Investigation and utilization of lactic acid bacteria for cider maturation processes.

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Investigation and utilization of Lactic Acid Bacteria for cider maturation processes

by

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February 2004

Thesis submitted in fulfillment of the requirements of the University of Wales, for the degree of Philosophiae Doctor

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Declaration

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Acknowledgements

I would like to thank the University of Wales Swansea (UWS) and the Chinese Government for their financial support, and UWS for the great research facilities they provided during this project.

In particular I would like to thank Dr. Lovitt for his professional support and friendship.

Thank you to my wife and son as well as my family for your special support, encouragement and care.

I wish to thank you to my friends and colleagues without whom this Ph. D. would not have been possible.

Finally, I would like to thank Dr. M. Jones of the Department of Chemical and Biological Process Engineering, University of Wales Swansea for his help in 100 litres fermentation.

Abstract

Investigation and utilization of Lactic Acid Bacteria for cider maturation processes

Cider maturation involves a number of transformations mediated by Lactic Acid Bacteria (LAB). A comparison of two heterofermentative lactic acid bacteria (*Oenococcus oeni 11648* and *Lactobacillus brevis* X_2), capable of malolactic fermentation, was made to assess the relative merits of these two organisms in cider maturation processes.

Initial work studied LAB propagation on safe (free of animal extracts) simple media. Optimum growth conditions were determined. The two strains were shown to be highly adapted to the cider environment being able to tolerate ethanol, up to 10% (v/v). *O. oeni* was shown to be more tolerant to ethanol and low pH condition compared to *L. brevis*. The growth of both strains was significantly stimulated by low levels of glucose (~ 1mM) in the presence of organic acids (5g/l malate) such that the specific growth rate and cell yield were stimulated by 167% and 260% respectively.

The growth of the organisms was studied in batch, continuous and membrane bioreactor (MBR) systems with pH control. The highest maximum specific growth rates of *O. oeni* with $0.066h^{-1}$ was obtained at pH 4.5 when the mixture of 5g/l glucose-5g/l fructose was added to the culture medium, while *L. brevis* with $0.110h^{-1}$ was obtained at pH 5.5 in the 10g/l glucose medium. Both strains were grown in continuous cultures at a variety of dilution rates and the precise growth parameters were determined and these data show that *L. brevis* gave higher biomass yield ($Y_{x/s}^{max}$), faster volumetric cell productivity (Q_x^{max}) and higher ATP yield (Y_{ATP}^{max}) than *O. oeni*.

In the MBR culture system, the growth of two LAB showed that high cell concentrations were possible, *O. oeni* cultures to 12 g/l of dry weight, this is over 32 times higher than batch (0.357 g/l) and continuous culture (0.37 g/l). The volumetric productivities of the three systems were 7.9 mg/l/h, 17.6 mg/l/h and 180mg/l/h in batch, continuous culture and MBR respectively. Similar results were obtained for the growth of *L. brevis*.

Having produced high cell concentrations of *O. oeni* and *L. brevis* in the MBR, the system was employed for rapid, continuous malolactic transformation of synthetic green cider medium. The levels of malate removed were a function of the residence time and the alcohol concentration. Starting with 2 g/l malate, over 85% could be removed using a 6 hour residence time and compared very favourably with conventional batch fermentations that take typically 20 - 40 days. The MBR system was run successfully for a period of over 9 days and with correct management could be run for much longer periods. In these conditions, *O. oeni* showed greater stability and viability than *L. brevis* in cider environment as it is more alcohol and acid tolerant.

These studies provide a basis for developing strategies for rapid maturation of cider using an MBR system. Future work for further optimization of the MBR operation with real cider is discussed. The economic viability of MBR in cider maturation needs to be assessed.

CONTENTS

Chapter 1 Introduction

This investigation is concerned with the growth of lactic acid bacteria (LAB) and cider maturation.

Chapter 1 introduces and outlines the principle aims and objectives of the project. The scientific background and relevant literature have been summarized to understand the characteristics and growth of LAB, the cider maturation process, the role of maturation and the possible solutions to the problems associated with maturation. Chapter 2 provides a detailed account of the methods and materials for the measurement of the growth of organisms, analysis of chemicals and the use of equipment.

The experimental results of the project are documented in Chapters 3 to 5. Chapter 3 reports the growth and characterization of LAB (*O. oeni* 11648). This focuses on the optimization of the growth medium and optimal growth conditions as well as the growth and metabolism of LAB with respect to the cider environment. Chapter 4 investigates the growth of both *O. oeni* 11648 and *L. brevis* X_2 in batch and continuous fermentation with pH control based on the results of Chapter 3. The emphasis was to monitor the cell growth, substrate consumption, product formation and determine the growth kinetics of two LAB so as to understand further the physiological and kinetic characteristics of the growth of two strains. Chapter 5 concerns studies involving a membrane bioreactor (MBR) system and the membrane performance within the system. The emphasis was on the fouling and cleaning of the membrane and on high density cell cultivation of *O. oeni* 11648 and *L. brevis* X_2 in the MBR. Finally, this chapter also presents a maturation process involving artificial 'green cider' under varied conditions. The discussion and conclusion to this study are presented in Chapter 6 together with suggestions for future work.

1.1 Cider making

Cider (UK), cidre (France), sidra (Spain) and 'hard cider' (North America) are terms that refer to fermented drink products made from apples (Lea 1995), and in German-speaking countries, cider is defined as 'Apfelwein'. Styles of cider are enormously diverse and not easily categorized, for example, British cider is defined as 'a beverage obtained by the partial or complete fermentation of the juice of apples or concentrated apple juice with or without the addition before or after fermentation of sugar or potable water' (NACM, 1992). In addition, French cider and German cider do not have much difference in either alcohol level or flavours, such as sweeter, tannic and acidic characteristics.

Cider making is very similar to wine making with fruit containing many of the same constituents and employing many of the same processing techniques. Of particular similarity and interest to this study is the maturation of wine and cider and in particular malo-lactic fermentation (MLF). Several good reviews on the cidermaking have been published (Carr & Whiting 1971; Beech[1,2] 1972, 1993; Beech & Carr 1977; Beech & Davenport 1983; Lea 1995). Also, many reviews of winemaking processes (Amerine & Kunkee 1968; Davis *et al.* 1985; Wibowo *et al.* 1985; Van Vuuren & Dicks 1993; Henschke 1993; Henick-Kling 1995; Versari *et al.* 1999) are relevant to the study of cider.

1.1.1 A brief history of cider

The fermentation of apple juice to produce an alcoholic beverage is believed to have been practiced for over 2000 years. Cider was recorded as being a common drink at the time of the Roman invasion of England in 55 BC. Cider was drunk throughout Europe in the third century AD and in the fourth century St. Jerome used the term *sicera* to describe drinks made from apples. Reputedly, cider was a more popular drink than beer in the 11th and 12th centuries in Europe. Cider was produced throughout the temperate regions of the world where apple trees flourished. In the Middle Ages, there were many references in monastic writings to cider. It was also clearly popular in the 14th century, as William of Shoreham reflected on the Church's concern for the niceties of sacramental rites by stating that 'young children were not to be baptized in cider'! (Jarvis *et al.* 1995; Lea 1995).

However, during the second half of the 18th century it became more profitable to produce corn and cattle, orchards were neglected and sales suffered accordingly. Much cider was sold cheaply to wholesalers who flavoured or fortified it. The production of cider and perry was also limited by taxation. The decline continued into the beginning of the 19th century when cider, which previously had contained 5 to 8.5% alcohol by volume, became more and more the drink of West Country farmers and their labourers. Being dry, completely still, 4-5% alcohol by volume and microbiologically safe, it was ideal for hard physical labourers. Farm workers often had a part of their wages paid in cider (Beech[1] 1993). Although, the part payment of wages was prohibited by The Agricultural Truck Act (1887), the practice did not cease completely until the First World War.

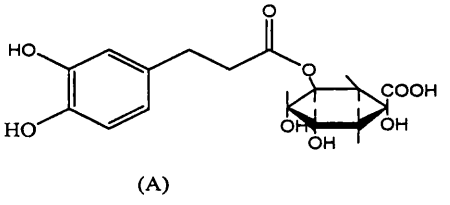
Cidermaking through the centuries was essentially a craft of cottage and farmhouse and was a hit-or-miss affair, and it was not until companies such as Bulmers were established that it became an industrialized process. Wild yeast gave an unpredictable fermentation and wide variations in the days of farmhouse cidermaking. The isolation by Bulmers of pure cider yeast that dominated the fermentation process made large-scale production a reality as the company developed in the 1890's. In England, cider production was estimated at 55 million gallons in 1900. By 1920 the level of production had decreased significantly; some 16 million gallons were produced on farms but only 5 million gallons by factory producers. By the 1980s cider sales had risen to over 60 million gallons per year. In the 12 months to December 1994, 98 million gallons were sold in the UK. Cider products range in alcoholic strength from less than 0.5% to 8.4% by volume, and in sweetness from very dry to sweet; and from 'white' (i.e. decolorized) to 'black' cider (a blend of cider with fermented malted barley). The cider market has almost doubled in size since 1989. Today the UK cider market is in excess of 113 million gallons, over 60% of this demand is met by Bulmers with a comprehensive range of ciders to suit all tastes.

1.1.2 Cider apples

Apples are the primary raw materials for cidermaking. Cider apples differ from both cooking and dessert apples in being generally smaller, very hard, unattractive and with an astringency (John 1984). Such apples have a high concentration of both sugars and tannins. Although these apples are high in sugar, they are not enjoyable to eat because

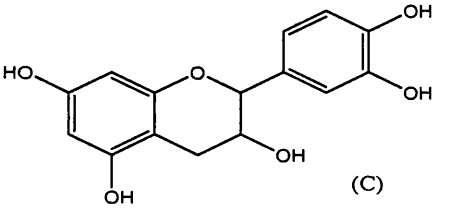
they also contain high levels of tannins. The former ferments to form the alcohol in the liquor whilst the latter imparts its characteristic flavour. Traditionally, the drink was made from cider apples of a type known as 'bittersharps' because of their high acid content, but nowadays, milder 'bittersweet' varieties are more to the public's taste. Cider can be made from cooking apples, but such cider does not keep well (John 1984).

A distinguishing feature of true cider fruit, particularly French and English bittersweets, is a relatively high concentration of these polyphenols, known as 'tannin', which confers bitterness and astringency on the finished beverage. Although modern ciders are generally lower in tannin than in the past, it still makes an important contribution to overall mouth-feel of the beverage and prevents it becoming too insipid (Jarvis *et al.* 1995). The nature of the 'tannin' is now established as a range of oligomeric procyanidins based on a flavanoid (-)-epicatechin structure (Figure 1.1). In cider apple juice, a range of oligomers up to the heptamer is present. In addition to the procyanidins, two other classes of polyphenol, which are not true tannins, are also present. These are the phenolic acids (chlorogenic and *p*-coumaroyl quinic) (Carr & Whiting 1971; Lea 1978), together with phloretin glucoside (phloridzin) and the xyloglucoside (Lea 1978). The polyphenols make a major contribution to flavour, colour, pressability and also have weak antimicrobial properties.



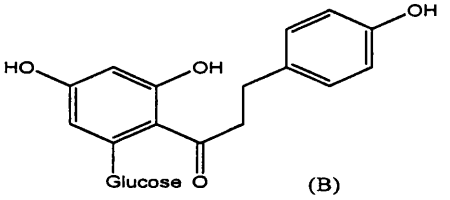
(A)

(A) chlorogenic acid



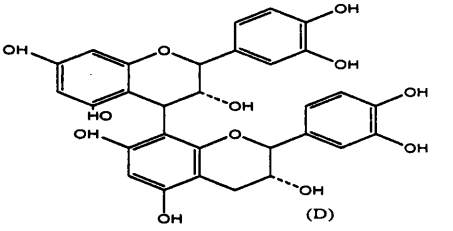
(C)

(C) (-)epicatechin



(B)

(B) phloridzin



(D)

(D) procyanidin B2

Figure 1-1 Typical phenolic components in cider apples (Lea 1995)

The traditional classification for English cider apples dates from Barker's early work, but is still a very useful guideline today (Table 1.1) (Lea 1995). Not all ciders are made from 'true' cider apples and many modern English ciders have a high proportion of dessert and culinary outgrades, or are reinforced with apple juice concentrate, for example, Bulmer recipes use both the juice from cider apples and normal eating apples to make a range of ciders. Sugar and malic acid are added to get the required taste, and the alcohol is adjusted to the correct strength for bottling (between 4% and 8.0%, depending on the product). In addition to apple juice, a little pear juice was blended into some of ciders. A proportion of pear has been traditionally used to provide a softer mouth-feel, in the same way that merlot grapes do in red wine.

Table 1.1 Classification of cider apples

	Acid (%) w/w	Tannin (%) w/w
Sharp	> 0.45	< 0.2
Bittersharp	> 0.45	> 0.2
Bittersweet	< 0.45	> 0.2
Sweet	< 0.45	< 0.2

1.1.3 The cidermaking process

Brewing beer is a science; making cider is an art, more akin to wine making. The foundation of the art is simple – to apple juice you add sugar and yeast and wait for fermentation. Cider is made from apple juice as a result of two fermentations. The first, carried out by yeasts derived providentially from the processing equipment or added deliberately, converts sugar to ethanol and the higher alcohols. The second, a conversion of L-malic acid to L-lactic acid and carbon dioxide by lactic acid bacteria can occur concurrently with the yeast fermentation or just after alcohol fermentation.

After fermentation, the rough cider is filtered, which removes the "lees" – the dead yeast cells – and it is moved to a storage vat. At this point the cider is high in alcohol and is completely "dry" – no residual sugar. The temperature is controlled and air is kept away from it. Sugar and malic acid are added to get the required taste, and the alcohol is

adjusted to the correct strength for bottling (4-8.0%). It is then very finely filtered to make it clear and "bright". Before filling, the cider is cooled to around 2°C and it carbonated by addition of CO_2, and then the bottles are pasteurized to kill any bacteria or remaining yeast that might affect the shelf life. This process involves heating the bottles to around 65°C, holding at that temperature, and then cooling again. The cycle of cider making operations is shown in Figure 1-2.

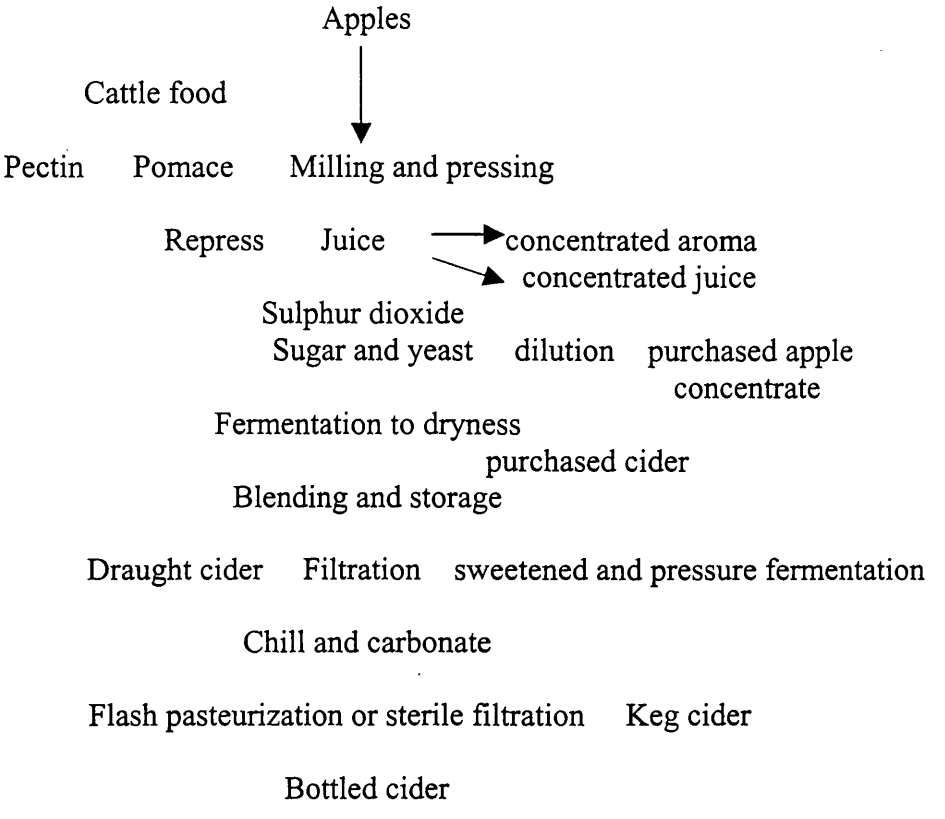


Figure 1-2 Flow diagrams of the cidermaking process (Beech[2] 1972)

1.1.4 Factors affecting cider flavour

Flavour is a complex sensation in which all factors interact. It is a combination of taste and aroma (Fredette 1970; Williams 1974; Williams & Tucknott 1971; Whiting 1975) and is one of the main factors which make alcoholic beverages enjoyable.

The primary characterization of cider is a 'spicy, aromatic, appley note' which occurs to a greater or lesser extent in all ciders (Jarvis *et al.* 1995). The sugars contribute to sweetness, the acids to sourness and the phenolics to bitterness and astringency. Non-

volatiles primarily give rise to taste sensations and the volatiles to aroma and mouth flavour other than the four basic tastes – sourness, sweetness, bitterness and astringency. Ciders can differ widely in taste and aroma, depending on the variety of apple used and method of manufacture (Williams *et al.* 1978; Williams & Tucknott 1978; Williams *et al.* 1980; Williams 1988), e.g. the juice concentration, the acidity of apple juice, the addition of sulphur dioxide and fermentation conditions (Temperature and hydrostatic pressure of fermentation vessel) (Jarvis *et al.* 1995). Also, the volatile flavour of cider is in most part qualitatively identical to that of all other fermented beverages and derives to a large extent from the selection of yeast (Durr 1986). Yeast species and strains can have a significant influence on the formation of volatile flavour components.

Table 1.2 Factors influencing the flavour of cider

Apple juice	Variety of fruits; Maturity and condition of fruit at pressing; Fresh juice or concentrate; Condition of concentrate
Other ingredients	Type of chaptalization sugars; Quantity of SO_2; Amelioration of pH by addition of acid; Yeast nutrients
Yeast	Natural or inoculated fermentation; Strain of yeasts; Condition of yeast when inoculated
Fermenter design and operation	Temperature / time of fermentation; Hydrostatic pressure; Operational pH / acidity level
Secondary fermentation	Natural or induced malo-lactic fermentation; Secondary yeast fermentation; Spoilage organisms
Maturation	Chemical and enzyme changes
Processing factors	Decolourization of juice or final cider; Dealcoholization
Final product make-up	Ingredients added (e.g. sugar, acid, colour); carbonation

There are many factors that combine to give a type of cider its particular flavour. Although the nature of the flavour constituents is not completely understood, modern

developments in cider brands have resulted in cider products with flavours ranging from 'wine-like' to 'malty' and 'hop-like'(Jarvis *et al.* 1995). Table 1.2 above shows the diversity of factors influencing the flavour of cider. A diversity of chemical compounds as contributors to the flavour of a cider have been found (Williams 1974; Williams and Tucknott 1971; Beech[2] 1993). Over 200 compounds which contribute to the flavour of ciders produced and stored in different ways have been identified in Bulmers, UK (Jarvis *et al.* 1995). This work helped to gain a better understanding of the way in which both desirable and undesirable flavour are produced and to control more effectively the production of ciders by traditional and modern fermentation processes.

1.2 Fermentation and Maturation

The microbiology of cider is complex (Bisson & Kunkee 1991; Bizeau *et al.* 1992), with microorganisms beneficially manipulated during two main phases of the cider making process: the primary fermentation (alcoholic) and the secondary bacterial fermentation (maturation). Yeast (*Saccaromyces cerevisiae, Kloeckera apiculata, Candida sp.*) carries out the alcoholic fermentation and lactic acid bacteria (*Oenococcus* (formerly *Leuconostoc*), *Lactobacillus, Pedicoccus*) the maturation. Acetic acid bacteria (*Acetobacter*) and other spoilage organisms may also occur at various times during the manufacture of the cider with detrimental effects on the product flavour. Acetic acid bacteria are strictly aerobic and will only occur in the presence of oxygen.

1.2.1 Primary fermentation

The primary fermentation mainly refers to the conversion of a range of sugars in apple juices to alcohol. Numerous factors are involved in the process of cider fermentation. There are changes in the yeast populations, both qualitative and quantitative, the chemical composition of the juice that controls the extent and rate of fermentation and the excretion of metabolic products by the yeast.

In traditional cider fermentation, no yeast is added and no sulphite is used, the non-*Saccharomyces spp.* are dominant and multiply quickly to produce a rapid evolution of

CO_2 and alcohol. Also, they generate a distinctive range of flavours, characterized by ethyl acetate, butyrate and related esters. When the alcohol level rises (2-4%), these initial fermenters begin to die out and the microbial succession is taken over by the alcohol tolerant organism *Saccharomyces uvarum*. This yeast continues to convert all the sugars into alcohol and to generate more wine-like flavour. If, however, the initial juice is treated with SO_2, which acts as both an antimicrobial agent and an antioxidant, the non-*Saccharomyces* yeasts and most bacteria are suppressed or killed. After a lag phase of several days, this enables the *Saccharomyces* spp. to multiply and allows the fermentation to continue to dryness with a more homogeneous and benign microflora than in the case of an unsulphited juice.

Nowadays, specific cultured yeasts are added to cider fermentation, the most important is their ability to ferment a wide range of sugars to alcohol in the presence of free SO_2, over a wide range of temperature, and to produce desirable flavour changes without the production of excessive amounts of H_2S or other mercaptans. Most yeast starter cultures used in the cider industry have been from culture collections of wines yeasts. Production of a sound wine, or indeed cider, requires the controlled exploitation of desired organisms while simultaneously discouraging the growth of unwanted microbes (Bisson & Kunkee 1991). By using sulphur dioxide, a selected pure culture yeast and fermenting ciders to dryness, followed by anaerobic storage, many undesirable microorganisms are eliminated (Buckle 1967). Dried cell preparations are increasingly being used commercially and added directly to a vat or used as inoculum to propagate starter cultures (Jarvis *et al.* 1995).

1.2.2 Malolactic fermentation

Malolactic fermentation (MLF) refers to the conversion of L-malate into L-lactate and CO_2 conducted by lactic acid bacteria (LAB). Malate is a dicarboxylic acid, containing two carboxyl groups, while lactate has a single carboxyl group and is monocarboxylic. Therefore the conversion of L-malate into L-lactate and CO_2 is a decarboxylation (Henick-Kling 1995) and leads to a natural decrease of acidity together with an enhancement of bacterial stability and flavour changes of alcoholic beverages. As with

wine, it is not unusual for cider to undergo MLF. As noted above, the MLF can reduce acidity in the cider, and also it can produce subtle flavour changes associated with the metabolism of tannin-derived substances as well as influence the microbiological stability (Davis *et al.* 1988; Kunkee 1997). Deacidification is important in regions where the fruit is high in acid at harvest, but may not be desired for all wines already low in acidity. In fact, in warmer areas grapes tend to be less acid and a further decrease in acidity by MLF may be deleterious for the sensory properties and biological stability of the wine (Davis *et al.* 1985; Henick-Kling 1995; Kunkee 1974; Renault *et al.*1988). Anyway, this conversion may occur whether desired or not if conditions support the growth of the organisms.

Malolactic fermentation is an important secondary reaction in winemaking and generally occurs just after the alcoholic fermentation has been completed. Different bacterial genera (*Lactobacillus, Pediococcus, Leuconostoc* and, principally, *Oenococcus*) have been reported to carry out MLF in wine produced worldwide (Edwards 1992; Izuagbe *et al.*1985; Juhasz *et al.* 1994; Patarata *et al.* 1994; Kosseva *et al.* 1998; Sieiro *et al.* 1990). *Oenococcus oeni* (formerly *Leuconostoc oenos*) is the major bacterial species found in wines during MLF, and is well adapted to the low pH and high ethanol concentration of wine (Wibowo *et al.* 1985; Tracey *et al.* 1987; Kunkee 1991). Several examples of vineries promoting MLF are found in the USA (Beelman *et al.* 1980; Henick-Kling *et al.* 1989; Delaquis *et al.* 2000), France (Lafon-Lafourcade[2] 1983; de Revel *et al.* 1999), Spain (Maicas[2] 1999; Sieiro *et al.* 1990; Pardo *et al.* 1992), Italy (Spettoli *et al.* 1982), and Australia (Costello *et al.* 1985; Davis *et al.* 1985).

A great deal of investigations into MLF has been carried out due to its economical importance and many reviews were published in the last two decades (Davis *et al.* 1985; Kunkee 1991; Van Vuuren & Dicks 1993; Versari *et al.* 1999). Influence of physico-chemical factors on the growth of *O. oeni* has been evaluated (Tracey & Van Rooyen 1988; Britz & Tracey 1990; Salou *et al.* 1991). MLF pathway and the mechanism of metabolic energy generation have been discussed (Salema *et al.* 1996; Miranda *et al.*

1997, Henick-Kling 1995). The metabolism of sugar and malic acid or citric acid by *O. oeni* was studied (Rodriguez *et al.* 1994; Firme *et al.* 1994; Salou *et al.* 1994, Vaillant *et al.* 1995).

There have been many studies on utilisation of *O. oeni* in wine or cider. Cell immobilized technology and simultaneous fermentation with yeast and lactic acid bacteria in apple juice have been developed around the world (Webb 1989; Scott & O'Reilly 1996; Laca *et al.* 1998). To increase the commercial production of *O. oeni* strains to be used for biological deacidification of wines, Maicas[2] *et al.* (1999) reported the related research results that production of *O. oeni* biomass induces malolactic fermentation in wine by control of pH and substrate addition (fructose alone, fructose and glucose). The use of this technological approach enables a high production of well-adapted biomass that can be used as an effective starter culture to induce the MLF in wines. Ca-alginate matrix was used to co-immobilize *Saccharomyces bayanus* and *Oenococcus oeni* in one integrated biocatalytic system in order to perform simultaneously alcoholic and malolactic fermentation of apple juice to produce cider (Herrero[2,3] *et al.*1999) or in a continuous packed bed bioreactor (Nedovic *et al.* 2000). Cells of *Oenococcus oeni* immobilized in alginate beads were used as starter culture to conduct malolactic fermentation in cider production (Durieux *et al.* 2000; Herrero *et al*, 2001). There are few studies involving cell retention by using cross flow filtration modules.

1.3 Microbiology of cider making

1.3.1 Lactic acid bacteria and their suitability to cider

Lactic acid bacteria (LAB) are a wide–ranging heterogeneous group of organisms that occur by design or by chance in many foods and drinks. LAB can be found in dairy products, meat, fish, water, sewage, beer, wine, fruits and fruit juice, pickled vegetables, sour dough and mash. They are also normal flora in the mouth, intestinal tract and vagina of many homothermal animals (Kandler *et al.* 1996; Bottazzi 1988). LAB have important roles in food fermentations, as probiotics and in beverage maturation. LAB are also able to perform several important biotransformation reactions. Most notable are the ability of

these systems to metabolize malic acid, tannins and related compounds to form important flavour and fragrance compounds in beers, ciders and wines. These biotransformation capacities also have uses in other areas of biotechnology including specific enzymes for sensors, diagnostics and biotransformation.

The MLF results from the growth of a diversity of LAB, containing strains of *'Lactobacillus pastorianus var. quinicus', L. mali, L. plantarum, Leuconostoc mesenteroides, L. oenos* and *'Streptobacterium'* spp. (Jarvis *et al.* 1995). The major desirable organism appears to be the heterofermentative coccus, *Oenococcus oeni* and other *Lactobacillus* spp. may also be present (Beech & Carr 1977; Carr 1983, 1987). There is only one species of *Leuconostoc* found in wine, *Leuconostoc oenos.* This organism is quite different from other members (pH and alcohol tolerance) of this genus, so a new genus and species name for this organism, *Oenococcus* was proposed (Dicks *et al.* 1995) and has now become the official designation. Therefore, both *Oenococcus oeni* and *Leuconostoc oenos* can be found in the literature. *Oenococcus oeni* (*O. oeni*) is an acidophilic behaviour and is able to grow in wine at an acidic pH, in the presence of ethanol and sulphite as well as most often responsible for the MLF in alcoholic beverages. Most heterofermentative lactic rods occurring throughout the cider industry are quite similar and form a fairly coherent group (Carr *et al.* 1972), presumably because of the years of natural selection to the harsh environment found in yeast-fermented cider. Heterofermentative LAB appear to be more acid tolerant than homofermenters, hence their predominance in cider. Cider LAB metabolize fructose in preference to glucose (Carr 1959), an advantage as fructose is the predominant sugar in apple juice. Most bacteria exhibit an inhibition of growth over a range of 1-5% (v/v) ethanol, except for organisms of the genus *Lactobacillus* (Couto *et al.* 1996), while the ability of LAB to survive in yeast fermented apple juice to over 12%. Indeed some organisms can tolerate over 20% ethanol in fortified rice wines.

In the absence of sugars, cider LAB must be able to utilize carbon sources not metabolized by yeast (Carr 1959). Heterofermentative *lactobacilli* can degrade organic acids such as citric acid, tartaric and malic acid to lactic and acetic acid and CO_2, as well as being able

to hydrolyse chlorogenic acid to quinic acid which in turn is reduced to dihydroshikimic acid. Shikimic acid may be reduced to catechol, while p-coumaric acid to p-ethylphenol (Kandler *et al.* 1996). The ability to utilize quinic acid is limited to a small group of herterofermentative rods and is not common to all LAB (Carr 1959), further explaining why LAB isolated from cider are so suited to a cider environment. But it should be noted that although suitable to cider and wine, LAB are the main spoilers of beer, and *L. brevis* is a common brewery contaminant (Yasui & Yoda 1997).

1.3.2 Metabolism of lactic acid bacteria

There are various strains of LAB and have been divided into different groups based upon the spectrum of end products produced. There are strict homofermenters, which only produce lactic acid and are unable to grow on pentoses. The strict heterofermenters generate several other compounds in addition to lactic acid. The final class is the facultative heterofermentative (or facultative homofermentative) LAB. These organisms are the most versatile and can switch between hetero- and homofermentative modes of metabolism, depending upon the carbon source (Zuniga *et al.* 1993).

- Homofermentative LAB degrade hexoses via glycolysis (Embeden-Meyerhof Pathway), producing lactic acid as the major end product.
- Heterofermentative LAB use the phosphoketolase pathway and yield lactic acid, CO_2, acetate and/or ethanol.

Four types of LAB have at some time been isolated from ciders, namely heterofermentative rods and cocci and homofermentative rods and cocci (Carr & Whiting 1971). In greater detail the main routes for catabolism of glucose by LAB have been determined (Romano *et al.* 1987; Thompson 1987; Salou *et al.* 1994; Miranda *et al.* 1997).

(1) Homofermentative metabolism

In homofermentative metabolism, the majority of the sugar present in the medium is converted to lactic acid. Lactic acid is produced from the reduction of pyruvate. Pyruvate

is formed via glycolysis (Embeden-Meyerhof Pathway, EMP) (Mandelstam *et al.* 1982) from hexose and then used directly as hydrogen acceptor to produce lactate. Theoretically, therefore, two lactic acid molecules are formed from each hexose molecule catabolized from the above equation. But in actuality, in homofermenting organisms the yield is frequently about 1.8 mol lactate/mol glucose, the rest being incorporated into cell structure. The ATP yield in the homofermentative pathway is 2 mol ATP per glucose metabolized (Gottschalk 1979).

Glucose → 2 Lactic acid

Glucose → 2 Pyruvate via glycolytic pathway

Pyruvate $\xrightarrow[\text{NAD}]{\text{NADH}}$ Lactate

(2) Heterofermentative metabolism

Heterofermentative organisms metabolize glucose via the phosphoketolase pathway (Fig. 1-3) which produces significant amounts of lactate together with other major products such as CO_2, acetate or ethanol (Mandelstam et al. 1982). They also ferment pentose with the formation of lactate and acetate. End products can vary depending upon level of aeration and presence of other proton and electron acceptors. Acetyl-phosphate can be converted to acetate and ATP or reduced to ethanol without ATP production. Five carbon sugars or pentose can be metabolized via this pathway. In this case the carbons appear as lactate and acetate.

In the course of this fermentation, the heterofermenting bacteria produce 0.8 mol lactate and some acetate in addition to ethanol and CO_2, the ATP yield is one per mol of glucose, half that of the homofermentative pathway (Gottschalk 1979).

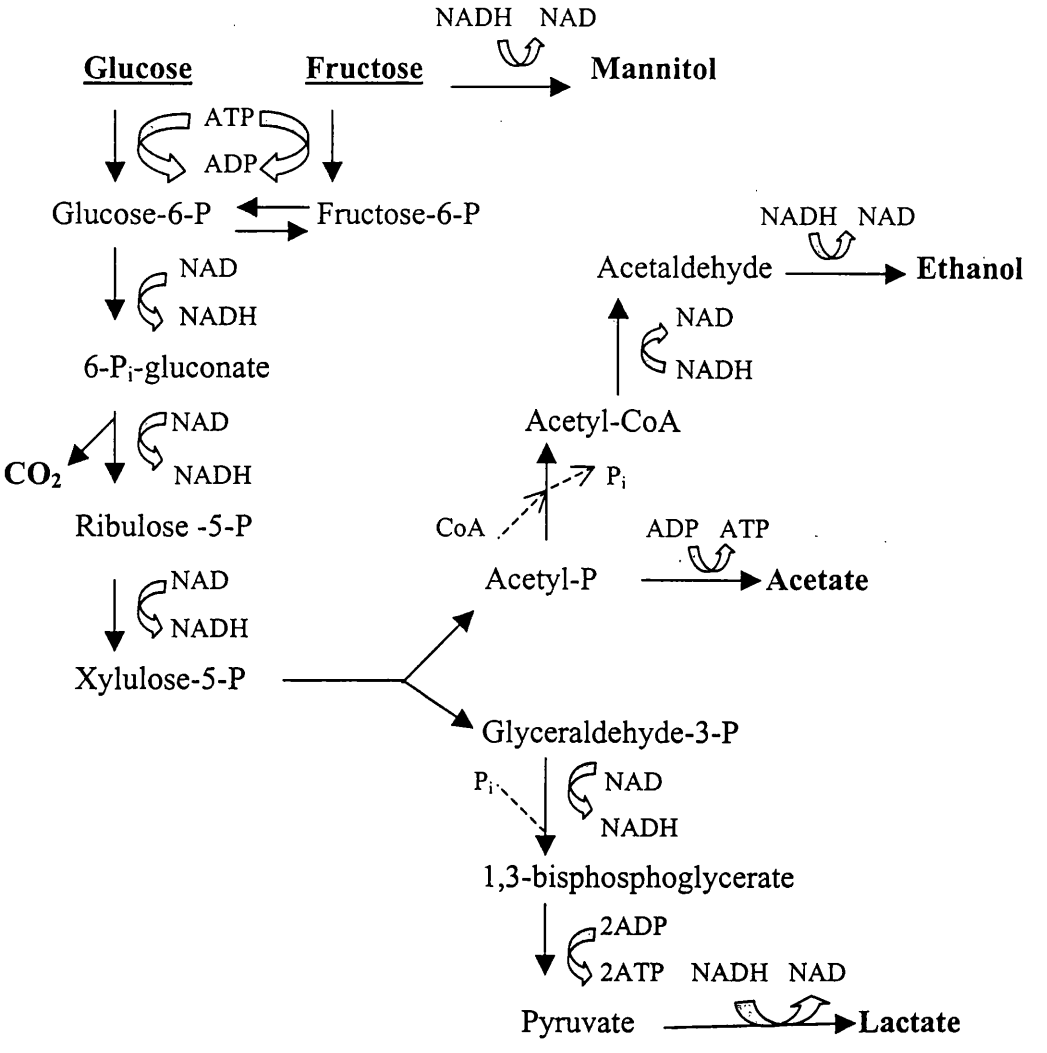


Figure 1-3 Metabolic pathways of glucose and fructose by the heterofermentative LAB

Glucose-6-P, glucose-6-phosphate; -P, -phosphate; 6-P_i-gluconate, 6-phosphogluconate; acetyl-CoA, acetyl coenzyme A; NAD, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form).

1.3.3 *Oenococcus oeni* and *Lactobacillus brevis*

These two bacteria that are of particular interest were employed in this research being undertaken at Swansea University.

O. oeni 11648: Gram Positive. Cells are spherical, but lenticular when grown on agar and usually occur in pairs or chains. Growth on surface plates is poor, and the smooth, round, grayish, white colonies are seldom larger than 1 mm in diameter. Growth in broth cultures is uniform, generally 0.5-0.8 × 0.5-1.4 μm, except when cells in long chains sediment.

This strain was supplied by UKNCC (the United Kingdom National Culture Collection) and was isolated from wine. A different name for the strain is *Leuconostoc oenos*. Type number *11648* is also assigned as *ATCC23279*, *DSM20252*, *NCDO1674* or *NRRLB3472*. It should be noted that *Leuconostoc oenos* is the only acidophilic member of the genus *Leuconostoc* and occurs naturally in wine and related habits. Therefore, *Leuconostoc oenos* has been assigned to a new genus as *Oenococcus oeni* (Dicks *et al.* 1995). Both names can be found in the wine literature but they are the same organism.

L. brevis X_2: Thick Gram-positive rods, heterofermentative, catylase–negative, oxidase – negative. Colony morphology on Rakka Ray Agar: cream/white, shiny, erose, flat and radically striated. *L. brevis* is obligate heterofermentative (Bottazzi 1988; Ye *et al.* 1995), generally short straight rods with rounded ends, generally 0.7-1.0 × 2-4 μm, occurring singly and in short chains. *L. brevis* is a type of LAB found commonly in sour dough (Gobbetti *et al.* 1995; Stolz *et al.* 1995) and as a spoilage organism in beer (Beverly *et al.* 1997; Sami *et al.* 1997). *L. brevis 8664* was collected by NCIMB (National Collection of Industrial and Marine Bacteria, NCIMB is a constituent body), equivalent-strains: NCFB2837, isolated from cider (Millis *et al.* 1954; Sharpe *et al.* 1972). This LAB strain used in this particular study was isolated from the traditional oak vats that were used for the uncontrolled maturation of cider at the old Ryeland Street vat house, HP.Bulmer Ltd.

1.4 Biochemistry of maturation

1.4.1 Biochemistry of malolactic fermentation

1.4.1.1 Mechanism of malic acid transformation

Several metabolic reactions have been proposed whereby malolactic bacteria convert L-malic acid to L-lactic acid (Kunkee 1975; Radler 1986). Among the lactic acid bacteria there are three enzymatic pathways for the conversion of L-malic to L-lactic acid and CO_2 (Figure 1-4): (1) by the malolactic enzyme directly to L-lactic acid (reaction 1); (2) by the malic enzyme to pyruvic acid and subsequent reduction by L-lactate dehydrogenase to L-

lactic acid (reaction 2) and (3) reduction by the malate dehydrogenase to oxaloacetate, then decarboxylation to pyruvate and finally reduction to L-lactic acid at low pH. At pH 5 part of the pyruvate is oxidized to acetate and hydrogen, even a small amount of succinate (reaction 3).

(1) L-MALATE $\xrightarrow[NAD^+, Mn^{2+}]{}$ L – LACTATE + CO_2

(2) L- MALATE $\xrightarrow[NAD \; NADH]{Mn^{2+}, \text{malic enzyme}}$ pyruvate + CO_2 $\xrightarrow[NADH \; NAD]{\text{L-LDH}}$ L-LACTIC ACID

L-LDH = L-lactate dehydrogenase

(3) L-MALATE $\overset{(1)}{\rightleftarrows}$ Oxalacetate + [2H] $\xrightarrow{(2)}$ Pyruvate + CO_2

LACTATE

(4)↑ [2H]

↓(5) NAD NADH

↓(3)

Fumarate + H_2O

Acetate + [2H] + CO_2

(6)↓ [2H]

Succinate

(1) malate dehydrogenase (MDH)
(2) oxalacetate decarboxylase (OAD)
(3) pyruvate dehydrogenase (PDH)
(4) lactate dehydrogenase (LDH)
(5) fumarase
(6) fumarate reductase

Figure 1-4 Mechanisms of malic acid metabolism to lactic acid (Radler 1986; Henick-Kling 1993)

Most malolatic bacteria, including *O. oeni*, contain the malolactic enzyme (Radler 1986; Saguir *et al.* 1996) and decarboxylates L-malate to L-lactate in the presence of NAD^+ and Mn^{2+} (reaction 1). *O. oeni* can not grow on L-malate as sole carbon source, which indicates that no malic enzyme (reaction 2) or malate dehydrogenase enzyme (reaction 3)

is present (Radler 1986; Lonvaud-Funel 1995). These results would seem to be accounted for by the following observation (Lonvaud-Funel 1995).

During MLF, the residual sugars which are not fermented by the yeasts, are fermented by bacteria. Glucose, fructose, xylose and arabinose are metabolized by *O. oeni* by the heterofermentative pathway to produce D-lactic acid, acetic acid, ethanol and carbon dioxide as main products. Heterofermentative cocci such as *O. oeni* produce only the D-isomer of lactic acid from sugars, so these strains contain only the D-lactate dehydrogenase which catalyses the reduction of pyruvate to D-lactic acid. It was known that one molecule of the L-isomer of malic acid, the natural form of the acid in wine, was observed to produce one molecule of L-lactic acid and one molecule of CO_2. Therefore, malic acid conversion does not involve either the well known malate dehydrogenase or the malic enzyme which produce pyruvic acid from L-malic acid. A novel mechanism for malic acid degradation was assumed based on the stoichiometry and stereochemistry of the MLF reaction. During MLF, a novel enzyme catalyses the decarboxylation of L-malic acid without production of pyruvic acid as an intermediary free product. This enzyme has been termed malolactic enzyme (MLE) (Lonvaud & Ribereau-Gayon 1975). It was first purified from LAB species usually present in grape must or wine (Schutz & Radler 1973; Lonvaud-Funel 1982; Spettoli[2] *et al.* 1984), then also described in other kinds of LAB from various origins (Chagnaud *et al.* 1989; Strasser *et al.* 1984). The malolactic enzyme isolated from *L. plantarum* follows the conversion outlined in reaction 1 (page 17), but also decarboxylates oxalacetate to pyruvate (reaction 2, page 17).

The second pathway was observed in following reports. *Lactobacillus casei* and *Lactococcus faecalis* possess a true malic enzyme which converts malate to pyruvate and enables their growth on malate as carbon source (London & Meyer 1969; London *et al.* 1971). A third pathway exists in *Lactobacillus fermentum*, which produces D- and L-lactate, acetate, succinate and carbon dioxide from malate (Radler 1986). At low pH (< 4) mainly lactate is formed via oxaloacetate and pyruvate, while at high pH (> 5) part of the pyruvate is oxidized to acetate and hydrogen, and then the excess hydrogen reduces fumarate to succinate. The conversion of malate to acetate and succinate plays a lesser role in the bacterial metabolism of L-malate due to the pH of wine, cider as well, below 4.0.

1.4.1.2 Energetics of malolactic fermentation

The growth rate of *O. oeni* increases when cells are grown in the presence of L-malate (Pilone & Kunkee 1972, 1976; Salou *et al.* 1991). The increase in biomass is accompanied by an increase in the levels of D-lactic acid (Pilone & Kunkee 1972) and the activity of D-lactate dehydrogenase (Lee & Pack 1980). The most usual comment was that in an acidic medium like wine, the decarboxylation of L-malic acid induced an increase in pH which was beneficial to bacterial growth (Kandler *et al.* 1973). However, Pilone & Kunkee (1976) demonstrated that, during MLF by *O. oeni*, the growth rate at low pH was increased, independent of the change of pH to the growth medium. Recent results have further demonstrated that in an acidic medium, the malolactic reaction induces ATP production via a chemiosmotic mechanism (Lonvaud-Funel 1995). The pathway of MLF includes the transport of L-malate, its decarboxylation to L-lactic acid and CO_2, and efflux of the end products and the concomitant efflux of a proton (Cox & Henick-Kling 1989, 1990; Olsen *et al.* 1991; Poolman *et al.* 1991; Garcia *et al.*1992; Kolb *et al.* 1992; Loubiere *et al.* 1992; Tourdot-Marechal *et al.* 1993). The decarboxylation reaction is catalyzed by the malolactic enzyme (L-malate:NAD^+ carboxylyase) in the presence of NAD and Mn^{2+} (Morenzoni 1974; Spettoli[2] *et al.*1984; Naouri *et al.* 1990). This reaction does not yield energy-rich phosphate bond intermediates directly, however the electrochemical energy can be conserved via an indirect electrical potential ($\Delta\Psi$). As a proton is consumed in the decarboxylation reaction the internal pH increases. Alkalinization of the cytoplasm results in creation of a chemical potential of protons across the membrane - a pH gradient (ΔpH) that, together with the $\Delta\Psi$, forms the proton motive force (PMF) across the cytoplasmic membrane (chemiosmotic mechanism). The proton motive force generated in *O. oeni* by L-malate fermentation has been studied further (Salema *et al.* 1996; Miranda *et al.* 1997).

During MLF by *O. oeni*, the PMF generated by efflux of L-lactic acid creates a ΔpH able to drive L-malate transport and generate ATP via the membrane ATPases. MLF provides the cell with additional metabolic energy (ATP) which probably is responsible for a stimulatory effect during the early stage of growth (Kunkee 1974, 1991). During MLF, *O. oeni* obtained an energetic advantage from an increased intracellular pH and from an increased PMF. Cox & Henick-Kling (1989) showed that ATP was produced during MLF

and that its production was linked to PMF across the cell membrane and functional ATPase. It can be used for transport processes or converted into ATP via the membrane-bound ATPase. At low pH (< 4.5) and with limited amounts of sugar the additional ATP allows increased growth yields (Renault *et al.* 1988; Olsen *et al.* 1991; Garcia *et al.* 1992; Henick-Hling 1993). This mechanism apparently works in all LAB that possess the malolactic enzyme (Cox & Henick-Kling 1990). In batch cultures of *O. oeni* at pH 5.0, improved growth rate was observed when both glucose and malate were used as energy sources compared with glucose alone (Loubiere *et al.* 1992). MLF allows *O. oeni* to take up nutrients by the chemiosmotic mechanism and maintain a suitable pH for enzymatic activity and cell growth, especially at lower pH values (Salema *et al.* 1996).

1.4.2 Biotransformation of organic acids

Organic acids play an important role in the sensory perception of alcoholic beverages, specifically in cider (Herrero[1,2] *et al.* 1999). Being a constituent of sourness group, each acid in alcoholic beverages conveys a characteristic flavor, aroma or taste. During MLF, malic acid is transformed into lactic acid, thus reducing the high acidity found in apple juice. Meanwhile, the other formation of products rather than lactic acid is also accompanied, and modifies both flavor and mouth-feel (Davis *et al* 1985; Henick-Kling 1993). Not only composition, but also concentration, of each acid is essential in the quality of the product.

Apple juice may contain 2-8 g/l of malate (Leguerinel *et al.* 1988, 1989; Cabranes *et al.* 1996, 1997, 1998; Duenas *et al.* 1997; Laplace *et al.* 1998), depending on a wide range of apple cultivars. During wine or cider maturation, malate is metabolized by LAB by either of three different pathways (Radler 1986) as described above (section 1.3.1). Most LAB obtained from wine or cider possess the malolactic enzyme which decarboxylates L-malate to L-lactate in the presence of NAD and Mn^{2+} without free intermediates (Caspritz & Radler 1983; Naouri *et al.* 1990).

Small amounts of citric acid and pyruvic acid are present in wine (Hegazi & Abo-Elnaga 1980; Cogan *et al.* 1981). In wine, citrate and pyruvate are mainly converted to lactate and acetate (Starrenburg & Hugenholtz 1991; Salou *et al.* 1994). Wine LAB, split citrate by

citrate lyase into acetate and oxaloacetate; oxaloacetate is then decarboxylated to pyruvate which is converted to acetate, ethanol, lactate, diacetyl, acetoin, or butylene glycol (Radler & Brohl 1984). The metabolism of citric acid and pyruvic acid is shown in Fig. 1-5. The utilization of citrate by wine LAB does not link to the citric acid cycle because it is not active under anaerobic conditions, wine LAB themselves contain the malolactic enzyme and do not produce malate dehydrogenase which is part of the citric acid cycle (Schutz & Radler 1973, 1974; Caspritz & Radler 1983; Spettoli *et al.* 1984; Naouri *et al.* 1990).

Pyruvate is generally converted to lactic acid by lactate dehydrogenase (Fig.1-5). However, *Leuconostocs* and *Lactobacillus casei* can also degrade pyruvate to acetate with the gain of one ATP (Kandler 1983). In this pathway, almost all pyruvate may be converted to acetate (and ethanol) under glucose limiting conditions. In *Lactococcus lactis*, as in *Oenococcus oeni*, and *Lactobacillus plantarum*, intracellular pH decreases as the pH of the growth medium decreases (Fitzgerald *et al.* 1990; Olsen *et al.*1991; Maicas[2] *et al.* 1999). In addition, it should be noted that pyruvate could be formed from organic acid catabolism as well as from sugar catabolism. Its formation will therefore be dependent upon the amount of these precursors present.

From above described, it is well known that there are several important flavour changes during MLF in addition to deacidification. Acetic acid produced by LAB is formed not only from sugar catabolism but from citrate as well. Acetic acid itself is pungent but not offensively smelling. However, acetic acid production can lead to the formation of other objectionable compounds. Diacetyl is another very important compound produced by the LAB and formed from metabolites of pyruvate. This compound is acknowledged to be a positive flavour component in dry red wines in amounts lower than 4 mg/l (Rankin 1972), and has a characteristic buttery note, which can become as strong as popcorn butter in high concentration, and may take on a rancid taint. Acetoin and 2,3-Butanediol are also produced by LAB (Fig. 1-5). Moreover, the LAB can produce some ester characters that contribute positively to the aroma profile of the wine, such as ethyl lactate, diethyl succinate, acrolein (Radler & Gerwarth 1971; Maicas[3] *et al.* 1999; Herrero *et al.* 2001).

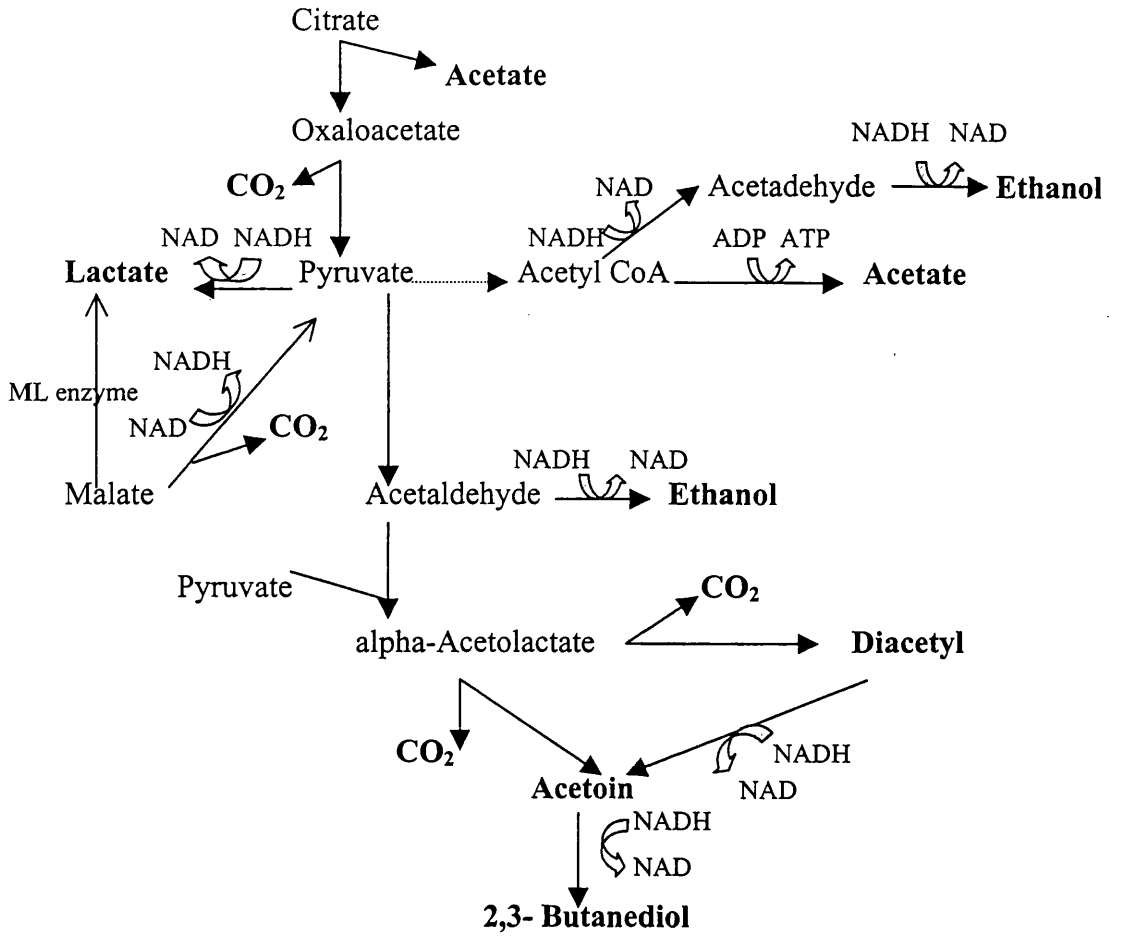


Figure 1- 5 Proposed pathway of metabolism of organic acids in the presence of glucose

1.4.3 Biotransformation of 'apple phenolics'

The term, tannins, covers a wide class of chemicals that are of particular interests in cider and wine. These chemical compounds are found within the traditional English bittersweet cider apples. Four classes of phenolic compounds may be distinguished in ciders (Lea[1,2] 1978):

(1) phenolic acids, e.g. chlorogenic;
(2) phloretin derivatives, e.g. phloridzin;
(3) catechins, e.g. epicatechin;
(4) procyanidins.

Only the procyanidins can be classed as true tannins and only they make any contribution to the bitterness and astringency of the cider product (Lea[1] 1978). The procyanidins may be regarded as polymers of the stereoisomeric (+) catechin and (-) epicatechin units (Lea[2] 1978). The analysis, isolation and structure of these phenolics have been described in detail (Lea[1,2] 1978; Lea & Arnold 1978; Lea & Beech 1978; Lea & Timberlake 1978). The number of true bittersweet cider apples available to cider-maker, unfortunately is diminishing year and year. It is important, therefore, to know something about the metabolism of cider phenolics so that the fruit available can be most effectively utilized.

Apple phenolics are metabolized by lactic acid bacteria during the maturation process based on quinic acid, cinnamic acid derivatives and volatile phenols which are involved in the complex flavor development of cider. Some of these compounds have been shown to have an important role on the growth and maintenance of the LAB stimulating growth in dry cider. Besides the malic acid, citric acid and pyruvic acid present in ciders that are metabolized by LAB, the metabolism of quinic acid, its esters chlorogenic and p-coumaryl-quinic acids and the related shikimic acid by *L. pastorianus* var. *quinicus* has been known (Carr *et al.* 1957; Whiting & Carr 1957, 1959).

Chlorogenic acid is hydrolyzed to caffeic and quinic acids first, and both are further metabolized by *Lactobacillus.* The metabolism of caffeic acid produced dihydrocaffeic acid and ethyl catechol, while quinic acid was reduced via shikimic acid to dehydroshikimic acid (Whiting & Carr 1957) which is further reduced to dihydroxycyclohexane-1-carboxylic acid by *L. plantarum.* Shikimic acid may be reduced to catechol by *L. plantarum* which also convert ρ-coumaric to ρ-ethylohenol. The electron source of these reactions is lactate which becomes oxidized to CO_2 and acetic acid (Whiting 1975).

