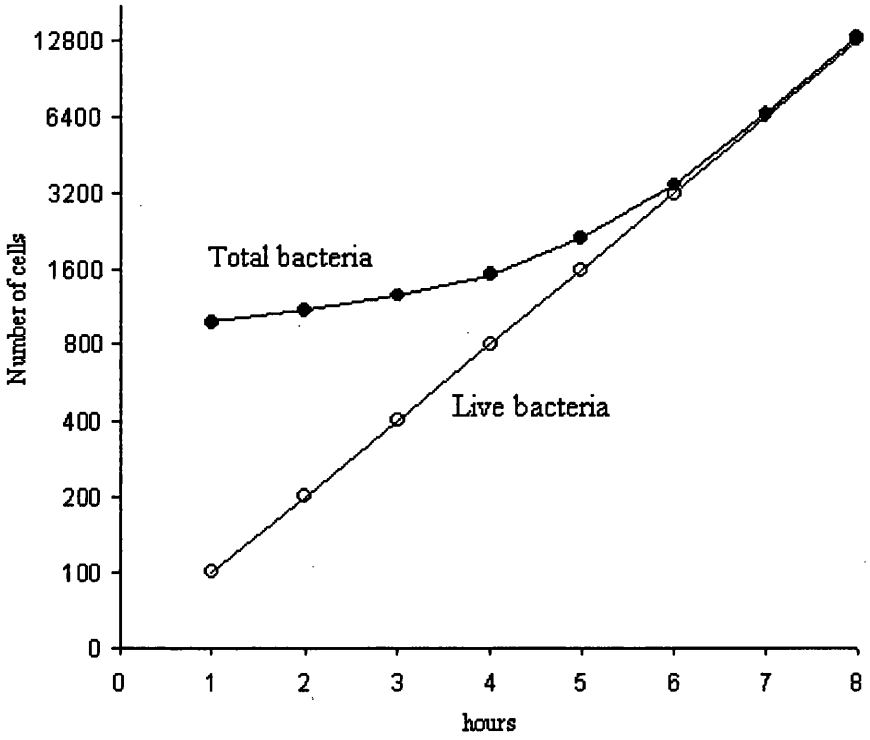


Figure 3.2 the measurement of live and total cells. If a population consisted initially of 100 living cells and 900 dead cells and growth is measured by estimating the total number of cells, an apparent lag will be seen. If living cells only are measured, there is no lag. This may happen when a 24-hour culture of *E. coli* has been transferred to fresh medium (After Mandelstam and McQuillen, 1973).

if the samples after stress are introduced as inoculum into the same fresh media and cultivated at the same conditions, the vitality of samples may be assessed by comparing observed growth curves of the treated cells. If the samples contain the same initial cell concentration, then the sample with higher growth rate has a higher vitality. Prior treatment of the inoculum will have a direct effect on the growth curve and further affect the assessment of vitality.

3.4 Analytic Methods for Viability and Vitality

To test the idea of using vitality as a measure of cultural quality, an experiment to compare vitality to viability was carried out.

3.4.1 Measurement of Cell Viability by Plate Counting

GY medium agar was used in this experiment: glucose 5 g/l, yeast extract 5 g/l, $(NH_4)_2SO_4$ 2 g /l, KH_2PO_4 2 g/l, agar 20 g/l and trace element solution (see Table 2.1) 20 ml/l. cell viabilities were determined after freezing by the methods described in Chapter 2 (section 2.7.2)

3.4.2 Measurement of Cell Vitality

To assess the cell vitality of LAB after freezing, the following general procedures were developed:

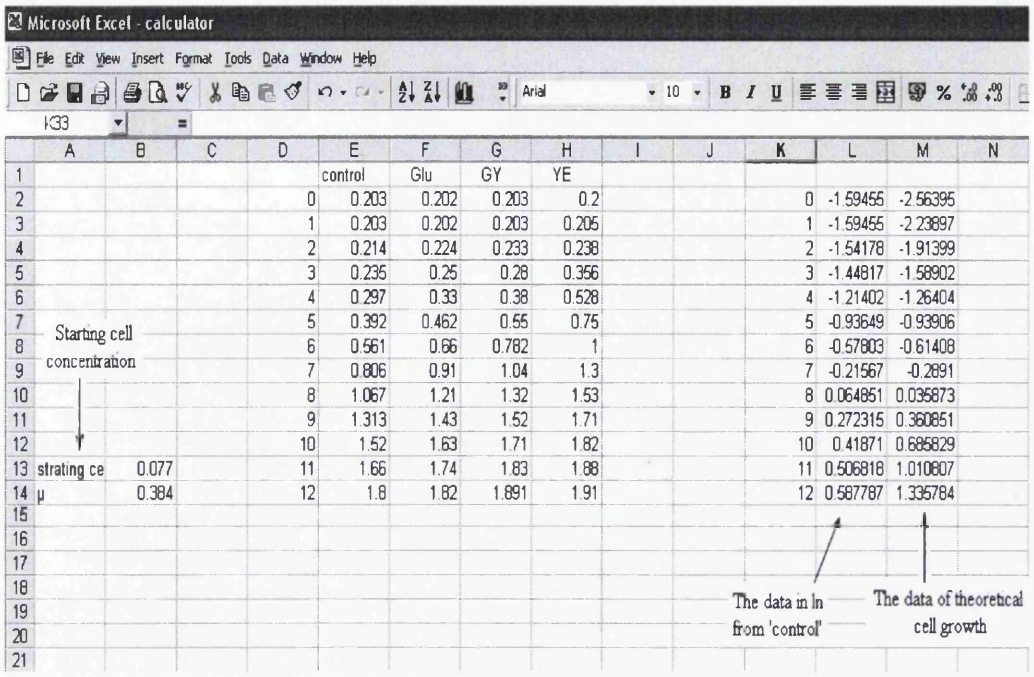
1. The bacterial cells were grown up according to the methods described in Chapter 2, section 2.3.1.
2. After cultivation, samples were taken from the cell cultures and added into 10 ml GY medium. The amount added was carefully controlled (measured by the optical densities) to give equal cell concentration. These cultures were then frozen without shaking at -20°C for about 48 hours.
3. After freezing the cultures were thawed for about 2 hours at room temperature (about 25°C).
4. These cell cultures were then incubated at 37°C (for *L. plantarum*) or 25°C (for *L. brevis* and *O. oeni*). The cell growth was monitored by the spectrophotometer at 660 nm. An example of the data observed is shown in Table 3.1. Initial cell concentration (A) was obtained.
5. The OD values of cell growth vs time were then input into an Excel spreadsheet. Firstly a group of data (e.g. 'control' in Table 3.1) were converted into the natural logs. These data were then compared with a group of data of theoretical cell growth as shown in Figure 3.3 where the population is assumed to have 100% vitality (i.e. no lag, but reduced population numbers). The starting cell concentration was varied until a good fit was obtained between the theoretical growth line and the linear segment of the growth curve (see Figure 3.4 and Figure 3.5).

6. The observed cell growth curve had a lag phase. It is assumed that there was no lag phase during the theoretical cell growth because all the population were either fully active or dead. When the theoretical cell growth curve was compared with the observed cell growth curves and the linear parts of the two curves were fitted with each other, the estimated active population (B) in the observed population was then obtained (see Figure 3.5).

Table 3.1 Table of observed optical densities (OD) of cell growth

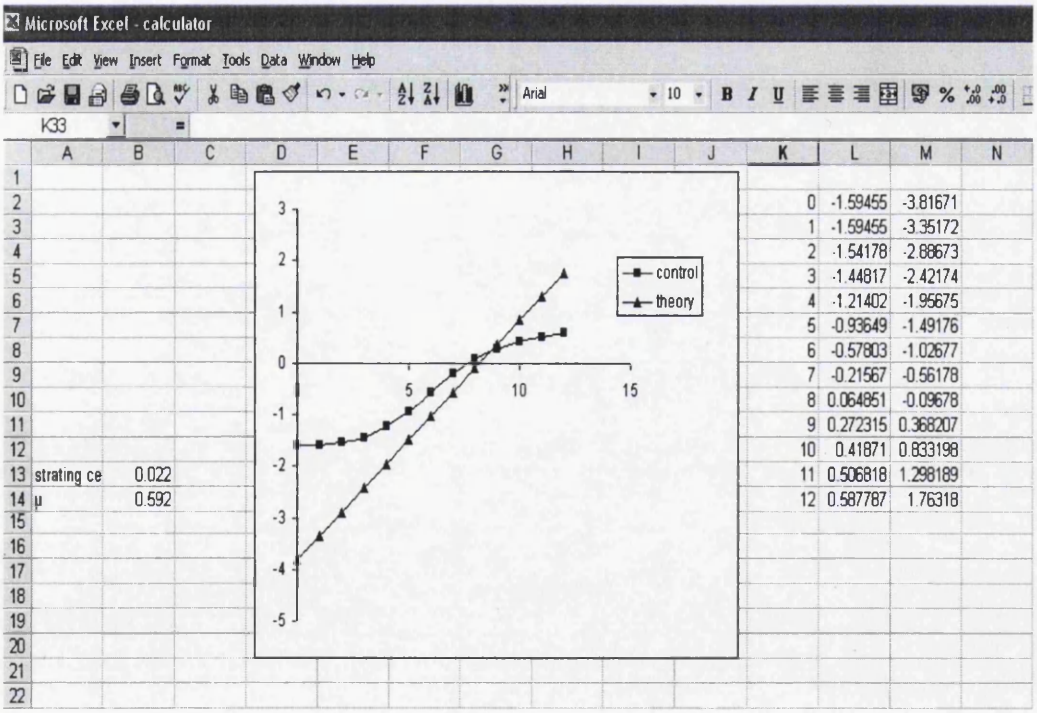
Preincubation medium	Control	Glucose	GY	YE
Time (hrs)				
0	0.203	0.202	0.203	0.200
1	0.203	0.202	0.203	0.205
2	0.214	0.224	0.233	0.238
3	0.235	0.250	0.280	0.356
4	0.297	0.330	0.380	0.528
5	0.392	0.462	0.550	0.750
6	0.561	0.660	0.782	1.000
7	0.806	0.910	1.040	1.300
8	1.067	1.210	1.320	1.530
9	1.313	1.430	1.520	1.710
10	1.520	1.630	1.710	1.820
11	1.660	1.740	1.830	1.880
12	1.800	1.820	1.891	1.910

Notes: Control: the cells were not pre-incubated before freezing, Glucose: 5 g/l glucose solution. YE: 5 g/l yeast extract solution, GY: GY medium. The data shown was average value for three samples.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1					control	Glu	GY	YE						
2				0	0.203	0.202	0.203	0.2			0	-1.59455	-2.56395	
3				1	0.203	0.202	0.203	0.205			1	-1.59455	-2.23897	
4				2	0.214	0.224	0.233	0.238			2	-1.54178	-1.91399	
5				3	0.235	0.25	0.28	0.356			3	-1.44817	-1.58902	
6				4	0.297	0.33	0.38	0.528			4	-1.21402	-1.26404	
7				5	0.392	0.462	0.55	0.75			5	-0.93649	-0.93906	
8				6	0.561	0.66	0.782	1			6	-0.57803	-0.61408	
9				7	0.806	0.91	1.04	1.3			7	-0.21567	-0.2891	
10				8	1.067	1.21	1.32	1.53			8	0.064851	0.035873	
11				9	1.313	1.43	1.52	1.71			9	0.272315	0.360851	
12				10	1.52	1.63	1.71	1.82			10	0.41871	0.685829	
13	strating ce	0.077		11	1.66	1.74	1.83	1.88			11	0.506818	1.010807	
14	μ	0.384		12	1.8	1.82	1.891	1.91			12	0.587787	1.335784	
15														
16														
17														
18														
19														
20														
21														

Figure 3.3 Screen from Excel program to assess cell vitality. Column D-H data from Table 3.1, column L is the data in ln from column E 'control', column M is the estimated data of theoretical cell growth, A13 is the starting cell concentration, A14 is the specific growth rate.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1														
2											0	-1.59455	-3.81671	
3											1	-1.59455	-3.35172	
4											2	-1.54178	-2.88673	
5											3	-1.44817	-2.42174	
6											4	-1.21402	-1.95675	
7											5	-0.93649	-1.49176	
8											6	-0.57803	-1.02677	
9											7	-0.21567	-0.56178	
10											8	0.064851	-0.09678	
11											9	0.272315	0.368207	
12											10	0.41871	0.833198	
13	strating ce	0.022									11	0.506818	1.298189	
14	μ	0.592									12	0.587787	1.76318	
15														
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Figure 3.4 The data shown here gave a poor fit so the starting cell concentration and μ were adjusted to give the best fit (see Figure 3.5).

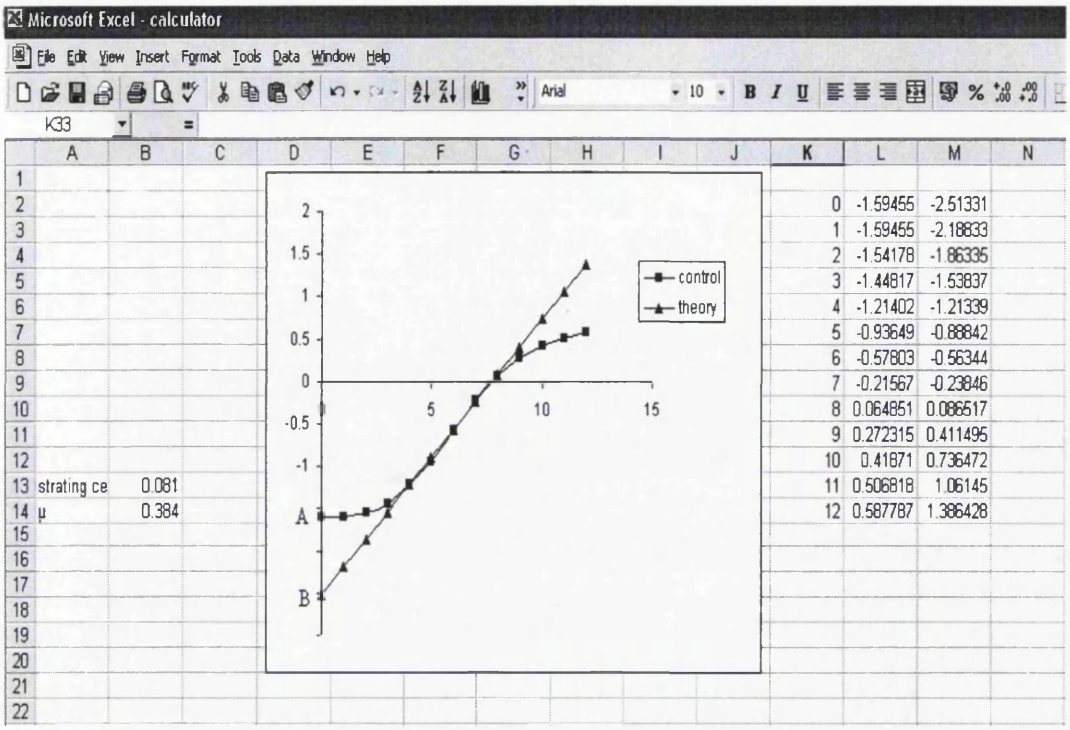


Figure 3.5 when starting cell concentration was 0.081 and μ was 0.384, the two curves fit best. Point B (0.081) obtained was the estimated active population; Point A (0.202) was the observed population.

7. The estimated active population (B) is compared with the observed population (A), and vitality index (VI) can be worked out.

$$\text{Vitality index} = B/A \times 100\%$$

For example, in Fig.3.5, B = 0.081, A= 0.202, then

$$\text{Vitality index} = 0.081/0.202 \times 100\% = 40.1\%$$

If VI is 80-100%, it indicates the vitality of the sample is high and the cells are very active after stress, if VI is >60 and <80%, the vitality of the sample is medium, if VI is < 60%, the vitality of the sample is low and the cells had been very stressed.

8. An estimate of sensitivity of the method was also made. A major source of error is that the fitting of two growth curves was by eye. For example, in Fig.3.5, when the starting cell concentration was 0.078, and 0.083, the two growth curves were also fit well, and vitality indices were 38.6% and 41.1%. So the sensitivity was quite low, e.g. for the sample 'control' was 40% ± 1.2.

3.5 Results

Using the above methods for viability and vitality, the following experiments were carried out to compare the two methods of assessment. In the experiments, the effect of preincubation in different media on cell viability and vitality after freezing was investigated.

The fresh cell culture of *L. plantarum* was introduced into the GY media, and cultivated at 25°C. When the cells had grown to early stationary phase, 1 ml aliquots of the cultures were taken into 1 ml sterile micro centrifugation tubes. The micro centrifugation tubes were centrifuged to remove the cultural media. The cell pastes obtained were re-suspended into 1 ml of the following solutions: glucose 5 g/l, yeast extract solution 5 g/l, and GY medium. Preincubation was carried out: the micro centrifugation tubes were kept at 25°C for 1 hour. When preincubation was complete the micro centrifugation tubes were centrifuged again and the preincubation media were removed. The cell pastes obtained were re-suspended in 1 ml of the GY media. 0.5 ml aliquot of the suspensions were introduced into 10 ml GY medium, and frozen at -20°C for about 48 hours. After these treatments, the cell viability and vitality were assessed by plate counting and comparison of the cell growth curves.

Table 3.2 shows the effect of preincubation in different media on viability of *L. plantarum*. When *L. plantarum* was directly frozen without preincubation, the cell survival rate was very low, only 5.2 %. When the cells were preincubated in yeast extract cell viability was the highest, 46.5%. The cell viability preincubated in GY medium was the second, 31.4%. While the cells were preincubated in glucose solution, cell viability increased slightly compared with the control.

The cell growth performance and calculated cell vitality index (VI) after freezing treatment are shown in Fig.3.6. When *L. plantarum* was directly frozen without preincubation, the cell growth was the slowest, and VI was 40.1%. When the cells were preincubated in yeast extract the cell growth rate was the highest and VI was also the highest, 73.5%, and the cells preincubated in GY medium were ranked second (60.0%).

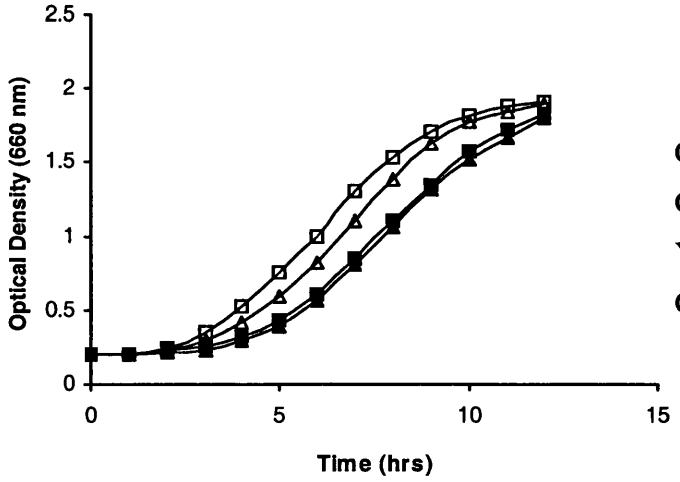
It is seen from the results above that the assessment of viability by plate counting and assessment of vitality by comparing growth curves gave very similar conclusions. It

indicates that assessment of vitality by comparing growth curves is feasible and reliable.

Table 3.2. Effect of preincubation in different media on the viabilities of *L. plantarum*

Preincubation medium	Control	Glucose	YE	GY
Viability (%)	5.2±3.5	6.3±2.8	46.5±4.2	31.4±3.2

The cells of *L. plantarum* were preincubated at 25°C for 1 hour in different media: 5g/l glucose, 5g/l yeast extract, the GY medium. Non-preincubated sample was control. The samples were frozen at –20°C for about 48 h. The cell viability was assessed by plate counting (CFU/ml). The data shown was average value for three plates.



	VI(%)	μ
Control	40.1±2.0	0.384
Glucose	42.5±1.8	0.384
YE	73.5±2.3	0.384
GY	60.0±2.1	0.384

Figure 3.6 Effect of preincubation in different media on vitality of *L. plantarum* after freezing. The cells were preincubated at 25°C for 1 hour in different media: 5g/l glucose (■), 5g/l yeast extract (□), the GY medium (△). Non-preincubated sample was as control (▲). Initial cell concentration (OD) was 0.30. Data shown was average value for three. VI: vitality index, μ: the specific growth rate.

3.6 Discussion

The traditional and typical method to evaluate cell viability is counting colony-forming units in cultures on solid media. This quantitative measurement provides much information about the quality of the preservation. But this method is time consuming and tedious to perform and is used only when it is necessary to estimate viability or in some statutory tests of food (Dawes and Sutherland, 1998).

In this work, a simple method to assess cell vitality by comparing growth curves was developed, and it was shown that this method was feasible and reliable. Moreover, this method was easier to do than plate counting method. Especially, vitality can be calculated by analysing the growth curves. This method was considered to be more useful that that providing counts of colony-forming units for the purpose of guiding species selection or qualitative experiments. However, the results obtained by comparing growth curves do not show cell survival rate after freezing and freeze-drying. For instance, the cell viability of the sample 'control' was 5.2% obtained by plate counting method whereas the vitality index was 40.1%. So comparing growth curves to assess vitality is a relative measurement method.

Chapter 4. Investigation of Effect of Culture Conditions on Vitality and Cryotolerance of LAB

4.1 Introduction

Freezing is one of the methods commonly used for the preservation of biological materials including starter cultures for the food industry (Murray, 1985; Bâati*et al.*, 2000; Konings*et al.*, 2000). However, usually microbial cells can be significantly damaged or killed during the freezing process, and it is difficult to keep microbial cultures in a highly active state. The optimal freezing performance of the strains depends on physiological state of the microbial cell and environmental conditions before freezing (Kenward and Brown, 1978).

It has been shown that the physiological state and cell composition of microorganisms are very important for cell survival after freezing. Microbial cells can adapt to environmental stresses by inducing specific proteins and other products (Jones and Inouye, 1994; Lorca and de Valdez, 1999; Sanders *et al.*, 1999); modifying the cell membrane composition (Marr and Ingraham, 1962; Mandelstam *et al.*, 1982; Panoff *et al.*, 1998); and accumulating compatible solutes within the cell (Da Costa *et al.*, 1998; Champomier-Vergès *et al.*, 2002). Culture conditions influence both physiological state and cell composition of microorganisms. Microorganisms have many different responses to freezing stress, some of which are species dependent (Palmfeldt and Hahn-Hägerdal, 2000).

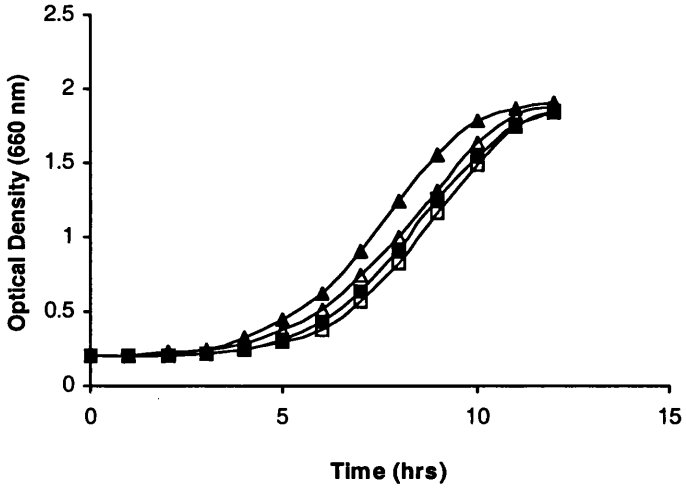
The process of preservation by freeze-drying consists of several interlinked processes. Firstly the cells have to be grown up and then are pre-treated to allow adaptation to freezing. They are then frozen and dried. All of these steps subject the organisms to significant stresses. The most significant stresses are due to freezing and osmotic pressure (Champagne *et al.*, 1991). Basic studies of cryo-tolerance in LAB may facilitate industry efforts to improve the activity and functionality of freeze-dried starter preparations.

In this chapter, several factors were investigated, including growth phase, growth temperature, medium composition, medium pH, and preincubation conditions, on the cryotolerance and vitality of *Lactobacillus plantarum, Lactobacillus brevis* and *Oenococcus oeni.* In the previous chapter, a method of estimating vitality was described. The method proved to be an easy and reliable method of assessing the effects of variety of treatments on vitality. In this section this method was applied to assess vitality of LAB after freezing.

4.2 Effect of Growth Phase

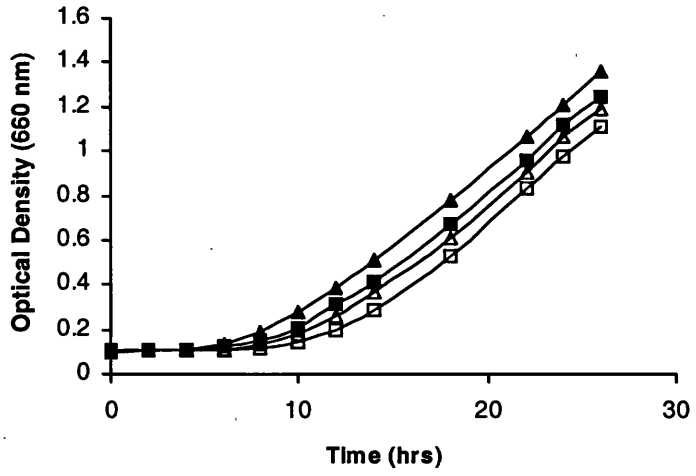
Lactobacillus plantarum, L. brevis and *O. oeni* were cultivated in the GY media at 25°C, 30°C for *L. brevis* and *O. oeni*, or 37°C for *L. plantarum*. The cells were grown and used at either exponential (OD = 0.8. ~ 1.0) or early stationary phase (OD =1.3 ~1.8). Samples were taken from the cell cultures and added into 10 ml GY media. The amount of cells added was carefully controlled (measured by the optical density) to give the same cell concentration. These cultures were then frozen at -20°C for 48 hours. After treatment the cultures were thawed for about 2 hours at room temperature (about 25°C) and the cell vitality was assessed by intercomparison of the cell growth curves.

The cell growth performance and calculated vitality indices after freezing treatment for three bacteria are shown in Fig.4.1, Fig.4.2, and Fig.4.3. It can be seen that the cells of all LAB species tested at stationary phase had higher vitality than those at exponential phase at the same culture temperature. For example, when the cells of *L. plantarum* grew at 25°C to early stationary phase (Fig.4.1, S25) the vitality index was 42.8% whereas the vitality index was 31.0% when the cells grew in exponential phase (Fig4.1, E25). But the individual performance of the three bacteria was different. For *L. plantarum*, the cell vitality at early stationary phase was higher than that at exponential phase, whatever the culture temperature was 25°C or 37°C. Similar results were obtained for *O. oeni.* However, for *L. brevis*, when the cells were cultivated at 25°C the cells at exponential phase had a higher vitality (VI, 39.2%) than those at the stationary phase cultivated at 30°C (VI, 35.3%). When the cells of three LAB species were cultivated at 25°C and grew at stationary phase, the cells had the highest cell vitality after freezing (VI, 42.8% for *L. plantarum*, 48.0% for *L. brevis*, and 41.8% for *O. oeni*).



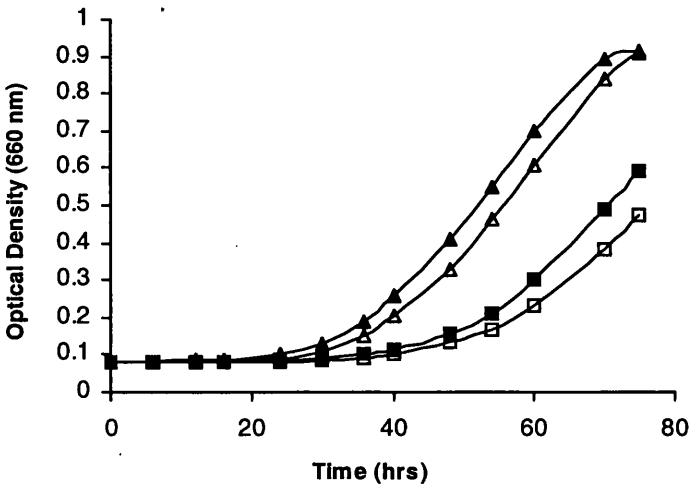
	VI (%)	μ
E25	31.0±1.8	0.387
E37	28.1±1.4	0.387
S25	42.8±1.6	0.387
S37	35.6±2.2	0.387

Figure 4.1 Effect of growth phase on the vitality of *L. plantarum* after freezing. E25 (■), E37 (□): the cells cultivated at 25, 37°C and grown at exponential phase; S25 (▲), S37 (△) the cells cultivated at 25, 37°C and grown at early stationary phase. The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.20 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.1



	VI (%)	μ
E25	39.2±1.4	0.180
E30	27.4±1.6	0.180
S25	48.0±1.6	0.180
S30	35.3±1.6	0.180

Figure 4.2 Effect of growth phase on the vitality of *L. brevis* after freezing. E25 (■), E30 (□): the cells cultivated at 25, 30°C and grown at exponential phase; S25 (▲), S30 (△) the cells cultivated at 25, 30°C and grown at early stationary phase. The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.10 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.2.



	VI (%)	μ
E25	16.1±2.3	0.053
E30	13.1±1.5	0.053
S25	41.8±1.7	0.053
S30	33.6±2.1	0.053

Figure 4.3 Effect of growth phase on the vitality of *O. oeni* after freezing. E25 (■), E30 (□): the cells cultivated at 25, 30°C and grown at exponential phase. S25 (▲), S30 (△) the cells cultivated at 25, 30°C and grown at early stationary phase. The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.08 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See chapter 3 for detail of the method, and the raw data is in Appendix-2.3.

4.3 Effect of Growth Temperature

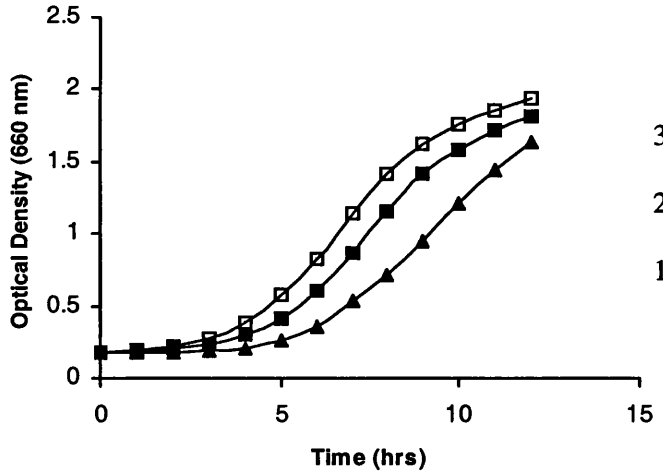
It has been shown that cells harvested in stationary phase showed a high resistance compared with those cells obtained in the exponential phase. Growth temperature is also an important cultural factor and affects the cell growth rate and the cell composition (Panoff *et al.*, 1998; Bâati *et al.*, 2000).

Lactobacillus brevis and *O. oeni* were cultivated in the GY media respectively at 18, 25 and 30°C, and *L. plantarum* was incubated at 18, 25 and 37°C. When the LAB cells grew up to early stationary phase, samples were taken from the cell cultures and added into 10 ml GY media. The amount of cells added was carefully controlled (measured by the optical density) to give equal cell concentrations. These cultures were then frozen at -20°C for 48 hours. After treatment the cell vitality was assessed by comparing the cell growth curves as described previously.

The growth curves and calculated vitality indices are shown in Fig.4.4, Fig.4.5, and Fig.4.6 for *L. plantarum*, *L. brevis* and *O. oeni* respectively. These results show that the cell vitality of *L. plantarum*, *L. brevis* and *O. oeni* was the highest (VI, 65.0%,

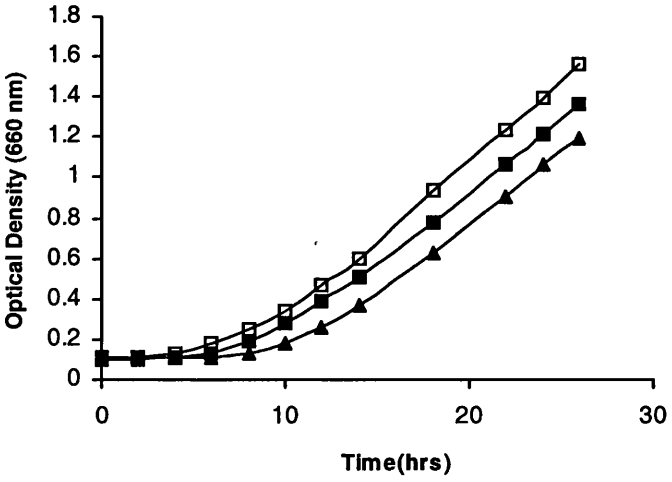
61.5% and 44.4% respectively) when the cells of the LAB were cultivated at 18°C, and the cell vitality cultivated at 25°C were ranked second. The cell vitality was the lowest when the cells grew at 37 or 30°C (VI, 30.6%, 34.3% and 35.0% respectively). The results showed that the culture temperature of cells had a significant effect on the survival after freezing, and the cells especially for *L. plantarum* and *L. brevis*, cultivated at relatively low temperature or sub-optimal temperature had higher vitality after freezing than those grown at optimal growth temperature, 37°C (*L. plantarum)* and 30°C (*L. brevis* and *O. oeni*).

However, when LAB were cultivated at low temperature (such as 18°C), the cells grew very slowly, especially for *O. oeni*. This is a disadvantage for production of starter culture because of low productivity.



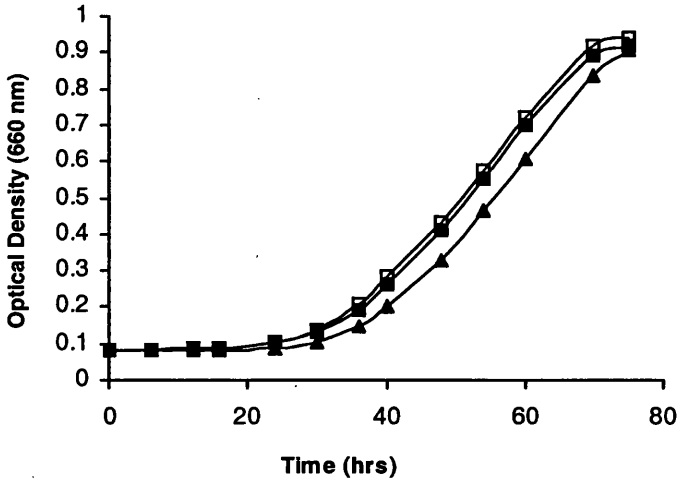
	VI (%)	μ
37°C	30.6±1.4	0.382
25°C	47.2±1.3	0.382
18°C	65.0±2.0	0.382

Figure 4.4 Effect of growth temperature on the vitality of *L. plantarum* after freezing. The cells were cultivated in the GY media at 18(□), 25 (■) and 30°C(▲), and grew to early stationary phase. The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.18 when the vitality was assessed. VI: Vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.4.



	VI(%)	μ
30°C	34.3±1.5	0.180
25°C	50.2±1.2	0.180
18°C	61.5±1.8	0.180

Figure 4.5 Effect of growth temperature on the vitality of *L. brevis* after freezing. The cells were cultivated in the GY media at 18(□), 25 (■) and 30°C(▲), and grew to early stationary phase. The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.10 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.5.



	VI(%)	μ
30°C	35.0±1.7	0.053
25°C	41.3±2.1	0.053
18°C	44.4±2.2	0.053

Figure 4.6 Effect of growth temperature on the vitality of *O. oeni* after freezing. The cells were cultivated in the GY media at 18(□), 25 (■) and 30°C(▲), and grew to early stationary phase. The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.08 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.6.

4.4 Effect of Medium Composition

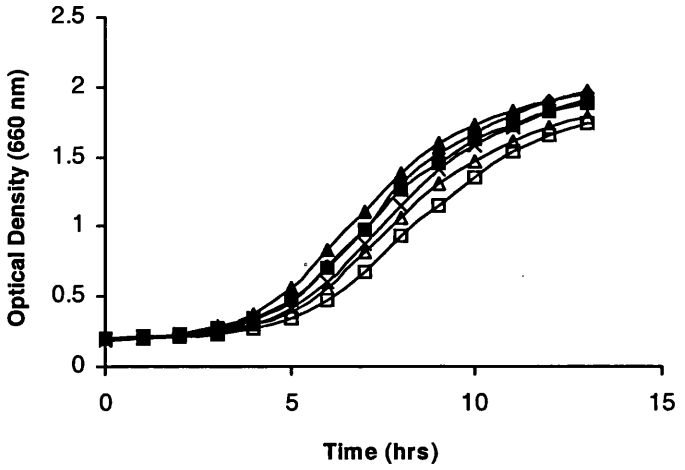
Medium composition is one of the most important factors that influence bacterial cell growth. Many components of growth medium have been shown to affect viability of bacterial cells after freezing (Wright and Klaenhammer, 1983a; Chang and Elander, 1986; Goldberg and Eschar, 1997).

4.4.1 Effect of Yeast Extract (YE)

The three types of bacteria were introduced into the GY media in which amount of yeast extract was 0, 1, 3, 5, 8, 10 g/l respectively and cultivated at 25°C. When the LAB cells grew to early stationary phase, samples were taken from the cell cultures and added into 10 ml GY medium. The amount of cells added was carefully controlled (measured by the optical density) to give the same and known cell concentrations. These cultures were then frozen at -20°C for 48 hours. After treatment the cell vitality was assessed by intercomparison of the cell growth curves.

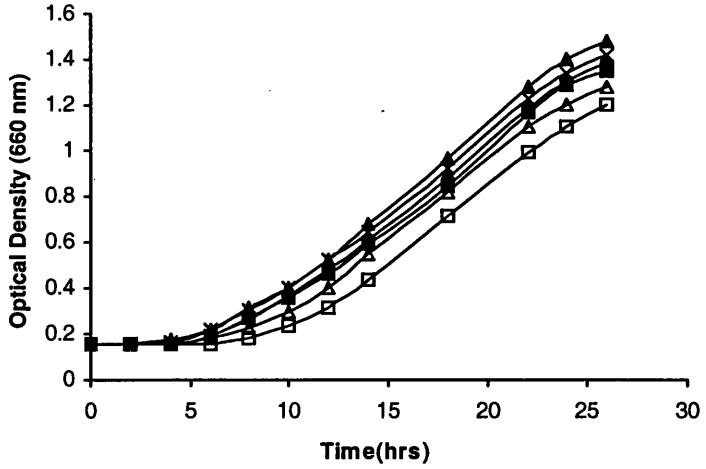
Fig.4.7, Fig.4.8 and Fig.4.9 show the cell growth curves and calculated vitality indices of *L. plantarum*, *L. brevis* and *O. oeni* after freezing. The cell vitality of the three bacteria increased as the content of yeast extract in the media increased, e.g. the vitality index of *L. plantarum* increased from 33.3% to 42.0% when the amount of yeast extract in the medium increased from 1 to 5 g/l. However, the optimal amount of yeast extract in the medium was different for the three bacteria. When the amount of yeast extract in the medium was 8 g/l, the cell vitality of *L. plantarum* was the highest, 53.2%, while *L. brevis* and *O. oeni* obtained the highest vitality, 49.7% and 44.6%, when yeast extract was 5 g/l in the medium. When yeast extract was over 8 g/l in the medium, the cell vitality of LAB remained constant, e.g. the vitality index of *L. brevis* was 42.5% when yeast extract in the medium was 10 g/l and 15 g/l.

These results indicate that medium composition influenced the cell vitality after freezing, and yeast extract as growth factor could enhance cell survival to freezing if the media contained more yeast extract. However, high concentration of yeast extract in the medium was not favourable to cell vitality after freezing in some cases.



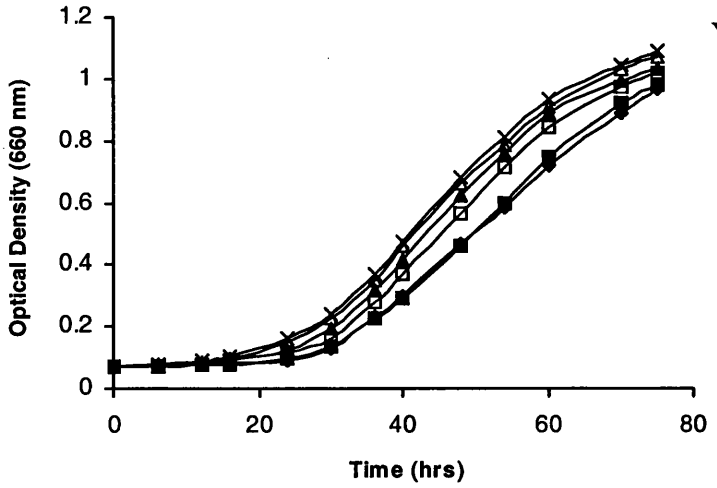
YE(g/l)	VI(%)	μ
15	48.3±2.1	0.387
10	48.3±2.3	0.387
8	53.2±1.5	0.387
5	42.0± 1.8	0.387
3	37.4±1.6	0.387
1	33.3±2.0	0.387

Figure 4.7 Effect of YE in the medium on the vitality of *L. plantarum* after freezing. The cells were cultivated in the GY media that contained respectively following amount of yeast extract (g/l): 1(□), 3 (△), 5 (×), 8(▲), 10 (■) and 15(◆). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.20 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.7.



YE(g/l)	VI(%)	μ
15	42.5±2.3	0.180
10	42.5±1.7	0.180
8	49.7±1.6	0.180
5	49.7±2.3	0.180
3	35.5±1.8	0.180
1	28.4±2.1	0.180

Figure 4.8. Effect of YE in the medium on the vitality of *L. brevis* after freezing. The cells were cultivated in the GY media that contained respectively following amount of yeast extract (g/l): 1(□), 3 (△), 5 (×), 8(▲), 10 (■) and 15(△). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.15 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.8.



YE(g/l)	VI(%)	μ
15	28.5 ±2.1	0.066
10	28.5±2.2	0.066
8	37.8±1.6	0.066
5	44.6±1.8	0.066
3	42.1±1.6	0.066
1	33.6±2.0	0.066

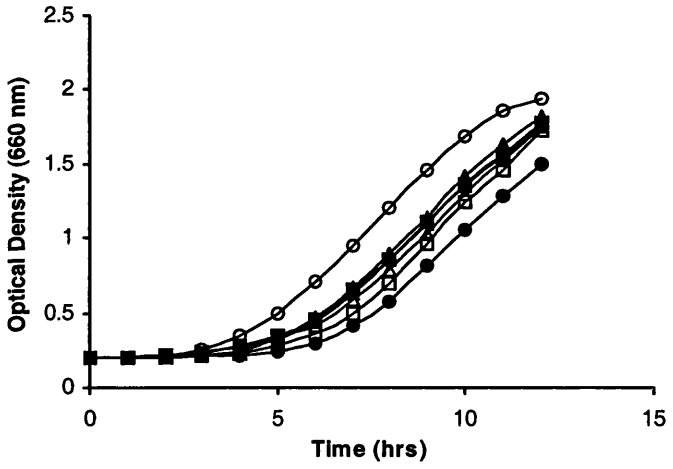
Figure 4.9 Effect off YE in the medium on the vitality of *O. oeni* after freezing. The cells were cultivated in the GY media that contained respectively following amount of yeast extract (g/l): 1(□), 3 (△), 5 (×), 8(▲), 10 (■) and 15(◆). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.07 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.9.

4.4.2. Effect of Calcium and Manganese

Divalent cations such as calcium are important components of the cell wall and membrane of lactic acid bacteria. Other cations such as manganese are important for catalytic activity of enzymes. When LAB are subjected to freezing stress, the cell wall and membranes may be damaged. Divalent cations in the culture medium possibly influence the structure of cell membranes and then cell survival rate after freezing (Champagne *et al.*, 1991).

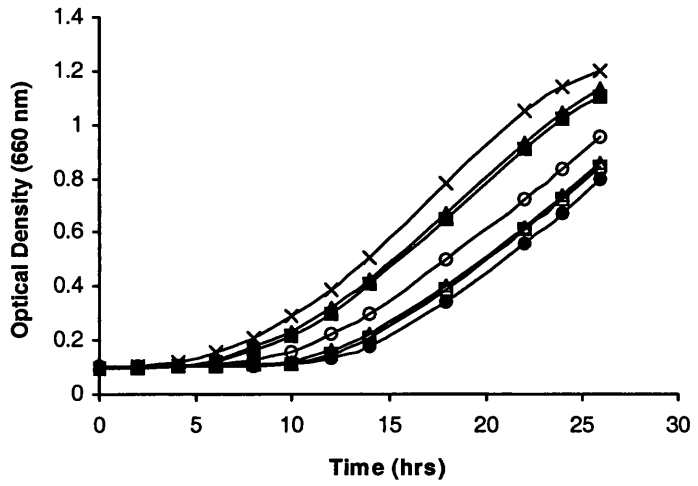
Active LAB were introduced into the GY media in which the trace element solution contained little calcium and manganese ions and the yeast extract used was salt-free (Oxoid Ltd, UK), and the medium was supplemented respectively with the following amounts of $CaCl_2$ (w/v %): 0.01, 0.03, 0.05, 0.08, 0.10, and $MnCl_2$ (w/v %): 0.01, 0.03, 0.05, 0.08, 0.10. The cultures of LAB were cultivated at 25°C. When the cells grew to early stationary phase, samples were taken from the cell cultures and added into 10 ml GY medium. The amount of cells added was carefully controlled (measured by the optical density) to give equal cell concentrations. These cultures were then frozen at -20°C for 48 hours. After treatment the cell vitality was assessed by intercomparison of the cell growth curves.

The effect of calcium concentration in the medium on vitality of *L. plantarum*, *L. brevis* and *O. oeni* are shown in Fig.4.10, Fig.4.11 and Fig.4.12 respectively. It is seen that the cells of three LAB had higher vitality when the media were supplemented with Ca^{2+}, Compared with the control (no added calcium and manganese in the medium). For example, the vitality of *L. brevis* increased from 25.1% to 66.7% when the concentration of calcium in the medium increased from 0% to 0.05%. When $CaCl_2$ in the medium was 0.08%, 0.05%, and 0.03% respectively, *L. plantarum*, *L. brevis* and *O. oeni* showed the highest cell vitality indices (33.5%, 66.7% and 50.7%), which showed that the three LAB had different sensitivities to calcium and the optimal concentration of calcium in the medium depended on the species. This result suggests that calcium is a factor that affects cell vitality after freezing, and more calcium in the media can improve the survival of three lactic acid bacteria. However, high concentration calcium (>0.05%) did not enhance the vitality. When $CaCl_2$ in the media was 0.08%, the vitality of *L. plantarum*, *L. brevis* and *O. oeni* did not increase further.



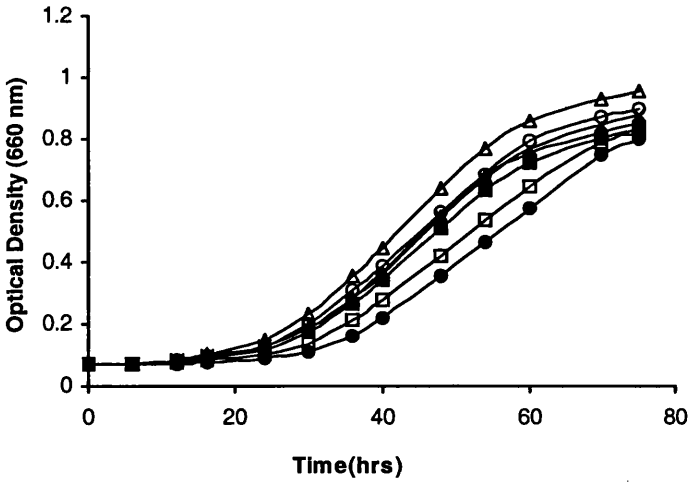
Ca^{2+}	VI(%)	μ
0%	21.0±1.5	0.380
0.01%	27.5±1.6	0.380
0.03%	30.0±1.4	0.380
0.05%	32.0±1.8	0.380
0.08%	33.5±1.7	0.380
0.10%	33.5±2.2	0.380
Ca+Mn	50.0±2.4	0.380

Figure 4.10 Effect of calcium in the medium on the vitality of *L. plantarum* after freezing. The cells were cultivated in the GY media in which trace elements solution did not contain Ca^{2+} and Mn^{2+}. The media were supplemented respectively following amount of $CaCl_2$ (% w/v): 0 (As control, •), 0.01(□), 0.03 (△), 0.05 (×), 0.08(▲), 0.10 (■), Ca + Mn: 0.08% $CaCl_2$ and 0.05%$MnCl_2$ (○). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.23 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.10.



Ca^{2+}	VI(%)	μ
0%	25.1±1.3	0.151
0.01%	29.4±1.8	0.151
0.03%	30.9±2.1	0.151
0.05%	66.7 ±2.5	0.151
0.08%	57.4±2.0	0.151
0.10%	57.0±1.8	0.151
Ca+Mn	41.6±2.1	0.151

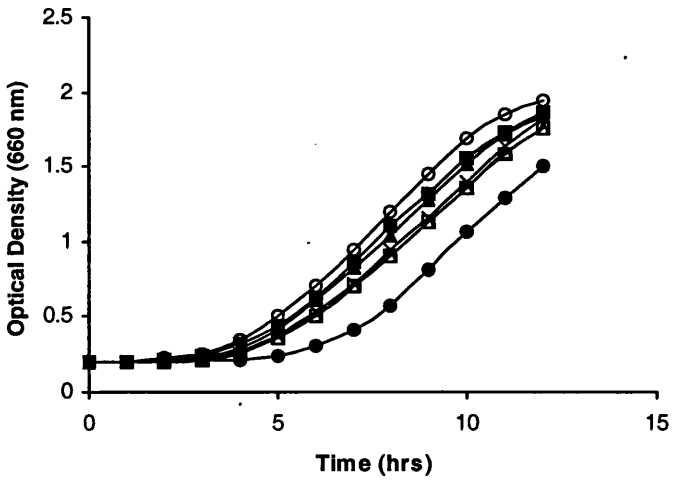
Figure 4.11 Effect of calcium in the medium on the vitality of *L. brevis* after freezing. The cells were cultivated in the GY media in which trace elements solution did not contain Ca^{2+} and Mn^{2+}. The media were supplemented respectively following amount of $CaCl_2$ (% w/v): 0 (As control, •), 0.01(□), 0.03 (△), 0.05 (×), 0.08(▲), 0.10 (■), Ca + Mn: 0.08% $CaCl_2$ and 0.05%$MnCl_2$ (○). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.10 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.11.