Quinic acid, together with malic acid is one of the major acids occurring in apples; in fact, quinic acid is high concentration in some varieties of cider apples. Quinic acid, shikimic acid and quinic acid esters are found at greater concentrations in cider apples than grapes. Quinic acid is reduced, after MLF (Whiting 1975), via shikimate to dihydroshikimic acid by heterofermentative LAB (Carr *et al.* 1957); the latter case can be enolized, dehydrated

and decarboxylated to catechol. The transformation of quinate esters chlorogenic and ρ-coumaryl-quinic acid has been observed (Whiting & Carr 1957). These reactions have an important effect on cider flavor and of wine as well.

1.5 Malolactic starter cultures

Starter cultures are being used to induce MLF in North and South America, Australia, New Zealand, South Africa and now more commonly in Europe. As the sanitary conditions were improved the production of wines with improved quality, free of microbial defects has been achieved, but this has also led to wines of 'simple' and 'unbalanced' character. In addition to this, the MLF may not occur spontaneously or can be very unpredictable in modern wineries (Henick-Kling 1993). The use of starter cultures to induce MLF can ensure more rapid maturation process, reduce the potential spoilage by other lactic acid bacteria, reduce potential interference by bacteriophages (Sandine 1979; Sozzi *et al.* 1982) and, furthermore, control the strain responsible for the MLF and its contribution to wine flavour (Henick-Kling 1988).

Many possible improvements for the reliability of malolactic starter cultures may be achieved. Very little about the exact nutrient requirements of the bacteria used for starter culture production is still known. Initially, media based on grape and apple juice were used for the production of malolactic starter cultures. The difficulty of obtaining and storing fresh juice has been a major factor in the high cost of producing malolactic cultures. Also, the quality of the juice is not consistent, varying with the supplier and the growing season. Differences in the quality of the juice can significantly change the rate of growth and cell yield. Later, a synthetic medium was used and had the advantages that dry ingredients with known quality can be used at any time of the year. The synthetic medium can give a large biomass, high cell viability and enhanced malolactic activity (Krieger *et al.* 1992). During the preparation of malolactic starter cultures, it should be considered separately that factors affect cell growth and viability and factors affect the activity of the malolactic system. Desired conditions must be chosen so as to give a large yield of biomass which retains high viability and malolactic activity. The use of a basal medium of

known composition and supplemented with specific nutrients at specific stages of growth is required in order to optimize cell viability and malolactic activity. Hayman & Monk (1982) evaluated the effect of adding wine to a medium for the production of *O. oeni* and found that a content of 40-80% wine in the medium improved survival and malolactic activity of the culture. The best time for a harvest of *O. oeni* was 18-24 h after the culture reached stationary phase; the cells had the highest survival and malolactic activity after inoculation into wine (Krieger & Henick-Kling 1991).

Inoculating the wine with a high concentration of *O. oeni* enhances the probability of obtaining rapid and complete MLF. The inoculation of wines or musts with optimal numbers of cells that perform the MLF requires the use of medium and cultural conditions that optimize growth rate, biomass yield, and the ability to conduct the MLF in wine. Under non-proliferating *O. oeni* conditions, the MLF can be conducted in wine at various pH values, malic acid concentration and densities of cells. Results shows the MLF can be successfully induced with inoculation of high densities of *O. oeni* G6 even in recalcitrant wines (Maicas *et al.* 2000). Cells of *O. oeni* immobilized in alginate beads were used as starter culture to conduct MLF in cider production (Herrero *et al.* 2001), the rates of malic acid consumption were similar with that by using free cells in the same conditions.

The use of starter cultures in cider fermentation might allow cider makers to produce a uniformly high quality product to be maintained during successive processes and seasons. Although the use of starter cultures to control industrial fermentations is well established in brewing and wine production, it has not been widely adopted in cider making. Several lyophilized starter cultures for malolactic fermentation are available on the market, but are only useful for wine fermentations (Herrero *et al.* 1999). Commercial starter cultures have great advantages over traditional inoculation methods. Hence, it is interesting to develop cider starter cultures to perform the MLF in a rapid and reproducible manner applicable to the cider industry, and wine industry as well.

1.6 Growth and kinetics of *O. oeni* and *L. brevis*

1.6.1 Growth of *O. oeni*

O. oeni is one of the main species of LAB present in wine and cider and is well adapted to perform MLF in a highly alcoholic and low pH environment of wine or cider (Lafon-Lafourcade[2,3] 1983, Wibowo *et al.* 1985). A number of studies have examined the effect of the major factors affecting the MLF performance of various strains (Bousbouras & Kunkee 1971; Davis *et al.* 1988; Ingram & Butke 1984; Wibowo *et al.* 1988; Salou *et al.* 1994). It was well known that wine has complex composition, such as carbohydrate, pH, SO_2, ethanol, phenolic compounds, fatty acids, amino acids, micronutrients, etc. and this composition varies depending on cultivar, season, and technology of wine production. The metabolic and energetic characterization of the growth of *O. oeni* on glucose-citrate or glucose-fructose mixtures enables the potential role of this bacterium in the winemaking process to be ascertained. Moreover, mixotrophic conditions remain a suitable means for improving biomass productivities of malolactic starter cultures (Salou *et al.*1991, 1994).

In addition to nutrient factors described above, the physicochemical properties that influence microbial growth are well known and are: pH, acidity, ethanol and sulfite concentration and temperature. Investigations indicate that ethanol showed the greatest inhibitory effect, followed by pH and SO_2 (Tracey & Van Rooyen 1988, Vaillant *et al.* 1995). *O. oeni* grows best in low pH media (pH 4.2-4.8) supplemented with tomato juice or grape juice (Dicks *et al.* 1990). Both cultural pH and temperature of incubation were the most important factors affecting bacterial growth, the rate of substrate consumption and the amount of metabolites produced by three strains of *O. oeni* (Pimentel *et al.* 1994). The optimum growth temperature is in a range 20°C to 30°C. Different optimal pH and temperatures for the growth of *O. oeni* were reported by different researchers (Beelman & Gallander 1979; Lafon-Lafourcade[1,2] 1983; Britz & Tracey 1990; Salou *et al.* 1991; Firme *et al.* 1994) and are largely strain dependent.

The effect of the mixture of carbohydrate, except for the mixture of glucose-fructose, on the growth of *O. oeni* has not been reported in previous investigations. Neither has the

effect of other factors, such as organic acids, ethanol and temperature in the presence of the mixture of carbohydrate on the growth of *O. oeni* been determined. Moreover, few studies have concerned the production of *O. oeni* starter cultures with the aim of optimizing growth rates and biomass yields (Champagne *et al.* 1989, Naouri *et al.* 1989), although a few investigations into growth, substrate utilization and product formation were reported in batch cultures of *O. oeni* (Salou *et al.* 1991; Maicas[2] *et al.* 1999) in recent years.

1.6.2 Growth of *L. brevis*

The growth and metabolism of *L. brevis* X_2 have been investigated by Donovan (2000). His project included four relevant topics and is summarized as follows.

Based on the optimal medium (Donovan's broth, D broth), X_2 grew better in the presence of fructose than glucose, it was believed that fructose contains its own internal electron accepter through the formation of mannitol, thus stimulating the growth of the LAB. The doubling time and OD of X_2 obtained in his experiment was 6.9 h and 0.86 when grown on 5 g/l fructose based on D broth, while a doubling time 8.7 h and OD 0.85 for 5 g/l glucose based on D broth.

When *L. brevis* X_2 was grown on glucose based on D broth, the growth of X_2 was significantly stimulated by the organic acid additions, such as malic, pyruvic, citric and fumaric acids. Malic acid is of particular interest because 'green cider' contains high concentration of malic acid that can be converted to lactic acid by MLF, and so develop the flavour of cider. It was observed that the addition of low concentration of glucose (1mM) in the presence of malic acid significantly stimulated the growth of X_2. This low concentration of glucose was enough to stimulate the growth of X_2 metabolizing the malic acid, but not enough to sustain and maintain growth on its own. The degradation of malic acid was slow and had not been completed in 80 h without the addition of low concentration glucose.

In addition the effect of 'apple phenolics' on the growth of X_2 was also investigated and was also shown to stimulate growth. Finally, the influence of temperature and ethanol on the growth of X_2 was studied and showed that ethanol tolerance was dependent on temperature and nutritional status. The important results are quoted in Chapter 3 to allow a comparison of the growth and performance of *O. oeni*. These experiments were not carried out in this work.

1.6.3 Growth kinetics of *O. oeni* and *L. brevis* with pH control

Studies on the utilization of the major hexoses (glucose, fructose) in wine have been reported in non-pH controlled experiments (Tracey & van Rooyen 1988; Firme *et al.* 1994; Salou *et at.* 1994). The benefits of the pH control in other LAB cultures have been demonstrated (Beal *et al.* 1989). Some studies have concerned the influence of cometabolism of substrates on the growth of *O. oeni,* such as glucose-citrate mixtures (Cogan *et al.* 1987), glucose-fructose mixtures without pH control (Salou *et al.* 1994), a glucose-malate mixtures with pH control (Salou *et al.* 1991) and a glucose-fructose mixture with pH control at pH 4.8 (Maicas[2] *et al.* 1999). However, studies on the effect of glucose-fructose mixture with pH control at various pH values, concentration of glucose-fructose mixture on the growth of *O. oeni* could not be found in literature. The aim of the present work in section 4.3 of Chapter 4 was to make a detailed study of the growth kinetics of *O. oeni* strain under various culture conditions. The influence of pH control at various values, concentration of glucose-fructose mixture on the biomass production, substrate utilization and product formation was determined in detail. Moreover, the growth parameters, product yields and energetic analysis were also examined at various pH and different glucose-fructose concentrations during the batch cultures of *O. oeni.*

The cells of *O. oeni* and *L. brevis* were grown in continuous cultures at constant pH at a variety of dilution rates (section 4.4 & 4.5, Chapter 4). Substrate consumption and product formation were determined for the steady state concentrations. It was helpful to understand the roles of physiological and environmental factors on the growth and metabolism, and to develop a rational scale-up procedure.

1.7 Membrane bioreactors

A membrane bioreactor (MBR) has a separation capability lacking in other types of bioreactors. Combining various functions of membrane separations and biocatalyst characteristics of enzymes, microbial cells, organelles, animal and plant tissues can generate quite a number of membrane bioreactor systems. The cell-retaining properties of membranes and selective removal of inhibitory by-products make high density cell culture possible and utilize enzyme catalytic activity more effectively, which leads to high productivity of bioreactors. Anaerobic cell culture may be efficiently carried out in membrane cell recycle systems.

The mild operating conditions of many membrane separations make them favourable for a number of biotechnological separation steps. The sterile filtration of fermentation media, purification buffers, and protein product pools is standard practice in industry. In the process of lactate fermentation, the fermentation system allowed continuous removal of lactate, an inhibitory product, while retaining cells completely in the fermenter with recycling of the cells in the MBR. Another term commonly used for such systems, especially with animal cell culture, is perfusion bioreactor.

A membrane is a crucial part of an MBR. Microfiltration is also used extensively for medium exchange, cell harvest and sterile filtration as the final step in the production of a protein product (Bowen & Gan 1991). Ultrafiltration can be found in virtually every biotechnology process (Van Reis & Zydney 2001). Biocatalytic membrane reactors combine a selective mass transport with chemical reactions, and a selective removal of products from the reaction site that increases the conversion of product-inhibited or thermodynamically unfavourable reactions. Membrane reactors using biological catalysts can be used in production, processing and treatment operations (Giorno & Drioli 2000). In the biotechnology sector microfiltration, mainly for sterilization and separation of cells, represents the largest application of membranes followed by ultrafiltration in the food and dairy industries (Bell & Cousins 1994).

1.7.1 Membrane performance

Microfiltration (MF) is the oldest membrane technology and is a pressure-driven membrane process. It started at the beginning of 20th century with the preparation of synthetic microporous membranes based on cellulose. Nowadays, cross-flow microfiltration (CMF) is widely used in many industries (Ripperger & Altmann 2002). In CMF, the fluid to be filtered flows parallel to the membrane surface and permeates through the membrane due to a pressure difference. The cross-flow reduces the formation of a filter cake and keeps it at a low level. So it is possible to get a quasi-steady filtrate flow for a long time. Nowadays, this operation mode is a standard operation in many medical, foods, beverages and chemical membrane applications. This operation mode is sometimes also called tangential or dynamic filtration. The following overview (Ripperger & Altmann 2002) demonstrates the scope of this separation technique for fine particles, droplets and macromolecular substances:

- Clarification of fruit juice, wine, cider and vinegar;
- Separation of fat and bacterial removal from milk;
- Removal of beer yeast;
- Clarification of whey before other membrane filtration steps, like ultrafiltration or electrodialysis;
- Cleaning of potable water without chemical conditioning (reduction of microbial load);
- Filtration of fermentation broths;
- Upgrading of cleaning solutions in food and metalworking industries;
- Wastewater treatment.

Although cross-flow membrane filtration is a very attractive option for harvesting cells and recovering enzymes from cell homogenates, the process is not without its problems. Foremost of these is the deposit of dissolved and suspended solutes onto the membrane surface during operation. The formation of these dense and, sometimes, compressive layers (often called cakes) offers an additional resistance to axial and permeate flows, results in the decline in the membrane performance. Current malolactic fermentation systems are generally operated at constant flux instead of constant trans-membrane

pressure to improve product yield and throughput (Van Reis & Zydney 1991; Kwon *et al.* 2000; Li *et al.* 2000). However, the trend towards high-density cell cultures, and the corresponding increase in the level of cell debris, can cause unacceptable fouling of membrane systems (Belfort *et al.* 1994). Different techniques are used to avoid or reduce the filter-cake formation on the membrane, for example, back flushing and back pulsing, shear-enhanced modules or working below the critical flux. With back flushing (or pulsing) the permeate flow is reversed with a certain time interval during the process to push the filter cake off the membrane surface back into the feed stream. Using shear filters, or rotary modules, high-shear rates are reached which reduce concentration polarization.

1.7.2 High density cell cultivation

High density cell culture systems generally include immobilized cell and film bioreactors (Vickroy *et al.* 1982; Inoes *et al.* 1983, 1985; Mehaia & Cheryan 1984; Nanba *et al.* 1985; Karel *et al.* 1989), fed-batch bioreactors (Yamane & Shimizu 1984) and cell recycle bioreactors (Ohleyer *et al.* 1985). The fed-batch method is the most commonly used in commercial practice and can be used if byproducts or products do not cause serious inhibition of growth or product formation. Immobilized cell bioreactors, film bioreactors and cell recycle methods are suitable for continuous cultures. Immobilization methods have advantages, such as protection of cells from shear or environment and improvement of recombinant DNA stability if immobilization is simple and the stability of the immobilized cells is maintained during the desired operation period. However, for aerobic cells the oxygen supply is a major limitation, scale-up difficulties exist as well. The recycle methods have been often implemented by sedimentation, centrifugation and microporous membranes. Membrane-based cell recycle technique, unlike sedimentation and filtration, allows complete cell recycling and inhibitory products removed. Nowadays, reactors equipped with hollow fibre membrane to retain cells have been used in general laboratories and pilot plants for ethanol and lactic acid fermentations and cultures of *Escherichia coli* (Lee *et al.* 1980, 1987, 1988, 1989, 1990; Nishizawa 1983; Vickroy *et al.* 1983). Thus, cell recycle cultures, besides improving productivity, have the benefit of increasing the average cell age in the reactor and this can be useful in production of

secondary metabolites (Chang *et al.* 1994). The performance of membranes used in cell recycle is important because the productivity of a bioreactor depends on product concentration and dilution rates. High dilution rates should be achieved so as to provide rich nutrients for the growth of cells. Membrane fouling, a major limitation, should be minimal to ensure long term stability of the reactor.

Cultures of cells in MBR with complete cell cycle have been effectively used to retain and/or propagate high cell concentrations. For example, a rotor fermenter equipped with a porous membrane was used to separate yeast cells from the medium in ethanol fermentation (Margaritis & Wilke 1978), the productivity of ethanol was 23-28 g/l/h. Rogers *et al.* (1980) used a micro-channel filter to retain *Zymomonas mobilis* for rapid ethanol production with ethanol productivity 120 g/l/h. Lafforgue *et al.* (1987) showed that continuous fermentation with total cell recycling enabled yeast concentration to reach 300 g/l with a dilution rate of 0.5 h^{-1} and a cell viability greater than 75%. In addition, membrane cell recycling has also been employed to produce lactic acid (Mehaia & Cheryan 1987; Taniguchi[1,2] *et al.* 1987; Major & Bull 1989; Bibal *et al.* 1991). The productivity was very high and continuous production of lactic acid was possible. The viability of *Lactobacillus delbrueckii* between 88% and 96% was obtained during all the run when using CSTR (continuous stirred tank reactor) with cell recycle produced lactic acid (Ohleyer *et al.* 1985). High concentration cultivation of lactic acid bacteria using two types of ceramic filter (tube type and multihole) was undertaken (Taniguchi[1,2] *et al.* 1987), and also the physiology of *Lactobacillus delbrueckii* in a continuous fermentor with cell recycle was studied (Major & Bull 1989). Actually, membrane recycle systems have widely been used to achieve high concentration of cells for the various enzyme and metabolic products, such as superoxide dismutase (SOD) (Holst *et al.* 1985), salicylate hydroxylase (Berg *et al.* 1989), β-galactosidase (Kawakatsu *et al.* 1993), penicillin acylase (Lee & Chang 1988, 1990), tryptophan (Lee *et al.* 1989), citric acid (Enzminger & Asenjo 1986).

It is known that the key factors governing the rate of MLF are said to be related directly to malic acid concentration, cell density and secondly to the specific malolactic activity within the LAB (Markides 1993). Generally, these systems require special technologies to confine or immobilize the cells in order to maintain them at high concentrations. High cell density reactors offer the following prospects for enology (Divies 1993), and cider making.

- Improved performance of alcoholic and malolactic fermentations.
- Adaptation to continuous processes that can be better optimized and controlled.
- Simplified systems for removing microbial cells from batch processes.
- Smaller scale fermentation facilities, with reduced capital expenditure.
- Possibilities of using a wider variety of microbial strains including genetically modified organisms.

There are two main systems for the confinement or immobilization of microbial cells (Divies 1993):

- Homogeneous systems where there is uniform distribution of the microbial biomass in the reaction medium. This result can be obtained using biomass recycling by centrifugation or maintenance of the biomass in a membrane reactor.
- Heterogeneous systems where two phases are distinguished; the liquid medium to be transformed and a solid phase containing the biomass.

However, high concentration cultivation of *O. oeni* and *L. brevis* in membrane bioreactor with complete cell recycle has not been described. In this research project, a new fermentation system with continuous separation of inhibitory metabolites by cross-flow filtration was employed for high concentration cultivation of *O. oeni* and *L. brevis* in order to improve the productivity of cell mass. This work was carried out in section 5.3 of Chapter 5.

1.7.3 Enhanced cider maturation

Traditionally, MLF, a natural process, in alcoholic beverages is slow and unreliable. It can take many weeks or months for the fermentation to be completed (Wibowo *et al.* 1985). Typically, these are in extreme environments of high alcoholic content (over 10%, w/v) and high acidity (below pH 3.5). In recent years, a starter culture technology, involving the inoculation of *O. oeni* into the wine, has been developed for inducing the MLF (King & Beelman 1986; Edwards & Beelman 1989). Although this technology increases the prospect of a rapid and reliable MLF, failures can still occur because the fermentation depends upon the batch growth of the bacterial cells inoculated into the wine which itself is a harsh and extreme environment. The condition of the cells at the time of inoculation and the properties of the wine will affect the growth of the MLF microbes and success of the biotransformation.

Conventional methods of fermentation that use free cells in batch processes have limitations, such as low productivity, product inhibition and batch to batch variation, which lead to high fermentation costs (Tejayadi & Cheryan 1995). Immobilized cell technology was used for cider maturation (Scott & O'Reilly 1996; Carbanes *et al.* 1998). Immobilized cell technology offers several important advantages as compared to the traditional fermentation by free cells, e.g. higher cell densities per unit bioreactor volume that result in very high fermentation rates, the reuse of the same biocatalysts for prolonged periods, the development of continuous process that may be operated beyond the nominal washing-out flow rate and smaller bioreactor volumes that may decrease capital costs (Pilkington *et al.* 1998). It also gives an opportunity to co-immobilize different kinds of microorganisms within the same porous matrix, allowing the integration of the two fermentation steps in one system. For example, in a continuous packed bed bioreactor, Ca-alginate matrix was used to co-immobilize *Saccharomyces bayanus* and *Leuconostoc oenos* in one integrated biocatalytic system in order to perform simultaneously alcoholic and malolactic fermentation of apple juice to produce cider (Nedovic *et al.* 2000). The continuous process permitted much faster fermentation compared with the traditional batch process. The flavour formation was also better controlled by adjusting the flow rate of feeding substrate through the bioreactor.

A membrane bioreactor improves the efficiency of enzyme-catalyzed bioconversions by simultaneously combining the two unit operations of enzymatic conversion and filtration of product. MBR can be charged with high densities of cells, the cells can then serve as biocatalysts, carrying out biochemical transformations. In the case of cider, it would be using a high density of LAB to carry out the complex biotransformation involved in the maturation of cider. MBR can also be easily scaled up. Such MBR can be used to maximize product yields, to facilitate multiphase processing and to permit reuse of expensive cofactors. The main advantages of an MBR system are that there can be an integration of fermentation and separation step and the use of a continuous mode. Continuous systems are inherently more efficient than batch systems, producing very rapid and predictable reactions, batch to batch variation leads to inconsistent products, high labour costs and the need to inactivate or remove the biocatalyst after the reactions, thus increasing bioprocessing costs (Mehaia & Cheryan 1990). Furthermore, an MBR does not have the mass transfer problems as previously described for matrix immobilized cells and so offers extremely fast reaction rates because the microbial cells are in direct contact with the reactant (alcoholic fruit juice) (Mehaia & Cheryan 1990).

Cider maturation is a slow process if allowed to occur naturally, however the use of an MBR has a potential to speed up the process by producing a reactor that contains a catalyst concentrate several orders of magnitude greater than the natural process. Preliminary work on a small scale has been carried out with MBR and has shown that maturation cider can be achieved only 5-6 hours compared to 1-3 months in the natural system (Donavan 2000). As discussed above, several LAB can catalyse these flavour biotransformation. Two main types are available: 1. *L. brevis,* isolated from cider; 2. *O. oeni*, a commercial strain used in wine maturation. At present there is little or no information comparing these organisms or deciding on a rational basis which would be an appropriate choice within the context of rapid cider maturation in the MBR. A purpose of the research is to address this situation.

1.8 Research aims and objectives

The aim of this research is to investigate the technology for the production and utilisation of intensified cultures of various LAB and the MBR in the production of inoculants and a commercial cider maturation system. The main benefits will be the safe and rapid growth and utilization of high concentrations of LAB so improving the quality, efficiency and reliability of LAB production and LAB induced maturation processes.

The main objectives for the project are as follows:

1. To compare and contrast the physiology and growth of two major types of LAB, *O. oeni* and *L. brevis*. This is to be carried out in small test tube cultures by studying growth conditions and stress factors on the kinetics of biotransformation and the production of organisms.
2. To investigate the LAB in batch and continuous cultures to evaluate their capabilities and fermentation characteristics, to provide data for process productivity and malolactic activity, so as to provide a method for the prediction of process operation and future operating process design.
3. To study the important interactions between the bacteria and the membrane under the environmental conditions of high density cell culture and of cider maturation. These data will be used to select the most appropriate membrane processing conditions to enhance membrane performance for the cell production and cell biotransformation systems (maturation).
4. To use the MBR for a comparison of the growth and characteristics of two organisms and to establish the optimal operation conditions for the MBR performance so as to improve the effectiveness of the MBR.
5. To make an overall comparison of the two organisms within the context of rapid cider maturation.

Chapter 2 Materials and Methods

All experimental work was carried out within the Centre for Complex Fluids Processing at the School of Engineering, the University of Wales Swansea and they provided all the equipment for this research. *Oenococcus oeni (O. oeni)* NCIMB *11648* strain used in this study was supplied by UKNCC (the United Kingdom National Culture Collection) and was isolated from wine. A different name for the strain is *Leuconostoc oenos*. Type number *11648* is also assigned as *ATCC23279*, *DSM20252*, *NCDO1674* or *NRRLB3472*. *Lactobacillus brevis* X_2 (*L. brevis* X_2), isolated from oak vats at H.P.Bulmers Ltd, was given by H.P.Bulmers Ltd. Chemicals required for the formulations of the various media were purchased from various companies - Fisher Scientific, Sigma Chemical Company, Oxoid and Aldrich.

2.1 Preparation of Microbial Growth Media

2.1.1 MRS medium

To culture *O. oeni* and *L. brevis*, it is necessary to use a complex nutrient medium for the growth requirements of LAB. MRS broth (de Man 1960) was used because this medium is rich in a wide variety of components that could be necessary for the growth. The composition of MRS is given in Table 2.1.

Table 2.1 Composition of MRS

Components *	Concentration (g/l)
Bacterial peptone	10.0
Lab-Lemco powder	8.0
Yeast extract	4.0
Dextrose (D- glucose)	20.0
Di-potassium hydrogen phosphate	2.0
Sodium acetate	5.0
Tri-ammonium citrate	2.0
'Tween' 80	1ml/l
"Trace elements"	20ml/l
Resazurin (1mg/ml)	1ml/l
Water	980ml

* Bacterial peptone, Lab-lemco powder and Yeast extract were purchased from Oxiod.
D-glucose, Di-potassium hydrogen phosphate, Sodium acetate and Tri-ammonium citrate from Fisher.
Chemicals used in the following study were purchased from the same company unless otherwise stated.

MRS was prepared and the pH was adjusted to the appropriate range with either HCl (1M or 6M) or NaOH (1M or 6M). A calibrated Dual Channel pH/Ion Meter AR25 (Fisher Scientific) was used to measure pH of medium. Prior to its distribution into pressure tubes MRS was boiled to expel any dissolved oxygen from medium.

2.1.2 Donovan's broth

To identify the essential components for the growth of the LAB in the MRS and to investigate medium composition, it is necessary to choose a simple medium. Donovan's broth (Donovan 2000) was used for the growth of LAB, and then the results of growth were compared with that in MRS so that it was possible to recognize whether or not which component was essential. Donovan's broth was used to investigate the effect of the main components on the growth of LAB.

Table 2.2 Composition of Donovan's broth

Components	Concentration (g/l)
Yeast extract	5.0
Lab-Lemco powder	5.0
Potassium dihydrogen phosphate [a]	5.0
Ammonium chloride [b]	2.0
"Trace elements"	20ml/l
Resazurin (1mg/ml)	1ml/l
Water	980ml
pH (before autoclaved)	5.0

a. Potassium dihydrogen phosphate (Fisher)
b. Ammonium chloride (A.C.S.Reagent)
Chemicals used in the following study were purchased from the same company unless otherwise stated.

2.1.3 Trace elements solution

The trace element solution contains elements needed for the bacterial growth. The composition of the trace element solution is shown in Table 2.3.

Table 2.3 Composition of the trace element solution

Component	Concentration (g/l)
$MgCl_2.6H_2O$	5.00
$MnCl_2.4H_2O$	0.50
$FeCl_2.6H_2O$	0.35
$ZnCl_2$	0.20
$CaCl_2$	0.10
$CoCl_2.6H_2O$	0.02
$CuCl_2.2H_2O$	0.02
$NaMoO_4$	0.01
$Na_2B_4O_7$	0.01
Water	1000ml
pH	4.0

2.1.4 Preparation of Zhang's broth

After investigating the essential components for the growth of LAB in pressure tube cultures, a new growth medium was formulated. This relative simple medium still maintained nutrients to support good consistent growth of the LAB, both *O. oeni* and *L. brevis*. The medium derived was less complex than those previously used. The medium was named as Zhang's broth or Z broth for short.

Table 2.4 Composition of Zhang's broth

Components	Concentration (g/l)
Yeast extract	5.0
Potassium dihydrogen phosphate	5.0
Ammonium chloride	2.0
"Trace elements"	20ml/l
Resazurin (1mg/ml)	1 ml/l
Water	980ml
pH*	5.0

* pH was adjusted to 5.0 in the Chapter 3, and while 4.5 in the other experiments unless otherwise stated.

2.2 Culture Techniques

2.2.1 Preparation of anaerobic liquid broth cultures

The anaerobic broth cultures were carried out on two sizes, in pressure test tubes (30ml) with 10ml broth for the growth experiments, and in serum bottles (150ml) with 100 ml broth for the inoculum cultures. The medium once prepared was boiled to expel any dissolved oxygen. After boiling, 10 ml of medium was added to each pressure tube via

10ml syringe from the flask containing the boiled medium that was flushed with nitrogen. A headspace of N_2 or N_2/CO_2 (95:5) mixture was placed on a top of the medium using a gas canular linked to manifold of rubber tubes linked to the gas cylinder. Each gas canular tube had a filter, constructed of a 1 ml syringe shell stuffed with fibre glass wool, with a needle at the end. The needle could then be placed in the bottle or tube and gas passed over the medium.

The gas manifold had a total of 8 cannula so that it was possible to flush 8 tubes or bottles at any one time. Resazurin, a redox indicator, was added to the medium to indicate whether or not the broth had become fully anaerobic. After autoclaving, if oxygen was present, the medium would take on a pink/red appearance, otherwise there was no colour present, which indicated the absence of oxygen. If oxygen was present in the medium, then a suitable amount of sterile cysteine solution (5%w/v) was added to remove any remaining dissolved oxygen, typically about 0.1ml per 10ml tube. Serum bottles of 50 or 150 ml volume were also used. Once filled with 30 or 100 ml respectively, they were then sealed with rubber bungs and retained with an aluminium cap. The aluminium cap prevented the rubber bung from being pushed out by the high pressure reached during the autoclave sterilization procedures. Once the tubes or bottles had been prepared, they were placed in the autoclave and autoclaved at 121°C for 15 minutes. Generally, the medium colour was yellow or brown possibly due to the presence of yeast extract or both yeast extract and sugar.

2.2.2 Anaerobic agar plate cultures

To confirm the purity of the cultures it was necessary to plate out samples taken from the liquid broth onto MRS agar. The agar was incubated at 25°C in completely anaerobic conditions for 4-5 days in order to try to isolate and identify any possible contaminants and to confirm the purity of the desired *O. oeni* or *L. brevis*. Dissolving 10g of nutrient agar in 500ml of MRS medium, and then autoclaved, poured out into petri dishes in the sterile air cabinet ensuring aseptic conditions as all times. The agar was left to dry with the lids resting on top at a slant for 5-10 minutes; the plates were then left to cool fully before a sample of the culture broth to be tested was streaked onto the surface of the agar.

The plates were then placed in anaerobic jars that were filled with nitrogen. The jars were linked to a vacuum pump to evacuate all the air present in the sealed jars creating a vacuum and then filling the vacuum with nitrogen. This procedure of sucking the gas out and blowing the oxygen-free nitrogen gas in was carried out four times before the jar was finally placed in the incubator at 25°C, which ensured that all the oxygen had been expelled. The final pressure of the jar was left just greater than the ambient atmosphere so that if there was a leak then gas would leak out and not air in.

2.2.3 Inoculation

After sterilization and cooling, the medium could then be inoculated with *O. oeni or L. brevis*. Inoculation was carried out by injecting 1ml of liquid culture from a previous liquid culture using a sterile needle and syringe, the needle being used to penetrate the rubber seal of the tubes. The 10ml tubes or 100ml bottles were placed in an incubator at 25°C or a constant temperature room at 25°C, until sufficient growth occurred, usually it took 60-72 h (hour). Then 1ml of inoculum was taken by syringe from an active culture tube and injected into fresh tubes.

In each case, aseptic conditions were maintained at all times with inoculation being carried out in the sterile air cabinet. The top of the rubber seal was flamed to kill any foreign microbes that may be present on the surface of the bottle, which could become a possible source of contamination.

In growth experiments where the microorganisms were grown in pressure tubes, 10ml medium were inoculated with 1ml of 60-72 h culture of *O. oeni*. pH value of all media was 5.0 and temperature maintained 25°C except where otherwise stated.

2.3 Test of Purity

To ensure that the organism present during the fermentation studies was the desired organism, tests of purity were made. The following tests were carried out at the start, end and during each investigation.

2.3.1 Microscopic examination

At the end of each fermentation, a sample of the culture was taken and placed under the microscope (Olympus) for analysis. This was a check against contamination, and a method of identifying presence of the desired *O. oeni* or *L. brevis*. When observing cells taken from the culture medium, the cells were placed onto a slide with a flamed loop and a cover slip was placed over the sample. The magnification used for microscopic examination was 400 times under phase contrast illumination. Cells of *O. oeni* grown in broth cultures are almost spherical, usually occurring in pairs or chains.

2.3.2 Agar plates

When the organisms were cultured on MRS agar, the colonies that had grown up on the agar plates were evaluated visually. If there were two different types of colonies located on a plate, sub-cultures were taken off the plates for isolation of contaminants or variants. This involved using a flamed loop to pick a single colony and then streak it onto a fresh plate. The colony morphology could easily be identified by simply looking at the surface of the agar. The cell morphology was obtained by placing cells taken from the colonies and studying them under the microscope. Cells of *O. oeni* grown on surface plate are smooth, round, grayish white colonies, seldom larger than 1mm in diameter.

2.4 Measurement of LAB Growth

Growth (i.e. cell density) was measured by change in optical density (OD) (Appendix A) of the liquid culture at wavelength 660nm. All the OD measurements were at a wavelength 660nm unless otherwise mentioned in subsequent description of diagrams. OD stands for optical density measured at 660nm.

With the 10ml pressure tube culture the OD was measured directly by placing the tube into a spectrophotometer (Pye Unicam 8625 UV/VIS Spectrophotometer) fitted with a test tube holder. The pressure tube had a 1.8cm light path. No correction has been made to convert these measurements to a 1 cm path length.

In batch fermentation and continuous fermentation the OD of cultures were measured by withdrawing 3 ml of culture from the fermentor and then putting into a quartz cuvette with 1-cm light path, which was then placed into a spectrophotometer (Pye Unicam SP8-400 UV/VIS Spectrophotometer).

The optical density of the culture was linear to the amount of growth, provided the OD of the culture did not exceed 1 with 1-cm light path at 660nm. If values above 1 were obtained, then they were diluted so avoiding non linear response at high concentration.

2.5 Data Analysis

The results of growth experiments could be expressed in two ways, that is, the specific growth rate (μ) and amount of growth (OD).

By analyzing graphical plots of the growth obtained the specific growth rate could be determined by a linear regression of ln(OD) vs. time of the log phase of growth. The biomass produced is obtained by subtraction of the start value (OD) from the highest value (OD) reached during the growth curve.

For determination of specific growth rate and amount of growth, measurements in experiments were carried out using duplicate samples to obtain better reproducibility. The results were shown graphically in each chapter, while the analysis of graphs (linear regression etc), which were used to determine the specific growth rate, are omitted. The examples of linear regression were shown in Appendix B. The tables of the original readings of OD are also omitted.

2.6 Relationship between Cell Dry Weight and Optical Density

A nominal dry weight (solids content) of bacterial cells originally in a liquid suspension is obtained by drying a measured wet weight or volume in an oven at 90-105°C to constant weight (Gerhardt 1981). Biomass may usually be expressed in optical density (OD) because the measurement of OD seems easy and straightforward in practice, so it is

necessary to determine the relationship between dry weight and optical density. As a result, the dry weight of cells was calculated easily from the optical density (OD) with a linear correlation factor provided the cell density of the amount cultured is below 1 (1 cm light path at 660nm).

An example of relationship between dry weight and OD of *O. oeni* was discussed as follows. A 1000 ml of cell culture was collected and distributed into six centrifuge bottles (260 ml each) with a stainless cap, and the total weight of each centrifuge bottle and cell culture was weighed so as to keep each bottle exactly same weight. There was 65 ml cell culture left, so a total volume of cell culture used was 935 ml (V_1 ml). The six bottles were placed into the rotor, centrifugated at 10,000×g for 15 min (MSE, High Speed 18), each centrifuge bottle was carefully removed, and the supernatant was then removed by decanting. The cell pellets were transferred to four lots of 1.5ml pre-weighed micro-centrifuge tubes (total weight W_1 g), and then centrifuged for 10 min (MSE, Micro Centaur), the supernatant was withdrawn by pipetting, washing with distilled water. The supernatant was removed after centrifugation and the tubes containing the cell pellet were dried for 24 h in a temperature-controlled oven at 95°C. The total weight (W_2 g) of four tubes was obtained by weighing tubes containing cell pellets. The difference ($W_2 - W_1$) was cell dry weight of 935 ml cell culture. If the optical density of this cell culture was N, then 1 OD value relevant to the dry weight was calculated by using following formula, in terms of gram dry weight per liter:

$$1\ OD = 1000\times (W_2 - W_1)/ (V_1 \times N)$$

Experimental data: $W_2 = 4.9851$ g, $W_1 = 4.6120$ g, $V_1 = 935$ ml, $N = 0.906$

$$1\ OD = 1000\times (4.9851 - 4.6120)/ (935\times 0.906) = 0.4404\ (g/l) = 0.44\ (g/l)$$

2.7 Calibration of the Spectrophotometer: Relationship between actual OD and measured OD

As mentioned previously, an accurate relationship between actual optical density and measured optical density obtained at 660nm using the spectrophotometer could be obtained only over a conservative range of 0-1.0. It therefore was necessary to calibrate the spectrophotometer in order that an actual optical density reading can be obtained at the

circumstance of high optical density, which was sure to be beyond the accurate range of spectrophotometer. *O. oeni* was taken as an example to evaluate the relationship between measured optical density and actual optical density (calculated OD).

Concentrated cells suspensions of *O. oeni* were obtained by harvesting cells of 100 l batch cultures (section 2.14), which the OD of concentrated cells equaled 13.84. A series of cell suspensions were prepared with distilled water of pH 4.5 in Appendix C. The dilution ratio was in a range of 5 to 50 fold (the reciprocal of dilution ratio 0.2 to 0.02), therefore the calculated OD should be in a range of 0 to 2.8. The optical densities (measured OD) were measured at 660nm in a quartz cuvette with a 1cm light path. From a comparison of OD calculated and OD measured (Fig. C-1 Appendix C), it was observed that there were a good fit of calculated OD and measured OD at less than OD 1.0 of cell concentration (the reciprocal of dilution ratio is less than 0.075). The difference between calculated OD and measured OD increased as the cell concentration increased under the condition of more than OD 1.0. The calculated optical densities versus the measured optical densities were plotted (Fig. C-2 Appendix C). The quadratic fit expressed the relationship between the actual optical densities and measured optical densities. Consequently, the measured OD should be calibrated using the equation given below if OD was more than OD 1.0.

$$OD_{actual} = 0.1748 \times OD^2_{measured} + 0.861 \times OD_{measured}$$

Alternatively, it is best to dilute high density cells up to less than OD 1.0, measured OD equals actual OD of cell suspensions.

2.8 HPLC Analysis of Sugars and Sugar Alcohols

Fermentation broths are complex mixtures of nutrients, waste products, cells, cell debris, and desired products. Many of these ingredients are non-chromophoric and cannot be detected by absorbance alone. Analysis of sugars and sugar alcohols were carried out using HPLC coupled to electrochemical detection. Carbohydrates, glycols, alcohols, amines, and sulfur-containing compounds can be oxidized and detected by amperometry. This detection method is specific for analytes that can be oxidized at a selected potential, leaving all other compounds undetected.

Pulsed amperometric detection (PAD) is a powerful detection technique with a broad linear range and very low detection limits. High-performance anion-exchange chromatography (HPAE) is capable of separating complex mixtures of carbohydrates. For complex samples such as fermentation broths, the high resolving power of HPAE and the specificity of PAD allow the determination of carbohydrates, glycols, sugar alcohols, and other alcohols such as ethanol and methanol, with little interference from other broth ingredients (Dionex Corporation, Application Note 122). HPAE-PAD provided the analytical capability to monitor a large number of different compounds simultaneously using a single instrument and chromatographic methods (Hanko and Rohrer 2000) and so was the method of choice.

2.8.1 Apparatus

HPLC system is shown in Figure 2-1 below.

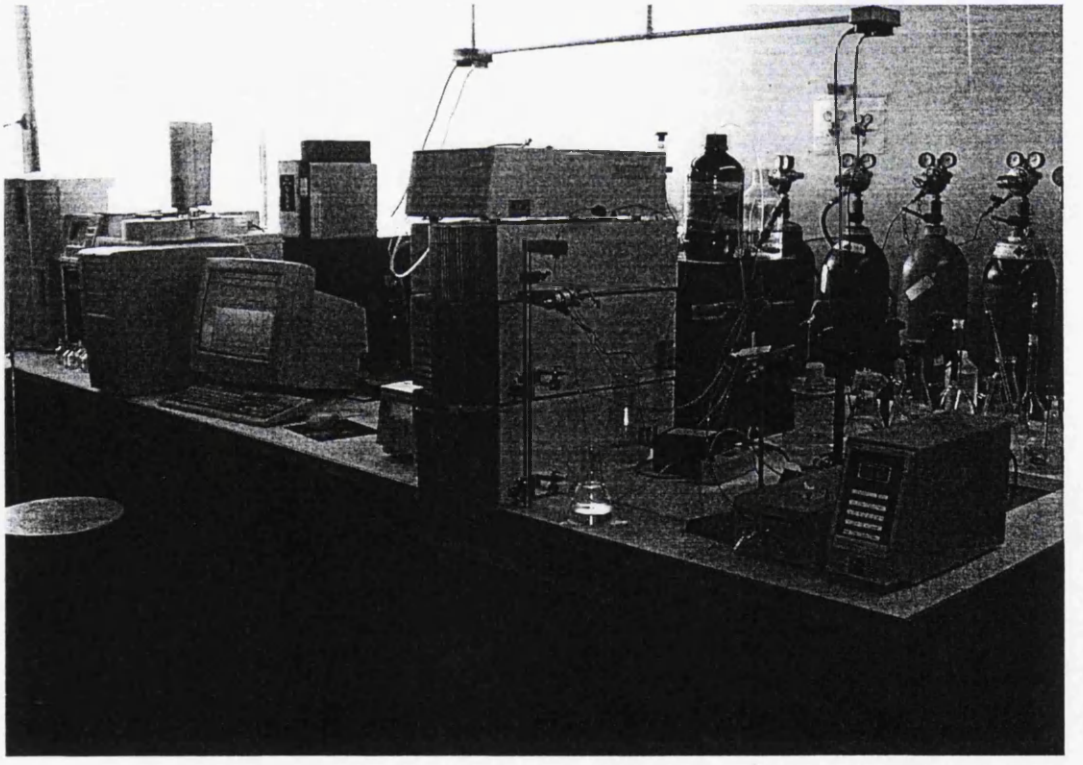


Figure 2-1 Diagram of HPLC system

The VARIAN ProStar HPLC with pump units (Solvent Delivery Module Pump A, Pump B) was connected to a computer workstation to control pumps and to measure and analyze data from electrochemical detector. A DIONEX ED40 Electrochemical Detector was used in Pulsed Amperometry mode with gold electrode.

2.8.2 Columns and Conditions

Columns: DIONEX Ion Chromatography

CarboPac™ MA1 (4×250mm) Analytical Column (P/N 44066)

CarboPac™ MA1 (4×50mm) Guard Column (P/N 44067)

Flow Rates: 0.4 ml/min

Eluent: 480mM Sodium hydroxide

An isocratic program was used as this allowed a rapid turn-round of samples and avoided pre-equilibration and reproducibility of the analysis. The injection volume was: 20μL at room temperature (20°C).

2.8.3 Reagents and Standards

Sodium hydroxide, 46-48% (w/w) (Fisher Scientific and J.T. Baker)

Deionized water (RiO_s and Elix Water purification system, MILLIPORE)

The following standards were used: D-Glucose (Fisher Chemicals, $C_6H_{12}O_6$, FW 180.16, 99%), D-Fructose (Sigma, $C_6H_{12}O_6$, FW 180.16, 99%), Mannitol (BDH Chemicals Ltd, $C_6H_{14}O_6$, FW 182.16), Erythritol (Sigma, $C_4H_{10}O_4$, FW 122.1, 99%) and Glycerol (BDH Chemicals Ltd, $C_3H_8O_3$, FW 92.10, 98%)

2.8.4 Preparation of Solutions and Reagents

480mM Sodium hydroxide

It is essential to use high-quality water of high resistivity as free of dissolved carbon dioxide as possible. It is extremely important to minimize contamination by carbonate, a divalent anion at high pH that binds strongly to the column, causing a loss of chromatographic resolution and efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used. A 46-

48% (w/w) sodium hydroxide solution is much lower in carbonate and is a preferred source for sodium hydroxide. Prior to use, 52 ml of above sodium hydroxide was diluted by 1948 ml of water, and then 2 litres 480 mM sodium hydroxide eluent was yielded.

Eluent Preparation

Weight method When formulating eluents from 46-48% (w/w) sodium hydroxide, the required amounts of 46-48% sodium hydroxide was weighed out from the sodium hydroxide bottle. For example, to make 1 l of 480mM NaOH use g of 46-48% sodium hydroxide: (0.480 mol/l × 40.0 g/mol)/48% = 40.0 g diluted to 1 l.

Volume method For gradient analysis volumetrically, the following formula will be used to determine the correct volume of 46-48% (w/w) sodium hydroxide to be diluted.

$v = g/(d \times \gamma)$

Where g is weight of sodium hydroxide required (g)

d is density of the concentrated solution (g/ml)

v is volume of the 46-48%(w/w) sodium hydroxide required (ml)

γ is % purity of the concentrated solution

For example: to make 1 l of 480mM use 26.14 ml of 48% sodium hydroxide: (0.480 mol/l ×40.0 g/mol ×1 l)/(1.53 g/l × 48%) = 26.14 ml diluted to 1 l.

Dilution of 48% NaOH to make standard AS11-HC eluents

Concentraton of NaOH Eluent (mM)	48% (w/w) NaOH (g)	48% (w/w) NaOH (ml)
0.5	0.042	0.027
1.0	0.083	0.054
100	8.33	5.45
480	40.00	26.14

2.8.5 Calibration of Carbohydrates and sugar alcohols

Calibration of the HPLC system was achieved by using different compounds as standards: glucose, fructose, mannitol, erythritol and glycerol. A 100 ml of standard solution of each compound was prepared. The solution was then diluted to a series of solutions with a range of different concentrations. The diluted solutions were analyzed and the calibration curve for each of them was then constructed. The calibration curves of peak area versus

concentration were given in Appendix D; good relationships over the range of concentrations used were obtained.

Relationships between peak area and concentration were given as follows:

- Glucose concentration (mM) $y = 6\times10^{-7}x$ (x is a peak area, mV sec)
- Fructose concentration (mM) $y = 3\times10^{-6}x$ (x is a peak area, mV sec)
- Mannitol concentration (mM) $y = 7\times10^{-7}x$ (x is a peak area, mV sec)
- Erythritol concentration (mM) $y = 1\times10^{-7}x$ (x is a peak area, mV sec)
- Glycerol concentration (mM) $y = 7\times10^{-7}x$ (x is a peak area, mV sec)

2.9 HPLC Analysis of Organic Acids

2.9.1 Apparatus

Again the VARIAN ProStar HPLC with pump units (Solvent Delivery Module Pump A, Pump B) connected to a Computer Workstation to control pumps and to measure and analyze data from the electrochemical detector. A DIONEX ED40 Electrochemical Detector was used in conductivity mode. The DS3 Detection stabilizer model DS3-1 (ED40 Conductivity Cell) with ion suppression. The Dionex Anion Self-Regenerating Suppressor-II (ASRS-Ultra 4mm) (P/N 53946) was used.

2.9.2 Columns and Conditions

DIONEX Chromatography IonPac^R ATC-1 (9×24mm) Anion Trap Column (P/N 37151) was located between pump and injection valve. IonPac^R AG11 – HC (4×50mm) guard column (P/N 52962) followed by IonPac^R AS11 – HC (4×250mm) analytical column (P/N 52960). The flow rate was set at 1.1 ml/min. The following gradient program (below) was used using two stock eluents. Eluent A: 0.5mM sodium hydroxide and eluent B: 100mM sodium hydroxide.

The injection volume was 20µL and the temperature was 35°C (Waters Temperature Control Module).

Time (min)	A%	B%
0:00	100	0
5:00	100	0
15:00	95	5
25:00	50	50
33:00	50	50
35:00	100	0

2.9.3 Reagents and Standards

Sodium hydroxide, 46-48% (w/w) (Fisher Scientific and J.T. Baker)

Deionized water (RiO_s and Elix Water purification system, MILLIPORE)

Acetate (Fisons Analytical Reagent, $C_2H_3O_2Na$, FM 82.04, 99%)

Lactate (Sigma, $C_3H_5O_3Li$, FW 96.01, Approx. 97%)

Malate (Sigma, monosodium salt, $C_4H_5O_5Na$, FW 156.1, 99%)

2.9.4 Preparation of Solutions and Reagents

Sodium hydroxide eluent is 0.5mM and 100mM. The preparation of eluents is discussed above in section 2.8.4, "preparation of solution and reagents".

2.9.5 Calibration of Organic Acids

Calibration of the HPLC system was achieved by using different compounds as standards: lactate, acetate and malate. A 100 ml of standard solution of each compound was made. The solution was then diluted to different concentrations required. The diluted solutions were analyzed and the calibration curve for each of them was then constructed. The calibration curves of peak area versus concentration were given in Appendix E; good relationships over the range of concentrations used were obtained.

Relationships between peak area and concentration were given as follows:

- Lactate concentration (mM) $y = 4\times10^{-6}x$ (x is a peak area, mV sec)
- Acetate concentration (mM) $y = 7\times10^{-6}x$ (x is a peak area, mV sec)
- Malate concentration (mM) $y = 5\times10^{-6}x$ (x is a peak area, mV sec)

2.10 Gas Chromatography of Volatile Compounds

Volatile compounds (alcohols, esters, and vicinal diketones) can be separated and quantified by gas chromatograph (GC) with FID. The GC was used here for the measurement of ethanol produced in the fermentation broth in this work.

2.10.1 Apparatus

A VARAIN ProStar GC-3800 (USA) fitted with CP-8400 AutoSampler and CP-8410 AutoInjector, Flame ionization detector (FID). The gases were either made in situ (Hydrogen generator: UHP-20H NITROX) or supplied as high pressure cylinders (air and nitrogen). A 50 m carbowax capillary column (Non-polar, 1μ film thickness, 4m×0.32mm, CP-Sil 5 CB) was fitted. GC system is shown Figure 2-2.

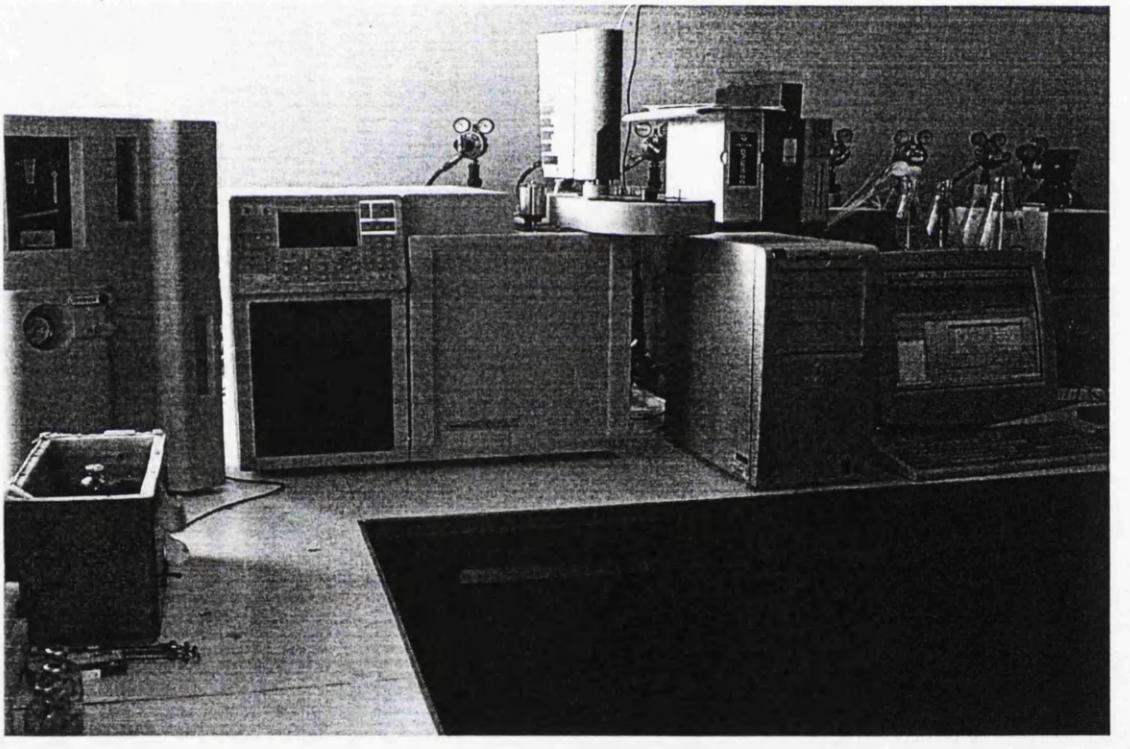


Figure 2-2 Gas chromatography system

2.10.2 Operation condition and procedure

The standard operating conditions were used. Flow/Pressure: Pressure 10.0 psi; Hold time (total) 12 min; Carrier gas nitrogen, 5ml/min; Injection (type 1079), 200°C; FI Detector temperature 220°C. The column oven temperature program is given below.

Column Temperature program:

Temperature (°C)	Rate (°C/min)	Hold (min)	Total (min)
30		2.00	2.00
100	20.0	1.00	6.50
200	40.0	3.00	12.00

To operate the GC, the gas sources were connected, i.e. H_2 from generator, N_2 and air from gas cylinder. Hydrogen generator was firstly turned on and allowed to reach operating pressure (6.5 psi), taking about 6 min, and then opened both nitrogen valve and air valve. Secondly, the GC was switched on, connected to the computer control system and the appropriate program loaded and run. After a waiting time of about 10 min for the system to equilibrate, the GC is ready for work.

2.10.3 Headspace analysis of samples

Headspace analysis was used for ethanol analysis. A 5 ml sample withdrawn was added into a serum bottle (30 ml), the bottle was then sealed and placed in 50°C of water in a water bath for 10 min. 0.5 ml gas was drawn in with a glass gas-tight syringe from the headspace of bottle, and the sample gas was injected into GC and analyzed.

2.10.4 Standard and calibration of ethanol

Absolute ethanol (HPLC grade, Scientific Fisher) was used as a standard. Firstly, weighing a 50 ml beaker containing some absolute ethanol, weight of W_1; secondly, sucking in ethanol using an accurate microsyringe, and then weighing the beaker again as quickly as possible, weight of W_2; finally, sucking out the ethanol into a measuring flask (100 ml), adding distilled water up to the level mark of 100 ml. The weight of ethanol withdrawn is equal to the difference of W_1- W_2. The ethanol concentration was calculated in terms of following method. W_1= 76.8167g, W_2= 75.3079g

The concentration of ethanol = (W_1-W_2)g /100ml= 1.5088g/100ml=15.088 g/l.

The above standard ethanol solutions were diluted 25, 50, 100, 133 and 200 fold and the following concentrations of ethanol solutions were made: 0.6035, 0.3018, 0.1509, 0.1134

and 0.0754 g/l. The diluted solutions were analyzed and the calibration curve was then constructed. The calibration curve of peak area versus concentration was given in Appendix F.

Ethanol concentration (mM) $y = 4\times10^{-5}x$

2.11 Batch Culture

2.11.1 Batch culture without pH control

2.11.1.1 200ml/400ml batch culture

A schematic diagram of 500 ml reactor is shown in Figure 2-3. A 500 ml reactor consists of two ports: one is gas exit (port A) on the top of the bottle, the other (port B) is used as both gas inlet and culture medium exit near the bottom. Each port has butyl rubber tubing and a filter that is made from 1 ml syringe tube shell stuffed by fibre glass wool. Both ports are clamped shut by clamping the rubber piping as required.

200ml/400ml fresh medium was transferred to 500 ml reactor, and autoclaved. The medium autoclaved was flushed with nitrogen which came from port B linked to nitrogen cylinder, port A is open to let air out, closed port A. the headspace of medium was filled with nitrogen, closed port B and disconnected to nitrogen cylinder.

30 ml inoculum reactivated in a 50 ml serum bottle was withdrawn three times with 10 ml syringe and added into 500 ml reactor through a needle penetrating the rubber bung on the port A. it was incubated at 25°C for about 60 h and ready for use as inoculum.

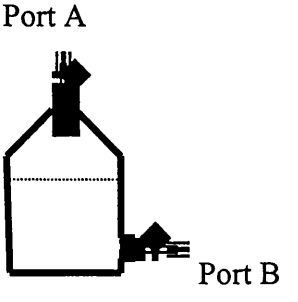


Figure 2-3 Schematic diagram of 500ml reactor

2.11.1.2 2 l/4 l batch culture

A 5 l bottle reactor has three ports: port A, port B and C. Each port consists of butyl rubber tubing connected to a filter (Figure 2-4) and can be closed by means of clamps. 2 l or 4 l fresh medium was transferred into the bottle reactor and autoclaved, the clamp of the top port was opened to let gases out. 200 ml or 400 ml inoculum was added in through the rubber tube in the port C. Nitrogen was let in through port A and filled in the headspace in bottle, and then the bottle was placed in the constant temperature room of 25°C for about 60 h. 2 l or 4 l inoculum was prepared for use below.

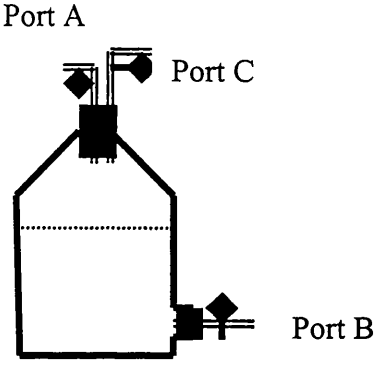


Figure 2-4 Schematic diagram of 2 l/4 l reactor

2.11.1.3 20 l batch culture

A 20 l batch culture was undertaken in 23 l glass carboy. The medium components were weighed and put in a 5 l plastic jar containing 4 l water, dissolved, then transferred to the carboy, adding distilled water up to 18 l of total volume, and then autoclaved at 121°C for 25 minutes, the clamp of the top port was opened to let gases out. 2 l inoculum was transferred into 18 l medium through a rubber tube in the top port. This carboy was placed in constant temperature room of 25°C, incubated for about 70 h.

2.11.2 2 l Batch culture with pH control

2.11.2.1 Preparation of culture medium

O. oeni was maintained on MRS liquid medium and sub-cultured before use. Investigation involved two aspects:

(1) The effect of pH on the growth. In the cultivation of *O. oeni* Z broth containing glucose: fructose (5g/l:5g/l) was used as the medium for batch cultures. The pH of media was adjusted to 3.5, 4.0, 4.5, 5.0 and 6.0, respectively. All media were autoclaved at 121°C for 15 min.

(2) The effect of concentration of glucose-fructose mixture on the growth. Z broth was used with the ratio around 1:1 of glucose: fructose, e.g. 1.35, 4.5, 9, 18 and 45g/l each. The media were adjusted to pH 4.5, and autoclaved at 121°C for 15 min.

In the cultivation of *L. brevis* Z broth with 10 g/l glucose was used as the initial medium for batch cultures.

2.11.2.2 Fermentation protocol

The batch fermentation assembly used in the present study is described as follows (Figure 2-5, 2-6), consisted of a 2 l tank reactor with magnetic stirrer, a heated water bath was used for temperature control, water from the bath was pumped through a coil within the fermenter before returning it to the water bath, a steam-sterilizable pH electrode in the fermenter was connected to an autotitration controller. In addition, oxygen-free nitrogen gas (from a high pressure cylinder) was added to maintain anaerobic conditions and a balance for weighing the consumption of 1M NaOH was also required (Figure 2-5).

A serial growth method was used in the preparation of inoculum. A pure culture inoculum from MRS liquid was used to seed a 30 ml medium in a 50 ml serum bottle, and incubated for 60 h at 25°C of constant temperature room. At the late log/early stationary phase of growth (about 60 h), the culture was used to seed a 300 ml culture (Fig. 2-3) with a filter sterilized gas out-in port (port A) at the top and a liquid exit (port B). After incubating about 60 h, finally 150 ml of this culture was aseptically transferred directly through the liquid exit into 1600 ml culture medium in 2 l fermentor.

After inoculation, a stirrer speed of 200rpm was set and N_2 was let in to the fermentor through sterile filter inlet up to gas out. The pH of the culture was measured with a pH probe and pH meter, and was controlled with pH controller/autotitrator and automatic addition of 1 M NaOH solution. The pH was maintained at desired values by using a pH

controller in each experiment, setting at pH 3.5, 4.0, 4.5, 5.0 and 6.0, respectively, by autotitration with 1M NaOH, until cell growth ceased and the cultivation was completed. Samples were taken regularly from the bioreactor for optical density determinations and quantitative assays. The first sample after inoculation should be withdrawn as quickly as possible and then subsequent samples were withdrawn at regular time intervals. After centrifugation of 8 min the supernatants were used for analytical measurements or stored at –20°C prior to analysis.

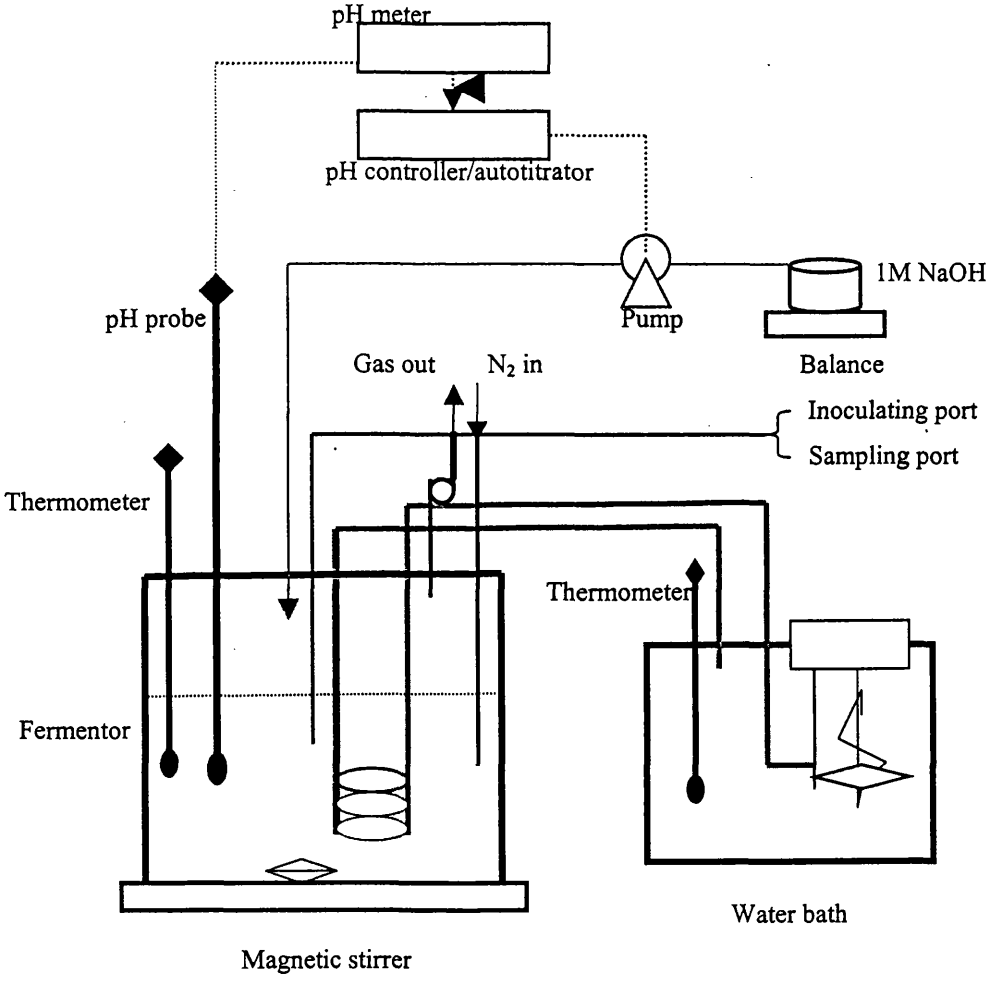


Figure 2-5 Schematic representation of batch fermentation with pH control

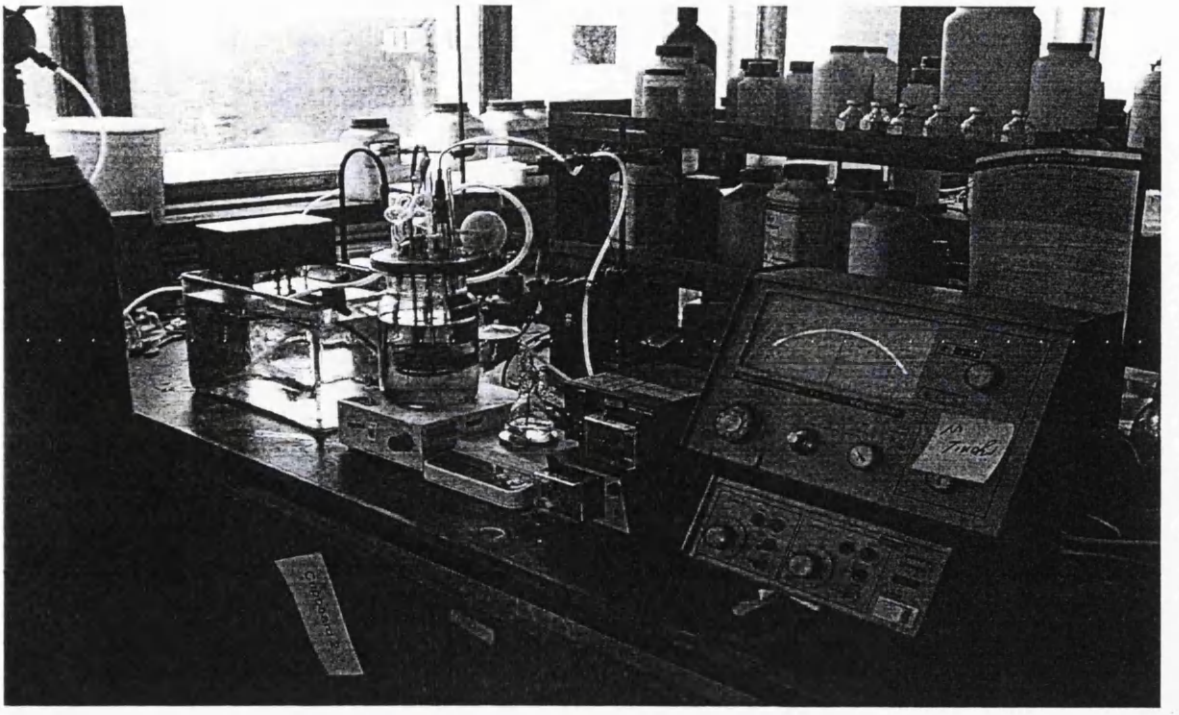


Figure 2-6 Experimental cell set-up of batch culture

2.12 2 l Continuous Culture

2.12.1 Growth media

The growth medium for *O. oeni* was Z broth with 5g/l glucose plus 5g/l fructose under pH= 4.5 and T = 25°C. The medium composition was the same throughout this study.

For *L. brevis*, the medium was Z broth with 10g/l glucose under pH = 5.5 and T = 27°C.

2.12.2 Fermentation protocol

Sterile culture medium (200 ml) was inoculated with 20 ml activation medium (60 h) and incubated for 60 h at 25°C in a 500 ml mini-reactor. 120 ml of this culture was aseptically transferred directly into 1150 ml culture medium in a 2 l fermentor with a steam-sterilizable pH electrode (Figure 2-7), blocking the exit port with a clamp.

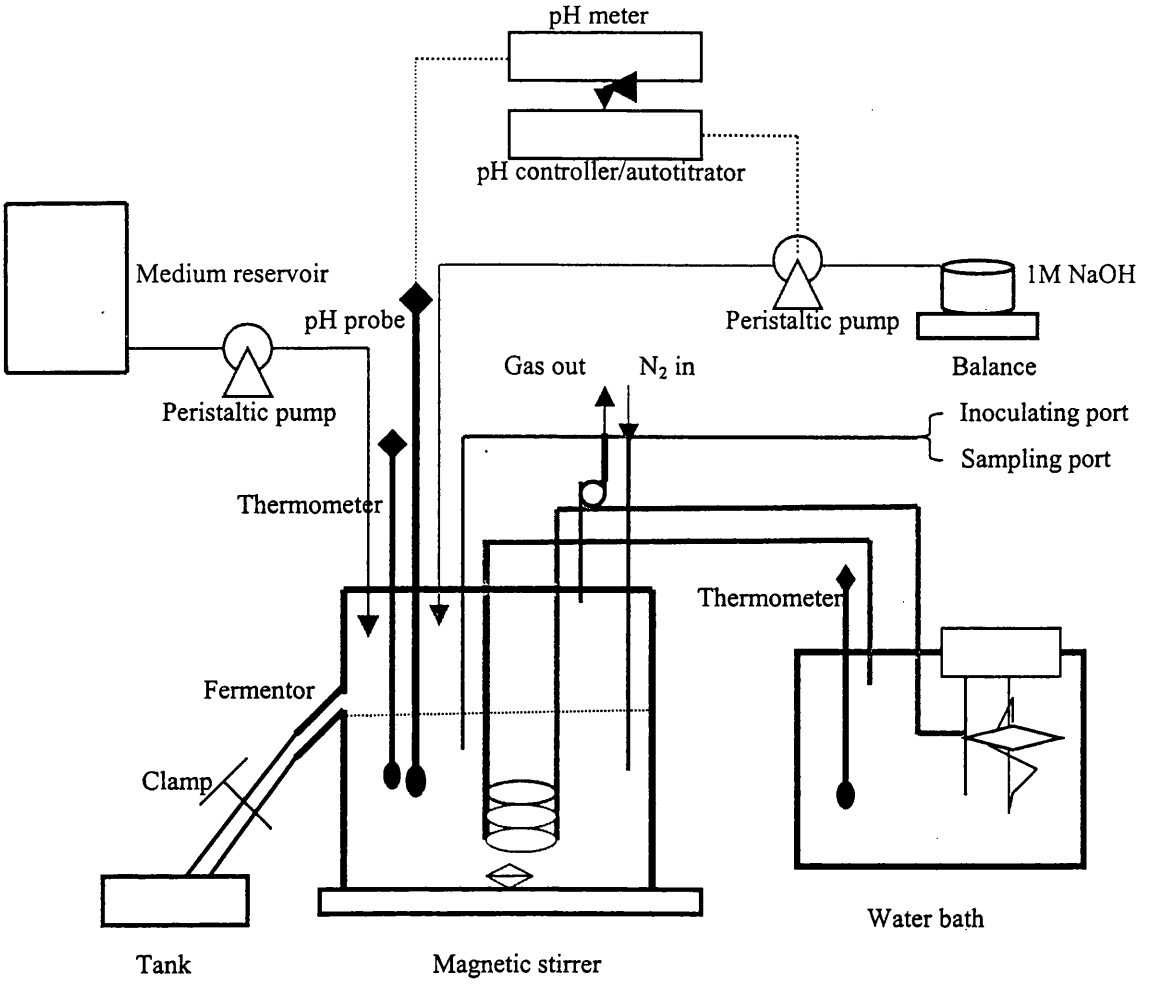


Figure 2-7 Schematic diagram of the single-stage chemostat

In the continuous culture (chemostat) (Figure 2-7) the inflow of medium was controlled with a peristaltic pump while the outflow was via a gravity weir. The outflow of the medium was measured periodically to determine the dilution rate D (h^{-1}). Prior to the feeding, the culture was grown in batch mode. Feeding was started at the late exponential phase/early stationary phase of the batch, and from 20 l fresh medium in carboys.

2.13 Membrane Bioreactor System

2.13.1 Construction of the membrane bioreactor

The membrane bioreactor (MBR) was constructed at the University of Wales, Swansea in the Centre for Complex Fluids Process Engineering and can be seen in the photograph (Figure 2-8).

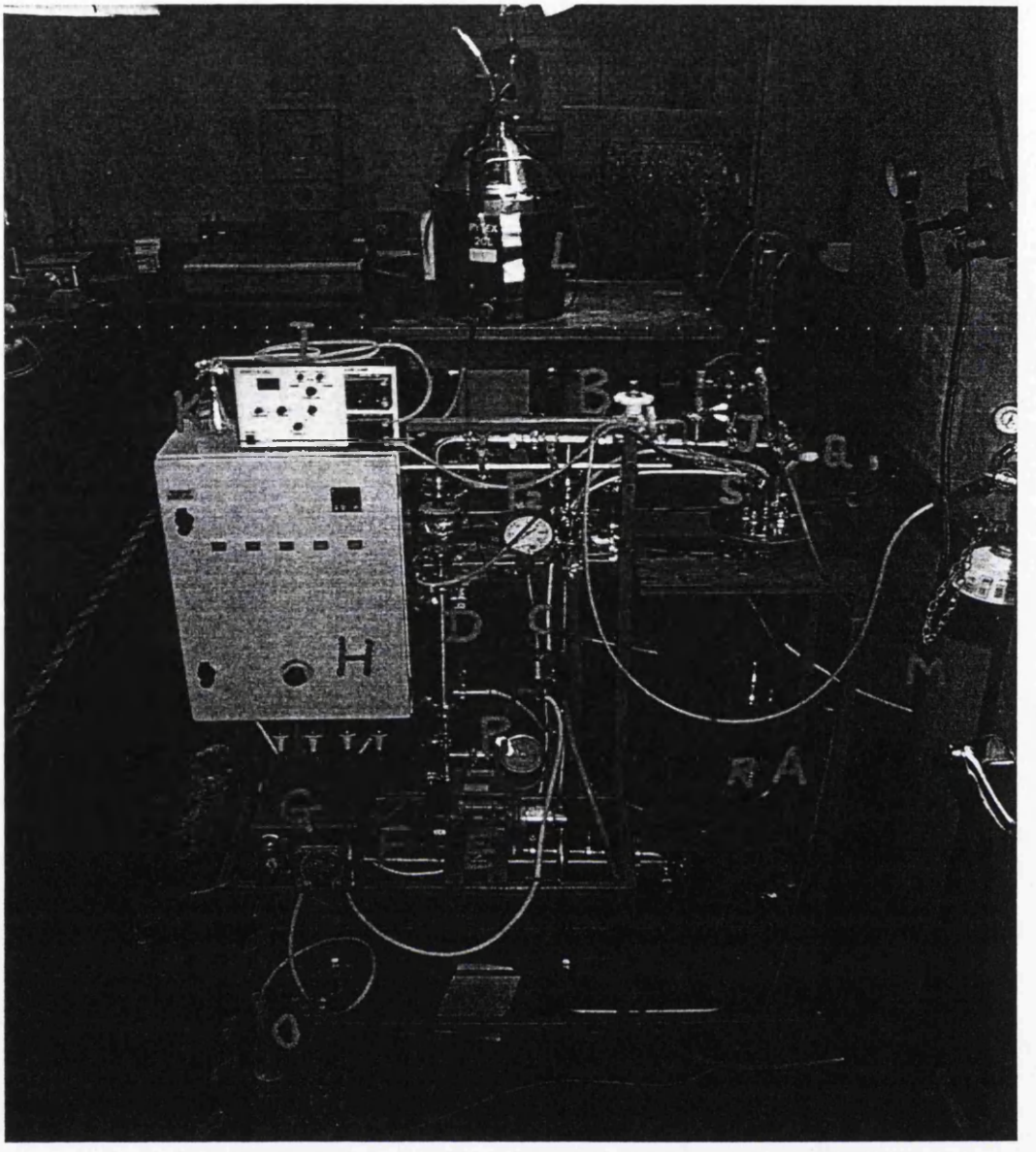


Figure 2-8 Experimental cell set-up of membrane bioreactor

The letters on Figure 2-8 are:

A-Fermentation vessel
B-Diaphragm valve
C-Heat exchanger
D-Ceramic membrane
E-Pump 2
F-Pump 1
G-Peristaltic Pump 3
H-Electrical control box
I-pH controller
J-pH probe
K-6M NaOH
L-Medium reservoir
M-N_2 cylinder
N-Steam pipe
O-measuring cup
P_1-Pressure gauge 1
P_2- Pressure gauge 2
Q- Sampling port
R- Cooling coil
S- Level controller

See Figure 2-10 for further schematic diagram.

2.13.1.1 Bioreactor vessel

The reaction vessel was glass and was operated under anaerobic conditions. The fermentation vessel employed was 5 l in operating volume, 760 mm depth and 120 mm in diameter. The vessel was sterilized by autoclaving at 121°C for 30 minutes. The top of the glass vessel was sealed with a stainless steel plate through which feed, recycled process fluid, level control, nitrogen, NaOH addition, cooling water in/out were made and gases were removed (Figure 2-9). The inlet for the feed was set above the process fluid level to ensure that its addition could also be observed. The inlet for the recycled process fluid had a 90° bend at its end to force the fluid into the side of the glass vessel causing vortexes to occur, ensuring good mixing of the fresh feed and recycled LAB. Finally, the outlet gas line passed out of the top of the reaction vessel and through a sterile water gas trap to prevent diffusion of air containing oxygen, from the atmosphere, back into the MBR.

The volume of fluid within the MBR was controlled by a level control, which is described later in this Chapter, section 2.13.1.6.

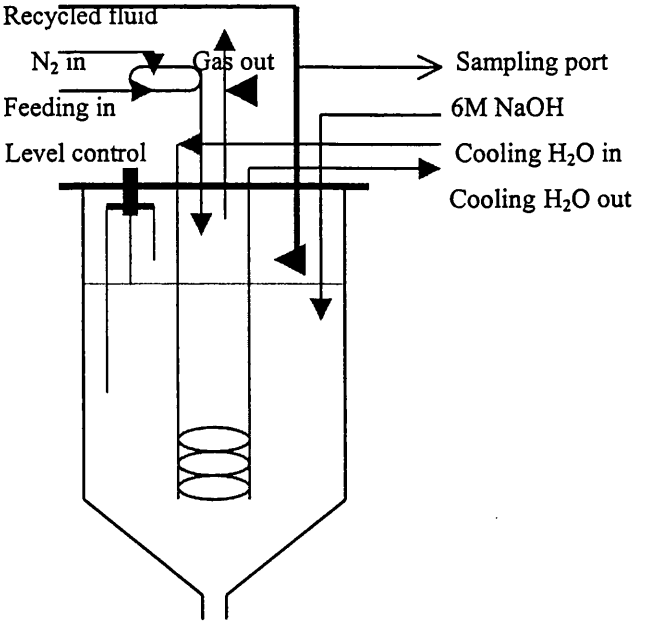


Figure 2-9 Schematic diagram of deep fermentor and stainless steel top plate

2.13.1.2 Pumps

Two pumps were originally purchased from Michael Smith Engineers Ltd., both of which were magnetically coupled stainless steel pumps. These magnetically coupled stainless steel pumps allowed for the entire membrane bioreactor system to be autoclaved in situ or disassembled, further reducing the chance of contamination.

Pump F: Pump F is a large centrifuge liquiflo pump (Brook Hansen from Michael Smith Engineers Ltd.), type ED 80BD, 0.37KW, 2820r/min, which was situated on the base of the frame. Pump F provided the large flow rate required, -100litre/min. The large flow rate was required to prevent fouling of the membrane by the scouring action of the process fluid.

Pump E: Pump E is a gear pump, gear pump model (Brook Hansen from Michael Smith Engineers Lid.), type PD 63SFH, 012KW, 2870r/min, capable of providing a flow rate of about 3 litre/min at 3 bar. Pump E is smaller and was situated on the shelf, and pressurized the MBR system. Pump E circulated the process fluid around the MBR. When pump F was not in operation the process fluids passed from the base of the glass reaction vessel through pump E into the stainless steel pipe work, through the heat exchanger and back into the top of the glass vessel.

Pump G: Pump G is a peristaltic pump (Watson Marlow Ltd. England) model 501UII10-0, 50Hz. Ti 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 its operation was to allow the procedure of back flushing of the membrane to take place by reversing its action to reverse the flow of products back through the membrane and into the bioreactor system. This reverse flow was believed to unblock the pores of ceramic membrane and reduce fouling.

2.13.1.3 Membrane

The membrane is a ceramic membrane, surface area 1.4 sq.ft., 0.13 m^2, and pore size 0.2 μm. the stainless steel membrane housing is 340 mm long, purchased from PCI-Memtec,

Swansea (formally Memtec). The ceramic membrane has a flow diameter 1.9 mm, maximum operating temperature 130°C, minimum pH 2, maximum pH 13, operating pressure 5-40 psi, flow rate 1 m/sec or 786 l/h (concentrate flow rate). The membrane was used to immobilize the lactic acid bacteria within the high cell cultivation and maturation process equipment. The ceramic membrane is encased within a stainless steel sheath. Cells could not pass through the membrane pores and were retained inside the membrane, whilst the process fluid was able to go through the membrane pores to the outside surface where it was removed through the outlet pipe. These fluids were fully clarified and so a further downstream filtering process was not required. The membrane filtration operated in a cross-flow mode, and was able to withstand high pressures. Fouling was also kept to a minimum by the natural scouring effects of the cross flow action of the membrane.

2.13.1.4 pH Controller

Model 260 pH controller (Electrolab Ltd. England, 115/230V, 50/60Hz, 50W) has the same functions as the normal bench pH meter and is calibrated in the same way. With the function switch in the RUN position, the cal control is adjusted using buffer pH 7 and the slope is set using buffer pH 4 or 9. The temperature compensation is set to the temperature of the buffer and then later to the vessel temperature. Ideally the meter should be calibrated at the same temperature that it would be run at. 260 pH control unit includes:

- pH set points – the function switch controls the acid and alkali set points. In the SET ALKALI position, the alkali set point (low) is set. As the pH drops (becomes more acidic) the alkali pump runs to correct the set point. The alkali pump is the bottom one. In the SET ACID position the acid set point (higher) can be set to pump acid to correct the pH as it becomes too high. The acid pump is the top one.
- pH electrode – fully autoclavable combination pH electrode (Thermo Russell, Russell pH Limited, Scotland), pH range 0-14, temperature range 0-130°C.
- Peristaltic pumps – there are two single speed Watson Marlow peristaltic pumps fitted as standard. They have a clutch which enables you to turn the pump manually in a clockwise direction. This can be used to load the tube or to give a manual shot.
- Pump flow rates – wall silicone tubing (bore 1.6mm), with standard 4RPM 50Hz motor, 0.87 ml/min.

2.13.1.5 Heat exchanger

Heat generation in the system was appreciable because of the two pumps. The operation of both pumps without temperature control resulted in the temperature of the process fluid rising to ~ 50°C, the system could not maintain itself at a constant temperature by simple interaction with its surroundings. To maintain the system at an optimum temperature for the lactic acid bacteria, it was necessary to cool the broth with a heat exchanger. The heat exchanger is a single pass hollow tube exchanger, the dimension of outer shell is 435mm by 50mm, with the inside diameter of the inner tube 22mm. The flow of the coolant was controlled by a solenoid valve linked to a thermocouple to ensure that the temperature of the process fluid in the MBR maintained constant. The process fluid passed through the inner tube and the coolant through the outer shell.

2.13.1.6 Instrumentation

- **Pressure gauge P_1 & P_2** The transmembrane pressure (TMP) was calculated in terms of the pressure difference of pressure gauge P_1 and pressure gauge P_2. TMP sometimes was approximately estimated from the reading of pressure gauge P_1 (sometimes called as membrane inlet pressure) situated at the base of the ceramic membrane on the outlet of pump E.

- **Level control** A system of electric level control was added so that the MBR could be run continuously without 24-hour observation. The level control consists of three electrodes which are situated inside the glass vessel and a pinch valve through which the feed tube passes. The first electrode is the earth and it is not insulated; it reaches down to –30cm from the base of the reactor vessel. The other two electrodes are both insulated and control the level of process fluid in the reactor. One electrode is about 5cm longer than the other electrode and when the level of the process fluid in the reactor drops below this electrode the pinch valve is opened. This allows the feed to enter the glass reactor vessel until the level reaches the top electrode (the shortest one), the pinch valve then shuts cutting off the flow of feed into the reactor. The level in the system then drops at the rate at which pump G is removing liquid through the membrane.

- **Cooling water flow** The flow of cooling water to the heat exchanger, which kept the process fluid within the bioreactor at a constant set temperature, was controlled by a solenoid valve. The solenoid valve was opened and shut depending on the temperature of the fluid in the process, which was measured by a thermocouple attached to the stainless steel pipe work and then fully lagged. The heat exchange kept the temperature of the process fluid within the pre-set constraints. When the temperature became high the valve would be opened to let cold water into the heat exchanger. Conversely, when the temperature was low the solenoid valve would be closed, preventing cold water from entering the heat exchanger.

2.13.2 Operation of the MBR

A large quantity of LAB, 20 l or 40 l, was grown up on an artificial medium (Z broth) and then added to the glass fermentation vessel through the pinch valve and stainless steel top plate. Gravity or an overhead CO_2 pressure of approximately 3 psi was used to push the inoculum into the reactor system. The cells were concentrated within the system, up to the operating volume of 6 l, using the membrane to remove the spent culture from the MBR. The pore size of the ceramic membrane was 0.2μm which allowed the process fluid to pass through the membrane but not the LAB, therefore immobilizing them within the MBR system. The level control was used to regulate the flow of inoculum into the MBR to prevent the system overfilling or emptying.

Once loaded with LAB, the fresh growth medium was driven into the fermentor by gravity through the pinch valve and stainless steel top plate. The rate at which the feed was added to the reactor system was governed by pump G, a peristaltic pump situated on the outlet of the membrane. Pump G therefore determined the feeding rate which entered the fermentor within the MBR. The residence time of the fluid was controlled by pump G for biotransformation by the LAB. Once the feed had entered the glass vessel it became in contact with the high density of LAB. The feed was drawn down through the glass reactor vessel and into the stainless steel piping by pump E.

Once in the piped system pump F pumped the mixture of bacteria and process fluid at a high velocity up through the ceramic membrane. This high velocity was designed to create

a scouring effect on the surfaces of the membrane which reduced the fouling that would have occurred. Some of the process fluid passed out through the membrane at the flow rate governed by pump G. The rest of the fluid plus the LAB were retained and passed up through the membrane and to the top of the MBR piping. The process fluid then divided into two paths. Some of the fluid was returned through the diaphragm valve to the glass reactor vessel. The proportion of process fluid taking each route was governed by the diaphragm valve. The heat exchanger was added to the MBR system to counteract the heat generated by the operation of the two pumps and the metabolism of the LAB.

The basic design of the MBR system is given in the schematic diagram of the process, Figure 2-10 below.

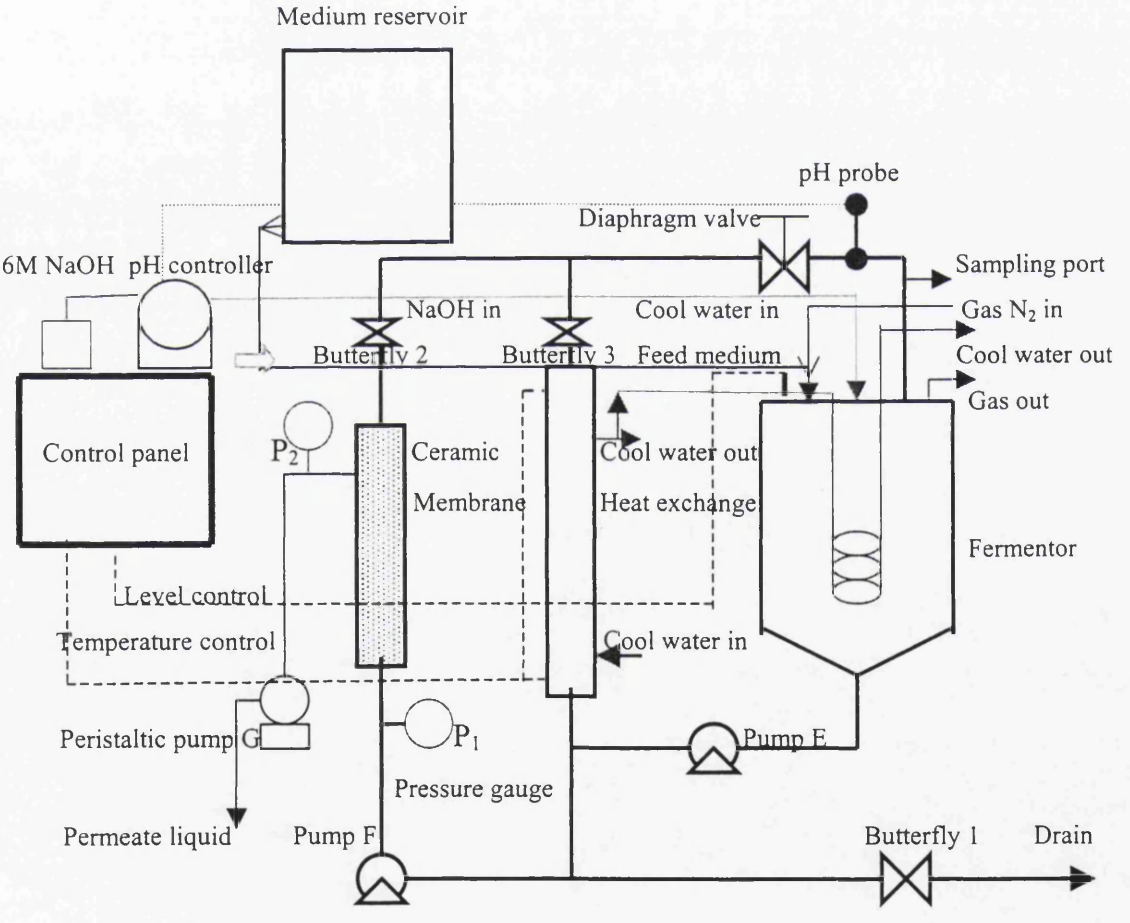


Figure 2-10 Schematic diagram of MBR system

(Please see Figure 2-8 for photograph)

2.13.3 Sterilization of the membrane bioreactor system

Before each operation the stainless steel sections of the MBR system were steam sterilized and the glass reactor vessel was autoclaved. First the glass reactor vessel and steel top plate were detached from the stainless steel pipe work of the MBR. All the openings and flexible pipe ends were plugged with cotton wool, covered in aluminum foil and the glass vessel was placed in the autoclave and autoclaved at 121°C for 30 minutes.

Prior to attaching a steam line to the MBR, allowing steam through the steam line for 5 minutes to remove any dirt and rust from the steam line which may damage the MBR was important. The steam line was attached to the top of the stainless steel pipe work close at the pH probe port and steam at less than 10 psi passed through the system by three separate routes, each for between ½ and 1 hour duration. The first route was from the top of the stainless steel line down through the heat exchanger and to drain. This was achieved by opening the bottom butterfly valve 1 to the drain and the butterfly valve 3 above the heat exchanger and closing the butterfly valve 2 to the membrane. The second route was from the top of the stainless steel piping down through the membrane and out to drain. This was carried out by closing the butterfly valve 3 above the heat exchanger and opening the butterfly valve 2 above the membrane housing. The final route allowed steam to pass through either the membrane or heat exchanger and out through pump E to drain. This was achieved by closing the bottom butterfly valve 1 to the drain and opening both butterfly valves 2 & 3 above the heat exchanger and membrane housing.

Once the autoclaved glass vessel (fermentor) was replaced and the flexible tubing reattached. The MBR was then sterile and ready for loading with the LAB.

2.13.4 Cleaning of the membrane bioreactor system

Effective cleaning methods are crucial for maintaining adequate long-term operation of all pressure-driven membrane processes. Frequent manipulation of the operating variables to induce a short-term increase in flux has proved successful. Thus, periodic flow reversal of the cleaning solutions is common practice. Other techniques include a sudden increase in axial flow rate or a decrease in transmembrane pressure, a change in the liquid

temperature and backflushing from the permeate side of the membrane with filtrate or cleaning solutions.

At the end of each operation the membrane and reactor must be cleaned in turn. In the MBR system used here, the following cleaning procedure was developed and operated as follows.

1. Rinse with cold distilled water, removing residual LAB and froth after the fermentation broth has been drained until all equipment was observed to be clean.
2. Add small amounts 6M NaOH into MBR system to adjust pH of 12-13 and run for ½ -1 h at 25°C. The techniques mentioned above were used here, such as a sudden increase in axial flow rate or a decrease in transmembrane pressure, and backflushing.
3. Wash with distilled water until pH goes down to about 7, the permeate flow was then measured and compared to the original permeate flow. If a recovery of permeate flow has taken place (95 to 100%) then the equipment has been cleaned. Generally the membrane had been clean after above processes were performed, repletion of the cleaning process was occasionally necessary.
4. Rinse with warm water of 40°C or/and HCl solution of pH 3 in case the membrane was not clean after above operation.
5. A scrubbing brush was used to remove LAB stuck to the internal and external surfaces of the glass vessel and the internal side of the cover of the glass vessel. It was then ready for autoclave for use.

2.14 100 litre fermentation

2.14.1 100 litre fermentor

The model PP100 liter fermentor designed by New Brunswick UK, constructed by Fabdec Ltd Shrewsbury, was controlled by a NBS model ML-4100 control console mounted adjacent to the fermentor skid. The console comprises of a drive system, manual override, power switches, the main electronic distribution point, and the control for Watson-Marlow peristaltic pumps for the addition of alkali. Figure 2-11 shows a highly schematic diagram of the fermentation system. The vessel is a cylinder. Vessel measurements: inside 0.4m diameter and 1.18m deep, exterior 0.516m diameter and 1.675m to top of headsplate; vessel capacities: total capacity 140 l, working capacity 100 l.

The agitation system, consists of a 2.2 kW variable speed D.C motor driving a bottom entry shaft with three Rushton-type 6 bladed turbine impellers (0.4 times vessel diameter), the agitation speed is closed loop controlled via the ML 4100 system and can be controlled at speeds between 50 and 1000 rpm, the margin of error of the selected speed constant is within +/- 2 RPM. To aid mixing further, there were four type 316L stainless steel removable baffles.

The temperature control uses a PID temperature sensor in the lower side-wall of the vessel. Temperature control range was from 5°C above water supply temperature to 60°C with an accuracy of +/- 2%. Temperature is displayed digitally on the controller.

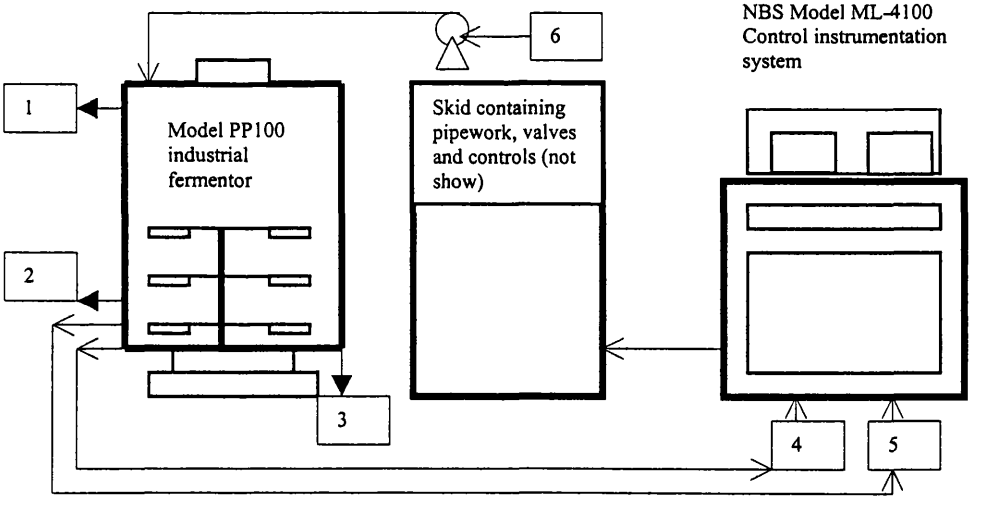
The pH control uses a PID controller with a pH probe sensor located in the lower side wall of the vessel. Since the pH was deemed to move in one direction, only the sodium hydroxide feed control is linked to the PID controller, the pH is set at 4.5 ± 0.5 pH unit and hence control the rate of NaOH feed input.

2.14.2 100 litre fermentation

2.14.2.1 Media preparation

The medium required for the fermentation was made up according to Z broth (Table 2.4), was dissolved in 10 l distilled water, then was filled through the filling port in the headplate into the fermentor containing 70 l distilled water, sealed and then raised to sterilization temperature (121°C for 30 minutes) whilst the contents of the vessel were agitated. Once sterilization of the medium had been accomplished, the temperature was reduced and then held at the operating temperature. Temperature of the fermentation broth was set at 25°C.

1M NaOH solution: 200g NaOH was dissolved in 5 l distilled water. A volume of the solution was used by topping up the NaOH feed bottle. The pH of the fermentation broth was set at 4.5 and was adjusted with 1M NaOH using an automated controller (ML-4100 instrument console, New Brunswick Scientific).



KEY

(1) Inoculum port
(2) Resterilisable sample port
(3) Resterilisable harvesting port
(4) pH sensor
(5) Temperature sensor
(6) 1M NaOH

Figure 2-11 Schematic diagram of 100 liter fermentor

2.14.2.2 Inoculation of the 100 l fermentor

The inoculation in the 2 l batch fermentation was carried out by 200 ml inoculum in a 500 ml bottle that was grown 70 h at 25°C. The 20 l fermentation was inoculated with 2 l of above inoculum, was undertaken at a 25°C constant temperature room for 60 h (details see 2.11.1). The inoculum was used for the inoculation of the 100 l fermentation broth. Z broth was used with 10g/l glucose-10g/l fructose mixture.

The content of the 20 l above was transferred to the 100 l fermentor via the inoculum port using a peristaltic pump. The connection between the sealed ending of the silicon tubing and the steam resterilizable inoculum port was made quickly after the tubing had been flamed with a Bunsen burner.

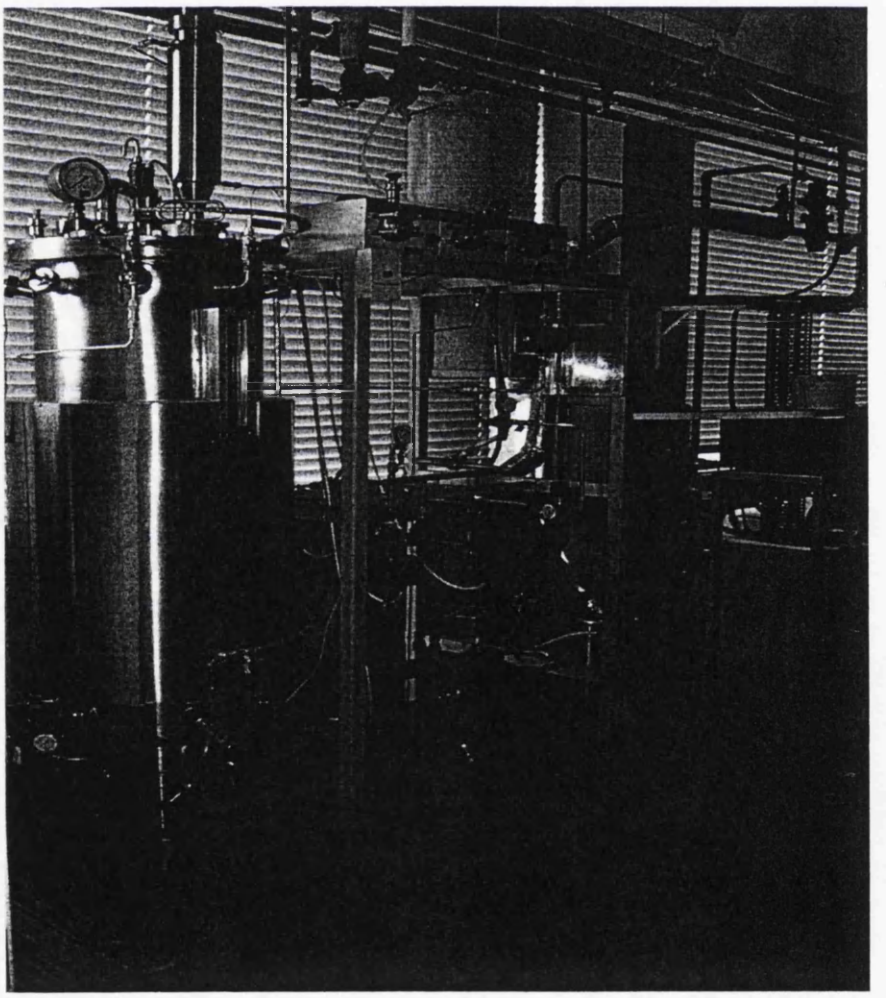


Figure 2-12 Experimental cell set-up of 100 l fermentor

2.14.2.3 Sampling and harvesting

O. oeni cells were withdrawn from the fermentor periodically via the steam resterilisable sample port and optical density measurement was carried out by using a SP8-400 UV/VIS Spectrometer in plastic cuvette of 1cm path length. Temperature was maintained at 25°C and pH at 4.5 throughout with automatic addition of 1M NaOH. The fermentation took about 70 h under these chosen conditions. At the end of the fermentation (OD more than 0.8), the cells were separated from fermentation broth by microfiltration membrane and harvested via the bottom harvest port and collected in 8 l bottle. About 6.5 l suspended cells were obtained and then stored in the refrigerator at 4°C for further processing and experiment.

Chapter 3 Metabolism and Growth

3.1 Introduction

Lactic acid bacteria (LAB) are used throughout the agriculture, food and beverage industries to impart flavour and preservative properties. Understanding how LAB can be grown more efficiently and their physiology and biochemistry associated with flavour transformations is not only fundamental to understanding the optimal condition for processing but is critical to food, beverage and inoculant producers.

As described in sections 1.2, 1.3, 1.4 & 1.5 of Chapter 1, *O. oeni* is one of the main species of LAB present in alcoholic beverages. It is well adapted to perform malolactic fermentation in a highly alcoholic and low pH environment of wine or cider (Lafon-Lafourcade[1,2] 1983; Wibowo *et al.* 1985), and many studies have been performed in these unfavourable conditions. The optimum growth temperature is in the range 20°C to 30°C. Different optimal pH and temperatures for growth of *O. oeni* were reported by researchers (Beelman & Gallander 1979; Lafon-Lafourcade[2,3] 1983; Salou *et al.* 1991; Britz & Tracey 1990; Firme *et al.* 1994) and are largely strain dependent. In recent years, a few investigations into growth, substrate utilization and product formation have been reported in batch cultures of *Leuconostoc oenos* (Salou *et al.* 1991, Maicas[2] *et al.* 1999).

Malic acid clearly stimulates bacterial growth in most studies, allowing an increase in the molar growth yield via additional ATP production. The increase of L-malic acid concentration prolonged the duration of MLF. The rate of MLF depends on the bacterial cell density, the specific malolactic activity (Nault *et al.* 1995) and the physiological state of the cells. The process of maturation in which LAB can catalyse a series of reactions that reduce acidity in the wine or cider as well as produce subtle flavour changes associated with the metabolism of tannin-derived substrates. The phenolic compounds in cider range from relatively simple compounds to complex tannin-type substrates, which have significant impact on the flavour of cider. The bacteria are also affected by the phenolic compounds of cider. Lea[1-5] (1978) investigated in detail the conversion of the phenolics of

cider, such as oligomeric and polymeric procyanidins, bitterness and astringency, and studied the effect of processing conditions and the effect of cultural conditions.

Little work has been done to evaluate the influence of the mixture of carbohydrate except for the mixture of glucose-fructose, and the influence of physiochemical factors in the presence of mixtures of sugars on the growth of *O. oeni*, e.g. organic acids, tannins, temperature and ethanol. It was therefore proposed to study the optimization of medium composition including carbon sources, nitrogen sources and buffer solutions for the growth of *O. oeni*, to investigate the influence of temperature and ethanol on the growth, and to recognize the degradation of organic acids, e.g. malate, citrate and pyruvate as well as the metabolism of tannin-derived substances. These studies improve our understanding of the growth, physiology and biochemistry of LAB for flavour transformations in cider and wine and to exploit this in the production of novel flavour and alcoholic beverages.

3.2 Materials and Methods

Oenococcus oeni (O. oeni) NCIMB *11648* used in this study was supplied by UKNCC and was isolated from wine.

Z broth (Table 2.4) with sugars was used in this chapter unless otherwise mentioned. All media were adjusted to pH of 5.0 before autoclave; 10ml medium was in each pressure test tube, and autoclaved at 121°C for 15 minutes.

1 ml cells of an active culture of *O. oeni* were inoculated into 10 ml growth medium in each pressure tube and incubated at 25°C in a constant temperature room with the exception of the study on the influence of temperature.

Growth measurements were described as in section 2.4 of Chapter 2. The maximum specific growth rate (μ_{max}) and maximum amount of growth (OD_{max}) were determined as described in section 2.5 Chapter 2. The specific growth rate and amount of growth were an average derived from two cultures.

3.3 Medium composition and formulation

Lactic acid bacteria (LAB) have a complex nutrient requirement for growth. De Man Rogosa Sharpe (MRS) (1960) medium has been used for the growth of LAB. However, because of its complex nutrient composition which will support the growth of a wide variety of organisms, the complex nutrient composition is very sensitive to contamination. Moreover, in the quest for developing a new large-scale culture process for LAB, a potential feedstock for the high density cultivation of selected LAB is a challenge to utilize safe cheap medium components.

A simplified medium was required because:

- A complex medium will contain all the nutrients required by a large range of organisms and therefore have a greater risk of becoming contaminated.
- MRS contains compounds derived from meat extracts, which give beefy aromas, and therefore MRS cannot be realistically considered for an applied utilization. A vegetable option was preferred by the beverage industry due to consideration of BSE (Bovine Spongiform Encephalopathy) contamination.
- A simpler and cheaper medium is a basic requirement for the intensive production of LAB to commercially useful amounts and the process is commercially viable.
- Analysis of the medium is made more straightforward by reducing its complexity. So reducing the risk of masking results, leading to false assay quantification.

3.3.1 Growth of *O. oeni* on MRS

It was necessary to grow the organisms up on MRS culture due to MRS being general culture medium for many organisms. The composition of MRS is given in Table 2.1. Here the liquid medium used was MRS with 5g/l glucose, 5g/l fructose, and 5g/l glucose plus 5g/l fructose. The results of this preliminary growth experiments are shown in Table 3.1.

Table 3.1 The growth of *O. oeni* on MRS

Carbohydrate	Maximum optical density OD_{max}	Maximum Specific growth rate μ_{max} (h^{-1})
5g/l glucose	0.817	0.021
5g/l fructose	0.915	0.033
5g/lglucose+5g/lfructose	1.193	0.035

Different specific growth rates and cell concentrations were observed in different carbohydrates from Table 3.1. Obviously, *O. oeni* preferred fructose to glucose in MRS, but the highest specific growth rate and amount of growth (OD_{max}) was in the mixture of glucose and fructose. This indicates that the mixture of 5g/l glucose-5g/l fructose is more suitable for growth of *O. oeni* than 5g/l glucose or 5g/l fructose alone.

3.3.2 Alternative nitrogen sources

An area of topical interest that has been raised through the beef crisis (BSE) and the *E. coli* poisonings that have occurred in Scotland, has been the desire to remove all traces of medium components originating from animal sources. Lab-Lemco in particular contains a large number of unidentified meat peptides. These also lead to an unwanted 'beefy' smell in the fermentation. Studies should be undertaken to replace or even remove bacterial peptone and Lab-Lemco in the synthetic medium. It was known that bacterial peptone, yeast extract (YE) and Lab-Lemco powder all are present in MRS medium. Donovan's broth is a simplified medium (section 2.1.2 Table 2.2) for the growth of LAB. The first stage is therefore to choose nitrogen sources and to simplify the medium, and then to determine nutrient requirements for the growth of LAB.

3.3.2.1 Influence of yeast extract in Donovan's broth on growth

A number of studies about the nutrients necessary for lactic acid fermentation have established two conclusions (Aeschlimann & von Stockar 1990; Olmos-Dichara *et al.* 1997; Payot *et al.* 1999; Kwon[2] *et al.* 2000): (1) The more supplemented the medium, the higher the productivity of the lactic acid could be obtained; (2) Among the various complex nitrogen sources, yeast extract (YE) is the best choice for both microbial growth and lactic acid production. A further investigation was required to assess the importance of YE. Experiments were performed in Donovan's broth with an addition of 5g/l glucose - 5g/l fructose at varying concentrations of YE. The results are shown in Table 3.2.

Table 3.2 The influence of yeast extract on the growth of *O. oeni*

[YE] (g/l)	OD_{max}	μ_{max} (h^{-1})	t_d (h)
0.0	0.012	0.002	385
0.5	0.015	0.002	385
1.0	0.091	0.025	28.2
2.5	0.993	0.051	13.5
5.0	1.340	0.069	10.0
7.5	1.338	0.065	10.6
10.0	1.270	0.062	11.2

YE had a remarkable influence on the growth rate and amount of growth of *O. oeni*. Maximum optical density of the culture (OD_{max}) and maximum specific growth rate (μ_{max}) were very low under less than 1.0g/l yeast extract. The greater the concentration of YE in the medium, the greater the amount of growth that was achieved, up to a maximum OD of 1.34 at 5g/l YE, and the higher the rate of growth, with a minimum doubling time of 10 h. More than 5.0g/l YE, the increase in the growth of *O. oeni* was ignored. At the concentration of YE reached up to 10g/l, OD_{max} and μ_{max} somewhat decreased possibly due to the inhibition of higher concentration of YE.

3.3.2.2 Influence of Lab-Lemco concentration in Donovan's broth on growth

A growth experiment was carried out in Donovon's broth, containing 5g/l YE and 5g/l glucose-5g/l fructose. The influence of Lab-Lemco concentrations on the growth of *O. oeni* is shown in Table 3.3.

Table 3.3 The influence of Lab-Lemco on the growth of *O. oeni*

[Lab-Lemco] (g/l)	OD_{max}	μ_{max} (h^{-1})	t_d (h)
0.0	1.365	0.062	11.1
0.5	1.405	0.065	10.7
1.0	1.340	0.062	11.2
2.5	1.444	0.055	12.7
5.0	1.370	0.058	12.0
7.5	1.357	0.058	12.0
10.0	1.336	0.052	13.3

It was observed that Lab-Lemco was not able to stimulate the amount of growth and the growth rate of *O. oeni* from above data. It possibly implied that Lab-Lemco did not

contain essential components for the growth of *O. oeni*. Therefore, removing Lab-Lemco from Donovan's broth reformulated a new broth - Z broth (section 2.1.4, Table 2.4). Removal of Lab-Lemco from the growth medium had two advantages, reduction of cost and promotion of safety.

In the forthcoming section, Z broth will be used in all experiments unless otherwise mentioned.