Ca^{2+}	VI(%)	μ
0%	25.1±1.2	0.064
0.01%	31.4±1.4	0.064
0.03%	50.7±1.7	0.064
0.05%	40.8±2.2	0.064
0.08%	40.8±1.4	0.064
0.10%	40.3±1.2	0.064
Ca+Mn	45.7±2.1	0.064

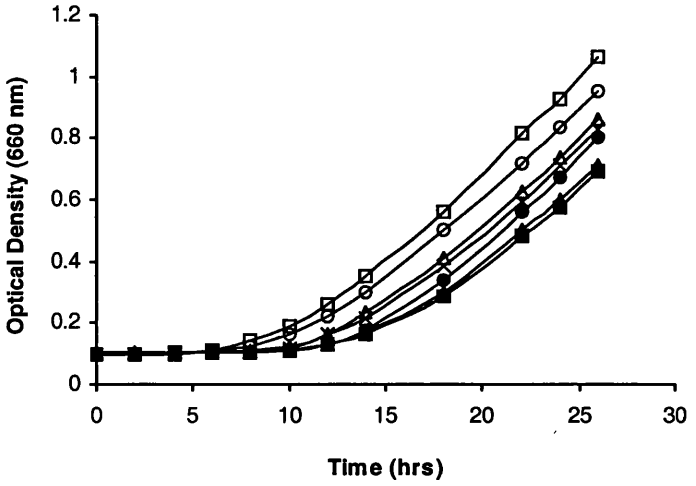
Figure 4.12 Effect of calcium in the medium on the vitality of *O. oeni* after freezing. The cells were cultivated in the GY media in which trace elements solution did not contain Ca^{2+} and Mn^{2+}. The media were supplemented respectively following amount of $CaCl_2$ (% w/v): 0 (As control, •), 0.01(□), 0.03 (△), 0.05 (×), 0.08(▲), 0.10 (■), Ca + Mn: 0.08% $CaCl_2$ and 0.05%$MnCl_2$ (○). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.07 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.12.

Fig.4.13, Fig 4.14 and Fig 4.15 show the effect of manganese in the medium on vitality of *L. plantarum*, *L. brevis* and *O. oeni*. These results show that manganese, like calcium, was also a factor that affected cell survival rate after freezing. When the media were supplemented with manganese, the cell vitality of three bacteria was enhanced to varying degrees. For example, comparing with the control, the vitality index of *L. plantarum* increased 17.0% when the medium was supplemented with 0.05% $MnCl_2$; the vitality indices of *L. brevis* and *O. oeni* increased 21.5% and 20.6% respectively when the media were supplemented with 0.01% $MnCl_2$. However, the three bacteria did not have the same sensitivity to manganese. When $MnCl_2$ in the medium increased from 0% to 0.1%, the cell vitality of *L. plantarum* was enhanced from 21.0% to 45.0%, while $MnCl_2$ in the medium was 0.01% *L. brevis* and *O. oeni* had the highest the cell vitality (46.6% and 45.7%). When $MnCl_2$ in the medium was 0.08%, the cell vitality of *L. brevis* declined (24.0%) and even was lower than the control (25.1%). When $MnCl_2$ in the medium was 0.08% the cell vitality of *O. oeni* was also decreased (40.4%), but was still much higher than the control (25.1%). These results indicated that *L. brevis* was more sensitive to manganese than *L. plantarum* and *O. oeni*. Optimum amounts of manganese in the medium depended on the bacterial species.



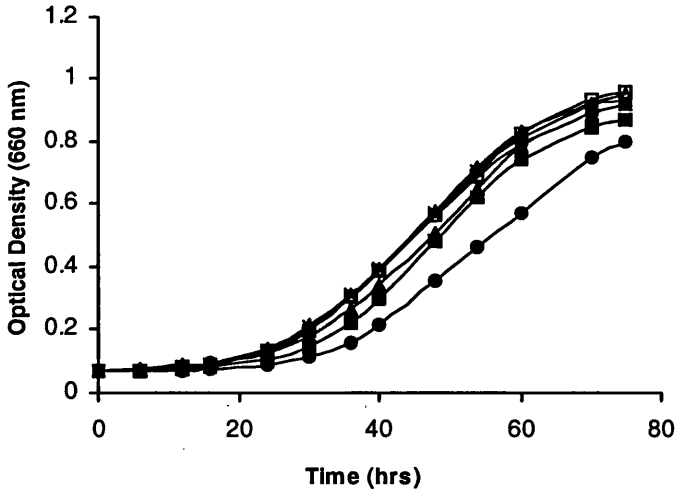
Mn^{2+}	VI(%)	μ
0%	21.0±1.3	0.380
0.01%	38.0±1.8	0.380
0.03%	38.0±1.7	0.380
0.05%	38.0±1.5	0.380
0.08%	42.5±2.3	0.380
0.10%	45.0±1.7	0.380
Ca+Mn	50.0±2.0	0.380

Figure 4.13 Effect of manganese in the medium on the vitality of *L. plantarum* after freezing. The cells were cultivated in the GY media in which trace elements solution did not contain $Ca^{2+}$and Mn^{2+}. The media were supplemented respectively following amount of $MnCl_2$ (%): 0 (as control •), 0.01(□), 0.03 (△), 0.05 (×), 0.08(▲), 0.10 (■), Ca + Mn: 0.08% $CaCl_2$ and 0.05%$MnCl_2$ (○). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.23 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.13.



Mn^{2+}	VI(%)	μ
0%	25.1±2.2	0.151
0.01%	46.6±2.7	0.151
0.03%	31.6±1.6	0.151
0.05%	29.4±2.4	0.151
0.08%	24.0±1.5	0.151
0.10%	23.2±2.3	0.151
Ca+Mn	41.6±2.4	0.151

Figure 4.14 Effect of manganese in the medium on the vitality of *L. brevis* after freezing. The cells were cultivated in the GY media in which trace elements solution did not contain $Ca^{2+}$and Mn^{2+}. The media were supplemented respectively following amount of $MnCl_2$ (%): 0 (as control •), 0.01(□), 0.03 (△), 0.05 (×), 0.08(▲), 0.10 (■), Ca + Mn: 0.08% $CaCl_2$ and 0.05%$MnCl_2$ (○). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.10 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.14.



Mn^{2+}	VI(%)	μ
0%	25.1±2.0	0.064
0.01%	45.7±1.5	0.064
0.03%	45.7±1.6	0.064
0.05%	45.3±1.3	0.064
0.08%	40.4±2.0	0.064
0.10%	34.3±1.8	0.064
Ca+Mn	45.7±2.2	0.064

Figure 4.15 Effect of manganese in the medium on the vitality *of O. oeni* after freezing. The cells were cultivated in the GY media in which trace elements solution did not contain $Ca^{2+}$and Mn^{2+}. The media were supplemented respectively following amount of $MnCl_2$ (%): 0 (as control •), 0.01(□), 0.03 (△), 0.05 (×), 0.08(▲), 0.10 (■), Ca + Mn: 0.08% $CaCl_2$ and 0.05%$MnCl_2$ (○). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.07 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.15.

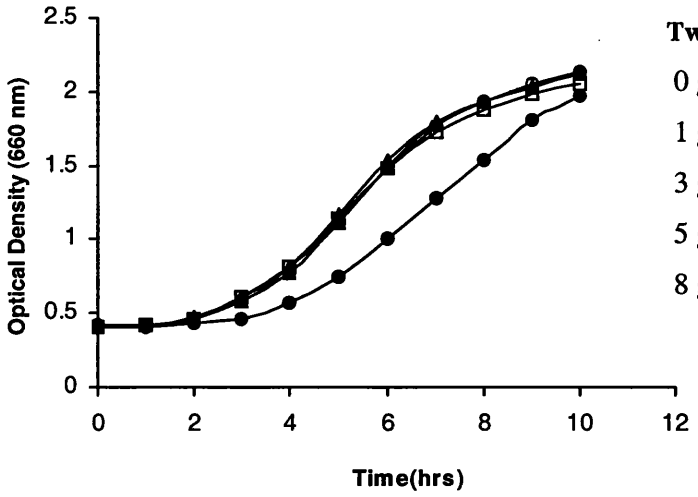
It was also noted that that calcium and manganese had a synergistic effect on *L. plantarum* because the cell vitality of *L. plantarum* was higher (50.0%) when the medium contained both 0.08% $CaCl_2$ and 0.05%$MnCl_2$ than those when the medium only contained 0.08% $CaCl_2$ (33.5%) or 0.05%$MnCl_2$ (38.0%). But this phenomenon was not observed from *L. brevis* and *O. oeni*.

4.4.3 Effect of Tween 80

Tween 80 is a surfactant and a nutrient for some LAB. Potentially it might influence the cell membrane of microoganisms and so affect the survival rate after freezing. Each of three LAB was introduced respectively into the GY medium in which Tween 80 was supplemented: 0, 1, 3, 5, 8 g/l respectively. The cells were cultivated at 25°C. When the LAB cells grew to early stationary phase, samples were taken from the cell cultures and added into 10 ml GY medium. The amount added was carefully controlled (measured by the optical density) to give equal cell concentrations. These cultures were then frozen at -20°C for 48 hours. After treatment the cell vitality was assessed by intercomparison of the cell growth curves.

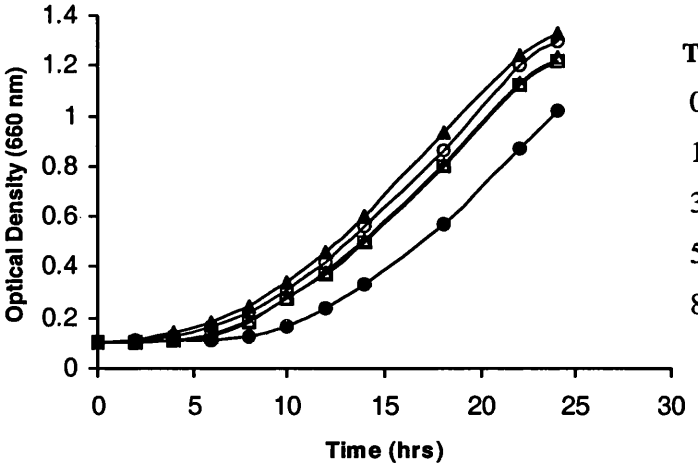
The cell vitality of *L. plantarum*, *L. brevis* and *O. oeni* are shown in Fig.4.16, Fig.4.17 and Fig.4.18. When the cultural media were supplemented with Tween 80, the cell vitality of all three bacteria were enhanced. For instance, the vitality index of *L. plantarum* increased 19.5% when the GY medium was supplemented with 1 g/l Tween 80, compared with the control (non Tween 80 in the medium). But the individual performances of the three bacteria were different. When Tween 80 in the medium was 1 g/l the cell vitality of *L. plantarum* and *O. oeni* were the highest (63.4% and 53.7%). When Tween 80 in the medium was over 1 g/l, the cell vitality of *L. plantarum* and *O. oeni* did not increase. However, the cell vitality of *L. brevis* increased from 67.4% to 81.1% with further addition of Tween 80 to the medium, reaching this maximum at 8 g/l Tween 80.

The results obtained showed that surfactants such as Tween 80 could improve the resistance of LAB to freezing stress, but the optimal amount in the medium depended on the species.



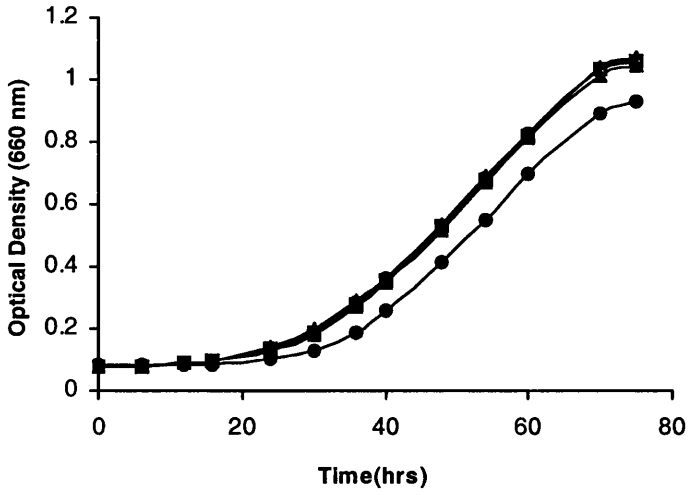
Tween 80	VI(%)	μ
0 g/l	43.9±1.9	0.368
1 g/l	63.4±2.1	0.368
3 g/l	63.4±1.6	0.368
5 g/l	62.5±2.0	0.368
8 g/l	62.5±2.5	0.368

Figure 4.16 Effect of Tween 80 on the vitality of *L. plantarum* after freezing. The cells were cultivated in the GY media that contained respectively following amount of Tween 80 (g/l): 1(□), 3 (△), 5 (×), 8(▲). The cell cultivated in the GY medium without Tween 80 was as control (●). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.41 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the methods, and the raw data is in Appendix-2.16.



Tween 80	VI(%)	μ
0 g/l	47.4±2.2	0.151
1 g/l	67.4±2.0	0.151
3 g/l	70.3±1.6	0.151
5 g/l	75.3±1.7	0.151
8 g/l	81.1±2.1	0.151

Figure 4.17 Effect of Tween 80 on the vitality of *L. brevis* after freezing. The cells were cultivated in the GY media that contained respectively following amount of Tween 80 (g/l): 1(□), 3 (△), 5 (○), 8(▲). The cell cultivated in the GY medium without Tween 80 was as control (●). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.12 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the methods, and the raw data is in Appendix-2.17.



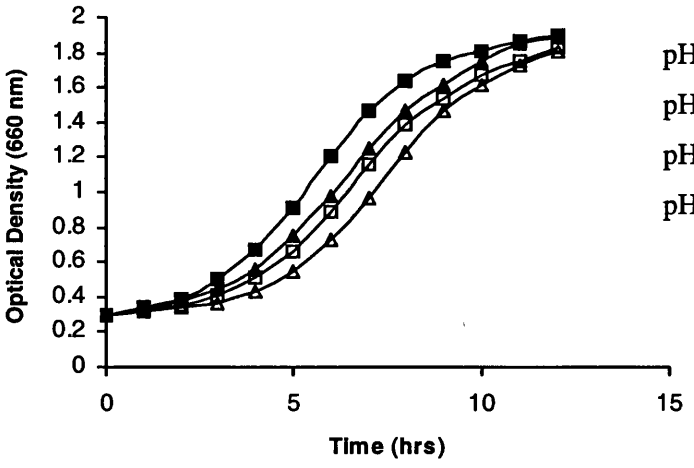
Tween80	VI(%)	μ
0 g/l	42.5±1.5	0.053
1 g/l	53.7±2.2	0.053
3 g/l	53.7±1.6	0.053
5 g/l	53.7±2.3	0.053
8 g/l	53.7±2.0	0.053

Figure 4.18 Effect of Tween 80 on the vitality of *O. oeni* after freezing. The cells were cultivated in the GY media that contained respectively following amount of Tween 80 (g/l): 1(□), 3 (△), 5 (○), 8(▲). The cell cultivated in the GY medium without Tween 80 was as control (●). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.07 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.18.

4.5 Effect of Culture pH

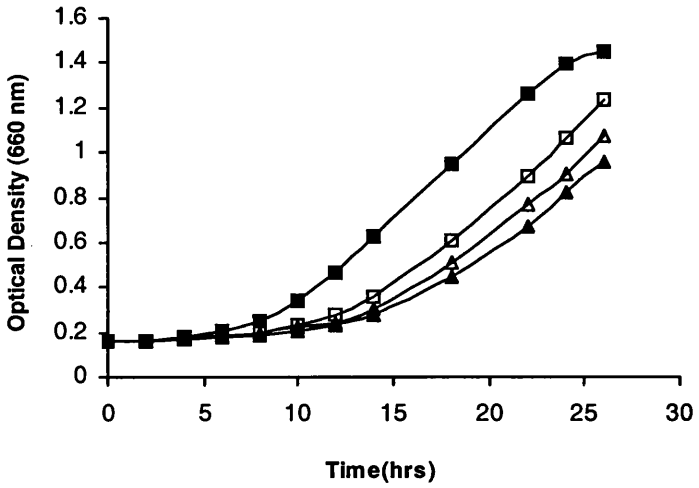
The value of pH is an important factor that affects the growth and physiological status of LAB. To check the effect of culture pH, LAB were cultivated in a 2 L pH-controlled reactor (see Figure.2.2) at 25°C. pH in the culture media was controlled by adding 1M NaOH automatically. The pH was maintained respectively at 4, 5, 6, and 7. The cell growth was monitored by regularly measuring OD of samples at 660 nm. When LAB had grown to the early stationary phase, samples were taken and centrifuged to remove the old media. The cells obtained were re-suspended in GY media. Samples were taken and added into 10 ml GY medium. The amount added was carefully controlled (measured by the optical density) to give equal cell concentrations. These cultures were then frozen at -20°C for 48 hours. After treatment the cell vitality was assessed by intercomparison of the cell growth curves.

Fig.4.19, Fig.4.20 and Fig.4.21 show the effect of culture pH on the vitality of *L. plantarum, L. brevis* and *O. oeni* after freezing. These results show that pH-controlled cultivations significantly affected the cell vitality. *L. plantarum, L. brevis* and *O. oeni* had their highest cell vitality (66.0%, 53.0% and 44.1% respectively) when the pH of culture medium was kept at pH 5. When culture pH was 4 or 6, *L. plantarum* had higher vitality after freezing than that when the culture pH was 7. *L. brevis* had higher vitality when the culture pH was controlled at pH 4 (30.9%) than pH 6 (23.3%) or 7 (26.0%). Because *O. oeni* does not grow in culture at pH 7, this test was not performed. *O. oeni* also showed higher vitality when culture pH was 4 (38.0%) than when culture pH was 6 (30.4%). These results suggest that acidic culture conditions may induce an adaptation to improve the resistance to freezing stress.



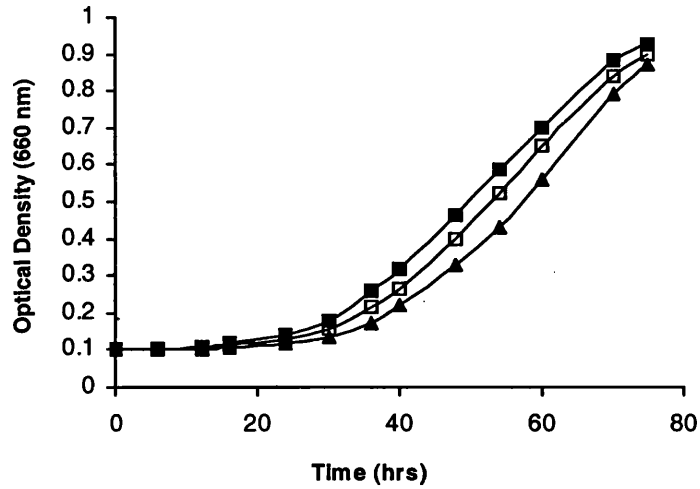
	VI(%)	μ
pH4	49.6±1.7	0.352
pH5	66.0±2.1	0.352
pH6	56.0±2.2	0.352
pH7	39.6±2.5	0.352

Figure 4.19 Effect of culture pH on the vitality of *L. plantarum* after freezing. The cells were cultivated in GY media in a 2L pH-controlled reactor. The culture pH was automatically controlled: pH4 (□), pH 5 (■), pH 6 (▲), and pH 7(△). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.30 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.19.



	VI(%)	μ
pH4	30.9±2.0	0.152
pH5	53.0±1.8	0.152
pH6	23.3±2.2	0.152
pH7	26.0±2.3	0.152

Figure 4.20 Effect of culture pH on the vitality of *L. brevis* after freezing. The cells were cultivated in the GY media in a 2L pH-controlled reactor. The culture pH was automatically controlled: pH4 (□), pH 5 (■), pH 6 (▲), and pH 7(△). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.16 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.20.



	VI(%)	μ
pH4	38.0±2.1	0.050
pH5	44.1±1.9	0.050
pH6	30.4±2.4	0.050

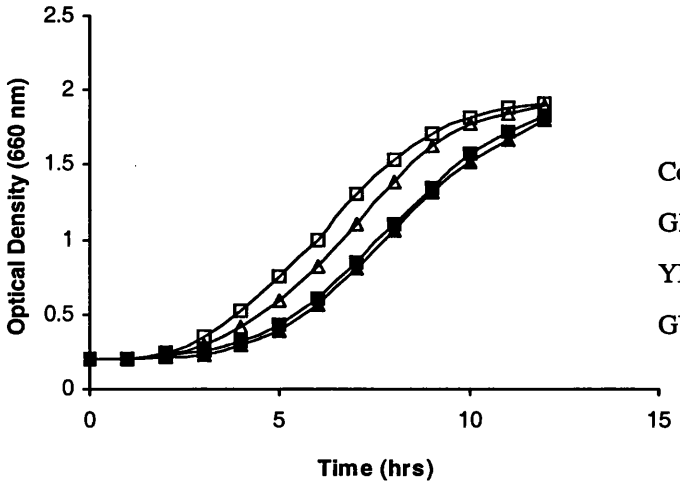
Figure 4.21 Effect of culture pH on the vitality of *O. oeni* after freezing. The cells were cultivated in the GY media in a 2L pH-controlled reactor. The culture pH was automatically controlled: pH4 (□), pH 5 (■), pH 6 (▲). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.10 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.21.

4.6 Effect of Preincubation

4.6.1 Effect of Preincubation in Different Media

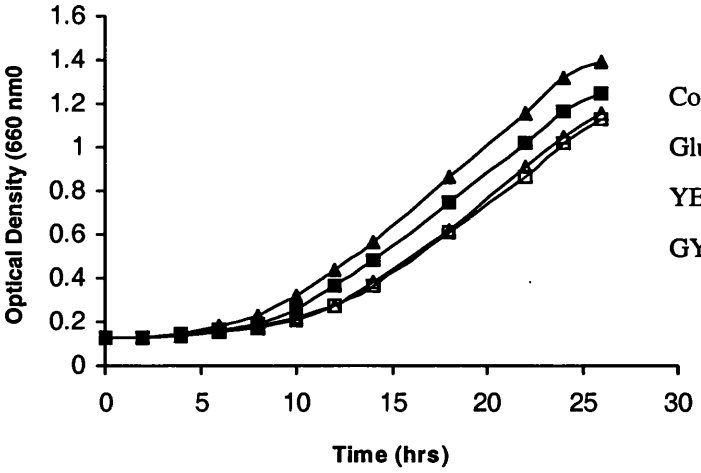
Preincubation is a term used to describe a treatment before freezing. The active LAB cultures were introduced respectively into the GY media, and cultivated at 25°C. When LAB had grown to early stationary phase, 1 ml aliquots of the cultures were placed in 1 ml sterile micro centrifugation tubes. The micro centrifugation tubes were centrifuged (13000 r/min for 8 min) to remove the culture medium. The cell pastes obtained were re-suspended into 1 ml of the following solutions: glucose 5 g/l, yeast extract solution 5 g/l, and GY medium. Preincubation was then carried out: the micro centrifugation tubes were kept at 25°C for 1 hour. When this period had elapsed the micro centrifugation tubes were again centrifuged and the preincubation media were removed. The cell pastes obtained were re-suspended into 1 ml of the GY medium. Samples were taken and added to 10 ml GY medium. The amount added was carefully controlled (measured by the optical density) to give equal cell concentrations. The cell culture without preincubation was the control. These cultures were then frozen at -20°C for 48 hours. After treatment the cell vitality was assessed by intercomparison of the cell growth curves.

The cell vitality of *L. plantarum*, *L. brevis* and *O. oeni* are shown in Fig.4.22, Fig.4.23 and Fig.4.24. The three bacteria had totally different responses to preincubation. For *L. plantarum*, the cell vitality increased after preincubation in all media tested, compared with the control. Especially, when the cells were preincubated in yeast extract solution the cell vitality was enhanced greatly, from 40.1% to 73.5%. In contrast with *L. plantarum*, the cell vitality of *L. brevis* declined after preincubation in all media. The cells of *L. brevis* without preincubation had the highest vitality (48.8%), and preincubation in YE gave the lowest cell vitality (30.2%). The vitality of *O. oeni* was also improved after any preincubation treatment. For example, when the cells were preincubated in 5g/l glucose solution the cell vitality was increased greatly, from 42.0% to 68.4%, compared with the control. But unlike *L. plantarum*, *O. oeni* preincubated in all of the three media had very similar vitality indexes. This results show that the effect of preincubation on the resistance of LAB to freezing stress depends on the species.



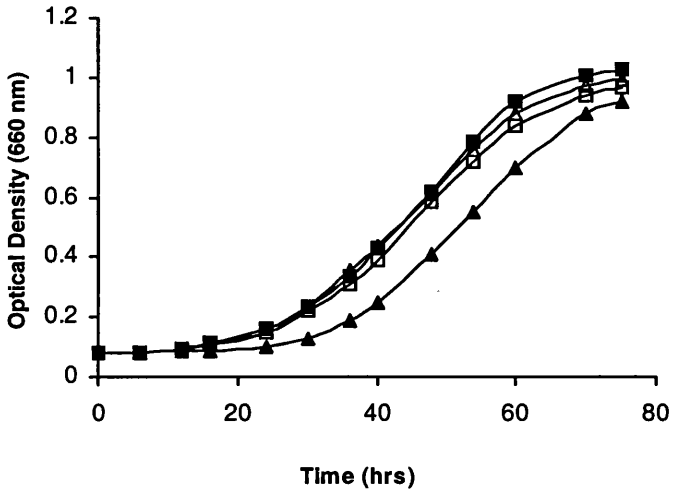
	VI(%)	μ
Control	40.1±2.0	0.384
Glucose	42.5±1.8	0.384
YE	73.5±2.3	0.384
GY	60.0±2.1	0.384

Figure 4.22 Effect of preincubation in different media on the vitality of *L. plantarum* after freezing. The cells were preincubated at 25°C for 1 hour in different media: 5g/l glucose (■), 5g/l yeast extract (□), the GY medium (△). The cells without preincubation were as control (▲). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.20 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.22.



	VI(%)	μ
Control	48.8±2.0	0.176
Glucose	41.6±1.8	0.176
YE	30.2±2.2	0.176
GY	32.0±2.7	0.176

Figure 4.23 Effect of preincubation in different media on the vitality of *L. brevis* after freezing. The cells were preincubated at 25°C for 1 hour in different media: 5g/l glucose (■), 5g/l yeast extract (□), the GY medium (△). The cells without preincubation were as control (▲). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.12 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.23.



	VI(%)	μ
Control	42.0±2.3	0.052
Glucose	68.4±2.2	0.052
YE	64.6±1.7	0.052
GY	68.4±2.1	0.052

Figure 4.24 Effect of preincubation in different media on the vitality of *O. oeni* after freezing. The cells were preincubated at 25°C for 1 hour in different media: 5g/l glucose (■), 5g/l yeast extract (□), the GY medium (△). The cells without preincubation were as control (▲). The samples were frozen at -20°C for about 48 hrs. Initial cell concentration (OD) was 0.12 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-2.24.

Table 3.2 shows the effect of preincubation in different media on viability of *L. plantarum* after freezing (see Chapter 3). Cell viability of *L. plantarum* after freezing was greatly enhanced by preincubating the cells in 5 g/l yeast extract solution at 25°C for 1 hour, compared with the cells without preincubation.

4.6.2 Effect of Preincubation in YE on Cryotolerance of *L. plantarum*

Cryotolerance of *L. plantarum* was improved through a preincubation treatment in yeast extract solution. So studies were carried out to investigate it further.

The cell pastes of *L. plantarum*, obtained as described above, were treated as follows: (1) resuspended in 1, 3, 5, 8 g/l yeast extract solution. The cell suspensions were preincubated at 25°C for 60 min. (2) resuspended in 5 g/l yeast extract solutions. The suspensions were preincubated respectively at 4, 25, 37°C for 30, 60, 120 minutes. When the preincubation was completed, the preincubation medium was removed by centrifugation, and the cell pastes were then re-suspended in the GY medium. Samples were then taken and added into 10 ml GY medium. These cultures were then frozen at –20°C for 48 hours. After treatment the cell viability was analysed by counting the

colony forming units (CFU/ml), and the cell vitality was assessed by intercomparison of the growth curves.

Table 4.1 shows that the effect of YE concentration in preincubation medium on the cell viability and vitality after freezing. The viability of *L. plantarum* increased from 17.3% to 46.0% as the concentration of yeast extract in the broth increased from 1 to 5 g/l. More YE in the preincubation medium was favourable to improve the viability after freezing. However, when the concentration of yeast extract in the medium was 8 g/l, the viability of *L. plantarum* did not increased (46.8%). The vitality indices showed very similar tendencies when compared with the viability, e.g. the vitality indices also increased from 48.5% to 73.5% when the concentration of yeast extract in the medium increased from 1 to 5 g/l.

Table 4.2 shows the effect of preincubation temperature and time on the cell viability and vitality of *L. plantarum* after freezing. It can be seen from Table 4.2 that these parameters also influenced the cell viability and vitality after freezing as well as the concentration of YE. The viability of *L. plantarum* preincubated in yeast extract at 25°C for 60 min was the highest, 46.5%. When the preincubation time was extended to 120 min, the cell survival rate decreased, and viability was 33%. The cell viability was the lowest when preincubated in YE at 37°C for 120 min, 21.1%. Nevertheless, the cell viability preincubated in YE at 4°C and 37°C was also much higher than the control (5.2%), no matter how long the preincubation time was. The similar conclusion was also obtained from the data shown in vitality indices.

When the viabilities shown in Table 3.2, Table 4.1 and Table 4.2 were plotted against the vitality indices, it was found that there was linear relationship between viability and vitality, where $y = 0.822x + 34.902$ (see Figure 4.25). This observation is somewhat problematic as the fuction must go through the origin, zero viability = zero vitality. This plot may indicate that non culturability in the viability assay would still have some vitality. If clumps occur during the viability assay, the viability obtained would be lower value; when there were a few cells such as 60 viable and culturable cells/ml in a culture, the colony forming units might 1 or 2, even 0 after serial dilutions and cultivation. However, the same case would not happen during the vitality measurement. Normally, vitality measurement involves viable-culturable cells and viable-non culturable cells whereas viability measurement only involves the viable-

culturable cells, namely vitality values were higher than viability values. So the results obtained were reasonable.

Table 4.1 Effect of YE concentrations in preincubation medium on the cell viability and vitality of *L. plantarum* after freezing.

YE concentration (g/l)	1	3	5	8
Viability (%)	17.3±2.8	32.1±3.0	46.0±4.8	46.8±4.2
Vitality index (%)	48.5±2.6	62.5±2.2	73.5 ±1.7	73.5 ±2.3

The cells were preincubated at 25°C for 1 hour in yeast extract solutions: 1, 3, 5, 8 g/l. The samples were frozen at –20°C for about 48 hours. The cell viability and vitality were assessed by plate counting (CFU/ml) and intercomparison growth curves. The data of viability shown was average value for three times. The raw data of vitality indices were in Appendix-2.25.

Table 4.2 Effect of preincubation temperature and time on the cell viability (V%) and vitality (VI%)of *L. plantarum* after freezing.

V% (VI%)		Preincubation Time (min)		
		30	60	120
Preincubation Temperature	4°C	25.9±3.0 (54.5±2.4)	33.2±3.8 (65.0±1.3)	32.3±4.5 (62.0±2.2)
	25°C	25.1± 2.2(54.0±1.8)	46.5±4.0 (73.5±2.0)	33.0±4.4 (64.0±1.6)
	37°C	23.6±4.2 (51.5±2.5)	31.1± 2.6(60.0±2.2)	21.1± 2.4(50.0±2.3)

The cells were preincubated in 5 g/l yeast extract solution at 4, 25 and 37°C for 30, 60 and 120 minutes respectively. The samples were frozen at –20°C for about 48 hours. The cell viability and vitality were assessed by plate counting (CFU/ml) and intercomparison of growth curves. The data of viability shown was average value for three times. The raw data of vitality indices were in Appendix-2.26.

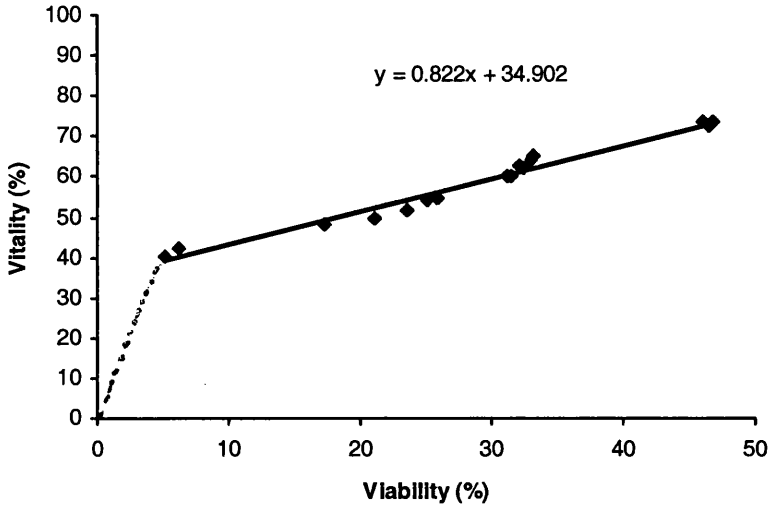


Figure 4.25 the relationship of viability and vitality. The data were from Table 3.2 and Figure 4.22, Table 4.2 and Table 4.3. Note that dashed lines represent possible extrapolation of data

4.6.3 Effect of Preincubation in Amino Acid Solutions on Cryo-Tolerance of *L. plantarum*

The results above have show that preincubation in yeast extract solution can enhance cryotolerance of *L. plantarum* to freezing. The composition of Yeast extract is mainly amino acids. Amino acid uptake and metabolism might play a role in the preincubation. In order to determine which amino acids were involved in this process, preincubations (25°C for 60 min), as described above, were carried out in single amino acid solutions. The concentration of each amino acid used was calculated according to the composition of yeast extract (Bridson, 1995; see Appendix-2.27). When the preincubation period was complete, the preincubation medium was removed by centrifugation, and the cell pastes were re-suspended into the GY medium. Samples were then taken and added into 10 ml GY medium. These cultures were then frozen at -20°C for 48 hours. After treatment the cell viability was analysed by counting the colony forming units (CFU/ml).

Figure 4.26 shows the effect of preincubation in amino acid solutions on the cell viability of *L. plantarum* after freezing. It is found that preincubation in all 13 amino

acid solutions could affect the cryotolerance of *L. plantarum* to freezing, and the cell viability preincubated in yeast extract was much higher than that in any one of the 13 amino acids. These results indicate that many amino acids act in combination during the process. However, not every amino acid had an equivalent effect. Preincubation in leucine, glycine, methionine, isoleucine, serine, and proline solutions showed greater improvement in cryotolerance. Preincubation in glutamic acid solution showed little improvement in cryotolerance, although the concentration of glutamic acid in yeast extract was the highest (13.49%, see Appendix-2.27).

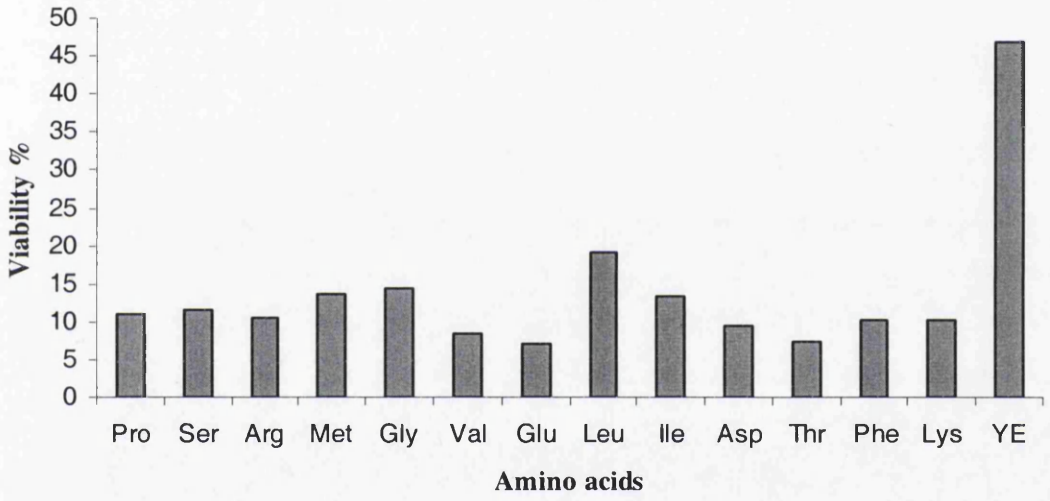


Figure 4.26 Effect of preincubation in amino acid solutions on the cell viability of *L. plantarum* after freezing. The cells were preincuabted at 25°C for 60 min in single amino acid solution (g/l): valine (Val) 0.05, threonine (Thr) 0.14, serine (Ser) 0.17, phenylalanine (Phe) 0.19, methionine (Met) 0.04, lysine (Lys) 0.27, leucine (Leu) 0.3, isoleucine (Ile) 0.24, glycine (Gly) 0.3, glutamic acid (Glu) 0.67, aspartic acid (Asp) 0.35, arginine (Arg) 0.17, proline (Pro) 0.05. The raw data of vabilities is in Appendix-4.28.

4.7 Discussion

The investigations in this work have shown that culture conditions, such as growth phase, composition of medium, pH and preincubation, can have a significant effect on the vitality and cryotolerance of LAB, and the optimum cultural conditions to improve them is species dependent.

In this work, it was found that LAB grown in stationary phases had higher vitality than those grown in exponential phases. Stationary phase is the most common state of bacteria cells in nature (Mandelstam *et al.*, 1982; Sanders *et al.*, 1999). When a microbial culture enters the stationary growth phase, physiological changes allow the cells to survive starvation conditions for extended periods. These physiological changes include: structural modifications of the cell wall providing resistance to autolysis, condensation of the cytoplasm, formation of cellular aggregates and condensation of the nucleoid. During the stationary phase, DNA synthesis continues for some time after net increase in mass has ceased, and many proteins are synthesised (Rensburg *et al.*, 2004). These changes may be favourable to develop multiple stress resistance. The cells in exponential phase are dividing. When a cell is dividing, not only do changes in the membrane exist, but also there are changes of critical molecules and structures that seem unprotected. This situation could cause more damage than normal because the membrane is more sensitive at the time. Sensitivity of the cells to environmental stresses increases during cell division (Graciela *et al.*, 1999; Rodrigo *et al.*, 2003).

Cultural temperature affects the cell composition and physiological state that are important for cell survival after freezing. Lorca and de Valdez (1999) researched the effect of growth temperature and the physiological age of *L. acidophilus* CRL639 on the development of cross-resistance to different kinds of environmental stresses. It was found that the cultures grown at sub-optimal growth temperature such as 25°C were the most resistant to freezing stress. In this study similar results were obtained. They found that *L. acidophilus* CRL 639 grown at 25°C synthesised over-expressed 'temperature specific' proteins, which were favourable for cells to survive during freezing. It has been found that a number of different microorganisms synthesize cold-shock-induced proteins when there was a sudden decrease in temperature (Panoff *et al.*, 1995; Graumann *et al.*, 1996; Kim *et al.*, 1998; Lorca and de Valdez 1998). These proteins were related to enhanced cryotolerance of the microorganisms. Apart from synthesis of proteins in response to cold shock, the proportion of unsaturated fatty acid residues in the membrane increases. Lipids containing unsaturated fatty acids melt at lower temperature than those containing the corresponding saturated acids, which is favourable for cells to resist to freezing. Another effect of temperature on the composition of cells is synthesis of polysaccharides. These processes can be seen as

mechanisms by which organisms protect themselves when exposed to cold temperature (Jeffery and Lin, 1999; Sanders *et al.*, 1999; Thammavongs *et al.*, 2000).

The composition of culture medium also affects the survival of LAB after freezing. In this study it was found that adding more yeast extract (5-8 g/l) in the media could enhance the three LAB cell vitality after freezing. The reason is not very clear. Yeast extract contains many amino acids, vitamins and minerals. If the culture medium contains more yeast extract and relatively less quantity of sugars, the cells might have a composition favourable to resist to freezing stress. Another reason could be that the cells absorb and accumulate amino acids as compatible solutes that protect the cells from the damage of freezing (Da Costa *et al.*, 1998).

Divalent cations have been reported to mediate cell division and separation during cellular growth. Calcium has an important role in the structure of cell walls (Smith 1995; Wright and Klaenhammer, 1983a). Wright and Klaenhammer (1983) researched the effect of calcium on the morphological state of *L. acidophilus RL8K*. They found that the cells grown in a medium containing low levels of calcium showed bleb-like protrusions from the cell wall. It was proposed that these blebs represent membrane evaginations through the cell wall and are thus susceptible targets for structural damage during freezing. Bacteria cells are sensitive to freezing, which usually leads to a slow rate of death. Membrane damage and DNA denaturation are the proposed causes of cell death after freezing (Panoff *et al.*, 1998; Wolfe and Bryant, 1999). When the cells are subjected to freezing stress, calcium might protect the cell wall and membrane and improve the cell vitality. The results in this study supported the hypothesis. Manganese is a cofactor for a few enzymatic reactions (Archibald, 1987). Manganese can stimulate the growth of LAB (Pirt, 1975; Archibald, 1987). In this study, when media were supplemented with manganese, the cell vitality was improved. But the mechanism is unclear and more research is needed. Magnesium, like calcium, is also a divalent cation and there is a high level of magnesium in the trace element solution (see Table 2.1). According to the results obtained, when the media contained calcium the cell vitality was improved, although the media as control contained magnesium. This result suggests that calcium and magnesium have different functions in the cells, although they are both divalent cations. Wright and Klaenhammer (1981) also found that MRS broth, which contains added Mn^{2+} and Mg^{2+} components, required added calcium to support growth of rod type cells of *L. acidophilus* NCFM.

The rod cells were more resistant to freezing and storage at conventional freezer temperatures.

It has been reported that the addition of Tween 80 increased the proportion of unsaturated fatty acids in the membrane and increased survival rate of LAB after freezing (Smittle *et al.*, 1974; Goldberg and Eschar, 1977; Gomez Zavaglia *et al.*, 2000). In this study, similar results were obtained. The enhanced vitality may be related to a modification of the membrane permeability that allowed better survival.

The culture pH of bacteria affects the cell wall composition, the nature of cell surface or envelope materials (Pirt, 1975). Resistance of LAB to freezing is better when the pH is controlled during culture and at a level below the optimal value for growth (Fonseca *et al.*, 2001). Palmfeldt and Hägerdal (2000) researched the influence of culture pH on survival of *Lactobacillus reuteri* subjected to freeze-drying. They found that the highest viability was obtained for cells from pH 5 cultures harvested 2.5 h after entering the stationary phase. The results in this study are in agreement with theirs. O'Sullivan and Condon (1997) observed that pH 5 can trigger a stress response in LAB. This could explain the high vitality observed in the present study for cells grown at pH 5.

Preincubation in YE could enhance the cryotolerance of *L. plantarum* to freezing. The reason was not clear. Yeast extract contains various kinds of amino acids. The cells might take up and accumulate amino acids during the preincubation. Amino acids can be used directly or indirectly as compatible solutes that preserve metabolism, and protect the cells and cell components from the damage of freezing. The uptake mechanism is found in most LAB (Sanders *et al.*, 1999). Another reason could be the synthesis of specific proteins, because exposure to low temperature above 0°C is usually associated with an active response by bacteria, typically synthesis of specific proteins, leading a transient metabolic adaptation (Jones and Inouye, 1994; Sanders *et al.*, 1999). Glaasker *et al* (1996) reported that *L. plantarum* accumulated glutamate as compatible solutes under osmotic stress. However, there was not osmotic stress during preincubation in yeast extract solution. Glutamic acid in yeast extract is the highest, 13.5%, while leucine and glycine in YE are 6.0%. But the cell viability (7.1%) preincubated in glutamic acid was much less than those (19.0% and 14.4%) preincubated in leucine and glycine. According to this, perhaps the enhanced resistance to freezing was mainly for the synthesis of specific proteins during preincubation in

yeast extract and amino acids, and accumulation of amino acids in the cells was minor. The effect of preincubation in YE was also related to the preincubation temperature and time. This may support the hypothesis above because the preincubation temperature and time would influence the synthesis of specific proteins.

Preincubation enhanced the cryotolerance of *O. oeni.* all of media tested provided similar cell vitality after freezing. According to the results, *L. plantarum* and *O. oeni* seem to have different mechanisms of improved cryotolerance when the preincubation were carried out before freezing.

When LAB are freeze-dried for preservation, the cells will also be subjected to freezing stress. In order to obtain higher cell vitality after freeze-drying, the recommended cultural conditions for *L. plantarum, L. brevis* and *O. oeni* are shown in Table 4.3.

Table 4.3 Recommended cultural conditions for *L. plantarum, L. brevis* and *O. oeni*

	L. plantarum	*L. brevis*	*O. oeni*
Medium	Glucose 5 g/l, Yeast extract 8 g/l, $(NH_4)_2SO_4$ 2 g/l, KH_2PO_4 2 g/l, Tween80 1g/l, $CaCl_2$ 0.08%, $MnCl_2$ 0.1%	Glucose 5 g/l, Yeast extract 5 g/l, $(NH_4)_2SO_4$ 2 g/l, KH_2PO_4 2 g/l, Tween80 8 g/l, $CaCl_2$ 0.05%, $MnCl_2$ 0.01%	Glucose 5 g/l, Yeast extract 5 g/l, $(NH_4)_2SO_4$ 2 g/l, KH_2PO_4 2 g/l, Tween 80 1g/l, $CaCl_2$ 0.03%, $MnCl_2$ 0.01%
Cultivation temperature	25°C	25°C	25°C
pH of culture medium	pH 5	pH 5	pH 5
Harvest	Stationary phase	Stationary phase	Stationary phase
Pre-incuation	5 g/l yeast extract solution at 25°C for 1 h	Non preincubation	5 g/l glucose solution at 25°C for 1 h

Chapter 5. Investigation on Freeze-Drying of Lactic Acid Bacteria

5.1 Introduction

Freeze-drying is one of widely used methods to preserve LAB. Freeze-drying is a process in which bacterial cell suspension is first frozen and then subjected to high vacuum. Under these conditions frozen water will sublime (vaporise) and the cells are dried. Freeze-drying is a complex process, and during such a treatment bacterial cells are exposed to freezing and drying processes that subject the cells to the stresses of high concentration of solutes including extremes of pH, to low temperature, to the formation of ice crystals and to the removal of water from within the cell. Freeze-drying might cause cell membrane damage, protein and DNA denaturation, and decreased cell survival (Jay, 1978; Panoff *et al.*, 1998; Wolfe and Bryant, 1999).

In this chapter, the cell suspensions of *L. plantarum*, *L. brevis* and *O. oeni* were freeze-dried. The freeze-drying conditions such as suspension medium, freezing temperature, rehydration, and storage test of the freeze-dried bacteria were investigated to obtain optimal cell vitality.

5.2 Effect of Freeze-Drying Conditions on the Vitality of LAB

The three lactic acid bacteria (*L. plantarum*, *L. brevis* and *O. oeni*) were cultured in GY media at 25°C. When the cells had grown to early stationary phase, the cultures were concentrated 3 times by centrifugation (13000 r/min for 8 min). Before freezing for freeze-drying, the concentrated cultures were placed in sterile serum bottles and mixed with the suspension media that are described below.

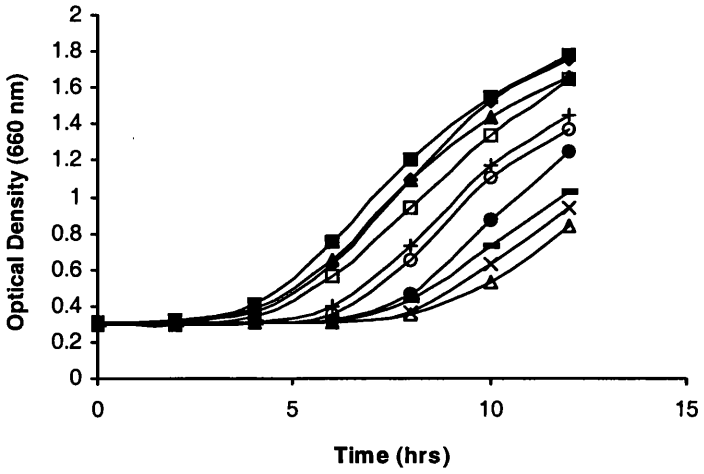
5.2.1 Effect of Suspension Medium

Aliquots of 5 ml of concentrated cell cultures of LAB were mixed in autoclaved serum bottles with 5 ml of the following suspension media: sugars (sucrose, trehalose,

maltose, lactose, glucose), polyols (sorbitol and mannitol), sodium glutamate, yeast extract. These substances were chosen for their known ability to enhance preservation of LAB (Champagne *et al.*, 1991). Different concentrations of suspension media were prepared. As a control, 5 ml of the concentrated culture was mixed with 5 ml of GY medium in an autoclaved serum bottle. These samples were freeze-dried according to methods described in Chapter 2 (Section 2.6). The cell vitality after freeze-drying was evaluated by intercomparison of the cell growth curves as described in Chapter 3 (section 3.4.2).