3.3.2.3 Influence of NH_4Cl or $(NH_4)_2SO_4$ on growth

Although nitrogen-fixing bacteria can acquire nitrogen directly from the atmosphere, most bacteria normally obtain this element in its most defined forms as inorganic salts such as NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, or KNO_3. Z broth with 5g/l glucose-5g/l fructose was used and the influence of NH_4Cl or $(NH_4)_2SO_4$ on the growth of *O. oeni* was carried out. The results are the following Table 3.4 and Table 3.5.

Table 3.4 The influence of NH_4Cl on the growth of *O. oeni*

[NH_4Cl] (g/l)	OD_{max} (120h)	μ_{max} (h^{-1})	t_d (h)
0	1.512	0.062	11.2
1	1.416	0.062	11.2
2	1.414	0.062	11.2
3	1.251	0.059	11.7
4	1.113	0.058	11.9
5	1.025	0.058	11.9

The specific growth rate maintained a certain level of 0.062 h^{-1} in a range 0-2g/l NH_4Cl, and OD_{max} was more than 1.4. Both of them decreased as the concentration of NH_4Cl was over 2 g/l. 2 g/l NH_4Cl would be used in the following experiments in order to compare with the results on the growth of *L. brevis* used by Donovan (2000).

O. oeni grew faster in the absence of $(NH_4)_2SO_4$ than in the presence of $(NH_4)_2SO_4$, OD_{max} achieved was higher as well (Table 3.5). It obviously indicated the inhibitory effect of $(NH_4)_2SO_4$ on the growth of *O. oeni*.

Table 3.5 The influence of $(NH_4)_2SO_4$ on the growth of *O. oeni*

$[(NH_4)_2SO_4]$ (g/l)	OD_{max} (100h)	μ_{max} (h^{-1})	t_d (h)
0.0	1.362	0.069	10.0
1.0	1.280	0.061	11.4
2.0	1.174	0.052	13.3
3.0	1.126	0.053	13.0
5.0	1.046	0.044	15.8

3.3.3 Influence of KH_2PO_4 on growth

Different concentrations of KH_2PO_4 were added to Z broth without KH_2PO_4 with 5g/l glucose plus 5g/l fructose, The influence of KH_2PO_4 on the amount of growth and growth rate of *O. oeni* are shown in Table 3.6.

Table 3.6 The influence of KH_2PO_4 on the growth of *O. oeni* on glucose-fructose

$[KH_2PO_4]$ (g/l)	OD_{max} (125h)	μ_{max} (h^{-1})	t_d (h)
0	0.978	0.048	14.5
1	1.265	0.057	12.2
2	1.248	0.054	12.8
3	1.287	0.060	11.6
4	1.254	0.059	11.7
5	1.378	0.057	12.1
6	1.171	0.055	12.6
8	1.266	0.057	12.1

There were low values of growth rate and of biomass formation in the absence of KH_2PO_4. The growth rate of *O. oeni* increased slightly as KH_2PO_4 concentration increased from 0 to 3g/l, and then declined slightly with a further increase. However, no exceptional change in amounts of growth took place in a range of KH_2PO_4 concentration tested. It is important for the pH of the fermentation to be buffered so as to reduce the effects of end product inhibition. Therefore, 3-5g/l KH_2PO_4 should be an appropriate concentration range in the medium.

3.3.4 Influence of metal elements – magnesium and manganese

The inorganic ion requirements of bacteria have been extensively studied (Knight 1951; Snell 1957; Hughes & Poole 1991). The nutritionally essential metal ions serve bacteria in a number of functions: (1) as activators or cofactors of a variety of enzymes (e.g.,

potassium, magnesium, and manganese), (2) in membrane transport (e.g., potassium and sodium), and (3) as components of molecules or structural complexes (e.g., calcium chelated within the spore protoplasts of gram-positive bacteria). For practical purposes, the mineral ion requirements for bacteria cultivated in defined media are satisfied by the addition of salts containing Ca^{2+}, K^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, Fe^{2+}or Fe^{3+}, PO_4^{3-}, and SO_4^{2-} in milligram-per-litre concentrations. Trace amounts of several other ions (e.g., Zn^{2+}, Cu^{2+}, Co^{2+}, and MoO_4^{2-}) are often included in micro- or nanogram proportions. Results of investigations demonstrating the roles of nickel, selenium, and tungsten in enzyme functions in anaerobes have led to the inclusion of these elements in complete trace-metal formulations.

Here attention was paid to the effect of Mg^{2+} and Mn^{2+} on the growth of *O. oeni* it was mainly due to high magnesium ion concentration (Table 2.3) and requirement of NAD and Mn^{2+} in malolactic enzyme that decarboxylates L-malate to L-lactate (Van Vuuren 1993).

3.3.4.1 Influence of Mg^{2+} or Mn^{2+} on growth

A solution of trace element was prepared for this experiment. Except for Mg^{2+} all other components of trace element were as same as that shown in Table 2.3. A 5g/l $MgCl_2$ solution of 100ml was prepared. 0ml, 0.1ml, 0.2ml, 0.4ml and 0.6ml of 5g/l $MgCl_2$ solution were added into different pressure tubes, respectively, and then different volumes of Z broth were added into above tubes up to total volume of 9ml. These tubes were inoculated with 1ml of 60 h inoculum after two sub-culture without Mg^{2+}, so that the inoculum contained only very small/without quantities of 'carry-over' Mg^{2+}.

The results are shown in Table 3.7 below. Complete inhibition of growth could not be achieved as the complex medium components also contained Mg. The lowest growth rate and biomass production indicated that the absence of magnesium ion led to the decrease in growth rate and biomass. Growth rate maintained 0.063 ± 0.003 h^{-1} and biomass of *O. oeni* reduced slightly with maximum OD of 1.154 to 1.079 in a range 0.05 to 0.30g/l magnesium ion. Hence, Mg^{2+} concentration would be maintained 0.10-0.20g/l in culture media.

Table 3.7 The influence of Mg^{2+} on the growth of *O. oeni* on glucose-fructose

$[Mg^{2+}]$ (g/l)	OD_{max} (70h)	μ_{max} (h^{-1})	t_d (h)
0	0.879	0.041	16.7
0.05	1.154	0.062	11.2
0.10	1.119	0.060	11.5
0.20	1.106	0.066	10.5
0.30	1.079	0.063	11.0

A similar experimental procedure was used in the preparation of solution of Mn^{2+} trace element. A 0.5g/l $MnCl_2$ solution of 100ml was prepared firstly. 0ml, 0.1ml, 0.2ml, 0.4ml and 0.6ml of 0.5g/l $MnCl_2$ solution were removed into different pressure tubes, respectively, and then different volumes of Z broth were added into above tubes up to total volume of 9ml, 1ml of 60 h old inoculum after two sub-culture without/very small Mn^{2+} were added into these tubes with the final volume 10ml.

The results shown in Table 3.8 indicated that the presence of Mn^{2+} had a significant effect on the growth rate and biomass of *O. oeni*. In the range of 0.005-0.010g/l the growth rate and biomass are a little higher than that in 0.020g/l Mn^{2+} or more. The results implied that more than 0.010g/l Mn^{2+} was not able to increase the growth of *O. oeni*. So the Mn^{2+} of 0.010g/l was an appropriate concentration in medium for the growth of *O. oeni*.

Table 3.8 The influence of Mn^{2+} on the growth of *O. oeni* on glucose-fructose

$[Mn^{2+}]$ (g/l)	OD_{max} (70h)	μ_{max} (h^{-1})	t_d (h)
0	0.460	0.028	24.4
0.005	1.127	0.067	10.4
0.010	1.143	0.065	10.6
0.020	1.098	0.062	11.1
0.030	1.020	0.062	11.1

3.3.4.2 Influence of Mn^{2+} in the presence of glucose and malate on growth

It was known from section 1.4 in the first chapter that L-malate was converted directly into L-lactate by malolactic bacteria in the presence of NAD^+ and Mn^{2+}. This experiment here aimed to investigate the influence of Mn^{2+} concentration on the growth of *O. oeni* in the presence of 2g/l glucose-2g/l malate mixture.

Similarly, the growth of *O. oeni*, either growth rate or biomass, was much lower in the absence of Mn^{2+} than that in the presence of Mn^{2+}. There was not much difference in the growth rate of *O. oeni* with the change in the Mn^{2+} concentration from 0.005 to 0.02g/l but OD slightly decreased in the range of this concentration. This suggested that 0.01g/l M_n^{2+} could meet the requirement of both growth and the degradation of malic acid.

Table 3.9 The influence of Mn^{2+} in the presence of glucose and malate on growth

$[Mn^{2+}]$ (g/l)	OD_{max} (70 h)	μ_{max} (h^{-1})	t_d (h)
0.000	0.294	0.030	23.4
0.005	0.433	0.042	16.5
0.010	0.442	0.044	15.8
0.020	0.424	0.044	15.8
0.030	0.410	0.043	16.1

3.4 The growth of *O. oeni* on different sugars

Sugars, as carbon sources and energy sources, can influence dramatically the growth of *O. oeni,* theoretically, as more glucose was added the greater amount of lactic acid produced increased the effect of end product inhibition. Therefore, this investigation was focused on influence of different sugars and varying concentrations on the growth of *O. oeni* so as to find the preferred sugar(s) in Z broth. Meanwhile, the results could be used in the preparation for the scale-up to larger cultures. The following sugars or mixtures of sugars were studied: (1) glucose, (2) fructose, (3) sucrose, (4) glucose –fructose mixtures, (5) glucose –sucrose mixtures and (6) fructose –sucrose mixtures.

3.4.1 Effect of glucose, fructose or sucrose on growth

Figure 3.1 shows the effect of varying glucose concentrations in Z broth on biomass formation of *O. oeni*. No growth occurred when glucose was omitted. Growth was stimulated when glucose was increased to 2g/l, higher concentrations from 5g/l to 15g/l did not modify greatly the growth rate and just increased OD_{max} to a small extent (Table 3.10). Maximum OD showed little difference in the presence of 15g/l glucose compared to 10g/l glucose. The results showed that all specific growth rates were small, less than 0.015 h^{-1}, corresponding to a doubling time of about 50 h. Both specific growth rate and biomass

production were slightly different when glucose was more than a concentration of 5g/l, this suggested that another factor apart from the substrate availability became growth limiting, perhaps the inhibition of end-products. The results were in agreement with those observed by Saguir *et al.* (1996).

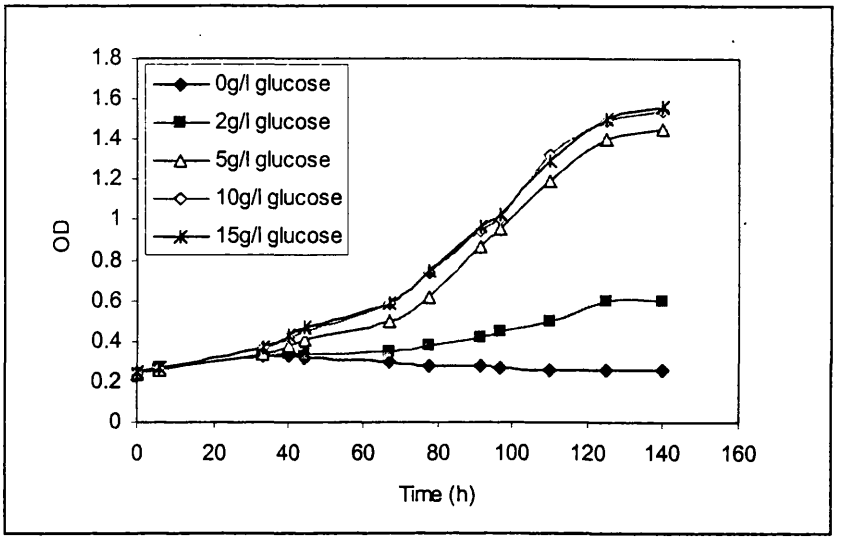


Figure 3-1 Effect of glucose concentration on the growth of *O. oeni*

(The measurement of OD was at a wavelength 660nm and with 1.8mm light path in pressure tubes; All OD are same in subsequent description of diagrams unless otherwise mentioned.)

Table 3.10 The effect of glucose concentration on the growth of *O. oeni*

[glucose] g/l	OD_{max}	μ_{max} (h^{-1})	t_d (h)
0	0.024	0.008	84.6
2	0.360	0.010	69.3
5	1.210	0.014	49.5
10	1.297	0.014	49.5
15	1.312	0.014	49.5

Figure 3-2 depicts the growth curve of *O. oeni* versus time as measured by OD. Fructose had an apparent effect on the specific growth rate and amounts of *O. oeni* in the presence of less than 5g/l fructose. The stimulated effect reduced as the fructose increased to more than 5g/l fructose, e.g. the growth curves were almost identical at 10g/l and 15g/l fructose, little variation in the values of specific growth rate and OD_{max} were observed. Compared

to the growth curves in glucose, the specific growth rates were also poor (Table 3.11), but the lag phase, OD being less than 0.5, was prolonged up to 100 h.

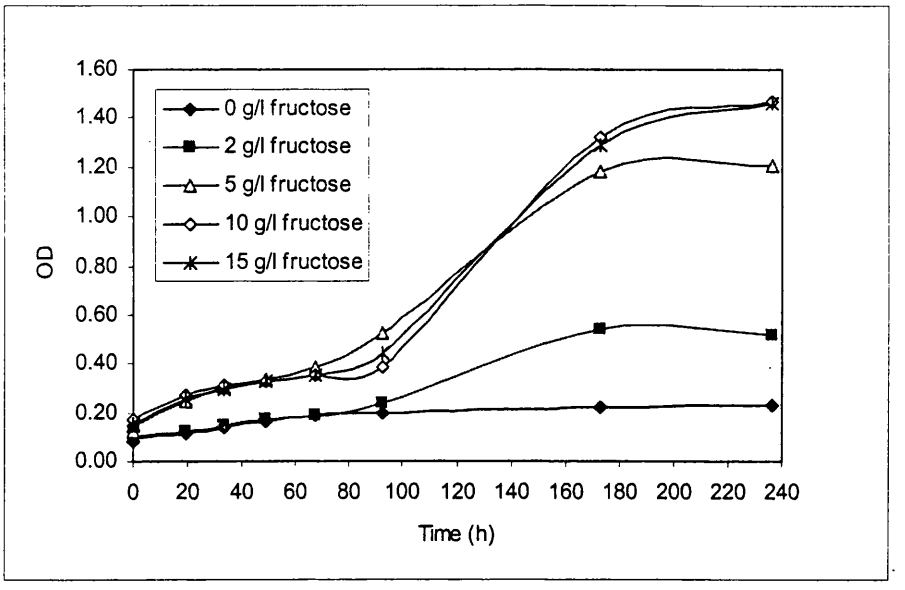


Figure 3-2 Effect of fructose concentration on the growth of *O. oeni*

(See Fig. 3-1 for note)

Table 3.11 The effect of fructose concentration on the growth of *O. oeni*

[fructose] g/l	OD_{max}	μ_{max} (h^{-1})	t_d (h)
0	0.128	0.008	84.6
2	0.430	0.010	69.3
5	1.085	0.012	57.8
10	1.302	0.015	46.2
15	1.311	0.014	49.5

In Figure 3.3 the growth of *O. oeni* in a medium containing different concentrations of sucrose is shown. The growth was enhanced as the addition of sucrose was increased. At 15g/l sucrose the maximum OD was obtained. The maximum OD increased from 0.088 to 1.031 when sucrose increased from 0 to 15g/l (Table 3.12). The inhibition was not observed at 15 g/l sucrose as in the same concentration of glucose or fructose.

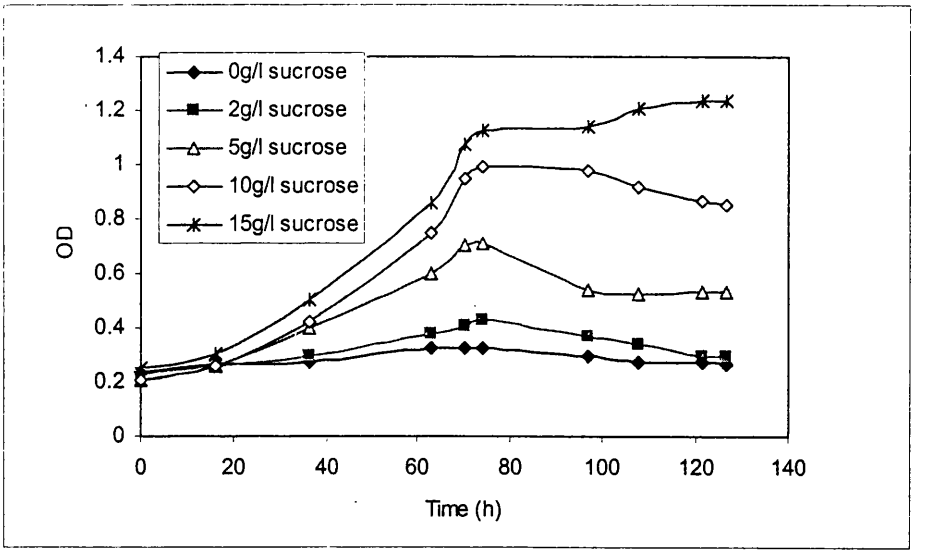


Figure 3-3 Effect of sucrose concentration on the growth of *O. oeni*

(See Fig. 3-1 for note)

Table 3.12 The effect of sucrose concentration on the growth of *O. oeni*

[sucrose] g/l	OD_{max}	μ_{max} (h^{-1})	t_d (h)
0	0.088	0.008	84.5
2	0.200	0.010	69.3
5	0.434	0.018	38.5
10	0.786	0.021	33.0
15	1.031	0.021	33.0

3.4.2 Influence of sucrose with 5g/l glucose on growth

The influence of sucrose-glucose mixtures on the growth of *O. oeni* is shown in Figure 3.4 and Table 3.13. In all cases, glucose concentration was a constant value of 5g/l. The maximum OD increased with the increase in sucrose concentration in a range 0 to 15 g/l, and specific growth rate also showed similar tendency in the presence of less than 10 g/l sucrose. When the medium contained 15g/l sucrose the specific growth rate declined slightly (Table 3.13).

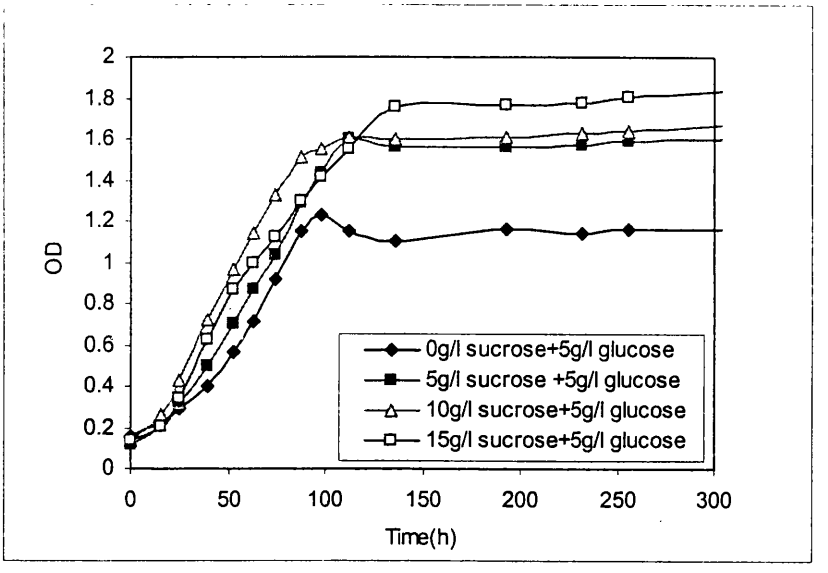


Figure 3-4 Effect of 5g/l glucose-sucrose concentration on the growth of *O. oeni*

(See Fig. 3-1 for note)

Table 3.13 The effect of 5g/l glucose-sucrose concentration on the growth of *O. oeni*

5g/l glucose+ [sucrose] g/l	OD_{max}	μ_{max} (h^{-1})	t_d (h)
0	1.000	0.024	28.9
5	1.466	0.034	20.5
10	1.503	0.044	15.9
15	1.671	0.041	17.1

3.4.3 Influence of sucrose with 5g/l fructose on growth

The growth of *O. oeni* in medium with mixtures of sucrose and fructose is shown in Figure 3-5. The growth of *O. oeni* was enhanced with the addition of sucrose. The second growth of *O. oeni* was observed after about 50 h. The growth rate and amount of *O. oeni* were increased slightly when concentration of sucrose varied from 5 to 15 g/l, but after 120 h the OD only showed very small differences. This indicated that higher concentration of sucrose did not result in the great increase of growth, and the growth was probably inhibited by end-products under conditions tested. These results are shown in Table 3.14.

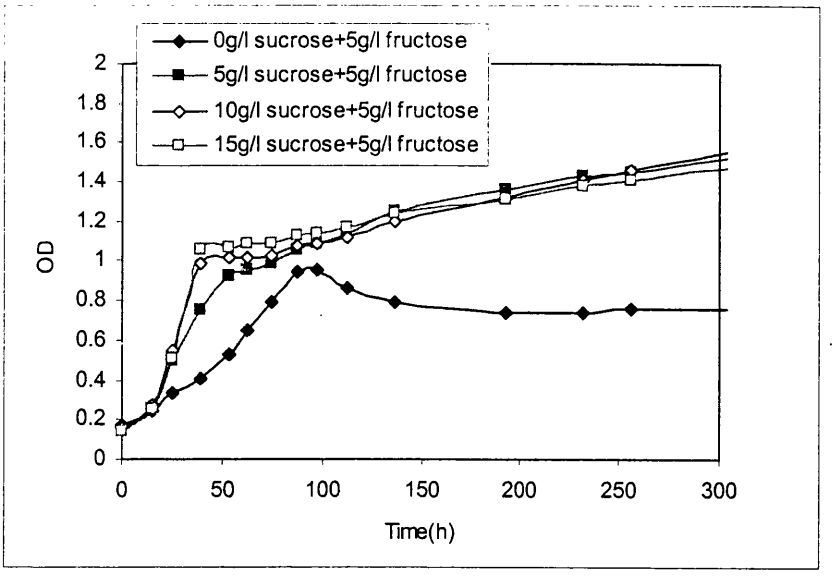


Figure 3-5 Effect of 5g/l fructose-sucrose concentration on the growth of *O. oeni*

(See Fig. 3-1 for note)

Table 3.14 The effect of 5g/l fructose-sucrose concentration on the growth of *O. oeni*

5g/l fructose+ [sucrose] g/l	OD_{max}	μ_{max} (h^{-1})	t_d (h)
0	0.595	0.021	33.0
5	1.296	0.042	16.5
10	1.289	0.048	14.4
15	1.268	0.050	13.9

3.4.4 Influence of glucose-fructose ratio on growth

As previously shown, the growth rate can be significantly improved when a mixture of sugars was used. It was expected that the mixture of glucose-fructose would promote an increase. The growth of *O. oeni* with glucose-fructose mixtures was further investigated. The ratio of glucose-fructose was expressed by G0/F10, G1/F9, etc, to G10/F0, respectively. G0, G1, etc stand for 0g/l and 1g/l of glucose respectively, while F0, F1 etc are 0g/l, 1g/l of fructose in media.

Table 3.15 shows that the ratio of glucose to fructose markedly affected the growth. The table also shows that the highest growth rate of 0.062 h^{-1} was with a mixture of 3g/l glucose-7g/l fructose. With 5g/l glucose-5g/l fructose the specific growth rate still remained in a certain level of 0.060 h^{-1}. Beyond a range of G3/F7 and G5/F5 the specific growth rate was less than 0.060 h^{-1}. The amount of growth was higher between G7/F3 and

G10/F0 than between G6/F4 and G0/F10, which suggested that higher ratio of glucose to fructose gave better amounts of growth, but with lower specific growth rates. Therefore, a mixture of G5/F5 could be considered as a reasonable choice of mixtures with an appropriate growth rate and OD_{max}. Figure 3-6 clearly shows this relationship between the growth and ratio of glucose-fructose mixtures.

Table 3.15 The influence of the ratio of glucose-fructose mixtures

Ratio of glucose-fructose (g/l)	OD_{max}	μ_{max} (h^{-1})	t_d (h)
G0/F10	1.299	0.024	29.1
G1/F9	1.399	0.030	23.5
G2/F8	1.358	0.052	13.3
G3/F7	1.289	0.062	11.3
G4/F6	1.356	0.061	11.4
G5/F5	1.431	0.060	11.6
G6/F4	1.445	0.053	13.2
G7/F3	1.604	0.050	13.9
G8/F2	1.676	0.046	15.2
G9/F1	1.698	0.027	25.3
G10/F0	1.563	0.023	30.3

Note: G0, G1...G10: zero to 10 g/l glucose, F0, F1...F10: zero to 10 g/l fructose.

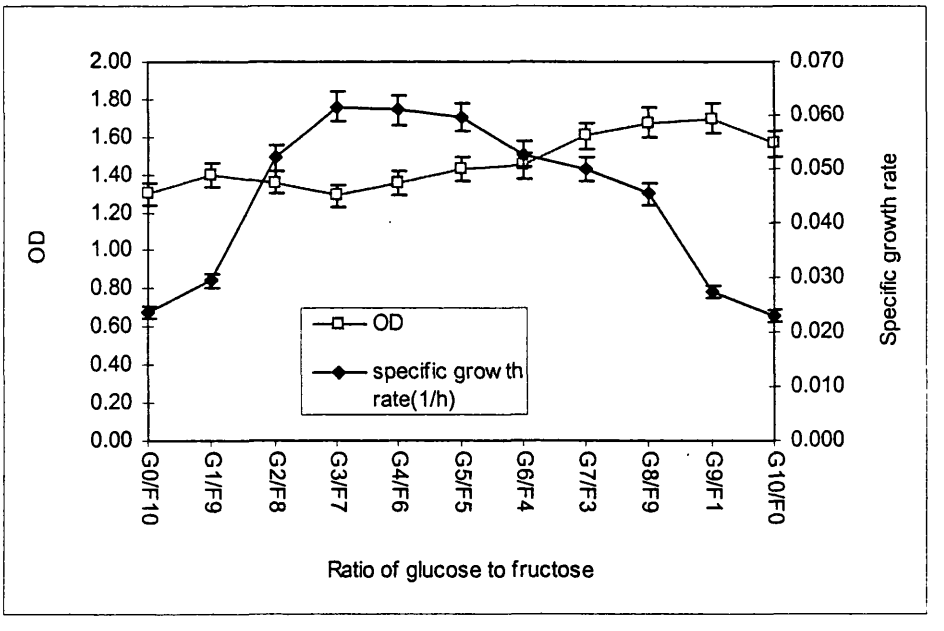


Figure 3-6 The growth of *O. oeni* in different ratios of glucose : fructose
(See Note, Table 3.15 above)

3.5 The influence of ethanol on the growth of *O. oeni*

Ethanol is generally regarded as a principal inhibitor of bacterial growth in wine or cider. The resistance to ethanol varies from strain to strain and is also influenced by other conditions (e.g. levels of SO_2 and pH) in the medium (Lafon-Lafourcade[3] 1983). High concentration of alcohol can be used as disinfectants or preservatives. Ethanol will also inhibit the growth of LAB, but indigenous LAB strains of cider and wine are significantly more tolerant to ethanol than other bacteria. However, few studies have attempted to systematically investigate the effect of ethanol on the growth of *O. oeni* in the presence of different sugars. The effect of ethanol on the growth of *O. oeni* with different sugars as carbon sources was evaluated in this section.

Z broth containing different sugars was used as culture media; ethanol concentration was expressed by volume concentration, that is, the percentage of ethanol volume to total medium volume (ethanol, %, v/v). The ethanol concentration refers to the volume concentration unless otherwise specified.

3.5.1 Growth of *O. oeni* in glucose, fructose or sucrose medium

Figure 3-7 shows the time curves of biomass formation in the presence of different concentrations ethanol. Large differences in the growth of *O. oeni* were observed at the different ethanol concentrations range from 0 to 12% (v/v). Ethanol had obvious inhibition on the growth of *O. oeni* in a medium with 5g/l glucose. The inhibitory effect was directly related to the amount of ethanol present. At an ethanol concentration of 6% the specific growth rate and amount of biomass of *O. oeni* decreased dramatically. The specific growth rate became quite small in the presence of 8% ethanol, the amount of growth was also small (less than 0.1) (Table 3.16). In addition, the relative values (with/without ethanol addition) of both specific growth rate and biomass were shown in Table 3.16 below. The relative values declined as the ethanol increased. The relative values revealed inhibition effects of ethanol on the growth of *O. oeni* in 5g/l glucose medium.

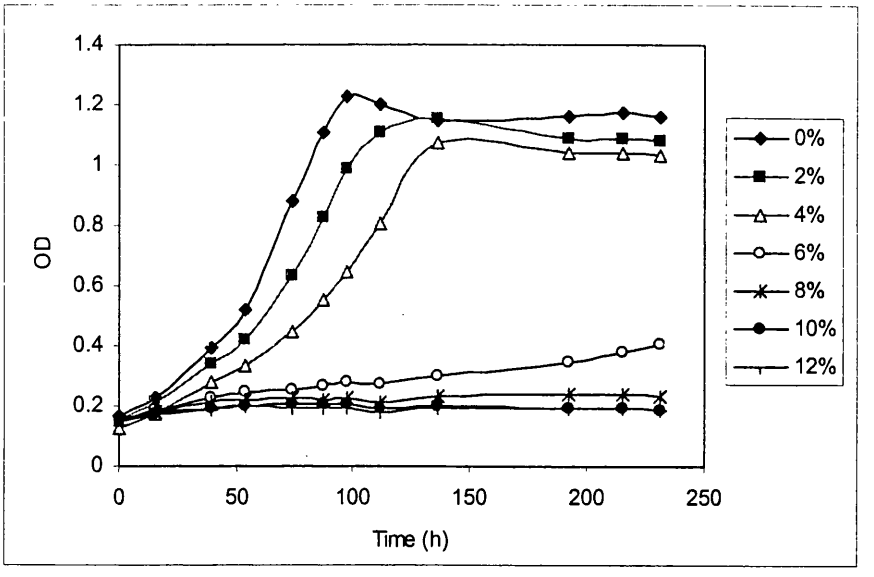


Figure 3-7 Effect of ethanol (v/v) in 5g/l glucose on the growth of *O. oeni* (see Table 3.16 for more details)

Table 3.16 The effect of ethanol on the growth of *O. oeni* in 5g/l glucose

Ethanol (%, v/v)	OD_{max}	Relative biomass [a]	μ_{max} (h^{-1})	t_d (h)	Relative specific rate (μ_i/μ_0) [b]
0	1.030	1.00	0.022	31.5	1.00
2	0.932	0.90	0.019	36.7	0.86
4	0.891	0.87	0.016	42.8	0.74
6	0.250	0.24	0.009	80.6	0.39
8	0.082	0.08	0.007	105	0.30
10	0.052	0.05	0.006	123	0.25
12	0.039	0.04	0.004	169	0.19

a relative biomass - the ratio of biomass with ethanol to biomass without ethanol;

b relative specific rate - the ratio of specific growth rate with ethanol to without ethanol, μ_0 specific growth rate without ethanol (0%,v/v); μ_i specific growth rate with concentration of i % (v/v) ethanol (i=0,2,4,6,8,10,12). All symbols are same in subsequent description of tables unless otherwise mentioned.

Similar curves (Figure 3-8) were obtained in 5g/l fructose compared to the growth curves in 5g/l glucose. The specific growth rate and biomass declined with the increase in initial ethanol addition. In the presence of 8% ethanol specific growth rate and biomass were higher in 5g/l fructose than in 5g/l glucose. However, in the presence of low ethanol contents (0-4%, v/v) OD_{max} was lower in 5g/l fructose with OD_{max} 0.59-0.77 than in 5g/l glucose with OD_{max} 0.89-1.03. Table 3.17 shows the growth of *O. oeni* in 5g/l fructose medium.

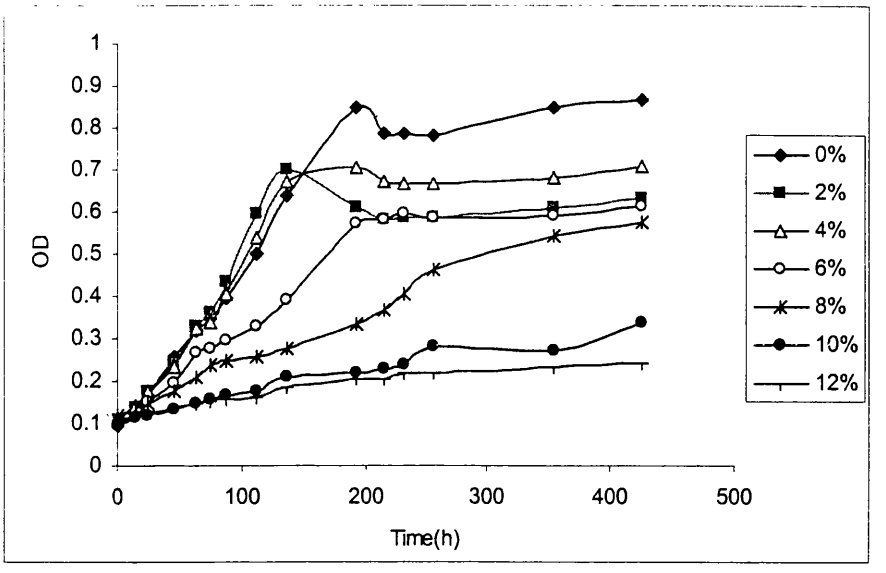


Figure 3-8 Effect of ethanol in 5g/l fructose on the growth of *O. oeni*
(see Table 3.17 for more details)

Table 3.17 The effect of ethanol in 5g/l fructose on the growth of *O. oeni*

Ethanol (% v/v)	OD_{max}	Relative biomass	μ_{max} (h^{-1})	t_d (h)	Relative specific rate $(\mu_i/\mu_0)^*$
0	0.772	1.00	0.020	34.8	1.00
2	0.624	0.81	0.020	34.8	1.00
4	0.596	0.77	0.020	34.8	1.00
6	0.521	0.67	0.017	40.8	0.85
8	0.431	0.56	0.009	77.0	0.45
10	0.247	0.32	0.007	99.0	0.36
12	0.132	0.17	0.005	138	0.25

* Symbols as in Table 3.16.

Figure 3-9 shows the time courses of biomass formation in 5 g/l sucrose and it was different from both glucose (Figure 3-7) and fructose (Figure 3-8). The ethanol had a strong inhibitory effect on the growth of *O. oeni* and much lower biomass was observed range from ethanol of 8%-12%, and biomass yields were less than OD_{max} of 0.3 with ethanol addition. The OD_{max} (Table 3.18) observed were remarkably low compared to the OD_{max} in glucose and fructose in the presence of 0-6% ethanol. This could be due to the inhibition of the expression or inactivation of the invertase enzyme. The cell membrane structure and transport properties may also be interfered with as the primary toxic effect of ethanol, so affecting the growth and viability of bacteria.

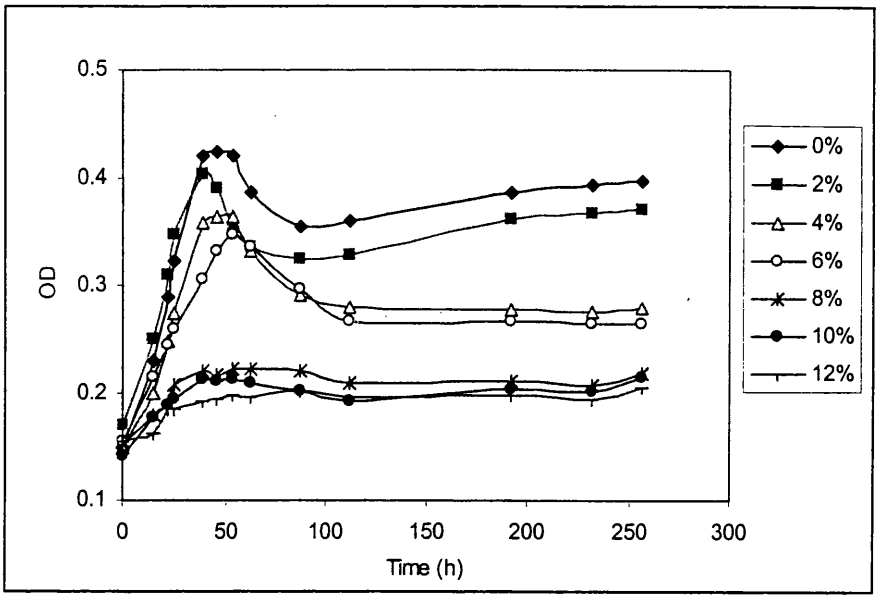


Figure 3-9 Effect of ethanol in 5g/l sucrose on the growth of *O. oeni*
(see further details in Table 3.18 below)

Table 3.18 The effect of ethanol in 5g/l sucrose on the growth of *O. oeni*

Ethanol (%, v/v)	OD_{max}	Relative biomass	μ_{max} (h^{-1})	t_d (h)	Relative specific rate (μ_i/μ_0)
0	0.312	1.00	0.027	25.4	1.00
2	0.251	0.80	0.023	30.1	0.84
4	0.225	0.72	0.022	31.5	0.80
6	0.200	0.64	0.016	43.3	0.59
8	0.082	0.33	0.012	57.8	0.42
10	0.073	0.30	0.009	77.0	0.34
12	0.042	0.17	0.006	115	0.22

Symbols as in Table 3.16.

3.5.2 Growth of *O. oeni* in glucose-sucrose medium

The curves for the growth of *O. oeni* with 5g/l glucose plus 5g/l sucrose containing different ethanol were plotted in Figure 3-10. The inhibitory effect of ethanol additions on growth of *O. oeni* can be clearly seen as measured growth. Specific rate or biomass produced was reduced by the addition of ethanol. However, the distinct difference in both biomass and specific growth rate were found in Figure 3-10 comparing with Figures 3-7, 3-8, and 3-9. Figure 3-10 shows much higher biomass formation with mixtures of glucose-sucrose than with glucose or fructose or sucrose alone, the OD_{max} of above 0.6 still achieved at 8% ethanol. The relative biomass (Table 3.19) decreasing from 1.00 to 0.79 in

a range of 0 to 6% ethanol with a mixed medium of glucose-sucrose compared with relative biomass of the individual sugar, glucose (Table 3.16), fructose (Table 3.17) and sucrose (Table 3.18) ranging from 0 to 6% ethanol with sole sugar medium, suggested that the *O. oeni* showed greater alcohol tolerance in the mixtures of 5g/l glucose-5g/l sucrose.

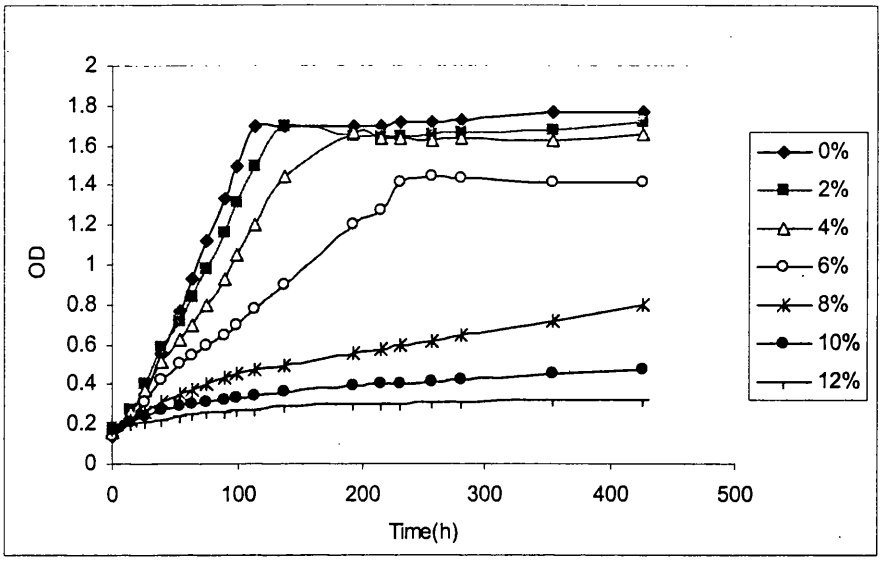


Figure 3-10 Effect of ethanol in 5g/l glucose-5g/l sucrose on the growth of *O. oeni* (details in Table 3.19)

Table 3.19 The effect of ethanol in 5g/l glucose-5g/l sucrose on the growth of *O. oeni*

Ethanol (%, v/v)	OD_{max}	Relative biomass	μ_{max} (h^{-1})	t_d (h)	Relative specific rate (μ_i/μ_0)
0	1.620	1.00	0.033	21.0	1.00
2	1.538	0.95	0.030	23.1	0.93
4	1.490	0.92	0.030	23.1	0.91
6	1.272	0.79	0.029	24.0	0.88
8	0.624	0.39	0.016	43.3	0.48
10	0.310	0.19	0.012	57.8	0.38
12	0.152	0.09	0.011	63.0	0.32

Symbols as in Table 3.16.

3.5.3 Growth of *O. oeni* in fructose-sucrose medium

The effects of added ethanol on the growth of *O. oeni* in a fructose-sucrose medium are shown in Figure 3-11 and in Table 3.20. Similarly, ethanol had negative effects on the specific growth rate and biomass formation. The interesting phenomena were observed in Figure 3-11 that in the presence of 6% ethanol or more, the OD nearly achieved the

highest value at 100 h and then maintained at constant value, whereas below 6% ethanol *O. oeni* grew rapidly at the beginning of fermentation (0-50 h), and after that, grew slowly. However, in glucose-sucrose medium (Figure 3-10) a different growth behaviour was observed. Below 6%, the growth almost reached maximum (OD 1.5) at about 110 h and was maintained constant up to 170 h, whereas above 8% ethanol the OD increased slowly during the period 0-50 h and then more slowly increased up to over 400 h (Figure 3-10). The comparison of relative biomass production in Table 3.19 and 3.20, in the range of 0-6% ethanol, lower relative biomass was observed (Table 3.20), whereas higher relative biomass was achieved ranging from 10%-12% ethanol. The mixture of fructose-sucrose possibly more suited for the growth of *O. oeni* than the mixture of glucose-sucrose at high ethanol content (>8%).

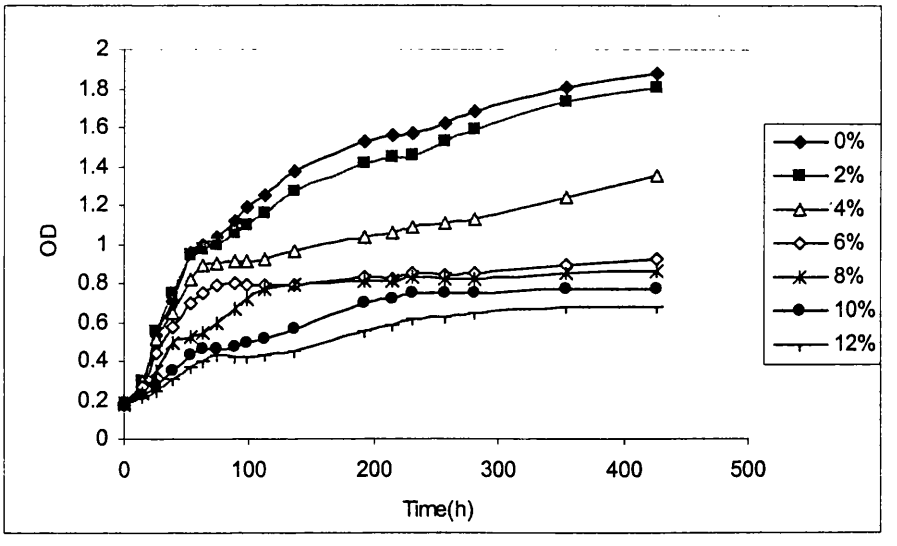


Figure 3-11 Effect of ethanol in 5g/l fructose-5g/l sucrose on the growth of *O. oeni*
(Note for more details in Table 3.20)

Table 3.20 Effect of ethanol in 5g/l fructose-5g/l sucrose on the growth of *O. oeni*

Ethanol (%,v/v)	OD_{max}	Relative biomass	$\mu_{max}(h^{-1})$	t_d (h)	Relative specific rate (μ_i/μ_0)
0	1.650	1.00	0.037	18.7	1.00
2	1.576	0.96	0.036	19.3	0.97
4	1.169	0.71	0.034	20.4	0.92
6	0.738	0.45	0.031	22.4	0.82
8	0.693	0.42	0.027	25.7	0.74
10	0.600	0.36	0.018	38.4	0.47
12	0.497	0.30	0.014	49.5	0.37

3.5.4 Growth of *O. oeni* in glucose-fructose medium

It was observed that the inhibitory effects of ethanol on the growth of *O. oeni* increased with maximum OD from 1.5 to 0.8 as more ethanol was added to the medium with ethanol from 0 to 12%. The relative biomass showed little variation at 0-6% ethanol concentration; up to 12% ethanol, relative biomass was still more than 0.5. This offered the highest alcohol tolerance of *O. oeni* in 5g/l glucose-5g/l fructose compared with other sugars tested previously as carbon sources (section 3.51 to 3.5.3). Moreover, not only relative specific growth rate but also specific growth rate was high. These indicated that the mixture of 5g/l glucose-5g/l fructose was appropriate in medium for the growth of *O. oeni* with/without high ethanol concentrations. Table 3.21 shows the results.

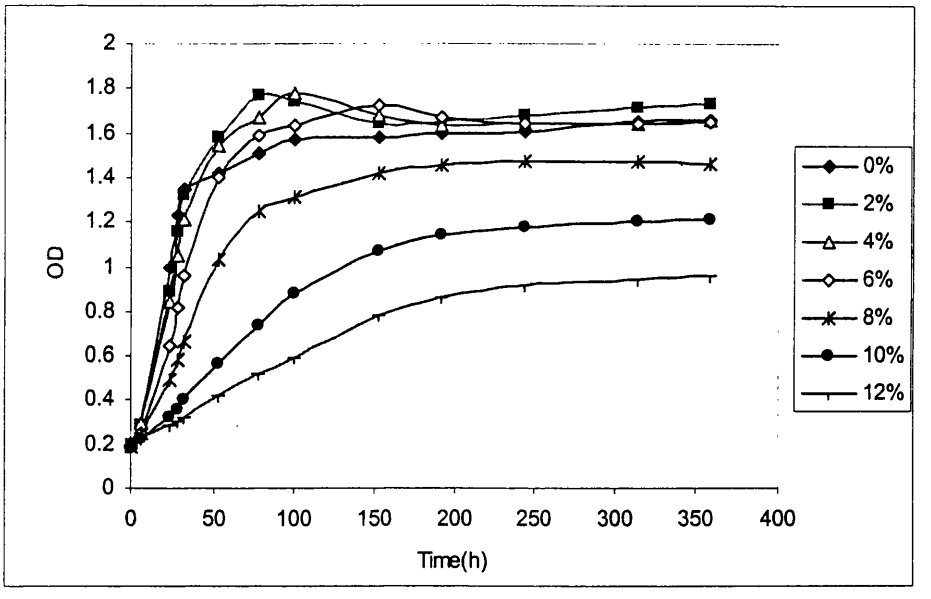


Figure 3-12 Effect of ethanol in 5g/l glucose-5g/l fructose on the growth of *O. oeni*
(Note for more details in Table 3.21)

Table 3.21 The effect of ethanol in 5g/l glucose-5g/l fructose on the growth of *O. oeni*

Ethanol (%, v/v)	OD_{max}	Relative biomass	μ_{max} (h^{-1})	t_d (h)	Relative specific rate (μ_i/μ_0)
0	1.501	1.00	0.063	11.0	1.00
2	1.483	0.99	0.057	12.1	0.91
4	1.451	0.97	0.057	12.2	0.90
6	1.462	0.97	0.052	13.3	0.82
8	1.267	0.84	0.039	17.8	0.62
10	1.030	0.69	0.024	29.1	0.38
12	0.765	0.51	0.015	46.5	0.24

3.5.5 Comparison of ethanol tolerance of *O. oeni* in different sugar media

From the experiments described above, ethanol had strongly inhibitory effects on the growth with different sugars as carbon sources. The aim was to optimize the growth of *O. oeni* in the presence of ethanol and to hopefully obtain the inoculants adapted well for the highly alcoholic environment and so perform MLF more efficiently in a cider maturation process.

Figure 3-13 (a) shows that the specific growth rates decreased as the ethanol increased, but these decreases in velocity were different in the different range of ethanol concentration. Under less than 6% ethanol, μ_{max} reduced slowly with the increase in ethanol with the exception of glucose, while in the range 6% to 12% ethanol μ_{max} diminished quickly. With glucose-fructose medium μ_{max} maintained the highest value in all media, even up to 12% (v/v) ethanol.

OD_{max} also dropped as the ethanol rose, and this trend was more significant when ethanol concentration exceeded 4% (v/v) (Figure 3-13 (b)). *O. oeni* grew much better with glucose-fructose than those with other sugars in the ethanol range tested. It indicated that the mixture of glucose-fructose was the best candidate as carbon source for the growth of *O. oeni* in highly alcoholic environment.

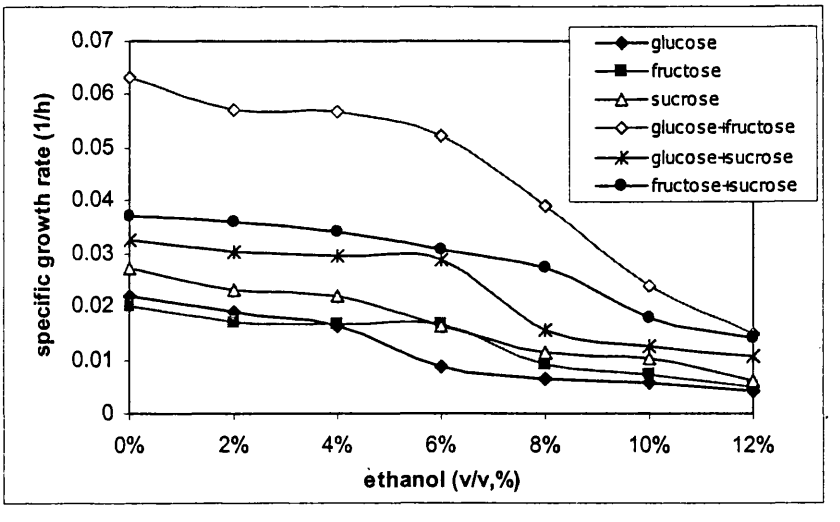


Figure 3-13 (a) Influence of ethanol on the specific growth rate of *O. oeni* in various sugars/mixtures

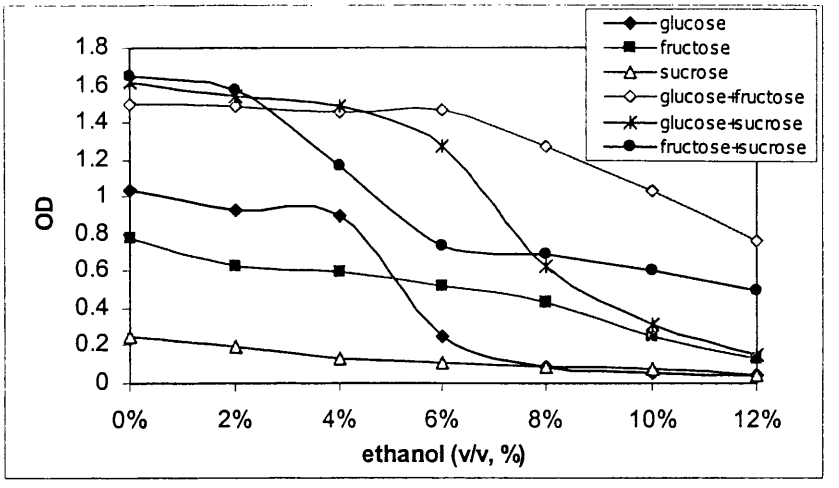


Figure 3-13 (b) Influence of ethanol on the amount of growth (OD) of *O. oeni* in various sugars/mixtures

3.6 Influence of temperature on the growth of *O. oeni*

The effect of temperature on the growth and ethanol tolerance for *O. oeni* was investigated. The aim was to understand the growth characteristics and find optimal temperature or temperature range for maximum growth rate (μ_{max}) and biomass (OD_{max}) for *O. oeni*. By means of the correlation between temperature, ethanol, μ_{max} and OD_{max}, future fermentations could be manipulated in order to optimize the operation conditions in the batch and continuous fermentations.

The experiments were undertaken with 5g/l glucose or 5g/l glucose-5g/l fructose in the absence or presence of ethanol (6% v/v) in pressure tubes. The 10°C and 15°C test tubes were placed individually in a refrigerator, the 20°C, 22°C, 25°C and 35°C test tubes in water baths and 27°C test tubes in a constant temperature oven.

3.6.1 Influence of temperatures in glucose medium on growth

Figure 3-14 illustrates the growth of *O. oeni* without ethanol in the temperature range 10 to 35°C. It was observed that temperature had an influence on the growth of *O. oeni* not only the specific growth rate but biomass yields. At 10°C and 15°C the lag phases were quite long, more than 120 h. Moreover, OD_{max} was still very low until 300 h. At below 22°C, biomass yields varied considerably as temperature changed; but at 22 to 35°C there

was small difference in the biomass yield apart from longer lag phase at 22°C. The maximum biomass yield was obtained at 25-27°C.

The maximum specific growth rate, 0.022 h^{-1} was observed at 25°C in the absence of ethanol. The specific growth rate rose steeply from the very low at 10°C to the highest at the optimum temperature 25°C and fell as the temperature was further increased. The inhibition percentage of temperature on the growth rates of *O. oeni* altered from 0 to 73% when temperature changed from 25°C to 15°C, but at the same temperature range of 25°C - 35°C inhibition percentage was only 0 - 18%. This suggested that temperature had greater inhibitory effect on the growth rate at below optimum temperature (25°C) than over 25°C. The results are shown in Table 3.22.

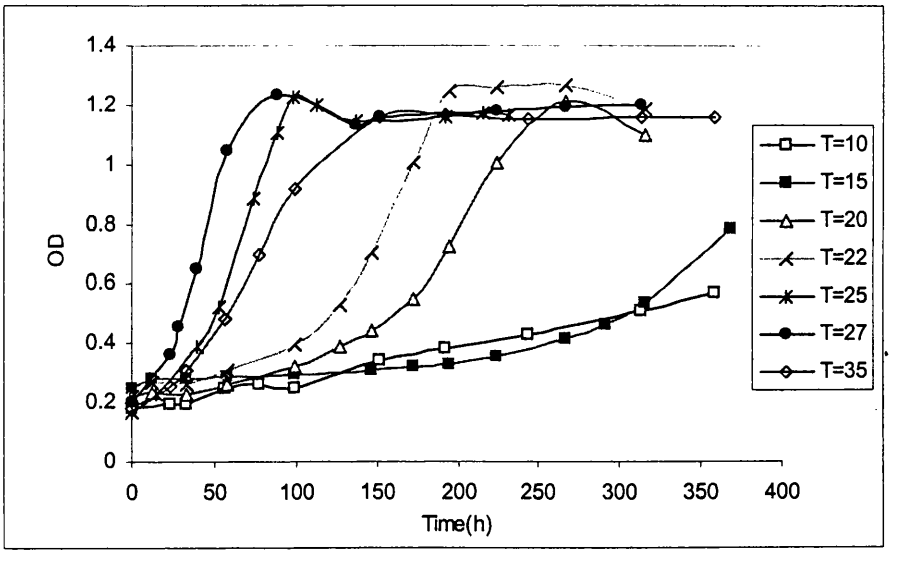


Figure 3-14 Effect of temperature with 5g/l glucose on the growth of *O. oeni*

(Note: more details in Table 3.22)

Table 3.22 The effect of temperature with 5g/l glucose on the growth of *O. oeni*

Temperature (°C)	OD_{max}	μ_{max} (h^{-1})	t_d (h)	Relative specific rate $\mu_{(T)}/\mu_{(25)}$	% Inhibition $100\mu_{(T)}/\mu_{(25)}-100$
10	0.388	0.005	138	0.23	-77.3
15	0.538	0.006	116	0.27	-72.7
20	0.880	0.008	86.6	0.36	-63.6
22	1.037	0.012	57.8	0.55	-45.5
25	1.064	0.022	31.5	1.00	0.0
27	1.002	0.020	34.7	0.91	-9.1
35	0.988	0.018	38.5	0.82	-18.2

3.6.2 Combined effect of temperature and ethanol in glucose on growth

Figure 3-15 illustrates the combined effect of temperature and ethanol on the growth of *O. oeni* in 5g/l glucose. Compared with the results obtained without ethanol, the much long lag phases were observed in the temperature range. The maximum OD_{max} and μ_{max} were obtained at 25-27°C (Table 3.23), but both OD_{max} and μ_{max} were much lower than those values without ethanol. Little growth took place at 35°C up to over 300 h, which implied that the inhibitory effect on the growth was especially striking under the combined effect of both temperature and ethanol. It has been demonstrated that *O. oeni* was more sensitive to ethanol in high temperature from the percent inhibition of specific rate 89% with 6% ethanol compared with 18% without ethanol at 35°C. Additions of ethanol also reduced the effects of temperature in suboptimal conditions (below 25°C) where the relative growth rate was higher than with glucose alone.