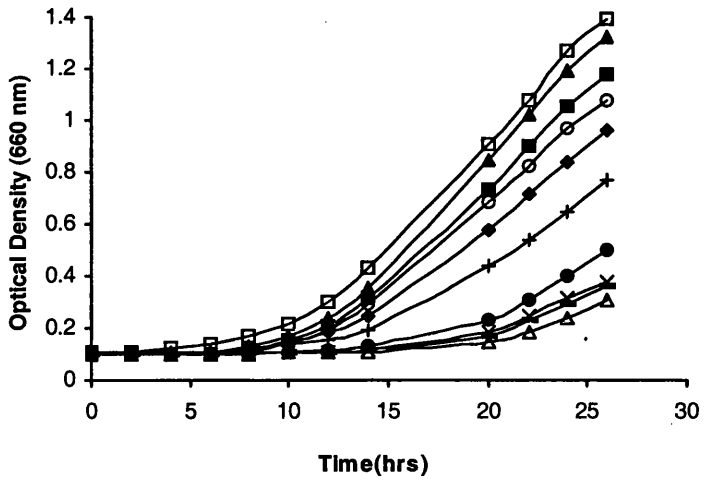
Fig.5.1, Fig.5.2 and Fig.5.3 show that the effect of suspension media on vitality of *L. plantarum*, *L. brevis* and *O. oeni* after freeze-drying. In this experiment, the sugar solutions were 10% (w/v), while polyol solutions and sodium glutamate solution were 5% (w/v), and the yeast extract solution was 4% (w/v). It can be seen from Fig.5.1, Fig.5.2 and Fig.5.3 that *L. plantarum*, *L. brevis* and *O. oeni* showed the lowest cell vitality (9.3%, 9.8% and 4.4% respectively) after freeze-drying when suspension medium was fresh GY medium (as control). It indicated that GY medium gave very poor protection. 10% lactose was found to be the best protectant for *L. plantarum*, giving cell vitality of 44.1%. 4% yeast extract gave *L. brevis* the highest vitality, 61.8%. While the suspension medium was 5% glutamate, *O. oeni* was at its highest vitality index, 60.6%.

Among the sugars tested, disaccharides (sucrose, trehalose, maltose and lactose) had significant protection on the vitality of *L. plantarum*, *L. brevis* and *O. oeni* after freeze-drying. For example, when the suspension media were sucrose, maltose and lactose the vitality indexes of *L. brevis* after freeze-drying were over 45.0%, and the cells with trehalose had vitality index 29.8%, which was much higher than the control (VI, 9.8%). It is also noted that the selection of optimum disaccharide depends on the species of LAB. For instance, among the sugars tested 10% lactose was the best protective agent for *L. plantarum* whereas 10% sucrose gave *O. oeni* a higher vitality than 10% lactose, although these differences are marginal in all cases. It was also found from Fig.5.1, Fig.5.2 and Fig.5.3 that monosaccharide, glucose, gave much lower vitality for the three LAB than disaccharides, but still gave some protection, e.g. *L. plantarum* with 10% glucose had 15.2% vitality index.



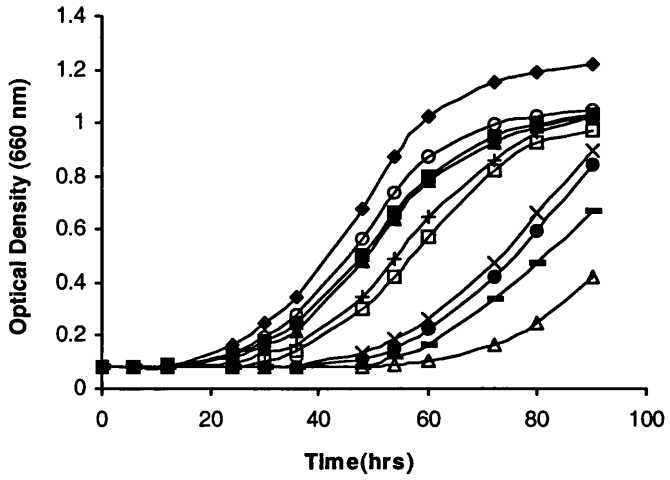
	VI(%)	μ
Control	9.3±3.1	0.335
Trehalose	22.8±2.5	0.335
Maltose	38.1±2.1	0.335
Lactose	44.1±2.4	0.335
Sucrose	21.2±2.6	0.335
Glucose	15.2±2.7	0.335
Glutamate	36.4±2.3	0.335
YE	31.7±2.3	0.335
Sorbitol	11.6±3.3	0.335
Mannitol	13.9±3.1	0.335

Figure 5.1. Effect of suspension medium on the vitality of *L. plantarum* after freeze-drying. Suspension media: 10% trehalose(+), 10% maltose(▲), 10%lactose(■), 10% sucrose(○), glucose(●), 4%yeast extract (□), 5% glutamate (◆), 5% sorbitol(×), 5% mannitol(—). Control (△): the suspension medium was GY medium. Initial cell concentration was 0.30 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.1.



	VI(%)	μ
Control	9.8±3.5	0.143
Trehalose	29.8±3.0	0.143
Maltose	54.2±1.8	0.143
Lactose	47.8±2.0	0.143
Sucrose	45.1±2.3	0.143
Glucose	16.2±3.6	0.143
Glutamate	39.2±2.2	0.143
YE	61.8±1.8	0.143
Sorbitol	12.6±3.2	0.143
Mannitol	11.8±3.5	0.143

Figure 5.2. Effect of suspension medium on the vitality of *L. brevis* after freeze-drying. Suspension media: 10% trehalose(+), 10% maltose(▲), 10% lactose(■), 10% sucrose(○), 10% glucose(●), 4%yeast extract (□), 5% glutamate(◆), 5% sorbitol(×), 5% mannitol(—). Control (△): the suspension medium was GY medium. Initial cell concentration was 0.10 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.2.



	VI(%)	μ
Control	4.4±3.3	0.054
Trehalose	34.0±2.5	0.054
Maltose	42.0±2.1	0.054
Lactose	43.4±2.7	0.054
Sucrose	50.0±2.0	0.054
Glucose	11.1±2.8	0.054
Glutamat	60.6±2.0	0.054
YE	28.9±3.0	0.054
Sorbitol	12.8±3.1	0.054
Mannitol	8.1±3.7	0.054

Figure 5.3 Effect of suspension medium on the vitality of *O. oeni* after freeze-drying. Suspension media: 10% trehalose(+), 10% maltose(▲), 10%lactose(■), 10% sucrose(○), 10% glucose(●), 4%yeast extract (□), 5% glutamate(◆), 5% sorbitol(×), 5% mannitol(—). Control (△): the suspension medium was GY medium. Initial cell concentration was 0.08 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See chapter 3 for detail of the method, and the raw data is in Appendix-3.3.

Comparing with the sugars tested, three LAB with polyols (sorbitol and mannitol) showed much lower cell vitality after freeze-drying. These results indicate that polyols tested offer poorer protection for LAB from freeze-drying than the sugars. The cell vitality with sorbitol and mannitol were slightly higher than that of the control, and it shows that sorbitol and mannitol slightly improved vitality of the LAB after freeze-drying.

With *O. oeni* 5% sodium glutamate gave the highest cell vitality, and was also effective in protection for *L. plantarum* and *L. brevis*. 4%Yeast extract was effective in the protection of all three LAB, especially *L. brevis*, which displayed the highest vitality when the suspension medium was 4% yeast extract.

In order to check the effect of concentration of protectants on the cell vitality of LAB after freeze-drying, the experiments shown in Fig.5.1, Fig.5.2 and Fig.5.3 were repeated but the concentration of the effective protectants were reduced into half. In

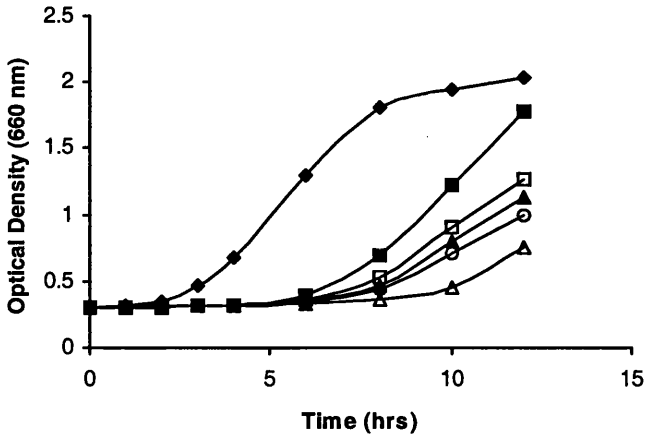
these experiments the effective protectants were selected according to the best results obtained in the experiments shown in Fig.5.1, Fig.5.2 and Fig.5.3. These samples were freeze-dried and the cell vitality was assessed by comparing the growth curves. Thus the vitality of *L. plantarum*, *L. brevis* and *O. oeni* treated with low concentration of protectants are shown in Figure 5.4, Figure 5.5, and Figure 5.6.

Comparing Figure 5.4 with Figure 5.1, only 2.5% sodium glutamate gave a better vitality with *L. plantarum*. The cell vitality with sucrose, maltose, lactose and YE all decreased as their concentration in the suspension medium decreased, although the cell vitality was still higher than the control.

In the case of *L. brevis*, comparing Fig.5.5 with Fig.5.2, the concentration of protectants when reduced to half gave lower vitality than the full strength of the protectants.

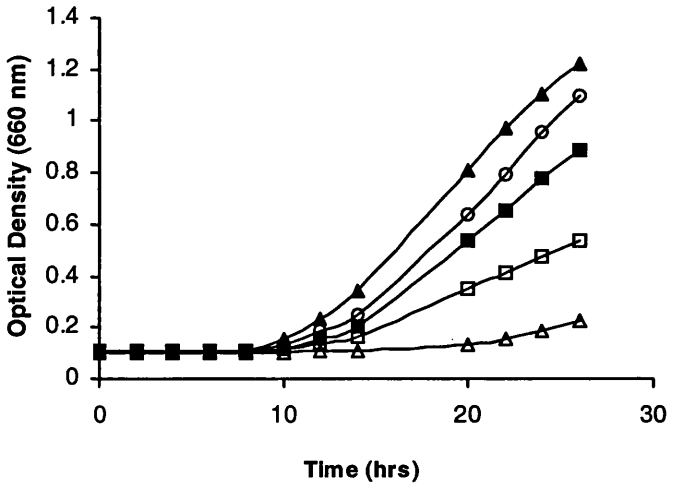
With *O. oeni*, it can be seen from Fig.5.6 and Fig.5.3 that sodium glutamate was still the best protectant for *O. oeni*, even though concentration of protectant was lower.

These results indicate that the protection offered by protectants was related to kinds of protectants as well as their concentrations.



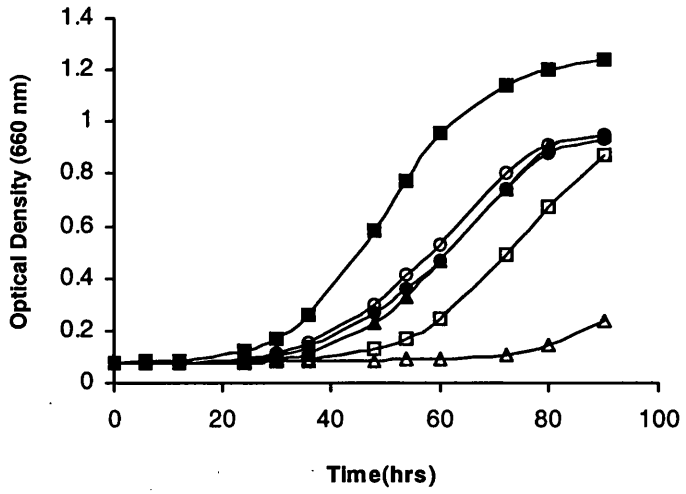
	VI(%)	μ
Control	8.3±4.2	0.335
Maltose	15.2±3.7	0.335
Lactose	28.8±3.5	0.335
Sucrose	13.9±3.7	0.335
Glutamat	65.5±2.2	0.335
YE	16.9±4.0	0.335

Figure 5.4 Effect of suspension medium at low concentration on the vitality of *L. plantarum* after freeze-drying. Suspension media: 5% maltose (▲), 5% lactose (■), 5% sucrose (○), 2.5% glutamate (◆), 2% yeast extract (□). Control (△): the suspension medium was GY medium. Initial cell concentration was 0.30 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.4.



	VI(%)	μ
Control	6.3±4.2	0.154
Maltose	41.4±2.5	0.154
Lactose	28.6±2.7	0.154
Sucrose	32.8±3.3	0.154
YE	23.5±3.6	0.154

Figure 5.5 Effect of suspension medium at low concentration on the vitality of *L. brevis* after freeze-drying. Suspension media: 5% maltose (▲), 5% lactose (■), 5% sucrose (○), 2% yeast extract (□). Control (△): the suspension medium was GY medium. Initial cell concentration was 0.10 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the methods, and the raw data is in Appendix-3.5.



	VI(%)	μ
Control	2.7±4.5	0.054
Maltose	23.9±3.2	0.054
Lactose	28.6±3.3	0.054
Sucrose	29.2±3.8	0.054
Glutamate	48.1±2.7	0.054
YE	13.6±3.7	0.054

Figure 5.6 Effect of suspension medium at low concentration on the vitality of *O. oeni* after freeze-drying. Suspension media: 5% maltose (▲), 5% lactose (●), 5% sucrose (○), 2.5% glutamate (■), 2% yeast extract (□). Control (△): the suspension medium was GY medium. Initial cell concentration was 0.08 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.6.

5.2.2 Effect of Washing the Cells With Phosphate Buffer

LAB can produce many metabolites, mainly lactate, when the cells are cultivated in GY medium containing glucose. These metabolites may influence the cell vitality during freeze-drying.

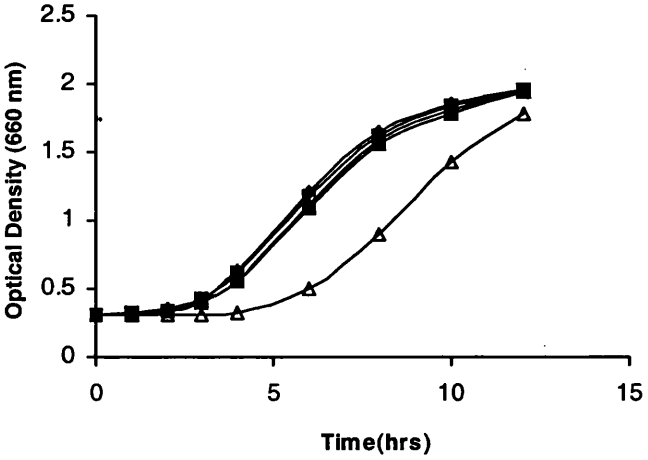
The concentrated LAB cultures were centrifuged, and the cultural media were removed in the sterile airflow cabinet. The cell pastes obtained were washed with 50 mM potassium phosphate buffer solution, the pH of which was either of 4.5, 5, 6 and 7. The buffer solutions were removed by centrifugation after washing. The cultures obtained were resuspended in 10 ml 5% lactose solution in sterile serum bottles. 5 ml of the concentrated culture without washing (as control) was mixed with 5 ml 10% lactose solution in a sterile serum bottle. The samples above were frozen and freeze-dried. The cell vitality of LAB after freeze-drying are shown in Fig.5.7, Fig.5.8 and Fig.5.9 respectively.

It is seen from Fig.5.7 that the cell vitality of *L. plantarum* washed with phosphate buffer was much higher than that as control (no washing), For example, vitality index of the cells without washing was 30.6% while the vitality index of cells washed with pH 5 phosphate buffer was 68.3%. But the cells washed with different pH phosphate buffers had very similar vitality indices.

The results obtained from *L. brevis* were contrary to those of *L. plantarum*. It is found from Fig.5.8 that the cell vitality of *L. brevis* washed with different pH phosphate buffers were lower than that of the control (no washing), and the control had the highest cell vitality (30.5%) after freeze-drying. The cell vitality indexes washed with pH 4.5, 5, 6 and 7 buffer were similar, e.g. the vitality obtained at pH 4.5 and 7 was 20.4% and 17.7% respectively.

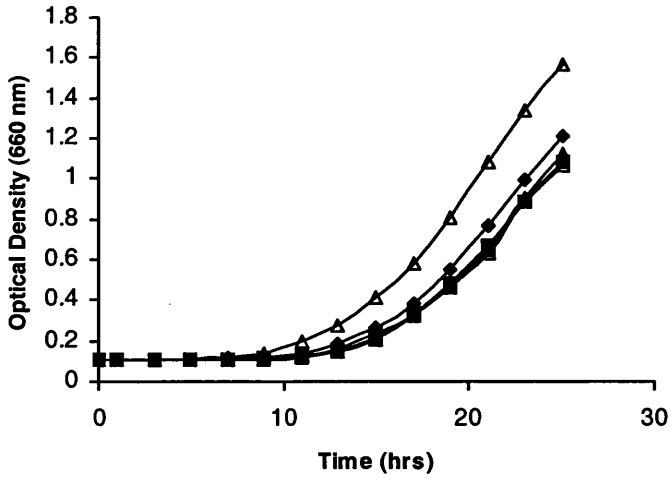
According to Fig.5.9, the results of *O. oeni* were similar with those of *L. brevis*. The control also had the highest cell vitality (31.0%) after freeze-drying. But the difference of cell vitality indices of *O. oeni* washed with pH 4.5, 5, 6 and 7 buffer were significant, and pH 6 of the phosphate buffer gave *O. oeni* the lowest vitality (12.0%).

These results suggest that the effect of washing the cells with phosphate buffer was species dependent.



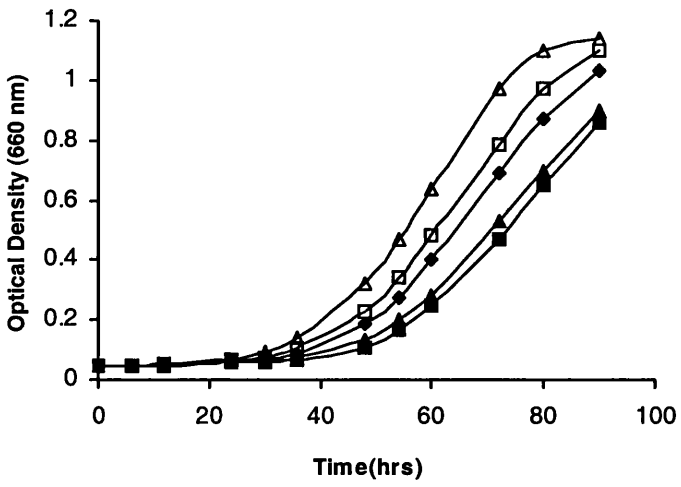
	VI(%)	μ
pH 4.5	68.3±2.4	0.331
pH 5.0	68.3±2.1	0.331
pH 6.0	63.1±2.2	0.331
pH 7.0	63.4±2.0	0.331
Control	30.6±2.7	0.331

Figure 5.7 Effect of washing by phosphate buffer in different pH on the vitality of *L. plantarum* after freeze-drying. pH of phosphate buffer: pH4.5 (◆), pH 5(□), pH 6 (■), pH 7 (▲), Control (△): no washing by phosphate buffer. Initial cell concentration was 0.30 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.7.



	VI(%)	μ
pH 4.5	20.4±2.3	0.177
pH 5.0	18.8±2.0	0.177
pH 6.0	17.7±2.2	0.177
pH 7.0	17.7±2.0	0.177
Control	30.5±1.7	0.177

Figure 5.8 Effect of washing by phosphate buffer in different pH on the vitality of *L. brevis* after freeze-drying. pH of phosphate buffer: pH4.5 (◆), pH 5(□), pH 6 (■), pH 7 (▲), Control (△): no washing by phosphate buffer. Initial cell concentration was 0.11 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.8.



	VI(%)	μ
pH 4.5	19.2±2.7	0.064
pH 5.0	23.0±2.2	0.064
pH 6.0	12.0±3.0	0.064
pH 7.0	13.8±2.4	0.064
Control	31.0±2.3	0.064

Figure 5.9 Effect of washing by phosphate buffer in different pH on the vitality of *O. oeni* after freeze-drying. pH of phosphate buffer: pH 4.5 (◆), pH 5(□), pH 6 (■), pH 7 (▲), Control (△): no washing by phosphate buffer. Initial cell concentration was 0.05 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-5.9.

5.2.3 Effect of pH of Suspension Medium

To examine the effect of pH of suspension medium on cell vitality, the concentrated LAB cultures were centrifuged, and the cultural media were removed in the sterile airflow cabinet. The cell pastes obtained were re-suspended into 10 ml 5% lactose solution in sterile serum bottles, pH of which was 3, 4, 5, 6 and 7 respectively. 5 ml of the concentrated culture without removal of the cultural medium (as control) was mixed with 5 ml 10% lactose solution in a sterile serum bottle. The samples above were frozen and freeze-dried. The cell vitality of LAB after freeze-drying are shown in Fig.5.10, Fig.5.11 and Fig.5.12 respectively.

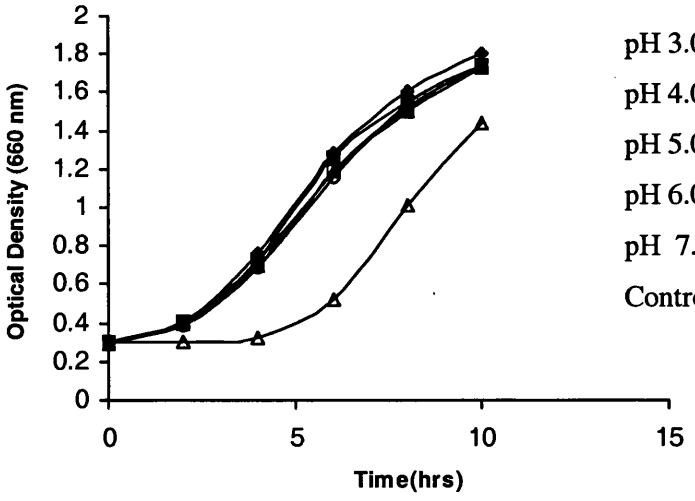
According to Fig.5.10, all of the samples treated had much higher cell vitality than the control (without removal of the cultural medium). But when pH of the suspension media increased from 3 to 7, the cell vitality obtained was very similar. It seems that pH of suspension medium had little effect on the cell vitality of *L. plantarum* after freeze-drying, even though the pH of suspension medium was lower such as pH 3.

Fig.5.11 shows the effect of pH of suspension medium on the cell vitality of *L. brevis*. Comparing to the control, the cell vitality of *L. brevis* decreased when pH of

suspension media was from 3 to 7, and vitality indices were similar. These results also suggest that pH of suspension medium had little effect on the cell vitality of *L. brevis* after freeze-drying.

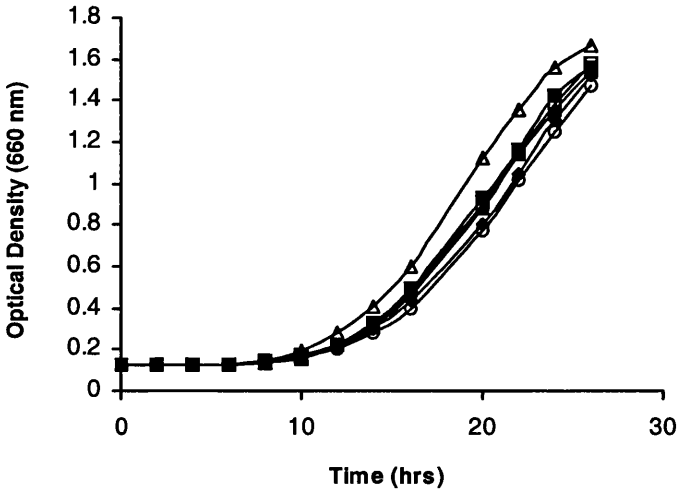
The case of *O. oeni* was similar to that of *L. brevis*. As shown in Fig.5.12, the cell vitality of *O. oeni* decreased when pH of suspension medium was from 3 to 7, compared with the control. The cell vitality was higher when the pH of suspension media was pH 5, 6, and 7 than pH 3 and 4. The cells of *O. oeni* freeze-dried at acidic condition had less survival rate than at neutral condition.

The results above suggest that the growth medium containing metabolites had an important influence on the cell vitality. The effect of old growth medium (metabolites) on the cell vitality, positive or negative, depended on the bacterial species. The pH of suspension medium was not important factor for LAB during freeze-drying.



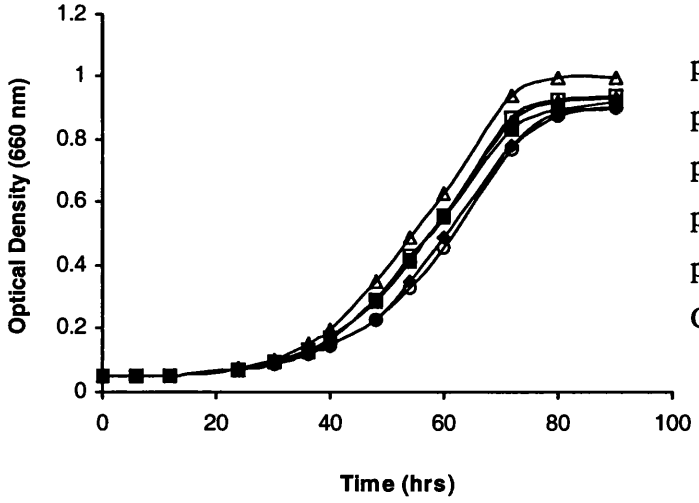
	VI(%)	μ
pH 3.0	74.4±1.6	0.331
pH 4.0	76.2±1.7	0.331
pH 5.0	74.4±2.0	0.331
pH 6.0	75.7±2.2	0.331
pH 7.0	74.4±1.8	0.331
Control	32.6±3.0	0.331

Figure 5.10 Effect of pH of suspension medium on the vitality of *L. plantarum* after freeze-drying. pH of suspension medium: pH3 (○), pH4 (◆), pH 5(□), pH 6 (■), pH 7 (▲), Control (△): without removal of the cultural medium. Initial cell concentration was 0.30 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.10.



	VI(%)	μ
pH 3.0	23.8±2.0	0.175
pH 4.0	24.8±2.2	0.175
pH 5.0	25.4±2.0	0.175
pH 6.0	25.7±2.1	0.175
pH 7.0	25.2±2.3	0.175
Control	31.7±2.0	0.175

Figure 5.11 Effect of pH of suspension medium on the vitality of *L. brevis* after freeze-drying. pH of suspension medium: pH3 (○), pH4 (◆), pH 5(□), pH 6 (■), pH 7 (▲), Control (△): without removal of the cultural medium. Initial cell concentration was 0.11 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.11.



	VI(%)	μ
pH 3.0	24.0±2.6	0.064
pH 4.0	24.0±2.3	0.064
pH 5.0	28.4±2.8	0.064
pH 6.0	28.4±3.0	0.064
pH 7.0	28.4±2.1	0.064
Control	32.8±2.5	0.064

Figure 5.12 Effect of pH of suspension medium on the vitality of *O. oeni* after freeze-drying. pH of suspension medium: pH3 (○), pH4 (△), pH 5(□), pH 6 (■), pH 7 (▲), Control (△): without removal of the cultural medium. Initial cell concentration was 0.05 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.12.

5.2.4 Effect of Preincubation

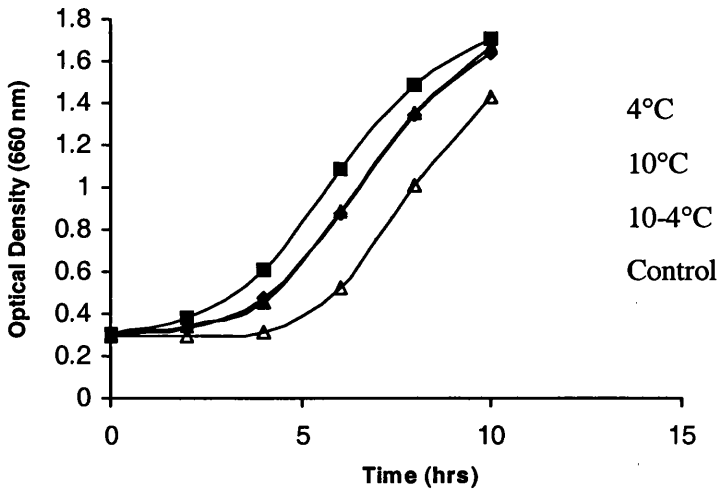
In order to examine the effect of preincubation before freezing, the concentrated LAB culture were mixed with the same volume of 10% lactose solution. 40 ml of such cultures were equally divided into 4 sterile serum bottles. The first one, as control, was directly frozen to –20°C overnight. The second and third were pre-incubated respectively at 10°C and 4°C for 3 hrs before freezing. The fourth was pre-incubated at 10°C for 1.5 hrs and then transferred at 4°C for 1.5 hrs. The samples above were then frozen for about 12 hours and freeze-dried. The cell vitality of *L. plantarum*, *L. brevis* and *O. oeni* after freeze-drying were assessed and shown in Fig.5.13, Fig.5.14 and Fig.5.15 respectively.

Fig. 5.13 shows the effect of preincubation on the cell vitality of *L. plantarum* after freeze-drying. The results showed that preincubation improved significantly the cell vitality of *L. plantarum* after freeze-drying, compared with the sample without preincubation (VI, 30.2%), e.g. the cell vitality index was 48.3% when the cells was preincubated at 4°C for 3 hrs before freezing. When the cells of *L. plantarum* were pre-incubated at 10°C for 3 hrs, the cells displayed the highest cell vitality (62.5%) after freeze-drying.

The situation with *L. brevis* was different from *L. plantarum*. Fig.5.14 shows that the control (non-preincubation) had higher vitality (VI, 33.3%) after freeze-drying than those with preincubation before freezing. Preincubation did not improve the resistance of *L. brevis* to freeze-drying. Surprisingly, when the cells of *L. brevis* were preincubated at 4°C for 3 hrs before freezing, the cells showed lower vitality (21.5%) than the control (33.3%).

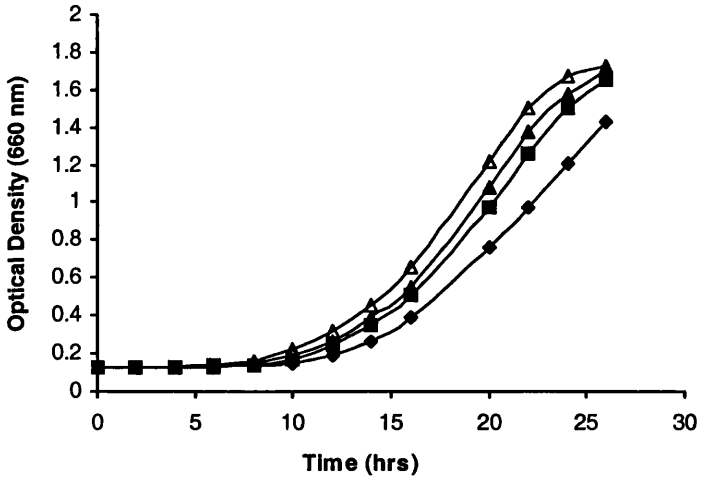
According to Fig.5.15, the vitality of *O. oeni* with and without preincubation were very similar, and preincubation had little effect on the cell vitality, although the cell vitality (VI, 37.2%) cooled at 10°C for 3 hrs before freezing was slightly higher than that of the control (31.0%).

These results show that the effect of preincubation on cell vitality after freeze-drying was related to the species of bacteria used.



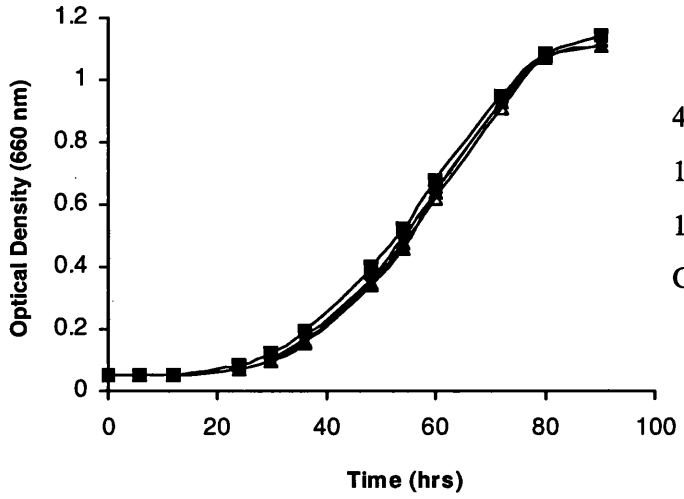
	VI(%)	μ
4°C	48.3±2.7	0.343
10°C	62.5±2.4	0.343
10-4°C	48.3±2.0	0.343
Control	30.2±3.1	0.343

Figure 5.13 Effect of preincubation on the vitality of *L. plantarum* after freeze-drying. 4°C (◆) = preincubation at 4°C for 3 hrs, 10°C (■) = preincubation at 10°C for 3 hrs, 10-4°C (▲)= preincubation at 10°C for 1.5 hrs and transferred at 4°C for 1.5 hrs. Control (△): without preincubation. Initial cell concentration was 0.30 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.13.



	VI(%)	μ
4°C	21.5±2.6	0.178
10°C	26.5±3.0	0.178
10-4°C	28.6±2.2	0.178
Control	33.3±2.7	0.178

Figure 5.14 Effect of preincubation on the vitality of *L. brevis* after freeze-drying. 4°C (◆) = preincubation at 4°C for 3 hrs, 10°C (■) = preincubation at 10°C for 3 hrs, 10-4°C (▲)= preincubation at 10°C for 1.5 hrs and transferred at 4°C for 1.5 hrs. Control (△): without preincubation. Initial cell concentration was 0.13 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.14.



	VI(%)	μ
4°C	32.8±2.2	0.065
10°C	37.2±2.3	0.065
10-4°C	32.0±3.0	0.065
Control	31.0±2.8	0.065

Figure 5.15 Effect of preincubation on the vitality of *O. oeni* after freeze-drying. 4°C (◆) = preincubation at 4°C for 3 hrs, 10°C (■) = preincubation at 10°C for 3 hrs, 10-4°C (▲)= preincubation at 10°C for 1.5 hrs and transferred at 4°C for 1.5 hrs. Control (△): without preincubation. Initial cell concentration was 0.05 when the vitality was assessed. VI: vitality, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.15.

5.2.5 Effect of Initial Cell Concentration

To obtain different initial cell concentration, when LAB cells had grown to early stationary phase, LAB cultures were concentrated by centrifugation, and approximately initial cell concentrations: 7.2×10^7 CFU/ml and 1.5×10^8 CFU/ml were obtained. LAB cultures that were not concentrated were used as the control (3.6×10^7 CFU/ml). 5 ml aliquots of the cultures obtained were mixed with 5 ml 10% lactose solution in sterile serum bottles. The samples above were frozen and freeze-dried. The cell vitality of *L. plantarum*, *L. brevis* and *O. oeni* after freeze-drying were evaluated and shown in Fig.5.16, Fig.5.17 and Fig.5.18 respectively.

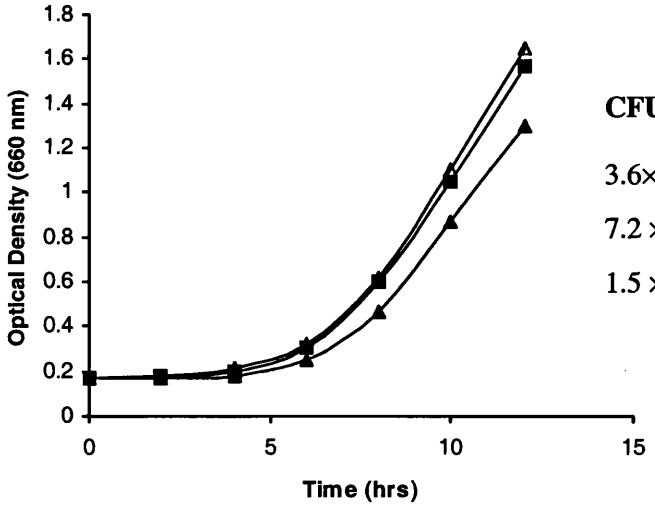
As shown in Fig.5.16, when the initial cell concentration of *L. plantarum* was 7.2×10^7 CFU/ml, the cell vitality (VI, 32.9%) after freeze-drying was similar with that of the control (3.6×10^7 CFU/ml; VI, 34.1%). However, when the initial cell concentration

was 1.5×10^8 CFU/ml, the cell vitality (VI, 26.5%) was obviously lower than the control.

The situation of *L. brevis* was different from *L. plantarum*. Fig.5.17 shows the effect of initial cell concentration on the cell vitality of *L. brevis* after freeze-drying. Comparing with the control (VI, 30.0%), the cell vitality of *L. brevis* increased when the initial cell density was 7.2×10^7 CFU/ml (VI, 45.1%). However, when the initial cell density was 1.5×10^8 CFU/ml, the cell vitality was lower (VI, 40.0%) than that with 7.2×10^7 CFU/ml.

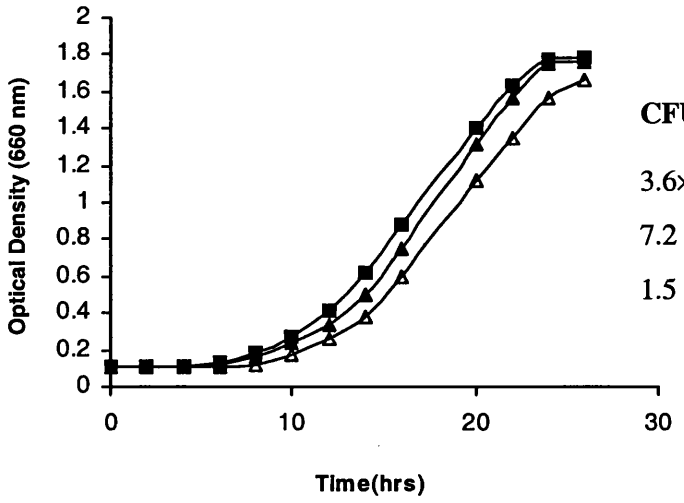
Fig.5.18 shows the effect of initial cell concentration on the cell vitality of *O. oeni* after freeze-drying. When the initial cell concentrations were 7.2×10^7 CFU/ml and 1.5×10^8 CFU/ml, the cell vitality index were the same, 18.0%, and significantly lower than that of the control, 30.4%.

According to these results, the effect of initial cell density on the survival after freeze-drying varied with the species of LAB investigated.



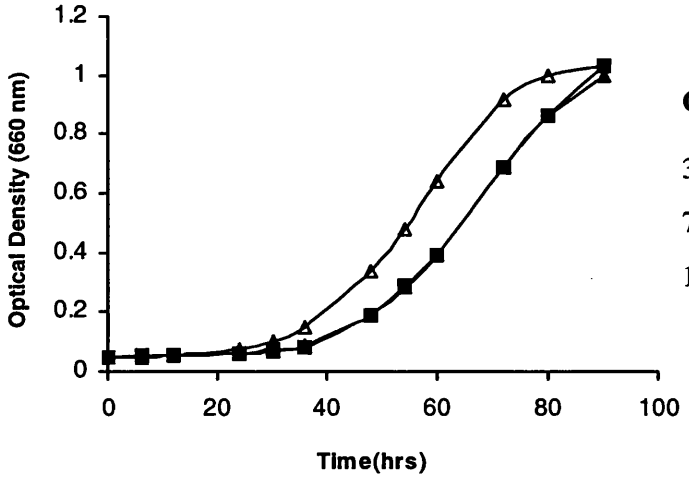
CFU/ml	VI(%)	μ
3.6×10^7	34.1±1.7	0.343
7.2×10^7	32.9±2.0	0.343
1.5×10^8	26.5±2.2	0.343

Figure 5.16 Effect of initial cell concentration on the vitality of *L. plantarum* after freeze-drying. Initial cell concentration: 3.6×10^7 CFU/ml (△, the control), 7.2×10^7 CFU/ml (■), 1.5×10^8 CFU/ml (▲). Initial cell concentration was 0.17 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.16.



CFU/ml	VI(%)	μ
3.6×10^7	30.0±2.3	0.183
7.2×10^7	45.1±1.8	0.183
1.5×10^8	40.0±2.0	0.183

Figure 5.17 Effect of initial cell concentration on the vitality of *L. brevis* after freeze-drying. Initial cell concentration: 3.6×10^7 CFU/ml (△, the control), 7.2×10^7 CFU/ml (■), 1.5×10^8 CFU/ml (▲). Initial cell concentration was 0.11 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.17.



CFU/ml	VI(%)	μ
3.6×10^7	30.4±2.7	0.065
7.2×10^7	18.0±2.3	0.065
1.5×10^8	18.0±2.5	0.065

Figure 5.18 Effect of initial cell concentration on the vitality of *O. oeni* after freeze-drying. Initial cell concentration: 3.6×10^7 CFU/ml (△, the control), 7.2×10^7 CFU/ml (■), 1.5×10^8 CFU/ml (▲). Initial cell concentration was 0.05 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.18.

5.2.6 Effect of Freezing Temperature

Different freezing temperature causes different freezing rate as heat transfer rate is changed. Certainly, the lower the temperature, the faster the freezing rate. Freezing rate influences the size of ice crystals that form intracellularly or extracelluarly, and then in turn may affect the bacterial cell survival during freeze-drying.

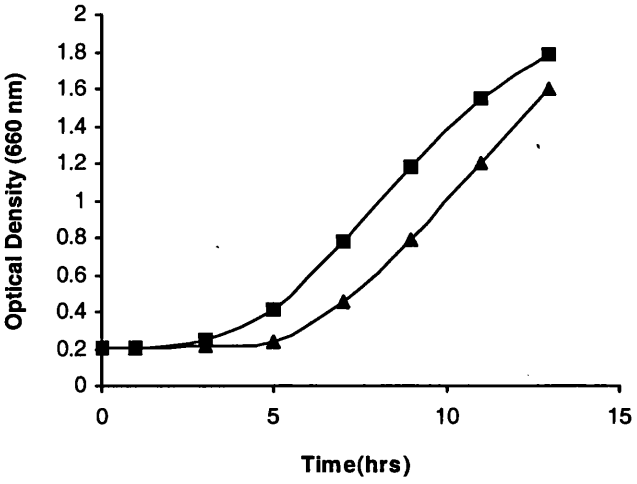
The concentrated LAB cultures were mixed with the same volume of 10% lactose solution. 20 ml of the cultures were equally dispensed into 2 sterile serum bottles. One bottle was directly frozen without shaking at –20°C overnight. Another was frozen fast by shaking it in the mixture of dry ice and methanol (-65°C), and kept it at -20°C overnight. The samples of LAB above were freeze-dried. The cell vitality of *L. plantarum*, *L. brevis* and *O. oeni* after freeze-drying were assessed and shown in Fig.5.19, Fig.5.20 and Fig.5.21 respectively.

According to the result shown in Fig.5.19, when the cells of *L. plantarum* were frozen slowly (-20°C), the cells had a lower vitality (VI, 27.1%) than that (VI, 48.7%) when the cells were frozen fast (-65°C). Fast freezing rate was favourable to obtain higher vitality after freeze-drying.

Fig.5.20 shows the effect of freezing temperature on the cell vitality of *L. brevis* after freeze-drying. The cells of *L. brevis* had a higher vitality (VI, 45.4%) when the cells were frozen quickly (-65°C) than that (VI, 26.4%) frozen slowly (-20°C), which was similar to that of *L. plantarum.*

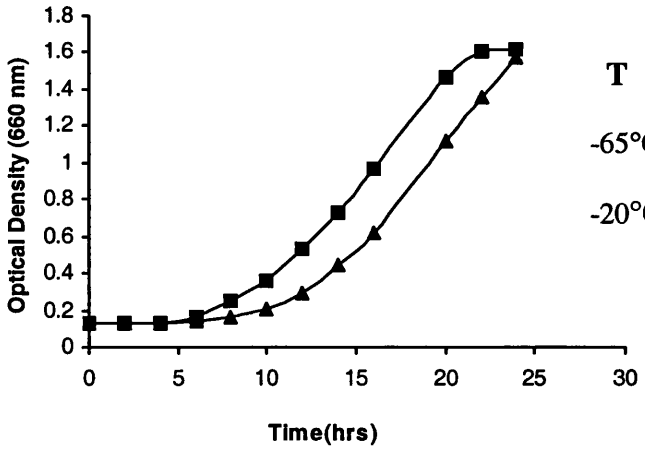
Effect of freezing temperature on the cell vitality of *O. oeni* after freeze-drying is shown in Fig.5.21. The cell vitality of *O. oeni* was much higher when the cells were frozen fast (-65°C) than slowly (-20°C).

These results show that freezing temperature (freezing rate) was an important factor that affects the cell vitality, and under the condition of fast freezing rate LAB obtained higher cell vitality after freeze-drying.



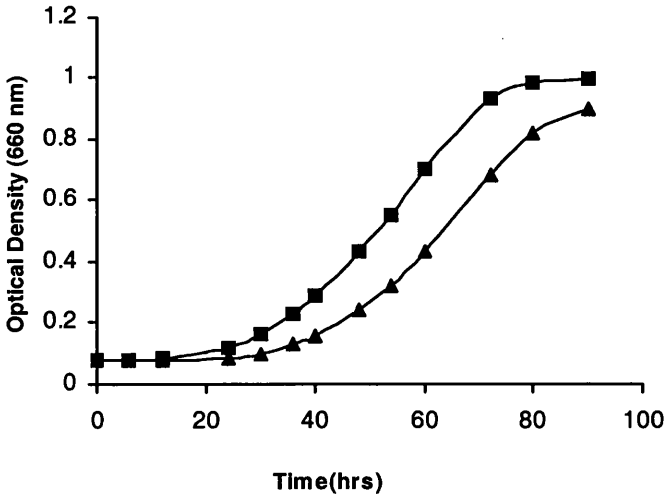
T	VI (%)	μ
-65°C	48.7±2.2	0.343
-20°C	27.1±2.6	0.343

Figure 5.19 Effect of freezing temperature on the vitality of *L. plantarum* after freeze-drying. Frozen temperature: –20°C (▲), –65°C (■). Initial cell concentration was 0.20 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix3.19.



T	VI (%)	μ
-65°C	45.4±2.1	0.148
-20°C	26.4±1.7	0.148

Figure 5.20 Effect of freezing temperature on the vitality of *L. brevis* after freeze-drying. Frozen temperature: –20°C (▲), –65°C (■). Initial cell concentration was 0.11 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.20.



T	VI (%)	μ
-65°C	48.5±2.1	0.050
-20°C	29.5±1.6	0.050

Figure 5.21 Effect of freezing temperature on the vitality of *O. oeni* after freeze-drying. Frozen temperature: –20°C (▲), –65°C (■). Initial cell concentration was 0.08 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.21.

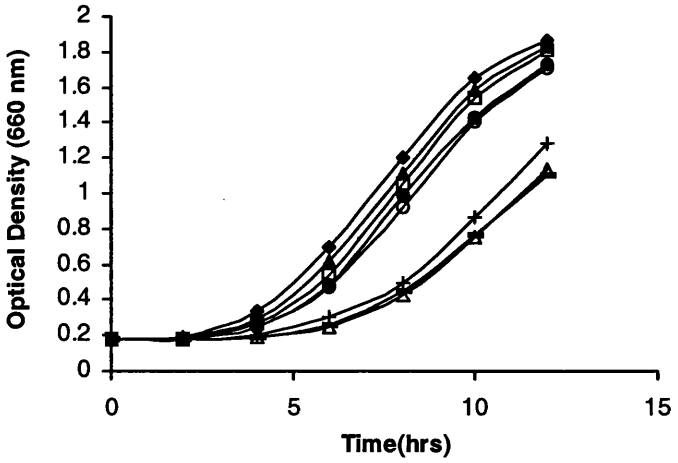
5.3 Effect of Rehydration Conditions

The concentrated LAB cultures were mixed with the same volume of 10% lactose and were freeze-dried. When freeze-drying was completed, the samples were re-hydrated to their original volume (10 ml) at room temperature (about 25°C) with the following solutions: Sucrose (10%), lactose (10%), maltose (10%), glutamate (5 g/l), $CaCl_2$ (2g/l), $MnCl_2$ (2 g/l), GY medium, distilled water (as control). The cell vitality after rehydration was evaluated.

Fig.5.22 shows the cell vitality of *L. plantarum* rehydrated in different media after freeze-drying. Among the rehydration solutions tested, distilled water gave the lowest vitality, 23.8%, whereas rehydration in 10% sucrose showed the highest vitality, 64.4%. 10% lactose and maltose also significantly improve the recovery of *L. plantarum* (VI, 48.8% and 47.2%), compared with that in distilled water. Rehydration in $MnCl_2$ (2g/l) and glutamate (5 g/l) gave higher cell vitality indices (57.7% and 61.6% respectively) and greatly improved the cell recovery. When rehydrated in GY medium, *L. plantarum* showed obviously higher vitality (VI, 30.0%) than the control (distilled water). However, rehydration in $CaCl_2$ (2 g/l) showed slight increase of vitality because its cell vitality index was 26.1%.

Fig.5.23 shows the cell vitality of *L. brevis* re-hydrated in different media after freeze-drying. When the freeze-dried cells of *L. brevis* were rehydrated in the rehydration solutions tested, the cell vitality was improved, compared with that in distilled water. Interestingly, all of the cell vitality except distilled water was similar. This suggests that sugars, minerals and amino acids had similar influences, the type of rehydration medium was not important for the recovery of *L. brevis*.

The cell vitality of *O. oeni* re-hydrated in different media after freeze-drying is shown in Fig.5.24. When the freeze-dried *O. oeni* were rehydrated in GY medium, the cell vitality was the highest (VI, 33.0%) and the cell recovery was improved greatly. GY medium was the best rehydration medium for *O. oeni*. Surprisingly, apart from 10% maltose, rehydration in 10% sucrose, 10% lactose, 5 g/l glutamate, 2 g/l $CaCl_2$, 2 g/l $MnCl_2$ made the cell vitality of *O. oeni* decrease or decrease drastically, compared with rehydration in distilled water (VI, 16.4%), e.g. the cell vitality index was only 5.6% when the freeze-dried cells were rehydrated in 10% lactose solution. These results seem indicate that those rehydration media were detrimental to the recovery of *O. oeni*.



	VI(%)	μ
Sucrose	64.4±2.7	0.330
Lactose	48.8±2.1	0.330
Maltose	47.2±2.4	0.330
Glutamate	61.6±2.6	0.330
$MnCl_2$	57.7±2.0	0.330
$CaCl_2$	26.1±3.0	0.330
Water	23.8±3.3	0.330
GY	30.0±2.2	0.330

Figure 5.22 The cell vitality of *L. plantarum* rehydrated in different media after freeze-drying. Rehydration solutions: 10% sucrose (◆), 10% lactose (●), 10% maltose (○), 5g/l sodium glutamat (▲), 2 g/l $MnCl_2$ (□), 2 g/l $CaCl_2$ (—), distilled water (△), GY medium(+). Initial cell concentration was 0.30 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.22.

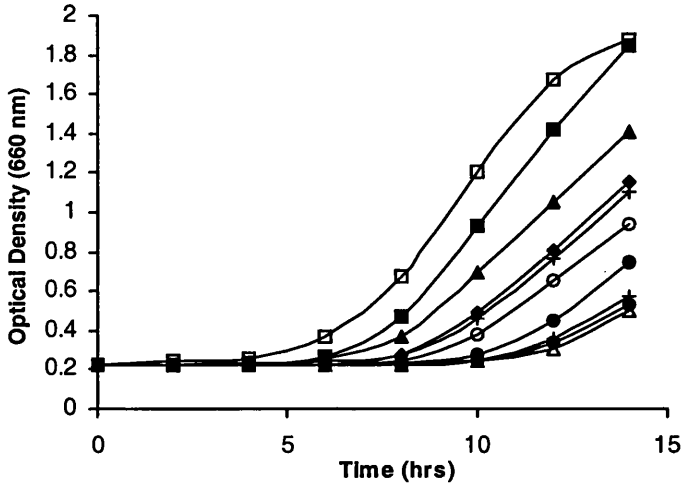
5.4 Storage Tests of Freeze-Dried LAB

In order to examine the effect of storage on the vitality of freeze-dried LAB, the experiments shown in Fig.5.1, Fig.5.2 and Fig.5.3 were repeated. Aliquots 5 ml of the concentrated LAB cultures were mixed respectively in autoclaved serum bottles with 5 ml of the following solutions: 20% sugars (sucrose, trehalose, maltose, lactose, glucose), 10% polyols (sorbitol, mannitol), 10% sodium glutamate, 8% yeast extract. As a control, 5 ml of the concentrated culture was mixed with 5 ml of GY medium. The samples above were frozen and freeze-dried. The freeze-dried samples were sealed under vacuum, and were stored at 4°C for 3 months. After storage, the vitality of *L. plantarum*, *L. brevis* and *O. oeni* were assessed, and the results are shown in Fig.5.25, Fig.5.26 and Fig.5.27 respectively.

Storage has a great influence on the stability of cell vitality of freeze-dried LAB. The effect of storage on the cell vitality of freeze-dried *L. plantarum* is shown in Fig.5.25. It was found that only 4% yeast extract gave *L. plantarum* an almost the same cell vitality after storage at 4°C for 3 months with that immediately after freeze-drying, compared Figure 5.25 with Figure 5.1. Other suspension media showed significantly loss in cell vitality after storage. For instance, the cell vitality with 10% lactose was 22.7% after storage at 4°C for 3 months while the cell vitality was 44.1% immediately after freeze-drying.

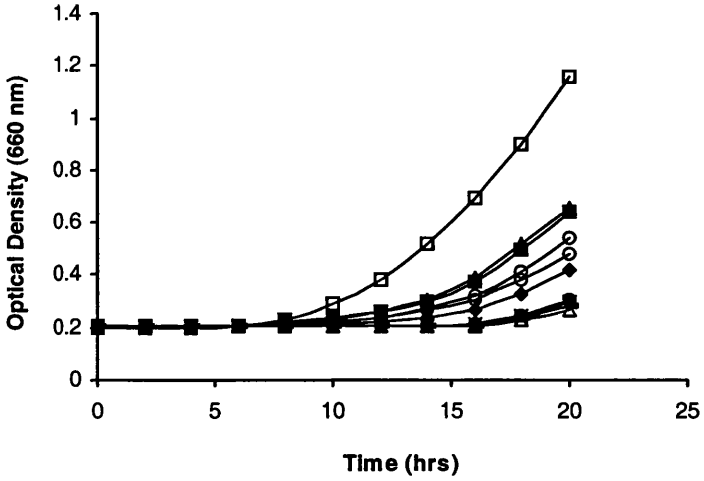
Fig.5.26 shows the growth curves and cell vitality indices of *L. brevis* freeze-dried samples after storage at 4°C for 3 months. Compared with the vitality immediately freeze-drying (see Figure 5.2), the cell vitality of *L. brevis* decreased, depending on the composition of the suspension media. Yeast extract made *L. brevis* keep the highest vitality (VI, 40.5%) after 3 months storage, was still the best protective agent. Maltose, lactose and sucrose also offer some protection. When suspension media were sorbitol, mannitol, glutamate and GY medium, the cell vitality was low after storage, e.g. the cell vitality index with 5% sodium glutamate was 15.3% while the VI was 39.2% immediately after freeze-drying.

According to Figure 5.27, the loss in cell vitality of *O. oeni* also occurred after 3 months storage at 4°C except the cells with 5% sodium glutamate. After storage, the freeze-dried cells of *O. oeni* with 5% sodium glutamate had very similar vitality (VI,



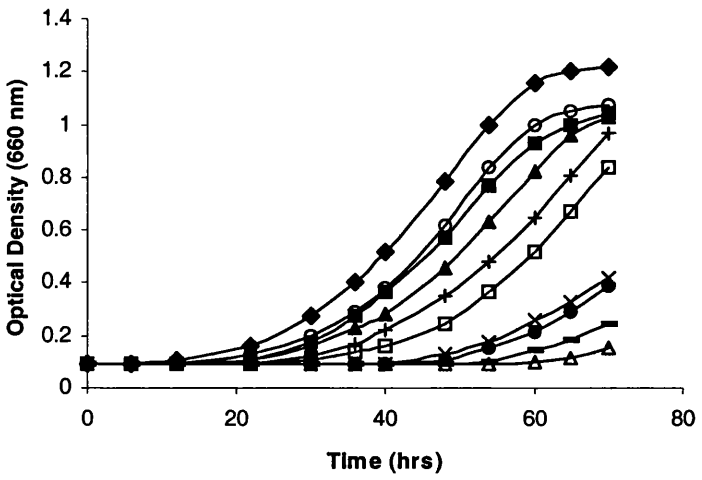
	VI(%)	μ
Control	4.3±4.5	0.331
Trehalose	12.3±3.7	0.331
Maltose	18.2±3.5	0.331
Lactose	22.7±3.3	0.331
Sucrose	10.0±3.6	0.331
Glucose	6.4±4.0	0.331
Glutamat	11.8±3.3	0.331
YE	30.0±2.6	0.331
Sorbitol	4.5±4.0	0.331
Mannitol	5.0±4.2	0.331

Figure 5.25. The cell vitality of freeze-dried *L. plantarum* after storage at 4°C for 3 months. Suspension medium: 10% trehalose (+), 10% maltose (▲), 10% lactose (■), 10%sucrose (o), 4% yeast extract (□), 5% glutamate (◆), 10% glucose (●), 5% sorbitol (×), 5% mannitol (–). Control (△): the suspension medium was GY medium. Initial cell concentration was 0.22 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.25.



	VI(%)	μ
Control	9.6±4.5	0.140
Trehalose	17.0±3.5	0.140
Maltose	24.2±3.2	0.140
Lactose	23.5±3.3	0.140
Sucrose	19.6±3.8	0.140
Glucose	11.0±4.0	0.140
Glutamate	15.3±3.6	0.140
YE	40.5±3.0	0.140
Sorbitol	10.9±4.1	0.140
Mannitol	10.3±4.2	0.140

Figure 5.26 The cell vitality of freeze-dried *L. brevis* after storage at 4°C for 3 months. Suspension medium: 10% trehalose (+), 10% maltose (▲), 10% lactose (■), 10%sucrose (o), 4% yeast extract (□), 5% glutamate (◆), 10% glucose (●), 5% sorbitol (×), 5% mannitol (–). Control (△): the suspension medium was GY medium. Initial cell concentration was 0.20 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.26.



	VI(%)	μ
Control	3.5±5.0	0.057
Trehalose	26.7±4.1	0.057
Maltose	34.6±3.4	0.057
Lactose	39.4±3.6	0.057
Sucrose	42.4±3.3	0.057
Glucose	8.6±4.6	0.057
Glutamate	59.3±2.6	0.057
YE	20.7 ±3.7	0.057
Sorbitol	9.8±4.4	0.057
Mannitol	5.6±4.6	0.057

Figure 5.27 The effect of storage on cell vitality of freeze-dried samples of *O. oeni* after 3 months at 4°C. Suspension medium: 10% trehalose (+), 10% maltose (▲), 10% lactose (■), 10%sucrose (o), 4% yeast extract (□), 5% glutamate (◆), 10% glucose (●), 5% sorbitol (×), 5% mannitol (–). Control (△): the suspension medium was GY medium. Initial cell concentration was 0.09 when the vitality was assessed. VI: vitality index, μ: the specific growth rate. See Chapter 3 for detail of the method, and the raw data is in Appendix-3.27.

59.3%) with that (VI, 60.6, see Figure 5.3) without storage, and sodium glutamate was excellent protective agent for *O. oeni*. Sucrose, lactose and maltose also gave *O. oeni* higher vitality (VI, > 30%). Glucose and sorbitol had a little protection for *O. oeni*. When suspension media were 5% mannitol, the cell vitality of *O. oeni* was the lowest, 5.6%.

5.5 Discussion

The industrial use of LAB as starter cultures for food industries depends on the concentration and preservation technologies employed, which are required to guarantee long-term delivery of stable cultures in terms of viability and functional activity (Carvalho *et al.*, 2003a). Freeze-drying has commonly been used for this purpose, but these techniques bring about undesirable side effects, such as denaturation of sensitive proteins and decreased viability and vitality of many cell types (Leslie *et al.*, 1995). Maximization of survival of LAB cultures during drying and subsequent storage for long periods is of vital importance, both technologically and economically.

Microbial cell survival throughout drying and storage is dependent on many factors, such as suspension medium, frozen temperature, and rehydration conditions (Champagne *et al.*, 1991).

In this work, the best conditions for freeze-drying of the LAB are:

- ***L. plantarum*:** when the cells have grown to early stationary phase the cell cultures are centrifuged and removed the growth medium. The cell pastes are then re-suspended in 4% yeast extract solution. The cell suspension is preincubated at 10°C for 3 hrs before freezing. The cell suspension is then frozen fast at -65°C. The frozen cell suspension is freeze-dried. When the freeze-dried cells are used, the cells are rehydrated in 10% sucrose solution to its original volume at room temperature.
- ***L. brevis*:** when the cells have grown to early stationary phase the cell cultures are concentrated to 2 folds and mixed with 8% yeast extract solution at the same volume. The cell suspension is then frozen fast at -65°C. The frozen cell suspension is freeze-dried. When the freeze-dried cells are used, the cells are rehydrated in 2 g/l $CaCl_2$ solution to its original volume at room temperature.
- ***O. oeni*:** when the cells have grown to early stationary phase the cell cultures are mixed with 10% sodium glutamate solution at the same volume. The cell suspension is then frozen fast at -65°C. The frozen cell suspension is freeze-dried. When the freeze-dried cells are used, the cells are rehydrated in GY medium to its original volume at room temperature.

Suspension Medium

In this work, it was found that 10% lactose, 4% yeast extract and 5% glutamate were the most effective protectants for *L. plantarum*, *L. brevis* and *O. oeni* respectively among the tested suspension media, and protection afforded by a given additive during freeze-drying varied with the species of LAB. The concentration of protectant also affects the protection of LAB during freeze-drying. Even though the protectant such as lactose, yeast extract was added into the suspension medium, loss in cell vitality still occurs. More substances and the combination between different substances should be investigated to get better vitality.

Washing with Phosphate Buffer

The cell vitality of *L. plantarum* washed with phosphate buffer was much higher than the control (no washing), no matter what the pH of phosphate buffers were. However, the results obtained from *L. brevis* and *O. oeni* were contrary to those of *L. plantarum*. The main difference between the cells washed and those without washing was whether the cultural medium had been removed or not. According to the results obtained, it is possible that *L. plantarum* produced some harmful metabolites, most probably sodium lactate during growth (Champagne *et al.*, 1991). These harmful metabolites were removed when washing cells with phosphate buffer. So the cell vitality was greatly improved. The pH of washing buffer had little effect the cell vitality of *L. plantarum*. It has been known that protection afforded by a given additive will vary with the species of LAB (Font de Valdez *et al.*, 1983a; Costa *et al.*, 2000). *L. brevis* and *O. oeni* also produced some metabolites such as sodium lactate. However, in contrast with *L. plantarum*, these metabolites might be not harmful but protective for *L. brevis* and *O. oeni* during freeze-drying. So the control (no washing) had higher cell vitality than those with washing with different pH buffers.

pH of Suspension Medium

The cells of LAB were centrifuged and resuspended in different pH media and then were freeze-dried. All of samples of *L. plantarum* treated had very similar vitality and much higher cell vitality than that of the control. pH of suspension media had little effect on cell vitality, even when the pH of suspension medium was pH 3. As shown in Fig.5.11 and Fig.5.12, comparing with the control, the cell vitality of *L. brevis* and *O. oeni* decreased when pH of suspension media were from 3 to 7. This situation was the same with that obtained above (Washing with phosphate buffer), and seems to support the assumption above, i.e. the metabolites in culture media influence the cell survival during freeze-drying. The effect of pH of suspension medium was minor for the survival of LAB during freeze-drying. Some authors reported that LAB survived better in neutral rather than in acidic medium (Lamprech and Foster, 1963; Champagne *et al.*, 1991). The similar phenomenon was only observed on *O. oeni*.

Preincubation

Preincubation the cells of *L. plantarum* at low temperature (10°C) improved greatly the cell vitality. This result was in agree with that obtained by Panoff *et al* (1994) and Bâati *et al* (2000). They found that cooling the bacterial cells at sup-optimal or low temperature before freezing could make the cells develop an adaptation to freezing stress, and the induction of specific proteins was observed during this process. The synthesis of specific proteins in the cell would increase the resistance to freezing stress. The level of induction depended on the incubation time and temperature. However, the results of *L. brevis* and *O. oeni* were different from those of *L. plantarum*. Preincubation had little or reverse effect on the cell survival of *O. oeni* and *L. brevis* after freeze-drying. The reason might be for different species of LAB. Woojin *et al* (1999) researched that *Lactococcus lactis* subspecies *lactis* and subspecies *cremoris* strains responded to stresses. They found that *L. lactis* subspecies *lactis* strains were shown to acquire freezing resistance by preadaptation at 10°C whereas strains of the subspecies *cremoris* are equally freezing sensitive with or without pre-adaptation.

Initial Cell Concentration

It has been reported that low initial cell concentration was detrimental for freeze-drying survival (Champagne *et al.*, 1991). Kilara *et al* (1976) researched the effect of initial bacterial load on survival after freeze-drying. They found that the cell viability of *L. bulgaricus* with 6×10^5 CFU/ml was much better than that with 7×10^4 CFU/ml. In this work, when LAB grew to early stationary phase, the cell cultures were concentrated rather than diluted. The suspension medium was 5% lactose. It was found that the cell vitality of *L. plantarum* and *O. oeni* decreased as the cell concentration increased. Only *L. brevis* was observed to show the cell vitality increase as the cell concentration increased. Even though, the cell survival with 1.5×10^8 CFU/ml initial concentration was lower than that with 7.2×10^7 CFU/ml. The initial cell concentration of the control (non-concentrated) in this test was about 3.6×10^7CFU/ml. So the initial cell concentration tested was much more than that tested by Kilara *et al* (1976). This maybe explains the reason why the results were different. Palmfeldt *et al* (2003) also researched the effect of initial cell concentration on freeze-drying tolerance of

Pseudomonas chlororaphis. They found that the optimal initial cell concentration was between 1×10^9 and 1×10^{10} CFU/ml when sucrose was used as protective solute, and the sucrose concentration influenced the optimal cell concentration. Costa *et al* (2000) also reported that the effect of initial cell concentration is related to the protective medium used. Bozoglu *et al.* (1987) suggested that the death of microorganisms is proportional to their area of contact with the external medium. The shielding effect of microorganisms with each other could increase the role of sucrose as a protectant. However, *Lactococcus lactis* had a survival of 100% when freeze-dried at an initial cell concentration as high as 2.5×10^{11} CFU/ml (Tsvetkov and Brankova 1983). So optimal initial cell concentration might be species-dependent.

Freezing Temeperature

Since freeze-drying is a change in state from the solid phase to the gaseous phase, the cell suspensions to be freeze-dried must first be adequately pre-frozen. The method of freezing can affect the cell vitality. In this work it was found that when the bacterial cells were frozen quickly (-65°C), three LAB showed increased vitality after freeze-drying. When bacterial cells were frozen fast, ice crystals were uniformly formed inside and outside the cell, which minimized the effect of solutes. However, fast cooling caused more intercellular ice crystals which might cause lethal damage. The optimum rate of freezing varies with one genus to another (Champagne *et al.*, 1991; Sanders *et al.*, 1999)

Rehydration

The results obtained also show that rehydration was an important step because rehydration had a great influence on the cell vitality. Freeze-dried bacteria suspensions usually contain dead cells and unharmed cells as well as those sub-lethally injured. The injured cells may repair and regain normal function if they are rehydated under appropriate conditions (Ray *et al.*, 1971; Font de Valdez *et al.*, 1985a and 1985b; Theunissen *et al.*, 1993). Rehydrations in 10% sucrose, 2 g/l $MnCl_2$ and 5 g/l glutamate greatly improve the vitality of *L. plantarum*, compared with distilled water. But the effect of rehydration in different media depends on the species. Rehydration in 10% sucrose slightly increased the cell vitality of *L. brevis*, not like *L. plantarum*.

Moreover, the cell vitality of *O. oeni* lost obviously when the freeze-dried cell was rehydrated in 10% sucrose. GY medium was the best rehydration medium for *O. oeni* whereas GY medium hardly showed any improvement of the recovery of *L. plantarum*. Surprisingly, when the freeze-dried cells of *O. oeni* were rehydrated in 10% sucrose, 10% lactose, 5 g/l glutamate, 2 g/l $CaCl_2$, 2 g/l $MnCl_2$, cell vitality of *O. oeni* decrease drastically. It might be that there was osmotic pressure shock and *O. oeni* was very sensitive to this shock. Font de Valdez *et al* (1985b) found that diluent medium (1.5% peptone, 1% tryptone and 0.5% meat extract) gave the best recovery for many LAB.

Storage Test of Freeze-Dried LAB

Many compounds have been tested to improve the survival of LAB during freeze-drying. But many compounds offer protection to freeze-drying itself, but do not prevent viability losses during storage (Kilara *et al.*, 1976, Font de Valdez *et al.*, 1983a and 1983b; Champagne *et al.*, 1996). The results of storage tests in this work gave a similar conclusion. 5% glutamate and 10% maltose were effective protectants for *L. plantarum* during freeze-drying. But after 3 months storage it was found that they offer less protections to prevent the loss in cell vitality. 10% lactose was the best protectant for *L. plantarum* during freeze-drying whereas 4% yeast extract became the best protectant after 3 months storage. However, 4% yeast extract and 5% glutamate offered the best protection for *L. brevis* and *O. oeni* after freeze-drying and after storage. So the test of storage after freeze-drying is necessary to decide which protectant is the best.

Chapter 6. Investigation on Ethanol Tolerance of *L. brevis* and *O. oeni*

6.1 Introduction

Winemakers use selected malolactic starter cultures to induce MLF (Davis *et al.*, 1985; Lonvaud-Funel, 1995). This gives better control over the timing and speed of MLF, and its consequent effect on wine aroma and flavour. Many workers have reported the sudden and rapid death of malolactic bacteria when inoculated directly into wines, and an initial levels of 10^6 to 10^8 cells/ml has fallen to a range ≤ 10 to 10^3 cells /ml after inoculation (Beelman, 1982; Liu and Gallander, 1982 and 1983; Nault *et al.*, 1995). Such loss in viability has been linked to the rather hostile environment found in wine, that is low pH, high ethanol and SO_2 concentration, and deficiency in nutrients (Davis *et al.*, 1985; Edwards and Bleeman, 1989). Among these factors, ethanol shows the greatest inhibitory effect on the growth and MLF of *O. oeni*, followed by pH and SO_2 (Britz and Tracey, 1990; Vaillant *et al.*, 1995). Therefore, ethanol tolerance of malolactic bacteria should be considered when malolactic starter is prepared and used. Malolactic bacteria with high ethanol tolerance are critical to induce successfully MLF.

In this chapter, investigations on ethanol tolerance of *L. brevis* and *O. oeni* were carried out, including: (1) The effect of ethanol concentration and temperature on the growth of *L. brevis* and *O. oeni*, (2) The effect of additives on the ethanol tolerance of *L. brevis* and *O. oeni*, (3) The effect of suspension media on the ethanol tolerance of freeze-dried *L. brevis* and *O. oeni*, and (4) The effect of preculture on the ethanol tolerance of freeze-dried *L. brevis* and *O. oeni*. The aim is to define the role and the utilization of LAB cultures in wines. *L. plantarum* is not malolactic bacterium and cannot carry out MLF. So *L. plantarum* was not investigated in this chapter.

6.2 Effect of Ethanol Concentration and Temperature on the Growth of *L. brevis* and *O. oeni*

In order to examine the effect of temperature and ethanol concentration on the growth of *L. brevis* and *O. oeni*, the active cultures of *L. brevis* and *O. oeni* were introduced in

test tubes that contained 10 ml GY media, the ethanol concentration of which was 0, 3, 7, 9, 11, 13 and 15% (v/v) respectively. The cell cultures were cultivated at 15, 20, 25, 30, and 37°C. The cell growth was monitored regularly by measuring OD of the cultures in the test tubes. The cell growth curves were plotted from the data obtained, and the specific growth rates of the bacteria were obtained by the methods described in Chapter 2 (section 2.8.3).

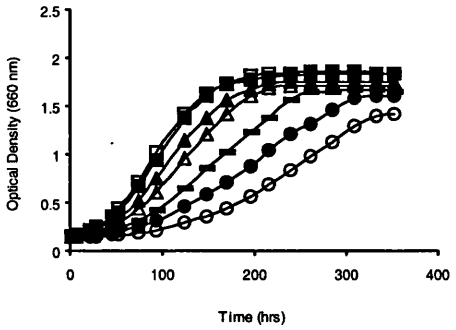
6.2.1 *L. brevis*

Figure 6.1 shows the growth curves of *L. brevis* cultivated at different ethanol concentrations and temperatures. The maximum OD (OD_{max}) values obtained from Figure 6.1 were plotted against the ethanol concentration in the media to obtain Figure 6.2. It can be seen from Figure 6.2 that temperature and ethanol concentration greatly affected the cell growth of *L. brevis*. When the cells of *L. brevis* grew in the medium without ethanol (0%), the maximum cell yields, OD_{max}, was the highest at any of the growth temperatures tested. When ethanol was added into GY medium, the OD_{max} declined as ethanol concentration increased, and the extent of decline varied with growth temperature. When growth temperature was 25°C and above, the declines of OD_{max} were faster than those grown at 20°C and 15°C, especially at 37°C.

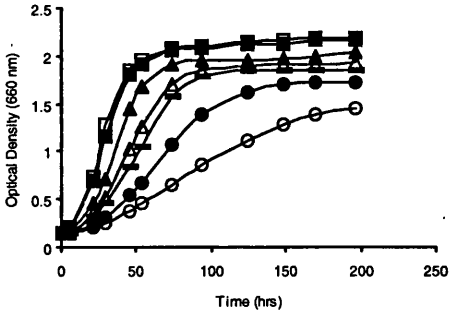
In order to give a clearer picture of the effect of ethanol and temperature on the relative cell growth ratio of *L. brevis*, OD_{max}/OD_{max0} (ratio of OD_{max} in the presence or absence of ethanol) was plotted against ethanol concentration (Figure 6.3). OD_{max}/OD_{max0} was a ratio that could represent the ability of the cells to resist ethanol stress. Higher OD_{max}/OD_{max0} means that the cells have higher growth ability and higher ethanol tolerance. As shown in Figure 6.3, the decline rate of OD_{max}/OD_{max0} became more rapid as growth temperature increased. These results indicated that growth temperature significantly affected the ethanol tolerance, and the cells of *L. brevis* grown at low temperature had relatively higher resistance to ethanol stress.

Figure 6.4 shows the effect of ethanol concentration and growth temperature on the specific growth rate (μ) of *L. brevis*. It was also found that temperature greatly affected the cell growth of *L. brevis*. For example, when the medium did not contain ethanol,

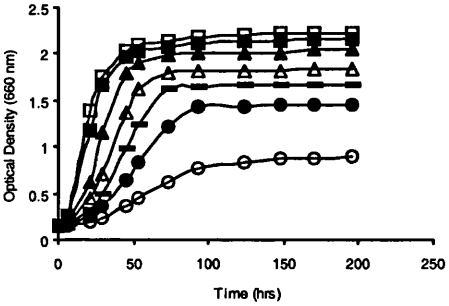
A. cultivated at 15°C



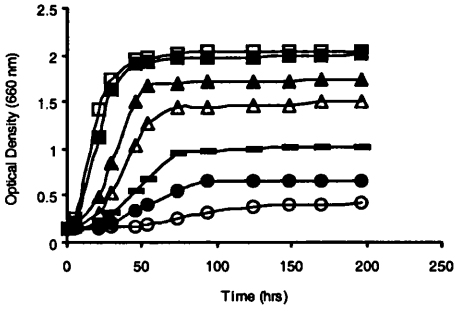
B. cultivated at 20°C



C. cultivated at 25°C



D. cultivated at 30°C



E. cultivated at 37°C

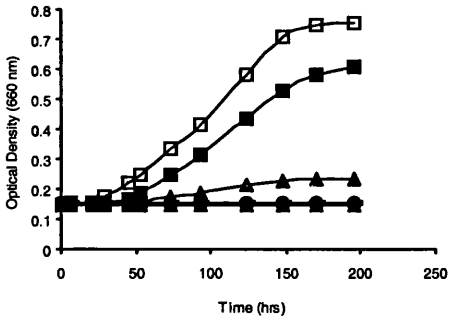


Figure 6.1 The growth curves of *L. brevis* cultivated at different ethanol concentration and temperature. The cells were cultivated at 15 (A), 20 (B), 25 (C), 30 (D), and 37°C (E) in the GY media that contained different ethanol concentrations (v/v): 0% (□), 3% (■), 7% (▲), 9% (□), 11% (—), 13% (●), and 15% (○). The raw data of the cell growth is in Appendix-4.1

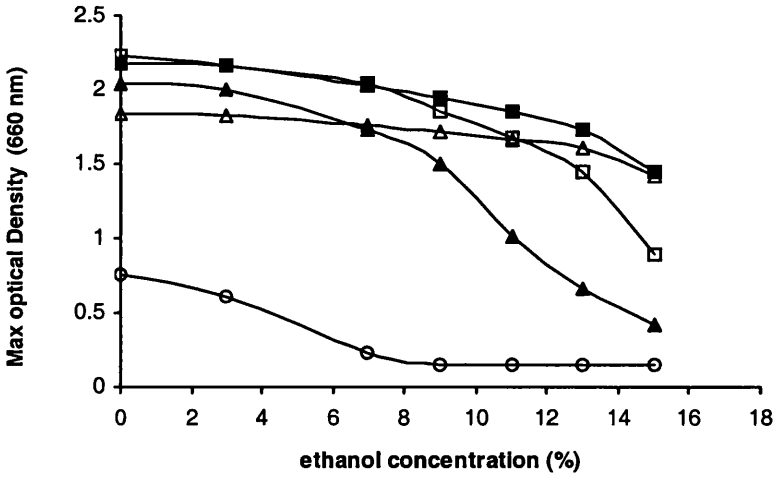


Figure 6.2 Effect of ethanol concentration and growth temperature on the maximum OD (OD_{max}) of *L. brevis*. The cells were cultivated in GY medium that contained the following ethanol concentrations (v/v): 0%, 3%, 7%, 9%, 11%, 13% and 15% at 15 (△), 20 (■), 25(□), 30 (▲), and 37 (o)°C.

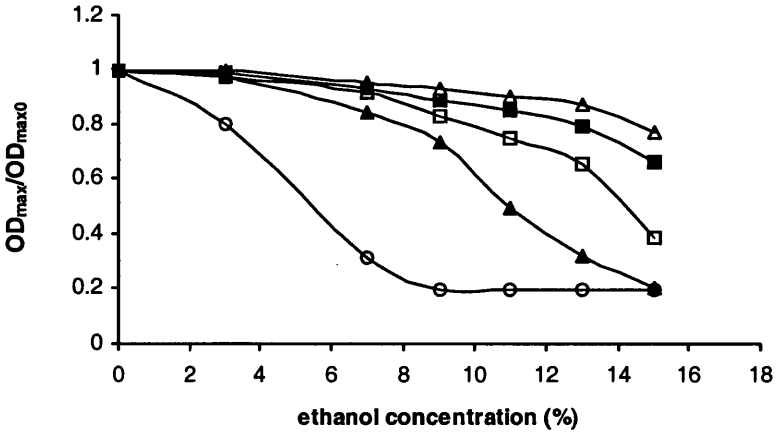


Figure 6.3 OD_{max}/OD_{max0} of *L. brevis* grown at different ethanol concentrations (%) and temperatures. $OD_{max0} = OD_{max}$ grown in the medium without ethanol, OD_{max} = the maximum OD grown in GY medium that contained the following ethanol concentrations (v/v): 3%, 7%, 9%, 11%, 13% and 15%. The cells were cultured at 15 (△), 20 (■), 25(□), 30 (▲), and 37 (○)°C in GY media that contained different ethanol concentrations.

the specific growth rate of cells grown at 15°C was 0.022 h^{-1} whereas the μ was 0.116 h^{-1} when the cells were grown at 30°C. Low temperature greatly inhibited the cell growth. However, when the cells of *L. brevis* were cultivated at 37°C the cell growth was also inhibited severely, and the μ was only 0.014 h^{-1}. As expected, ethanol showed an inhibitory effect on the cell growth, and the specific growth rate declined as ethanol concentration increased in GY media. But growth temperature affected the performance of the cells to ethanol stress. The decline of μ grown at 30°C was significantly faster than those grown at 20°C or 25°C. When ethanol concentration in GY medium was over 7% and growth temperature was 37°C, *L. brevis* did not grow.

When μ/μ_0 (ratio of growth rate in the presence or absence of ethanol) values were plotted against ethanol concentration, Figure 6.5 was then obtained. The μ/μ_0 rate could also represent the ability of the cell to resistant to ethanol stress. The greater μ/μ_0 is, the more resistant the cell is to ethanol stress. Figure 6.5 shows that the higher the growth temperature was, the faster the μ/μ_0 decreased as ethanol concentration increased. When the cells grown at 37°C, the μ/μ_0 declined the fastest, and the μ/μ_0 of the cells grown at 30°C was the second. The cells of *L. brevis* grown at 15°C showed the highest μ/μ_0, and were the most resistant to ethanol stress.

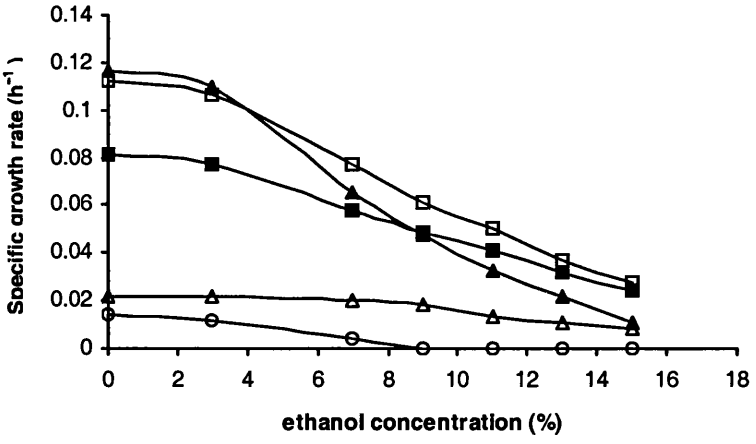


Figure 6.4 Effect of ethanol concentration and growth temperature on the specific growth rate of *L. brevis*. The cells were cultivated in GY medium that contained the following ethanol concentrations (v/v): 0%, 3%, 7%, 9%, 11%, 13% and 15% at 15 (△), 20 (■), 25(□), 30 (▲), and 37 (○)°C.

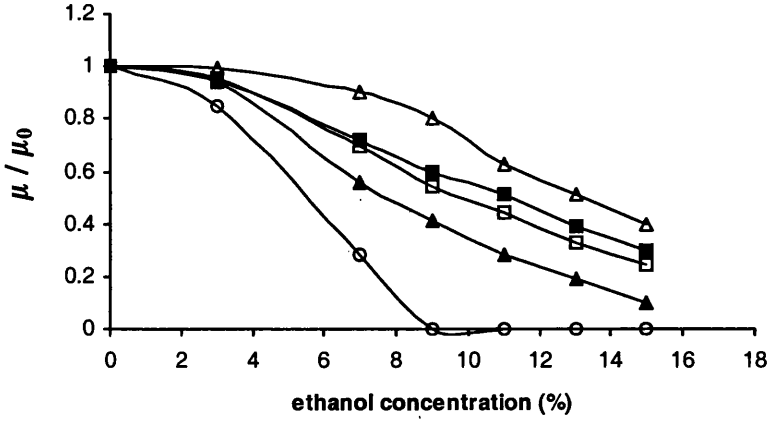


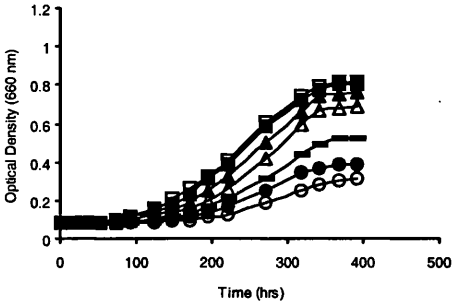
Figure 6.5 The growth ration (μ/μ_0) of *L. brevis* grown at different ethanol concentrations (%) and temperatures. μ_0 = Specific growth rate of the cells grown in GY medium without ethanol, μ = Specific growth rate of the cells grown in GY medium that contained following ethanol concentrations (v/v): 3%, 7%, 9%, 11%, 13% and 15%. The cells were cultured at 15 (△), 20 (■), 25(□), 30 (▲), and 37 (○)°C

6.2.2 *O. oeni*

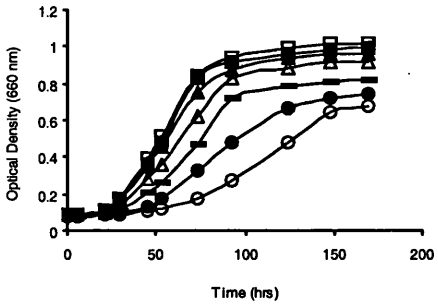
Figure 6.6 shows the growth curves of *O. oeni* cultivated at different ethanol concentrations and temperatures. According to OD_{max} shown in Figure 6.6, Figure 6.7 was then plotted. The results in Figure 6.7 show that growth temperature significantly affects the maximum cell yield (OD_{max}). For example, when the medium did not contain ethanol, cells of *O. oeni* grown at 37°C had 0.73 OD_{max} whereas the OD_{max} of the cells grown at 25°C was 1.087. As expected, ethanol also showed an inhibitory effect on the cell growth, and the OD_{max} declined as ethanol concentration in the media increased, and the extent of decline varied with growth temperature, which was similar with *L. brevis*. Figure 6.7 also clearly shows that the OD_{max} was almost constant when the ethanol concentration in the media was below 7%, whatever the growth temperature. When ethanol concentration was over 7%, the OD_{max} decline grown at 30°C was significantly more rapid than those cultures grown at 20 °C and 25°C.

When OD_{max}/OD_{max0} values of *O. oeni* were plotted against ethanol concentration (see Figure 6.8), it was found that OD_{max}/OD_{max0} was almost constant when ethanol

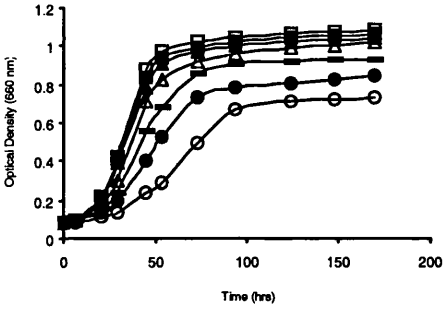
A. cultivated at 15°C



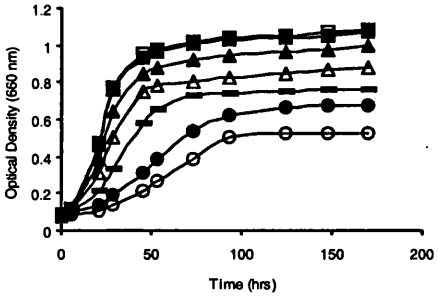
B. cultivated at 20°C



C. cultivated at 25°C



D. cultivated at 30°C



E. cultivated at 37°C

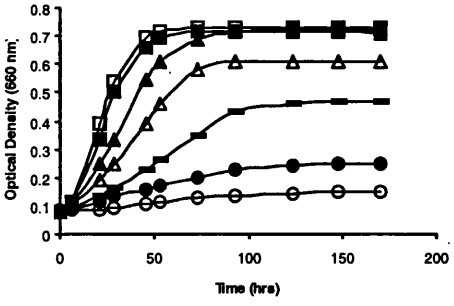


Figure 6.6 The growth curves of *O. oeni* cultivated at different ethanol concentrations and temperatures. The cells were cultivated at 15 (A), 20 (B), 25 (C), 30 (D), and 37°C (E) in the GY media that contained different ethanol concentrations (v/v): 0% (□), 3% (■), 7% (▲), 9% (□), 11% (—), 13% (●), and 15% (○). The raw data of the cell growth is in Appendix-4.2

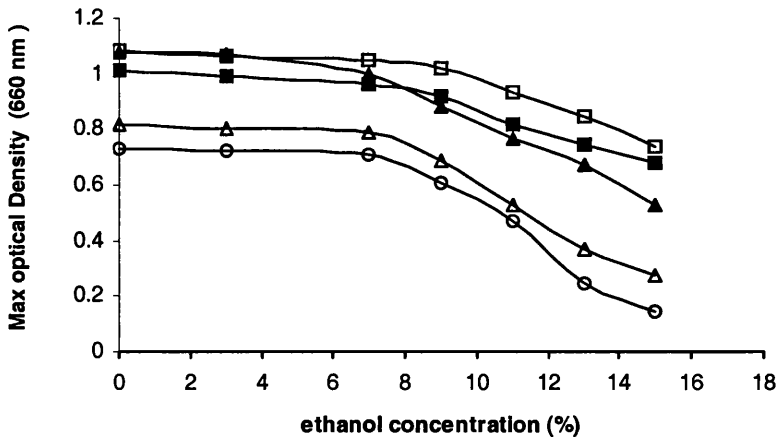


Figure 6.7 Effect of ethanol concentration and growth temperature on the maximum OD (OD_{max}) of *O. oeni*. The cells were cultivated in GY medium that contained following ethanol concentrations (v/v): 0%, 3%, 7%, 9%, 11%, 13% and 15% at 15 (△), 20 (■), 25(□), 30 (▲), and 37 (○)°C.

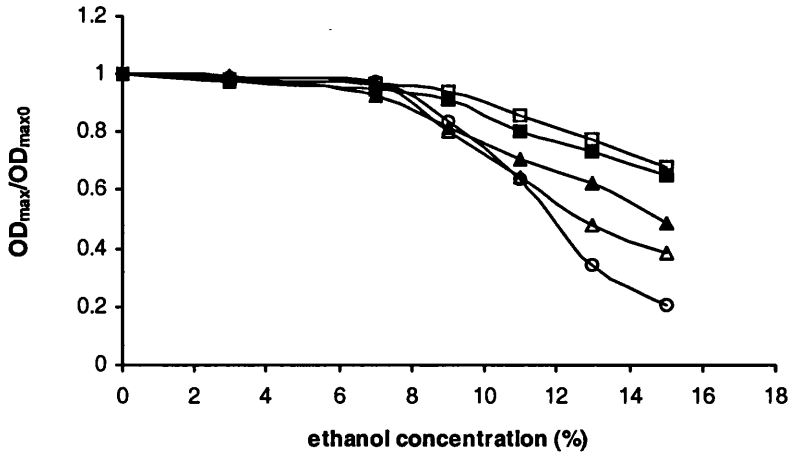


Figure 6.8 OD_{max}/OD_{max0} of *O. oeni* grown at different ethanol concentrations (%) and temperatures. $OD_{max0} = OD_{max}$ grown in the medium without ethanol, OD_{max} = the maximum OD grown in GY medium that contained following ethanol concentration (v/v): 3%, 7%, 9%, 11%, 13% and 15%. The cells were cultured at 15 (△), 20 (■), 25(□), 30 (▲), and 37 (○)°C in GY media that contained different ethanol concentrations.

concentration in the medium was below 7%, and OD_{max}/OD_{max0} declined as more ethanol was added into the medium, varying with growth temperature. When the cells of *O. oeni* were grown at 25°C, the OD_{max}/OD_{max0} declined the slowest, which means the cells were the most resistant to ethanol stress.

The effect of ethanol concentration and growth temperature on the specific growth rate of *O. oeni* is shown in Figure 6.9. Under conditions where the medium did not contain ethanol, the specific growth rate (μ) of *O. oeni* was 0.010 h^{-1} when the cell growth temperature was 15°C whereas the μ was 0.058 $h^{-1}$when the cells grew at 25°C. Low temperatures greatly inhibited the cell growth of *O. oeni*. The cells grown at 30°C showed the highest μ (0.081 h^{-1}), and under the same growth conditions *L. brevis* grew faster ($\mu \approx 0.117\ h^{-1}$) than *O. oeni*. When ethanol was added into GY medium the cell growth was inhibited, as a function of ethanol concentration and growth temperature. When growth temperature was over 30°C, the μ of *O. oeni* declined rapidly. Below optimal growth temperature, the μ of *O. oeni* declined more slowly. The μ of *O. oeni* was unaffected when ethanol concentration in the medium was below 9%. This was different compared with *L. brevis* (Figure 6.4), and *O. oeni* was significantly more tolerant to ethanol stress at the same growth temperature.

When growth rate ratio (μ/μ_0) was plotted against ethanol concentration in the medium (see Figure 6.10), it can be seen that growth temperature significantly influenced the decline of growth rate ratio of *O. oeni*. When the cells grew at 37°C, the μ/μ_0 declined the fastest as ethanol concentration increased, and the cells grown at 30°C were the second. The cells of *O. oeni* grown at 25°C had the least decline in μ/μ_0, and they were the most resistant to ethanol stress. The cells grown at 15°C and 20°C had similar decline in μ/μ_0, and also had higher ethanol tolerance.

The results above indicated that the effect of ethanol on the cell growth of malolactic bacteria not only depend on ethanol concentration, but also was affected by growth temperature and species of bacteria.

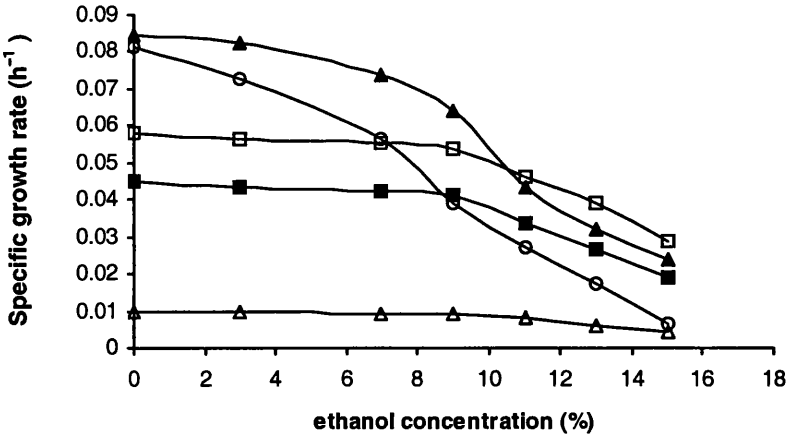


Figure 6.9 Effect of ethanol concentration and growth temperature on the specific growth rate of *O. oeni*. The cells were cultivated in GY medium that contained following ethanol concentrations (v/v): 0%, 3%, 7%, 9%, 11%, 13% and 15% at 15 (△), 20 (■), 25(□), 30 (▲), and 37 (○)°C.

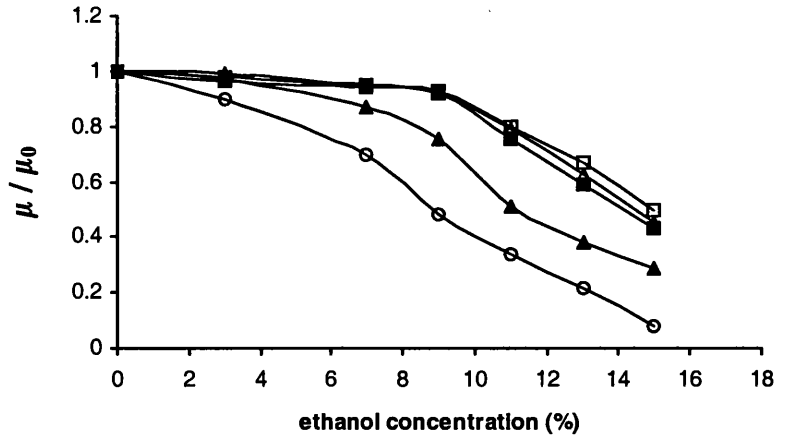


Figure 6.10 The growth rate ration (μ/μ_0) of *O. oeni* grown at different ethanol concentrations (%) and temperatures. μ_0 = Specific growth rate of the cells grown in GY medium without ethanol, μ = Specific growth rate of the cells grown in GY medium that contained following ethanol concentrations (v/v): 3%, 7%, 9%, 11%, 13% and 15%. The cells were cultured at 15 (△), 20 (■), 25(□), 30 (▲), and 37 (○)°C

6.3 Effect of Additives on Ethanol Tolerance of *L. brevis* and *O. oeni*

Ethanol in wine inhibits the malolactic bacteria growth, and therefore affects MLF (Davis *et al.*, 1985; Maicas *et al.*, 1999a and 1999b). Components of wine may affect the cell growth and ethanol tolerance of malolactic bacteria.

In this experiment, a synthetic wine was used: 4 g/l yeast extract, 2 g/l glucose, 1 g/l fructose, 3 g/l malic acid, 10% (v/v) ethanol and 20 ml/l trace elements solution. The pH of medium was adjusted by 1M NaOH to pH 4.0. The following additives were supplemented in the synthetic wine respectively: pyruvic acid 1 g/l, citric acid 1 g/l, and glycerol 1 g/l, gallic acid 100 mg/l, ferulic acid 100 mg/l. The active cultures of *L. brevis* and *O. oeni* were introduced in the synthetic wine, and then cultivated at 25°C. The synthetic wine that had none of any additives above was used as the control. The cell growth was monitored by measuring OD of the test tubes at 660 nm.

6.3.1 *L. brevis*

The growth curves of *L. brevis* in the synthetic wine are shown in Figure 6.11. It can be seen that the cells of *L. brevis* grew much faster than the control when the synthetic wines were added pyruvic acid or citric acid. The cell growth rate with ferulic acid was also slightly faster than that of the control. These results indicated pyruvic acid, citric acid and ferulic acid could stimulate the cell growth in the synthetic wine, and enhanced the ethanol tolerance of *L. brevis*. When gallic acid and glycerol was added into the synthetic wine, the cells of *L. brevis* showed similar growth rates to the control, which means that gallic acid and glycerol did not enhance the resistance of *L. brevis* to ethanol stress.

6.3.2 *O. oeni*

Figure 6.12 shows the growth curves of *O. oeni* in the synthetic wines at 25°C. When the synthetic wine was supplemented with pyruvic acid, citric acid and gallic acid,

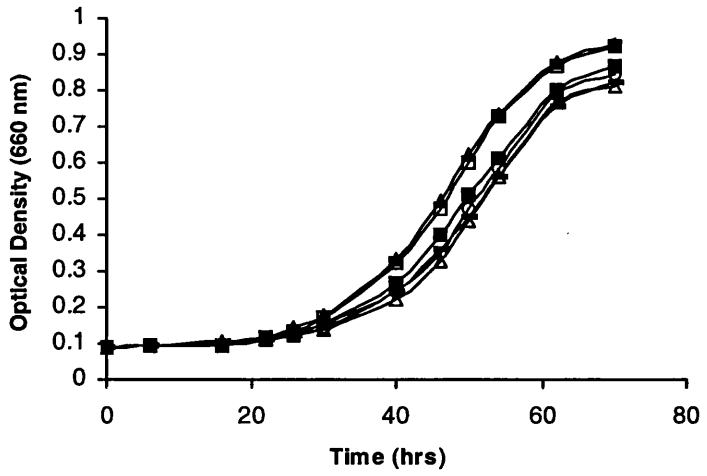


Figure 6.11 The growth curves of *L. brevis* in the synthetic wine. The synthetic wines that contained 10% (v/v) ethanol were supplemented: pyruvic acid (□) 1 g/l, citric acid (▲) 1 g/l, and glycerol (—) 1 g/l, gallic acid (○) 100 mg/l, ferulic acid (■) 100 mg/l. the control (△) was the synthetic wine that was not supplemented any additives above. The cells were cultivated in the synthetic wine at 25°C. The raw data of the cell growth is in Appendix-4.3

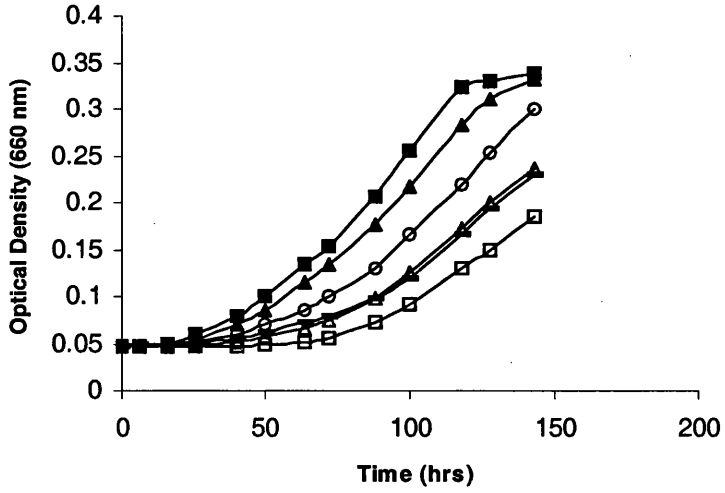


Figure 6.12 The growth curves of *O. oeni* in the synthetic wine. The synthetic wines that contained 10% (v/v) ethano were supplemented: pyruvic acid (■) 1 g/l, citric acid (▲) 1 g/l, and glycerol (—) 1 g/l, gallic acid (○) 100 mg/l, freulic acid (□) 100 mg/l. the control (△) was the synthetic wine that was not supplemented any additives above. The cells were cultivated in the synthetic wine at 25°C. The raw data of the cell growth is in Appendix-4.4

the cells of *O. oeni* grew significantly faster than the control. Especially, when the synthetic wine contained pyruvic acid the cells of *O. oeni* grew the fastest. It is also found in Figure 6.12 that gallic acid could increase the ethanol tolerance of *O. oeni* whereas ferulic inhibited the cell growth, which was different from the observation of *L. brevis* above (Figure 6.11). Glycerol also did not influence the cell growth and ethanol tolerance of *O. oeni*.