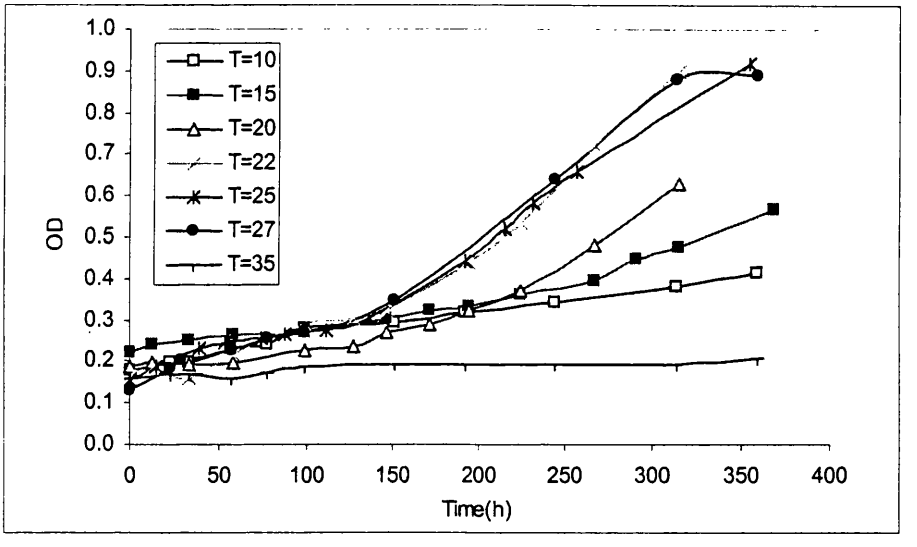


Figure 3-15 Combined effect of temperature and ethanol in 5g/l glucose medium on the growth of *O. oeni*

Table 3.23 The combined effects of temperature and ethanol with 5g/l glucose

Temperature (°C)	OD_{max} (6%, v/v)	μ_{max} (h^{-1}) (6%, v/v)	t_d (h)	Relative growth rate $\mu_{(T)}/\mu_{(25)}$	% Inhibition $100\mu_{(T)}/\mu_{(25)}$ -100
10	0.235	0.004	173	0.44	-55.6
15	0.349	0.004	173	0.44	-55.6
20	0.444	0.005	138	0.56	-44.4
22	0.747	0.007	99	0.78	-22.2
25	0.765	0.009	77	1.00	0.0
27	0.778	0.007	99	0.78	-22.2
35	0.048	0.001	693	0.11	-88.9

3.6.3 Influence of temperature in glucose-fructose with/without ethanol

The influence of temperature on the growth of *O. oeni* in the mixture of 5g/l glucose-5g/l fructose with 6% ethanol or without ethanol is shown in Figure 3-16. The optimal growth temperature was in the range of 25-27°C which was independent of the presence of ethanol. The biomass concentration (OD_{max}) was only slightly different (1.4-1.6) from 20°C to 27°C (Table 3.24 a). This indicated that the inhibitory effect of temperature with 6% ethanol on the biomass in the range of 20-27°C was negligible when the mixture of glucose-fructose was taken as carbon source.

The specific growth rate was more greatly influenced by both temperature and ethanol than the biomass yield. The specific growth rate increased as temperature tested increased in the absence of ethanol, i.e. $0.019h^{-1}$ at 20°C and $0.068h^{-1}$at 25°C; whereas in the presence of 6% ethanol the specific growth rate firstly rose in the temperature range 20°C to 25°C and then descended slightly (Table 3.24 b).

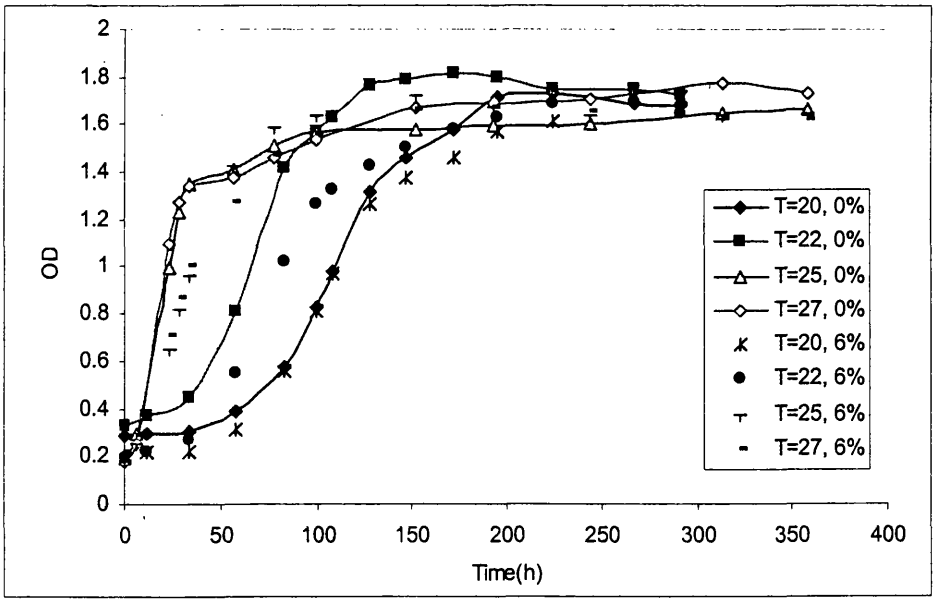


Figure 3-16 Effect of temperature in 5g/l glucose-5g/l fructose medium on the growth of *O.oeni*

Note: T- Temperature (Celsius degree, °C), 20 to 27°C; ethanol concentration (v/v): 0% and 6%. For further details in Table 3.24.

Table 3.24 Effect of temperature in 5g/l glucose-5g/l fructose medium on growth

(a) Cell concentration (OD_{max})

Temperature (°C)	OD_{max} (0%, v/v)	$OD_{(T)}/OD_{(25)}$* (0%, v/v)	OD_{max} (6%, v/v)	$OD_{(T)}/OD_{(25)}$* (6%, v/v)
20	1.394	0.95	1.525	1.04
22	1.397	0.95	1.452	0.99
25	1.468	1.00	1.462	1.00
27	1.560	1.06	1.404	0.96

* $OD_{(25)}$ and $OD_{(T)}$ are the maximum OD in 25°C and T°C (T=20, 22, 25, 27°C); ethanol (v/v) 0% and 6%.

(b) Specific growth rate (μ_{max})

Temperature (°C)	μ_{max} (h^{-1}) (0%, v/v)	t_d (h) (0%,v/v)	$\mu_{(T)}/\mu_{(25)}$ (0%,v/v)	μ_{max} (h) (6%, v/v)	t_d (h) (6%, v/v)	$\mu_{(T)}/\mu_{(25)}$ (6%, v/v)
20	0.019	36.5	0.28	0.020	34.7	0.38
22	0.023	29.7	0.34	0.024	28.9	0.46
25	0.068	10.2	1.00	0.052	13.3	1.00
27	0.068	10.2	1.00	0.049	14.1	0.94

*$\mu_{(25)}$ and $\mu_{(T)}$ are the maximum μ in 25°C and T°C (T=20, 22, 25, 27°C); ethanol (v/v) 0% and 6%.

3.6.4 Comparison of influence of temperature on growth

Section 3.6.1-3.6.3 describes the influence of temperature on the growth of *O. oeni* without/with ethanol in the presence of glucose alone and glucose-fructose as carbon sources, respectively. Figures 3-14, 3-15 & 3-16 clearly show the effect of every factor-temperature, ethanol and carbon source on the growth. Figure 3-17 (a) below shows that in the presence of glucose-fructose mixture maximum biomass (OD_{max}) varied slightly in the range of 20°C to 27°C, independent of the presence of ethanol, which indicated that 6% ethanol had no explicit inhibitory effect on the biomass yield. However, in the presence of glucose alone, greater biomass yields were observed without ethanol than with ethanol in the temperature range of 10°C to 35°C, indicating that ethanol had an apparent inhibitory effect on the biomass yield in glucose alone as carbon source. In Figure 3-17 (b), the specific growth rate was at a peak at 25°C in all cases. With a medium containing glucose alone the curve of specific growth rate without ethanol was above the curve with 6% ethanol over the range of 10°C to 35°C. In comparison, with a mixture of glucose-fructose little difference in the specific growth rate was observed at 20 to 22°C. Figure 3-17 (c) depicts that relative specific growth rate of *O. oeni* in the presence of glucose alone or mixture of glucose-fructose without/with ethanol. The relative growth rate was higher

with ethanol than without ethanol when below 25°C in glucose alone, while completely adverse when above 25°C. Similar results were observed in the mixture of glucose-fructose. It indicated that the synergistic effect of temperature with ethanol was enhanced significantly in high temperature (above 25°C).

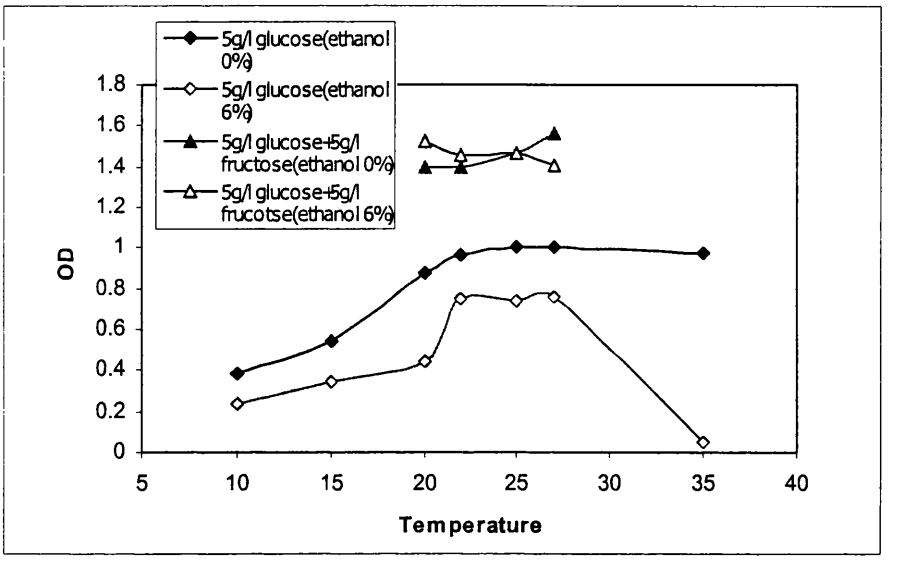


Figure 3-17 (a) Influence of temperature and ethanol on biomass yield with glucose alone (10°C to 35°C) and a mixture of glucose-fructose (20°C to 27°C)

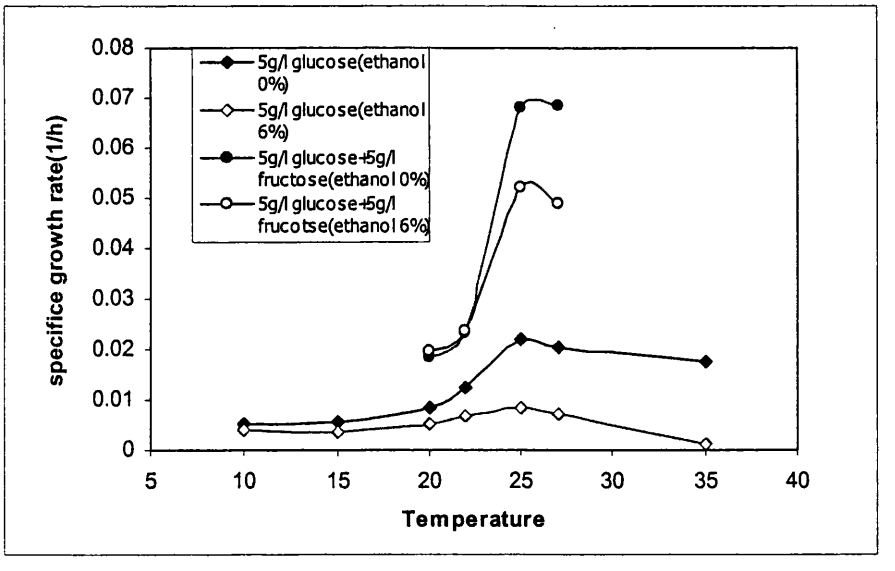


Figure 3-17 (b) Influence of temperature and ethanol on specific growth rate with glucose alone (10°C to 35°C) and a mixture of glucose-fructose (20°C to 27°C)

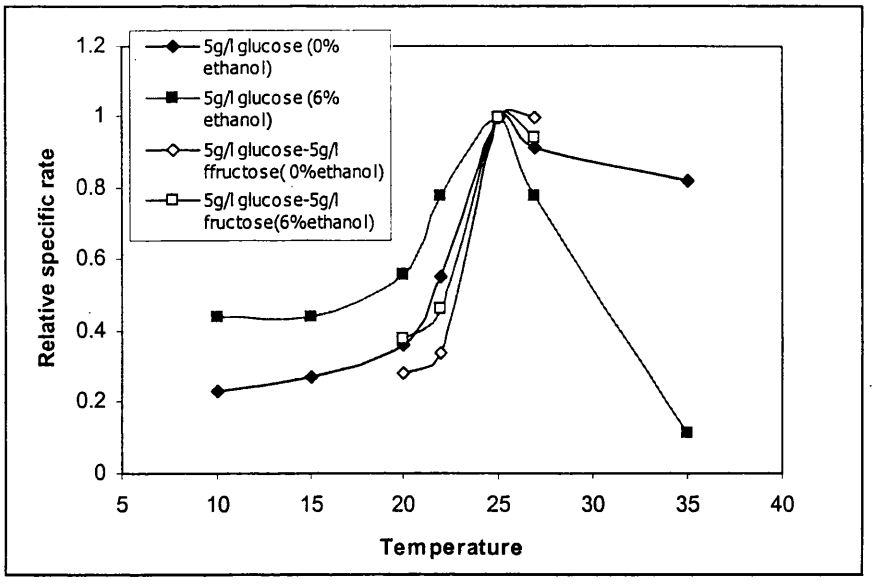


Figure 3-17 (c) Influence of temperature and ethanol on relative growth rate with glucose alone (10°C to 35°C) and a mixture of glucose-fructose (20°C to 27°C)

(Note: Relative specific rate refers to a ratio of $\mu_{(T)}/\mu_{(25)}$, $\mu_{(T)}$ is specific growth rate at T°C , $\mu_{(25)}$ is at 25°C).

3.6.5 Calculation of *Arrhenius* activation energy of growth

Temperature has a significantly kinetic effect on reactions. Variation of the rate constant k with temperature is described by the *Arrhenius equation:*

$$k = Ae^{-E/RT} \qquad (3.1)$$

Where k is the rate constant, A is the *Arrhenius constant* or *frequency factor*, E is the *activation energy* for the reaction, R is the ideal gas constant, and T is absolute temperature. According to the *Arrhenius* equation, as T increases, k increases exponentially. Taking the natural logarithm of both sides of Eq. (3.1)

$$\ln k = \ln A - E/RT \qquad (3.2)$$

Thus, a plot of lnk versus 1/T gives a straight line with slope –E/R. From an *Arrhenius* plot (Appendix G) the activation energy (E) could be determined.

Two phases of activation energy were observed as shown in Appendix G, Linear regression equation (ethanol 0%, v/v):

$\ln\mu_{01} = -11704/T + 35.31$ ($288.2K \leq T < 298.2K$) (3.3)

$\ln\mu_{02} = 1846.5/T - 10.024$ ($298.2K < T \leq 308.2K$) (3.4)

$E_{01} = 11704R = 97.31$ KJ/mol ($288.2K \leq T < 298.2K$)

E_{02} = -1846.5R = -15.35 KJ/mol (298.2K< T ≤ 308.2K)

Where μ_{01} is specific growth rate and E_{01} activation energy without ethanol at 15-25°C; μ_{02} is specific growth rate and E_{02} activation energy without ethanol at 25-35°C.

The cross point of two straight lines is T = 298.9 K = 25.7°C, μ_{max} = 0.021 h^{-1}. This data agreed with the experimental data (0.022h^{-1}, 25°C) in Table 3.22.

It was known from activation energy that specific growth rate increased as temperature increased from 15 to 25°C, but specific growth rate decreased as temperature increased from 25 to 35°C. Furthermore, in the absence of ethanol, the calculated activation energy (97.31KJ/mol) below 25°C was more than 6 times higher than that (15.35KJ/mol) above 25°C, so the influence of temperature on the growth of *O. oeni* was much greater at below 25°C than above 25°C. The result was in agreement with the conclusion made in section 3.6.1.

In the presence of 6% v/v ethanol,

$\ln\mu_{61}$ = -7864.4/T +21.624 (288.2K ≤ T < 298.2K) (3.5)

$\ln\mu_{62}$ = 18085/T –65.298 (298.2K< T ≤ 308.2K) (3.6)

E_{61} = 7864.4R = 65.38 KJ/mol (288.2K ≤ T < 298.2K)

E_{62} = -18085R = -150.36 KJ/mol (298.2K< T ≤ 308.2K)

Where μ_{61} is specific growth rate and E_{61} activation energy with 6% ethanol at 15-25°C; μ_{62} is specific growth rate and E_{62} activation energy with 6% ethanol at 25-35°C.

The cross point of two straight lines is T = 298.5K = 25.3°C, μ_{max} = 0.009 h^{-1}. This data was in good agreement with the experimental data (0.009h^{-1}, 25°C) in Table 3.23.

Similarly, it was known from activation energy that specific growth rate increased as temperature increased from 15 to 25°C, but decreased as temperature increased further from 25 to 35°C. Moreover, in the presence of 6% ethanol, the activation energy (150.35KJ/mol) above 25°C was about 2.5 times higher than that (65.38KJ/mol) below 25°C, so much greater influence was observed with respect to specific growth rate at

above 25°C than below 25°C, and it suggested that a synergistic effect of temperature and ethanol was enhanced above 25°C.

From above results the specific growth rate increased with increase in temperature below 25°C in the absence or presence of 6% ethanol. But the change in specific growth rate was uncoupled with change in temperature (Fig.3-17 b). In order to understand the different effect of temperature on the growth without/with ethanol 6%, equation (3.3) subtracted from equation (3.5) and equation (3.4) subtracted from equation (3.6):

Eq.(3.3) – Eq.(3.5): $\ln(\mu_{01}/\mu_{61}) = -3839.6/T + 13.686$ (288.5-298.5K) (3.7)

Eq.(3.4) – Eq.(3.6): $\ln(\mu_{02}/\mu_{62}) = -16238.5/T + 55.274$ (298.5-303.5K) (3.8)

Formula (3.7) was used to calculate the value of μ_{01}/μ_{61} with the temperature change interval of 2°C. The results are shown Table 3.25. At 15.3°C (288.5K), a ratio of 1.5, μ_{01}/μ_{61} of specific rate without ethanol to with 6% ethanol, increased up to a ratio of 2.3 at 25.3°C (298.5K). It indicated that specific growth rate increased slightly quicker without ethanol than with 6% ethanol although both increased with a rise in temperature. In terms of linear regression, the relationship between μ_{01}/μ_{61} and temperature was as follows:

$\mu_{01}/\mu_{61} = 0.0818t + 0.1857$ (15.3°C – 25.3°C) (3.9)

It was observed that the ratio (μ_{01}/μ_{61}) increased linearly with the increase in temperature below 25.3°C. t is expressed by centigrade degree. The calculated data was in agreement with experimental data (Table 3.25).

Table 3.25 The calculated data for the influence of temperature on ratio of specific growth rate without /with ethanol* (15.3-25.3°C)

Temperature		Calculated data [a]		Experimental data [b]		
(K)	(°C)	$\ln(\mu_{01}/\mu_{61})$	μ_{01}/μ_{61}	μ_{01}	μ_{61}	μ_{01}/μ_{61}
288.5	15.3	0.377	1.5	0.006(15°C)	0.004(15°C)	1.5
290.5	17.3	0.469	1.6			
292.5	19.3	0.559	1.8	0.008(20°C)	0.005(20°C)	1.6
294.5	21.3	0.648	1.9	0.012(22°C)	0.007(22°C)	1.7
296.5	23.3	0.736	2.1			
298.5	25.3	0.823	2.3	0.022(25°C)	0.009(25°C)	2.4

*μ_{01} is specific growth rate in absence of ethanol and μ_{61} is in the presence of 6% ethanol at 15.3-25.3°C, medium containing 5g/l glucose.

a. Calculated on Eq. (3.7); b. see Table 3.22 and Table 3.23.

Similarly, Formula (3.8) was used to calculate the value of μ_{02}/μ_{62} with the temperature change in the interval of 2°C. The result is in Table 3.26. The ratio (μ_{02}/μ_{62}) increased as temperature increased e.g. 2.4 at 25.3°C and 14.0 at 35.3°C, that is, at 25.3°C specific growth rate is 2.4 fold higher without ethanol than with 6% ethanol, while at 35.3°C this is 14.0 fold higher although both specific growth rates were reduced with the further increase of temperature (above 25.3°C). It implied that the inhibitory effect of temperature and ethanol on the growth rate was enhanced by a synergistic interaction of temperature and ethanol above the optimal growth temperature of 25.3°C. In terms of exponential regression the relationship between μ_{02}/μ_{62} and temperature was below:

$\mu_{02}/\mu_{62} = 0.0279e^{0.1763t}$ (t-centigrade degree) (3.10)

This indicated that a ratio (μ_{02}/μ_{62}) rose exponentially with the increase in temperature above 25.3°C. The experimental data offers evidence (Table 3.26).

Table 3.26 The calculated data for the influence of temperature on ratio of specific growth rate without /with ethanol* (25.3-35.3°C)

Temperature		Calculated data [a]		Experimental data [b]		
(K)	(°C)	$\ln(\mu_{02}/\mu_{62})$	μ_{02}/μ_{62}	μ_{02}	μ_{62}	μ_{02}/μ_{62}
298.5	25.3	0.874	2.4	0.022(25°C)	0.009(25°C)	2.4
300.5	27.3	1.236	3.4	0.020(27°C)	0.007(27°C)	2.9
302.5	29.3	1.593	4.9			
304.5	31.3	1.946	7.0			
306.5	33.3	2.294	9.9			
308.5	35.3	2.637	14.0	0.018(35°C)	0.001(35°C)	18.0

*μ_{02} is specific growth rate in absence of ethanol and μ_{62} is in the presence of 6% ethanol at 25.3-35.3°C, medium containing 5g/l glucose.
a. Calculated on Eq. (3.8); b. see Table 3.22 and Table 3.23.

3.7 Influence of organic acids on the growth of *O. oeni*

Organic acids are important in the cider making and wine making process in two main ways:

1. As a carbon and energy source for the LAB;
2. In development of cider and wine flavour (i.e. MLF).

Apple juice is first fermented to dryness by the yeast. The cider as described previously in Chapter 1 contains only residual levels of sugars (approximately 1 or 2mM), which is not enough energy source to support significant growth of the LAB. As such, the sugars can act as a carbon source while LAB metabolise organic acids present in the cider, e.g. malic, citric and pyruvic acid for energy (Radler 1975).

Secondly, the breakdown of organic acids leads to various flavour changes within the cider and are very important as previously described (section 1.4 Chapter 1). Malic acid is of particular importance because it is found in such high concentration in 'green' cider, malic acid is converted to lactic acid by LAB, and so this is called the malo-lactic fermentation (MLF). The influence of organic acids, particularly malic acid, on the LAB needs to be studied further to characterize their effect on the LAB under investigation.

Investigations on how organic acids, e.g. malic acid, citric acid and pyruvic acid, make a contribution to the growth of *O. oeni* have been carried out by many authors (Cogan *et al.* 1981; Salou *et al.* 1991, 1994; Firme *et al.* 1994; Saguir *et al.* 1996). As mentioned above, residual sugars were at low concentrations after the primary alcoholic fermentation of sugars. The main objectives of the work here were to investigate the effect of organic acids on the growth of *O. oeni* at low concentration of sugars, e.g. glucose, glucose-fructose and glucose-sucrose, at a typical concentration found in cider or dry wine.

Malic acid, citric acid and pyruvic acid were respectively added to Z broth, a total volume of 10ml in pressure tubes. Malic acid, citric acid and pyruvic acid were 5g/l respectively and 0.2g/l, 0.5g/l, 1.0g/l glucose, 0.2g/l, 0.5g/l, 1.0g/l mixtures (1:1, w/w) of glucose-fructose and 0.2g/l, 0.5g/l, 1.0g/l mixtures (1:1, w/w) of glucose-sucrose were added. The pH was then adjusted to 5.0 before autoclave sterilization.

3.7.1 Influence of organic acids in media with low sugar concentrations

Figure 3-18 shows the influence of organic acids on the growth of *O. oeni* by small additions of other supplementary sugars. It was observed that the growth was enhanced by a small addition of 0.2g/l sugar. It could be also observed that the different amounts of growth were obtained with the different sugars. For example, Figure 3-18 (a) shows that in

the presence of malic acid the maximum biomass was obtained by the addition of glucose compared to the addition of glucose-fructose or glucose-sucrose. Similar results are shown in the presence of citric acid in Figure 3-18 (b). However, in the presence of pyruvate the similar amounts of growth were achieved (Figure 3-18 (c)) in all cases. It indicated that the presence of malic acid or citric acid inhibited to some extent the growth of *O. oeni* in a mixture of glucose-fructose. Previously, this was proved to be the most favourable sugar for the growth in the absence of malate and citrate (section 3.4, 3.5 and 3.6).

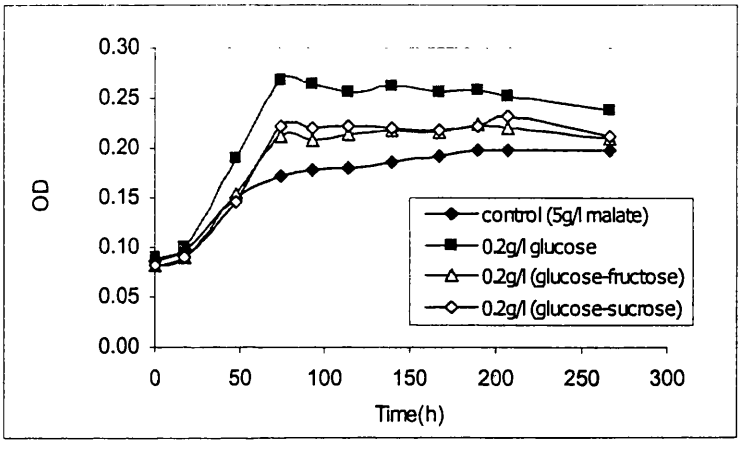


Figure 3-18 (a) Influence of malic acid and small addition of 0.2g/l sugar on the growth of *O. oeni*

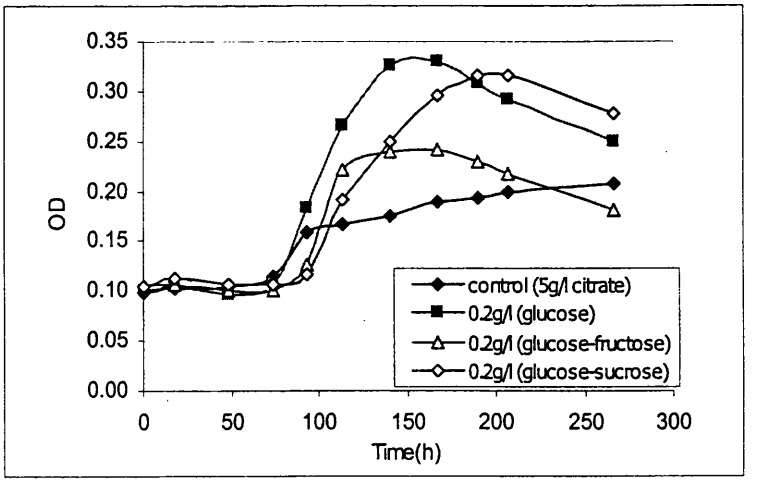


Figure 3-18 (b) Influence of citric acid and small addition of 0.2g/l sugar on the growth of *O. oeni*

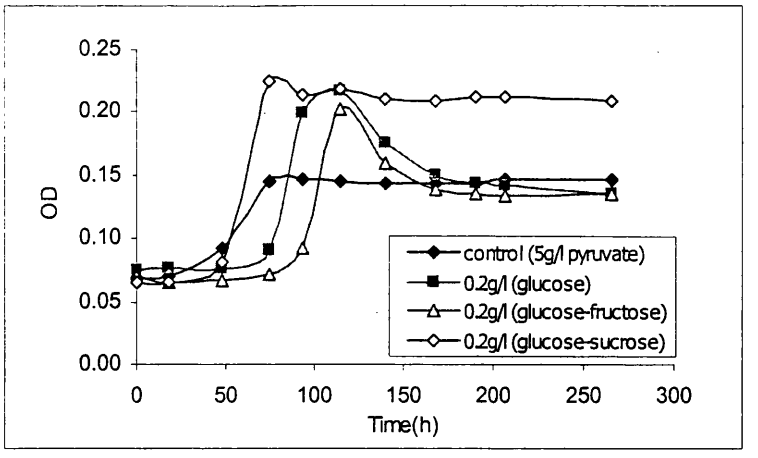


Figure 3-18 (c) Influence of pyruvlic acid and small addition of 0.2g/l sugar on the growth of *O. oeni*

This result was very interesting and worthy of further consideration. From our previous experimental results, it was known the mixture of glucose-fructose was the best carbon source for the growth of *O. oeni* under conditions tested, such as temperature and ethanol (section 3.5 & 3.6 in this Chapter). However, this result was opposite in the presence of organic acids. The growth was poorer in glucose-fructose than that in glucose in the presence of malate. Other workers have not investigated or found this behaviour. This result could have implications as to the timing of MLF. The timing of the MLF obviously depended upon the composition of the juice. The present results indicated that *O. oeni* could not grow well or MLF could not be performed efficiently until the glucose-fructose mixture concentration is low. Post yeast fermentation inoculation would be the most probably best option.

The results revealed that organic acids increased the growth of *O. oeni*, but neither L-malic nor citric acid alone supported the growth in the absence of sugar, confirming that under the experimental conditions, glucose was the main or sole carbon source (Saguir *et al.*1996). Enhanced growth rates were obtained for other *O. oeni* strains when L-malic or citric acids were used in addition to glucose (Schmitt & Divies 1990; Loubiere *et al.*1992; Salou *et al.* 1994). In the presence of glucose, L-malic or citric acids were simultaneously utilized and the L-malic acid was metabolized at a higher rate than glucose and citric acid for the growth of *O. oeni* (Saguir *et al.* 1996; Tracey & Van Roogen 1988). The different results that L-malic acid was utilized before sugar were also found (Pilone & Kunkee 1972; Radler 1975). Under unfavourable medium conditions (pH 3.5 and poor nutrient

levels), malic acid was quickly used by *O. oeni* with very slight sugar degradation and no noticeable cell growth. It seemed that at low pH values the sugars were not metabolized and the catabolism of malate is enhanced in order to change the pH to a level more favourable to the cell (i.e. more alkaline). Cell growth is not necessary to achieve malic acid degradation (Firme *et al.* 1994). These conditions showed the degradation of malic acid can take place with a small addition of glucose (1mM) without cell growth of *O. oeni.*

Table 3-27 clearly shows the effects of organic acids on the growth of *O.oeni* in the presence of low glucose concentration. The specific growth rates were dependent on the concentrations of glucose and the types of organic acids. The specific growth rate and the OD increased as glucose increased in a glucose range of 0.2 to 1.0 g/l. The highest specific growth rate was obtained with pyruvate and the lowest with malate at the same glucose concentration. Moreover, it was observed the highest OD occurred with glucose compared with the same concentrations of other sugars (glucose-fructose mixtures, glucose-sucrose mixtures), as presented in Appendix H, which may imply that the presence of glucose is an energetically more favourable environment for the metabolism of organic acids. The OD increased with the increase in the additive amounts of sugars.

Table 3.27 The influence of low glucose concentration on the growth of *O. oeni*

	OD_{max}*	μ_{max} (h^{-1})	Relative value $\mu_{glucose+malate}/\mu_{glucose}$
5g/l malate alone	0.11	0.016	-
0.2g/l glucose **	0.10	0.012	1.00
0.2g/l glucose+5g/l malate	0.26	0.021	1.75
0.5g/l glucose+5g/l malate	0.27	0.031	2.58
1.0g/l glucose+5g/l malate	0.39	0.034	2.83
5g/l citrate alone	0.13	0.015	-
0.2g/l glucose	0.10	0.012	1.00
0.2g/l glucose+5g/l citrate	0.23	0.028	2.33
0.5g/l glucose+5g/l citrate	0.29	0.038	3.17
1.0g/l glucose+5g/l citrate	0.37	0.042	3.50
5g/l pyruvate alone	0.11	0.020	-
0.2g/l glucose	0.10	0.012	1.00
0.2g/l glucose+5g/l pyruvate	0.14	0.042	3.50
0.5g/l glucose+5g/l pyruvate	0.34	0.055	4.58
1.0g/l glucose+5g/l pyruvate	0.48	0.065	5.42

* OD_{max} = maximum OD – starting OD

** Glucose concentration: 0.2 g/l (1.1mM), 0.5 g/l (2.7mM) and 1.0 g/l (5.5mM)

3.7.2 Influence of malate in media with low sugar concentrations

Figures 3-19 a, b & c present the results for the growth of *O. oeni* in media containing malic acid in the presence of different sugars. They showed that malic acid alone hardly supported any growth, but enhanced growth was obtained in the presence of 0.2g/l sugar/sugar mixture only which was not enough for the growth. Also, the amounts of growth (OD_{max}) increased as the sugar/sugar mixtures concentration increased.

Figure 3-19 (a) indicates that glucose stimulated the growth, e.g. 0.2g/l glucose doubled the growth on malic acid. Similar results were observed in the presence of 0.2g/l glucose-fructose or 0.2g/l glucose-sucrose (Figure 3-19 b & c). Also, the highest OD_{max} was achieved with malate-glucose medium and it suggested that the malic acid may degrade more completely by the addition of glucose than other sugars. Conditions for the degradation of malic acid in the presence of glucose-fructose or glucose-sucrose were not optimal. The experimental observations clearly showed that by small additions of glucose to the medium malolactic fermentation may be enhanced considerably over malolactic fermentation without additions.

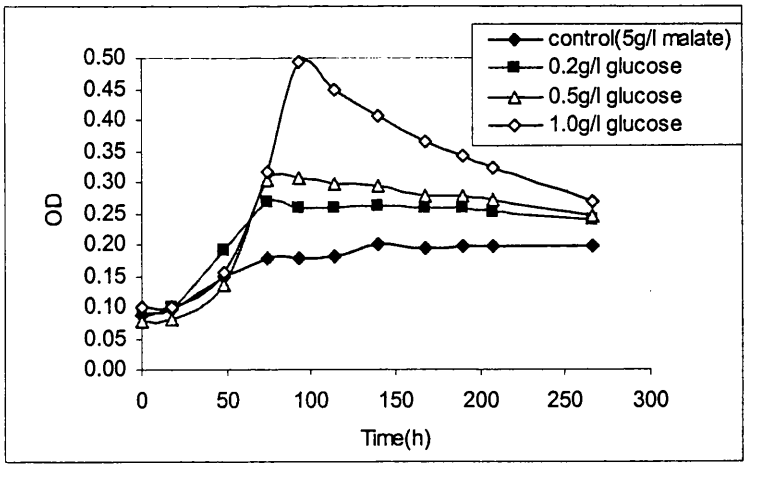


Figure 3-19 (a) malic acid with various low concentrations of glucose (0 to 1.0 g/l)

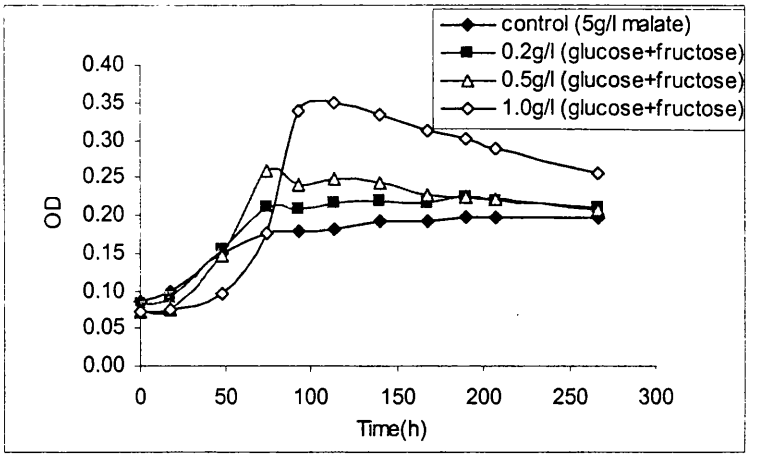


Figure 3-19 (b) malic acid with various low concentrations of glucose-fructose (0 to1.0 g/l, 1:1 w/w)

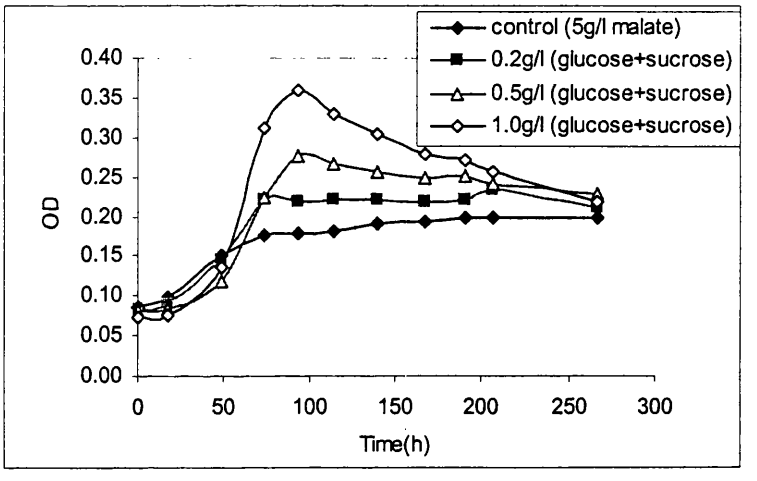


Figure 3-19 (c) malic acid with various low concentrations of glucose-sucrose (0 to 1.0 g/l, 1:1 w/w)

Figure 3-19 Influence of malic acid on the growth of *O. onei* at low concentrations of sugars

3.8 Influence of apple phenolics on the growth of *O. oeni*

The presence of phenolic compounds other than organic acids described above in section 3.7 can influence the growth of LAB as well as the malolactic fermentation. The phenolic compounds can inhibit or stimulate the growth of LAB. The stimulation by anthocyanins and gallic acid has been reported (Vivas *et al.* 1997). It is important to understand the influence of these complex compounds on the biochemistry of cider, not only in flavour development but also their effects on the growth of LAB present during maturation of the alcoholic beverage.

The metabolism of phenolic acids was therefore investigated in pressure tube cultures. 10ml Z broth was used in the presence of 5g/l glucose, 5g/l glucose-5g/l fructose and 5g/l glucose-5g/l sucrose, respectively. Phenolic acids used: 0.2g/l chlorogenic acid; 0.1g/l quinic acid, shikimic acid, coumaric acid, caffeic acid and gallic acid. pH was adjusted to 5.0 before autoclave sterilization.

The results showed that with three types of sugar/sugar mixtures coumaric and caffeic acid can inhibit the growth of *O. oeni* (Appendix I) while additions of other compounds had only small effects.

Table 3.28 shows how phenolic acids affected the growth of *O. oeni*. The relative OD value (%) was expressed as the ratio of biomass with sugar-phenolics to biomass with sugar alone. From Table 3.28, it could be observed that with the addition of chlorogenic acid, the ratio of OD is about 92% with these sugars used as well as the ratio of specific growth rate was 100% or a little more. It indicated that chlorogenic acid slightly reduced biomass, independent of the types of sugars and slightly increased or did not affect the specific growth rate. Similar results were obtained with quinic acid or shikimic acid or gallic acid. However, coumaric acid and caffeic acid had a markedly inhibitory influence on the specific growth rate and inhibition of the yield of the culture (OD). For example, in the presence of 0.1g/l coumaric acid, the ratio of OD_{G+F+P}/OD_{G+F} was only 78% in glucose-fructose and the ratio of μ_{G+P}/μ_G was only about 62% in glucose after 400 h. Both coumaric acid and caffeic acid depicted the same effect on the growth of *O. oeni*.

Table 3.28 The influence of phenolic acids on the growth of *O. oeni*

Relative value (%)*	sugar alone	Chlorogenic acid	Quinic acid	Shikimic acid	Coumaric acid	Caffeic acid	Gallic acid
OD_{G+P}/OD_G	100	92	92	94	92	90	95
OD_{G+F+P}/OD_{G+F}	100	93	93	96	78	80	95
OD_{G+S+P}/OD_{G+S}	100	93	92	95	86	85	90
μ_{G+P}/μ_G	100	100	101	103	62	64	101
μ_{G+F+P}/μ_{G+F}	100	103	106	113	86	89	105
μ_{G+S+P}/μ_{G+S}	100	107	104	109	70	70	101

*OD_{G+P}/OD_G – the ratio of max OD with glucose-phenolic acid to max OD with glucose alone after 400 h.
μ_{G+P}/μ_G – the ratio of specific growth rate with glucose-phenolic acid to specific growth rate with glucose alone.
G+F – Glucose- Fructose; G+F+P – Glucose-Fructose-Phenolic acid;
G+S – Gructose- Sucrose; G+S+P – Gructose-Sucrose-Phenolic acid.

From Donovan's work (Donovan 2000) it was known that on glucose the growth rate of *L. brevis* X_2 was stimulated up to 20-200% by the addition of shikimic, chlorogenic, quinic and gallic acids, while the amount of growth obtained was stimulated by the additions of chlorogenic, shikimic acids (35-55%), and to a lesser extent by quinic and caffeic acids (10%). Ferulic and caffeic acids had no observed stimulatory effects on the growth of *L. brevis* X_2 and inhibited the growth rate of *L. brevis* X_2. Stimulation of the growth rate and biomass yield of LAB by quinic acid may be related to their ability to reduce quinic acid to dihydroshikimic acid. The chlorogenic acid also stimulated the growth of *L. brevis* X_2, possibly because LAB can hydrolyse this ester to caffeic and quinic acids and subsequently metabolise the products (Stead 1994). This stimulation was greater in the presence of glucose compared with fructose (Whiting & Coggins 1971).

Similar work by Whiting & Carr (1957) has showed that chlorogenic acid disappeared during fermentation, and caffeic acid also disappeared if it was present. It indicated that the first stage in the metabolism of chlorogenic acid was hydrolysis to caffeic and quinic acids, both products were further metabolized. Quinate was metabolized via shikimate to dihydroshimimate, changes brought about by the induced enzymes quinate dehydrogenase and shikimate reductase and the enzymes of the aromatic pathway dehydroquinase and dehydroshikimate reductase (Carr & Whiting 1971).

3.9 Conclusion

This chapter has presented data from the pressure tube growth experiments. These have produced a great deal of information about how *O. oeni* grew and the influence of medium components and physicochemical factors on the growth. A number of important conclusions could be drawn here. Meanwhile, the characteristics of two LAB, *O. oeni* and *L. brevis* (Donovan 2000) can be also compared.

Based on the MRS and Donovan's broth (Table 2.2) investigations into the influence of nitrogen sources, metal elements and KH_2PO_4 on the growth of *O. oeni* have been carried out. Yeast extract was the most important nutrient nitrogen sources; Lab-Lemco was removed from Donovan's broth from the safely point of view (animal organ) and had little effect on the growth. 2g/l NH_4Cl, 0.1g/l Mg^{2+} and 0.01g/l Mn^{2+} were used in culture media. 5g/l KH_2PO_4 was used as buffer for the decrease in inhibition of end-products at low pH. Composition of an optimised broth (Z broth) for the growth of *O. oeni* has been formulated. For detailed information see Table 2.3 and Table 2.4.

The specific growth rate of *O. oeni* on glucose as a sole carbon source was poor and similar results were obtained on fructose as a sole carbon source. However, a mixture of sugars (glucose-fructose, glucose-sucrose and fructose-sucrose) improved the specific growth rate, but increased in biomass yield to a limited extent. The mixture of glucose-fructose as carbon sources remarkably enhanced the growth rate, the highest μ_{max} occurred as the ratio of glucose to fructose was at 3g/l glucose-7g/l fructose and the highest OD_{max} was obtained on a 8:2 ratio of glucose and fructose. The 1:1 ratio of the mixture gave a reasonable growth rate and OD_{max}, for example 5g/l glucose-5g/l fructose. The composition of medium, Z broth with 5g/l glucose-5g/l fructose could be used in the 2 l batch cultures and continuous cultures in Chapter 4. *L. brevis* grew well in Donovan's broth with glucose or fructose, but grew better in the presence of fructose than glucose as reported in Chapter 4 (Donovan 2000).

It was also shown that sugars have considerable influence on the ethanol tolerance of *O. oeni*. A concentration up to 4% (v/v) ethanol did not apparently decrease the growth rate

and growth yield while 10% ethanol seriously inhibited the growth. The ethanol tolerance was improved when a mixture of sugars (glucose/fructose, glucose/fructose and fructose/sucrose) was used as compared with glucose or fructose or sucrose alone (Fig.3-7 to 3-12), as measured by the change in relative growth rate or growth yield (Table 3.16 to 3.21). Similarly, a mixture of glucose-fructose of 5g/l-5g/l gave the best tolerance; growth was possible even to 12% (v/v) ethanol in these conditions (Fig.3-12 and Table 3.29 below).

The ethanol tolerance of two LAB was compared in Table 3.29 below. *L. brevis* showed higher specific growth rate than *O. oeni*, but the ratio of specific growth rate is reversed at up to 10-12% ethanol. It showed greater ethanol tolerance of *O. oeni* in high alcoholic contents.

Table 3.29 Comparison of tolerance to ethanol of two LAB in varied sugars

Ethanol (%, v/v)	Specific growth rate (μ_{max}) on different sugars				
	Glucose		Fructose		Glucose-fructose
	O. oeni	*L. brevis**	*O. oeni*	*L. brevis**	*O. oeni*
0	0.022	0.070	0.020	0.132	0.063
5	-	0.046	-	0.086	-
6	0.009	-	0.017	-	0.052
10	0.006	0.018	0.007	0.045	0.024
12	0.004	0.003	0.005	0.022	0.015
	Ratio of $\mu_{(i)}/\mu_{(0\%)}$ (i = 10, 12% v/v, ethanol)				
$\mu_{(10\%)}/\mu_{(0\%)}$	0.27	0.26	0.35	0.34	0.38
$\mu_{(12\%)}/\mu_{(0\%)}$	0.18	0.04	0.25	0.17	0.24

* the specific growth rate (μ_{max}) of *L. brevis* derived from Chapter 7 in Donovan (2000).

The optimum growth temperature for *O. oeni* was about 25°C which was independent of the presence of ethanol. Growth with glucose was very slow at 15°C. The effect of temperature on the growth was greater under below 25°C than over 25°C in the absence of ethanol; it implied that *O. oeni* was more sensitive to low temperature. However, by adding 6% (v/v) ethanol, this trend was completely reversed. The inhibitory effect of temperature on the growth rate was markedly promoted at high temperatures of more than

25°C. It showed that the temperature dependence of *O. oeni* was reduced by the addition of ethanol below 25°C, while it was increased significantly above 25°C. The calculated activation energy supported this view (Table 3.30). In addition, the experimental results also showed that the growth of *O. oeni* was related to sugars present in the medium. For example, in the presence of glucose-fructose, OD_{max} obtained in the presence or absence of 6% ethanol was nearly identical in the range of 20-27°C, while the OD_{max} with 6% ethanol was smaller than OD_{max} without ethanol in the presence of glucose alone (Fig.3-17 a).

The optimum temperature for the maximum growth rate of *L. brevis* in the absence of ethanol was 36°C and was reduced to 28°C in the presence of 5% (v/v) ethanol. Similarly, the temperature dependence of *L. brevis* was reduced by the addition of ethanol below optimal temperature of 28°C.

Table 3.30 The calculated activation energy of two strains

	O. oeni (KJ/mol)		*L. brevis* * (KJ/mol)
	15-25°C	25-35°C	10-25°C
0% ethanol	97.3	-15.4	127.4
5% (v/v) ethanol	-	-	118.5
6% (v/v) ethanol	65.4	-150.4	-

* The data derived from Chapter 7 (Donovan 2000), the activation energy above 25°C was not reported.

The organic acids, such as malic acid, citric aicd and pyruvic acid, as a sole carbon source did not simulate the growth of *O. oeni*, but with small additions of sugar, the growth was considerably enhanced. It was thought that small supplemental sugar was very useful for the degradation of organic acids and helped to shorten the period of malolactic fermentation. This was most probably due to the use of the sugars as a carbon source for synthesis of structure rather than energy generation. The specific growth rates of *O. oeni* were different in the presence of different organic acids (Table 3.27). For example, μ_{max} (0.042 h^{-1}) with pyruvate was the largest, μ_{max} (0.028 h^{-1}) with citric acid was second, and μ_{max} (0.021 h^{-1}) was the smallest in the presence of 0.2 g/l glucose (1.1mM). As for the types of sugars, glucose alone was the favourable for the growth of *O. oeni* (Appendix H) and the degradation of organic acids as well.

The biotransformation ability of *L. brevis* to metabolize organic acids was also improved by small additions of glucose. The OD_{max} increased from 0.20 to 0.46 (by up to 230%) in media containing 1mM glucose alone and 0.2g/l glucose-5g/l malate mixture (Donovan 2000). Growth curves showed that the growth rate was increased by an addition of 1mM glucose in the presence of malate. Similar results were observed by additions of other organic acids such as citrate and pyruvate.

The phenolics had only a small effect on the growth of *O. oeni*. The experimental results showed that phenolics increased slightly the growth rate, while coumaric acid and caffeic acid reduced the growth rate. They can also influence the biomass yield. These results implied that *O. oeni* is not involved in the metabolism of tannins and the product formation of very potent flavour components of mature cider. In comparison, quinic and cinnamic acid derivatives are metabolized by *L. brevis* X_2 in glucose and fructose medium. Chlorogenic, shikimic and quinic acids stimulate the growth of *L. brevis* X_2. Ferulic, caffeic and gallic acids although do not stimulate the growth of *L. brevis* X_2 were still metabolised by *L. brevis* X_2 in the presence of glucose and fructose.

The transformation of tannins by *O. oeni* needs to be confirmed by further experiment and analysis. Moreover, the analytical method of both tannin-derived substances and volatile phenols formed need to develop in order to monitor the biotransformation of tannin-derived substances during the maturation process in cider and to control the production of good quality cider.

Chapter 4 Batch and Continuous Culture Studies

4.1 Introduction

In the winemaking and cidermaking process, malolactic fermentation (MLF) is generally considered to be a desirable reaction. In recent years the wine and cider manufacturing industry has moved towards using pure starter cultures of selected lactic acid bacteria including *O. oeni* with a view to promoting a reliable and rapid malic acid bioconversion so ensuring better control of the reaction and enhancing quality and reliability of product. MLF in wine by direct inoculation with freeze-dried *O. oeni* cultures has been developed (Nielsen *et al.* 1996). Although a number of studies have examined the effect of the main factors affecting the MLF performance of various strains (Davis *et al.* 1988; Wibowo *et al.* 1988; Champagne *et al.* 1989; Hayman & Monk 1982), these studies were mostly performed in wine or grape juice, an unfavourable environment for the growth of LAB. Few studies have focused on the efficient growth of *O. oeni* starter cultures with reference to optimizing growth rates and biomass yields of cell production (Salou *et al.* 1991).

The influence of various main growth factors, e.g. temperature, ethanol concentration and a wide range of sugars, on the growth of *O. oeni* has been carried out in Chapter 3. In this Chapter, the influence of pH on the growth of *O. oeni* and the growth kinetics of *O. oeni* under pH control at a variety of values was investigated in detail. Moreover, the influence of glucose-fructose concentration on biomass yields under pH control was also examined. The growth parameters, product yields and energy yields were determined at various pH values and at different glucose-fructose concentrations in batch cultures.

The principal characteristics of batch culture are continuous changes in the culture environment i.e. cells, substrate and end-product concentrations are changing with respect to time. It is virtually impossible to study the physiological effects of nutrient limitation in batch culture (Stanbury *et al.* 1995). In contrast, the major feature of continuous culture is 'the steady state' i.e. the biomass, substrate and product concentrations should remain constant over very long periods of time. The specific growth rate is controlled by dilution rate and the growth is typically nutrient limited. Once a steady state is reached neither the

properties of the culture, not those of the environment undergo further change. The culture then has become time-independent, and conditions are under full control. Under these conditions the physiological state of the cells should also be constant and directly attributed to the environmental conditions.

The continuous cultures of *O. oeni* and *L. brevis* have not been studied in literature. The continuous cultures were also carried out in this chapter so as to obtain the precise kinetic properties of *O. oeni* and *L. brevis* respectively, so producing a detail comparison of the kinetics and physiology of growth.

4.2 Parameters of growth and analysis of growth data

The quantitative observation of whether or not growth occurs in a culture is useful for many purposes. There are various growth parameters that are used in literature and these parameters cited in this section mainly derived from Pirt[1] (1975) and/or Doran (1995) who summarized the use of Monod growth equations.

4.2.1 Growth rate and doubling time

Specific growth rate The following equation can be used to describe the growth of culture:

$$ln\, x = \mu t + ln\, x_0 \qquad (4.1)$$

where μ is termed the specific growth rate which represents the rate of growth per unit amount of biomass $(1/x)(dx/dt)$ and has the dimension of reciprocal time $(1/t)$, e.g. h^{-1}; x_0 is the biomass when $t = 0$; x is the biomass at time t. According to equation (4.1), a plot of lnx versus time gives a straight line with slope μ.

Biomass doubling time (t_d)

Cell growth rate is often expressed in terms of the doubling time t_d. An expression for doubling time can be derived from equation (4.1), the concentration $x = 2x_0$ at $t = t_d$, substituting these values into equation (4.1) and cancelling x_0 gives:

$$t_d = ln2/\mu \qquad (4.2)$$

4.2.2 Yield coefficient

Biomass yield $Y_{x/s}$ Mass or moles of biomass produced per unit mass or mole of substrate consumed can be described by the following equation:

$$Y_{x/s} = -\Delta X/\Delta S \text{ (g cell / g substrate)} \qquad (4.3)$$

where $Y_{x/s}$ is biomass yield from substrate;

ΔX is the amount of biomass produced, g/l;

ΔS is the amount of substrate consumed, g/l.