6.4 Effect of Suspension Media on the Ethanol Tolerance of Freeze-Dried *L. brevis* and *O. oeni*

Previous work has shown that a great deal of cell viability is lost greatly when freeze-dried malolactic bacteria were inoculated directly into wine (Beelman *et al.*, 1982; Liu and Gallander, 1982 and 1983; Nault *et al.*, 1995). It is known that suspension media such as sugars can enhance the resistance of LAB to freeze-drying stress and improve the vitality (Section 5.2.1, Chapter 5). Suspension media may also influence ethanol tolerance of freeze-dried malolactic bacteria when they are introduced into wines.

The cell cultures of *L. brevis* and *O. oeni* were cultivated in GY media at 25°C. When the cells had grown to early stationary phase, the cultures were concentrated 3 times by centrifugation (13000 r/min for 5 min). Aliquots of 5 ml the concentrated cell cultures of LAB were mixed in autoclaved serum bottles with 5 ml of the following suspension media: 3% (w/v) gelatine, 10% (w/v) glutamate, 8% (w/v) YE, 20% (w/v) sugars (sucrose, lactose maltose and trehalose). These cell cultures were freeze-dried. After freeze-drying, the freeze-dried cells were rehydrated in GY medium, and then the bacteria were inotroduced immediately in GY medium that contained 10% (v/v) ethanol. In order to examine the effect of freeze-drying on ethanol tolerance of LAB, the fresh cells of LAB were also introduced in GY medium that contained 10% (v/v) ethanol. After introduction, the cell cultures were incubated at 25°C, and the cell growth was monitored by measuring the OD of test tubes at 660 nm. Figure 6.13 summarizes graphically the experiments above.

6.4.1 *L. brevis*

Figure 6.14 shows the effect of suspension media on the ethanol tolerance of freeze-

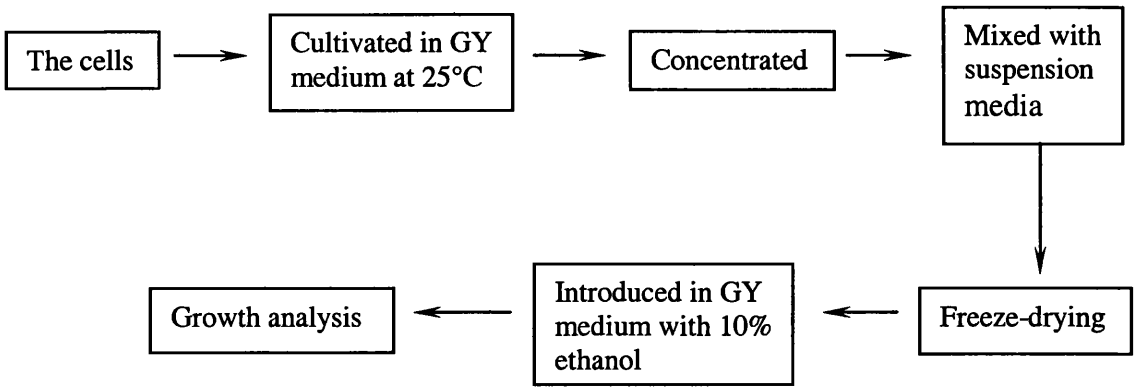
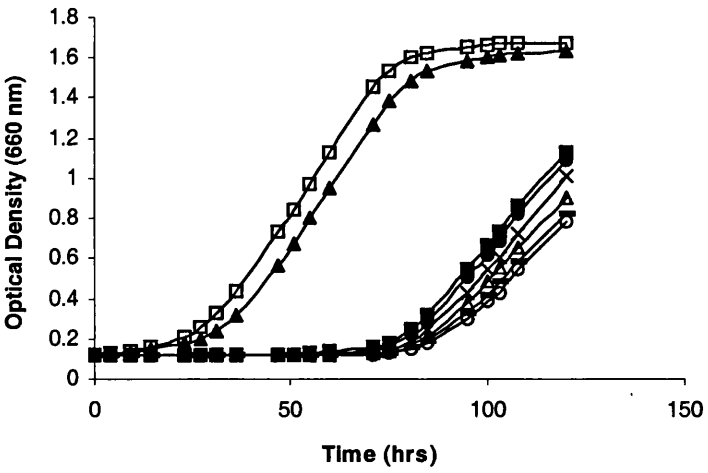


Figure 6.13 Summary of cultures handling procedure for investigation on effect of suspension media on the ethanol tolerance of freeze-dried *L. brevis* and *O. oeni*



	VI(%)	μ
Fresh cell	57.4±1.7	0.052
Glutamate	42.5±2.0	0.052
Sucrose	3.2±2.7	0.052
Maltose	3.5±3.0	0.052
Trehalose	3.8±2.7	0.052
Lactose	3.0±3.1	0.052
YE	2.2±3.3	0.052

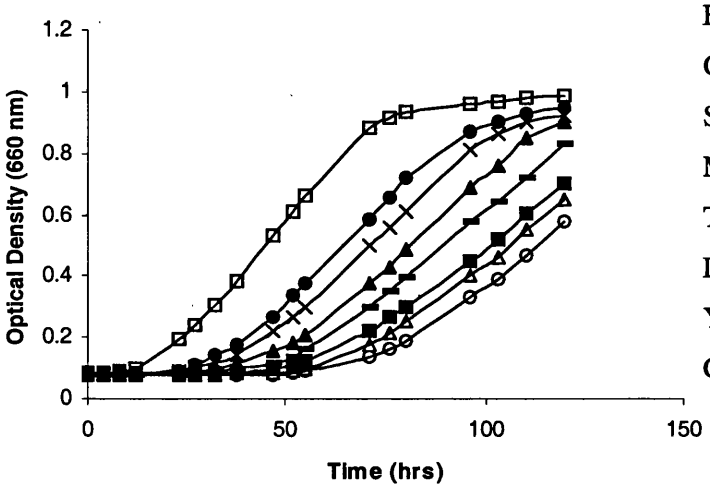
Figure 6.14 The effect of suspension media on the ethanol tolerance of freeze-dried *L. brevis*. The cells were initially cultivated in GY media at 25°C. when the cells grew to early stationary phase, 5 ml of the cell cultures were concentrate and mixed 5 ml of with the following suspension media (w/v): 10% Glutamate (▲), 20% sucrose (●), 20% maltose (×), 20% trehalose (■), 20% lactose (△), 8%YE (—), 3% gelatine (○). These samples were freeze-dried. The freeze-dried cells were introduced directly into the GY media that contained 10% (v/v) ethanol. The fresh cells (□) also were introduced into the GY media that contained 10% (v/v) ethanol. The test tubes were incubated at 25°C, and the cell growth was monitored by measuring the OD of the test tubes at 660 nm. The raw data is in Appendix-4.5

dried *L. brevis*. The cells with glutamate grew much faster than those with other suspension media tested, and vitality index of the cells with glutamate was 42.5%, which was far higher than those with other suspension media tested. These results showed that suspension media had great effects on the ethanol tolerance of *L. brevis*, and glutamate was an excellent protective agent for *L. brevis* and greatly improved the ethanol tolerance. Among the suspension media, YE and gelatine gave the cell vitality indices below 3.0% and showed the poorest ability to enhance ethanol tolerance. It is also found in Figure 6.14 that the fresh cells grew faster than all freeze-dried cells, whatever the suspension media. Apart from glutamate treated cells, these results suggest that freeze-dried cells of *L. brevis* had less ethanol tolerance than those without freeze-drying. Compared Figure 6.14 with Figure 5.2 (the conditions of freeze-drying were the same, but the medium for assessing vitality was GY without ethanol), it could also be noted that the cells of *L. brevis* grew very slowly when freeze-dried cells were directly inoculated in GY medium that contained 10% (v/v) ethanol. For example, lag phase of the cells freeze-dried with sucrose was about 70 h and the specific growth rate (μ) was 0.052 h^{-1} (see Figure 6.14) whereas the lag phase was about 8 h and the μ was 0.143 (see Figure 5.2) when the freeze-dried cells were inoculated in GY medium. These results indicated that the freeze-dried cells of *L. brevis* became very sensitive to ethanol stress and had lower ethanol tolerance.

6.4.2 *O. oeni*

A similar experiment described in section 6.4.1 was carried out on *O. oeni*. The freeze-dried cells of *O. oeni* with different suspension media were directly introduced in GY media that contained 10% (v/v) ethanol, and cultivated at 25°C. Figure 6.15 shows the growth curves of *O. oeni*. When the cells were freeze-dried with sucrose, the cell growth rates were the highest; the second was that with maltose. Among the suspension media investigated, sucrose gave *O. oeni* the highest vitality index 41.3%, and was the best protective agent to improve ethanol tolerance of *O. oeni*. Maltose and glutamate were also good protectants, and gelatine gave the poorest protection of *O. oeni*. It was noted that the vitality index of fresh *O. oeni* (86.5%) was very higher than that of *L. brevis* (57.4%), which means that *O. oeni* was more resistant to ethanol stress than *L. brevis*. Comparing with the fresh cells, the growth rates of freeze-dried cells of *O. oeni* were significantly low, whatever suspension media were used. The freeze-

dried cells of *O. oeni*, like *L. brevis*, also showed less ethanol tolerance than the cell without undergoing freeze-drying. When the results shown in Figure 6.15 were compared with those shown in Figure 5.3, it was also found that the cell growth of freeze-dried *O. oeni* became slower when the freeze-dried cells were directly inoculated in GY medium that contained 10% (v/v) ethanol.



	VI(%)	μ
Fresh cell	86.5±2.2	0.047
Glutamate	21.9±3.2	0.047
Sucrose	41.3±3.0	0.047
Maltose	31.1±3.4	0.047
Trehalose	10.6±3.7	0.047
Lactose	8.4±3.6	0.047
YE	15.8±4.0	0.047
Gelatine	6.3±4.3	0.047

Figure 6.15 The effect of suspension media on the ethanol tolerance of *O. oeni*. The cells were initially cultivated in GY media at 25°C. when the cells grew to early stationary phase, 5 ml of the cell cultures were concentrate and mixed with 5 ml of the following suspension media (w/v): 10% Glutamate (▲), 20% sucrose (●), 20% maltose (×), 20% trehalose (■), 20% lactose (△), 8%YE (—), 3% gelatine (○). These samples were freeze-dried. The freeze-dried cells were directly introduced into the GY media that contained 10% (v/v) ethanol. The fresh cells (□) also were introduced into the GY media that contained 10% (v/v) ethanol. The test tubes were incubated at 25°C, and the cell growth was monitored by measuring the OD of the test tubes at 660 nm. The raw data is in Appendix-4.6

6.5 Effect of Preculture on the Growth of Freeze-Dried Cultures of *L. brevis* and *O. oeni* in Ethanol Medium

As shown above (Figure 6.14 and Figure 6.15), the freeze-dried *L. brevis* and *O. oeni* have less ethanol tolerance than the fresh cells. This is primarily due to the destructive effect of freeze-drying processes. So pre-culture or reactivation should be considered to obtain high ethanol tolerance so that MLF is induced successfully. This option was therefore investigated.

The cells of *L. brevis* and *O. oeni* were cultivated in GY media at 25°C. When the cells had grown to early stationary phase, the cultures were concentrated 3 times by centrifugation. Aliquots 5 ml of the concentrated cell cultures of LAB were mixed with 5 ml 10%(w/v) lactose in autoclaved serum bottles. These cell cultures were freeze-dried according to methods described in Chapter 2 (section 2.4). After freeze-drying, the freeze-dried cells were re-hydrated in the following media:

1. CME medium: 1 g/l citrate, 1 g/l malic acid, 5 g/l YE, 5 g/l fructose, 5% ethanol
2. YFE medium: 5 g/l YE, 5 g/l fructose, 5% ethanol
3. YGE medium: 5 g/l YE, 5 g/l glucose, 5% ethanol
4. GY medium

After rehydration, preculture was carried out: the cell cultures were incubated at 25°C for about 48 hrs. When the preculture was complete, some of the cell cultures were introduced under standard conditions in 10 ml GY media that contained 10% (v/v) ethanol, and incubated at 25°C. The cell growth was monitored. The experimental procedure is summarized graphically in Figure 6.15.

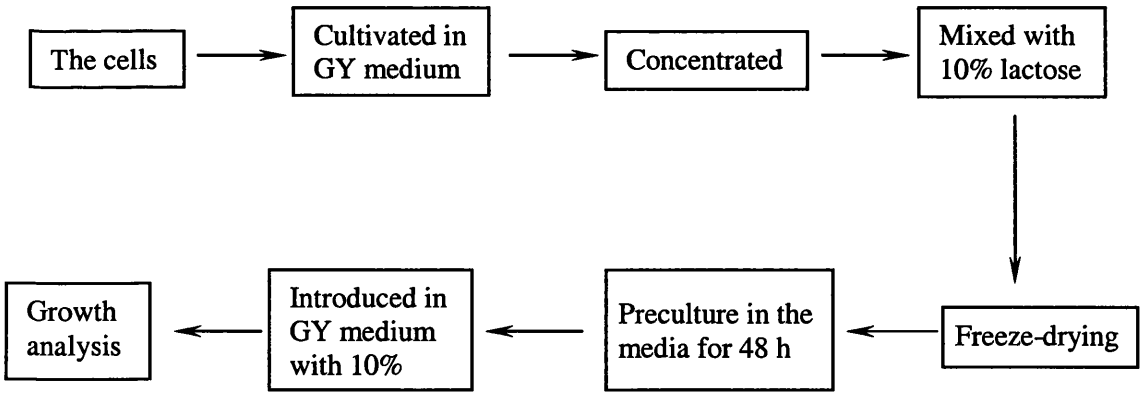
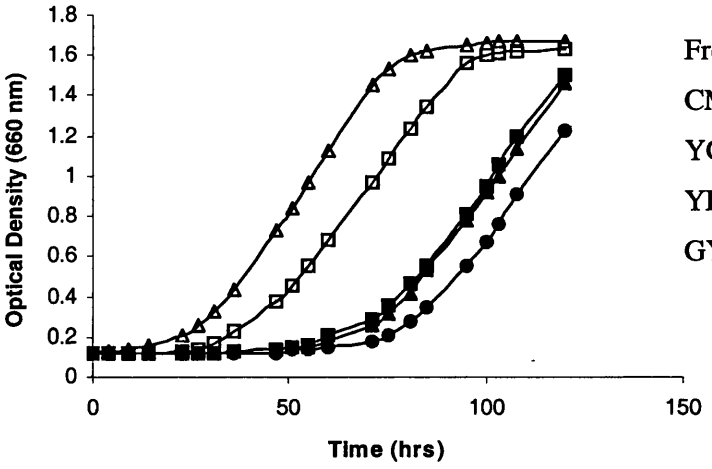


Figure 6.16 Summary of cultures handling procedure for investigation on effect of preculture on the ethanol tolerance of freeze-dried *L. brevis* and *O. oeni*.

6.5.1 *L. brevis*

Figure 6.17 shows the effect of different preculture media on the growth of *L. brevis* in GY medium with 10% ethanol (ethanol medium). When the freeze-dried cells of *L. brevis* were precultured in CME medium, the cell growth rate was the highest, and

much higher than other preculture medium, which suggests presence of citrate and malate was favourable to repair and recover the cell damage caused by freeze-drying. The cells precultured in YGE and YFE medium had very similar growth rates, which means the kind of sugars in the preculture media did not influence ethanol tolerance of *L. brevis*. When the preculture medium was GY medium the cells showed the poorest growth rate. CME, YGE and YFE media all contained 5% (v/v) ethanol whereas GY medium did not. Ethanol adaptation could improve ethanol tolerance of *L. brevis*. Even though undergoing reactivation for 48 h, the growth rates of pre-cultured cells were still slower than that of the fresh cells, and preculture for a longer time was needed.



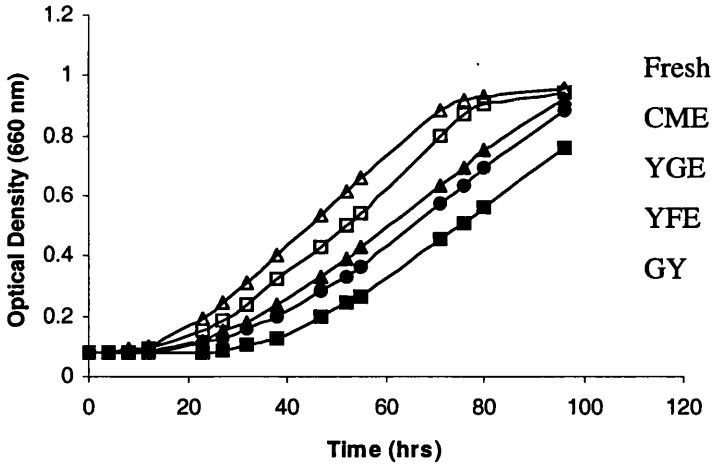
	VI(%)	μ
Fresh cell	57.6±2.1	0.051
CME	31.4±2.3	0.051
YGE	7.2±3.2	0.051
YFE	6.6±3.5	0.051
GY	4.0±2.0	0.051

Figure 6.17 Effect of different preculture media on the growth of *L. brevis* in GY medium with 10% ethanol (ethanol medium). The cells were initially cultivated in GY media at 25°C. When the cells had grown to early stationary phase, the cultures were concentrated and freeze-dried with 10%(w/v) lactose. The freeze-dried cells were precultured in the following medium: CME medium (□), YFE medium (▲), YGE medium (■), GY medium (●). After preculture for 48 h the cell cultures were introduced in the GY media that contained 10% (v/v) ethanol, and the cell growth was monitored. The control (△): the fresh cells were introduced directly into GY medium that contained 10% (v/v) ethanol. The raw data is in Appendix-4.7

6.5.2 *O. oeni*

The effect of different preculture media on the growth of *O. oeni* in GY medium with 10% ethanol (ethanol medium) is shown in Figure 6.18. Those results also show that the preculture medium significantly affects ethanol tolerance of *O. oeni*. When the freeze-dried cells of *O. oeni* were precultured in CME medium, the cells displayed the

highest growth rate in GY medium that contained 10% (v/v) ethanol, which was similar to *L. brevis*. However, the cells precultured in YFE medium had much higher growth rate than those precultured in YGE medium. The type of sugars in the preculture medium affected the preculture of *O. oeni*, and the cells of *O. oeni* recovered quickly when the sugar in preculture medium was fructose. When preculture medium was ethanol free, the cells of *O. oeni* showed a higher growth rate than those precultured in YGE medium that contained 5% ethanol, which was unlike *L. brevis*. But the cell growth rate precultured in GY medium was slower than that precultured in YFE medium. It seems that the effect of type of sugars (fructose and glucose) on ethanol tolerance of *O. oeni* was greater than that of ethanol adaptation. It was noted in Figure 6.18 that the growth rate of freeze-dried cells of *O. oeni* precultured in CME was slightly slower than the fresh cells, and a longer reactivation time was still needed.



	VI(%)	μ
Fresh cell	86.5±2.4	0.047
CME	70.3±2.1	0.047
YGE	27.1±2.5	0.047
YFE	54.1±3.0	0.047
GY	42.4±3.1	0.047

Figure 6.18 Effect of different preculture media on growth of *O. oeni* in GY medium with 10% ethanol (ethanol medium). The cells were initially cultivated in GY media at 25°C. When the cells had grown to early stationary phase, the cultures were concentrated and freeze-dried with 10%(w/v) lactose. The freeze-dried cells were precultured in the following medium: CME medium (□), YFE medium (▲), YGE medium (■), GY medium (●). After preculture for 48 h the cell cultures were introduced in the GY media that contained 10% (v/v) ethanol, and the cell growth was monitored. The control (△): the fresh cells were introduced directly into GY medium that contained 10% (v/v) ethanol. The raw data is in Appendix-4.8

6.6 Discussion

Ethanol tolerance is an important factor that should be considered when malolactic starter is prepared and used. Ethanol shows the greatest inhibitory effect on the growth and MLF of *O. oeni*, followed by pH and SO_2 (Vaillant *et al.*, 1995).

In this work, it was found that temperature not only affected the cell growth in wines, but also the cell resistance to ethanol stress. *O. oeni* grown at 25°C displayed the most resistance to ethanol stress, and *L. brevis* was the most resistant to ethanol stress when the cell grew at 15°C. Lorca *et al* (1999) also found that the cells of *Lactobacillus acidophilus* grown at sub-optimal temperature (25°C) had higher survival after exposure to ethanol stress (20% ethanol). However, when the cells of *L. brevis* grew at 15°C the growth rate was very low. This is a big disadvantage if *L. brevis* is cultivated at 15°C because the productivity is too low. As expected, more ethanol in wines displayed larger inhibitory effect on the cell growth. But the inhibitory effect was not a linear function of ethanol concentration, which means malolactic bacteria had some tolerance to ethanol stress. The inhibitory effect of ethanol depends on species of bacteria, ethanol concentration and growth temperature.

Apart from ethanol, wine also contains many other components such as organic acids and amino acids. These components may affect the growth and metabolism of malolactic bacteria. In this work, it was found that pyruvic acid and citrate were effective in improving the ethanol tolerance of *L. brevis* and *O. oeni*. The mechanism of tolerance seems to be related to many factors. Additives that are electron acceptors all improve tolerance, suggesting that high ethanol causes imbalances in metabolism. More generally, the effect of reduced temperature on tolerance is commonly found in both yeast and bacteria, suggesting that the membrane and its function are the primary target. It has been reported that citrate stimulated microbial growth with fermentable carbohydrate in the absence of ethanol (Schmitt *et al.*, 1992; Salou *et al.*, 1994). Glycerol did not improve the ethanol tolerance of *L. brevis* and O. *oeni*, although wine contains much glycerol. Red wines contain large amounts of phenolic compounds: phenolic acids, anthocyanins, flavonols and tannins (Vivas *et al.*, 1997). Only two phenolic acids: gallic acid and ferulic acid were tested here. It was found that gallic acid could activate the cell growth of *O. oeni* whereas ferulic acid showed an inhibitory effect. A similar result was obtained by Vivas *et al* (1997) and Reguant *et*

al (2000). However, ferulic acid stimulated the cell growth of *L. brevis*. It indicates that the effect of phenolic compounds on ethanol tolerance of malolactic bacteria depends on the species and the type of phenolic compound itself. Other independent work in the Department has shown that ethanol tolerance was also improved with chlorogenic acid, butanediol and malate (Donovan, 2000)

The suspension medium had a great influence on cell vitality after freeze-drying (Chapter 5). In this work, it was also found that suspension medium greatly affected ethanol tolerance of *L. brevis* and *O. oeni*. 5% glutamate was an excellent protective agent for *L. brevis* because the cells freeze-dried with 5% glutamate had a slightly lower growth rate than that of the fresh cells. The cells of *L. brevis* freeze-dried with 5% glutamate may be directly inoculated into wines if the ethanol concentration of wine is not over 10%. Other suspension media such as sugars and YE showed poor ability to improve ethanol tolerance of *L. brevis*. However, sugars (maltose, lactose and sucrose) and YE displayed effective protection on the vitality of *L. brevis* after freeze-drying (see section 5.2.1, Chapter 5). The reason is not clear. The case with *O. oeni* was different from that of *L. brevis*. Among the suspension media, 10% sucrose was the best to enhance ethanol tolerance of *O. oeni*. Maltose and glutamate also showed good ability to improve ethanol tolerance. It had been found that sucrose, maltose and glutamate provided effective protection on the vitality of *O. oeni* after freeze-drying (see section 5.2.1, Chapter 5). Comparing with the fresh cells, the freeze-dried cells showed lower growth rate, although protective agents were used. This may prove that the cells were damaged or injured during freeze-drying, and the cell survival decreased (Jay, 1978; Panoff *et al.*, 1998; Wolfe and Bryant, 1999). So preculture or reactivation is necessary for freeze-dried LAB starter cultures before inoculation in wines.

The effect of preculture on the growth of LAB in GY medium with 10% ethanol (ethanol medium) was investigated in this work. When the freeze-dried cells of *L. brevis* and *O. oeni* were precultured in CME media, the cells had the highest ethanol tolerance. The possible reasons were: (1) CME medium contained citrate and malate. Citrate and malate can stimulate the cell growth (Schmitt *et al.*, 1992; Salou *et al.*, 1994) and were favourable to repair and recover the damage or injure caused by freeze-drying. (2) CME medium contained 5% ethanol, and the cells had an ethanol stress adaptation which can improve the ethanol tolerance of malolactic bacteria (Nault

et al., 1995; Garbay and Lonvaud-Funel, 1996; Teixeira *et al.*, 2002). The ethanol tolerance of *O. oeni* precultured in YFE medium was higher than that precultured in YGE medium. It may be explained by the fact that fructose is the most rapidly and efficiently metabolized sugar, and when glucose was used as sole substrate, the cell growth was poor (Krieger *et al.*, 1992; Maicas *et al.*, 1999c).

The productivity of freeze-dried preparations can be enhanced with preculture. For example, the lag phase of cells of *L. brevis* freeze-dried with 5% lactose was about 81 h, while the same cells undergoing 48 h preculture in CME medium had a about 23 h lag phase. On other hand, after alcoholic fermentation and as long as MLF has not begun, wine cannot be treated by sulphite which would inhibit LAB growth. During this period the wine is exposed to chemical oxidation or development of spoilage microorganisms such as acetic acid bacteria. Therefore, the delay between the two fermentations must be as short as possible. After preculture the growth rate of malolactic bacteria was increased and MLF occurred early, the risk of spoilage was reduced. So preculture is necessary in the view of practical wine production. Even though undergoing preculture in CME medium for 48 h, the freeze-dried cells of *L. brevis* and *O. oeni* still had lower growth rate than the fresh cells, and longer preculture time was required to activate them.

In conclusion, high ethanol tolerance is an important indicator for the quality of malolactic starter cultures. The attention should be paid throughout the whole production of malolactic starter cultures.

Chapter 7. Investigation on Malolactic Fermentation

7.1 Introduction

In wine production, malolactic fermentation (MLF) is an important process. MLF is the bioconversion of the malic acid to lactic acid and CO_2, causing a decrease in acidity of the wine. MLF results from the metabolism of certain lactic acid bacteria in wines, mainly *O. oeni*. MLF is essential for nearly all red wines and some white wines (Davis *et al.*, 1985; Maicas *et al.*, 1999a and 1999b). As well as decreasing wine acidity, MLF can improve the flavour of wines and increase the microbiological stability of wines. Traditionally, MLF is allowed to develop spontaneously by the growth of lactic acid bacteria naturally present in wines. However, this natural process is slow and unreliable. It may take weeks or months, and the results are unpredictable because MLF is working in extreme environments of high alcohol and SO_2, low pH such that growth is marginal. In recent years starter culture technology, involving the inoculation of *O. oeni* into wines, has been developed for inducing MLF. Although this technology increases the prospect of a rapid, reliable MLF, failures can still occur. The process of MLF is only partially understood and difficult to predict. The performance of different strains in wines may be greatly affected by the composition of wines, vinification procedures and the interrelationship with other organisms (Martineau, 1995). Therefore, an improved knowledge of MLF is essential to control this important process.

Both traditional (natural fermentation) and modern (inoculated fermentation) approaches to MLF are currently used by winemakers throughout the world. However, these fermentations depend upon the growth of malolactic bacteria in wines as a batch culture and are strongly influenced by environmental conditions, often leading to delay or failure of MLF. Such problems have inspired the search for alternative technologies that enable more rapid and reliable MLF (Gao and Fleet, 1995). Membrane bioreactor (MBR) charged with high densities of malolactic bacteria cells can be used to carry out MLF. In this system, the cells serve as biocatalysts that conduct the biochemical transformations without the need for cell growth. Such bioreactor systems offer many

advantages over batch fermentation, including very rapid and predictable reactions and use in a continuous mode. Because production of high concentration of cells of *O. oeni* is a significant cost, long-term stability of the malic acid degrading activity of the cells in MBR is necessary for the process to be commercially acceptable.

In this chapter, the effect of wine composition on batch MLF of *O. oen* and *L. brevis* was investigated. A 5 L cross-flow microfiltration MBR was used to carry out continuous MLF. The performance of membrane was assessed, and the factors that affected the stability of malic acid degrading activity were investigated in a synthetic wine. Measures to improve the stability of malic acid degrading activity were also carried out. In order to conveniently perform the research, a defined alcoholic solution (synthetic wine) was used.

7.2 Effect of Wine Composition on Batch MLF of *L. brevis* and *O. oeni*

It has been known that many wine components such as ethanol, citrate, and other nutrients affect growth of malolactic bacteria in wines, and then influence MLF (Wibowo *et al.*, 1985; Henschke, 1993; Lonvaud-Funel *et al.*, 1995). The malolactic activity of malolactic bacteria is also related to the species (Davis *et al.*, 1985). In order to examine the wine composition on MLF of *L. brevis* and *O. oeni*, a synthetic wine was used: yeast extract, 4 g/l; glucose, 2 g/l; fructose 1g/l, malic acid, 3 g/l; and ethanol 10% (v/v); trace elements solution (see Table 2.1), 20 ml/l. Except where indicated, the pH of medium was normally adjusted by 1M NaOH to pH 4.0.

When MLF of *L. brevis* and *O. oeni* were stopped, the malic acid concentration in the samples were analysed by HPLC (see Chapter 2, section 2.8.4 for details of the methods). The malic acid degradation (%) was calculated by malic acid concentration of the synthetic wine after MLF divided by its initial concentration.

7.2.1 Effect of Sugars (Glucose and Fructose) on MLF

Aliquots 1 ml of the active cultures of *L. brevis* and *O. oeni* were introduced in 10 ml of the synthetic wine, which contained respectively the following components:

glucose: 2, 4, 6 g/l; fructose: 2, 4, 6 g/l; glucose + fructose: 2 + 2 g/l. the synthetic wine that did not contain any sugars was used as the control. All the cultures above were cultivated at 25°C. After 48 hrs (*L. brevis*) or 72 hrs (*O. oeni*), MLF were stopped and the malic acid concentration was determined.

Table 7.1 shows the effect of sugars in the synthetic wine on MLF of *L. brevis* and *O. oeni*. It is seen from Table 7.1 that sugars in the synthetic wine greatly affected MLF of the malolactic bacteria. When the synthetic wine did not contain any sugars the malic acid degradation of *L. brevis* was only 30%. While in the wine containing 2 g/l glucose, the malic acid degradation was 63.9%. However, when the concentration of glucose in the synthetic wine increased from 2 g/l to 6 g/l the malic acid degradation slightly decreased. Comparing with the wine in which available sugar was only glucose, malic acid degradation of *L. brevis* significantly increased when available sugar in the wine was fructose. For example, the malic acid degradation of *L. brevis* with 2 g/l fructose was higher (73.4%) than that with 2 g/l glucose (63.9%). The fructose concentration in the wine also affected the malic acid degradation. When fructose concentration increased from 2 g/l to 4 g/l the malic acid degradation increased. However, when fructose was over 6 g/l malic acid degradation of *L. brevis* declined. When the wine both contained glucose and fructose, malic acid degradation of *L. brevis* decreased, compared with the wine that contained only fructose. These observations suggest that glucose was inhibitory for malolactic activity of *L. brevis* when the wine contained both glucose and fructose.

The performance of MLF of *O. oeni* was different from that of *L. brevis*. As shown in Table 7.1, when the synthetic wine did not contain any sugars *O. oeni* still displayed higher malic acid degradation, 94.4%. But when available sugar in the synthetic wine was glucose, the malolactic activity decreased drastically. Furthermore, malic acid degradation of *O. oeni* decreased as glucose levels in the wine increased. These results also showed that glucose was inhibitory to the malolactic activity of *O. oeni*. In comparison to the synthetic wine that did not contain any sugars, malic acid degradation increased slightly when the concentration of fructose in the synthetic wine was 2 g/l. But when fructose levels in the synthetic wine increased from 2 g/l to 6 g/l, the malic acid degradation was almost the same. When the synthetic wine contained both glucose and fructose, the malic acid degradation was the same as that of the synthetic wine that just contained fructose. It seems that glucose was not inhibitory for

the malolactic activity of *O. oeni* when the wine contained both glucose and fructose, and the MLF was unaffected.

Table 7.1 Effect of sugars on the malolactic fermentation in the synthetic wine

Sugars		Degraded mali acid g/l (*L. brevis*)	Malic acid degradation % (*L. brevis*)	Degraded malic acid g/l (*O. oeni*)	Malic acid degradation % (*O. oeni*)
Sugars	0 g/l	0.9±0.02	30.0	2.832±0.02	94.4
Glucose	2 g/l	1.917±0.05	63.9	1.704±0.04	56.8
Glucose	4 g/l	1.854±0.03	61.8	1.248±0.01	41.6
Glucose	6 g/l	1.767±0.02	58.9	1.071±0.02	35.7
Fructose	2 g/l	2.202±0.03	73.4	2.865±0.03	95.5
Fructose	4 g/l	2.397±0.04	79.9	2.865±0.02	95.5
Fructose	6 g/l	2.055±0.02	68.5	2.871±0.01	95.7
Glucose + Fructose	2 g/l 2 g/l	1.965±0.02	65.5	2.865±0.02	95.5

The synthetic wine (3 g/l malic acid and 10% ethanol, pH4.0) contained respectively the following amounts: glucose: 2, 4, 6 g/l; fructose: 2, 4, 6 g/l; 2 g/l glucose + 2 g/l fructose. The synthetic wine that did not contain any sugars was used as the control. All the samples above were cultivated at 25°C. After 48 hrs (*L. brevis*) or 72 hrs (*O. oeni*), MLF were stopped.

7.2.2 Effect of Ethanol Concentration on MLF

Aliquots 1 ml of the cultures of *L. brevis* and *O. oeni* were introduced in 10 ml of the synthetic wine, which contain respectively the following amounts of ethanol: 0, 3, 5, 8, 10, 12 % (v/v). All the samples above were cultivated at 25°C. After 48 hrs (*L. brevis*) or 72 hrs (*O. oeni*), MLF were stopped, and the malic acid concentration in the samples were analysed.

As expected, ethanol inhibited MLF of *L. brevis* (Table 7.2). But the inhibitory effect was not a linear function of ethanol concentration. When ethanol concentration increased from 3% to 10%, the malic acid degradations of *L. brevis* were similar. These experiments showed that ethanol concentrations below 10% had a little inhibitory effect on the malolactic activity of *L. brevis*. However, when ethanol

concentration was over 12% the malic acid degradation decreased greatly, showing that it was very inhibitory to MLF of this species.

The performance of *O. oeni* was similar to *L. brevis*. As shown in Table 7.2, the malic acid degradations of *O. oeni* did not decrease when the ethanol concentration increased from 3% to 10%. Moreover, the malic acid degradation only slightly declined when ethanol in the wine was 12%. Ethanol concentrations below 12% had a negligible effect on the malolactic activity of the cells. These results indicate that malolactic bacteria have different ethanol tolerances, and *O. oeni* was more tolerant to ethanol stress than *L. brevis*.

Table 7.2 Effect of ethanol concentration on MLF in the synthetic wine

Ethanol concentration % (v/v)	Degraded malic acid g/l (*L.* brevis)	Malic acid degradation % (*L. brevis*)	Degraded malic acid g/l (*O. oeni*)	Malic acid degradation % (*O. oeni*)
0	1.929±0.02	64.3	2.865±0.03	95.5
3	1.905±0.03	63.5	2.856±0.04	95.2
5	1.917±0.02	63.9	2.862±0.05	95.4
8	1.899±0.03	63.3	2.859±0.03	95.3
10	1.893±0.04	63.1	2.865±0.02	95.5
12	0.564±0.05	18.8	2.781±0.04	92.7

The synthetic wine (3 g/l malic acid, pH4.0) contained respectively the following amounts of ethanol: 0, 3, 5, 8, 10 and 12% (v/v). All the samples above were cultivated at 25°C. After 48 hrs (*L. brevis*) or 72 hrs (*O. oeni*), MLFs were stopped.

7.2.3 Effect of Malic Acid Concentration on MLF

Aliquots 1 ml of the cultures of *L. brevis* and *O. oeni* were introduced in 10 ml of the synthetic wine, which contained respectively the following amounts of malic acid: 1 g/l (pH 3.91), 2 g/l (pH 3.72), 3 g/l (pH 3.08), and 4 g/l (pH 2.9). All the samples above were cultivated at 25°C. After 48 hrs (*L. brevis*) or 72 hrs (*O. oeni*), MLFs were stopped, and the malic acid concentration in the samples were analysed. The results are shown in Table 7.3.

Table 7.3 Effect of malic acid concentration on MLF in synthetic wine

Malic acid Concentration	Degraded malic acid g/l (*L. brevis*)	Malic acid degradation % (*L. brevis*)	Degraded malic acid g/l (*O. oeni*)	Malic acid degradation % (*O. oeni*)
1 g/l	0.154±0.02	15.4	0.911±0.03	91.1
2 g/l	0.114±0.03	5.7	1.890±0.03	94.5
3 g/l	0.165±0.02	5.5	2.775±0.02	92.5
4 g/l	0.204±0.02	5.1	1.508±0.05	37.7
Control	1.893±0.03	63.1	2.865±0.03	95.5

The synthetic wine (10% ethanol) contained respectively the following amounts of malic acid: 1 g/l (pH3.91), 2 g/l (pH 3.72), 3 g/l (pH 3.08), and 4 g/l (pH 2.9). All the samples above were cultivated at 25°C. After 48 hrs (*L. brevis*) or 72 hrs (*O. oeni*), MLFs were stopped. The synthetic wine that contained 3g/l malic acid and 10% ethanol was used as the control (pH 4.0).

Malic acid concentration in wine affects the pH of wine, and then affects the malolactic bacterial growth and MLF. As shown in Table 7.3, when the concentration of malic acid was 1 g/l (pH3.91), 0.154 g/l malic acid was degraded and the malic acid degradation of *L. brevis* was very low, 15.4%. When malic acid was 2 g/l (pH 3.72) in the wine, 0.114 g/l malic acid was degraded, and the malic acid degradation of *L. brevis* decreased strongly, only 5.7%. For higher malic acid concentrations, the malic acid degradations continued to decrease progressively. When the malic acid concentration was 3 g/l (pH 3.08), the malic acid degradation of *L. brevis* was 5.5%, which was much lower than that (63.1%) of the control (pH 4.0). These results showed that *L. brevis* was very sensitive to pH stress.

The situation with *O. oeni* was very different. When the concentration of malic acid was 1 g/l (pH3.91), 0.911 g/l malic acid was degraded and the malic acid degradation of *O. oeni* was high, 91.1%. Moreover, when the malic acid concentration increased from 1 g/l (pH 3.91) to 3 g/l (pH 3.08) the malic acid degradations of *O. oeni* were slightly lower than that of the control. It also can be noted that when the malic acid concentration was 3 g/l (pH 3.08) the malic acid degradation of *O. oeni* was 92.5%, and was slightly lower than that (96.5%) of the control (pH 4.0). These results indicated that *O. oeni* was much more resistant to pH stress than *L. brevis*. But when

the concentration of malic acid was over 4 g/l (pH 2.9), the malic acid degradation of *O. oeni* decreased drastically.

When the malic acid concentration was 1 g/l, the synthetic wine (pH 3.91) had a similar pH to that of the control (pH 4.0), but the malic acid degradation of *L. brevis* was much lower than that of the control. *O. oeni* gave a similar result. This suggests that malate may have a stimulating effect on MLF of the bacteria.

7.2.4 Effect of Phenolic Compounds on MLF

Aliquots 1 ml of the cell cultures of *L. brevis* and *O. oeni* were inoculated in 10 ml of the synthetic wine, which contained respectively the following amounts of gallic and ferulic respectively: 50, 100, 200 mg/l. All the samples above were cultivated at 25°C. After 48 hrs (*L. brevis*) or 72 hrs (*O. oeni*), MLFs were stopped, and the malic acid concentration in the samples were analysed.

Table 7.4 shows the effect of phenolic compounds (gallic and ferulic) on MLFs. When the synthetic wine was supplemented gallic, malic acid degradation by *L. brevis* increased, compared with the control (no phenolic compounds in the synthetic wine). Furthermore, the malic acid degradation by *L. brevis* slightly increased as gallic increased from 50 mg/l to 200 mg/l. Ferulic also showed a stimulatory effect on MLF by *L. brevis* as the ferulic concentration increased from 50 mg/l to 100 mg/l. However, when ferulic in the wine was over 200 mg/l an inhibitory effect on MLF by *L. brevis* was observed.

It is also seen form Table 7.4 that neither gallic nor ferulic influenced MLF by *O. oeni.* When gallic and ferulic concentration in the synthetic wine increased from 0 mg/l to 200 mg/l, the malic acid degradation by *O. oeni* almost kept constant. This was not too surprising since there was over 90% malic acid degradation in the control. These results indicate that the effect of phenolic compounds (gallic and ferulic) tested on MLF depends on the species of malolactic bacteria.

Table 7.4 Effect of phenolic compounds in the synthetic wine on MLF

Phenolic compounds	Degraded malic acid g/l (*L.brevis*)	Malic acid degradation % (*L. brevis*)	Degraded malic acid g/l (*O. oeni*)	Malic acid degradation % (*O. oeni*)
Gallic 50 mg/l	2.022±0.02	67.4	2.892±0.04	96.4
Gallic 100 mg/l	2.055±0.03	68.5	2.856±0.02	95.2
Gallic 200 mg/l	2.076±0.03	69.2	2.868±0.03	95.6
Ferulic 50 mg/l	2.046±0.02	68.2	2.859±0.05	95.3
Ferulic 100 mg/l	2.232±0.05	74.4	2.862±0.02	95.4
Ferulic 200 mg/l	1.572±0.04	52.4	2.883±0.03	96.1
Control	1.893±0.05	63.1	2.865±0.05	95.5

The synthetic wine (3 g/l malic acid and 10% ethanol, pH 4.0) contained respectively the following amounts of gallic: 50, 100, 200 mg/l, and ferulic: 50, 100, 200 mg/l. All the samples above were cultivated at 25°C. After 48 hrs (*L. brevis*) or 72 hrs (*O. oeni*), MLFs were stopped. The control was the synthetic wine.

7.2.5 Effect of Yeast Extract Concentration on MLF

Aliquots 1 ml of the active cultures of *L. brevis* and *O. oeni* were introduced in 10 ml of the synthetic wine, which contained respectively the following amounts of yeast extract: 0, 2, 4 g/l. All the samples above were cultivated at 25°C. After 48 hrs (*L. brevis*) or 72 hrs (*O. oeni*), MLFs were stopped, and the malic acid concentration in the samples were analysed.

The nutritional status of wine greatly affects MLF. It can be seen from Table 7.5 that the malic acid degradation by *L. brevis* was low, only 6.1%, when the synthetic wine did not contain any amount of yeast extract. Shortage of nutrients in wine inhibited MLF of malolactic bacteria. When yeast extract in the synthetic wine was 2 g/l, malic acid degradation of *L. brevis* strongly increased, 62.8%. However, when the concentration of yeast extract increased from 2 g/l to 4 g/l the malic acid degradation by *L. brevis* slightly increased.

The case with *O. oeni* was similar to *L. brevis*. As shown in Table 7.5, when the synthetic wine did not contain any yeast extract, like *L. brevis*, the malic acid

degradation by *O. oeni* was low, 54.3%. When yeast extract in the synthetic wine was 2 g/l, malic acid degradation by *O. oeni* greatly increased, 72.2%. Furthermore, when the concentration of yeast extract increased from 2 g/l to 4 g/l, malic acid degradation by *O. oeni* continued to increase, from 72.2% to 95.5%. It is also found from Table 7.5 that the ability of *O. oeni* to degrade malic acid was much higher than that of *L. brevis*.

Table 7.5 Effect of yeast extract in the synthetic wine on MLF

Yeast extract	Degraded malic acid g/l (*L.brevis*)	Malic acid degradation % (*L.brevis*)	Degraded malic acid g/l(*O. oeni*)	Malic acid degradation % (*O. oeni*)
0 g/l	0.183±0.03	6.1	1.629±0.05	54.3
2 g/l	1.884±0.05	62.8	2.166±0.03	72.2
4 g/l	1.893±0.03	63.1	2.865±0.03	95.5

The synthetic wine (3 g/l malic acid and 10% ethanol, pH 4.0) contained respectively the following amounts of yeast extract: 0, 2, 4 g/l. All the samples above were cultivated at 25°C. After 48 hrs (*L. brevis*) or 72 hrs (*O. oeni*), MLFs were stopped.

7.3 MLF of *O. oeni* in MBR

7.3.1 Performance of the Membrane

The performance of the membrane within a MBR is extremely important to future development of a full-scale plant. It allows the upper limits of operation to be calculated, allowing predictions to be made about how such a system might operate on a full industrial scale.

7.3.1.1 Operation with Water

The operation of the MBR with distilled water was taken to give a benchmark flow rate for cleaning of the MBR system after each operation. The MBR had been well cleaned before operation. Distilled water was fed to the MBR and passed through the membrane. The permeate flow rates were measured at various pressures across the

ceramic membrane. The range of pressure employed was from 0-40 psi. A stopwatch and measuring cylinder were used to collect a certain volume of water over a period of time, from which a flow rate was calculated. Measurements were taken five times for each pressure and the mean results are presented in Figure 7.1, please refer also to Appendix-5.1 for experimental data. For this investigation it was not necessary to operate pump (3) (see Figure 2.4).

Figure 7.1 shows the actual flow rates when the membrane was well cleaned. These were the conditions that were aimed for during the cleaning of the bioreactor system.

It is seen from Figure 7.1 that the permeation across the membrane was pressure dependent, responding linearly to an increase in pressure, where $y = 0.0368x - 0.1091$.

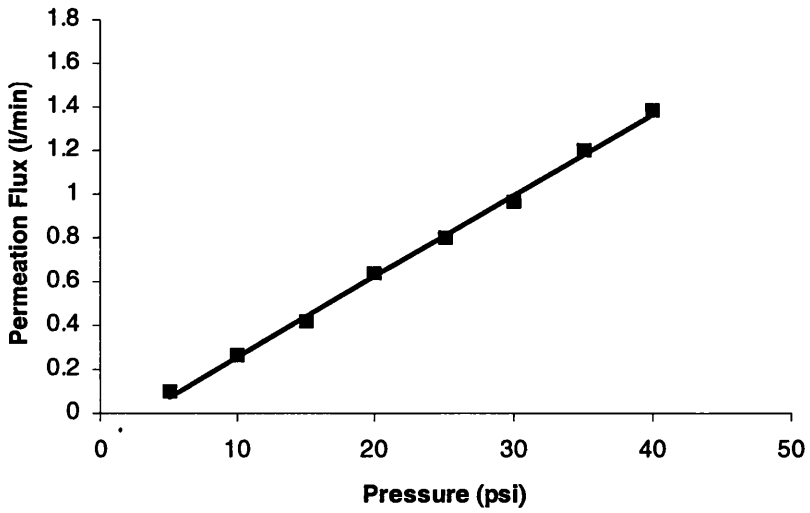


Figure 7.1 Performance of the membrane operated with water. The raw data is in Appendix-5.1.

7.3.1.2 Operation with GY Medium

The performance of the membrane operated with GY medium was assessed by the same method as above. Figure 7.2 shows the flow rates of the permeation with GY medium. The permeation across the membrane increased linearly as the pressure increased, where y = 0.0167x –0.0431. Comparing with the results operated with distilled water, the GY medium reduced the permeation flux significantly at any pressure tested. For instance, under pressures 20 psi, the flux rate was 0.276 l/min when the fluid in the MBR was GY medium, whereas the flux rate was 0.64 l/min when the fluid was distilled water. The reason may be that the viscosity of GY medium is higher than distilled water.

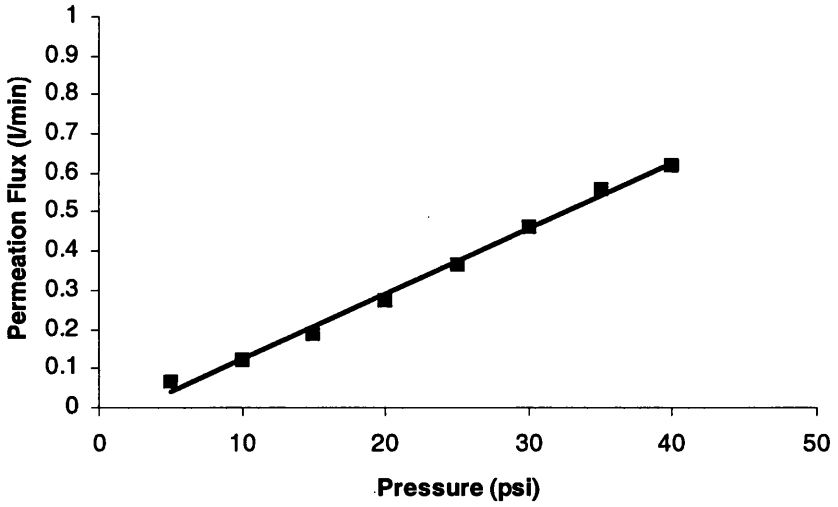


Figure 7.2 Performance of the membrane operated with GY medium. The raw data is in Appendix-5.2.

7.3.1.3 Operation with Bacterial Cells

When the MBR is charged with the bacterial cells, the flow rate of permeation through the membrane decreases because of the increased effect of fouling. To assess how much fouling increased due to cell density and pressurisation of the membrane system,

harvested *O. oeni* cells were added into distilled water in the bioreactor system, and the flow rates through the membrane were measured at different pressures. The cell densities (OD) used were 1.2 and 7.4. Each flow rate at each set optical density and pressure was measured five times. The results are given in Figure 7.3.

When increasing the pressure across the membrane from 5 to 30 psi, the flow rate of fluid through the membrane was increased. Increasing the cell density of LAB in the system reduced the flow rate of the fluid through the membrane. For example, at the pressure 15 psi, the permeation rate was 0.186 l/min when the cell density was at zero; when the cell densities were OD 1.2 and 7.4 the permeation rates decreased, 0.148 and 0.126 l/min, which were decreases in flow rate through the membrane of 20.4% and 32.2%. Increasing the cell concentration in the system increased the effect of fouling simply because there were more cells to block the pores of the membrane and to form a fouling layer through concentration polarisation.

Increasing the pressure across the membrane not only increased the flow rate across the membrane but it also increased the effect of fouling by forcing more cells into the pores and onto the surface of the membrane. For instance, comparing the permeation rates of cell density OD 7.4 with those of OD 0 (distilled water), when the operational

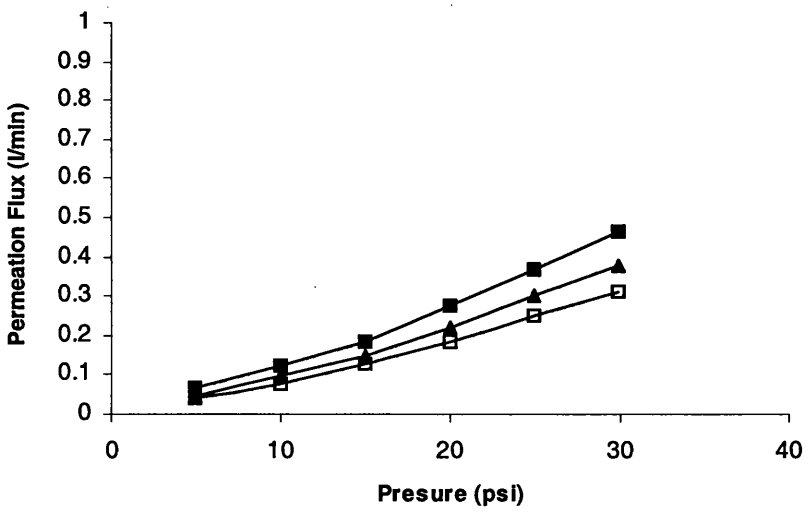


Figure 7.3 Fluxes through membrane at different pressures with varying cell densities
The cell densities (OD) were: 0 (■), 1.2 (▲) and 7.4 (□). The raw data is in Appendix-5.3.

pressure was 15 psi the drop in permeation rate was 0.06 l/min; while the operational pressure was 25 psi the drop in permeation rate was 0.118 l/min, which was a reduction in flux of about 32%.

7.3.2 Malolactic Fermentation in MBR

A cross-flow microfiltration MBR with high cell density was used to carry out continuous MLF. The construction of MBR has been described in Chapter 2, Section 2.6. In order to obtain high cell densities a great mass of *O. oeni* cells were required. To get enough cells, two 20 L fermentors were filled with HGY medium, sterilized, introduced and incubated (for detail, see 2.4.2 Large-Scale Cultivation, Chapter 2). A synthetic wine was used in this experiment: yeast extract, 4 g/l; glycerol, 2 g/l; glucose, 2 g/l; fructose 1 g/l; malic acid, 2 g/l; and ethanol 10 % (v/v); trace elements solution, 20 ml/l; pH 4.0.

7.3.2.1 Effect of Ethanol Concentration in synthetic Wine

In order to examine the effect of ethanol concentration in wines on stability of the malic acid degrading activity of *O. oeni*, different ethanol concentrations of synthetic wine were made respectively: 5%, 10% (as control) and 13%. *O. oeni* had been cultivated before carrying out MLF in the MBR. The cells of *O. oeni* were put into the MBR, and the cell densities of cell suspension in the reactor were about 1.8×10^{8}CFU/ml (OD= 8.3, 5% ethanol), 1.6×10^{8}CFU/ml (OD=8.0, 10% ethanol) and 1.62×10^{8}CFU/ml (OD=8.1, 13% ethanol) respectively. When the cell charging was complete the MBR was fed continuously with synthetic wine at 2.4 l/h for 2 hrs, and then at 0.48 l/h for 10 hrs. It was thought that synthetic wine had replaced most of cultural medium in the reactor. The flow rate of synthetic wine was controlled at 0.48 l/h during the experiment. The MBR was operated at 25°C. After running MBR continuously for 72 hrs, the experiment was stopped. Samples were taken regularly throughout the experiment. Malic acid concentration in the samples were analysed by HPLC, and malic acid degradation was calculated by the malic acid concentration after MLF divided by the malic acid concentration before MLF.

The malic acid consumptions (in natural logarithms, ln) in synthetic wine of various ethanol concentrations are shown in Figure 7.4. Ethanol concentration in wine had a

great effect on the stability of malic acid degrading activity of *O. oeni* in the MBR. As shown in Figure 7.4, when the ethanol concentration in synthetic wine was 5%, the malic acid degradation of *O. oeni* almost did not decline during 72 hrs continuous operations. When the ethanol concentration in synthetic wine was 10%, the cells of *O. oeni* gave over 1.9g/l (0.64 in ln) malic acid consumption during the first 42 hrs of continuous operation. While running time was over 42 hrs the malic acid consumption of *O. oeni* decreased progressively. By 72 hours, the malic acid consumption was 1.802 g/l (0.59 in ln), and the loss in ability to degrade malic acid was 5%. When the ethanol concentration in synthetic wine increased to 13%, the malic acid consumption did not decrease during the first 23 h of continuous operation. However, when operation time was over 37 hrs the ability of malic acid degrading activity of *O. oeni* declined fast. By running 72 hrs, the malic acid consumption of *O. oeni* was only 1.608 g/l (0.47 in ln), and the loss in ability to degrade malic acid was 14.8 %. The linear decay (on a log scale) suggests a first order process.

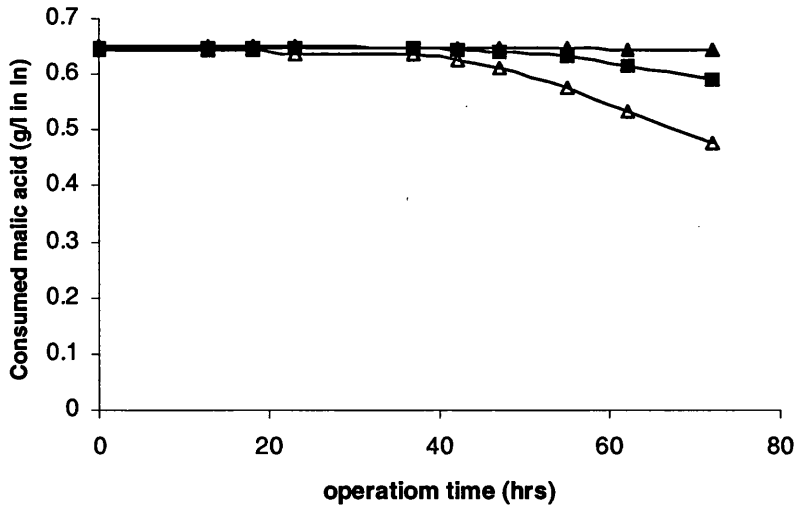


Figure 7.4 Malic acid consumption in synthetic wines of various ethanol concentrations. The synthetic wine passed continuously through the membrane bioreactor charged with the cell of *O. oeni.* Operating conditions: temperature 25°C; malic acid in synthetic wine 2.0g/l; the flow rate 0.48 l/h; the cell density: 1.8×10^8 CFU/ml (5% ethanol, ▲), 1.6×10^8 CFU/ml (10% ethanol, ■) and 1.62×10^8 CFU/ml (13% ethanol, Δ). The raw data is in Appendix-5.4.

7.3.2.2 Effect of Flow Rate

The effect of variable flow rate was investigated. Cells of *O. oeni* were grown to early stationary phase, as described above. The cells were put into the MBR, and the cell density of *O. oeni* suspension in the reactor was about 1.6×10^8 CFU/ml (OD=8.0). The synthetic wine was then passed continuously through the MBR. The MBR was operated at 25°C. The following flow rates of synthetic wine were set: 0.48, 0.6, 1.2, 1.8, 2.4 l/h. 5 L of synthetic wine was passed at each flow rate. Samples were taken regularly. Malic acid concentration in the samples were analysed by HPLC, and malic acid degradation was calculated.

Figure 7.5 shows the malic acid consumption in synthetic wine at various flow rates. The consumed malic acid declined linearly as the flow rate increased. When the flow rate was 0.6 l/h the malic acid consumed in synthetic wine was 1.87 g/l whereas the malic acid consumption was 1.534 g/l at 2.4 l/h.

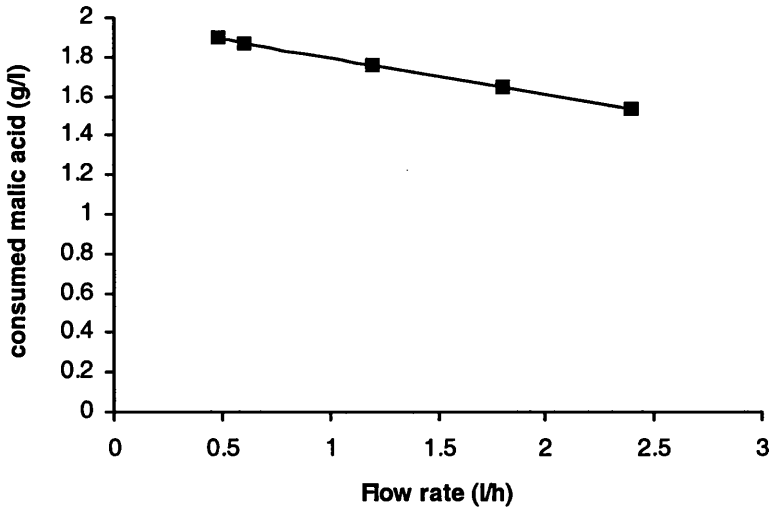


Figure 7.5 Malic acid consumptions in the synthetic wine at various flow rates within the MBR. The synthetic wine passed continuously through MBR charged with cells of *O. oeni.* Operating conditions: cell density 1.6×10^8CFU/ml; temperature 25°C; malic acid in synthetic wine 2.0 g/l. 5 L flow per condition. The raw data is in Appendix-5.5.

The degradation of malic acid at various flow rates is shown in Figure 7.6. Malic acid degradation of *O. oeni* decreased as the flow rate of the wine increased. A flow rate of less than 0.48 l/h was needed to obtain greater than 95% degradation of malic acid. Thus, the wine needed a minimum residence time of 10.4 h in the reactor to undergo 95% or more degradation of its malic acid. Faster flow rates reduced the residence time and reduced degradation of malic acid. At a flow rate of 2.4 l/h, only 79.7% of the malic acid in the wine was degraded.

Decarboxylication of malic acid to lactic acid is catalysed by malolactic enzyme. So the rate at which malic acid was consumed should follow Michaelis-Menten kinetics. When the reciprocal of malic acid degradation (g/l/h) was plotted against the reciprocal of residual malic acid in synthetic wine, a linear relationship was obtained (see Figure 7.7), where $y = 0.1045x + 0.039$. The kinetic parameters can be calculated through the equation: V_{max} was 25.64 g/l/h, and K_m was 2.68 g/l. So the Michaelis-Menten equation of the reaction was obtained:

$$V = \frac{Vmax \times S}{Km + S} = \frac{25.64 \times S_{malate}}{2.68 + S_{malate}}$$

It was noted that the Km is relatively high when typical value of malate is 2 to 3 g/l.

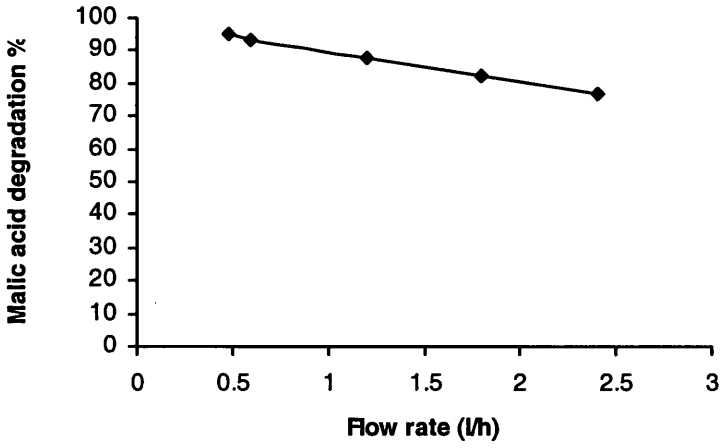


Figure 7.6 The degradation of malic acid at various flow rates within the MBR. The synthetic wine passed continuously through the MBR charged with the cell of *O. oeni.* Operating conditions: cell density 1.6 $\times 10^8$CFU/ml; temperature 25°C; malic acid in synthetic wine 2.0g/l. 5 L flow per condition. The raw data is in Appendix-5.6.

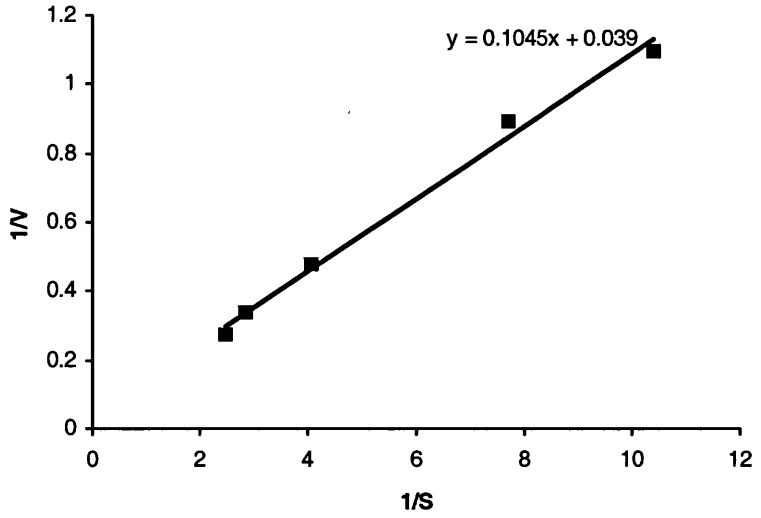


Figure 7.7 The relationship between 1/V and 1/S. V is the malic acid degradation (g/l/h), and S is the residual malic acid in synthetic wine (g/l).

7.3.2.3 Effect of pH of Synthetic Wine

The pH of wine is also one of the main factors that influence the cell growth and MLF. In this experiment, the pH of synthetic wine was adjusted to pH 2.9 by malic acid. The cells of *O. oeni* cultured in advance were then added to MBR, and cell density of the cell suspension in the bioreactor was about 1.6×10^{8}CFU/ml (OD=8.0). When the cell charging was complete, the membrane bioreactor was fed continuously with synthetic wine (ethanol 10%, pH 2.9) at 2.4 l/h for 2 hrs, and then at 0.48 l/h for 10 hrs to flush out the growth medium. The flow rate of synthetic wine was controlled at 0.48 l/h during later experiment. The MBR was operated at 25°C. Samples were taken regularly and analysed by HPLC. After running the MBR continuously for 72 hrs, the fermentation was stopped.

The malic acid consumption (g/l) is shown in Figure 7.8. It can be seen that low pH (2.9) inhibited the malic acid degrading activity of *O. oeni*. For instance, when pH of the synthetic wine was 4.0 and at the beginning of the operation, the malic acid consumption of *O. oeni* were 1.912 g/l, whereas when the pH was 2.9 the consumed malic acid was 1.84 g/l. But low pH did not cause the malic acid degrading activity of *O. oeni* to decline faster than that of the control (pH 4.0). The malic acid degradation

decreased slightly over the experiment, whatever the pH of the synthetic wine was, 4.0 or 2.9.

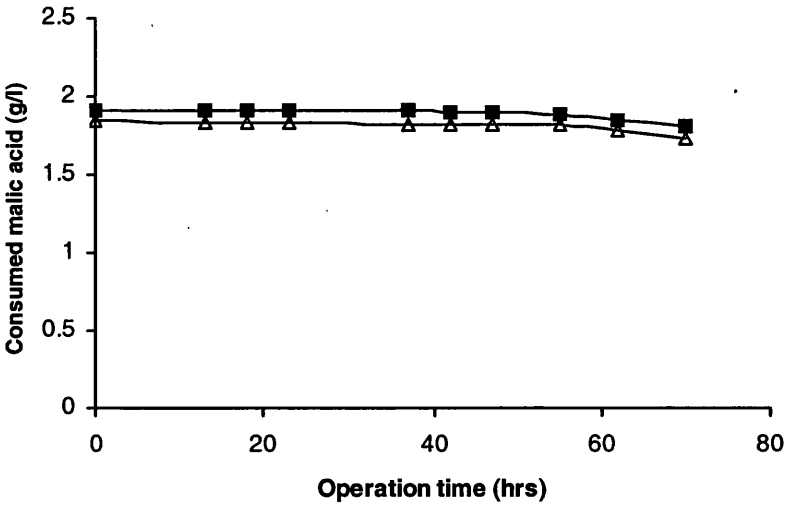


Figure 7.8 Malic acid consumption in the synthetic wines of various pH. The synthetic wine passed continuously through the membrane bioreactor charged with the cell of *O. oeni.* Operating conditions: temperature 25°C; malic acid in the synthetic wine 2.0 g/l; the flow rate 0.48 l/h; the cell density: 1.6×10^8CFU/ml (the control, pH4.0 and 10% ethanol, ■) and 1.6×10^8CFU/ml (the synthetic wine, pH 2.9 and 10% ethanol, △). The raw data is in Appendix-5.7.

7.3.2.4 Effect of Yeast Extract Concentration in Synthetic Wine

The cells of *O. oeni* at high cell density may need more nutrients to keep them active. In order to examine the effect of nutritional status in the wine on the stability of malic acid degrading activity of *O. oeni*, the concentration of yeast extract in synthetic wine was reduced from 4.0 g/l to 0.2 g/l. Other component concentrations in synthetic wine were not changed. The cells of *O. oeni* cultured in advance were charged into the MBR, and the cell density in the bioreactor was about 1.7×10^8CFU/ml (OD=8.4). When the cell charging was finished the MBR was fed continuously with synthetic wine at 2.4 l/h for 2 hrs, and then at 0.48 l/h for 10 hrs to flush out the growth medium. The flow rate of synthetic wine was then controlled at 0.48 l/h during later experiment. The MBR was operated at 25°C. Samples were taken regularly and analysed by HPLC. After running continuously for 72 hrs, the fermentation was stopped.

Figure 7.9 show the malic acid consumptions (in natural logarithms, ln) in synthetic wine that contained different concentrations of yeast extract. As shown in Figure 7.10, when the concentration of yeast extract in synthetic wine was 0.2 g/l, the malic acid degradation of *O. oeni* was very similar to that of the control (4.0 g/l YE in synthetic wine) during the 72 hours of continuous operation. At the end of 72 hours continuous operation, the malic acid consumption of *O. oeni* in synthetic wine with 0.2 g/l YE was 1.772 g/l (0.57 in ln), and was slightly lower than that (1.806 g/l, 0.59 in ln) of the control. The stability of malic acid degrading activity of *O. oeni* in synthetic wine with poor nutritional condition (0.2 g/l YE) was slightly lower than that in synthetic wine with rich nutritional condition (4.0 g/l YE).

These results indicate that the nutritional status in synthetic wine hardly affect the stability of malic acid degrading activity of *O. oeni* in MBR under the experimental conditions, although yeast extract was important for the cell growth.

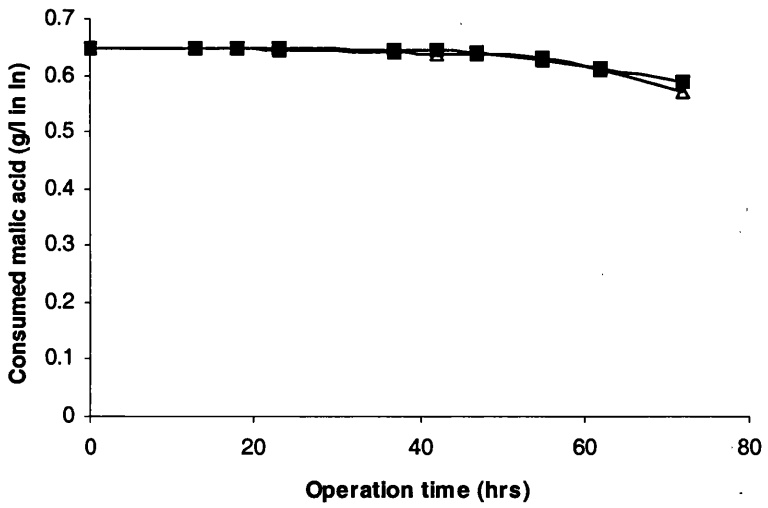


Figure 7.9 Malic acid consumptions in synthetic wine that contained various concentrations of yeast extract. The synthetic wine passed continuously through MBR charged with the cells of *O. oeni.* Operating conditions: temperature 25°C; malic acid in synthetic wine 2.0 g/l; the flow rate 0.48 l/h; the cell density: 1.6×10^8CFU/ml (the control, 10% ethanol and 4 g/l YE, ■) and 1.7×10^8CFU/ml (the synthetic wine that contained 10% ethanol and 0.2 g/l YE, △). The raw data is in Appendix-5.8.

7.3.2.5 Effect of Additives

It has been known that pyruvic acid and citrate can improve the ethanol tolerance of *O. oeni* (Chapter 6). In order to check if pyruvic acid and citrate also improve the stability of malic acid degrading activity of *O. oeni*, the cells were cultivated in the medium supplemented with 1 g/l citrate, and 1g/l pyruvic acid. When the cells had grown to stationary phase the cells of *O. oeni* were charged into the MBR, and the cell density in the bioreactor was about 1.6×10^{8}CFU/ml (OD=8.07). When the cell charging had finished the MBR was fed continuously with the synthetic wine at 2.4 l/h for 2 hrs, and then at 0.48 l/h for 10 hrs to flush out the growth medium. The flow rate of synthetic wine was controlled at 0.48 l/h for the rest of experiment. The MBR was operated at 25°C. Samples were taken regularly and analysed by HPLC. After running continuously the MBR for 72 hrs, the fermentation was stopped.

According to Figure 7.10, during the first 55 hours of continuous operation the malic acid consumption of *O. oeni* cultured in the medium that contained pyruvic acid and citrate (PC) was very similar with those of the control. But after running 55 hours, the stability of malic acid degrading activity of the control was slightly lower than that of PC. When operation time was 72 hours, the malic acid consumption of the control was 1.802 g/l whereas that of PC was 1.876 g/l. Figure 7.11 shows the effect of additives (pyruvic acid and citrate) on the malic acid degradation (in natural logarithms) in synthetic wine. A similar conclusion with Figure 7.11 was obtained. However, at the end of 72 continuous operations, the malic acid degradation of *O. oeni* cultured in the medium that contained pyruvic acid and citrate was obviously higher than that of the control without the additives. If operation time had been longer, it is believed that the difference would be more obvious.

Pyruvic acid and citrate are commonly found in green (young) wines. The results above seem to suggest that pyruvic acid and citrate slightly improve the stability of the malic acid degrading activity of *O. oeni*, although pyruvic acid and citrate were effective to improve the ethanol tolerance of *O. oeni*.

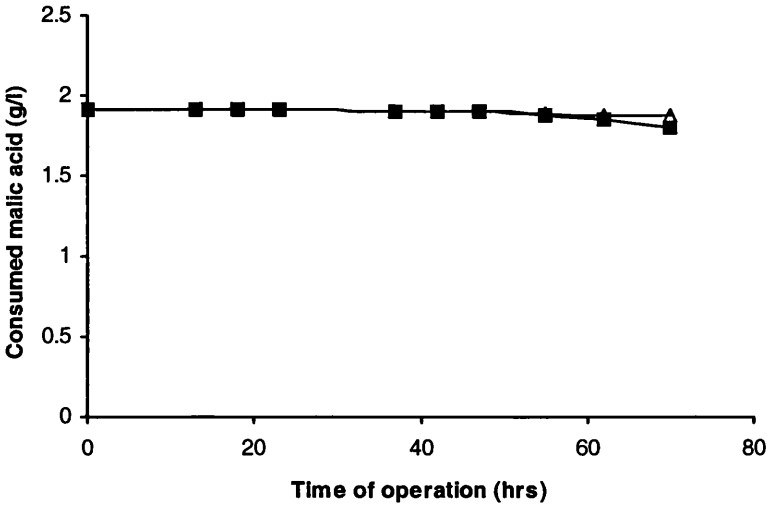


Figure 7.10 Effect of additives on the malic acid consumptions of *O. oeni* in synthetic wine. The synthetic wine passed continuously through the MBR charged with the cells of *O. oeni.* Operating conditions: temperature 25°C; malic acid in synthetic wine 2.0 g/l; the flow rate 0.48 l/h; the cell density: 1.6 × 10^8CFU/ml (the control, ■) and 1.6 × 10^8CFU/ml (the cells cultivated in the medium that supplemented with 1 g/l citrate, 1g/l pyruvic acid, Δ). The raw data is in Appendix-5.9.

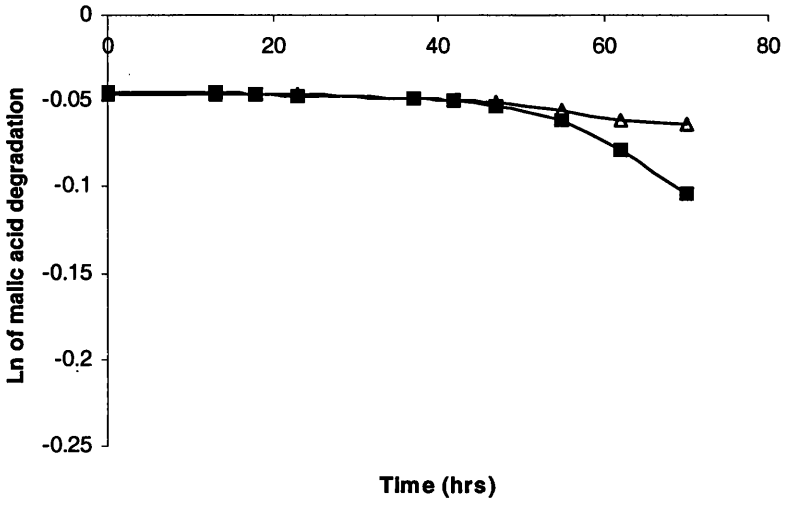


Figure 7.11 Effect of additives on the maic acid degradations of *O. oeni* in synthetic wine. The synthetic wine passed continuously through the MBR charged with the cells of *O. oeni.* Operating conditions: temperature 25°C; malic acid in synthetic wine 2.0 g/l; the flow rate 0.48 l/h; the cell density: 1.6 × 10^8CFU/ml (the control, ■) and 1.6 × 10^8CFU/ml (the cells cultivated in the medium that supplemented with 1 g/l citrate, 1g/l pyruvic acid, Δ).

7.3.2.6 Effect of Ethanol Stress Adaptation

The cells subjected to ethanol stress adaptation might show higher stability of malic acid degrading activity. According to this assumption, the cells were cultivated in the medium that was supplemented with 5% ethanol. When the cells had grown to stationary phase, the cells of *O. oeni* were then used in the MBR, and the cell density in the bioreactor was about 1.58×10^8CFU/ml (OD=7.85). When the cell charging was complete the MBR was fed continuously with synthetic wine at 2.4 l/h for 2 hrs, and then at 0.48 l/h for 10 hrs to flush out the growth medium. The flow rate of synthetic wine was controlled at 0.48 l/h for the rest of experiment. The MBR was operated at 25°C. Samples were taken regularly and analysed by HPLC. After running the MBR continuously for 72 hrs, the fermentation was stopped.

Figure 7.12 shows the effect ethanol stress adaptation on the malic acid consumption of *O. oeni* in synthetic wine. The cells of *O. oeni* that had undergone ethanol stress adaptation showed similar malic acid consumption with those of the control during the

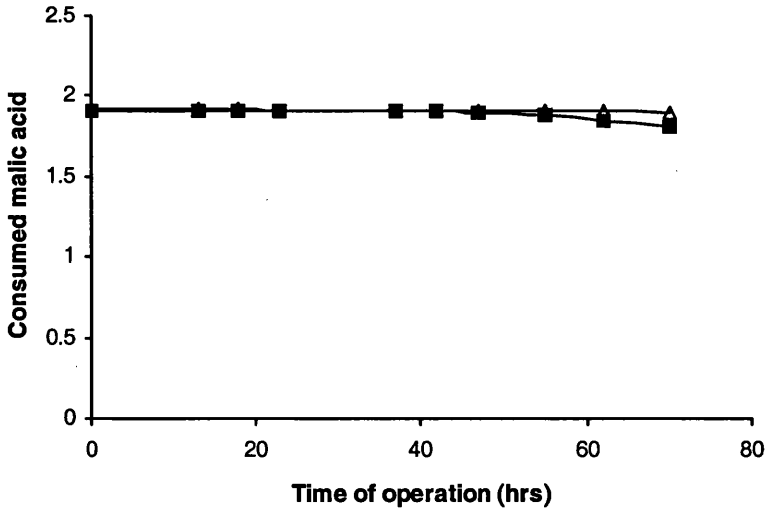


Figure 7.12 Effect of ethanol stress adaptation on malic acid consumptions of *O. oeni* in synthetic wine. The synthetic wine passed continuously through the MBR charged with the cells of *O. oeni.* Operating conditions: temperature 25°C; malic acid in synthetic wine 2.0 g/l; the flow rate 0.48 l/h; the cell density: 1.6×10^8CFU/ml (the control, ■) and 1.6×10^8CFU/ml (the cells underwent ethanol stress adaptation, Δ). The raw data is in Appendix-5.10.

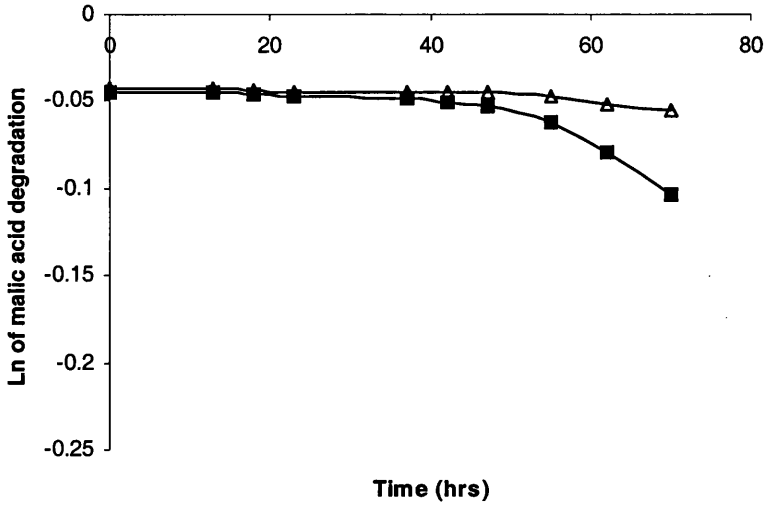


Figure 7.13 Effect of ethanol stress adaptation on maic acid degradations of *O. oeni*. The synthetic wine passed continuously through the MBR charged with the cells of *O. oeni*. Operating conditions: temperature 25°C; malic acid in synthetic wine 2.0 g/l; the flow rate 0.48 l/h; the cell density: 1.6 × 10^8CFU/ml (the control, ■) and 1.58 × 10^8CFU/ml (the cells underwent ethanol stress adaptation, Δ).

first 37 hours of continuous operation. However, after running 62 hours the malic acid degrading activity of the control was lost faster than that of cells which underwent ethanol stress adaptation. At the end of 72 hours continuous operation, the malic acid consumption of the control was 1.802 g/l whereas that of cells with the ethanol stress adaptation was 1.892 g/l. The effect of ethanol stress adaptation on the malic acid degradation (in natural logarithms) is shown in Figure 7.13. It was clearly shown that the malic acid degradation of *O. oeni* with ethanol stress adaptation was obviously higher than that of the control after 72 h continuous operation, and ethanol stress adaptation could enhance the stability of malic acid degrading activity of *O. oeni* in the MBR. If the operating time had been longer, the difference may be enhanced.

7.4 Discussion

Batch MLF

Wine has a complex composition with variable levels of carbohydrates, pH, SO_2, ethanol, phenolic compounds, fatty acids, amino acids, micronutrients, which depend on several variables, such as cultivation, season, technology of wine production, etc

(Versari *et al.*, 1999). The composition of wine affects the ability of malolactic bacteria to carry out MLF.

After alcoholic fermentation, wine normally contains very low amounts of sugars (glucose, fructose, arabinose, xylose, and galactose). Sugars in wine significantly affect the MLF by malolactic bacteria. It was found in this work that when the synthetic wine did not contain any sugars malic acid degradation of *L. brevis* was low, only 30%. While in wine containing small amounts of glucose (2 g/l), malic acid degradation by *L. brevis* was 63.9%. The type of sugar in wine also influenced the MLF. When the available sugar was fructose, malic acid degradation by *L. brevis* and *O. oeni* was much higher than that when the available sugar was glucose. In addition, the effect of sugars in wine on MLF depended on the species of malolactic bacteria. When the synthetic wine did not contain any sugars, malic acid degradation by *O. oeni* was still high (94.4%), whereas malic acid degradation by *L. brevis* was low (30.0%).

Miranda *et al* (1997) reported that glucose had an inhibitory effect on the malolactic activity of *O. oeni*. When fructose was present the inhibition of malolactic activity caused by glucose was completely relieved. In this work similar results were obtained from *O. oeni*. However, when synthetic wine contained both glucose and fructose, the malolactic activity of *L. brevis* still decreased, compared with synthetic wine that contained only fructose. Miranda *et al* (1997) also examined four other strains of *O. oeni* (M3, 8A, LOD004, and LOD017) for glucose-induced inhibition of malolactic activity. Glucose inhibited the malolactic activity in the first two organisms, whereas no effect was found in strains LOD004 and LOD017. So glucose-induced inhibition of malolactic activity depends on the species or strains of malolactic bacteria.

Malic acid is one of main acids in wines. Low pH is considered one of the main inhibitory factors on the cell growth and malolactic activity (Pimentel and Silva, 1994). It was observed in this work that *L. brevis* was very sensitive to pH stress. When wine pH was below 3.72, the MLF by *L. brevis* was inhibited strongly. However, pH 3.08 only slightly inhibited the MLF by *O. oeni*. *O. oeni* was more resistant to pH stress than *L. brevis*. But as reported (Lonvaud-Funel, 1995), wines with pH below 3.0 displayed a strong inhibitory effect on the MLF by *O. oeni*.

The synthetic wine (1 g/l malic acid, pH 3.91, see Table 7.3) had a pH similar to that of the control (3 g/l malic acid, pH 4.0), but the malic acid degradations of *L. brevis* and *O. oeni* were lower than those of the control. This suggests that malate stimulates

malolactic activity, and the extent of the stimulation depends on the malate concentration and the species. Loubiere *et al* (1992) and Henschke (1993) also found that malate stimulated cell growth and malolactic activity of *O. oeni.*

Malolactic bacteria are known to have complex nutritional requirements. The nutritional conditions in wine depend on grape varieties, harvest season, processing technology and so on. It has been reported that amino acids are important for growth, both as nitrogen and carbon sources (Tracey and Britz, 1989). Depending on the bacterial strains, the amino acids arginine, glutamic acid, cystine, valine, isoleucine, tryptophan and tyrosine are essential for cell growth of *O. oeni.* Glycine, phenylalanine, proline and tyrosine stimulate MLF but not cell growth (Henschke, 1993; Versari *et al.*, 1999). In this work, it was observed that the nutritional status affected MLF of *L. brevis* and *O. oeni* (Table 7.5). When synthetic wine was low in nutrients (0 g/l YE), malic acid degradations were low. However, when yeast extract was added to synthetic wine malic acid degradations by *L. brevis* and *O. oeni* increased drastically. The increase in malic acid degradation might be due to two reasons: (1) the increase in cell concentration stimulated by yeast extract. Addition of yeast extract to wine would generally ensure that sufficient nutrients were available for cell growth of malolactic bacteria. (2) Yeast extract contained amino acids that could stimulate MLF. When concentrations of these amino acids were low in wines, the malolactic activity was inhibited. Fourcassie *et al* (1992) reported that deficiencies of leucine, histidine, or valine decreased malic acid degradation by *O. oeni*, although deficiency such as proline or tyrosine had no influence on cell growth. The lack of these amino acids could limit transport of lactic acid across the cell membrane and influences malolactic conversion.

Continuous MLF in MBR

In this work, rapid and continuous malolactic fermentation was achieved using high cell density (greater than 10^8CFU/ml) in the MBR. More than 95% degradation of malic acid was obtained at 0.48 l/h flow rate and 10.4 h residence time. It can be expected that the flow rate of wine may be increased and the residence time shortened if a higher cell concentration (such as 1×10^{10}CFU/ml) is used.

It is well documented that the conduct of MLF by conventional fermentation processes is influenced by factors such as the strains, temperature, yeast species associated with the wine, and wine properties, especially pH and ethanol concentration (Wibowo *et al.*, 1988; Henschke, 1993; Versari *et al.*, 1999). These factors may also be significant in the performance of a MBR. To better understand which wine properties affect the stability of a MBR system, a synthetic wine was used, and its composition was varied for this work.

Because production of high cell concentrations of *O. oeni* is a significant cost, long-term stability of the malic acid degrading activity of cells in a bioreactor is very important for economical use of this technology in practical wine production. How to maintain the activity of the reactor is a significant problem.

According to results obtained in this work, high ethanol concentration in synthetic wine caused significant loss in stability of malic acid degrading activity by *O. oeni* in the MBR whereas low pH and poor nutritional status did not under the conditions investigated.

The membrane bioreactor used a high cell density (OD=8.04, about 1.7×10^8 CFU/ml). So supply of nutrients may be needed to keep the cells in an active state. Surprisingly, it was found that when the concentration of yeast extract in synthetic wine was reduced from 4.0 g/l to 0.2 g/l, the malic acid degrading activity of *O. oeni* hardly changed (see Figure 7.10) under the conditions investigated. The possible reasons were: (1) as there were high cell densities in the MBR, and cell growth was not necessary for MLF, the nutrients (0.2 g/l yeast extract in synthetic wine) were sufficient to keep the cells in an active state; (2) the energy generated form MLF was used to keep the cells active.

The cells of *O. oeni* with ethanol stress adaptation showed higher stability of malic acid degrading activity in the MBR (Figure 7.14). Teixeira *et al* (2002) observed that cell membrane composition of *O. oeni* was dependent on ethanol concentration and cell physiological state. The protein electrophoretic profile in cell membrane of *O. oeni* was modified when the cells were cultured in the presence of 8 and 10% ethanol. *O. oeni* maintained a high level of phospholipid biosynthesis via the relative increased biosynthesis of phosphoethanolamine and sphingomyelin in the presence of ethanol. Conversely, ethanol induced an increase in membrane lactobacillic acid at the expense of cis-vaccenic acid. This increased synthesis of lactobacillic acid appears as the more significant change induced by ethanol in *O. oeni* membrane. The increase of

lactobacillic acid in the membrane of *O. oeni* clearly appears as a factor that provides protection against the toxic effect of ethanol, balancing the increase of membrane fluidity normally attributed to ethanol. Lactobacillic acid may have a part in the survival and/or adaptive mechanisms developed by *O. oeni* under adverse conditions, allowing these bacteria to maintain their MLF activity in the presence of ethanol. Ethanol (10%) also decreased the unsaturated/saturated fatty acid ratio in the microbial membrane (Versari *et al.*, 1999). Da Silveira *et al* (2002) reported that the subpopulation of *O. oeni* cells that maintained their membrane integrity was three times larger in the population grown in the presence of ethanol, reflecting the protective effect of ethanol adaptation. So prior adaptation to ethanol stress can be beneficial to maintain the stability of the MBR.

There is considerable shear stress created by centrifugal pumps when pumping the cell suspensions in a MBR. As shown in Figure 7.4, when the ethanol concentration in the wine was 5%, the malic acid degradation of *O. oeni* almost did not decline during 72 hrs continuous operations. This result suggests that the shear stress had little influence on malic acid degrading activity of *O. oeni* under the conditions investigated.

In this work, the stability of malic acid degrading activity of *O. oeni* was good (malic acid degradation $\geq$ 80% after 72 hrs of continuous operation), even at an ethanol concentration of 13%. The main reasons may be: (1) the big volume of the reactor (5 L). The residence time in the reactor was 10.4 h (at the flow rate 0.48 l/h). Longer residence time corresponds with more reaction time. (2) The operation time was not long enough (72 hours) to observe the performance of MBR.

In conclusion, use of membrane bioreactor technology for the continuous, rapid malolactic fermentation in wines is attractive to the wine industry, as it offers the potential for more reliable and predictable control over the MLF in wines. Long-term stability of malic acid degrading activity is very necessary for commercial application. The results in this work clearly showed that the ethanol concentration in wine was the main factor causing loss in malic acid degrading activity by *O. oeni*. Although ethanol stress adaptation can improve the stability of malic acid degrading activity by *O. oeni*, other measures are still needed. Small concentrations of sugars, organic acid and tannins may enhance the stability (Donovan, 2000). In addition, a synthetic wine, not real wine was used in this experiment. So more research is still required.

Chapter 8. General Discussion

8.1 Introduction

The primary aim of this investigation is to study the preservation and utilization of the lactic acid bacteria (*L. plantarum*, *L. brevis* and *O. oeni*), especially those involved in MLF. These LAB have industrial significance associated with silage and maturation of alcoholic beverages. Effective utilization of these bacteria is based upon the use of high vitality cultures so as to produce good quality of products rapidly and economically. Normally, such cultures are preserved and handled as freeze-dried preparations. Much of the work in this thesis concerns the investigation of the factors that influence vitality, especially those associated with preservation by freezing and freeze-drying.

In this context, the following work has been carried out:

1. Development of a simple method to assess cell vitality
2. The effect of cultural conditions on cryotolerance of LAB so as to produce the cells that had high vitality after freezing and freeze-drying
3. Investigation of conditions to produce the cultures of LAB with high vitality after freeze-drying
4. Investigation on ethanol tolerance of *L. brevis* and *O. oeni*
5. Investigation of MLFs of *L. brevis* and *O. oeni* in batch culture to obtain more knowledge on MLF
6. Rapid and continuous MLFs in synthetic wine were carried out in a microfiltration MBR. This investigation focused on the stability of malic acid degrading activity by *O. oeni* in the MBR.

The results of this investigation obtained have significances in practical application.

8.2 A Simple Method to Assess Cell Vitality after Freezing and Freeze-Drying

Freezing and freeze-drying are used to preserve lactic acid bacteria during production of starter cultures. During these processes the bacterial cells may be damaged or killed. So it is necessary to evaluate the quality of the starter culture after freezing and freeze-drying. There are many methods that have been developed to assess viability. Table 8.1 (from Breeuwer and Abee, 2000) shows criteria and methods for the assessment of viability of microorganisms. However, these methods are time consuming and tedious to perform, and require high cost equipment such as flow cytometry. Development of simple and reliable method for evaluation was still necessary.

Viability is typically defined as the ability to reproduce and form colonies in a suitable medium under favourable conditions. For example, viability means what proportions of the cells are viable after freeze-drying. While cell vitality as described here is the capacity to overcome and recover from freezing and freeze-drying, such as the growth ability and acidification capacity. For the user of the starter cultures, vitality may be a better indicator than viability because the cells with high viability may not have high growth ability or acidification capacity.

Generally, under the same cultural conditions such as cell concentration, medium composition, pH, and temperature, the more viable cells present in a medium, the faster the cell cultures will grow. A simple method to assess cell vitality after freezing and freeze-drying was developed in this work according to the principle above.

The cells after freezing and freeze-drying were inoculated in the same fresh media and cultivated at the same conditions. The cell growth was monitored by measuring the optical density in a spectrophotometer. The cell vitality was assessed by intercomparing of observed growth curves of the treated cells. If the samples contain the same cell concentrations, then the sample with higher growth rate has a higher vitality. An experiment to compare vitality with viability has been done. The results (see Chapter 3) proved that assessment of vitality by intercomparing growth curves was feasible and reliable. The advantage of this method is that it is simple and easy to do, needing simple culture tubes and a spectrophotometer, which are available in almost all laboratories. Another

Table 8.1 Criteria and methods for the assessment of viability of microorganisms

Criterion	Method	Time	Comments
Reproduction	Plate count method	2–5 days	High sensitivity
Cell morphology (cell elongation)	Inhibition of cell division by nalidixic acid or other antibiotics	6 h	Only for antibiotic-sensitive bacteria, microscopic analysis elongated cells
Membrane integrity	Dye exclusion methods, e.g., methylene blue labeling, influx DNA probes	30 min	Viable cells with an intact cytoplasmic membrane will not be stained
Respiration	Reduction tetrazolium dyes in cells with an active electron transfer chain	1–4 h	Accumulation of insoluble formazan products. Not applicable for fermentative microorganisms
Enzyme activity	Fluorescein diacetate method (esterase activity)	30 min	Fluorescein is accumulated in intact cells
Membrane potential (negative inside)	Distribution of Rhodamine 123, carboxycyanine dyes, and oxonols	1 h	Potential dependent uptake or exclusion of dye
pH gradient	Intracellular pH measurement using, e.g., fluorescein derivatives	1 h	Viable cells maintain pH gradient

major advantage is that vitality can be represented in numbers by analysing the growth curves, and it is very convenient to compare the results. But intercomparing growth curves to assess vitality is a measure of relative performance against a control. This method was used throughout this project and demonstrated directly the potential of the cultures.

8.3 Production of the Freeze-Dried LAB with High Vitality

As stated in the introduction, many factors can affect the vitality of LAB during freeze-drying process. Freeze-drying is an expensive process because of its low drying rate, high capital and energy cost due to refrigeration and vacuum units. So it is very

important to produce freeze-dried starter cultures of LAB with high vitality. Many methods have been investigated to enhance the survival rate after freeze-drying and are summarized in Table 8.2. It is hard to see a coherent general strategy for LAB preservation, rather specific treatments for each bacterium seem to be more effective.

8.3.1 Enhancing the Cryotolerance of the LAB

Cells of LAB may be damaged or killed during freezing and freeze-drying processes. Sometimes the mortality is very high. For example, the viability of cells of *L. plantarum* was only 5.2% (see Table 3.1) after freezing at -20°C for about 48 hrs. So it is critical to produce cells with high cryotolerance to obtain highly productivity for the process. It has been known that many factors such as growth phase status, growth temperature and the medium composition influence the cryotolerance of microorganisms, and different species of microorganisms have different responses to freezing stress.

In this work, the effect of cultural conditions on the cryo-tolerance and vitality of LAB were investigated. The cells of three LAB cultivated at sub-optimal temperature such as 25°C and grown in stationary phase possessed higher vitality after freezing, which agrees with other authors (Lorca and de Valdez, 1999; Graciela *et al.*, 1999). Culture pH significantly affected cell vitality. The three LAB had the highest cell vitality after freezing when the pH of culture medium was kept at pH 5. O'Sullivan *et al* (1997) observed that pH 5 could trigger a stress response in some LAB. This could explain the high vitality observed in this work.

The composition of culture medium also affected the vitality of LAB after freezing. When calcium and Tween 80 were added to the culture media, the LAB showed higher vitality. Other authors (Goldberg and Eschar, 1977; Wright and Klaenhammer, 1981 and 1983a; Gomez Zavaglia *et al.*, 2000) obtained similar results from other species of LAB. Little research has been carried out on the effect of manganese on the cryotolerance of LAB. In this work, when the media were supplemented with manganese, the cell vitality was improved. Yeast extract contains many amino acids and vitamins. When the media contained relatively high concentration of yeast extract, the cell survival

Table 8.2 Methods to enhance cryo-tolerance and viability of microorganisms

Methods	Treatments	Microorganisms	Authors
Application of preincubation treatment	preincubated at suboptimal temperature with a fermentable sugar.	*L. delbrueckii*	Urraza and Antoni, 1997
Application of cold shock	Kept at low temperature for some time such as10°C for 2 hours	*Lactococcus lactis*	Broadbentl and Lin, 1999
Manipulation of Growth conditions	1. Grown at sub-optimal temperature e.g. 25-30°C	*Streptococcus lactis*, *L. acidophilus*	Goldberg and Eschar, 1977; Lorca and de Valdez, 1999; Murga *et al.*, 2000;
	2. Supplement in the medium with Ca^{2+}, Tween 80	*L. acidophilus*, *L. bulgaricus*	Wright and Klaenhammer, 1981, 1983a and 1983b
	3. pH-controlled cultivation	*L. reuteri*	Palmfeldt and Hahn-Hägerdal, 2000
Addition of Protective agents	1. Polysaccharides: pectin 5%	*L. acidophilus*, *L. bulgaricus*	Kilara *et al.*, 1976
	2. Disaccharides: trehalose, lactose, maltose, sucrose e.g. 10-15%, w/v	*S. cerevisiae*, *L. salivarius*, *E. coli*, *L. bulgaricus*, *L. acidophilus*, *Pantoea agglomerans*; *L. salivarius*,	Lodato *et al.*, 1999; Zayed and Roos, 2004; Leslie *et al.*, 1995; Carvalho *et al.*, 2003b; Kilara *et al.*, 1976; Costa *et al*, 2000; Zayed and Roos, 2004
	3. polyols: glycerol, sorbitol, mannitol, adonitol. e.g. 5-10%, w/v	*L. acidophilus*, *L. bulgaricus*, *Strep. Thermophilus*, *Lb. Plantarum*, *Ent. faecalis*	Font de Valdez *et al*, 1983a; 1983b; Kilara *et al.*, 1976; Carvalho *et al.*, 2003c
	4. Proteins: skimmed milk, gelatin e.g. 5-10%, w/v	*Lactococcus lactis*, *lactic streptococci*, *Candida sake*, *S. platensis*	Cárcoba and Rodríguez, 2000; El-Sadek *et al*, 1975; Abadias, 2001a; Takano, 1973;
	5. Amino acids: glutamate, proline e.g. 1-5%, w/v	*Lactococcus lactis*, *L. acidophilus*, *Candida sake*, *E. bicyclis*	Cárcoba and Rodríguez, 2000; Kilara *et al*, 1976; Abadias *et al*, 2001b Kono *et al.*, 1998
Lower frozen temperature	Frozen at liquid N_2 (-79°C)	Root nodule bacteria	Safronova and Novikova, 1996

rates after freezing were significantly increased. Such research has not been reported elsewhere.