The negative sign is required because ΔS for a consumed substrate is negative in value, yield is calculated as a positive quantity.

Product yield $Y_{p/s}$ Mass or moles of product formed per unit mass or mole of substrate consumed can be described by the following equation.

$$Y_{p/s} = -\Delta P/\Delta S \text{ (g product /g substrate)} \qquad (4.4)$$

Product yield $Y_{p/x}$ Mass or moles of product formed per unit mass or mole of biomass formed can be described by the following equation.

$$Y_{p/x} = \Delta P/\Delta X \text{ (g product /g biomass)} \qquad (4.5)$$

Theoretical (maximum) and observed yield

Distinguishing between theoretical and observed yield is particularly important for cell metabolism because there are always many reactions occurring at the same time; theoretical and observed yield are therefore very likely to differ. Consider the example of biomass yield from substrate, $Y_{x/s}$. If the total mass of substrate consumed is ΔS, some proportion of ΔS equal to ΔS_G will be used for growth while the remainder, ΔS_R, is channelled into other products and metabolic activities not related to growth. Therefore, the observed biomass yield based on total substrate consumption is:

$$Y_{x/s} = -\Delta X/\Delta S = -\Delta X/ (\Delta S_G + \Delta S_R) \qquad (4.6)$$

Where ΔX is the amount of biomass produced and $Y_{x/s}$ is the observed biomass yield from substrate.

In comparison, the true or theoretical biomass yield from substrate is:

$$Y_{x/s}^{max} = -\Delta X/\Delta S_G \qquad (4.7)$$

where ΔS_G is the mass of substrate actually directed into biomass production. Theoretical yield is sometimes referred to as maximum possible yield because it represents the yield in the absence of competing reactions. $Y_{x/s}^{max}$ is theoretical or true biomass yield, also called maximum biomass yield.

4.2.3 Specific metabolic rate and cell productivity

Specific rate of substrate uptake, Q_s, can be described by the following equation:

$$Q_s = -\Delta S/\Delta X/\Delta t \quad \text{(g substrate/g cell/h)} \qquad (4.8)$$

ΔS and ΔX are the amount of substrate uptake and amount of biomass formation (dry weight) at the duration of fermentation (Δt).

Specific rate of product formation, Q_p, can be described by the following equation:

$$Q_p = \Delta P/\Delta X/\Delta t \quad \text{(g product/g cell/h)} \qquad (4.9)$$

ΔP and ΔX are the amount of product formation and amount of biomass formation (dry weight) at the duration of fermentation (Δt).

Cell productivity, *CP*, can be described by the following equation:

$$CP = \Delta X/\Delta t \quad \text{(g/l/h)} \qquad (4.10)$$

ΔX is the amount of growth formation (dry weight) at time interval (Δt).

4.2.4 Monod equation

Monod in 1942 first showed empirically the relationship between specific growth rate of cell and substrate concentration.

$$\mu = \mu_{max} S/(S + K_s) \qquad (4.11)$$

where μ is specific growth rate, μ_{max} is maximum specific growth rate, S is substrate concentration, K_s is called saturation constant, $K_s = S$ at $\mu = 0.5\mu_{max}$.

4.3 2 l batch culture of *O. oeni* with pH control

4.3.1 Cell culture of *O. oeni* at various pH values in 2 l batch culture

4.3.1.1 The fermentation of glucose-fructose mixture

The fermentation of *O. oeni* was first investigated in 2 l (litre) batch culture. The system was prepared according to that described in materials and methods (section 2.11.2 Chapter 2). After autoclaving, the system was inoculated with cells. As the culture grew, samples were taken for further analysis. The results of these studies are summarized as follows.

Figure 4-1 (a) – (e) show the time-courses of biomass formation and glucose-fructose uptake when the mixture of glucose and fructose was used as carbon sources at different pH values. The residual glucose and residual fructose were detected after 100 h at pH 3.5 and pH 6.0 (Figure 4-1 a & e), whilst at pH 4.0, 4.5 and 5.0 the glucose was completely consumed in 50 h (b, c & d). These results indicated that the rates of glucose and fructose uptake were inhibited at low pH or high pH. Moreover, the simultaneous uptake of glucose and fructose was observed in the range pH 3.5-6.0, but the rates of glucose uptake and of fructose uptake were different. The specific rates of fructose uptake were higher than specific rates of glucose uptake in the range pH from 3.5 to 6.0 (Table 4.1), while a ratio of specific rate of glucose to fructose uptake remained 0.88 ± 0.02 with the exception of pH 3.5. In addition, different amounts of growth and the growth rates were observed in the range 3.5-6.0 (Table 4.2), the highest growth rate of 0.066 h^{-1} was obtained at pH 4.5 (section 4.3.1.2).

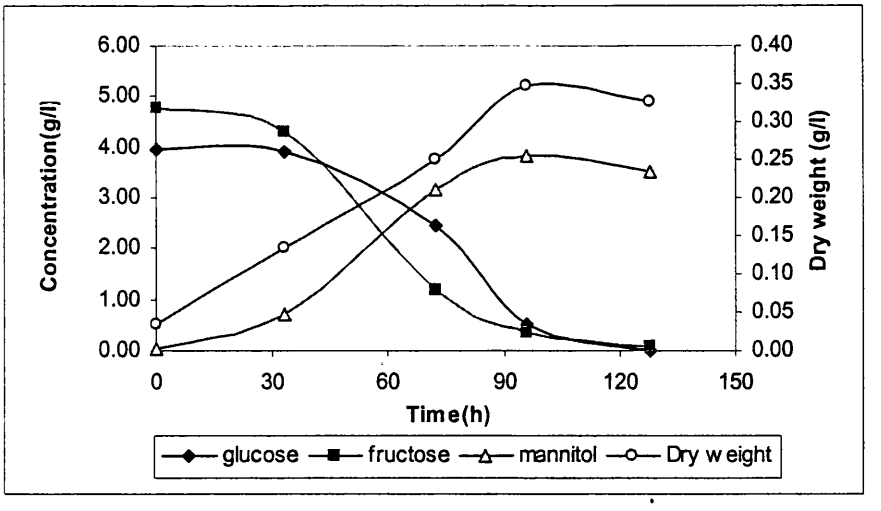


Figure 4-1 (a) The growth of *O. oeni* and consumption of glucose-fructose at pH=3.5 in 2 l batch culture

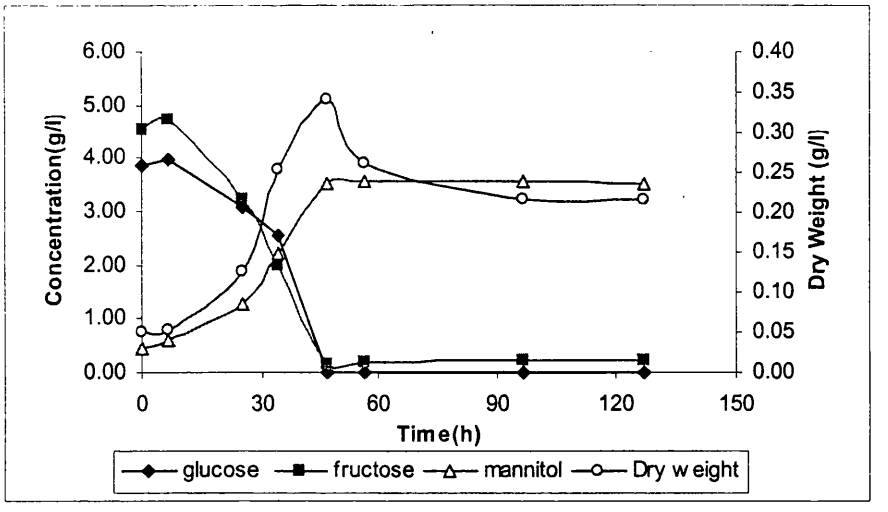


Figure 4-1 (b) The growth of *O. oeni* and consumption of glucose-fructose at pH=4.0 in 2 l batch culture

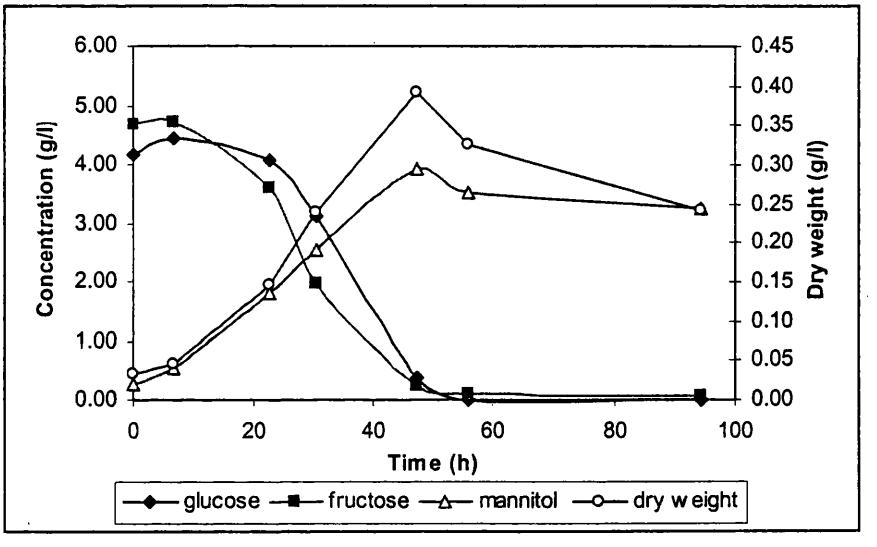


Figure 4-1 (c) The growth of *O. oeni* and consumption of glucose-fructose at pH=4.5 in 2 l batch culture

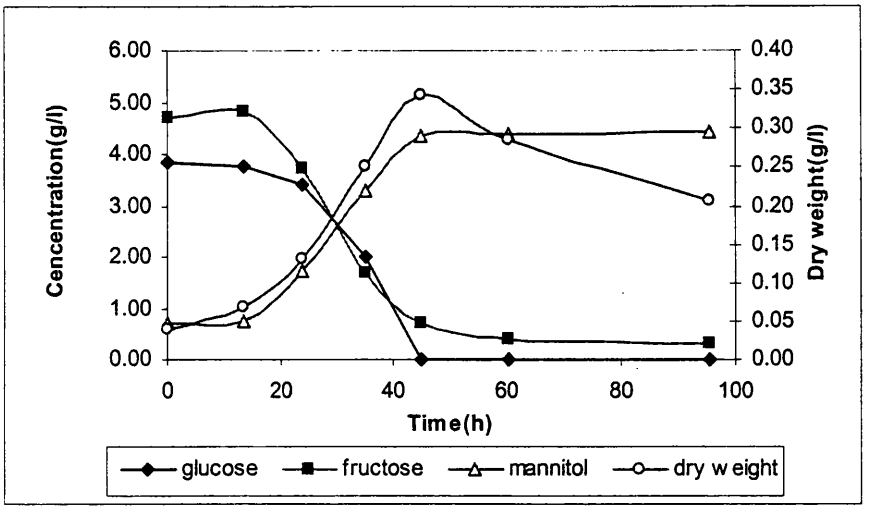


Figure 4-1 (d) The growth of *O. oeni* and consumption of glucose-fructose at pH=5.0 in 2 l batch culture

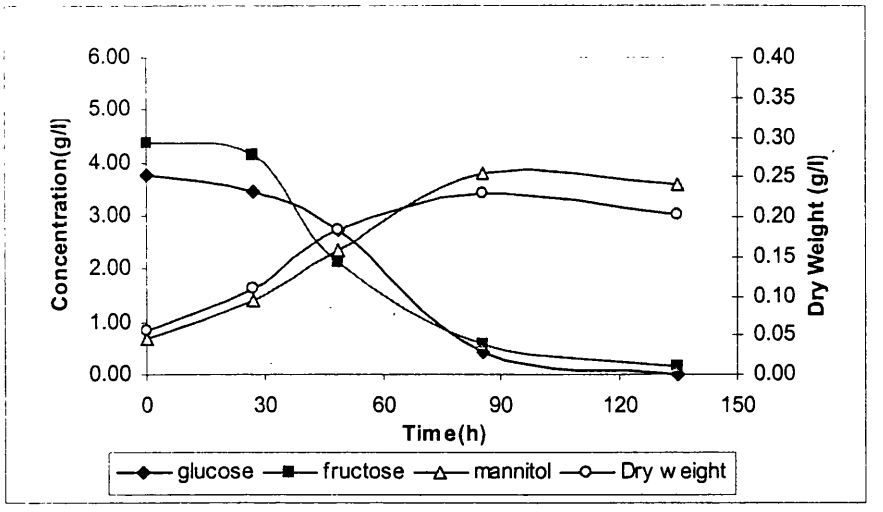


Figure 4-1 (e) The growth of *O. oeni* and consumption of glucose-fructose at pH=6 in 2 l batch culture

Table 4.1 Specific rate of glucose and fructose uptake during the culture of *O. oeni*

pH	$Q_{s\ (total\ sugar)}$ (g sugar/g cell/h)	$Q_{s\ (glucose)}$ (g glucose/g cell/h)	$Q_{s\ (fructose)}$ (g fructose/g cell/h)	ratio of $Q_{glucose}$ to $Q_{fructose}$
3.5	0.26	0.11	0.15	0.73
4.0	0.51	0.24	0.27	0.89
4.5	0.52	0.24	0.28	0.86
5.0	0.53	0.25	0.28	0.89
6.0	0.48	0.23	0.26	0.88

4.3.1.2 The effect of pH on the growth parameters of *O. oeni*

The specific growth rate and biomass formation at various pH values are shown in Figure 4-2 and Table 4.2. The specific growth rate was related to the pH in batch culture. The maximum specific growth rate, 0.066 h^{-1} (10.5 h), was obtained at pH 4.5 (Table 4.2), and low pH showed more inhibitory effect on the growth of *O. oeni* than high pH, e.g. relative growth rate 0.36 at pH 3.5 and 0.48 at pH 6.0. The amount of growth had little variation in the range pH 3.5-5.0, while only half the amount of growth at pH 6.0 was observed as compared to pH 4.5 (Fig.4-2), and the delay of the growth of *O. oeni* was also observed.

The cell productivity and observed biomass yield are also shown in Table 4.2 (the calculation shown in Appendix J). The cell volumetric productivity observed was significantly different. The minimum 2.0 mg/l/h obtained at pH 6.0 is only around ¼ of the maximum 7.9 mg/l/h obtained at pH 4.5. No significant variation in biomass yield was observed in the range pH 3.5 – 5.0, but at pH 6.0 the biomass yield is 0.024 g cell/g sugar,

about 60% of 0.041 g cell/g sugar of the biomass yield at pH 4.5. Hence, the optimal growth of *O. oeni* was achieved at pH 4.5, with the maximum cell volumetric productivity, biomass yield and maximum specific growth rate (0.066 h^{-1}).

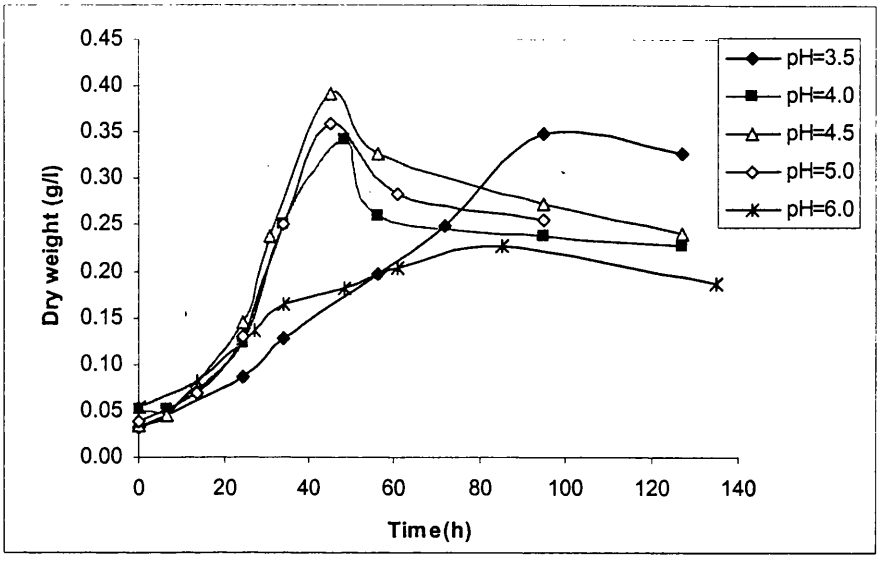


Figure 4-2 The growth of *O. oeni* with pH control in the mixture of glucose-fructose in 2 l batch culture

Table 4.2 Batch growth of *O. oeni* on a mixture of glucose- fructose

pH	3.5	4.0	4.5	5.0	6.0
μ_{max} (h^{-1})	0.024	0.056	0.066	0.060	0.031
Relative growth rate $\mu_{(i)}/\mu_{(4.5)}$ [a]	0.36	0.84	1.00	0.92	0.48
t_d (h)	28.9	12.4	10.5	11.6	22.4
OD_{max} [b]	0.714	0.713	0.810	0.760	0.394
Dry weight [c] (g/l)	0.314	0.314	0.357	0.335	0.174
Time[d] (h) (achieved maximum dry weight)	96	49	45	45	85
Cell productivity (mg cell/l/h)	3.3	6.4	7.9	7.4	2.0
$Y_{x/s}$					
(g cell /g sugar)	0.040	0.040	0.041	0.042	0.024
(g cell/mol sugar)	7.2	7.2	7.4	7.6	4.3

a $\mu_{(i)}/\mu_{(4.5)}$ relative value of growth rate, $\mu_{(i)}$ at pH i, i = 3.5, 4.0, 4.5, 5.0, 6.0; $\mu_{(4.5)}$ at pH 4.5,
b OD_{max} = maximum OD obtained – initial OD, so OD_{max} is a maximum amount of biomass formation.
c dry weight is a maximum cell dry weight produced, excluding initial cells inoculated.
d time refers to the duration of fermentation from the beginning to the maximum cell concentration.

A relationship between μ_{max} and pH obtained by polynomial regression (R^2 = 1) (Appendix K) is below:

$\mu_{max} = 0.0089 \times pH^3 - 0.1504 \times pH^2 + 0.8155 \times pH - 1.3696$ (4.12)

The equation is in good agreement with experimental data. In terms of the relation (4.12) the calculated maximum growth rate 0.066 h^{-1} at pH 4.54. This is in good agreement with the experimental data.

4.3.1.3 The effect of pH on product formation

The products, including lactate, acetate, ethanol, glycerol, erythritol and mannitol, were determined during the fermentation of 5 g/l glucose-5 g/l fructose at different pH. The time-courses of all product formation are shown in Appendix M apart from mannitol shown in Figure 4-1.

The results showed that pH had an obvious effect on the product formation (Appendix L). In order to compare these relations of product formation, the ratio of product formation at several pH to pH 3.5 is shown in Figure 4-3 below. The plot presents the influence of pH on the various product formations. Lactate and acetate concentration showed little variation in the range pH 3.5 to 5.0, but both were halved at pH 6.0. Ethanol increased from pH 3.5 to 4.5 and then decreased with increase in pH. Glycerol declined from pH 3.5 to 4.5, then rose and reached the highest value at pH 6.0. Erythritol showed the same trend as glycerol at pH 3.5 – 5.0, but reduced significantly at pH 6.0, a half of the concentration obtained at pH 3.5.

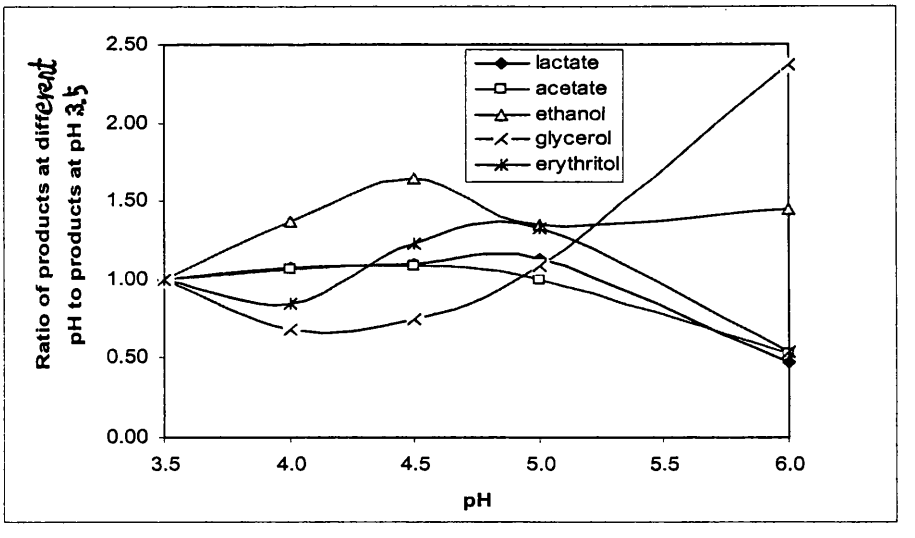


Figure 4-3 Formation of organic acids and sugar alcohols during the growth of *O. oeni* in 5g/l glucose-5g/l fructose mixture at pH 4.5 in 2 l batch culture

The product yields of sugar to product ($Y_{p/s}$), expressed as relationship between sugar consumption and product formation, are shown in Table 4.3. Small difference in mannitol yields was observed in a pH range 3.5 to 6.0. The product yields of lactate and acetate were approximately constant with average 0.27±0.01 g lactate/g sugar and 0.10 g acetate/g sugar under pH 4.0, 4.5 and 5.0; The lowest value of lactate and acetate yield, 0.13 g lactate/g sugar and 0.06 g acetate/g sugar, occurred at pH 6.0, but with the highest value of ethanol and glycerol yield. The calculations of product yield ($Y_{p/s}$, $Y_{p/x}$) see Appendix M.

Table 4.3 The product yields at different pH values

pH	$Y_{p/s}$ (g product/g sugar)					
	mannitol	lactate	acetate	ethanol	glycerol	erythritol
3.5	0.44	0.23	0.09	0.031	0.002	0.002
4.0	0.40	0.28	0.10	0.045	0.001	0.002
4.5	0.43	0.26	0.10	0.051	0.001	0.002
5.0	0.44	0.27	0.09	0.043	0.002	0.002
6.0	0.44	0.13	0.06	0.054	0.005	0.001

4.3.1.4 The fermentation balance of glucose-fructose mixture

Stoichiometric data

Glucose and fructose were consumed simultaneously at pH 4.5 (Fig. 4-1 c). 82% of the fructose consumed was reduced to mannitol (Appendix N). The glucose and the remaining fructose were catabolized via the phosphoketolase pathway, produced lactate, acetate, ethanol, CO_2 and very small amounts of glycerol and erythritol. Assuming cell carbon (carbon content of the cells) accounts for 50% of cell dry weight (Atkinson & Mavitunna 1983), and CO_2 is equal to the amount of acetate plus ethanol (Richter *et al.* 2001), from the product formation observed, the overall stoichiometry (in mole) could be established as follows:

1 glucose + 1.06 fructose 0.87 mannitol + 1.07 lactate+ 0.60 acetate + 0.41 ethanol + 0.005 glycerol + 0.007 erythritol + 1.01 CO_2

Similarly, the stoichiometry could be established at pH 3.5, 4.0, 5.0 and 6.0. These results are represented below in Table 4.4.

Table 4.4 The stoichiometry of fermentation of glucose-fructose*

pH	glucose	fructose	mannitol	lactate	acetate	ethanol	glycerol	erythritol	CO_2
3.5	1	1.19	0.95	1.02	0.58	0.26	0.007	0.006	0.84
4.0	1	1.11	0.84	1.18	0.67	0.39	0.005	0.005	1.06
4.5	1	1.06	0.87	1.07	0.60	0.41	0.005	0.007	1.01
5.0	1	1.16	0.95	1.19	0.60	0.37	0.008	0.008	0.97
6.0	1	1.13	0.92	0.57	0.37	0.45	0.019	0.004	0.82

* CO_2 was not measured assuming CO_2 equals the addition of acetate and ethanol.

Due to 82% of fructose consumed was transformed into mannitol at pH 4.5, the total glucose plus fructose that was not channeled into mannitol (about 18%) were metabolized via the phosphoketolase pathway ("central sugar" metabolism) (Salou *et al.* 1994) and this stoichiometry, expressed relative to the part of sugar, was as follows:

1 central sugar 0.50 acetate + 0.90 lactate + 0.34 ethanol + 0.004 glycerol + 0.006 erythritol + 0.84 CO_2

This stoichiometry indicated a carbon recovery of 97%.

Table 4.5 shows the product formation, carbon content of cells formed per molar central sugar and carbon recovery (%), as calculated in Appendix N.

Table 4.5 Product formation (mol) and carbon content (mol) of cells per central sugar * and carbon recovery

pH	central carbon	lactate (mol)	acetate (mol)	ethanol (mol)	glycerol (mol)	erythritol (mol)	CO_2 (mol)	C (mol) in cells	Carbon recovery (%)
3.5	1.0	0.82	0.47	0.21	0.005	0.005	0.68	0.48	84
4.0	1.0	0.93	0.52	0.31	0.004	0.004	0.83	0.51	97
4.5	1.0	0.90	0.50	0.34	0.004	0.006	0.84	0.54	97
5.0	1.0	0.98	0.49	0.30	0.006	0.007	0.79	0.54	98
6.0	1.0	0.47	0.30	0.37	0.016	0.003	0.67	0.32	63

* central sugar refers to the total amounts of glucose plus fructose not converted into mannitol (Salou *et al.* 1994).

Good carbon recoveries were obtained with the exception of pH 6 where only 63% was recovered. Under theses conditions the levels of glycerol increase significantly with the reduction of lactate and acetate formation. However, the balance would indicate that a new undetected product has been formed at pH 6.0. Possible products are diols (propane diol or butane diols) both common products formed by LAB in stressful conditions. *O. oeni* is an

acidophile capable of good growth and activity at pH 3.5 as indicated by the data above in Table 4.5 and Table 4.2.

Energetic calculations

Taking into account the heterofermentative pathway of glucose and fructose metabolism in *O. oeni*, ATP synthesis by substrate-level phosphorylation is 1 mol/mol lactate and 1 mol/mol acetate. The global organic acid conversion yields enabled the calculation of the yield of ATP production. For example, at pH 4.5 the amount of ATP produced was 1.40 (0.50 + 0.90) mol/mol central sugar (Table 4.5), and was associated with the production of 12.9 g cell/mol central sugar [0.357 g cell $l^{-1}/(27.60\times10^{-3})$ mol l^{-1} central sugar], resulting in a biomass yield produced relative to ATP consumed of 9.2 g cell/mol ATP [Y_{ATP} = 12.9 g cell mol^{-1} central sugar/1.40 mol ATP mol^{-1} central sugar = 9.2 g cell/mol ATP]. The value was in good agreement with that determined by Salou *et al.* (1994), e.g. 10 ± 2 g /mol carbohydrate. The results of energetic calculations at different pH are shown in Table 4.6 below (details refer to Appendix N). Moreover, the μ_{max} (Table 4.2) was related to the rate of ATP formation.

Table 4.6 Energetic calculations at different pH

pH	n [a] (mol ATP/mol central sugar)	$Y_{x/s}$ (g cell/mol central sugar)	Y_{ATP} [b] (g cell/mol ATP)	The rate of ATP formation [c] (mmol ATP/g cell/h)
3.5	1.29	11.5	8.91	1.06
4.0	1.46	12.1	8.30	1.25
4.5	1.40	12.9	9.25	1.93
5.0	1.47	12.9	8.81	1.73
6.0	0.77	7.69	10.0	1.17

a. n is moles of ATP made available to the organism by the metabolism of one mole of energy source;
b. Y_{ATP} is ATP yield of biomass.
c. the rate of ATP formation = $n/Y_{x/s}/t$ (t is time at maximum lactate (acetate) concentration)

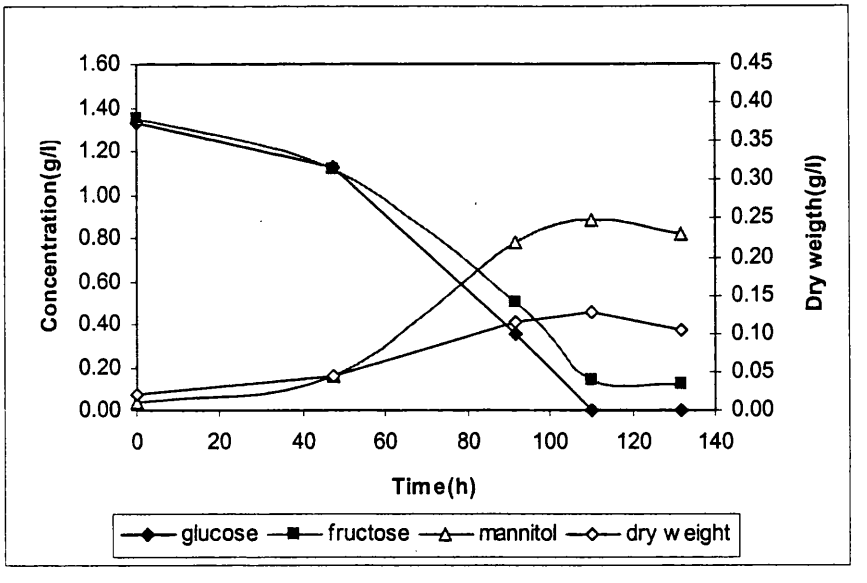
The data above show that even at pH 3.5 the yields remained high and substantiated previous observation that this organism is well adapted to an acid environment. The data for pH 6 is somewhat suspect due to the poor carbon recovery.

4.3.2 The effect of concentrations of glucose-fructose on the culture of *O. oeni*

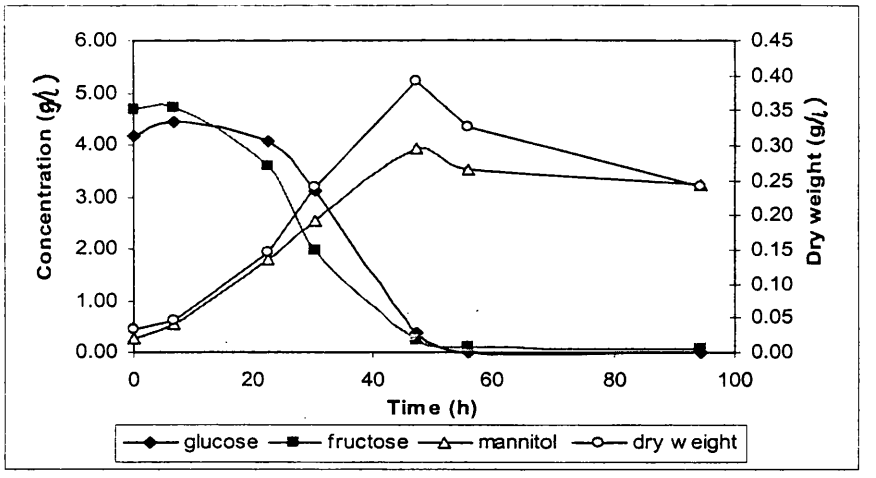
4.3.2.1 Substrate consumption during fermentation of glucose-fructose

Fermentations of glucose and fructose of different concentrations by *O. oeni* at pH 4.5 are shown in Figure 4-4 (a)-(e). Simultaneous consumption of glucose and fructose were observed at low concentration of glucose and fructose, e.g. 2.7 g/l and 8.5 g/l (1:1, w/w) of glucose and fructose, both sugars were almost completely consumed at the same period (Fig. 4-4 a & b). At the high concentrations of glucose and fructose, the specific uptake rate was much faster for fructose than glucose. Fructose was consumed completely at a relative short period compared with glucose (Figure 4-4 c, d, e). In Figure 4-4 (e) using a high concentration of sugar (about 90 g/l) a stationary phase was observed from 25 to 140 hours. After this although only poor growth was observed the glucose and fructose were consumed until after 400 h, 92mM glucose still remained in the culture.

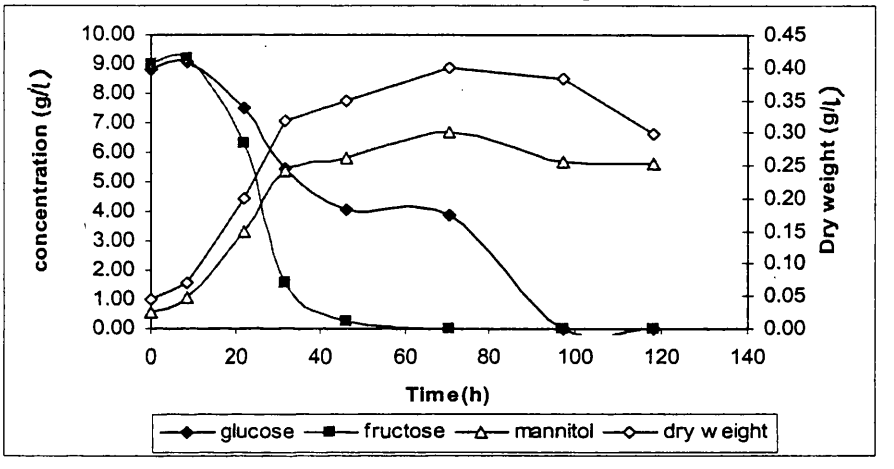
The glucose uptake and growth of *O. oeni* of two phases were observed (Fig. 4-4 c). A similar trend was also apparent in Fig. 4-4 (d) & (e). The specific rates of glucose and fructose uptake are presented in Table 4.7 below. The results showed that the specific rates of glucose and fructose uptake increased as the glucose and fructose concentration increased suggesting that system had relatively low affinity for the sugars.



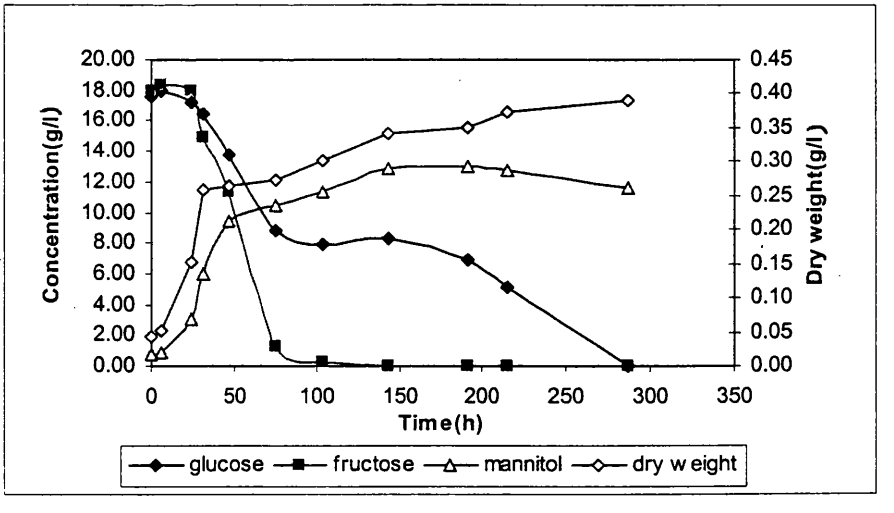
(a) The growth of *O. oeni* and consumption of glucose-fructose at $[glucose]_0$ = 7.4 mM = 1.33 g/l, $[fructose]_0$ = 7.5 mM = 1.35 g/l



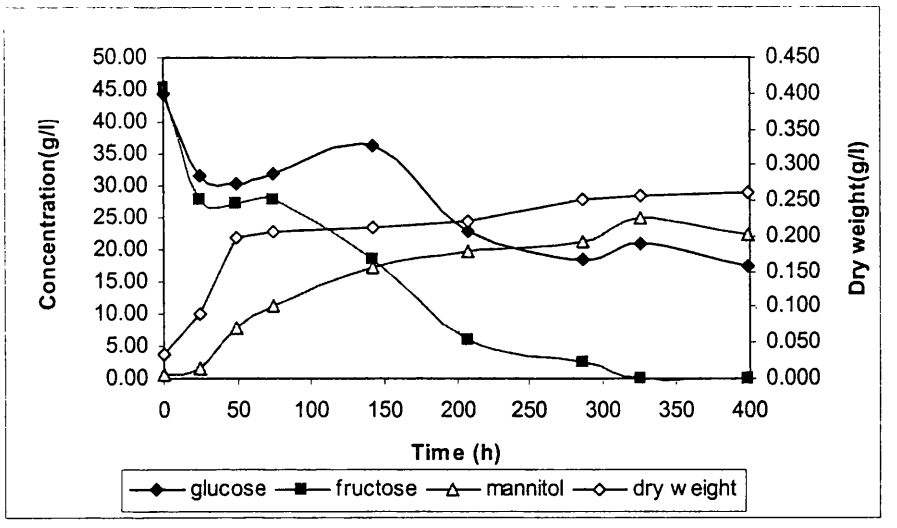
(b) The growth of *O. oeni* and consumption of glucose-fructose at $[glucose]_0$ = 23.2 mM= 4.2 g/l, $[fructose]_0$ = 26.0 mM = 4.3 g/l



(c) The growth of *O. oeni* and consumption of glucose-fructose at $[glucose]_0$ = 48.8 mM = 8.8 g/l, $[fructose]_0$ = 50.0 mM =9.0 g/l



(d) The growth of *O. oeni* and consumption of glucose-fructose at $[glucose]_0$ = 97.8 mM= 17.6 g/l, $[fructose]_0$ = 99.8 mM= 18.0 g/l



(e) The growth of *O. oeni* and consumption of glucose-fructose at $[glucose]_0$ = 246.8 mM = 44.5 g/l, $[fructose]_0$ = 251.0 mM = 45.2 g/l

Figure 4-4 The growth of *O. oeni* and consumption of glucose-fructose at different concentrations of glucose-fructose in 2 l batch culture

Table 4.7 The specific rate of glucose and fructose uptake during the culture of *O. oeni*

[glucose]+ [fructose] (g/l)	Q_s^* (total sugar) (g sugar/g cell/h)	$Q_{s\,(glucose)}$ (g glucose/g cell/h)		$Q_{s\,(fructose)}$ (g fructose/g cell/h)		Ratio of $Q_{glucose}$ to $Q_{fructose}$ ** (phase 1)
		Phase 1	Phase 2	Phase 1	Phase 2	
3	0.21	0.11	-	0.10	-	1.10
9	0.51	0.24	-	0.27	-	0.89
18	1.03	0.39	0.29	0.64	-	0.61
36	1.52	0.53	0.20	0.99	-	0.54
90	3.98	1.75	0.42	2.23	0.56	0.78

* the addition of $Q_{s\,(glucose)}$ and $Q_{s\,(fructose)}$ during the first growth phase (phase 1).
** the ratio of $Q_{s\,(glucose)}$ to $Q_{s\,(fructose)}$ during the first growth phase (phase 1).
- no variation was observed (no second growth phase).

4.3.2.2 Analysis of growth kinetics

The specific growth rate varied with the change in concentrations of glucose and fructose mixture. A single growth phase was observed up to 9 g/l glucose-fructose mixture. At higher concentrations a biphasic curve was observed this was most pronounced at concentration of 18 g/l of sugar or more, a second slower growth phase was observed (Figure 4-5 & 4-4 c, d, e). The slower growth phase correlated well with the exhaustion of fructose in the cultures. The growth rates were also dependent on the substrate concentration (Table 4.8). Slow growth rates were observed when sugar concentration was low and increased when the sugar levels were high, however this trend was reversed at

very high sugar concentrations where substrate and/or product inhibition were affecting the growth rate, i.e. a specific growth rate was significantly lower at 0.025 h^{-1} in the presence of 3 g/l of sugar, but 0.066 h^{-1} was observed between 9 g/l and 36 g/l of glucose-fructose mixture, up to 90 g/l sugar with a lower growth rate 0.060 h^{-1}.

Table 4.8 The specific growth rate and dry weight at varied concentration

Concentration * (g/l) [glucose]+[fructose]	μ_{max} (h^{-1})		Dry weight ** (g cell /l)		Time to achieve to dry weight (h)	
	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2
3	0.025	-	0.108	-	110	-
9	0.066	-	0.357	-	45	-
18	0.064	0.007	0.318	0.354	31	71
36	0.066	0.004	0.220	0.370	31	215
90	0.060	0.002	0.165	0.229	31	398

* The rough value of addition of glucose and fructose concentration (glucose: fructose around 1:1).
** Dry weight = 0.44 × OD_{max} (g/l) (OD_{max} = maximum OD obtained – initial OD)
- no second growth phase was observed.

A direct comparison of the effect of sugar concentration on the growth curves of *O. oeni* in the glucose-fructose medium is shown in Figure 4-5. With the initial total concentration of 3 g/l (1:1, w/w) and 9 g/l (1:1) glucose and fructose, after the growth of *O. oeni* reached a peak of the growth (OD), the cell concentration declined; while at 18 and 36 g/l glucose

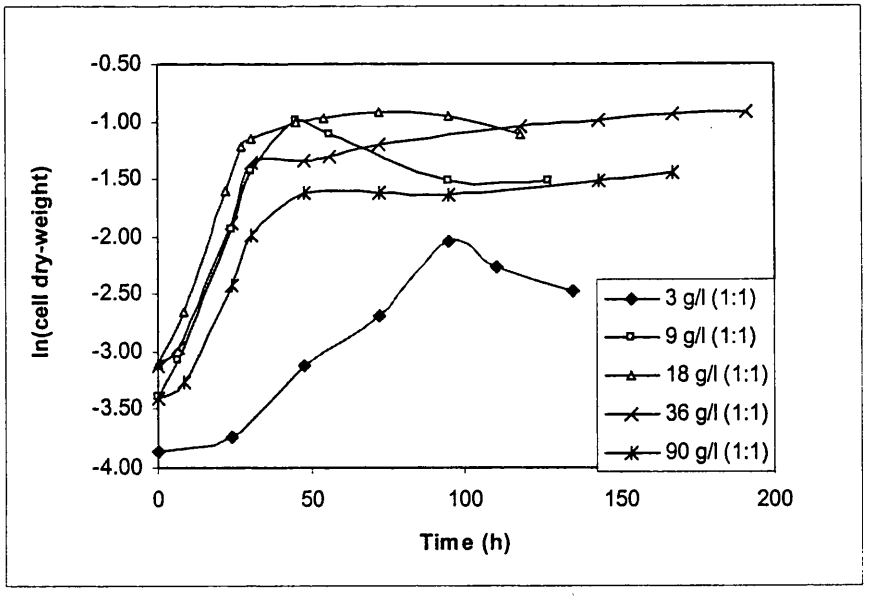


Figure 4-5 The growth of *O. oeni* at different concentrations of glucose-fructose.
(ln(cell dry-weight) vs Time), glucose: fructose 1:1 (w/w)

and fructose, the OD rose rapidly during the period 0-31 h and then went slowly up to a maximum. Similarly, with 90 g/l sugar, the OD increased quickly at the beginning of growth and then increased slowly and in this case the final cell concentration was lower. Table 4.8 shows these observations. These observations are consistent with the initial nutrient limitation at low sugar concentrations, to nutrient sufficient conditions at intermediate sugar concentrations and to final substrate/product inhibition at high sugar concentrations.

The maximum cell productivity for these cultures was obtained at 9 g/l of concentration of glucose and fructose (Table 4.9). When sugar was more than 9 g/l, both cell productivity and biomass yield declined as the concentration increased. Little variation of biomass yield was observed at 3 g/l and 9 g/l of sugar (1:1), while $Y_{x/s}$ reduced half with the doubled sugar of 18 g/l. The calculation of biomass yield is presented in Appendix O.

Table 4.9 Influence of the concentration of glucose and fructose on the cell productivity and biomass yield

Concentration (g/l)* [glucose]+[fructose]	Time (h) (achieved maximum dry weight)	Cell productivity (mg/l/h)	$Y_{x/s}$ Biomass yield (g cell/g sugar)
3	110	0.98	0.042
9	45	7.93	0.041
18	71	4.99	0.022
36	215	1.53	0.010
90	398	0.58	0.003

* glucose: fructose 1:1, w/w

4.3.2.3 Formation of organic acids and sugar alcohols

The time-courses of product formation, lactate, acetate, ethanol, glycerol and erythritol, at the various sugar concentrations are shown in Appendix P, but time-courses of mannitol is shown previously in Figure 4-4 (a)-(e). The final concentrations of lactate, acetate, ethanol, glycerol and erythritol of the cultures are shown in Figure 4-7.

Under the range of sugar concentrations tested, an increased concentration of glucose and fructose led to an increase in lactate, acetate, and glycerol. However, the concentration of end products formation was not directly related to sugar concentration. Initially at low sugar concentration there are proportionate increases in end product. For example, as

shown in Fig. 4-7, all products (within experimental error) increased linearly with the increase in sugar at low sugar concentrations (< 36 g/l). Above 36 g/l the ratio between the end products changes with increase or decline in the amounts of products formed. Most notable here is the continued formation of acetate, with the declines in ethanol and erythritol while lactate and glycerol increased slightly. Another indication of the shift in metabolism is that the ratio of acetate to lactate was around 0.53 ± 0.05 at 3 -36 g/l, while the ratio increased up to1.12 at 90 g/l of glucose and fructose.

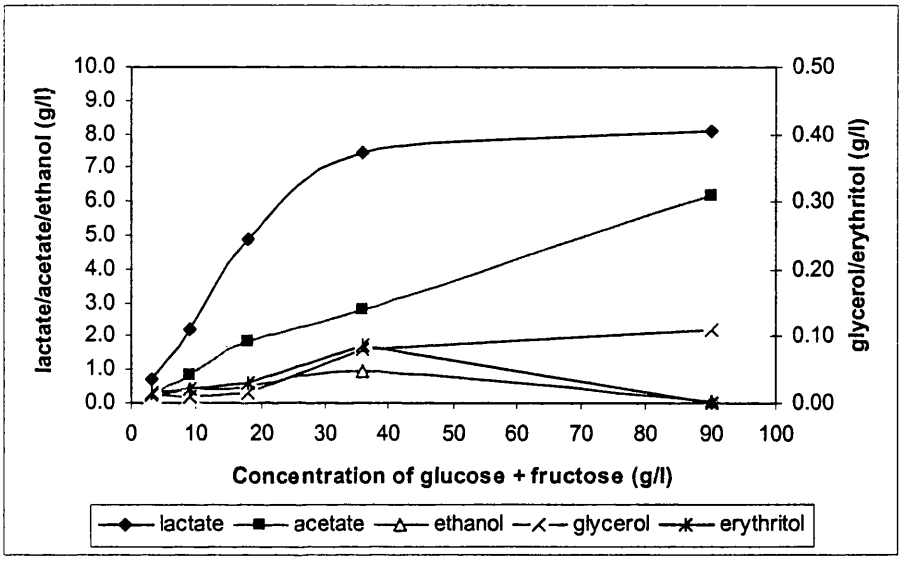


Figure 4 - 7 The influence of concentration of glucose-fructose mixture on the end-product formation in 2 litre batch culture with pH control of 4.5

The influences of concentrations of glucose and fructose on the product yield are summarized in Table 4.10. Small difference in mannitol yield and acetate yield was observed in the whole range tested with 0.35±0.06 g/g and 0.09± 0.01 g/g, respectively. The lactate yield of $Y_{p/s}$ showed little variation (0.28±0.02g/g) at 3-18 g/l sugar, and declined with the further increase of sugar. Ethanol yield of $Y_{p/s}$ decreased when the sugar increased especially decreased significantly at 90 g/l sugar. Glycerol and erythritol yield were very low as a whole, but it should be noted that erythritol could not be detected at 90g/l sugar.

Table 4.10 The product yields at various concentrations of glucose plus fructose

Concentration (g/l)	$Y_{p/s}$ (g product/g sugar)					
	Mannitol	Lactate	Acetate	Ethanol	Glycerol	Erythritol
3	0.33	0.29	0.09	0.066	0.004	0.001
9	0.43	0.26	0.10	0.051	0.001	0.002
18	0.32	0.30	0.11	0.030	0.001	0.002
36	0.37	0.22	0.08	0.028	0.002	0.003
90	0.29	0.11	0.08	0.001	0.002	0.000

4.3.2.4 Fermentation balances for glucose-fructose

Stoichiometric data

As previously described in section 4.3.1.4, glucose and fructose were consumed simultaneously. A part of fructose was converted into mannitol, while glucose and the remaining fructose were catabolized via the phosphoketolase pathway, produced lactate, acetate, ethanol, glycerol and erythritol (section 4.3.2.3). The overall stoichiometry and stoichiometry, expressed as 'central sugar' (in mole) could then be established and the specific calculations are included in Appendix Q.

An example was given previously in section 4.3.1.4. Similarly, the other stoichiometries for per molar 'central sugar' (glucose plus fructose not channeled to mannitol) at each sugar concentration are summarized in Table 4.11. All overall stoichiometries from which "central sugar stiochiometries" were derived can be seen in Appendix Q. The main effect of increased central sugar concentration is the poor recovery and it may be that additional unmeasured products may be produced.

Table 4.11 Stoichiometry for per molar 'central sugar' and carbon recovery

Central sugar /end product (mol)	Sugar concentration (g/l)				
	3	9	18	36	90
Central sugar	1.00	1.00	1.00	1.00	1.00
Lactate	0.85	0.90	0.87	0.70	0.31
Acetate	0.42	0.50	0.48	0.40	0.36
Ethanol	0.39	0.34	0.17	0.17	0.004
Glycerol	0.012	0.004	0.003	0.007	0.004
Erythritol	0.012	0.006	0.004	0.006	0.000
CO_2 *	0.81	0.84	0.65	0.57	0.36
C** (in cells)	0.47	0.54	0.23	0.12	0.03
Carbon recovery (%)	92	97	80	66	34

*CO_2 was not examined assuming CO_2 is equal to the amount of acetate plus ethanol.

**cell content of cells was based on 50% carbon of cell dry-weight (mol carbon/mol central sugar).

Calculation of the fermentation energetics

Taking into account the heterofermentative pathway of glucose and fructose metabolism by *O. oeni*, ATP synthesis by substrate-level phosphorylation theoretically is 1 mol/mol lactate and 1 mol/mol acetate. The overall organic acid conversion yields enabled the calculation of the yield of ATP production. For example, at 3 g/l of glucose and fructose the amount of ATP produced was 1.27 (0.42 + 0.85) mol/mol central sugar, and was associated with the production of 11.4 g cell/mol central sugar [0.108 g cell $l^{-1}/(9.50\times10^{-3})$ mol l^{-1} central sugar], resulting in a biomass yield produced relative to ATP consumed of 8.94 g cell/mol ATP [Y_{ATP} = 11.4 g cell mol^{-1} central sugar/1.27 mol ATP mol^{-1} central sugar = 8.94 g cell/mol ATP]. The results of energetic calculations at different initial concentrations of sugar are shown in Table 4.12 below.

Table 4.12 Energetic calculations at different concentration of sugar

Total initial concentration (g/l)	n [a] (mol ATP/mol central sugar)	$Y_{x/s}$ (g cell/mol central sugar)	Y_{ATP} [b] (g cell/mol ATP)
3	1.27	11.4	8.94
9	1.40	12.9	9.25
18	1.35	5.63	4.18
36	1.10	2.78	2.53
90	0.67	0.79	1.19

a. n is moles of ATP made available to the organism by the metabolism of one mole of energy source;
b. Y_{ATP} is ATP yield of biomass.

These Y_{ATP} values at low sugar concentration are typical of organisms growing on hexose sugars. However, as the sugar concentrations increase the Y_{ATP} drops. This is another indicator that the systems become increasingly stressed by high sugar concentrations.

4.4 Continuous culture of *O. oeni*

This section involves the study of continuous culture of *O. oeni* and the first part of this section introduces the basic theory that will be utilized to determine growth kinetics and provide a comparison of performance in different conditions.

4.4.1 Background theory

Depending on the control parameters and the operation mode, continuous culture can be classified into four general types: a. Chemostat (steady state conditions under substrate limitation); b. Auxostat (steady state conditions under nutrients sufficient condition and constant cell concentration); c. Multi-stage continuous culture, which in the presence of several vessels, mimic tubular flow (or plug flow) reactor systems i.e. steady state conditions with spatial/time distribution of culture. d. Continuous cell recycle, an unsteady state system where biomass accumulates, except where the maintenance energy (substrate consumption rate) for growth equals the feed rate.

The general concept and theory of these types of continuous culture were described by Zeng[1] (1999). A common feature of these cultures is that they consist of one or more culture vessels into which fresh medium or culture from a preceding vessel is continuously introduced at a rate, F, expressed in liters per hour (l/h), and that the culture volume, V, expressed in liters (l), is kept constant by continuous removal of the culture. Chemostat is shown in Fig. 4-8 and described in more detail as follows.

The chemostat is defined as a continuous culture system in which the feed rate is set externally and cell growth is limited by a selected nutrient. The second condition means the specific growth rate, μ (h^{-1}), of the organism is a function of a single growth-limiting nutrient. However, this definition may be relaxed to include continuous culture limited simultaneously by more than one nutrient component (Gottschal 1992). Continuous cultures that are fed with an inhibitory nutrient or that form toxic products can be limited by growth inhibition under the condition of excess of all nutrients. Strictly speaking, they cannot be referred to as chemostat cultures.

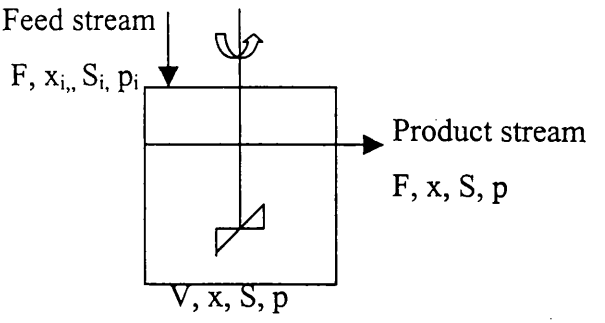


Figure 4-8 Continuous stirred-tank reactor (Chemostat)

A chemostat is usually started as a batch culture. Before a nutrient becomes limiting, the nutrient feed is started. Cells grow until the chosen nutrient becomes limiting. After this, cell growth is limited by the rate of medium addition. The specific growth rate of a chemostat culture can be determined from a material balance for biomass:

net increase in biomass = biomass in incoming medium + growth - output – death

in mathematical form this is:

$$dx/dt = x_i F/V + \mu x - x F/V - k_d x \qquad (4.13)$$

where dx/dt is the rate of accumulation of biomass per unit of time and per volume (g cell/l/h); x_i is the biomass concentration (g cell dry weight/l) in the incoming medium; x is biomass concentration (g cell dry weight/l) in the bioreactor; and k_d is specific death rate of cells (h^{-1}). This expression can be simplified if one assumes that the chemostat is at a steady state, that there are no cells in the incoming medium, and that cell death is negligible. The assumption of steady state implies that there is no net accumulation of cells in the bioreactor, so the left-hand side of the equation is equal to zero. Furthermore, if the dilution rate, D, is introduced defined as F/V, equation 4.13 becomes:

$$\mu x = Dx \qquad (4.14)$$

$$\text{hence } \mu = D \qquad (4.15)$$

This is one of the most important properties of a chemostat. The specific growth rate of a culture over a range up to the maximum specific growth rate (μ_{max}) can be precisely controlled by the nutrient feed rate if maintaining a constant volume.

In a steady-state chemostat with sterile feed and negligible cell death, the specific growth rate μ is equal to the dilution rate D. The relationship is useful for determining kinetic and yield parameters in cell culture. Pirt (1975) and Doran[1] (1995) both give a detailed discussion of these principles.