Preincubation before freezing is another approach to improve the cryotolerance of lactic acid bacteria. Urraza and Antoni (1997) found that the protective effect of preincubation (30°C, 60 min) for *Lactobacillus delbrueckii subsp. bulgaricus* was obtained only when it was performed in medium with a fermentable sugar, such as glucose or lactose. But it was found in this work that when the cells of *L. plantarum* were preincubated in 5 g/l yeast extract solution at 25°C for 1 hour, the survival rate greatly increased, from 5.2% to 46.5%. Similar results were not obtained from *L. brevis* and *O. oeni*. It has been demonstrated that amino acids in yeast extract played a role in the preincubation of *L. plantarum* to increase the cryotolerance. Preincubation in yeast extract solution could be applied to practical starter production to enhance the vitality of *L. plantarum* during frozen storage.

8.3.2 Enhancing Vitality of the Freeze-Dried LAB

Freeze-drying has been studied as a dehydration process for bacteria in order to achieve a solid, stable formulation. Freeze-drying is a more complex process, including freezing and drying. The bacterial cells may be damaged or killed during these processes. A number of factors, such as cultural conditions, freezing medium, freezing rate and initial cell density, influence the vitality of freeze-dried cell cultures, together with subsequent storage conditions including temperature, storage atmosphere, exposure to light and relative humidity. In order to obtain freeze-dried LAB with high vitality, screening such as suspension medium, preincubation and frozen temperature was carried out.

As previously described, the freeze-drying medium should prevent damage, improve storage stability, and facilitate rehydration. In this work, sugars (sucrose, trehalose, maltose, lactose, glucose), polyols (sorbitol, mannitol), amino acids (sodium glutamate, yeast extract) were tested for their protective actions. It was found that 10% lactose, 4% yeast extract and 5% glutamate were the most effective protectant for *L. plantarum*, *L. brevis* and *O. oeni* respectively, and their protection varied with the species of LAB. The results proved that for any given microorganism, there are only

one or two suitable cryoprotective agents (Theunissen *et al.*, 1993). The concentration of these agents influenced their protection for ALB during freeze-drying.

Champagne *et al* (1991) reported that freeze-drying of acid cultures is detrimental to survival. Similar results were obtained in this work. But the pH of sugar suspension had little effect on the vitality of *L. plantarum* and *L. brevis*. Removal of the growth medium greatly influenced the vitality after freeze-drying. When the growth medium was removed the vitality of *L. plantarum* after freeze-drying increased greatly. This was probably due to the relatively high concentration of sodium lactate that was toxic for the cells (Champagne *et al.*, 1991). However, the situations with *L. brevis* and *O. oeni* were opposite. The reasons for this were not clear. Perhaps, the effect of sodium lactate on the cell vitality, positive or negative, was the species dependent.

Research in recent years has demonstrated that bacteria possess remarkable resilience and adaptability in unfavorable environments and that survival under adverse conditions is frequently enhanced by the induction of a stress response (Jones and Inouye, 1994; Broadbentl and Lin, 1999; Lorca and de Valdez, 1999; Sanders *et al.*, 1999). Induction of cold shock response may enhance microbial resistance to freeze-drying. As expected, preincubation of the cells of *L. plantarum* at low temperature (10°C) before freezing greatly improved cell vitality after freeze-drying. However, the effect of preincubation was species dependent as well as preincubation temperature and time. *L. brevis* and *O. oeni* did not acquire freezing resistance by preincubation before freeze-drying.

It has been reported that low initial cell concentration is detrimental for freeze-drying survival of microorganisms (Kilara *et al.*, 1976; Tsvetkov and Brankova, 1983; Costa *et al.*, 2000; Palmfeldt *et al*, 2003). As high cell concentrations are necessary for starter production, it is useful to increase the initial concentration as much as possible to optimize the industrial process. In this work, the cell cultures of LAB were concentrated rather than diluted to examine the effect of initial cell concentration on the vitality after freeze-drying. When the cell cultures were concentrated, the vitality of *L. plantarum* and *O. oeni* decreased. Only *L. brevis* was observed to show increase of the cell vitality as the cell concentration increased. According to this result, too high initial cell concentration is harmful for vitality of the LAB after freeze-drying. Costa *et al* (2000) and Palmfeldt *et al* (2003) reported that the optimal initial cell concentration

of microorganism was related to the protective medium used. The effect of suspension medium on the optimum initial cell density of LAB was not investigated in this work.

When cells of *L. plantarum*, *L. brevis* and *O. oeni* were frozen at -65°C, the vitality obtained was higher than those frozen at -20°C after freeze-drying. Rapid cooling minimized the solute concentration effects as ice forms uniformly; intracellular ice crystals formed were very fine and might cause less damage. So cell vitality was enhanced when the cells of LAB were frozen at -65°C.

Rehydration is a critical step in the recovery of freeze-dried bacteria. If using appropriate rehydration conditions the injured cells may be able to repair the damage (Champagne *et al.*, 1991). In this work, it was also found that rehydration had a great influence on the cell recovery. For instance, rehydration in 10% sucrose significantly improved the recovery of freeze-dried cells of *L. plantarum*. However, cell recovery of *O. oeni* was reduced when freeze-dried cells were rehydrated in 10% sucrose. These results proved that the medium itself, its molarity and the rehydration conditions significantly affect the rate of recovery (Ray *et al.*, 1971; Font de Valdez *et al.*, 1983a). No one rehydration medium was suitable for all LAB. So for a given lactic acid bacterium, the optimum rehydration medium should be selected by testing.

After freeze-drying, the storage of freeze-dried cells should receive serious consideration. It was found in this work that 5% glutamate and 10% sucrose were very effective protectants for *L. plantarum* during freeze-drying. But after 3 months storage it was found that their protection was reduced. This agreed with the findings that many compounds offer protection to freeze-drying itself, but do not prevent viability losses of LAB during storage (Kilara *et al.*, 1976; Font. de Valdez *et al.*, 1983a and 1983b; Champagne *et al.*, 1996). So the storage test after freeze-drying was necessary to decide which protectant was the best, although it was also found that 4% yeast extract and 5% glutamate were still the best protectants for *L. brevis* and *O. oeni* after freeze-drying and storage.

8.3.3 Productivity of Preservation Processes

As stated in the introduction, freeze-drying is an expensive dehydration process. High productivity is very important in practical production of starter cultures. However, the

microbial cells can be damaged or killed during freeze-drying process, and cell viability and vitality decreases. Low vitality means low productivity. For example, if 0.5 kg of active starter cultures is produced by freeze-drying, when the viability after freeze-drying is 100%, if for example, the 20 L cell cultures are needed; when the viability is 50% 40 L cell cultures are then needed; when the viability is 10% 200L cell cultures are required. The lower the viability and vitality, the more cell culture is required to obtain the same productivity. It can be seen from the example above that high viability and vitality are key factors to obtain high productivity in the preservation processes. As summarized in Figure 1.3, many factors affect the viability and vitality of the microbial cells after freeze-drying. Optimisation of freeze-drying conditions of LAB is therefore very important for economic production.

8.4 Understanding More about MLF

As stated in introduction, MLF is an important process in wine production. Although intensive researches on MLF have been carried out, the process of MLF is only partially understood and difficult to predict. The performance of different strains in wines may be greatly affected by the composition of wines, vinification procedures and the interrelationship with other organisms (Martineau and Henick-Kling, 1995). Therefore, an improved knowledge of MLF is essential to control this important process.

8.4.1 Improving Ethanol Tolerance of the Malolactic Bacteria

A factor to be considered important is ethanol tolerance when freeze-dried malolactic bacteria are used because the loss of viability was very high if the cultures are inoculated directly into wines (Nault *et al.*, 1995; Nielsen *et al.*, 1996). The freeze-dried malolactic bacteria with high ethanol tolerance are important to induce rapid and successful MLF in wines.

In this work, it was found that temperature not only affected the cell growth in wines, but also the cell resistance to ethanol stress. *L. brevis* and *O. oeni* grown at sub-optimal growth temperature (such as 25°C) displayed the most resistance to ethanol stress. The inhibitory effect of ethanol depended on species of bacteria, ethanol concentration and

growth temperature. Apart from ethanol, wine also contains many other components such as organic acids and amino acids. It was also found that the presence of pyruvic acid and citrate could improve the ethanol tolerance of *L. brevis* and *O. oeni*. This might be directly related to electron flow pathways in those organisms such that pyruvic and citrate represent material that can be reduced.

The effect of suspension medium on ethanol tolerance of *L. brevis* and *O. oeni* was investigated in this work. It was found that the suspension medium affected greatly the ethanol tolerance of *L. brevis* and *O. oeni*. Among the suspension media investigated, 5% glutamate and 10% sucrose showed the greatest ability to improve the ethanol tolerance of *L. brevis* and *O. oeni* respectively. Especially, the freeze-dried cells of *L. brevis* with 5% glutamate had a similar growth rate with the fresh cells (see section 6.4, Chapter 6), and the cells of *L. brevis* freeze-dried with 5% glutamate may be introduced directly into wine if the ethanol concentration of wine is not over 10%. Compared with the fresh cells, the freeze-dried cells showed lower growth rate, even when protective agents were used. It proved that cells were damaged or injured during freeze-drying, and the cell survival decreased (Jay, 1978; Panoff *et al.*, 1998; Wolfe and Bryant, 1999). So pre-culture or reactivation is necessary.

Several methods for pre-culture of freeze-dried malolactic bacteria have been proposed to restore the loss of viability resulting from freeze-drying (Beelman *et al.*, 1977; Davis *et al.*, 1985; Krieger *et al.*, 1992; Nault *et al.*, 1995). The media recommended are normally based on grape juice or apple juice containing yeast extract. The use of fruit juice in the industrial production of starter cultures is cumbersome and expensive since large volumes of juice must be obtained, stored, and protected against microbial spoilage. Also, the supply of fresh juice without preservatives is seasonal and the quality varies over the course of the growing season. The use of a synthetic medium composed of dry ingredients of consistent quality was investigated in this work. Among the preculture media investigated, it was found that when freeze-dried cells of *L. brevis* and *O. oeni* were precultured in CME media (1 g/l citrate, 1 g/l malic acid, 5 g/l YE, 5 g/l fructose, 5% ethanol) for 48 h, the cells had the highest ethanol tolerance. The possible reasons were: (1) CME medium contained citrate and malate. Citrate and malate could stimulate the cell growth (Schmitt *et al.*, 1992; Salou *et al.*, 1994) and were favourable to repair and recover the damage or injury caused by freeze-drying. (2) CME medium contained 5% ethanol, and the cells had an ethanol stress adaptation

that could improve the ethanol tolerance of malolactic bacteria (Nault *et al.*, 1995; Teixeira *et al.*, 2002). Even though undergoing preculture for 48 h, the precultured cells of *L. brevis* and *O. oeni* still had a lower growth rate than fresh cells. The reasons may be that the preculture time was not long enough and the cells were not recovered totally.

8.4.2 MLF in Alcoholic Environment

The process of MLF in wine is very complex. It is only partially understood and difficult to predict (Versari *et al.*, 1999). Individual strains exhibit variable MLF performance in different wines (Wibowo *et al.*, 1988). This phenomenon can be explained by the fact that survival and growth of strains of *O. oeni* vary considerably in response to aspects of wine composition, such as pH, and the concentration of ethanol and total SO_2. This phenomenon encouraged research into the effect of components of wine on MLF. In order to conveniently investigate the effect of wine components on MLF, a synthetic wine was used (see section 7.2, Chapter 7).

After alcoholic fermentation, malolactic bacteria can utilize the residual sugars for cell growth. When glucose was used as sole substrate, the cell growth of *O. oeni* was poor while *O. oeni* grew fast when available sugar was fructose (Maicas *et al.*, 1999c, Krieger *et al.*, 1992). In this work the same phenomenon was also observed. However, *L. brevis* still grew well in synthetic wine that just contained glucose. MLF was also found to be sensitive to the type of sugar present. When the available sugar in synthetic wine was fructose, malic acid degradations of *L. brevis* and *O. oeni* was much higher than those when the available sugar was glucose. Glucose-induced inhibition of malolactic activity reported by Miranda *et al* (1997) was also observed in this work. When fructose was present, the inhibition of malolactic activity of *O. oeni* caused by glucose was completely relieved. However, similar phenomenon did not happen with *L. brevis*. The effect of the ratio between glucose and fructose needs be further investigated. The ratio might be important for controlling malolactic fermentation.

Ethanol not only affected cell growth, but also affected malolactic activity of the cells. When ethanol concentration in synthetic wine was 12%, the malic acid degradation of *L. brevis* after MLF was very low (see Table 7.4). But 12% ethanol hardly influenced malolactic activity of *O. oeni*. This phenomenon is complex and depends on the

contribution of a number of underlying mechanisms. According to Jiménez and van Uden (1985), the interference of ethanol with membrane-bound processes plays a dominant role. Moreover, the lipids are main site of ethanol toxicity.

Malic acid is one of main acids present in wines. Low pH is considered one of the main inhibitory factors on the cell growth and malolactic activity (Pimentel and Silva, 1994). Similar results were obtained in this work. So pH is more important than malic acid concentration for controlling MLF.

Phenolic compounds are abundant in wines being extracted from the initial grape material (skins, seeds and stalks) and from wood used for storage. Compounds of this group contribute to the sensory characteristics and chemical qualities of wine both directly and indirectly, through their interactions with other molecule types, e.g. proteins, polysaccharides and other polyphenols (Reguant *et al.*, 2000; Campos *et al.*, 2003; Rozes *et al.*, 2003). The effect of phenolic compounds can be either stimulatory or inhibitory in terms of the bacterial growth and MLF, depending on bacterial species, specific phenolic acids and their concentrations.

Malolactic bacteria have complex nutritional requirements (Tracey and Britz, 1989; Versari *et al.*, 1999;). Shortage of nutrients in wine will affects the bacterial growth and MLF. In this work, as expected, when the synthetic wine with no added yeast extract, malic acid degradation of *L. brevis* and *O. oeni* was low. When yeast extract was added in synthetic wine, the malic acid degradation increased drastically. So nutritional status is an important factor affecting MLF. To stimulate MLF, some measurements should be done such as extended contact time with yeast lees to encourage autolytic release of nutrients for bacterial growth, and long skin contact time (Davis *et al.*, 1985).

8.5 Rapid and Continuous MLF in Membrane Bioreactor

MLF depends upon the growth of LAB in wines as a batch culture and is strongly influenced by environmental conditions, so that the process is often delayed or fails. Those problems have driven the search for alternative technologies that enable more rapid and reliable MLF to take place (Gao and Fleet, 1995). The induction of MLF with high levels of cells enables better control of the process as well as guaranteeing

that it takes place at the required time during wine production (Gao and Fleet, 1994; Maicas *et al.*, 1999c). MBR may achieve the aim.

In this work, the MBR charged with high densities of cells of *O. oeni* (about 1.6 × 10^8CFU/ml) could carry out a rapid MLF. When the synthetic wine flow rates were less than 0.48 l/h, rapid and continuous MLF were achieved, and the degradation of malic acid was greater than 95%.

If this technology is applied in commercial wine production, the long-term stability of malic acid degrading activity of *O. oeni* in the MBR is a major consideration because the production of high concentration of cells of *O. oeni* is a significant cost. The stability of malic acid degrading activity of *O. oeni* in the MBR was investigated. The results in this work clearly showed high ethanol concentration of synthetic wine was the main factor that caused loss in malic acid degrading activity of *O. oeni*. Low pH (less than pH 3.0) inhibited the malic acid degradation of *O. oeni* but hardly influenced stability of malic acid degrading activity of *O. oeni* in the MBR. Poor nutritional conditions (yeast extract concentration) also had no significant effect on the stability under the conditions tested. According to the results, improvement of ethanol tolerance of *O. oeni* should be main measurement to enhance the stability of the system. The cells of *O. oeni* cultivated in the medium that contained 5% (v/v) ethanol showed higher stability of malic acid degrading activity. A supplement of pyruvic acid and citrate (also commonly found in green wine) in the medium slightly improved the stability of malic acid degrading activity of *O. oeni*.

In this work, the stability of malic acid degrading activity of *O. oeni* was good under the conditions investigated, even though the ethanol concentration was 13% (the loss was 14.8% in 72 h). The main reasons may be: (1) the large volume of the reactor (5 L), the residence time in the reactor was 10.4 h (at the flow rate 0.48 l/h). Longer residence time gave more reaction time. The volume of reactor should be important aspect for the design of MLF to carry out rapid and continuous MLF. (2) The operation time was not long enough (72 hours) to observe the change in stability. In addition, the malic acid degradation should increase if the MBR is charged with higher cell density such as 1×10^{10} CFU/ml.

The bioreactor used in this work was a cross-flow microfiltration MBR. The membrane is made from ceramic and can be sterilized conveniently by steam. The

style of cross-flow of membrane module can minimize the membrane fouling. The use of two pumps in the MBR was also to minimize the membrane fouling by increasing the flow rate in the membrane module. A synthetic wine was used in this work. Serious membrane fouling did not occur, and the bioreactor could operate at flow rates from 0.48-2.4 l/h. However, the composition of real wines is far more complex than the synthetic wine used. Crude wine following alcoholic fermentations is a multi-component system including numerous solutes (organic acids, salts and polyphenols), macromolecules and colloidal size-range aggregates and particles, microorganisms (yeast and LAB) and various large particles such as cell debris (Belfort *et al.*, 1994; Vernhet *et al.*, 2003). Membrane fouling would most probably be a significant problem. The consequence of it is a reduction in permeation rates, affecting the economic viability of the process, and a risk of excessive polysaccharide and polyphenol retention together likely with changed in product organoleptic quality (Czekaj *et al.*, 2000). Fouling not only depends greatly on the wine processed, but also on the membrane and process conditions.

Limitations of recycle bioreactors include possible shear stress on the cells entering the filtration unit (Maicas, 2001). Shear can obviously be a disrupting factor in microorganism culture. Shear effect will depend on the resistance of the cell to mechanical forces and the extent of deformation it can stand undamaged, on the levels of requirements of nutrients and on the potentially detrimental effects of metabolites in each organism (Merchuk, 1991). In this work, when ethanol concentration in synthetic wine was 5%, the malic acid degradation of *O. oeni* almost did not decline during 72 h continuous operations. This result suggests that the shear stress have little influence on the stability of malic acid degrading activity of *O. oeni*. Perhaps, *O. oeni* used was more resistant to shear stress.

Another consideration needed to take into account is the flavour. This aspect was not examined in this work. Gao and Fleet (1995) reported that the wine processed through the bioreactor for 56 h with 1×10^{10} CFU/ml exhibited a slight off-flavour described by wine makers as 'over-cooked' or 'burnt'. Such taints would be a significant barrier to commercial acceptability of the process. The biochemical mechanism of this flavour change is not understood. It could involve the production of volatiles such as esters and diketones and the metabolism of organic acids such as citric acid and acetic acid (Davis *et al.*, 1985). However, Maicas *et al* (2000) reported that the inoculation of

high density of non-proliferating cells into wine did not produce off-flavours. Another possible source of off-flavour could be the autolysis or physical breakdown of the cells in the reactor releasing intracellular constituents into the wine. Further studies are needed.

8.6 Conclusions

The following conclusions can be made:

1. In order to obtain higher cell vitality after freeze-drying the LAB should be cultivated under the following conditions:

   - ***L.* plantarum:** The medium consists of: glucose 5 g/l, yeast extract 8 g/l, $(NH_4)_2SO_4$ 2 g /l, KH_2PO_4 2 g/l, Tween 80 1 g/l, and a trace element solution ($CaCl_2$ 0.08%; $MnCl_2$ 0.1%) 20 ml/l. The cells are cultivated at 25°C and cultural pH is controlled at pH 5.0. When growth is at early stationary phase the cells are harvested. Preincubation in 5 g/l yeast extract solution at 25°C for 60 minutes is carried out before freezing.

   - ***L. brevis*:** The medium is: glucose 5 g/l, yeast extract 5 g/l, $(NH_4)_2SO_4$ 2 g/l, KH_2PO_4 2 g/l, Tween80 8 g/l, and a trace element solution ($CaCl_2$ 0.05%; $MnCl_2$ 0.01%) 20 ml/l. The cells are cultivated at 25°C and cultural pH is controlled at pH 5.0. When growth is at early stationary phase the cells are harvested.

   - ***O. oeni*:** The medium consists of: glucose 5 g/l, yeast extract 5 g/l, $(NH_4)_2 SO_4$ 2 g /l, KH_2PO_4 2 g/l, Tween 80 1 g/l, and a trace element solution ($CaCl_2$ 0.03%; $MnCl_2$ 0.01%) 20 ml/l. The cells are cultivated at 25°C and cultural pH is controlled at pH 5.0. When growth is at early stationary phase the cells are harvested. Preincubation in 5 g/l glucose at 25°C for 60 minutes is carried out before freezing.

2. The freeze-drying of *L. plantarum*, *L. brevis* and *O. oeni* should be carried out under the following conditions:

- ***L. plantarum*:** when the cells have grown to early stationary phase the cell cultures are centrifuged and removed the growth medium. The cell pastes are then resuspended in 4% yeast extract solution. The cell suspension is preincubated at 10°C for 3 hrs before freezing. The cell suspension is then frozen fast at -65°C. The frozen cell suspension is freeze-dried. When the freeze-dried cells are used, the cells are rehydrated in 10% sucrose solution to its original volume at room temperature.

- ***L. brevis*:** when the cells have grown to early stationary phase the cell cultures are concentrated to 2 folds and mixed with 8% yeast extract at the same volume. The cell suspension is then frozen fast at -65°C. The frozen cell suspension is freeze-dried. When the freeze-dried cells are used, the cells are rehydrated in 2 g/l $CaCl_2$ solution to its original volume at room temperature. Before inoculation into wines, the freeze-dried cells are precultrued in CME medium at 25°C for 48 hrs or more.

- ***O. oeni*:** when the cells have grown to early stationary phase the cell cultures are mixed with 10.0% sodium glutamate at the same volume. The cell suspension is then frozen fast at -65°C. The frozen cell suspension is freeze-dried. When the freeze-dried cells are used, the cells are rehydrated in GY medium to its original volume at room temperature. Before inoculation into wine, the freeze-dried cells are precultured in CME medium at 25°C for 48 hrs or more.

3. Wine composition has a great effect on batch MLF of *L. brevis* and *O. oeni*. When the available sugar in wine is fructose, malic acid degradation of *L. brevis* and *O. oeni* are much higher than that when the available sugar was glucose. Glucose induces an inhibitory effect on MLF. Fructose can relieve the glucose-induced inhibition on MLF of *O. oeni* but *L. brevis*. Ethanol not only affects the cell growth, but also affects the malolactic activity of the cells. 12% ethanol shows a great inhibition on the malolactic fermentation of *L. brevis* but hardly influenced the malolactic activity of *O. oeni*. Malate is stimulatory for MLF. Shortage of nutrients in wine inhibits bacterial growth and MLF of *L. brevis* and *O. oeni*. The effect of phenolic compounds can be either stimulatory or inhibitory in terms of the bacterial growth and MLF, depending on bacterial species, specific phenolic acids and their concentrations.

4. When the MBR is charged with high densities of cells of *O. oeni* (about 1.6 × 10^8CFU/ml), rapid, prolonged and continuous MLF can be achieved. When the wine flow rate is less than 0.48 l/h and the residence time is 10.4 h, the degradation of malic acid is greater than 95%. The ability of malic acid degradation of *O. oeni* will decrease during continuous operation. High ethanol concentration of wine is the main factor that causes loss in malic acid degrading activity of *O. oeni*. Poor nutritional condition does not cause significant loss in the stability under the conditions tested. Low pH inhibits the malic acid degrading activity of *O. oeni* but hardly influenced the stability of malic acid degrading activity of *O. oeni* in MBR. Shear stress seems to have little influence on the stability of malic acid degrading activity of *O. oeni* under the conditions investigated. Ethanol stress adaptation can improve the stability of malic acid degrading activity of *O. oeni* in the MBR.

8.7 Recommendations and Further Work

Based on the work carried out in this study the following areas should be investigated further:

- A combination of different protective substances such as amino acids and sugars may allow higher cell vitality after freeze-drying.

- The effect of additives on the optimal initial cell density should be investigated. As discussed above, when the cell cultures were concentrated, the vitality of *L. plantarum* and *O. oeni* decreased, and suspension medium is related to the optimal initial cell density. Suitable additives may allow use of higher initial cell densities during freeze-drying. High cell concentrations are necessary for starter production.

- The effect of storage on freeze-dried LAB should be investigaed because loss in vitality during storage occurs.

- A mathematical model for the analysis of preservation process to obtain high productivity should be developed for study of the optimisation of the processes.

- The selection of malolactic bacteria strains with high ethanol tolerance should be done. As described previously, high ethanol tolerance of malolactic bacteria is important for successful induction of MLF in wines.

- The stability of malic acid degrading activity of malolactic bacteria in MBR should be further investigated by using longer periods, say, 2 weeks or even several months.

- Using real wine to carry out malolactic fermentation in a MBR. Effect of continuous MLF in MBR on the quality of wine such as flavour and effect of wine composition on membrane fouling should be investigated.

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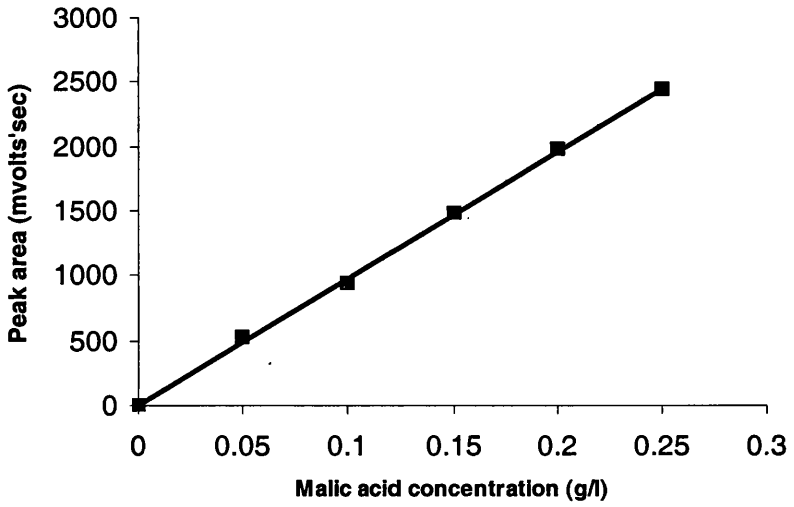
Appendix-1. Assay of Malic Acid

1.1. The sodium hydroxide gradient elution

The sodium hydroxide gradient elution used when the malic acid concentration in the samples was analysed in the HPLC.

Time [Min : Sec]	% Pump A 0.5 mM NaOH	% Pump B 100 mM NaOH
0:00	100	0
5:00	100	0
15:00	95	5
25:00	50	50
33:00	50	50
35:00	100	0

1.2. The calibration curve for determination of malic acid



The relationship between malic acid concentration and peak area in the HPLC.

Appendix-2. Experimental Data Presented in Chapter 4

2.1 Experimental Data for Figure 4.1

Time (h)	Cell growth (OD_{660}) of *L. plantarum*			
	E25	E37	S25	S37
0	0.2	0.203	0.205	0.205
1	0.2	0.203	0.208	0.205
2	0.206	0.203	0.226	0.219
3	0.218	0.218	0.25	0.243
4	0.238	0.239	0.318	0.278
5	0.312	0.295	0.441	0.372
6	0.428	0.383	0.626	0.509
7	0.631	0.562	0.903	0.737
8	0.923	0.825	1.243	0.998
9	1.257	1.157	1.56	1.312
10	1.534	1.487	1.782	1.63
11	1.756	1.744	1.865	1.83
12	1.846	1.832	1.91	1.88

2.2 Experimental Data for Figure 4.2

Time (h)	Cell growth (OD_{660}) of *L. brevis*			
	E 25	E 30	S 25	S 30
0	0.103	0.103	0.105	0.102
2	0.103	0.103	0.105	0.103
4	0.105	0.103	0.108	0.106
6	0.121	0.106	0.132	0.106
8	0.15	0.12	0.188	0.134
10	0.21	0.142	0.276	0.178
12	0.311	0.2	0.386	0.26
14	0.411	0.29	0.51	0.368
18	0.67	0.53	0.78	0.61
22	0.96	0.83	1.064	0.905
24	1.12	0.97	1.21	1.064
26	1.24	1.108	1.36	1.19

2.3 Experimental Data for Figure 4.3

Time (h)	Cell growth (OD_{660}) of *O. oeni*			
	S25	S30	E25	E30
0	0.08	0.082	0.081	0.08
6	0.08	0.082	0.081	0.08
12	0.085	0.082	0.081	0.08
16	0.087	0.084	0.081	0.08
24	0.102	0.09	0.084	0.082
30	0.13	0.106	0.089	0.084
36	0.19	0.15	0.1	0.093
40	0.26	0.203	0.114	0.1
48	0.41	0.329	0.156	0.132
54	0.55	0.463	0.21	0.168
60	0.7	0.608	0.3	0.23
70	0.89	0.84	0.488	0.38
75	0.916	0.906	0.589	0.475

2.4 Experimental Data for Figure 4.4

Time (h)	Cell growth (OD_{660}) of *L. plantarum*		
	37°C	25°C	18°C
0	0.18	0.181	0.183
1	0.18	0.183	0.186
2	0.183	0.202	0.218
3	0.188	0.233	0.275
4	0.209	0.298	0.383
5	0.265	0.414	0.576
6	0.364	0.6	0.826
7	0.532	0.865	1.136
8	0.72	1.156	1.418
9	0.952	1.413	1.627
10	1.202	1.582	1.754
11	1.445	1.715	1.86
12	1.633	1.82	1.939

2.5 Experimental Data for Figure 4.5

Time (h)	Cell growth (OD_{660}) of *L. brevis*		
	30°C	25°C	18°C
0	0.102	0.105	0.106
2	0.103	0.105	0.106
4	0.106	0.108	0.128
6	0.106	0.132	0.176
8	0.134	0.188	0.252
10	0.178	0.276	0.341
12	0.26	0.386	0.471
14	0.368	0.51	0.599
18	0.63	0.78	0.93
22	0.905	1.064	1.234
24	1.064	1.21	1.394
26	1.19	1.36	1.56

2.6 Experimental Data for Figure 4.6

Time (h)	Cell growth (OD_{660}) of *O. oeni*		
	25°C	30°C	18°C
0	0.08	0.082	0.08
6	0.08	0.082	0.08
12	0.085	0.082	0.087
16	0.087	0.084	0.089
24	0.102	0.09	0.106
30	0.13	0.106	0.138
36	0.19	0.15	0.21
40	0.26	0.203	0.283
48	0.41	0.329	0.432
54	0.55	0.463	0.573
60	0.7	0.608	0.723
70	0.89	0.834	0.92
75	0.916	0.906	0.94

2.7 Experimental Data for Figure 4.7

Time (h)	Cell growth (OD_{660}) of *L. plantarum*					
	15 g/l	10 g/l	8 g/l	5 g/l	3 g/l	1 g/l
0	0.207	0.206	0.205	0.191	0.203	0.201
1	0.207	0.21	0.205	0.206	0.203	0.201
2	0.225	0.227	0.233	0.219	0.219	0.214
3	0.26	0.267	0.288	0.251	0.249	0.236
4	0.333	0.339	0.376	0.298	0.297	0.276
5	0.465	0.474	0.557	0.414	0.392	0.352
6	0.714	0.707	0.827	0.6	0.561	0.475
7	0.979	0.972	1.108	0.876	0.814	0.68
8	1.303	1.259	1.386	1.156	1.067	0.93
9	1.521	1.458	1.594	1.413	1.313	1.153
10	1.663	1.619	1.729	1.582	1.47	1.352
11	1.795	1.731	1.818	1.715	1.613	1.531
12	1.891	1.822	1.902	1.82	1.713	1.655
13	1.96	1.885	1.963	1.904	1.788	1.734

2.8 Experimental Data for Figure 4.8

Time (h)	Cell growth (OD_{660}) of *L. brevis*					
	15 g/l	10 g/l	8 g/l	5 g/l	3 g/l	1 g/l
0	0.154	0.153	0.155	0.155	0.156	0.156
2	0.154	0.153[a]	0.156	0.156	0.156	0.156
4	0.158	0.156	0.171	0.168	0.156	0.156
6	0.193	0.195	0.22	0.214	0.179	0.157
8	0.265	0.273	0.309	0.306	0.226	0.182
10	0.367	0.354	0.402	0.4	0.295	0.231
12	0.481	0.463	0.526	0.518	0.402	0.317
14	0.607	0.587	0.676	0.645	0.544	0.435
18	0.876	0.846	0.964	0.918	0.82	0.71
22	1.186	1.165	1.28	1.23	1.1	0.992
24	1.306	1.285	1.4	1.34	1.202	1.106
26	1.38	1.35	1.48	1.42	1.28	1.197

2.9 Experimental Data for Figure 4.9

Time (h)	Cell growth (OD_{660}) of *O. oeni*					
	15 g/l	10 g/l	8 g/l	5 g/l	3 g/l	1 g/l
0	0.07	0.07	0.07	0.07	0.07	0.07
6	0.072	0.072	0.075	0.08	0.073	0.072
12	0.08	0.076	0.086	0.09	0.082	0.08
16	0.082	0.08	0.098	0.104	0.095	0.09
24	0.093	0.097	0.126	0.16	0.148	0.118
30	0.132	0.135	0.192	0.242	0.23	0.165
36	0.231	0.225	0.318	0.372	0.352	0.278
40	0.3	0.295	0.415	0.476	0.46	0.367
48	0.465	0.463	0.62	0.68	0.662	0.567
54	0.585	0.597	0.76	0.814	0.786	0.712
60	0.718	0.745	0.887	0.934	0.908	0.843
70	0.887	0.919	0.99	1.045	1.034	0.976
75	0.965	0.982	1.03	1.09	1.07	1.02

2.10 Experimental Data for Figure 4.10

Time (h)	Cell growth (OD_{660}) of *L. plantarum*						
	Ca + Mn	0.10% Ca	0.08% Ca	0.05% Ca	0.03% Ca	0.01% Ca	Control
0	0.2	0.2	0.198	0.203	0.2	0.202	0.202
1	0.206	0.203	0.202	0.203	0.203	0.202	0.202
2	0.22	0.212	0.21	0.216	0.208	0.207	0.204
3	0.256	0.223	0.225	0.234	0.214	0.215	0.208
4	0.346	0.276	0.276	0.265	0.241	0.23	0.21
5	0.5	0.342	0.338	0.32	0.328	0.282	0.234
6	0.71	0.46	0.463	0.44	0.418	0.363	0.3
7	0.95	0.649	0.671	0.63	0.593	0.501	0.41
8	1.197	0.852	0.897	0.862	0.792	0.701	0.57
9	1.452	1.104	1.14	1.1	1.03	0.967	0.811
10	1.684	1.35	1.42	1.36	1.3	1.24	1.06
11	1.852	1.55	1.63	1.57	1.526	1.462	1.285
12	1.94	1.76	1.82	1.78	1.75	1.72	1.5

2.11 Experimental Data for Figure 4.11

Time (h)	Cell growth (OD_{660}) of *L. brevis*						
	Ca + Mn	0.10%Ca	0.08% Ca	0.05% Ca	0.03% Ca	0.01% Ca	Control
0	0.1	0.1	0.1	0.1	0.102	0.1	0.101
2	0.1	0.1	0.101	0.101	0.102	0.1	0.101
4	0.101	0.105	0.107	0.117	0.103	0.102	0.101
6	0.108	0.122	0.13	0.156	0.104	0.106	0.102
8	0.123	0.165	0.176	0.21	0.11	0.108	0.106
10	0.16	0.216	0.23	0.288	0.12	0.115	0.108
12	0.22	0.3	0.318	0.387	0.163	0.152	0.132
14	0.3	0.412	0.426	0.51	0.22	0.205	0.176
18	0.5	0.65	0.67	0.78	0.4	0.386	0.34
22	0.72	0.91	0.93	1.05	0.62	0.607	0.56
24	0.835	1.023	1.04	1.14	0.74	0.726	0.67
26	0.95	1.1	1.13	1.2	0.856	0.842	0.8

2.12 Experimental Data for Figure 4.12

Time (h)	Cell growth (OD_{660}) of *O. oeni*						
	Ca + Mn	0.10% Ca	0.08% Ca	0.05% Ca	0.03% Ca	0.01% Ca	Control
0	0.07	0.07	0.07	0.07	0.07	0.07	0.07
6	0.072	0.07	0.07	0.07	0.074	0.07	0.07
12	0.082	0.078	0.08	0.083	0.087	0.075	0.073
16	0.096	0.091	0.093	0.097	0.105	0.082	0.078
24	0.132	0.116	0.13	0.128	0.147	0.106	0.092
30	0.2	0.172	0.188	0.186	0.234	0.138	0.112
36	0.307	0.265	0.285	0.286	0.356	0.216	0.16
40	0.386	0.34	0.364	0.365	0.445	0.276	0.22
48	0.563	0.507	0.55	0.547	0.636	0.42	0.354
54	0.687	0.635	0.678	0.67	0.765	0.534	0.465
60	0.795	0.725	0.756	0.77	0.856	0.643	0.572
70	0.87	0.802	0.82	0.845	0.928	0.786	0.75
75	0.896	0.83	0.854	0.875	0.956	0.82	0.8

2.13 Experimental Data for Figure 4.13

Time (h)	Cell growth (OD_{660}) of *L. plantarum*						
	Ca + Mn	0.10%Mn	0.08%Mn	0.05%Mn	0.03%Mn	0.01%Mn	Control
0	0.2	0.202	0.2	0.203	0.201	0.202	0.202
1	0.206	0.203	0.201	0.203	0.201	0.202	0.202
2	0.22	0.21	0.207	0.204	0.202	0.203	0.204
3	0.256	0.243	0.23	0.213	0.21	0.208	0.208
4	0.346	0.32	0.292	0.265	0.25	0.252	0.21
5	0.5	0.438	0.412	0.368	0.354	0.356	0.234
6	0.71	0.63	0.607	0.534	0.5	0.503	0.3
7	0.95	0.86	0.823	0.720	0.7	0.703	0.41
8	1.197	1.1	1.04	0.942	0.9	0.902	0.57
9	1.452	1.32	1.28	1.153	1.13	1.132	0.811
10	1.684	1.55	1.52	1.392	1.36	1.363	1.06
11	1.852	1.73	1.718	1.634	1.6	1.58	1.285
12	1.94	1.86	1.83	1.82	1.76	1.755	1.5

2.14 Experimental Data for Figure 4.14

Time (h)	Cell growth (OD_{660}) of *L. brevis*						
	Ca + Mn	0.10%Mn	0.08%Mn	0.05%Mn	0.03%Mn	0.01%Mn	Control
0	0.1	0.101	0.101	0.101	0.103	0.101	0.101
2	0.1	0.101	0.101	0.101	0.103	0.101	0.101
4	0.101	0.101	0.101	0.102	0.103	0.103	0.101
6	0.108	0.102	0.102	0.104	0.105	0.112	0.102
8	0.123	0.104	0.104	0.108	0.11	0.143	0.106
10	0.16	0.112	0.113	0.119	0.126	0.186	0.108
12	0.22	0.13	0.132	0.16	0.165	0.26	0.132
14	0.3	0.16	0.167	0.217	0.232	0.35	0.176
18	0.5	0.285	0.3	0.388	0.41	0.56	0.34
22	0.72	0.483	0.5	0.592	0.628	0.815	0.56
24	0.835	0.576	0.6	0.712	0.74	0.923	0.67
26	0.95	0.69	0.71	0.83	0.86	1.06	0.8

2.15 Experimental Data for Figure 4.15

Time (h)	Cell growth (OD_{660}) of *O. oeni*						
	Ca + Mn	0.10%Mn	0.08%Mn	0.05%Mn	0.03%Mn	0.01%Mn	Control
0	0.07	0.07	0.07	0.07	0.07	0.07	0.07
6	0.072	0.07	0.071	0.072	0.074	0.073	0.07
12	0.082	0.078	0.078	0.08	0.086	0.083	0.073
16	0.096	0.083	0.087	0.092	0.095	0.09	0.078
24	0.132	0.11	0.124	0.132	0.14	0.132	0.092
30	0.2	0.148	0.18	0.207	0.213	0.2	0.112
36	0.307	0.22	0.265	0.308	0.312	0.307	0.16
40	0.386	0.3	0.34	0.391	0.395	0.39	0.213
48	0.563	0.48	0.51	0.572	0.578	0.564	0.354
54	0.687	0.623	0.65	0.705	0.72	0.693	0.465
60	0.795	0.743	0.786	0.812	0.834	0.826	0.572
70	0.886	0.844	0.886	0.913	0.923	0.932	0.75
75	0.92	0.872	0.923	0.935	0.952	0.96	0.8

2.16 Experimental Data for Figure 4.16

Time (h)	Cell growth (OD_{660}) of *L. plantarum*				
	0 g/l	1 g/l	3 g/l	5 g/l	8 g/l
0	0.411	0.413	0.415	0.417	0.421
1	0.411	0.415	0.418	0.419	0.423
2	0.432	0.468	0.478	0.466	0.475
3	0.467	0.617	0.606	0.588	0.58
4	0.567	0.816	0.821	0.771	0.772
5	0.753	1.138	1.168	1.109	1.112
6	1	1.479	1.53	1.48	1.498
7	1.28	1.728	1.791	1.771	1.784
8	1.537	1.871	1.929	1.924	1.926
9	1.811	1.981	2.034	2.046	2.025
10	1.97	2.051	2.122	2.133	2.128

2.17 Experimental Data for Figure 4.17

Time (h)	Cell growth (OD_{660}) of *L. brevis*				
	0 g/l	1 g/l	3 g/l	5 g/l	8 g/l
0	0.1	0.102	0.1	0.1	0.101
2	0.1	0.105	0.106	0.108	0.11
4	0.107	0.11	0.113	0.126	0.14
6	0.109	0.13	0.136	0.163	0.18
8	0.126	0.18	0.188	0.224	0.246
10	0.17	0.273	0.278	0.312	0.34
12	0.24	0.37	0.38	0.423	0.456
14	0.334	0.5	0.505	0.56	0.6
18	0.57	0.8	0.81	0.86	0.93
22	0.87	1.12	1.13	1.2	1.24
24	1.02	1.22	1.23	1.3	1.33

2.18 Experimental Data for Figure 4.18

Time (h)	Cell growth (OD_{660}) of *O. oeni*				
	0 g/l	1 g/l	3 g/l	5 g/l	8 g/l
0	0.08	0.08	0.082	0.082	0.081
6	0.08	0.08	0.082	0.082	0.081
12	0.085	0.09	0.092	0.092	0.091
16	0.087	0.095	0.096	0.095	0.094
24	0.102	0.134	0.143	0.135	0.132
30	0.13	0.186	0.2	0.19	0.18
36	0.19	0.278	0.288	0.282	0.27
40	0.26	0.356	0.36	0.364	0.348
48	0.41	0.53	0.536	0.532	0.518
54	0.55	0.68	0.693	0.686	0.673
60	0.7	0.82	0.826	0.824	0.812
70	0.89	1.03	1.04	1.033	1.01
75	0.93	1.06	1.07	1.054	1.045

2.19 Experimental Data for Figure 4.19

Time (h)	Cell growth (OD_{660}) of *L. plantarum*			
	pH4	pH5	pH6	pH7
0	0.302	0.301	0.3	0.303
1	0.326	0.345	0.335	0.318
2	0.356	0.385	0.37	0.342
3	0.405	0.5	0.44	0.365
4	0.51	0.675	0.56	0.431
5	0.662	0.905	0.755	0.549
6	0.887	1.203	0.98	0.725
7	1.156	1.462	1.254	0.97
8	1.386	1.641	1.462	1.232
9	1.536	1.753	1.614	1.462
10	1.674	1.81	1.752	1.613
11	1.75	1.86	1.847	1.724
12	1.83	1.9	1.89	1.806

2.20 Experimental Data for Figure 4.20

Time (h)	Cell growth (OD_{660}) of *L. brevis*			
	pH4	pH5	pH6	pH7
0	0.162	0.162	0.16	0.163
2	0.164	0.162	0.162	0.165
4	0.172	0.175	0.169	0.17
6	0.186	0.204	0.182	0.183
8	0.195	0.251	0.192	0.195
10	0.234	0.337	0.21	0.22
12	0.278	0.468	0.23	0.243
14	0.36	0.628	0.274	0.3
18	0.61	0.951	0.45	0.51
22	0.89	1.258	0.67	0.77
24	1.065	1.396	0.82	0.907
26	1.234	1.45	0.96	1.07

2.21 Experimental Data for Figure 4.21

Time (h)	Cell growth (OD_{660}) of *O. oeni*		
	pH 4	pH 5	pH 6
0	0.101	0.1	0.102
6	0.101	0.102	0.102
12	0.108	0.11	0.103
16	0.112	0.116	0.108
24	0.13	0.14	0.118
30	0.156	0.18	0.132
36	0.216	0.256	0.172
40	0.264	0.315	0.22
48	0.4	0.46	0.326
54	0.52	0.587	0.432
60	0.65	0.7	0.56
70	0.84	0.88	0.79
75	0.9	0.923	0.872

2.22 Experimental Data for Figure 4.22

Time (h)	Cell growth (OD_{660}) of *L. plantarum*			
	Control	Glucose	GY	YE
0	0.203	0.202	0.203	0.2
1	0.203	0.202	0.203	0.205
2	0.214	0.224	0.233	0.238
3	0.235	0.253	0.3	0.356
4	0.297	0.326	0.42	0.528
5	0.392	0.432	0.592	0.75
6	0.561	0.601	0.822	1
7	0.806	0.846	1.103	1.3
8	1.067	1.1	1.38	1.53
9	1.313	1.35	1.62	1.71
10	1.52	1.57	1.77	1.82
11	1.66	1.72	1.84	1.88
12	1.8	1.83	1.891	1.91

2.23 Experimental Data for Figure 4.23

Time (h)	Cell growth (OD_{660}) of *L. brevis*			
	Control	Glucose	GY	YE
0	0.125	0.128	0.126	0.129
2	0.126	0.128	0.127	0.129
4	0.149	0.143	0.138	0.14
6	0.18	0.165	0.152	0.156
8	0.225	0.194	0.175	0.181
10	0.314	0.255	0.21	0.214
12	0.435	0.364	0.274	0.276
14	0.567	0.486	0.385	0.368
18	0.862	0.743	0.62	0.608
22	1.156	1.021	0.905	0.862
24	1.321	1.165	1.045	1.021
26	1.39	1.25	1.156	1.123

2.24 Experimental Data for Figure 4.24

Time (h)	Cell growth (OD_{660}) of *O. oeni*			
	Control	Glucose	GY	YE
0	0.08	0.08	0.08	0.08
6	0.08	0.082	0.082	0.08
12	0.085	0.096	0.095	0.094
16	0.087	0.115	0.11	0.108
24	0.102	0.164	0.16	0.15
30	0.13	0.234	0.237	0.22
36	0.188	0.337	0.355	0.31
40	0.25	0.43	0.438	0.39
48	0.41	0.62	0.61	0.58
54	0.55	0.786	0.76	0.72
60	0.7	0.92	0.875	0.835
70	0.88	1.004	0.97	0.94
75	0.916	1.025	0.992	0.965

2.25 Experimental Data for Table 4.1

Time (h)	Cell growth (OD_{660}) of *L. plantarum*			
	1 g/l YE	3 g/l YE	5 g/l YE	8 g/l YE
0	0.203	0.203	0.2	0.202
1	0.203	0.203	0.205	0.205
2	0.214	0.22	0.238	0.23
3	0.245	0.303	0.358	0.33
4	0.35	0.443	0.532	0.51
5	0.475	0.623	0.763	0.752
6	0.662	0.865	1.03	0.992
7	0.896	1.176	1.33	1.293
8	1.182	1.42	1.55	1.534
9	1.453	1.612	1.72	1.708
10	1.65	1.753	1.83	1.83
11	1.782	1.845	1.89	1.88
12	1.843	1.88	1.922	1.91