True biomass yield ($Y_{x/s}^{max}$) and maintenance coefficient (m_s)

The observed yield of a culture and its relationship to maintenance and growth can be defined by the following (Doran[2] 1995).

$$Y_{x/s} = \mu /(\mu/Y_{x/s}^{max} + m_s) \qquad (4.16)$$

where $Y_{x/s}$ is the observed biomass yield from substrate; $Y_{x/s}^{max}$ is the true biomass yield from substrate, which is the maximum possible value of the biomass yield from substrate; and m_s is the maintenance coefficient. The effect of maintenance on yields can be ascertained graphically by inverting Eq.(4.16) to produce the expression:

$$1/Y_{x/s} = 1/Y_{x/s}^{max} + m_s/\mu \qquad (4.17)$$

Eq.(4.17) can be written in the form:

$$\mu/Y_{x/s} = \mu/Y_{x/s}^{max} + m_s \qquad (4.18)$$

Defining the specific rate of substrate uptake q_s with dimensions, e.g. h^{-1}:

$$q_s = \mu/Y_{x/s} \qquad (4.19)$$

In chemostat culture with $\mu = D$, Eq.(4.18) becomes:

$$q_s = D/Y_{x/s}^{max} + m_s \qquad (4.20)$$

Therefore, if $Y_{x/s}^{max}$ and m_s are relatively constant, a plot of q_s versus D gives a straight line with slope $1/Y_{x/s}^{max}$ and intercept m_s. S allowing these constant to be determined.

In a chemostat with sterile feed, the observed biomass yield from substrate $Y_{x/s}$ is obtained as follows:

$$Y_{x/s} = x/(S_i - S) \qquad (4.21)$$

Where x and S are steady-state cell and substrate concentration, respectively, and S_i is the inlet substrate concentration.

Maximum specific growth rate (μ_{max}) and substrate saturation constant (K_s)

Cell growth can be modelled using Monod kinetics equation, then in a chemostat culture,

$$D = \mu_{max}S/(K_s + S) \qquad (4.22)$$

Where μ_{max} is the maximum specific growth rate, K_s is the substrate saturation constant and S is the steady-state substrate concentration in the reactor.

Rearrangement of Eq.(4.22) gives the following linear equation which provides the determination of the constants via a reciprocal plot:

$$S/D = K_s/\mu_{max} + S/\mu_{max} \qquad (4.23)$$

Linearisation of data for the reciprocal plot minimizes distortions in experimental error especially of the substrate (Moser 1985). Accordingly, Eq.(4.23) is used to evaluate μ_{max} and K_s. Therefore, a reciprocal plot of S/D versus S should give a straight line with slope $1/\mu_{max}$ and intercept K_s/μ_{max}.

Theoretical calculation of cell concentration

Thus a steady-state cell concentration, x can be predicted if the parameters on the right hand side of the equation are known:

$$x = D(S_i - S)/(D/Y_{x/s}^{max} + q_p/Y_{p/s}^{max} + m_s) \quad (4.24)$$

- If there is no product synthesis or if production is directly linked with energy metabolism then this simplifies:

$$x = D(S_i - S)/(D/Y_{x/s}^{max} + m_s) \qquad (4.25)$$

- If, in addition, maintenance effects can be ignored, then Eq. (4.25) becomes:

$$x = (S_i - S)\ Y_{x/s}^{max} \qquad (4.26)$$

During balanced growth, the specific growth rate is related to the concentration of growth –limiting substrate by the Monod equation:

$$\mu = \mu_{max} S/(K_s + S) \qquad (4.27)$$

The steady–state concentration of substrate limiting in the reactor:

$$S = DK_s/(\mu_{max} - D) \qquad (4.28)$$

The steady-state cell concentration in Chemostat can be described in terms of dilution rate, kinetic and yield parameters only:

$$x = [S_i - DK_s/(\mu_{max} - D)]\ Y_{x/s}^{max} \qquad (4.29)$$

Critical dilution rate (D_{crit})

In continuous operation if products are either absent or directly linked to energy generation and maintenance effects can be neglected, the chemostat is represented by Eqs (4.28) and (4.29).

As D increases, S increases slowly at first and then more rapidly as D approaches μ_{max}. Cells will washout when the rate of cell removal in the reactor outlet stream is greater than the rate of generation by growth. For systems with negligible maintenance requirements and either energy-associated or zero product formation, the critical dilution rate D_{crit} can be estimated when the steady-state biomass concentration just becomes zero. Substituting $x = 0$ into Eq. (4.29) and solving for D:

$$D_{crit} = \mu_{max} S_i /(K_s + S_i) \qquad (4.30)$$

To avoid washout of cells from the chemostat, the operating dilution rate must always be less than D_{crit}. Near washout, the system is very sensitive to small changes in D which cause relatively large shifts in x and s.

Biomass productivity (Q_x)

The rate of biomass production in chemostat is equal to the rate at which cells leave the reactor: Fx. The volumetric productivity is therefore equal to Fx divided by V:

$$Q_x = Fx/V = Dx \qquad (4.31)$$

where Q_x is the volumetric rate of biomass production.

When maintenance requirement are negligible and product formation either absent or energy associated, x can be replacd by Eq.(4.29), and then Eq. (4.31) becomes:

$$Q_x = DY_{x/s}^{max}[S_i - K_sD/(\mu_{max}-D)] \quad (4.32)$$

From this relationship between Q_x and D there is an optimum dilution rate, D_{opt}, at which rate of biomass output reaches a maximum of biomass productivity. Therefore, at D_{opt} the slope $dQ_x/dD = 0$. Differentiating Eq. (4.32) with respect to D and equating to zero provides an expression for D_{opt}:

$$D_{opt} = \mu_{max}[1 - sqrt(K_s/(K_s+S_i))] \quad (4.33)$$

4.4.2 Influence of dilution rate on the growth of *O. oeni*

Oenococcus oeni was cultured in a chemostat the cells were first growth as a batch culture on Z broth with 5 g/l glucose and 5 g/l fructose (Table 2.4 Chapter 2), feeding medium is the same medium. pH was controlled at 4.5 and temperature 25°C.

The growth and metabolism were investigated as a function of dilution rate. Samples were taken periodically and analyzed to determine the behavior of the culture. Five steady states were established between D 0.007 and 0.052 h^{-1}. This is a good range considering that μ_{max} (as determined in batch) was 0.066 h^{-1}.

The biomass concentrations obtained at various dilution rates are shown in Appendix R. At steady-state the cell dry weight achieved at dilution rate 0.007 h^{-1} was lower than that at D 0.013 h^{-1}, whereas the cell dry weight maintained around 0.367 g cell/h at D 0.013-0.044 h^{-1}. With the further increase in dilution rate the cell dry weight declined. The time-courses of substrate consumption (glucose and fructose) are also shown in Appendix S.

4.4.3 Evaluation of kinetic and yield parameters

4.4.3.1 Maximum biomass yield and maintenance coefficient

Equation 4.20, $q_s = D/Y_{x/s}^{max} + m_s$, can be used to determine maximum biomass yield and maintenance coefficient. In this equation, $D/Y_{x/s}^{max}$ represents the rate of substrate consumption for the growth, and m_s the rate of consumption for maintenance purposes. If

$Y_{x/s}^{max}$ and m_s remain constant, a plot of q_s versus D gives a straight line with slope $1/Y_{x/s}^{max}$ and intercept m_s. In addition q_s and $Y_{x/s}$ can be calculated by equations of 4.19 and 2.21. An example of the graphical determination is shown in Figure 4-9 below and values of biomass yield $Y_{x/s}$ determined at different dilution rates are in Table 4-13.

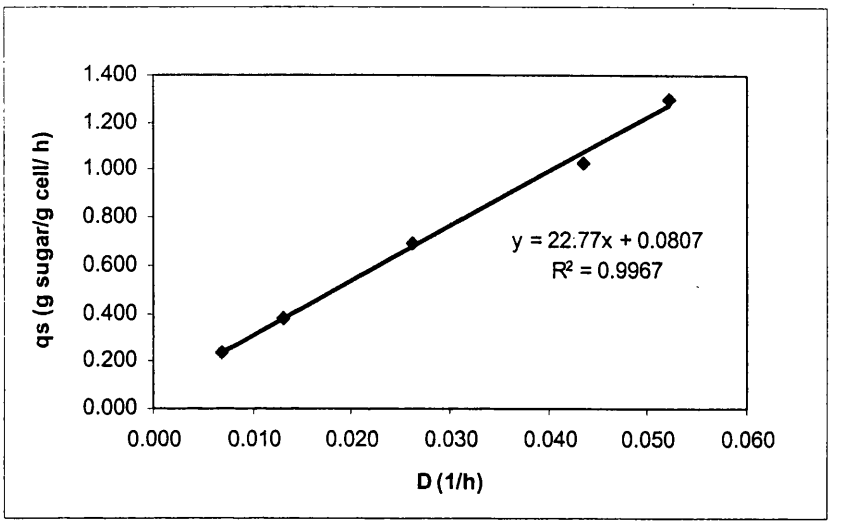


Figure 4-9 Graphical determination of the maintenance coefficient m_s and true biomass yield $Y_{x/s}^{max}$ using data from chemostat culture of *O. oeni*

$q_s = D/Y_{x/s}^{max} + m_s$ (4.20); $q_s = \mu/Y_{x/s} = D/Y_{x/s}$ (4.19); $Y_{x/s} = x/(S_i - S)$ (4.21)

In Figure 4-9 there is a straight line with the slope of 22.77 and the intercept of 0.0807, respectively, hence,

$$1/Y_{x/s}^{max} = 22.77$$

$$Y_{x/s}^{max} = 0.044 \text{ (g cell/g sugar uptake)}$$

$$m_s = 0.081 \text{(g sugar uptake/g cell/h)}$$

True yield or maximum possible yield that represents the yield is obtained in the absence of competing reactions. Maximum possible biomass yield $Y_{x/s}^{max} = 0.044$ (g cell/g sugar uptake) as shown in Table 4.13.

Table 4.13 Yield parameters of *O. oeni* at steady state in Chemostat

D (h^{-1})	q_s (g sugar/g cell/h)	$Y_{x/s}$ (g cell/g sugar)	$Y_{x/s}^{max}$ (g cell/g sugar)	Percentage yield *(%)
0.007	0.235	0.029		67
0.013	0.377	0.035		78
0.026	0.692	0.038	0.044	86
0.044	1.031	0.042		96
0.052	1.295	0.040		92

* Percentage yield (%) = $Y_{x/s}/Y_{x/s}^{max} \times 100\%$

The influence of dilution rate on the biomass yield is shown Figure 4-10. It is seen that the experimental data is in accordance with theoretical calculation based on the equation (4.16) in the dilution rate range tested.

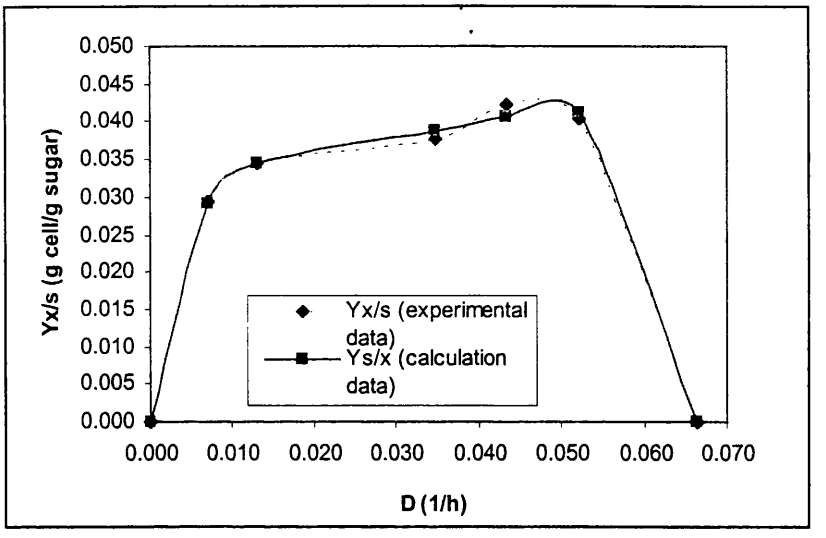


Figure 4-10 The observed biomass yield as a function of dilution rate during continuous culture of *O. oeni*
Calculation data based on equation (4.16) $Y_{x/s} = \mu / (\mu/Y_{x/s}^{max} + m_s)$ and (4.15) μ=D.
$Y_{x/s}^{max}$ = 0.044 (g cell/g sugar uptake), m_s = 0.081(g sugar uptake/g cell/h)

4.4.3.2 Maximum specific growth rate and substrate saturation constant

The Monod equation for specific growth rate, equation (4.27), is analogous mathematically to the Michaelis-Menten expression for enzyme kinetics.

$$\mu = \mu_{max} S/(K_s + S) \qquad (4.27)$$

Typical values of K_s are very small, of the order of mg per liter for carbohydrate substrates and μg per liter for other compounds such as amino acids. The level of growth-limiting substrate in culture media is normally much greater than K_s. As a result, μ μ_{max} provided S is greater than about $10K_s$. That is why the measurement of μ_{max} can be made from batch culture. However, because values of K_s in cell culture are usually very low, accurate determination of this parameter from batch data is difficult. Better estimation of μ_{max} can be made using continuous culture of cells.

If cell growth can be modeled using Monod kinetics equation, for chemostat culture, reciprocal equation above (Eq.4.23) $S/D = S/\mu_{max} + K_s/\mu_{max}$ can be used to graphically determine the specific growth rate and the saturation constant and are given Appendix T, there is a slope of 13.739 and a intercept of 19.02, and hence

$1/\mu_{max} = 13.739$ $K_s/\mu_{max} = 19.02$

$\mu_{max} = 0.073\ h^{-1}$ $K_s = 1.384$ g/l

4.4.3.3 Biomass concentration and sugar uptake

The effect of dilution rate on the steady-state biomass and sugar uptake in a chemostat is shown in Figure 4-12 when a maintenance requirement is taken into consideration. At low dilution rate, cell concentration was low. As D increased, cell concentration increased, but in the range 0.130-0.044 h^{-1}cell concentration remained at a steady level of 0.367 g/l cell dry weight, and then cell concentration declined at 0.052 h^{-1}. As expected, the cells will wash out at critical dilution rate of 0.065 h^{-1} (section 4.4.3.4). The results are in good agreement with the data represented by Eq. (4.25): $x = D(S_i - S)/(D/Y_{x/s}^{max} + m_s)$, as shown in Figure-12.

At $D \rightarrow 0$, nearly all glucose and fructose were consumed, $S \rightarrow 0$. As D increased, fructose concentration increased very slowly, while glucose was nearly consumed up to D 0.026 h^{-1}, and then both sugars increased in the range tested. Similarly, both will more rapidly increase as D approaches the critical dilution rate 0.065 h^{-1}.

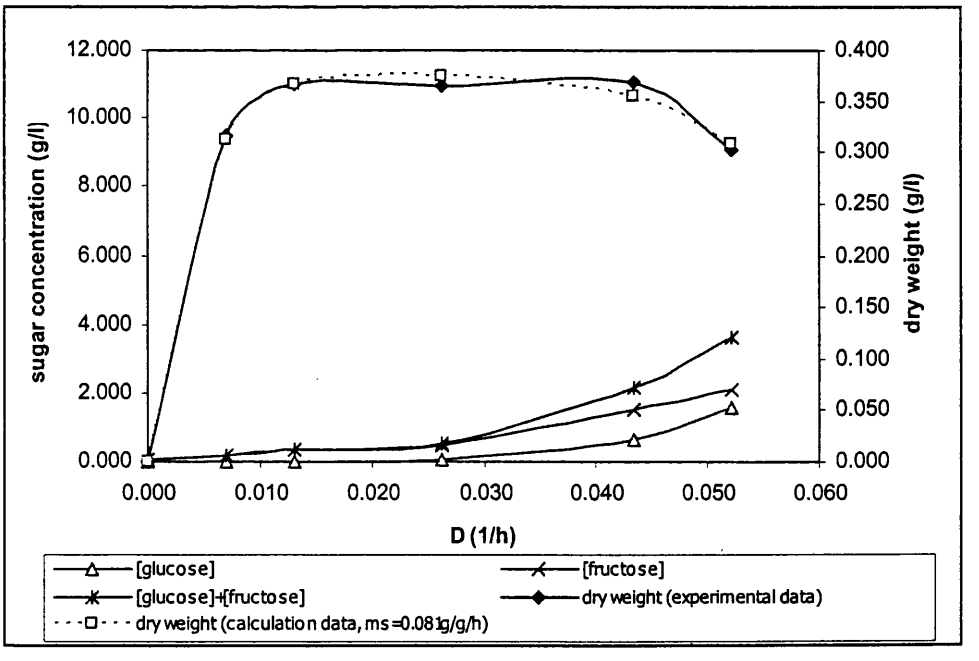


Figure 4-12 The steady-state cell and substrate concentration as a function of dilution rate in a chemostat when maintenance requirement is considered for the growth-limiting substrate.
Theory calculation of dry weight based on Eq. (4.25) $x = D(S_i - S)/(D/Y_{x/s}^{max} + m_s)$
$S_{i,\ glucose} = 5$ g/l, $S_{i,\ fructose} = 6$ g/l, $Y_{x/s}^{max} = 0.044$ g cell/g sugar,
$m_s = 0.081$ g sugar/g cell/h, $D_{crit.} = 0.065\ h^{-1}$, $K_s = 1.384$ g/l.

4.4.3.4 Critical dilution rate ($D_{crit.}$) and cell productivity (Q_x)

In systems with negligible maintenance requirements and either energy-associated or zero product formation, the critical dilution rate $D_{crit.}$ at which the steady-state biomass concentration just becomes zero can be estimated by Eq. (4.30).

$$D_{crit} = \mu_{max} S_i / (K_s+S_i) \qquad (4.30)$$

$$D_{crit} = 0.073*10.967/(1.384+10.967) = 0.065\ (h^{-1})$$

For most cell cultures K_s is very small, and $K_s << S_i$, therefore D_{crit} μ_{max}. The data of D_{crit} (0.065 h^{-1}) obtained is smaller than the μ_{max} (0.073 h^{-1}) obtained from continuous culture (section 4.4.3.2), but is equal to the μ_{max} (0.066 h^{-1}) from batch culture (section 4.3.1.2) under the same experimental conditions. The difference between the D_{crit} and μ_{max} calculated from the data of continuous culture may possibly be considered that K_s is not very small and here the assumption of $K_s << S_i$ is not acceptable. The additional possibility is that Eq. (4.30) is in the conditions that maintenance requirement is negligible, so the data of D_{crit} calculated by Eq. (4.30) may be different from the μ_{max} from the data of continuous culture. However, a margin of error is less than 10%.

The volumetric productivity, Q_x, the volumetric rate of biomass production, is estimated by Eq. (4.31) with maintenance requirement or (4.32) without maintenance requirement:

$$Q_x = Fx/V = Dx \qquad (4.31)$$

$$Q_x = DY_{x/s}[S_i - K_sD/(\mu_{max}-D)] \qquad (4.32)$$

The results are shown in Figure 4-13. The calculation values of volumetric productivity by Eq. (4.31) were in good agreement with the experimental data. The calculation values by Eq. (4.32) were higher when the maintenance requirement was negligible. A rate of biomass output reached a sharp maximum at the optimum dilution rate D_{opt} for biomass productivity. D_{opt} is expressed by Eq. (4.33), $D_{opt} = \mu_{max}\ [1- sqrt(K_s/(K_s+S_i))]$. Hence, the optimum feed rate, 56.4 ml/h, was obtained by Eq. $F = D_{opt} \times V$ for the reactor with 1.15 liter working volume used in my study, a maximum volumetric cell productivity, 17.6 mg/l/h, can be achieved by Eq. (4.31) under conditions tested.

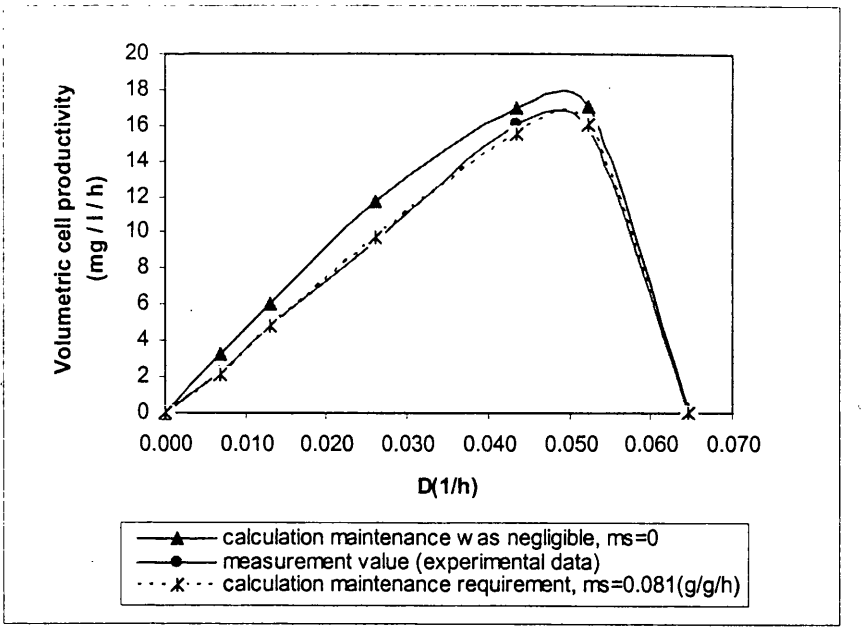


Figure 4-13 Comparison of calculation value and experimental data of volumetric cell productivity at varied dilution rate, curves correspond to $D_{crit.} = 0.065\ h^{-1}$, Ks =1.384 g/l, $S_{i,\ glucose} = 5$ g/l, $S_{i,\ fructose} = 6$ g/l, $Y_{x/s}^{max} = 0.044$ g cell/g sugar. $\mu_{max} = 0.073\ h^{-1}$.

4.4.4 Product formation

The time-course of product formation i.e. mannitol, lactate, acetate and ethanol was determined and is shown in Appendix U. Glycerol and erythritol could also be detected in the medium (no show of data) but only at low levels.

The steady-state concentrations of products obtained at different dilution rates are shown in Figure 4-14. Lactate and acetate decreased as D increased from 0.007 to 0.044 h^{-1} and then increased slightly at 0.052 h^{-1}. Ethanol and mannitol declined slightly when the dilution rate increased from 0.007 to 0.026 h^{-1} and then increased as D increased further to 0.44 h^{-1}. A small decline is then noted at the highest dilution rate (0.052 h^{-1}), this point corresponds to poor substrate utilization at the culture nears the critical dilution rate and the substrate concentration begins to rise in the culture (Figure 4.12).

The stoichiometric data in batch culture for lactate, acetate and ethanol were described previously in section 4.3.1.4. Table 4.14 shows stoichiometric data for products and carbon recovery in chemostat. With increasing D from 0.007 to 0.026 h^{-1}, the production

of acetate and lactate per mol 'central sugar' decreased gradually, while increased as D increased further from 0.026 to 0.052 h^{-1}. The carbon recovery was low, less than 80% in the D range 0.007 to 0.044 h^{-1}.

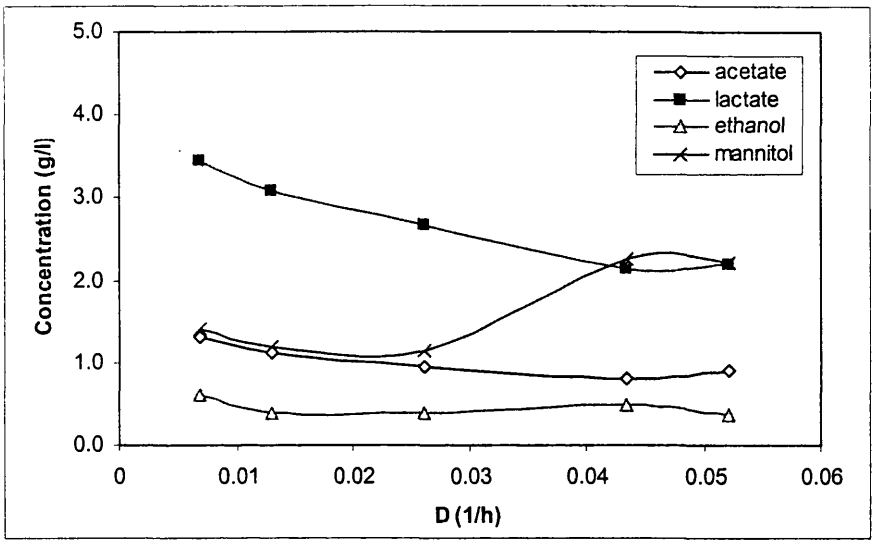


Figure 4-14 The steady-state concentrations of products at different dilution rates when the continuous culture of *O. oeni* performed in chemostat containing 1.15 liter culture. Feed medium: Z broth with 5 g/l glucose - 6 g/l fructose; pH 4.5; Temperature 25°C.

Table 4.14 Fermentation results for *O. oeni* in a substrate-limited chemostat at various dilution rates

Product and carbon recovery	D (h^{-1})				
	0.007	0.013	0.026	0.044	0.052
Central sugar	1.00	1.00	1.00	1.00	1.00
Lactate (mol)	0.73	0.72	0.62	0.68	0.82
Acetate (mol)	0.42	0.37	0.33	0.38	0.51
Ethanol (mol)	0.26	0.16	0.18	0.30	0.26
CO_2 [a] (mol)	0.68	0.53	0.51	0.68	0.77
C [b] in cells (mol)	0.25	0.29	0.32	0.44	0.43
C recovery (%)	74	67	62	76	87

a. CO_2 was not examined, assuming CO_2 is equal to the amount of acetate plus ethanol.
b. C carbon content of cell dry-weight assuming dry-weight contains 50% carbon.

In addition, molar biomass yields, $Y_{x/s}$ and Y_{ATP} relative to 'central sugar', increased progressively from 5.37 to 7.61 g cell per mol 'central sugar' and from 4.68 to 7.24 g dry weight per mol ATP, respectively, in the D range between 0.007 h^{-1} and 0.044 h^{-1} (Table 4.15). As the dilution rate is further increased, the molar biomass yields $Y_{x/s}$ and ATP yield Y_{ATP} decreased, for example $Y_{x/s}$ and Y_{ATP} declined to 7.31 g cell/mol 'central sugar' and 5.44 g cell/mol ATP respectively when D was in the highest dilution rate (D = 0.052 h^{-1}).

Table 4.15 Molar biomass yields at various dilution rates

D (h^{-1})	mol ATP produced (mol ATP/mol 'central sugar')	$Y_{x/s}$ (g cell/mol 'central sugar')	Y_{ATP} (g cell/mol ATP)
0.007	1.15	5.37	4.68
0.013	1.25	6.21	4.97
0.026	0.96	6.79	7.10
0.044	1.05	7.61	7.24
0.052	1.35	7.31	5.44

The maximum ATP yield, that is, Y_{ATP}^{max}, is given by the following relation derived from Eq. (4.20):

$$q_{ATP} = \mu/Y_{ATP}^{max} + m_{ATP} \qquad (4.34)$$

In a chemostat culture with $\mu = D$, Eq.(4.34) becomes:

$$q_{ATP} = D/Y_{ATP}^{max} + m_{ATP} \qquad (4.35)$$

$$q_{ATP} = D/Y_{ATP} \qquad (4.36)$$

The observed Y_{ATP} is g cell/mol of ATP used, the maximum Y_{ATP}^{max} is g cell/mol ATP, m_{ATP} is moles ATP required for maintenance/g cell/h and q_{ATP} is the specific rate of ATP utilization, mol ATP/g cell/h. If Y_{ATP}^{max} and m_{ATP} are relatively constant, a plot of q_{ATP} versus D gives a straight line with slope $1/Y_{ATP}^{max}$ and intercept m_{ATP}.

Figure 4-15 below is plotted by the data of Table 4.15 above, a straight line with slope $1/Y_{ATP}^{max} = 0.1245$ and intercept 0.0006.

$$Y_{ATP}^{max} = 8.03 \text{ (g cell/mol ATP)}$$

$$m_{ATP} = 0.0006 \text{ (mol ATP/g cell/h)}$$

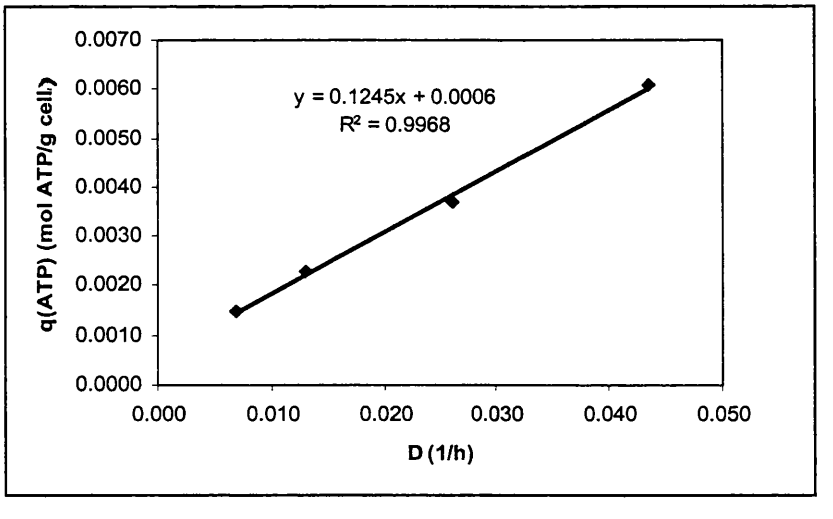


Figure 4-15 Graphical determination of the energy coefficient m_{ATP} and maximum Y_{ATP} yield using data from chemostat culture of *O. onei* Ks =1.384 g/l, $S_{i,\,glucose}$ = 5 g/l, $S_{i,\,fructose}$ = 6 g/l, $Y_{x/s}^{max}$ = 0.044 g cell/g sugar, m_s= 0.081 g sugar/g cell/h

4.5 Batch and Continuous cultures of *L. brevis*

Batch data for the growth and performance of *L. brevis* has been researched elsewhere (Donovan, 2000). However, in order to compare *O. oeni* and *L. brevis* continuous cultures of *L. brevis* were performed.

4.5.1 Influence of pH on the growth of *L. brevis* in batch culture

Lactobacillus brevis (*L. brevis*, X_2), given by H.P.Bulmer Ltd., was isolated from cider and used in this investigation.

To choose a suitable pH for the growth of *L. brevis*, the influence of initial pH on the growth of *L. brevis* was investigated in test tubes. Medium contained Z broth (g/l): Yeast extract 5, KH_2PO_4 5, NH_4Cl 2, Trace element 20ml/l, and addition of glucose 5. Fermentation temperature was in 27°C.

The results are shown in Table 4.16. The specific growth rates increased as the pH increased in the range 3.5 to 5.0, and remained about 0.080 h^{-1} at 5.0-6.0, finally decreased with a further increase in pH. OD was in the level 1.58 ± 0.07 h^{-1} in the range pH 3.5-5.5, afterwards, declined as pH increased further. Final pH in the culture increased progressively in the range 3.5-7.0 except pH 5.0 and 5.5. So the optimal pH for the growth of *L. brevis* would be in the range 5.0-6.0.

Table 4.16 the growth of *L. brevis* in batch culture

pH	OD_{max}	μ_{max} (h^{-1})	t_d (h)	end pH
3.5	1.545	0.055	12.6	3.02
4.0	1.650	0.068	10.2	3.15
4.5	1.627	0.072	9.6	3.27
5.0	1.516	0.080	8.7	3.36
5.5	1.632	0.080	8.7	3.36
6.0	1.417	0.081	8.6	3.52
6.5	1.284	0.075	9.2	3.73
7.0	1.124	0.073	9.5	4.04

On the bases of above experiments, the influence of pH (5.0, 5.5 and 6.0) on the growth of *L. brevis* was investigated further with pH control in 2 liter batch culture so as to achieve an optimal condition for the growth and a maximum specific growth rate. Medium

composition and condition were same as above. In addition, the concentration of both glucose and yeast extract in medium was increased from 5 g/l to 10 g/l, the growth of *L. brevis* was carried out under this medium composition and the optimal pH 5.5.

Table 4.17 shows the growth of *L. brevis* under these conditions. The higher growth rate was obtained at pH 5.5 compared to 5.0 and 6.0 under same medium. OD observed was greater at 10 g/l glucose and 10 g/l yeast extract than at 5 g/l glucose and 5 g/l yeast extract under pH 5.5. The relationship between cell dry weight and OD at 660nm was obtained, 1 OD corresponds to 0.42 g dry weight/l.

Table 4.17 The growth of *L. brevis* under pH control

pH	OD_{max} (28 h)	Dry weight (g/l)	μ_{max} (h^{-1})	t_d (h)
5.0	0.902	0.379	0.091	7.6
5.5	0.898	0.377	0.112	6.2
6.0	0.880	0.370	0.099	7.0
5.5 *	1.520	0.638	0.110	6.3

* medium contained 10 g/l yeast extract and 10 g/l glucose. Others contained 5 g/l yeast extract and 5 g/l glucose.

4.5.2 Continuous culture of *L. brevis* at different dilution rates

In continuous culture of *L. brevis*, feeding medium composition consisted of (g/l): yeast extract 10, KH_2PO_4 5, NH_4Cl 2, Trace element 20ml/l, glucose 10. Prior to feeding medium *L. brevis* grew as a batch culture, medium composition was same as the feeding medium. pH and temperature were controlled at 5.5 and 27°C respectively. The equipment used was described previously in Chapter 2, Figure 2-7.

In continuous culture of *L. brevis*, residual glucose concentration was below 0.6 g/l at dilution rate 0.010-0.070 h^{-1}. As D increased from 0.070 to 0.089 h^{-1}, glucose concentration was about 0.9 g/l. The cell dry weight increased slightly as the dilution rate increased from 0.010 to 0.070, and then declined slightly at D 0.089 h^{-1} (Figure 4-16).

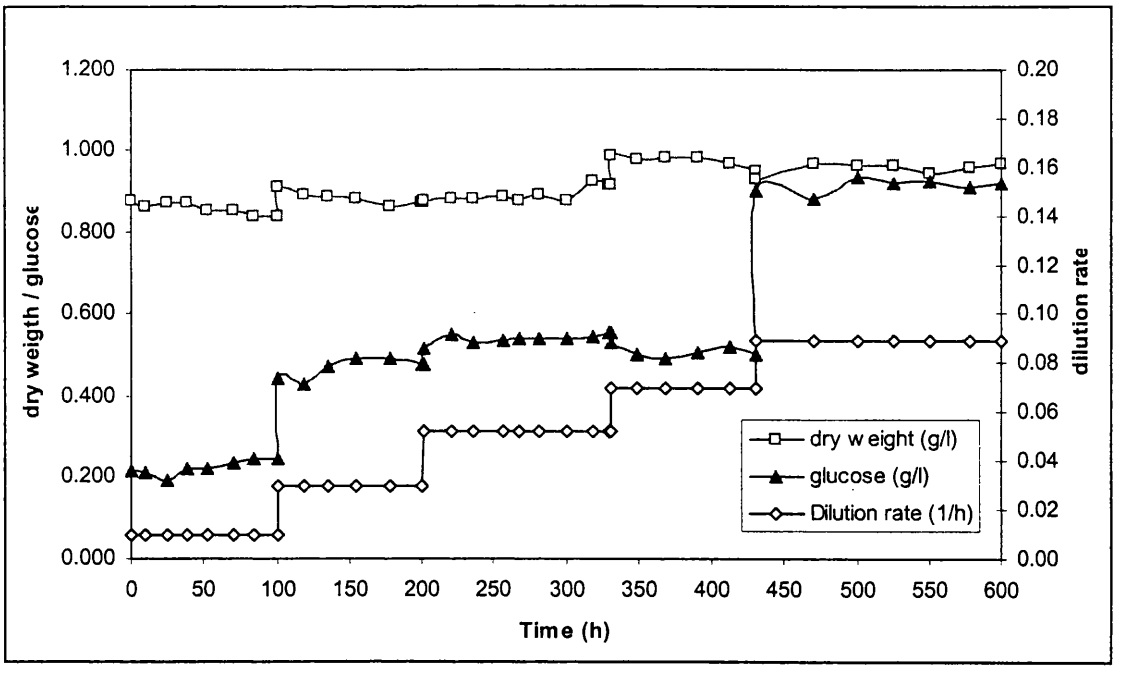


Figure 4-16 Representative results of continuous culture of *L. brevis* in Chemostat Medium containing glucose 10 g/l, temperature 27°C, pH 5.5.

4.5.3 Evaluation of kinetic and yield parameters

4.5.3.1 Maximum biomass yield and maintenance coefficient

Equation 4.20 $q_s = D/Y_{x/s}^{max} + m_s$ can be used to determine maximum biomass yield $Y_{x/s}^{max}$ and maintenance coefficient m_s. If $Y_{x/s}^{max}$ and m_s are relatively constant, a plot of q_s versus D gives a straight line with slope $1/Y_{x/s}^{max}$ and intercept m_s.

Specific rate of glucose uptake q_s and observed biomass yield $Y_{x/s}$ at different dilution rates were calculated (Appendix V) and are shown below in Table 4.18. The graphical determination for $Y_{x/s}^{max}$ and m_s is shown in Figure 4-17 and the result is:

$$m_s = 0.037 \text{ g glucose/g cell/h}$$

$$Y_{x/s}^{max} = 1/8.0898 = 0.124 \text{ g cell/g glucose}$$

Figure 4-18 shows that the biomass yield determined was in agreement with theoretical calculation based on the Eq.(4.16) $Y_{x/s}=\mu/(\mu/Y_{x/s}^{max} + m_s)$ and these increased as the dilution rate increased, and relative yield was also improved from 80 to 96% when the dilution rate increased in the range from 0.010 to 0.089 h^{-1} (Table 4.18).

Table 4.18 The specific rate of glucose uptake and biomass yield of *L. brevis*

D (h^{-1})	q_s [a] (g glucose/g cell/h)	$Y_{x/s}$ [b] (g cell/g glucose)	$Y_{x/s}^{max}$ (g cell/g glucose)	Relative yield [c] (%)
0.010	0.105	0.099		80
0.030	0.288	0.106		85
0.052	0.487	0.107	0.124	86
0.070	0.594	0.117		94
0.089	0.742	0.120		96

a. $q_s = \mu/Y_{x/s} = D/Y_{x/s}$
b. $Y_{x/s} = x/(S_i - S)$
c. Relative yield = $Y_{x/s}/Y_{x/s}^{max} \times 100\ \%$

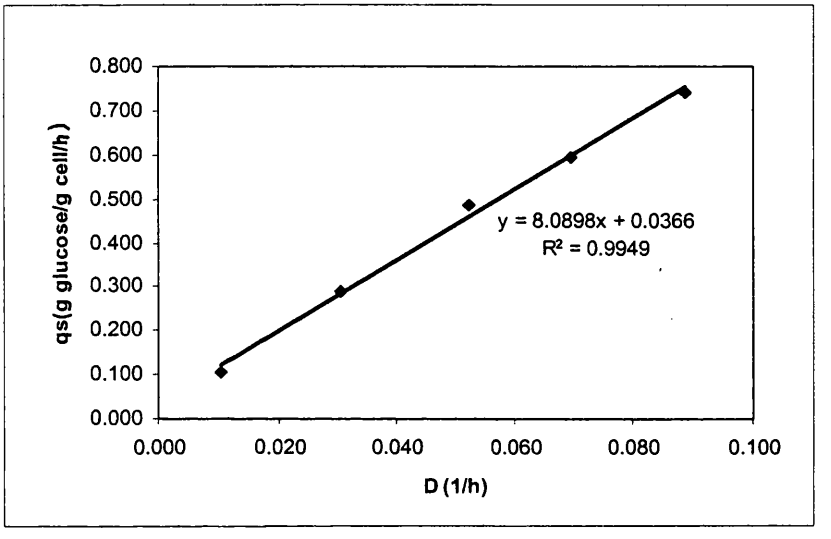


Figure 4-17 Graphical determination for $Y_{x/s}^{max}$ and m_s in continuous culture of *L. brevis*

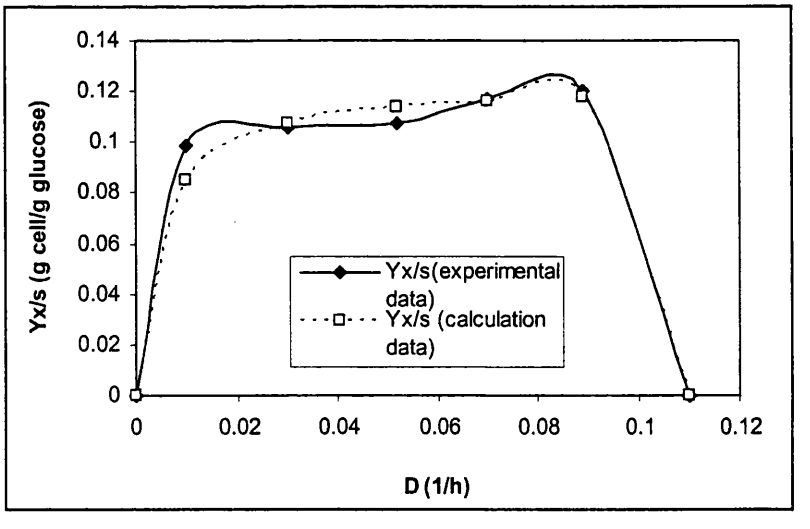


Figure 4-18 Observed biomass yield as a function of dilution rate during continuous culture of *L. brevis*
Calculation based on $Y_{x/s} = D/(D/Y_{x/s}^{max} + m_s)$, m_s=0.037g glucose/g cell/h, $Y_{x/s}^{max}$ = 0.124 g cell/g glucose

4.5.3.2 Maximum specific growth rate and substrate saturation constant

The reciprocal equation (4.23) $S/D = S/\mu_{max} + K_s/\mu_{max}$ is normally used for the graphically determination of the maximum specific growth rate and substrate saturation constant. Unfortunately, according to the experimental data of S and D, a plot of S/D versus S did not give a straight line, so both μ_{max} and K_s for *L. brevis* could not be obtained by this method. The data of μ_{max} obtained from batch culture (section 4.5.1) could be taken as the maximum specific growth rate (0.110 h^{-1}) for *L. brevis*. The K_s calculated by equation 4.23 is shown in Table 4.19. The K_s decreased as the D increased, this is an unusual observation. This may possibly be explained by adaptation of the cells to environmental conditions. At low dilution rates, the percentage of available ATP needed for maintenance purposes becomes high and energy status of the cells is relatively low, this in turn results in an increase in K_s as substrate transport systems become more passive as the high affinity active transport processes are shutdown. This is in contrast to *O. oeni* where there is a constant value for K_s (Appendix T).

Table 4.19 The effect of dilution rate on the substrate saturation constant

D (h^{-1})	0.010	0.030	0.052	0.070	0.089
K_s (g/l)	2.04	1.27	0.61	0.29	0.22

4.5.3.3 Biomass concentration and glucose uptake

The effect of dilution rate on the steady-state biomass and glucose uptake in the chemostat is shown in Figure 4-19.

Observed cell concentration slightly increased as the dilution rate increased in the range 0.010 to 0.089 h^{-1}, and also in accordance with the data calculated on Eq. (4.25): $x = D(S_i - S)/(D/Y_{x/s}^{max} + m_s)$. Glucose concentration slowly increased with the increase in dilution rate tested.

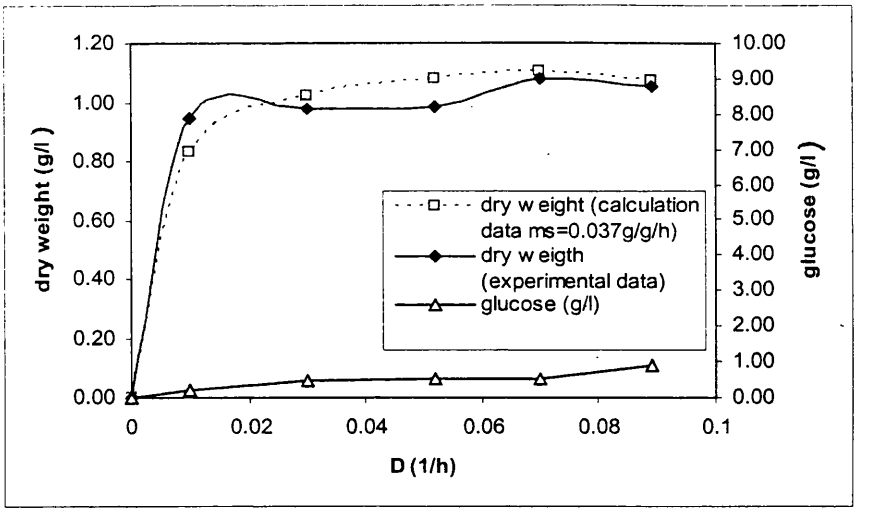


Figure 4-19 12 The steady-state cell and substrate concentration as a function of dilution rate in a chemostat when maintenance requirement is considered for the growth-limiting substrate.
S_i, glucose = 10 g/l, $Y_{x/s}^{max}$ = 0.124 g cell/g sugar, m_s= 0.037 g glucose/g cell/h

4.5.3.4 Volumetric biomass productivity

Volumetric productivity increased as the dilution rate increased in the range of 0.010 – 0.089 h^{-1} (Table 4.20 & Figure 4-20). These results observed were in accordance with theoretical calculation based on Eq. (4.31) $Q_x = Dx$ and (4.25) $x = D(S_i-S)/(D/Y_{x/s}^{max} + m_s)$ with the requirement coefficient being taken into account. When requirement coefficient was ignored (m_s = 0), the data calculated were higher than experimental data observed in the dilution rate range tested (Table 4.20).

These results for *L. brevis* are much larger than volumetric productivity for *O. oeni* with the maximum volumetric productivity of 17.6 mg/l/h (Figure 4-13).

Table 4.20 Volumetric productivity at various dilution rates

D (h^{-1})	Dry weight (g/l) (experimental data)	Q_x [a] (mg/l/h) (experimental data)	Q_x [b] (mg/l/h) (calculation data, m_s=0.037 g/g/h)	Q_x [c] (mg/l/h) (calculation data, m_s=0)
0.010	0.944	9.44	8.31	12.1
0.030	0.975	29.7	30.7	35.5
0.052	0.982	51.2	56.0	61.0
0.070	1.079	75.1	77.3	82.4
0.089	1.051	93.2	95.3	100.3

a. Eq. (4.31) $Q_x = Dx$ with maintenance requirement.
b. Eq. (4.31) $Q_x = Dx$ and (4.25) $x=D(S_i-S)/(D/Y_{x/s}^{max} + m_s)$, m_s= 0.037 g glucose/g cell/h, $Y_{x/s}^{max}$ = 0.124 g cell/g glucose.
c. Eq. (4.26) $Q_x = (S_i-S)Y_{x/s}^{max}$ when requirement coefficient is ignored (m_s = 0).

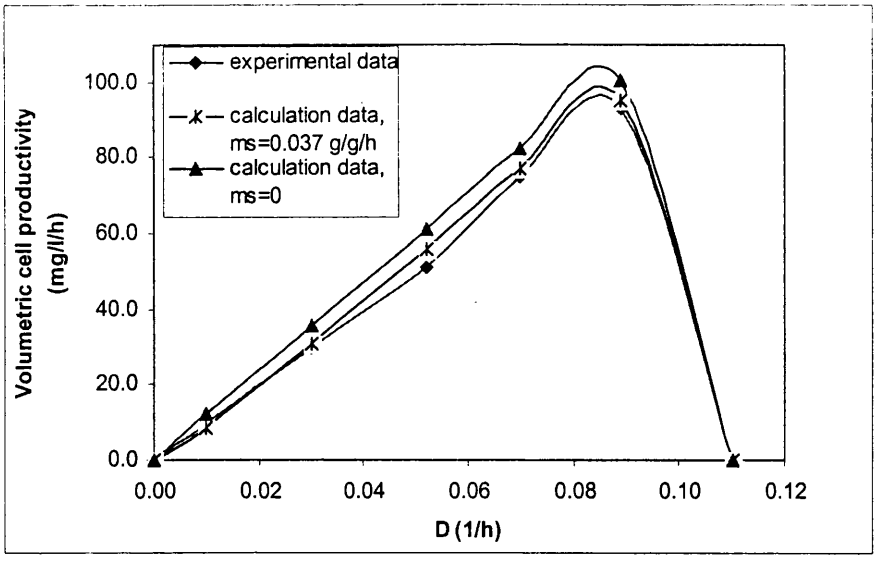


Figure 4-20 Comparison of calculation value and experimental data of volumetric cell productivity at various dilution rates. μ_{max} = 0.110 h^{-1}, S_i = 10 g/l, m_s= 0.037 g glucose/g cell/h, Y_{xs}^{max} = 0.124 g cell/g glucose.

4.5.4 Product formation

Lactate and ethanol were determined and the steady-state concentrations of products obtained at different dilution rates are shown in Figure 4-21 and also shown in Appendix V. CO_2 was not determined during the fermentation. Lactate decreased slowly as D increased from 0.010 to 0.089 h^{-1}. Ethanol maintained at about 1.3 g/l when the dilution rate was the variety of 0.010- 0.052 h^{-1} and then declined as D increased further.

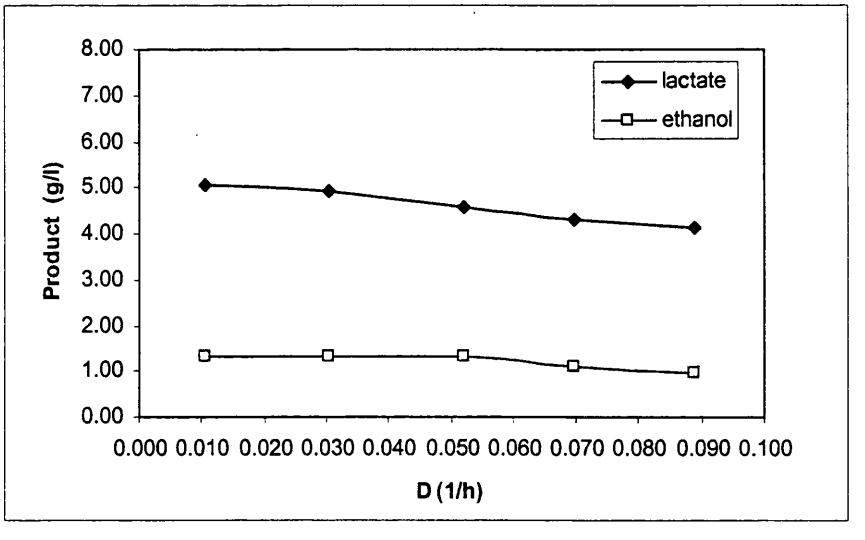


Figure 4-21 The steady-state concentrations of products at different dilution rates when the continuous culture of *L. brevis* in chemostat Feeding medium: Z broth with yeast extract 10 g/l and 10 g/l glucose; pH 5.5; Temperature 27°C

Table 4.21 shows the stoichiometric data for lactate and ethanol. With increasing D from 0.010 to 0.089 h^{-1}, lactate decreased gradually. Ethanol remained 0.55 mol/mol glucose when D increased from 0.010 to 0.052 h^{-1} and then decreased with the further increase of dilution rate.

Table 4.21 Product formation per molar glucose in continuous culture of *L. brevis*

D (h^{-1})	Glucose (mol)	Lactate (mol)	Ethanol (mol)	CO_2 [a] (mol)	C [b](mol) in cells	C% [c]
0.010	1.00	1.06	0.55	0.55	0.75	93
0.030	1.00	1.07	0.55	0.55	0.79	94
0.052	1.00	1.00	0.56	0.56	0.80	92
0.070	1.00	0.93	0.46	0.46	0.88	84
0.089	1.00	0.94	0.42	0.42	0.90	83

a. CO_2 is supposed to be associated with ethanol formation, 1 mol CO_2 per mol ethanol.
b. carbon content of cell dry-weight based on an assumption that the 50% dry weight is carbon.
c. carbon recovery (%).

In addition, molar biomass yields, $Y_{x/s}$ and Y_{ATP} relative to glucose, increased progressively from 17.8 to 21.6 g cell/mol glucose and from 16.8 to 22.8 g cell/mol ATP, respectively in the dilution rate range 0.010 h^{-1} to 0.089 h^{-1} (Table 4.22).

Table 4.22 Molar biomass yields in continuous culture of *L. brevis*

D (h^{-1})	mol ATP produced (mol ATP/mol glucose)	$Y_{x/s}$ (g cell/mol glucose)	Y_{ATP} (g cell/mol ATP)
0.010	1.06	17.8	16.8
0.030	1.07	19.1	17.8
0.052	1.00	19.3	19.3
0.070	0.93	21.1	22.6
0.089	0.94	21.6	22.8

The maximum ATP yield could be obtained from Eq. (4.35) as given in section 4.4.4

$q_{ATP} = D/Y_{ATP}^{max} + m_{ATP}$

The observed Y_{ATP} is g cell/mol of ATP used, the maximum Y_{ATP}^{max} is g cell/mol ATP, m_{ATP} is moles ATP required for maintenance/g cell/h and q_{ATP} is the specific rate of ATP utilization, mol ATP/g cell/h.

Therefore, if Y_{ATP}^{max} and m_{ATP} are relatively constant, a plot of q_{ATP} versus D gives a straight line with slope $1/Y_{ATP}^{max}$ and intercept m_{ATP}.

From Figure 4-22, $1/Y_{ATP}^{max} = 0.0405$, $Y_{ATP}^{max} = 24.7$ (g cell/mol ATP)

$m_{ATP} = 0.0004$ (mol ATP/g cell/h)

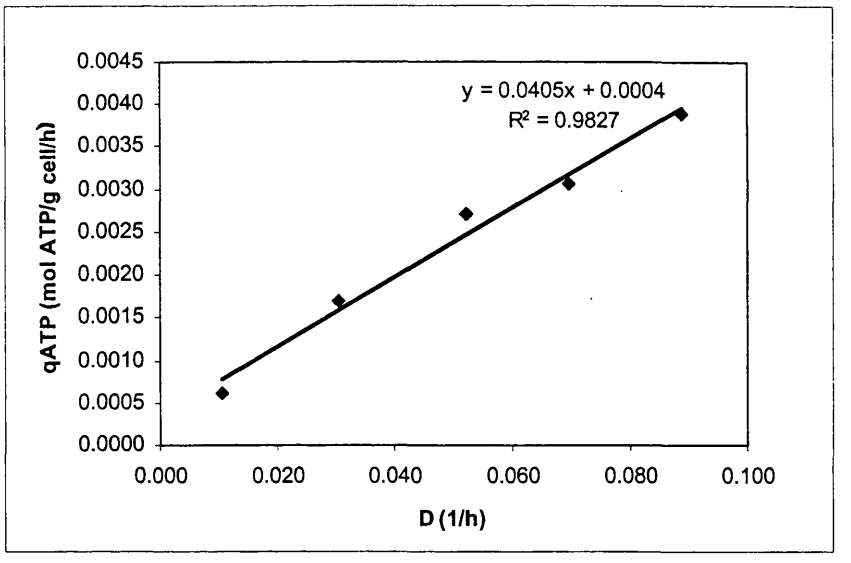


Figure 4-22 Graphical determination of the energy coefficient m_{ATP} and maximum Y_{ATP} yield using data from chemostat culture of *L. brevis* $Y_{x/s}^{max} = 0.124$ g cell/g glucose/h, $m_s = 0.037$ g glucose/g cell/h
Conditions: medium contained 10 g/l glucose, temperature 27°C and pH 5.5

4.6 Conclusion

In Chapter 3 studies were restricted to test tube culture so as to broadly characterize the growth and growth characteristics. These culture data also suffer from a lack of pH control. This chapter extends these studies to fermentor with pH control. The purpose of studying batch and continuous cultures was to characterize the kinetics of growth in some detail so that a comparison of the two organisms could be made. In batch culture the majority of the work was on *Oenococcus oeni* as much work of *L. brevis* has already been done by Donovan (2000). Continuous cultures for both organisms were studied in the same system to give a very good kinetic comparison. Also, from literature, no work has been carried out with these organisms in chemostat culture. From previous studies in Chapter 3, a mixture of glucose-fructose was employed as the carbon and energy source for the culture of commercial strain *O. oeni*. The effect of pH and of concentrations of glucose-fructose mixture on the growth of *O. oeni* was investigated in order to optimize cell starter cultures in 2 liter batch culture with Z broth (Table 2.4) under pH control (section 4.3.1 and 4.3.2).