2.26 Experimental Data for Table 4.2

Time (h)	Cell growth (OD_{660}) of *L. plantarum*								
	25°C			37°C			4°C		
	120min	60min	30min	120min	60min	30min	120min	60min	30min
0	0.2	0.2	0.201	0.2	0.2	0.201	0.203	0.2	0.201
1	0.2	0.205	0.201	0.2	0.2	0.201	0.203	0.2	0.201
2	0.232	0.235	0.206	0.204	0.218	0.208	0.226	0.232	0.216
3	0.312	0.352	0.273	0.237	0.272	0.25	0.288	0.316	0.267
4	0.45	0.527	0.378	0.33	0.4	0.354	0.426	0.454	0.38
5	0.64	0.757	0.523	0.478	0.593	0.52	0.6	0.643	0.53
6	0.9	1.02	0.723	0.662	0.82	0.714	0.856	0.902	0.745
7	1.16	1.3	0.966	0.89	1.1	0.95	1.12	1.162	0.974
8	1.373	1.53	1.208	1.12	1.35	1.188	1.34	1.375	1.206
9	1.58	1.712	1.435	1.35	1.56	1.42	1.55	1.584	1.434
10	1.73	1.821	1.638	1.57	1.715	1.62	1.712	1.733	1.63
11	1.84	1.882	1.786	1.72	1.816	1.77	1.82	1.845	1.792
12	1.89	1.916	1.857	1.8	1.867	1.84	1.88	1.902	1.865

2.27 Amino Acids in the Yeast Extract (YE)

Amino acid	% (w/w)
Valine	1.00
Tyrosine	4.95
Tryptophan	0.85
Threonine	2.73
Serine	3.42
Proline	0.88
Phenylalanine	3.78
Methionine	0.80
Lysine	5.40
Leucine	6.04
Isoleucine	4.81
Glycine	5.95
Glutamic acid	13.49
Cyctine	0.76
Aspartic acid	7.07
Arginine	3.31
Alanine	0.91

2.28 Experimental Data for Figure 4.2

Amino acid solutions	Viability (%)
Proline	11±3.2
Serine	11.45±2.6
Arginine	10.48±3.1
Methionine	13.71±4.3
Glycine	14.35±3.7
Valine	8.45±2.2
Glutamic acid	7.09±2.0
Leucine	19.03±4.2
Isoleucine	13.38±3.4
Aspartic acid	9.35±2.6
Threonine	7.3±2.0
Phenylalanine	10.16±4.1
Lysine	10.32±4.4
Yeast extract	46.8±4.2

Appendix-3. Experimental Data Presented in Chapter 5

3.1 Experimental Data for Figure 5.1

T (h)	Cell growth (OD_{660}) of *L. plantarum*									
	Control	Trehalose 10%	Maltose 10%	Lactose 10%	Sucrose 10%	YE 4%	Glutamate 5%	Glucose 10%	Sorbitol 5%	Mannitol 5%
0	0.305	0.3	0.302	0.305	0.304	0.303	0.306	0.305	0.305	0.303
2	0.305	0.303	0.303	0.319	0.304	0.305	0.323	0.305	0.305	0.303
4	0.306	0.312	0.392	0.414	0.305	0.338	0.365	0.306	0.306	0.305
6	0.307	0.397	0.651	0.75	0.357	0.56	0.63	0.324	0.308	0.316
8	0.359	0.729	1.089	1.2	0.656	0.943	1.095	0.465	0.37	0.428
10	0.532	1.17	1.431	1.552	1.105	1.338	1.53	0.873	0.635	0.724
12	0.842	1.45	1.658	1.782	1.368	1.645	1.76	1.25	0.936	1.024

3.2 Experimental Data for Figure 5.2

T (h)	Cell growth (OD_{660}) of *L. brevis*									
	Control	Trehalose 10%	Maltose 10%	Lactose 10%	Sucrose 10%	YE 4%	Glutamate 5%	Glucose 10%	Sorbitol 5%	Mannitol 5%
0	0.102	0.103	0.105	0.103	0.102	0.104	0.102	0.102	0.102	0.102
2	0.102	0.103	0.105	0.103	0.102	0.107	0.102	0.102	0.102	0.102
4	0.102	0.103	0.106	0.103	0.102	0.122	0.102	0.102	0.102	0.102
6	0.102	0.11	0.111	0.103	0.102	0.14	0.108	0.102	0.102	0.102
8	0.103	0.12	0.13	0.118	0.115	0.166	0.11	0.106	0.104	0.104
10	0.104	0.135	0.171	0.156	0.145	0.214	0.144	0.11	0.108	0.107
12	0.105	0.156	0.242	0.208	0.196	0.3	0.188	0.116	0.11	0.11
14	0.108	0.196	0.356	0.312	0.292	0.433	0.25	0.127	0.118	0.115
20	0.145	0.442	0.848	0.732	0.681	0.908	0.58	0.234	0.185	0.172
22	0.186	0.541	1.02	0.901	0.824	1.075	0.712	0.304	0.246	0.232
24	0.242	0.645	1.193	1.056	0.967	1.266	0.84	0.4	0.312	0.295
26	0.31	0.766	1.32	1.176	1.08	1.39	0.965	0.503	0.38	0.36

3.3 Experimental Data for Figure 5.3

T (h)	Cell growth (OD_{660}) of *O. oeni*									
	Control	Trehalose 10%	Maltose 10%	Lactose 10%	Sucrose 10%	YE 4%	Glutamate 5%	Glucose 10%	Sorbitol 5%	Mannitol 5%
0	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
6	0.08	0.082	0.081	0.085	0.082	0.08	0.082	0.08	0.08	0.08
12	0.08	0.086	0.086	0.091	0.086	0.08	0.086	0.08	0.08	0.08
24	0.08	0.098	0.117	0.128	0.132	0.086	0.165	0.08	0.08	0.08
30	0.082	0.132	0.165	0.178	0.194	0.104	0.245	0.082	0.083	0.08
36	0.083	0.167	0.22	0.25	0.276	0.145	0.346	0.084	0.088	0.08
48	0.085	0.35	0.478	0.503	0.566	0.3	0.68	0.11	0.136	0.083
54	0.088	0.487	0.642	0.66	0.736	0.42	0.876	0.153	0.188	0.118
60	0.104	0.65	0.782	0.8	0.872	0.572	1.02	0.224	0.267	0.162
72	0.166	0.86	0.924	0.952	0.995	0.82	1.15	0.42	0.477	0.335
80	0.245	0.963	0.983	0.992	1.02	0.925	1.19	0.592	0.662	0.471
90	0.42	1.024	1.02	1.03	1.05	0.973	1.22	0.845	0.893	0.67

3.4 Experimental Data for Figure 5.4

Time (h)	Cell growth (OD_{660}) of *L. plantarum*					
	YE 2 %	Maltose 5%	Glutamate 5%	Sucrose 5%	Lactose 5%	Control
0	0.3	0.303	0.302	0.306	0.305	0.304
1	0.3	0.303	0.316	0.306	0.306	0.307
2	0.307	0.308	0.345	0.31	0.307	0.31
3	0.31	0.309	0.46	0.314	0.31	0.31
4	0.317	0.313	0.673	0.32	0.318	0.312
6	0.362	0.355	1.3	0.343	0.39	0.328
8	0.532	0.464	1.8	0.436	0.688	0.354
10	0.908	0.8	1.95	0.704	1.22	0.45
12	1.264	1.124	2.026	0.987	1.78	0.75

3.5 Experimental Data for Figure 5.5

Time (h)	Cell growth (OD_{660}) of *L. brevis*				
	YE 2%	Maltose 5%	Lactose 5%	Sucrose 5%	Control
0	0.102	0.105	0.107	0.102	0.102
2	0.102	0.105	0.107	0.102	0.102
4	0.102	0.105	0.107	0.102	0.102
6	0.102	0.106	0.107	0.105	0.102
8	0.107	0.112	0.112	0.108	0.103
10	0.12	0.158	0.12	0.136	0.104
12	0.137	0.23	0.152	0.185	0.106
14	0.167	0.342	0.206	0.25	0.109
20	0.35	0.812	0.54	0.64	0.136
22	0.415	0.972	0.65	0.79	0.152
24	0.476	1.102	0.78	0.96	0.186
26	0.54	1.223	0.89	1.093	0.224

3.6 Experimental Data for Figure 5.6

Time (h)	Cell growth (OD_{660}) of *O. oeni*					
	Sucrose 5%	Lactose 5%	Maltose 5%	Glutamate 2.5%	YE 2%	Control
0	0.08	0.08	0.08	0.08	0.08	0.08
6	0.08	0.08	0.08	0.082	0.08	0.08
12	0.08	0.08	0.08	0.087	0.08	0.08
24	0.09	0.083	0.085	0.12	0.08	0.08
30	0.116	0.106	0.09	0.17	0.082	0.081
36	0.153	0.14	0.116	0.26	0.09	0.083
48	0.302	0.27	0.23	0.58	0.128	0.085
54	0.412	0.36	0.33	0.77	0.172	0.088
60	0.53	0.47	0.465	0.96	0.246	0.093
72	0.8	0.74	0.74	1.14	0.493	0.108
80	0.91	0.88	0.9	1.2	0.67	0.143
90	0.948	0.93	0.95	1.24	0.87	0.236

3.7 Experimental Data for Figure 5.7

Time (h)	Cell growth (OD_{660}) of *L. plantarum*				
	pH 4.5	pH 5	pH 6	pH 7	Control
0	0.306	0.308	0.308	0.307	0.304
1	0.32	0.318	0.315	0.32	0.304
2	0.354	0.34	0.336	0.338	0.306
3	0.43	0.42	0.4	0.408	0.31
4	0.628	0.62	0.564	0.557	0.33
6	1.204	1.17	1.095	1.122	0.503
8	1.641	1.617	1.561	1.587	0.902
10	1.854	1.832	1.782	1.812	1.432
12	1.963	1.957	1.935	1.944	1.78

3.8 Experimental Data for Figure 5.8

Time (h)	Cell growth (OD_{660}) of *L. brevis*				
	pH 4.5	pH 5	pH 6	pH 7	Control
0	0.113	0.11	0.11	0.11	0.11
1	0.113	0.11	0.11	0.11	0.11
3	0.113	0.11	0.11	0.11	0.11
5	0.113	0.11	0.11	0.11	0.11
7	0.115	0.112	0.11	0.11	0.114
9	0.118	0.116	0.11	0.116	0.138
11	0.134	0.125	0.122	0.121	0.194
13	0.186	0.158	0.156	0.152	0.278
15	0.264	0.233	0.221	0.21	0.41
17	0.388	0.329	0.335	0.32	0.576
19	0.546	0.46	0.48	0.462	0.802
21	0.764	0.63	0.667	0.65	1.08
23	0.998	0.886	0.89	0.902	1.34
25	1.21	1.06	1.086	1.12	1.56

3.9 Experimental Data for Figure 5.9

Time (h)	Cell growth (OD_{660}) of *O. oeni*				
	pH 4.5	pH 5	pH 6	pH 7	Control
0	0.05	0.05	0.05	0.05	0.05
6	0.05	0.05	0.05	0.05	0.05
12	0.05	0.051	0.052	0.05	0.053
24	0.063	0.07	0.058	0.059	0.07
30	0.072	0.076	0.064	0.063	0.095
36	0.088	0.107	0.07	0.073	0.14
48	0.19	0.23	0.11	0.134	0.32
54	0.274	0.34	0.17	0.2	0.47
60	0.4	0.48	0.25	0.28	0.64
72	0.69	0.785	0.47	0.53	0.97
80	0.87	0.97	0.65	0.7	1.1
90	1.03	1.1	0.86	0.9	1.14

3.10 Experimental Data for Figure 5.10

Time (h)	Cell growth (OD_{660}) of *L. plantarum*					
	pH3	pH4	pH 5	pH 6	pH 7	Control
0	0.303	0.302	0.294	0.3	0.301	0.3
2	0.39	0.394	0.395	0.406	0.398	0.3
4	0.69	0.761	0.716	0.731	0.702	0.32
6	1.152	1.284	1.186	1.263	1.192	0.524
8	1.537	1.609	1.522	1.548	1.499	1.008
10	1.743	1.806	1.73	1.739	1.732	1.433

3.11 Experimental Data for Figure 5.11

Time (h)	Cell growth (OD_{660}) of *L. brevis*					
	pH3	pH4	pH 5	pH 6	pH 7	Control
0	0.125	0.126	0.125	0.127	0.125	0.126
2	0.125	0.126	0.125	0.127	0.125	0.126
4	0.126	0.126	0.125	0.127	0.125	0.126
6	0.128	0.128	0.13	0.13	0.128	0.13
8	0.143	0.146	0:15	0.15	0.134	0.15
10	0.163	0.165	0.169	0.171	0.158	0.195
12	0.2	0.215	0.225	0.223	0.22	0.276
14	0.28	0.302	0.325	0.323	0.31	0.41
16	0.4	0.438	0.488	0.498	0.462	0.6
20	0.77	0.801	0.903	0.93	0.88	1.118
22	1.015	1.048	1.148	1.166	1.14	1.353
24	1.25	1.302	1.386	1.42	1.356	1.56
26	1.47	1.52	1.58	1.56	1.538	1.66

3.12 Experimental Data for Figure 5.12

Time (h)	Cell growth (OD_{660}) of *O. oeni*					
	pH3	pH4	pH 5	pH 6	pH 7	Control
0	0.05	0.05	0.05	0.05	0.05	0.05
6	0.05	0.05	0.05	0.05	0.05	0.05
12	0.052	0.052	0.052	0.052	0.052	0.053
24	0.07	0.068	0.07	0.073	0.072	0.075
30	0.09	0.087	0.096	0.095	0.096	0.1
36	0.12	0.12	0.136	0.132	0.13	0.15
40	0.145	0.15	0.17	0.165	0.167	0.196
48	0.23	0.23	0.29	0.287	0.284	0.35
54	0.33	0.35	0.43	0.42	0.41	0.488
60	0.46	0.492	0.556	0.55	0.56	0.63
72	0.77	0.78	0.87	0.83	0.86	0.94
80	0.874	0.885	0.926	0.894	0.92	0.995
90	0.9	0.9	0.94	0.92	0.934	1

3.13 Experimental Data for Figure 5.13

Time (h)	Cell growth (OD_{660}) of *L. plantarum*			
	4°C	10°C	10-4°C	Control
0	0.304	0.306	0.308	0.3
2	0.335	0.379	0.343	0.3
4	0.479	0.614	0.455	0.311
6	0.88	1.088	0.883	0.524
8	1.342	1.485	1.357	1.008
10	1.642	1.705	1.665	1.433

3.14 Experimental Data for Figure 5.14

Time (h)	Cell growth (OD_{660}) of *L. brevis*			
	4°C	10°C	10-4°C	Control
0	0.13	0.13	0.13	0.13
2	0.13	0.13	0.13	0.13
4	0.13	0.13	0.13	0.13
6	0.134	0.136	0.131	0.138
8	0.136	0.14	0.15	0.16
10	0.146	0.172	0.189	0.22
12	0.187	0.245	0.268	0.32
14	0.265	0.35	0.39	0.456
16	0.387	0.51	0.552	0.66
20	0.76	0.976	1.08	1.22
22	0.97	1.26	1.38	1.5
24	1.21	1.5	1.58	1.67
26	1.43	1.65	1.7	1.73

3.15 Experimental Data for Figure 5.15

Time (h)	Cell growth (OD_{660}) of *O. oeni*			
	4°C	10°C	10-4°C	Control
0	0.05	0.05	0.05	0.05
6	0.05	0.05	0.05	0.05
12	0.052	0.053	0.051	0.052
24	0.072	0.086	0.07	0.07
30	0.105	0.125	0.1	0.1
36	0.171	0.193	0.157	0.16
48	0.36	0.4	0.34	0.35
54	0.5	0.524	0.46	0.48
60	0.64	0.68	0.64	0.62
72	0.93	0.95	0.93	0.91
80	1.07	1.087	1.078	1.07
90	1.11	1.14	1.108	1.108

3.16 Experimental Data for Figure 5.16

Time (h)	Cell growth (OD_{660}) of *L. plantarum*		
	3.6×10^7	7.2×10^7	1.5×10^8
0	0.172	0.17	0.17
2	0.182	0.175	0.17
4	0.215	0.2	0.183
6	0.32	0.306	0.254
8	0.62	0.6	0.47
10	1.1	1.05	0.87
12	1.65	1.57	1.3

3.17 Experimental Data for Figure 5.17

Time (h)	Cell growth (OD_{660}) of *L. brevis*		
	3.6×10^7	7.2×10^7	1.5×10^8
0	0.11	0.11	0.11
2	0.11	0.11	0.11
4	0.11	0.11	0.11
6	0.114	0.126	0.116
8	0.121	0.182	0.158
10	0.176	0.276	0.236
12	0.256	0.412	0.342
14	0.384	0.62	0.5
16	0.6	0.877	0.75
20	1.118	1.404	1.31
22	1.353	1.632	1.57
24	1.56	1.775	1.746
26	1.66	1.778	1.765

3.18 Experimental Data for Figure 5.18

Time (h)	Cell growth (OD_{660}) of *O. oeni*		
	3.6×10^7	7.2×10^7	1.5×10^8
0	0.05	0.05	0.05
6	0.05	0.052	0.05
12	0.052	0.055	0.055
24	0.073	0.06	0.06
30	0.103	0.072	0.07
36	0.15	0.084	0.088
48	0.336	0.192	0.192
54	0.48	0.287	0.28
60	0.64	0.39	0.39
72	0.92	0.69	0.69
80	1	0.864	0.86
90	1.034	1.03	1

3.19 Experimental Data for Figure 5.19

Time (h)	Cell growth (OD_{660}) of *L. plantarum*	
	-20°C	-65°C
0	0.203	0.203
1	0.204	0.204
3	0.214	0.245
5	0.243	0.415
7	0.45	0.78
9	0.793	1.18
11	1.2	1.545
13	1.6	1.78

3.20 Experimental Data for Figure 5.20

Time (h)	Cell growth (OD_{660}) of *L. brevis*	
	-20°C	-65°C
0	0.128	0.128
2	0.128	0.129
4	0.129	0.135
6	0.138	0.168
8	0.166	0.246
10	0.208	0.36
12	0.292	0.53
14	0.45	0.73
16	0.62	0.967
20	1.116	1.46
22	1.36	1.6
24	1.57	1.62

3.21 Experimental Data for Figure 5.21

Time (h)	Cell growth (OD_{660}) of *O. oeni*	
	-20°C	-65°C
0	0.08	0.08
6	0.08	0.08
12	0.08	0.085
24	0.083	0.12
30	0.098	0.164
36	0.131	0.23
40	0.16	0.288
48	0.24	0.43
54	0.32	0.55
60	0.43	0.7
72	0.68	0.93
80	0.82	0.985
90	0.9	1

3.22 Experimental Data for Figure 5.22

T (h)	Cell growth (OD_{660}) of *L. plantarum*							
	Sucrose 10%	Lactose 10%	Maltose 10%	Glutamate 0.5%	$MnCl_2$ 2 g/l	$Ca Cl_2$ 2 g/l	Water	GY
0	0.181	0.181	0.18	0.182	0.183	0.182	0.182	0.18
2	0.19	0.183	0.18	0.185	0.186	0.18	0.18	0.18
4	0.34	0.25	0.243	0.27	0.287	0.188	0.188	0.2
6	0.701	0.486	0.467	0.541	0.614	0.26	0.25	0.3
8	1.204	0.985	0.92	1.061	1.107	0.45	0.43	0.5
10	1.65	1.43	1.4	1.54	1.58	0.76	0.75	0.86
12	1.87	1.73	1.71	1.81	1.83	1.103	1.14	1.28

3.23 Experimental Data for Figure 5.23

T (h)	Cell growth (OD_{660}) of *L. brevis*							
	Sucrose 10%	Lactose 10%	Maltose 10%	Glutamate 0.5%	$MnCl_2$ 2 g/l	$Ca\ Cl_2$ 2 g/l	Water	GY
0	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
2	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
4	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
6	0.116	0.116	0.118	0.119	0.119	0.11	0.118	0.118
8	0.148	0.136	0.142	0.153	0.146	0.136	0.137	0.146
10	0.217	0.185	0.2	0.21	0.205	0.203	0.176	0.212
12	0.326	0.278	0.3	0.31	0.3	0.32	0.25	0.31
14	0.462	0.409	0.435	0.444	0.423	0.497	0.368	0.44
16	0.634	0.567	0.6	0.596	0.576	0.694	0.516	0.607
18	0.844	0.767	0.821	0.796	0.765	0.91	0.708	0.829
20	1.061	0.98	1.023	1.024	0.934	1.12	0.89	1.05

3.24 Experimental Data for Figure 5.24

T (h)	Cell growth (OD_{660}) of *O. oeni*							
	Sucrose 10%	Lactose 10%	Maltose 10%	Glutamate 0.5%	$MnCl_2$ 2 g/l	$Ca\ Cl_2$ 2 g/l	Water	GY
0	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
6	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
12	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
24	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.055
30	0.05	0.05	0.052	0.05	0.05	0.05	0.05	0.073
36	0.05	0.05	0.056	0.05	0.05	0.05	0.054	0.094
48	0.052	0.052	0.093	0.06	0.05	0.051	0.08	0.172
54	0.056	0.054	0.127	0.072	0.052	0.054	0.106	0.245
60	0.072	0.06	0.183	0.096	0.055	0.06	0.15	0.35
70	0.12	0.083	0.33	0.16	0.063	0.086	0.256	0.57
76	0.168	0.11	0.45	0.22	0.08	0.12	0.362	0.71
80	0.217	0.142	0.55	0.286	0.1	0.15	0.45	0.786
86	0.318	0.192	0.676	0.4	0.143	0.21	0.6	0.86
96	0.516	0.34	0.834	0.6	0.245	0.35	0.8	0.934
100	0.61	0.43	0.86	0.68	0.3	0.44	0.84	0.946
105	0.732	0.552	0.876	0.78	0.376	0.54	0.87	0.96

3.25 Experimental Data for Figure 5.25

T (h)	Cell growth (OD_{660}) of *L. plantarum*									
	Sucrose 10%	Trehalose 10%	Glucose 10%	Maltose 10%	Lactose 10%	Sorbitol 10%	Mannitol 10%	YE 4%	Glutamate 5%	Control
0	0.223	0.222	0.224	0.226	0.226	0.223	0.222	0.22	0.22	0.22
2	0.223	0.222	0.224	0.226	0.226	0.223	0.222	0.243	0.221	0.22
4	0.223	0.223	0.224	0.228	0.231	0.223	0.222	0.256	0.228	0.22
6	0.226	0.228	0.225	0.256	0.267	0.223	0.222	0.368	0.241	0.22
8	0.245	0.273	0.23	0.371	0.47	0.223	0.226	0.673	0.28	0.224
10	0.374	0.46	0.276	0.692	0.931	0.246	0.25	1.2	0.488	0.24
12	0.65	0.77	0.45	1.05	1.42	0.34	0.36	1.67	0.803	0.31
14	0.94	1.1	0.75	1.413	1.842	0.53	0.576	1.88	1.15	0.5

3.26 Experimental Data for Figure 5.26

T (h)	Cell growth (OD_{660}) of *L. brevis*									
	Sucrose 10%	Trehalose 10%	Glucose 10%	Maltose 10%	Lactose 10%	Sorbitol 10%	Mannitol 10%	YE 4%	Glutamate 5%	Control
0	0.205	0.202	0.202	0.203	0.201	0.202	0.202	0.202	0.202	0.202
2	0.205	0.202	0.202	0.203	0.201	0.202	0.202	0.202	0.202	0.202
4	0.205	0.202	0.202	0.203	0.201	0.202	0.202	0.202	0.202	0.202
6	0.205	0.202	0.202	0.203	0.208	0.202	0.202	0.208	0.202	0.202
8	0.211	0.206	0.202	0.21	0.218	0.202	0.202	0.228	0.206	0.202
10	0.22	0.218	0.2026	0.228	0.236	0.202	0.202	0.287	0.212	0.202
12	0.238	0.235	0.204	0.261	0.26	0.202	0.202	0.382	0.223	0.202
14	0.274	0.265	0.207	0.308	0.3	0.205	0.204	0.518	0.235	0.203
16	0.323	0.308	0.214	0.39	0.374	0.213	0.208	0.695	0.265	0.202
18	0.408	0.382	0.24	0.515	0.491	0.246	0.233	0.898	0.33	0.225
20	0.54	0.483	0.302	0.654	0.637	0.293	0.28	1.157	0.42	0.267

3.27 Experimental Data for Figure 5.27

T (h)	Cell growth (OD_{660}) of *O. oeni*									
	Sucrose 10%	Trehalose 10%	Glucose 10%	Maltose 10%	Lactose 10%	Sorbitol 10%	Mannitol 10%	YE 4%	Glutamate 5%	Control
0	0.092	0.091	0.091	0.09	0.09	0.09	0.09	0.091	0.09	0.09
6	0.092	0.091	0.091	0.09	0.09	0.09	0.09	0.091	0.094	0.09
12	0.094	0.091	0.091	0.09	0.09	0.09	0.09	0.091	0.108	0.09
22	0.126	0.091	0.091	0.108	0.11	0.09	0.09	0.091	0.162	0.09
30	0.2	0.123	0.091	0.156	0.178	0.09	0.09	0.108	0.273	0.09
36	0.288	0.168	0.091	0.225	0.276	0.09	0.09	0.134	0.4	0.09
40	0.381	0.223	0.093	0.284	0.362	0.094	0.09	0.16	0.52	0.09
48	0.618	0.35	0.11	0.46	0.567	0.13	0.092	0.24	0.782	0.091
54	0.835	0.476	0.154	0.63	0.77	0.177	0.1	0.365	1.00	0.092
60	1.00	0.65	0.21	0.823	0.93	0.255	0.142	0.52	1.16	0.098
65	1.05	0.81	0.288	0.96	1.00	0.33	0.185	0.672	1.2	0.112
70	1.07	0.966	0.386	1.03	1.04	0.42	0.243	0.835	1.22	0.15

Appendix-4. Experimental Data Presented in Chapter 6

4.1 Experimental Data for Figure 6.1

A. Cultivation at 15°C

Time (h)	Cell growth (OD_{660}) of *L. brevis* in GY media with ethanol						
	0%	3%	7%	9%	11%	13%	15%
0	0.152	0.15	0.153	0.152	0.15	0.15	0.152
6	0.17	0.168	0.156	0.153	0.152	0.154	0.153
21	0.218	0.204	0.182	0.167	0.155	0.16	0.154
29	0.252	0.238	0.208	0.191	0.175	0.163	0.155
45	0.354	0.324	0.275	0.245	0.219	0.184	0.164
53	0.431	0.401	0.338	0.295	0.236	0.199	0.168
73	0.704	0.673	0.519	0.435	0.316	0.245	0.19
93	1.018	0.93	0.74	0.602	0.423	0.308	0.218
124	1.408	1.378	1.14	0.962	0.642	0.467	0.287
148	1.633	1.6	1.38	1.21	0.855	0.587	0.345
170	1.735	1.72	1.555	1.42	1.018	0.703	0.434
196	1.819	1.78	1.671	1.6	1.23	0.873	0.556
216	1.83	1.8	1.73	1.68	1.37	1.04	0.682
240	1.842	1.817	1.752	1.713	1.56	1.2	0.843
260	1.844	1.823	1.754	1.716	1.656	1.32	0.976
284	1.846	1.831	1.755	1.716	1.656	1.46	1.12
308	1.846	1.831	1.755	1.716	1.658	1.575	1.286
332	1.843	1.831	1.752	1.718	1.657	1.6	1.394
352	1.841	1.831	1.752	1.718	1.656	1.606	1.423

B. Cultivation at 20°C

Time (h)	Cell growth (OD_{660}) of *L. brevis* in GY media with ethanol						
	0%	3%	7%	9%	11%	13%	15%
0	0.15	0.153	0.15	0.153	0.15	0.152	0.15
6	0.202	0.201	0.171	0.156	0.152	0.153	0.15
21	0.728	0.701	0.467	0.348	0.31	0.241	0.204
29	1.281	1.156	0.713	0.528	0.459	0.32	0.244
45	1.847	1.803	1.458	1.022	0.84	0.543	0.382
53	1.949	1.91	1.687	1.263	1.06	0.662	0.452
73	2.077	2.065	1.919	1.709	1.58	1.061	0.643
93	2.1	2.087	1.946	1.858	1.777	1.39	0.868
124	2.133	2.128	1.952	1.89	1.856	1.626	1.121
148	2.16	2.13	1.984	1.906	1.842	1.696	1.278
170	2.179	2.16	1.992	1.922	1.853	1.718	1.392
196	2.182	2.165	2.03	1.94	1.858	1.727	1.445

C. Cultivation at 25°C

Time (h)	Cell growth (OD_{660}) of *L. brevis* in GY media with ethanol						
	0%	3%	7%	9%	11%	13%	15%
0	0.15	0.151	0.15	0.153	0.152	0.153	0.15
6	0.258	0.235	0.193	0.178	0.168	0.154	0.155
21	1.385	1.167	0.626	0.439	0.326	0.257	0.194
29	1.742	1.676	1.144	0.697	0.492	0.36	0.229
45	2.02	1.956	1.79	1.37	0.978	0.65	0.372
53	2.095	2.036	1.907	1.62	1.232	0.836	0.453
73	2.144	2.077	1.983	1.803	1.616	1.218	0.623
93	2.184	2.109	2.003	1.814	1.645	1.423	0.765
124	2.205	2.142	2.005	1.823	1.661	1.439	0.835
148	2.215	2.145	2.012	1.826	1.666	1.445	0.868
170	2.23	2.166	2.044	1.828	1.67	1.452	0.885
196	2.231	2.166	2.043	1.829	1.67	1.452	0.887

D. Cultivation at 30°C

Time (h)	Cell growth (OD_{660}) of *L. brevis* in GY media with ethanol						
	0%	3%	7%	9%	11%	13%	15%
0	0.153	0.15	0.15	0.153	0.152	0.153	0.15
6	0.246	0.213	0.19	0.164	0.155	0.154	0.15
21	1.417	1.132	0.497	0.322	0.222	0.18	0.154
29	1.73	1.628	0.854	0.52	0.315	0.212	0.16
45	1.94	1.902	1.51	1.036	0.552	0.345	0.18
53	1.968	1.932	1.67	1.277	0.674	0.406	0.201
73	2.012	1.965	1.692	1.435	0.951	0.543	0.26
93	2.043	1.978	1.71	1.45	0.98	0.647	0.322
124	2.043	1.98	1.72	1.455	0.994	0.653	0.374
148	2.043	1.99	1.72	1.465	1.008	0.656	0.402
170	2.044	2	1.73	1.495	1.015	0.661	0.412
196	2.043	2.01	1.73	1.495	1.015	0.662	0.416

E. Cultivation at 37°C

Time (h)	Cell growth (OD_{660}) of *L. brevis* in GY media with ethanol						
	0%	3%	7%	9%	11%	13%	15%
0	0.15	0.15	0.152	0.15	0.151	0.152	0.15
6	0.152	0.151	0.152	0.15	0.151	0.152	0.15
21	0.156	0.151	0.152	0.15	0.151	0.152	0.15
29	0.17	0.152	0.152	0.15	0.151	0.152	0.15
45	0.218	0.168	0.156	0.15	0.151	0.152	0.15
53	0.248	0.185	0.16	0.15	0.151	0.152	0.15
73	0.332	0.247	0.171	0.15	0.151	0.152	0.15
93	0.416	0.312	0.189	0.15	0.151	0.152	0.15
124	0.577	0.431	0.215	0.15	0.151	0.152	0.15
148	0.71	0.528	0.23	0.15	0.151	0.152	0.15
170	0.747	0.58	0.235	0.15	0.151	0.152	0.15
196	0.752	0.604	0.236	0.15	0.151	0.152	0.15

4.2 Experimental Data for Figure 6.6

A. Cultivation at 15°C

Time (h)	Cell growth (OD_{660}) of *O. oeni* in GY media with ethanol						
	0%	3%	7%	9%	11%	13%	15%
0	0.08	0.08	0.08	0.08	0.08	0.08	0.08
6	0.08	0.08	0.08	0.08	0.08	0.08	0.08
21	0.08	0.08	0.08	0.08	0.08	0.08	0.08
29	0.08	0.08	0.08	0.08	0.08	0.08	0.08
45	0.081	0.08	0.081	0.08	0.08	0.08	0.08
53	0.084	0.083	0.084	0.082	0.081	0.08	0.08
73	0.095	0.092	0.092	0.086	0.083	0.082	0.08
93	0.116	0.11	0.102	0.092	0.087	0.086	0.081
124	0.157	0.147	0.121	0.108	0.095	0.095	0.086
148	0.206	0.181	0.153	0.132	0.115	0.104	0.092
170	0.257	0.228	0.187	0.152	0.128	0.113	0.098
196	0.322	0.308	0.248	0.203	0.165	0.132	0.11
221	0.409	0.387	0.321	0.263	0.207	0.165	0.13
270	0.606	0.581	0.502	0.422	0.316	0.25	0.187
318	0.742	0.723	0.66	0.593	0.442	0.33	0.252
342	0.795	0.771	0.736	0.663	0.493	0.354	0.265
368	0.812	0.8	0.756	0.682	0.523	0.367	0.272
392	0.814	0.806	0.758	0.684	0.525	0.368	0.276

B. Cultivation at 20°C

Time (h)	Cell growth (OD_{660}) of *O. oeni* in GY media with ethanol						
	0%	3%	7%	9%	11%	13%	15%
0	0.082	0.085	0.086	0.087	0.08	0.08	0.08
6	0.082	0.085	0.086	0.087	0.08	0.08	0.08
21	0.11	0.11	0.108	0.1	0.095	0.086	0.083
29	0.178	0.171	0.162	0.136	0.11	0.095	0.092
45	0.398	0.361	0.346	0.28	0.202	0.132	0.107
53	0.512	0.475	0.458	0.363	0.261	0.172	0.122
73	0.839	0.824	0.75	0.624	0.472	0.322	0.171
93	0.94	0.913	0.875	0.826	0.722	0.482	0.271
124	0.992	0.965	0.943	0.884	0.786	0.665	0.478
148	1.01	0.978	0.956	0.914	0.812	0.723	0.646
170	1.012	0.988	0.96	0.92	0.815	0.745	0.678

C. Cultivation at 25°C

Time (h)	Cell growth (OD_{660}) of *O. oeni* in GY media with ethanol						
	0%	3%	7%	9%	11%	13%	15%
0	0.086	0.084	0.082	0.085	0.08	0.082	0.08
6	0.095	0.096	0.095	0.088	0.093	0.096	0.083
21	0.222	0.214	0.208	0.189	0.159	0.137	0.118
29	0.427	0.411	0.39	0.3	0.238	0.196	0.139
45	0.88	0.84	0.788	0.713	0.557	0.406	0.233
53	0.972	0.946	0.912	0.825	0.682	0.526	0.291
73	1.02	0.998	0.975	0.925	0.854	0.732	0.499
93	1.045	1.024	1	0.966	0.912	0.788	0.672
124	1.065	1.043	1.02	0.988	0.924	0.812	0.71
148	1.078	1.053	1.03	1	0.932	0.824	0.725
170	1.087	1.065	1.05	1.02	0.934	0.845	0.739

D. Cultivation at 30°C

Time (h)	Cell growth (OD_{660}) of *O. oeni* in GY media with ethanol						
	0%	3%	7%	9%	11%	13%	15%
0	0.08	0.082	0.089	0.083	0.084	0.082	0.08
6	0.12	0.112	0.119	0.117	0.109	0.096	0.083
21	0.471	0.465	0.385	0.316	0.21	0.137	0.11
29	0.771	0.758	0.638	0.506	0.335	0.196	0.139
45	0.95	0.932	0.846	0.745	0.576	0.315	0.213
53	0.98	0.967	0.882	0.78	0.657	0.388	0.265
73	1.02	1.012	0.922	0.805	0.726	0.534	0.389
93	1.034	1.024	0.94	0.827	0.741	0.62	0.502
124	1.052	1.041	0.964	0.849	0.754	0.662	0.526
148	1.067	1.052	0.978	0.863	0.764	0.673	0.528
170	1.08	1.072	1	0.882	0.766	0.673	0.528

E. Cultivation at 37°C

Time (h)	Cell growth (OD_{660}) of *O. oeni* in GY media with ethanol						
	0%	3%	7%	9%	11%	13%	15%
0	0.08	0.08	0.087	0.085	0.08	0.084	0.081
6	0.115	0.112	0.106	0.102	0.089	0.088	0.082
21	0.388	0.333	0.247	0.188	0.135	0.112	0.088
29	0.536	0.503	0.335	0.248	0.166	0.132	0.092
45	0.693	0.66	0.548	0.389	0.224	0.158	0.103
53	0.715	0.695	0.612	0.457	0.262	0.168	0.11
73	0.728	0.712	0.689	0.582	0.35	0.197	0.124
93	0.73	0.718	0.712	0.607	0.434	0.226	0.138
124	0.732	0.72	0.713	0.61	0.462	0.238	0.145
148	0.73	0.722	0.712	0.61	0.468	0.245	0.146
170	0.73	0.722	0.711	0.61	0.468	0.248	0.148

4.3 Experimental Data for Figure 6.11

Time (h)	Cell growth (OD_{660}) of *L. brevis* in the synthetic wine					
	Citrate 1 g/l	Pyruvic 1 g/l	Glycerol 1 g/l	Ferulic 100 mg/l	Gallic 100 mg/l	control
0	0.09	0.09	0.09	0.09	0.09	0.09
6	0.094	0.093	0.092	0.093	0.093	0.092
16	0.106	0.097	0.104	0.096	0.096	0.096
22	0.118	0.115	0.11	0.112	0.112	0.11
26	0.142	0.135	0.124	0.126	0.124	0.123
30	0.18	0.175	0.153	0.157	0.143	0.14
40	0.335	0.32	0.245	0.268	0.245	0.22
46	0.492	0.47	0.36	0.4	0.35	0.33
50	0.62	0.6	0.45	0.51	0.475	0.44
54	0.734	0.73	0.56	0.61	0.582	0.56
62	0.876	0.867	0.754	0.8	0.788	0.766
70	0.93	0.925	0.82	0.867	0.845	0.81

4.4 Experimental Data for Figure 6.12

Time (h)	Cell growth (OD_{660}) of *O. oeni* in the synthetic wine					
	Pyruvic 1 g/l	Citrate 1 g/l	Glycerol 1 g/l	Gallic 100 mg/l	Ferulic 100 mg/l	Control
0	0.046	0.046	0.047	0.046	0.046	0.046
6	0.047	0.047	0.048	0.046	0.047	0.046
16	0.048	0.049	0.049	0.046	0.048	0.046
26	0.054	0.059	0.052	0.046	0.051	0.048
40	0.071	0.079	0.056	0.047	0.06	0.051
50	0.086	0.1	0.061	0.049	0.07	0.057
64	0.115	0.134	0.072	0.052	0.086	0.067
72	0.134	0.154	0.078	0.056	0.1	0.075
88	0.176	0.207	0.098	0.073	0.13	0.097
100	0.216	0.256	0.12	0.092	0.165	0.126
118	0.284	0.323	0.167	0.13	0.22	0.172
128	0.31	0.33	0.193	0.15	0.254	0.2
143	0.332	0.338	0.23	0.186	0.3	0.237

4.5 Experimental Data for Figure 6.14

Time (h)	Freeze-dried cell growth (OD_{660}) of *L. brevis* in GY with 10% ethanol							
	glutamate 5%	Fresh cell	Gelatine 1.5%	YE 4%	Lactose 10%	Maltose 10%	Sucrose 10%	Trehalose 10%
0	0.12	0.12	0.121	0.122	0.121	0.121	0.12	0.12
4	0.12	0.126	0.121	0.122	0.121	0.121	0.12	0.12
9	0.124	0.138	0.121	0.122	0.121	0.121	0.12	0.12
14	0.145	0.156	0.121	0.122	0.121	0.121	0.12	0.12
23	0.178	0.21	0.121	0.122	0.121	0.121	0.12	0.12
27	0.2	0.258	0.121	0.122	0.121	0.121	0.12	0.12
31	0.237	0.326	0.121	0.122	0.121	0.121	0.12	0.12
36	0.312	0.44	0.121	0.122	0.121	0.121	0.12	0.12
47	0.56	0.73	0.121	0.122	0.121	0.121	0.12	0.12
51	0.67	0.843	0.121	0.122	0.121	0.121	0.12	0.12
55	0.8	0.967	0.121	0.122	0.121	0.121	0.124	0.126
60	0.946	1.13	0.121	0.122	0.121	0.124	0.132	0.137
71	1.27	1.45	0.121	0.122	0.125	0.128	0.143	0.16
75	1.38	1.53	0.126	0.13	0.136	0.143	0.165	0.182
81	1.487	1.6	0.145	0.155	0.176	0.187	0.216	0.244
85	1.53	1.62	0.176	0.186	0.21	0.243	0.276	0.312
95	1.58	1.65	0.3	0.33	0.376	0.43	0.503	0.546
100	1.6	1.66	0.387	0.42	0.487	0.545	0.612	0.665
103	1.61	1.67	0.43	0.482	0.554	0.607	0.687	0.733
108	1.62	1.67	0.54	0.583	0.65	0.723	0.817	0.857
120	1.63	1.67	0.78	0.82	0.9	1.008	1.087	1.13

4.6 Experimental Data for Figure 6.15

Time (h)	Freeze-dried cell growth (OD_{660}) of *O. oeni* in GY with 10% ethanol							
	Fresh cell	Sucrose 10%	YE 4%	Trehalose 10%	Lactose 10%	Gelatine 1.5%	Maltose 10%	glutamate 5%
0	0.08	0.081	0.08	0.082	0.08	0.08	0.081	0.082
4	0.08	0.081	0.08	0.082	0.08	0.08	0.081	0.082
8	0.093	0.081	0.08	0.082	0.08	0.08	0.081	0.082
12	0.1	0.084	0.08	0.082	0.08	0.08	0.081	0.082
23	0.193	0.093	0.08	0.082	0.08	0.08	0.09	0.086
27	0.243	0.113	0.084	0.082	0.08	0.08	0.102	0.09
32	0.308	0.143	0.09	0.083	0.08	0.08	0.118	0.1
38	0.38	0.176	0.1	0.087	0.082	0.08	0.14	0.12
47	0.532	0.267	0.12	0.095	0.084	0.08	0.22	0.154
52	0.611	0.335	0.135	0.108	0.088	0.083	0.268	0.18
55	0.662	0.378	0.163	0.123	0.1	0.093	0.3	0.21
71	0.881	0.587	0.3	0.22	0.178	0.134	0.5	0.376
76	0.915	0.652	0.35	0.267	0.213	0.162	0.56	0.43
80	0.931	0.72	0.393	0.3	0.253	0.186	0.61	0.484
96	0.958	0.87	0.576	0.45	0.4	0.33	0.81	0.687
103	0.965	0.9	0.643	0.52	0.46	0.387	0.86	0.76
110	0.98	0.93	0.72	0.6	0.55	0.47	0.9	0.85
120	0.987	0.945	0.83	0.7	0.65	0.58	0.92	0.9

4.7 Experimental Data for Figure 6.16

Time (h)	Pre-cultured cell growth (OD_{660}) of *L. brevis* in GY with 10% ethanol				
	Fresh cell	CME	YFE	YGE	GY
0	0.12	0.12	0.121	0.122	0.121
4	0.126	0.12	0.121	0.122	0.121
9	0.138	0.12	0.121	0.122	0.121
14	0.156	0.12	0.121	0.122	0.121
23	0.21	0.128	0.121	0.122	0.121
27	0.258	0.135	0.121	0.122	0.121
31	0.326	0.167	0.122	0.122	0.121
36	0.44	0.23	0.124	0.127	0.121
47	0.73	0.38	0.139	0.142	0.122
51	0.843	0.456	0.144	0.148	0.135
55	0.967	0.553	0.153	0.158	0.142
60	1.13	0.686	0.176	0.203	0.152
71	1.45	0.967	0.253	0.287	0.176
75	1.53	1.087	0.32	0.354	0.21
81	1.6	1.24	0.42	0.46	0.278
85	1.62	1.35	0.53	0.554	0.345
95	1.65	1.56	0.784	0.812	0.55
100	1.66	1.6	0.92	0.95	0.67
103	1.67	1.612	0.997	1.06	0.763

4.8 Experimental Data for Figure 6.17

Time (h)	Pre-cultured cell growth (OD_{660}) of *O.oeni* in GY with 10% ethanol				
	Fresh cell	CME	YFE	YGE	GY
0	0.08	0.08	0.081	0.082	0.08
4	0.08	0.08	0.081	0.082	0.08
8	0.093	0.08	0.081	0.082	0.08
12	0.102	0.094	0.084	0.082	0.08
23	0.193	0.15	0.12	0.11	0.08
27	0.243	0.187	0.15	0.123	0.085
32	0.308	0.24	0.18	0.156	0.103
38	0.405	0.32	0.238	0.2	0.127
47	0.532	0.43	0.332	0.282	0.2
52	0.611	0.5	0.387	0.332	0.243
55	0.662	0.54	0.43	0.362	0.267
71	0.881	0.8	0.633	0.573	0.452
76	0.915	0.87	0.692	0.634	0.51
80	0.931	0.9	0.751	0.693	0.56
96	0.958	0.94	0.92	0.885	0.76

Appendix-5. Experimental Data Presented in Chapter 7

5.1 Experimental Data for Figure 7.1

The performance of the membrane operated with distilled water

Pressure (psi)	5	10	15	20	25	30	35	40
Permeation flux (l/min)	0.13 ±0.02	0.28 ±0.03	0.42 ±0.04	0.62 ±0.03	0.81 ±0.05	0.98 ±0.05	1.20 ±0.06	1.44 ±0.06

5.2 Experimental Data for Figure 7.2

The performance of the membrane operated with GY medium

Pressure (psi)	5	10	15	20	25	30	35	40
Permeation flux l/min)	0.086 ±0.03	0.182 ±0.02	0.265 ±0.04	0.356 ±0.05	0.452 ±0.06	0.52 ±0.07	0.62 ±0.07	0.72 ±0.08

5.3 Experimental Data for Figure 7.3

The performance of the membrane with varying cell densities

Pressure (psi)	Permeation flux (l/min)		
	OD0	OD 1.2	OD 5.4
5	0.045±0.03	0.035±0.04	0.033±0.05
10	0.082±0.03	0.07±0.03	0.058±0.06
15	0.15±0.05	0.134±0.03	0.11±0.05
20	0.264±0.04	0.23±0.04	0.185±0.08
25	0.368±0.06	0.32±0.04	0.28±0.05
30	0.51±0.08	0.42±0.06	0.35±.07

5.4 Experimental Data for Figure 7.4

Operation time (hrs)	Malic acid consumed in synthetic wine (13% ethanol) g/l	Malic acid consumed in synthetic wine (10% ethanol) g/l	Malic acid consumed in synthetic wine (5% ethanol) g/l
0	1.904±0.02	1.912±0.03	1.914±0.01
13	1.904±0.01	1.912±0.01	1.914±0.01
18	1.902±0.02	1.91±0.02	1.914±0.02
23	1.89±0.03	1.908±0.01	1.913±0.01
37	1.888±0.02	1.906±0.01	1.912±0.01
42	1.87±0.01	1.902±0.02	1.910±0.02
47	1.84±0.02	1.896±0.03	1.908±0.03
55	1.78±0.03	1.88±0.05	1.906±0.03
62	1.706±0.03	1.848±0.04	1.904±0.03
72	1.608±0.04	1.802±0.02	1.902±0.04

5.5 Experimental Data for Figure 7.5

Flow rate (l/h)	Consumed malic acid (g/l)
0.48	1.904±0.02
0.6	1.87±0.03
1.2	1.754±0.03
1.8	1.65±0.04
2.4	1.534±0.02

5.6 Experimental Data for Figure 7.6

Flow rate (l/h)	Degradation of malic acid (g/l)
0.48	95.2±0.02
0.6	93.5±0.03
1.2	87.7±0.03
1.8	82.5±0.04
2.4	76.7±0.02

5.7 Experimental Data for Figure 7.8

Operation time (hrs)	Malic acid consumed in synthetic wine (pH2.9) g/l	Malic acid consumed in synthetic wine (pH 4.0) g/l
0	1.84±0.02	1.912±0.03
13	1.836±0.03	1.912±0.01
18	1.834±0.03	1.91±0.02
23	1.832±0.04	1.908±0.01
37	1.826±0.02	1.906±0.01
42	1.823±0.03	1.902±0.02
47	1.82±0.02	1.896±0.03
55	1.816±0.03	1.88±0.05
62	1.784±0.03	1.848±0.04
72	1.728±0.04	1.802±0.02

5.8 Experimental Data for Figure 7.9

Operation time (hrs)	Malic acid consumed in synthetic wine (0.2 g/l YE) g/l	Malic acid consumed in synthetic wine (4.0 g/l YE) g/l
0	1.914±0.02	1.912±0.03
13	1.914±0.04	1.912±0.01
18	1.908±0.01	1.91±0.02
23	1.902±0.04	1.908±0.01
37	1.896±0.02	1.906±0.01
42	1.894±0.01	1.902±0.02
47	1.89±0.02	1.896±0.03
55	1.87±0.03	1.88±0.05
62	1.836±0.02	1.848±0.04
72	1.772±0.04	1.802±0.02

5.9 Experimental Data for Figure 7.10

Operation time (hrs)	Malic acid consumed in synthetic wine (containing additive) g/l	Malic acid consumed in synthetic wine (the control) g/l
0	1.91±0.02	1.912±0.03
13	1.91±0.04	1.912±0.01
18	1.91±0.02	1.91±0.02
23	1.91±0.02	1.908±0.01
37	1.906±0.02	1.906±0.01
42	1.904±0.04	1.902±0.02
47	1.9±0.03	1.896±0.03
55	1.892±0.04	1.88±0.05
62	1.88±0.03	1.848±0.04
72	1.876±0.03	1.802±0.02

5.10 Experimental Data for Figure 7.12

Operation time (hrs)	Malic acid consumed in synthetic wine (containing additive) g/l	Malic acid consumed in synthetic wine (the control) g/l
0	1.916±0.03	1.912±0.03
13	1.916±0.05	1.912±0.01
18	1.914±0.04	1.91±0.02
23	1.912±0.03	1.908±0.01
37	1.912±0.02	1.906±0.01
42	1.912±0.04	1.902±0.02
47	1.912±0.03	1.896±0.03
55	1.908±0.03	1.88±0.05
62	1.90±0.04	1.848±0.04
72	1.892±0.05	1.802±0.02