These results are discussed as follows.

The results show that pH has influence on the growth parameters and growth kinetics of *O. oeni*. In the range pH 3.5 to 6.0, smaller specific uptake rate of glucose was observed than that of fructose (Table 4.1). Maicas[2] *et al.* (1999) and Salou *et al.* (1994) reported qualitatively that the rate of consumption of fructose is larger than that of glucose at pH 4.8 and 5.0, respectively. At low pH (pH 3.5), the specific uptake rate of glucose and fructose with 0.26 g sugar/g cell/h and was about half, compared with 0.52 g sugar/g cell/h at pH 4.5 (Table 4.1). This result is a corresponding increase in maximum specific growth rate (μ_{max}) with 0.024 h^{-1} at pH 3.5 compared with 0.066 h^{-1} at pH 4.5 (Table 4.2). At high pH (6.0), the specific uptake rate of glucose and fructose, 0.48 g sugar/g cell/h, is higher than 0.26 at pH 3.5 whereas lower than 0.52 at pH 4.5. Moreover, the specific growth rate of *O. oeni* was found to be related to the specific uptake rate of sugar. The maximum specific growth rate (μ_{max}) was obtained at pH 4.5 with 0.066 h^{-1}, doubling time 10.5 h; higher cell dry weight was also achieved at pH 4.5 than other pH (Table 4.2). Furthermore, both specific growth rate of *O. oeni* and specific uptake rate of sugar are also associated with the rate of ATP formation in the range pH 3.5 to 6.0 (Table 4.23 below). The higher the rate of ATP formation was, the higher the maximum specific growth rate. Finally, biomass yields, relative to the total sugar consumed, are nearly identical (7.4 ± 0.2 g cell/mol sugar) at pH 3.5-5.0 (Table 4.2 & 4.23). The data is slightly high compared with 7.1 g of cell/mol of sugar (Salou *et al.* 1994) and 6.89 g of cell/mol of sugar (Maicas[2] *et al.* 1999). These data are summarized below in Table 4.23.

Table 4.23 The effect of pH on the growth of *O. oeni*, specific rate of sugar uptake, rate of ATP formation and biomass yields*

pH	μ_{max} (h^{-1})	Q_s (g sugar/g cell/h)	The rate of ATP formation (mmol ATP/g cell/h)	$Y_{x/s}$ (cell/sugar) (g/g)	(g/mol)
3.5	0.024	0.26	1.06	0.040	7.2
4.0	0.056	0.51	1.25	0.040	7.2
4.5	0.066	0.52	1.93	0.041	7.4
5.0	0.060	0.53	1.73	0.042	7.6
6.0	0.031	0.48	1.17	0.024	4.3

* Q_s derived from Table 4.1; μ_{max} and $Y_{x/s}$ from Table 4.2; the rate of ATP formation from Table 4.6.

The products, including lactate, acetate, ethanol, glycerol, erythritol and mannitol, were produced by *O. oeni* during the fermentation of glucose-fructose mixture at different pH with pH control. From stoichiometric data of fermentation of glucose-fructose, it is seen that a small difference in lactate formation with 0.94 ± 0.04 mol/mol central sugar and acetate formation with 0.50 ± 0.01 mol/mol central sugar (glucose plus fructose not converted to mannitol) was observed between pH 4.0 and 5.0 (Table 4.5). However, at low pH 3.5, lactate and acetate production rates are low. This suggests that the enzymes involved in lactate production may be partially inhibited by low pH. Previous work (Ramos *et al.* 1995) described that *O. oeni* lactate dehydrogenase activity declined at low pH. At pH 6.0 lactate and acetate production are the lowest in a range pH 3.5 to 6.0, but correspondingly, ethanol and glycerol production are the highest. This result is probably due to the effect of pH on enzyme activities involved and indicates that too high pH (6.0) inhibits the lactate and acetate formation and benefits the ethanol and glycerol formation, but with a dramatically low carbon recovery of 63% compared to that of 97-98% in a range pH 4.0 to 5.0. The yield of ATP production is 10 ± 2 g/mol 'central sugar' at varied pH (Table 4.6). The value is in good agreement with that determined by Salou *et al.* (1994). The constant value reflects the fact that Y_{ATP} is independent of the nature of the organism and the environmental conditions, such as physicochemical parameters (pH, for example) and nutrient availability. Any increase in ATP synthesis stimulated anabolic reactions, the biomass yield is directly proportional to the ATP produced per mole of energy source (Pirt[2] 1975).

It is observed in this study that *O. oeni* produced small amounts of erythritol and glycerol anaerobically from the fermentation of glucose-fructose in addition to the end products, lactate, acetate, and ethanol. The amount of erythritol produced is the same level as glycerol produced (Table 4.4 & 4.5). The pathway of formation of erythritol from glucose in *O. oeni* was shown to involve the isomerization of glucose 6-phosphate to fructose 6-phosphate by a phosphoglucose isomerase, the cleavage of fructose 6-phosphate by a phosphoketolase, the reduction of erythritol 4-phosphate by an erythritol 4-phosphate dehydrogenase and, finally, the hydrolysis of erythritol 4-phosphate to erythritol by a phosphatase. This regulation of erythritol formation in *O. oeni* was studied and a similar observation was reported by Maria *et al.* (1993).

The influence of the concentration of glucose-fructose mixture on the growth of *O. oeni* in pH controlled batch culture was investigated. The results show that the optimal concentration was 5 g/l glucose-5 g/l fructose and 9 g/l glucose-9 g/l fructose for the growth rate and amount of growth of *O. oeni* (Table 4.8). At low concentration of mixture, glucose and fructose were almost consumed completely at the same period (Fig.4-4 a & b). As the concentration increased to total concentration of 18 g/l, the specific uptake rate was larger fructose than glucose (Table 4.7). The specific uptake rate of both glucose and fructose increased as the concentration increased. The specific rate of glucose uptake went down after fructose was consumed (Fig.4-4 c & d). The second growth phase was observed at total concentration of sugar more than 18 g/l (Fig.4-5). However, it is interesting that there is only small difference in growth rate of 0.065 h^{-1} in the first growth phase in a range of sugar concentration 9 to 36 g/l, a slight decrease (0.060 h^{-1}) at 90 g/l and a significant decline (0.025 h^{-1}) at 3 g/l. Biomass yield $Y_{x/s}$ is significantly reduced from 0.042 to 0.003 g cell/g sugar relative to the total consumption of sugar as the mixture concentration increased from 3 g/l to 90 g/l (Table 4.9).

The concentration of glucose-fructose mixture also had significant influence on the product formation (Figure 4-7). Figure 4-23 below clearly shows that the increase in acetate concentration was proportional to the increase in the substrate concentration, whereas lactate formation was inhibited at 36 g/l sugar, in particular inhibited markedly at 90 g/l sugar. Ethanol and erythritol also exhibits the significant inhibition at 90 g/l sugar. This result possibly indicates that enzyme activities involved or cell physiologies of these reactions are influenced by the substrate concentration or end products. The carbon recovery in the fermentation declines from 97% to 34% when glucose-fructose was 9 g/l to 90 g/l (Table 4.11), the production of other compounds could not be ascertained.

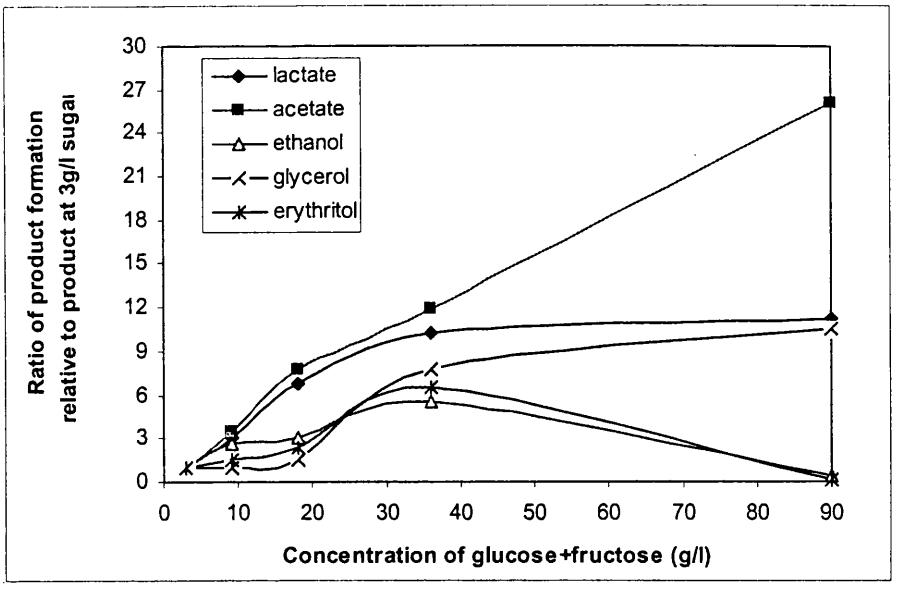


Figure 4-23 The relative amount of product formed at various concentrations of glucose-fructose mixture compared to that formed at 3 g/l of glucose-fructose

Assuming that no other energy forming reaction is occurring, the yield of ATP declined as the concentration of glucose-fructose mixture increased. This was different from results that Y_{ATP} remains 10 ± 2 g cell/mol central sugar in a range pH 3.5 to 6.0 shown in Table 4.6. It is probably due to a number of products, the uncoupling of ATP production from energy source dissimilation or changes in metabolic pathway or maintenance energy that influence $Y_{x/s}$ and then $Y_{x/s}$ will affect Y_{ATP} (Pirt[2] 1975).

The growth kinetics and yield parameters of *O. oeni* and *L. brevis* were evaluated by the continuous culture with pH control. A comparison of growth parameters and conditions of two strains is shown below in Table 4.24. The maximum specific growth rate of two strains was higher under pH control as compared to no pH control, while *L. breivs* has more benefit. *O. oeni* has lower μ_{max}, lower dry-weight than *L. brevis* in batch culture. Two strains were grown in continuous culture in a wide variety of dilution rates and *O. oeni* has lower volumetric cell productivity (Q_x), lower maximum biomass yield ($Y_{x/s}^{max}$) and lower maximum energy yield (Y_{ATP}^{max}), higher maintenance coefficient (m_s) and higher energy coefficient (m_{ATP}) compared with *L. brevis*.

Table 4.24 A comparison of growth parameters and growth conditions of two LAB strains

	O. oeni	*L. brevis*
μ_{max} (no pH control in test tubes) (h^{-1})	0.063	0.080
μ_{max} (pH control in batch culture) (h^{-1})	0.066	0.110
cell dry-weight formed [a] (g/l)	0.357	0.638
T optimum (°C)	25	27
pH optimum	4.5	5.5
Substrate [a] (g/l)	10 g/l	10 g/l
Q_x [b] (mg cell/l/h)	17.6	93.2
$Y_{x/s}^{max}$ (g cell/mol sugar) [c]	7.93	22.3
(g cell/g sugar)	0.044	0.124
m_s (mmol sugar/g cell/h)	0.45	0.21
(g sugar/g cell/h)	0.081	0.037
Y_{ATP}^{max} (g cell/mol ATP)	8.03	24.7
m_{ATP} (mol ATP/g cell/h)	0.0006	0.0004

a. 10 g/l (5 g/l glucose-5 g/l fructose) for the growth of *O. oeni*; 10 g/l glucose for *L. brevis*.
b. maximum volumetric cell productivity in dilution range: *O oeni* 0.007-0.052 h^{-1}, *L.brevis* 0.010-0.089 h^{-1}.
c. sugar refers to glucose-fructose mixture for *O. oeni*, glucose for *L. brevis*.

The end product of fermentation of glucose-fructose mixture by heterofermentative *O. oeni* includes (1) mannitol, lactate, acetate, ethanol determined; (2) glycerol, erythritol (data not shown) and CO_2 undetermined. Carbon recovery is in the range 62% to 87% in the D = 0.007 to 0.052 h^{-1}. This result is low, compared with 97% in batch culture under the same conditions. The lactate/(acetate+ethanol) ratio remains 1.2 ± 0.2 at all dilution rates tested. The end product of fermentation of glucose by *L. brevis* includes lactate, ethanol measured and CO_2 unmeasured. Acetate was not detected during the fermentation of glucose. Carbon recovery varied from 83% to 94% in the dilution rate range 0.010 to 0.089 h^{-1}. The lactate/ethanol ratio remains 2.0 ± 0.2 in the dilution rate range tested. It indicates that there is no change in the fermentation product profile over the growth rates studied.

Clearly there are significant differences between the growth kinetics of the two organisms and the choice of which system to apply to cider maturation very much depends on the conditions of the cider and the process environment in which maturation is to take place.

Chapter 5 Membrane Bioreactor System Studies

5.1 Introduction

A membrane bioreactor (MBR) has a separation capability lacking in many other types of bioreactor system. Combining various functions of membrane separations and the biocatalyst characteristics of enzymes, microbial cells, organelles or animal and plant tissues can generate quite a number of useful membrane bioreactor systems (Chang *et al.* 1991). The cell retaining property of membranes and selective removal of inhibitory by-products makes high cell density culture possible and generally makes more efficient use of enzymes, which leads to more highly product of bioreactors. Enzyme reactions utilizing cofactors and hydrolysis of macromolecules can be carried out in membrane bioreactors. Anaerobic cell culture may be efficiently carried out in membrane cell recycle systems, while aerobic cell culture can also perform well if correctly designed. In membrane reactors animal and plant cells have much successful opportunity due to the protective environment of the reactor and the small oxygen uptake rate of these cell (Chang *et al.* 1991).

In many microbial processes the desired products are the cells themselves. The cells often contain valuable compounds such as enzymes, lipids or cell-bound materials. An MBR is a device in which enzymes, organelles, microbial, animal and plant cells are retained by means of membranes for production of valuable materials, for processes such as wastewater treatment and for analysis used in biosensors (Jean-Marc 1988; Lee & Chang 1988, 1990; Chang *et al.* 1991; Donato *et al.* 1995; Yuya *et al.* 1998; Michele *et al.* 1999). An MBR also has been widely used in high concentration cell cultivation (Olle *et al.* 1985; Taniguchi *et al.* 1987; Borch *et al.* 1991; Hiroshi *et al.* 1991; Kenichi *et al.* 1992; Chang *et al.* 1994) in order to obtain desired products. Two typical examples are studies on ethanol fermentation using an MBR and on lactic acid production and there are many reports of such work (Vickroy *et al.* 1983; Ohleyer *et al.* 1985; Lee *et al.* 1987; Cho *et al.* 1991; Karel *et al.* 1991).

Although MBR are used widely nowadays, such processes are not without problems. Foremost of these is the deposit of dissolved and suspended solutes onto the membrane surface during operation.

In view of the complex nature of the cake formation process, the first stage was to determine the main factors responsible for the decline in performance by using cross-flow membrane microfiltration by LAB. Thus, the effects of pH of medium, the concentration of cells, pH of medium containing cells on membrane performance have been investigated. Based on the understanding of this membrane performance, the high density propagations of *O. oeni* and *L. brevis* in the MBR were then carried out. The fermentation system with continuous separation of inhibitory metabolites by cross-flow filtration was employed in order to improve the productivity of cell mass. The fermentation system allowed continuous removal of acids in end products, such as inhibitory lactic acid, whilst retaining the cells completely in the fermentor.

The cells produced can then be used in the process of cider maturation, in which microbes are used to rapidly transform the alcoholic and acidic raw material into a flavoursome drinks. As natural maturation can take up to three months the MBR is already used to try and speed up the process so enhancing process viability. So finally a study of the degradation of artificial 'green cider' by high density of *O. oeni* and *L. brevis* respectively, was carried out in the MBR system.

5.2 Membrane Performance

One of the main advantages of membrane separation techniques compared with centrifugation is more efficient separation of small cells. Moreover, a bioreactor, growing LAB, coupled with the cell recycling membrane process gives high cell viability and high permeate rate so as to remove lactic acid, the spent broth medium from the fermentation. In such a way a significant increase in lactic acid and cell mass production is possible as compared with other culture systems (Taniguchi *et al.* 1987; De Raucourt[1,2] *et al.* 1989; Anna *et al.* 2001). Work on organic acid production has underlined the importance of medium composition (Nagata *et al.* 1989), microbial strain (Junker *et al.* 1994) and the

type of membrane to efficient cross-flow filtration of cell suspensions. However, the available information is scarce concerning the optimization of operating conditions during microfiltration or ultrafiltration of LAB (Zhang *et al.* 1994; Xavier *et al.* 1995). A better management of the cell filtration operation is undoubtedly commercially important in improving membrane bioreactor performance (Boyaval *et al.* 1996).

The performance of the membrane within an MBR is extremely important to future development of a full scale plant, for example, knowing the permeation rate allows the upper limits of operation to be determined so enabling prediction to be made about how such a system might operate on a full industrial scale. This section focuses on the permeate flow rates through the membrane under different operating conditions, assessing the performance of the membrane in relation to cleaning and the fouling caused by LAB cultures.

5.2.1 Theory

Flux decline accompanied by increasing retention of materials expected to pass in the permeation, has been observed and reported by many researchers in the field of cross-flow microfiltration (Bowen *et al.* 1991; Bell & Cousins 1994; Anna *et al.* 2000). To an extent, this deterioration in membrane performance is irreversible, leading to the phenomenon known as fouling. Irreversible adsorption of materials to walls of the membrane pores and/or on the membrane surface, has been assumed to be the first stage leading either to pore plugging or to the building up of a cake layer in the membrane surface. The phenomena involved in fouling are complex and are not yet well understood and both theoretical and experimental work is needed in order to increase knowledge of this to progress our knowledge of CMF. In this section membrane fouling that influences performance of microfiltration membrane is discussed. The flux of liquid through a microfiltration membrane can be described by Darcy's law; this can be further subdivided into a series of reaction parameters (Boyaval *et al.* 1996).

$$Jv = TMP/\mu_L R \qquad (5.1)$$

Where Jv is the permeate flux, TMP is transmembrane pressure, μ_L is the dynamic viscosity, R is the hydraulic resistance.

$$R = R_m + R_f = R_m + R_{if} + R_{rf} \quad (5.2)$$

With: R_m : clean membrane hydraulic resistance

R_f: overall fouling hydraulic resistance

R_{rf}: reversible fouling hydraulic resistance (fouling removed with a water rinsing of the membrane); this resistance includes concentration polarization

R_{if}: irreversible fouling hydraulic resistance (fouling not removed after water rinsing).

When a cake builds up, R_{rf} can be defined as R_d, the hydraulic resistance of the deposit. R_d can be defined using:

$$R_d = \alpha M \quad (5.3)$$

Where M is the mass of deposited layer per unit of membrane area (kilograms per square meter) and α is the intrinsic resistance of the deposit and is defined by the Carman-Kozeny expression as used (Belfort 1979):

$$\alpha = 180\ (1-\varepsilon)/\rho_p d^2_p \varepsilon^3 \quad (5.4)$$

d_p is the mean diameter of particles being filtered, ρ_p is the particle density, and ε is the porosity of the deposit.

5.2.2 The permeate flux and transmembrane pressure - operation with water

The operation of an MBR with pure water was undertaken to give a benchmark flow rate for cleaning of the membrane reactor system after each operation. Once the reactor had been cleaned the performance of the membrane was measured with water and compared to the initial flow rates or fluxes first calculated. The MBR had been confirmed whether or not to be clear after and before a run. If the performance was significantly reduced, then the reactor required further cleaning.

All experiments were performed in the MBR system described in detail in Figure 2-5. Temperature was set at 25°C, transmembrane pressures (TMP) was first set at 5 psi and regulated by adjusting the diaphragm valve (Figure 2-10). TMP was increased step by step

up to a maximum value of 40 psi. At each given value of TMP, a flow rate of the filtrate was measured over 4 minutes by a measuring cylinder after a run of 1 hour (time assumed to be close to the steady state). The run took about one hour to get a steady state after changing the TMP, the following operation was identical unless otherwise specifically stated. At steady state, the permeate flux (J_v) was determined at various TMP. Pure water flux was determined in order to study the water flux recovery associated with fouling of the membrane.

Figure 5-1 shows the influence of transmembrane pressure on the permeate flux with pure water. By increasing the TMP by 40 psi, the flow rate of fluid through the membrane was increased by up to 64.56 l/h (0.497 $m^3/m^2/h$) by using pure water as a fluid, which was 11 fold higher than that at 5 psi. The flux was increased as the TMP increased. The flux of pure water is pressure dependent, responding linearly to an increase. From linear regression equation the relationship between flux and pressure was obtained: $J_v = 0.0126 \times$ TMP, where J_v is the flux ($m^3/m^2/h$), TMP is transmembrane pressure (psi). The flux obtained is the actual flux expected when the membrane is very clean, which act as an indicator whether the membrane is clean.

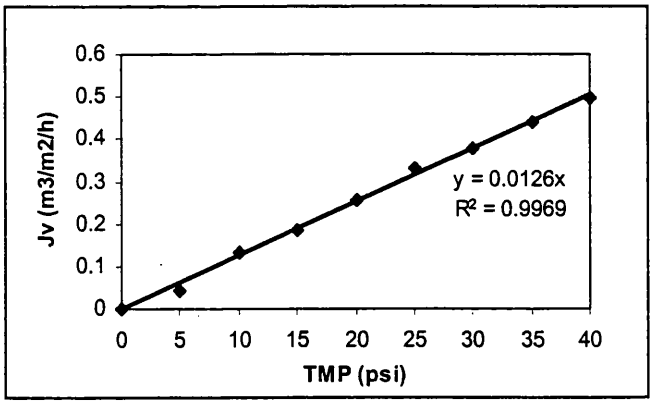


Figure 5-1 Flux obtained during the operation of MBR with pure water

5.2.3 Effect of medium pH on the membrane performance

The effect of media pH on the membrane flux was investigated so as to assess how the fouling of membrane changed over the range of pH 3-7 in the growth medium of *O. oeni*. Firstly, 10 l of the medium was prepared and then adjusted pH to 3, 4, 5, 6 and 7

respectively. The medium was then added to the MBR system and recycled for 1 hour at pressure of 5psi and the membrane flux was measured three times. Secondly, the pressure was increased from 5psi to 10psi, recycled for one hour the flux was measured three times under TMP given. Finally, the flux was determined at different TMP. The membrane flux for each pH condition over the pressure range of 5psi and 40psi were measured and the mean results are given below in Table 5.1.

Table 5.1 The effect of medium pH on the flux at different TMP

TMP (psi)		5	10	15	20	25	30	35	40
flux Jv ($m^3/m^2/h$)									
pure water		0.044	0.130	0.184	0.255	0.332	0.378	0.442	0.497
growth medium (pH)	3.0	0.045	0.114	0.165	0.197	0.230	0.267	0.296	0.326
	4.0	0.042	0.111	0.162	0.200	0.230	0.263	0.299	0.333
	5.0	0.038	0.099	0.156	0.193	0.229	0.263	0.299	0.324
	6.0	0.035	0.107	0.170	0.216	0.241	0.275	0.303	0.323
	7.0	0.043	0.107	0.168	0.209	0.245	0.270	0.299	0.332

The data are also presented in Figure 5-2 where cell growth medium is compared with pure water. Pure water as shown above (Fig. 5-1) gives a linear relation between pressure and flux. However, this is not observed with the medium which showed a non-linear increase in flux with pressure presumably as the degrees of fouling increased. For example, at pH 3.0 the medium is compared with pure water the flux decreased 34% at 35-40 psi. Similar results were obtained with other media over the range of pH. As little variation in the permeate flux was observed under the different pH of the medium and suggests that medium pH in the absence of cells had no apparent effect on the flux. From the practical view of point, these results suggest that a low TMP should be maintained so as to reduce the membrane fouling during the operation, although high TMP could improve the flux to some extent.

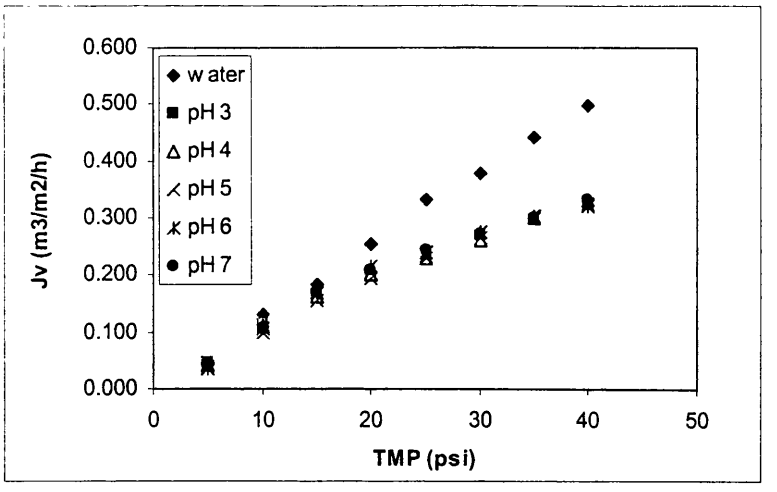


Figure 5-2 Flux obtained at different pH medium at various TMP
Medium components: yeast extract 5g/l, KH_2PO_4 5g/l, NH_4Cl 2g/l, glucose 5g/l, fructose 5g/l, trace element 20ml/l. autoclaved at 121°C for 15 minutes.

5.2.4 Effect of cell suspension on the membrane performance

5.2.4.1 Cell suspension preparation

O. oeni was used throughout this section (5.2.4.1) for the measurement of membrane performance. 100 liters fermentation was carried out at pH 4.5 and temperature 25°C and cells were harvested by microfiltration at the end of the exponential growth phase (section 2.14). 6.5 liters of concentrated cells, OD 13.84, were obtained and stored in a refrigerator prior to use.

The cell suspension had the following components: 2.9 liter concentrated cells of OD 13.84 and 1.1 liter distilled water mixed in 5 liter plastic jar, pH was adjusted to 4.0 with 1M HCl. A cell concentration of 4.4 g/l (OD 10) was obtained. The flux was measured at each given TMP.

The effect of cell concentration was investigated by making solutions of different cell concentrations. A known concentration of concentrated cells was diluted with distilled water at pH 5.0. A range of OD was from OD 1.4 to 19.7. Flux, J_v, was determined at each given concentration.

The effect of pH of medium containing cells was also investigated. Here quantities of the stock cells were diluted with water to OD 10 and the pH adjusted to the required pH using 1M HCl, the membrane flux was measured at pH 3.0. Similarly, experiments were carried out at pH 4.0, 6.0 and 7.0, respectively. J_v was measured at each given pH. After each experiment the equipment was cleaned to obtain original flux.

5.2.4.2 Effect of medium containing cells on the membrane performance

The effect of medium containing cells (OD 10) on the membrane performance is presented in Figure 5-3. The flux increased with the increase of TMP when the TMP was at or below 10 psi, whereas the flux had variation when the TMP was more than 20 psi. For each TMP increment, the flux rose up to a maximum value at TMP = 20 psi, and the flux was not further improved by TMP increase. The almost-steady flux obtained at each TMP increment, was around 0.128 $m^3/m^2/h$ (Figure 5.3). This implies that the flux depends on the TMP at low TMP (< 20 psi) and was independent of TMP (TMP>20 psi). This indicates that the higher the transmembrane pressure was, the more serious the membrane fouling. Hence, TMP was proposed to be within the range of low pressure to reduce the membrane fouling. The TMP should be as low as possible provided the rate of permeate can meet the level required.

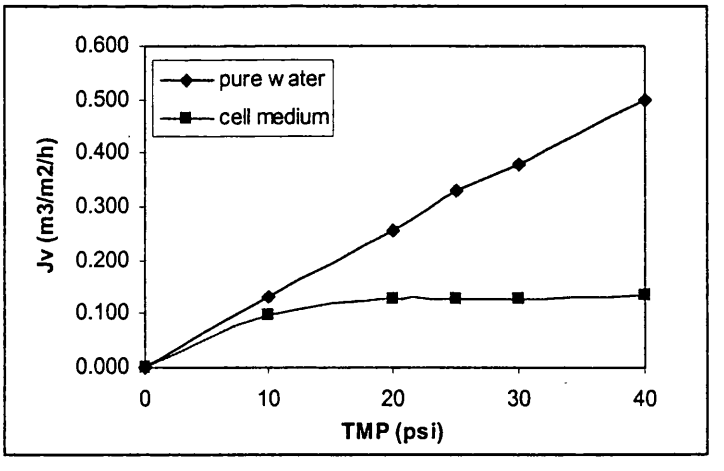


Figure 5-3 Flux obtained with medium containing cells (dry weight 4.4g/l) in pH = 5.0, Jv were measured after 1 hour at each given TMP value.

A comparison of the decline in flux with different fluids (Figure 5-2 compared to 5-3) is shown in Figure 5-4. Clearly, the flux declined the growth medium and medium

containing cells were compared to pure water. Among these three fluids, the addition of cells to the medium resulted in serious membrane fouling. The fouling observed in the three system shows that both the medium and cells cause fouling and that once a cell cake has built up the flux is largely governed by this and not TMP.

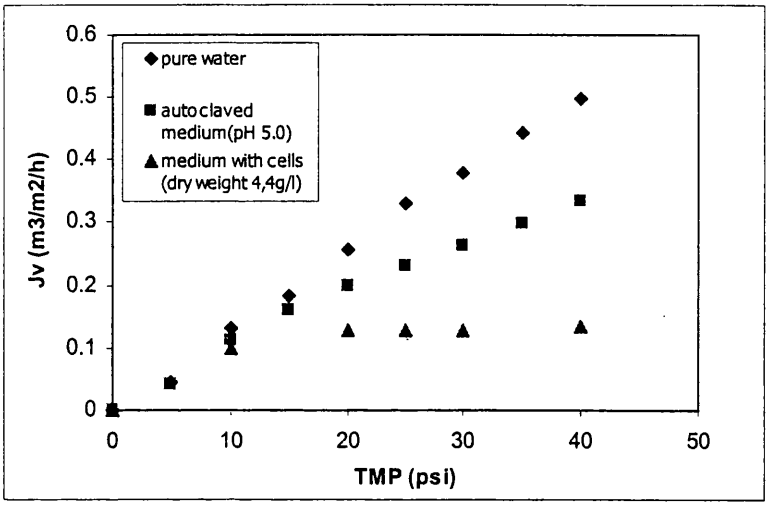


Figure 5-4 Comparison of fluxes of pure water, growth medium and medium containing cells

5.2.4.3 Effect of cell concentration on the membrane performance

The effect of cell concentration on membrane performance was investigated by increasing cell concentration (as measured by OD) at a constant TMP. Figure 5-5 shows the results of the study. The figure shows that the flux is slightly depended on cell concentration with the exception of low cell concentration (<1g/l). The flux changed rapidly at cell concentration from 0 to 1 g/l, and then was only affected slightly with a further increase in cell concentration above 1 g/l. This result has significance in the practical utilization during the operation of an MBR and indicates that the increase in cell concentration did not result in a significant increase in the membrane fouling within the range of 1- 8.8 g/l cell dry weight (OD 2.5 – 20).

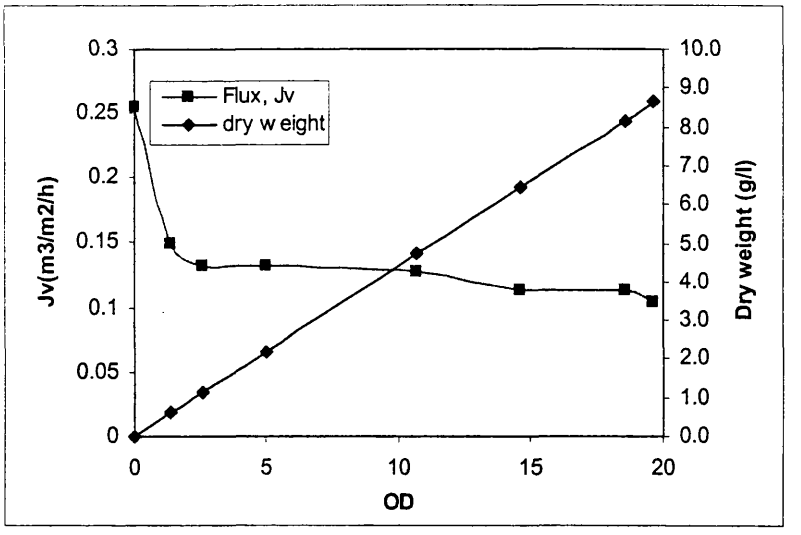


Figure 5-5 The effect of cell concentrations on membrane permeate flux.

TMP = 20psi, pH = 5.0. *Jv* was measured after 1 hour at a given cell concentration

5.2.4.4 Effect of pH of medium with cells on the membrane performance

The dependence of the membrane flux on pH of the medium containing constant cell concentration is shown in Figure 5-6. The flux gradually increases from pH 3 to 7. The lowest flux was 0.076 $m^3/m^2/h$ at pH 3, which was only 50% of the flux at pH 7. It indicates that loss of flux was slightly more rapid at low pH possibly due to some change in the physiology of the microorganisms or the cell surface chemistry.

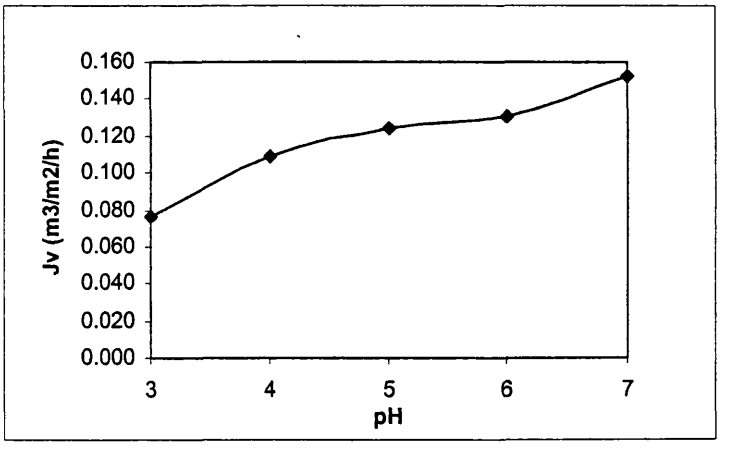


Figure 5-6 The effect of pH of filtration medium containing cells (4.4g/l dry weight) on permeate flux (TMP = 20psi, *Jv* was measured after 1 hour at given pH).

5.2.5 Fouling mechanism

Membrane fouling can be divided into two different types: a reversible one and an irreversible one. Irreversible fouling could be attributed simultaneously to the adsorption and entrapment of fermentation broth components. Such as yeast extract, sugars, mineral salts, cell debris (such as cell walls, nucleic acids, etc.), and cells. These components have been shown to be foulants of ceramic membrane (Nagata *et al.* 1989).

Because cells were totally rejected by the membrane, due to their size, compared to the largest pore size of the membrane (0.20 μm), it was likely that reversible fouling was as a cake of microbial particles accumulated on the membrane surface. In addition, some molecules (such as proteins, peptides, salts, etc) could induce concentration polarization and participate to form the reversible fouling, but this effect was assumed to be insignificant (Boyaval 1996).

As to the flux with pure water, the dynamic viscosity μ_L and the hydraulic resistance R were assumed to be constant, from equation (5.1), J_v increased linearly with the increase of TMP. The increase of TMP resulted in the increase of overall fouling hydraulic resistance R_f most probably due to the presence of yeast extract absorbing on to the surface of the membrane. Comparing medium containing cells to medium alone, cells were easier to absorb and plug the pore, leading to the larger overall fouling R_f compared to R_f without cells, so reducing J_v when cells are present than with autoclave medium alone at 10 psi.

When a cake builds-up, thus $R_{rf} = R_d = \alpha M$ (equation 5.3). A cell cake was compressible when TMP increased, both the intrinsic resistance α and the mass per unit area M (kg/m^2) were supposed to increase, so reversible fouling hydraulic resistance R_{rf} increases (equation 5.3). Moreover, entrapment of medium components within the cake structure increased M (Arora & Davis 1994), and reduced the porosity of the deposit ε, leading to a further improvement in α (see equation 5.4 section 5.2.1). The thicker and more disorganized deposit allowed more macromolecules from the medium to be trapped and to compact the cake, leading to a fast fouling increase. Thus the enhanced TMP could not result in the further increase of J_v (Figure 5-3). After the cake growth had stabilized, M

was assumed to be unchanged and ε to be reduced to some extent mainly due to particle rearrangement from disorder to order-state rather than the net deposit of additional cells within the cake, so reversible fouling hydraulic resistance R_{rf} (defined as the hydraulic resistance of the deposit $R_d = \alpha M$) increased, leading to the decrease of J_v, but the flux declined rather slowly at an OD above 2.5 (Figure 5-5). The change in pH of medium containing cells causes two significant changes: (1) the change of physical structure of the cell and surface of bacteria and (2) changes the properties of the membrane surface. It seems from the data above that higher pH reduces the influence of cell fouling layer as J_v increased with the higher pH. The flux was not pH dependent with medium alone but due to changes in either cell-cell interaction within the cake or cell-membrane interaction at the membrane surface.

5.3 High density Cultivation of *O. oeni* in MBR

For high productivity in microbial processes a high cell concentration in the fermenter is normally desired. However, some microorganisms produce growth inhibitory substances that are excreted to the medium and in such cases it may thus be difficult to achieve a high cell density. If, however, conditions are set to remove inhibitory products a considerable increase in cell productivity is possible.

LAB are typical of microorganisms that excrete inhibitory metabolites (lactic acid) while growing. These organisms are also very valuable to the food process industries for food fermentation as starter cultures. This is especially true in dairy processing where the direct inoculation of milk is challenged by indigenous microbes. They are also important in alcoholic beverages where LAB starter cultures are used to enhance mature speed and reliability.

Microfiltration is one of the most promising methods of removing inhibitory metabolites from bacterial cultures. High density cell cultivation of LAB in MBR has been reported (Masayuki *et al.* 1987; Bibal *et al.* 1991). The fermentation system allowed continuous removal of lactate, while retaining cells completely in the fermenter. By maintaining a low

concentration of lactate in the medium, a high cell concentration (88 g/l dry weight) was achieved (Bibal *et al.* 1991).

After intensive cultivation, an MBR charged with a high density of cells of particular microbial species can be used to carry out biotransformation such as MLF. In this section, LAB serve as biocatalysts which conduct biochemical transformations in the absence or presence of cell growth. Such bioreactor systems offer many advantages over traditional batch fermentation and these include very rapid and predictable reactions, continuous operation and reuse of biocatalyst (Belfort *et al.* 1989; Mehaia & Cheryan 1990; Divies *et al.* 1993). High densities of resting cells of *O. oeni* have been used to catalyze the biotransformation of L-malate to L-lactate in wine (Lafon-Lafourcade *et al.* 1975). However, high density cultivation of *O. oeni* and *L. brevis* in an MBR has not been reported in the literature. In the present study, high cell cultivations of these two organisms were undertaken in the MBR with complete cell recycle.

5.3.1 Materials and methods

Microorganism and inoculum medium *O. oeni* 11648, a heterofermentative lactic acid bacterium, was used in the investigation. For cultivating the inoculum, the medium used is shown in Table 2.4, Z broth (Chapter 2) contained 10 g/l glucose-10 g/l fructose. pH was adjusted to 4.5, autoclaved at 121°C for 15 minutes. The medium was used in 0.4 l, 4 l and 20 l batch cultures.

MBR feeding medium The feeding medium for growth of cells in the MBR is shown in Table 5.2. All materials were dissolved in distilled water; pH was adjusted to 4.6-4.7, stored in glass carboys. This medium was autoclaved at 121°C for 25 minutes and cooled for use. The concentrations of medium components were twice higher than those used in both batch and continuous cultures previously studied (Chapter 4) whilst in some cases different ratios of glucose-fructose were used (Table 5.2).

Analysis Samples were periodically withdrawn through the sample port at duration of fermentation. After diluting these samples, cell concentration was determined by measuring optical density at 660nm (OD). Each time 15 ml of sample of permeate was

collected and stored at -20°C until analyzed. The concentrations of glucose, fructose, mannitol, lactate, acetate were determined by HPLC. Ethanol was analyzed by GC.

Table 5.2 The growth medium composition

Composition	Concentration [d] (g/l)		
	Experiment 1	Experiment 2	Experiment 3
Yeast extract [a]	16	16	16
KH_2PO_4	6	6	6
Trace element [b]	20ml/l	20ml/l	20ml/l
Glucose [c]	14	12	10
Fructose	6	8	10

a. Yeast extract, salt-free, 25kg package, Bio Springer.
b. See Table 2.3, doubled $MgCl_2 .6H_2O$ and $MnCl_2 .4H_2O$.
c. D-glucose monohydrate, 25kg Packages, Cargill.
d. The same materials will be used in the following experiments, but composition and concentrations are subject to each experiment requirement.

Setting the feeding rate for the MBR The specific growth rate (μ) was found to be 0.066 h^{-1} from batch culture (Chapter 4), a doubling time 10.6 h and this value was used to estimate the feeding rate required to obtain the desired increase in cell mass. Prior to start of feeding medium in the MBR, the OD was around 6, so dilution rate should be approximately six times higher than this value of 0.066h^{-1} and was 0.4h^{-1}. Feeding rate therefore was approx equal to 2.4 l/h (0.40h^{-1} multiplying volume of 6.0l). If doubling time was calculated in 11 h, then feeding rate should be 4.8 l/h from 11 to 22 h and 9.6 l/h at 22 to 33 h when the medium contained 5 g/l glucose-5 g/l fructose. In the MBR system a maximum permeate rate was 15.6 l/h with distilled water at membrane inlet pressure 10psi and 25°C (no show of data). Considering the cell fouling of membrane, a feeding rate of 9.6 l/h could not be achieved in the apparatus as the membrane area was too small. Therefore, the concentration of medium components was doubled in order to halve the feeding rate and to meet the nutrient requirements for the increasing cell mass. The details of the feed medium components are shown above in Table 5.2.

5.3.2 The MBR fermentation system

A diagram of the MBR system is shown in Figure 5-8 (details refer to section 2.13 Chapter 2). The headspace of the medium in the vessel was continuously sparged by N_2 gas from a nitrogen (oxygen-free) cylinder. A steam-sterilizable pH electrode fixed in the stainless top pipe and connected to a pH controller (Electrolab Ltd). This was used to

measure the pH of the culture medium and the pH controller automatically controlled the titration of alkali (6M NaOH). The pH was set at 4.5 for the propagation of *O. oeni* or 5.5 for *L. brevis*. Temperature was set at 25°C. Foaming was controlled by adding the small amount of antifoam, 3ml of polypropylene glycol autoclaved prior to addition. It was injected manually through sampling port into the fermentor when needed.

The vessel was sterilized by autoclaving at 121°C for 30 minutes and, the other parts of the MBR system were sterilized by directly passing steam through the system in situ before use. After sterilization, the vessel and the piping of the membrane unit were then assembled. Sterile medium or LAB cultures could then be added to the system as described below.

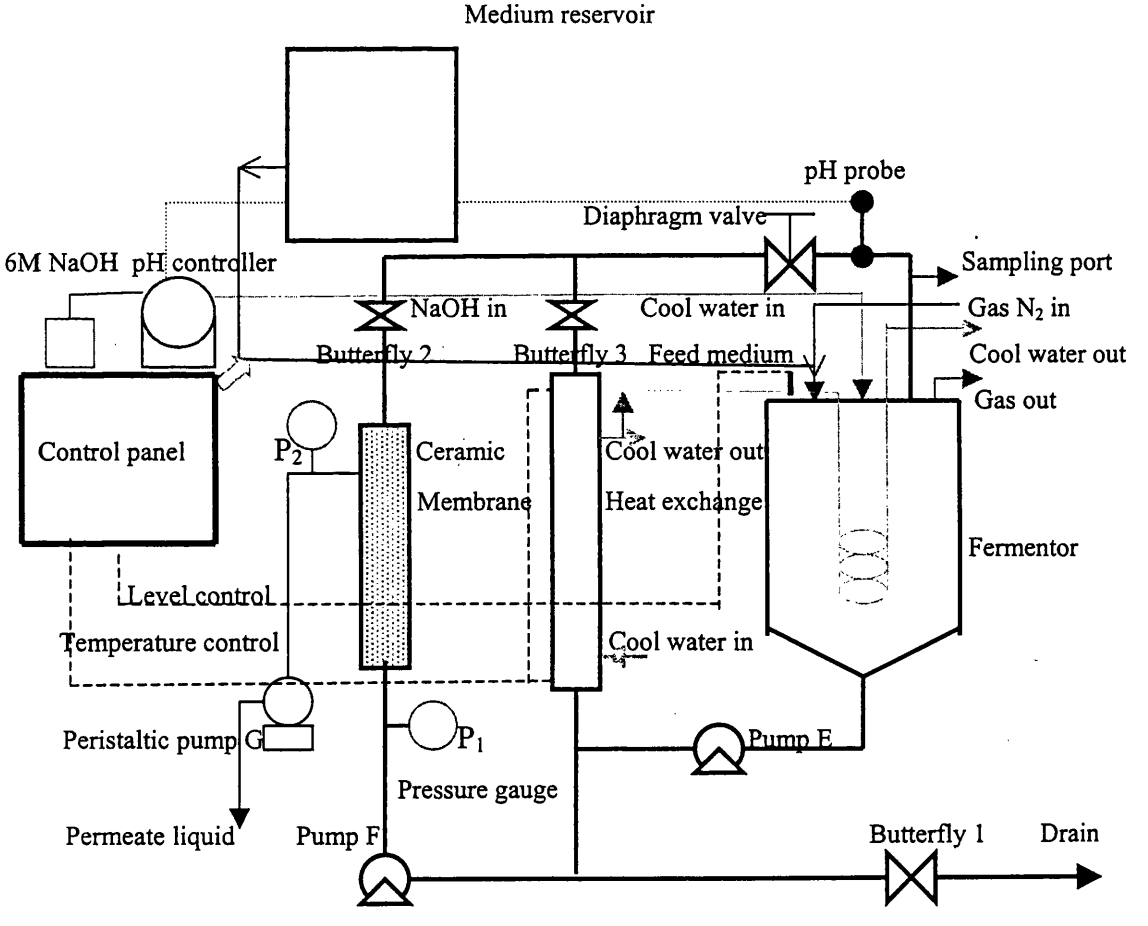


Figure 5-8 schematic diagram of the MBR with complete cell recycle

Photo of Figure 2-8 in section 2.13 of Chapter 2.

5.3.3 Propagation of *O. oeni* with complete cell recycle

40 l (two of 20 l) batch culture (prepared as in section 2.11.1 Chapter 2) was first transferred to the vessel through a feeding pipe by gravity feed. As the added medium was filtered, more of the batch culture was added until all 40 litres had passed into the system. This step effectively concentrated the cells by a filtration by about a factor of over 6. Once the 40 l batch culture was added to the fermentor, a sample was withdrawn to measure OD and this was taken as initial OD at time of zero hour. The permeate rate was then initially set at 1.2 l/h (see section 5.3.1) and was controlled by using a peristaltic pump (pump G). At this stage fresh medium was added to maintain a constant volume made to the system via the level control of gravity feed. The cells were circulated from the fermentor via the ceramic membrane unit and heat exchanger and returned to the fermentor using a large magnetically centrifugal pump. The permeate rate could be changed with the stepwise change in a speed of the peristaltic pump. Under these conditions, the membrane permeate rate was equal to the feeding rate of growth medium from medium reservoir.

5.3.4 MBR culture using 14 g/l glucose:6 g/l fructose

The first experiment investigated the growth of *O. oeni* with 14 g/l glucose-6 g/l fructose mixture. Samples were taken periodically from the reaction system and the optical density, the concentration of products and substrates were determined. The results are shown in Figure 5-10 and 5-11.

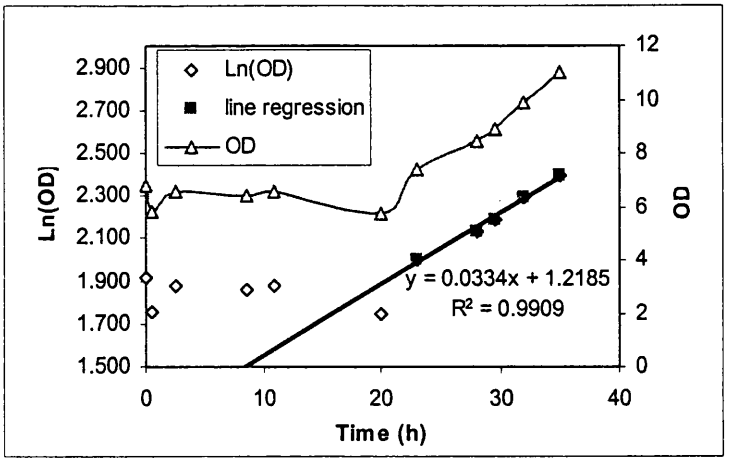


Figure 5-10 Growth curve of *O. oeni* in the MBR with complete cell recycle
A line is a linear regression of the plots of Ln(OD) vs. time, μ_{max} 0.033 h^{-1} was obtained in the medium containing glucose 14 g/l- fructose 6 g/l

After a long lag period of 20 h (Figure 5-10), the cells started growing, and the μ_{max} of biomass formation was 0.033 h^{-1} (calculated by linear regression). Cell productivity of 0.133 g cell/l/h was achieved in the logarithmic growth phase from 23 h to 35 h. The final OD was 11, an equivalent dry cell weight (DCW) was 4.8 g/l.

Time-courses of the concentration of biomass, glucose, fructose and mannitol are shown in Figure 5-11. Glucose concentration was found to be in the range of 4.5 g/l-9.0 g/l during the whole period of cell culture. Glucose gradually rose from about 5 h to 20 h, during this period cell growth and activity were low. Glucose then declined from 8.8 g/l at 20 h to 4.8 g/l at 28 h as the cells actively grew. After 28 h a subsequent phase, glucose rose again due to the higher feeding rate. Fructose concentration was below 1g/l throughout the progress of the culture and could not be detected in many samples taken from the fermentation.

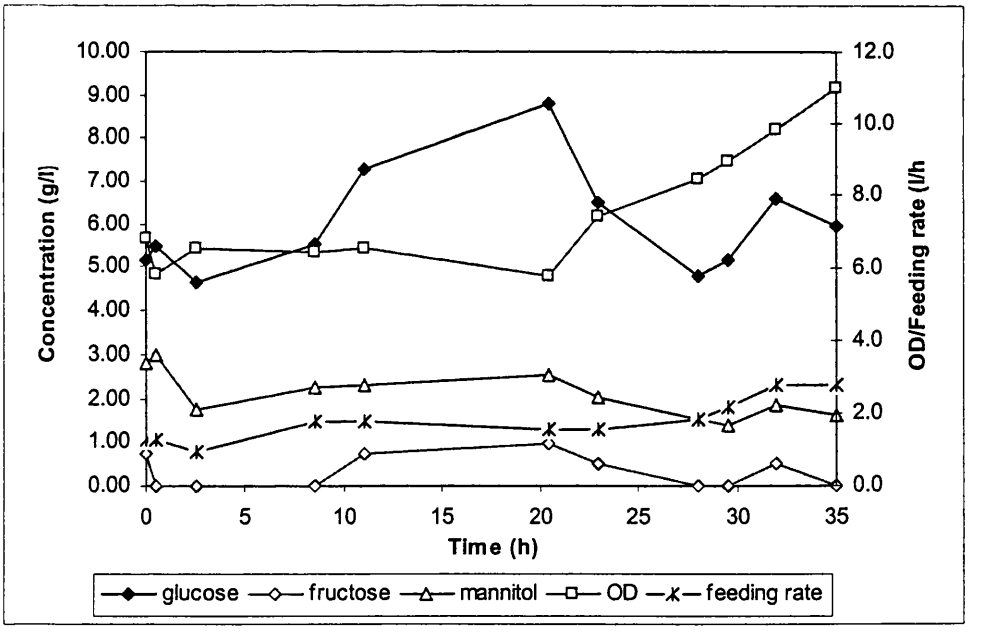


Figure 5-11 The high concentration cultivation of *O. oeni* in the MBR with complete cell recycle
Temperature 25°C, pH = 4.5, feeding medium: glucose 14 g/l, fructose 6 g/l

From the above results, this experiment was not very successful due to the long initial lag phase of 20 hours. The main reason for this was the possibility that the system was not properly anaerobic and this would influence significantly the growth and activity of *O. oeni*. In addition, residual glucose concentration was high and so initial concentrations of

glucose and fructose were then appropriately adjusted for the next run. From these results about equimolar proportions of glucose and fructose were consumed so the relative level of glucose was reduced.

5.3.5 MBR culture using 12g/l glucose: 8g/l fructose

The experiment above was then repeated with a change in the ratio of glucose to fructose in the medium. 12:8 of glucose: fructose, otherwise the starting concentrations of other medium components were the same.

5.3.5.1 Cell growth and substrate uptake

Figure 5-12 shows the growth of *O. oeni* in the MBR with complete cell recycle. Cell density increased from initial OD 5.5 to maximal OD 28 at 56 hours, and then the cell growth stopped. Specific growth rate of biomass formation (μ) was 0.035 h^{-1} (doubling time near 20 h), a little higher than in the previous experiment (section 5.3.4). The *O. oeni* grew at maximum specific growth rate of 0.035 h^{-1} from start to about 42 h with an overall reactor cell productivity 0.18 g cell/l/h. These data indicate that it would be possible to maintain the growth rate for longer periods providing there is plenty of nutrients supplied. A cell productivity of 0.175 g cell/l/h was obtained if the whole fermentation time from 0 to 56 h, is taken into account.

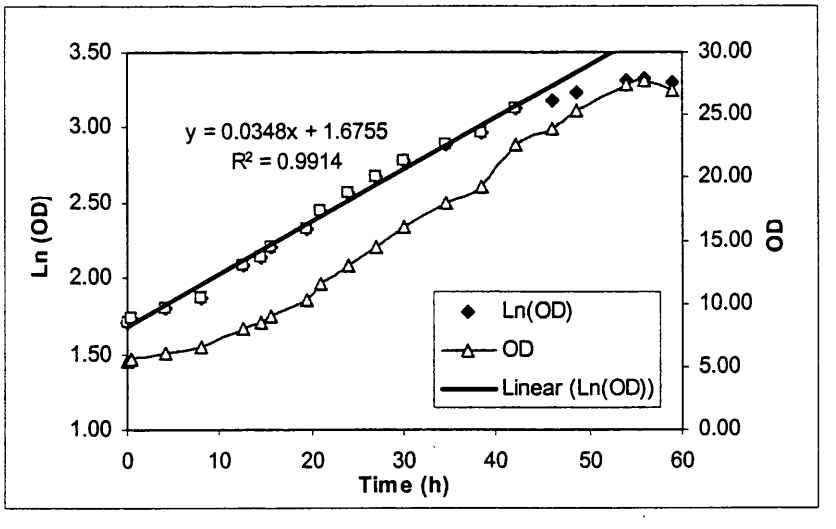


Figure 5-12 Growth curve of *O. oeni* in the MBR culture with complete cell recycle
Feeding medium: glucose 12 g/l, fructose 8 g/l

Figure 5-13 (a) shows the glucose, fructose and mannitol concentration with time. Using this feeding strategy the glucose was maintained in the range of 5.5 g/l to 3 g/l. However, the glucose fell below 2 g/l at the end of the fermentation. Fructose was almost completely consumed throughout the whole run with only small levels detected in the early stage of the fermentation. Over the majority of the fermentation, the ratio of glucose-fructose consumed was around 1:1 (around 8:8 g/l of each). Mannitol produced was in the range 1.5 g/l to 2.5 g/l. Once the glucose had declined below 1 g/l, the cell growth was slow or had stopped. It was estimated that the higher cell density could be achieved if an adequate

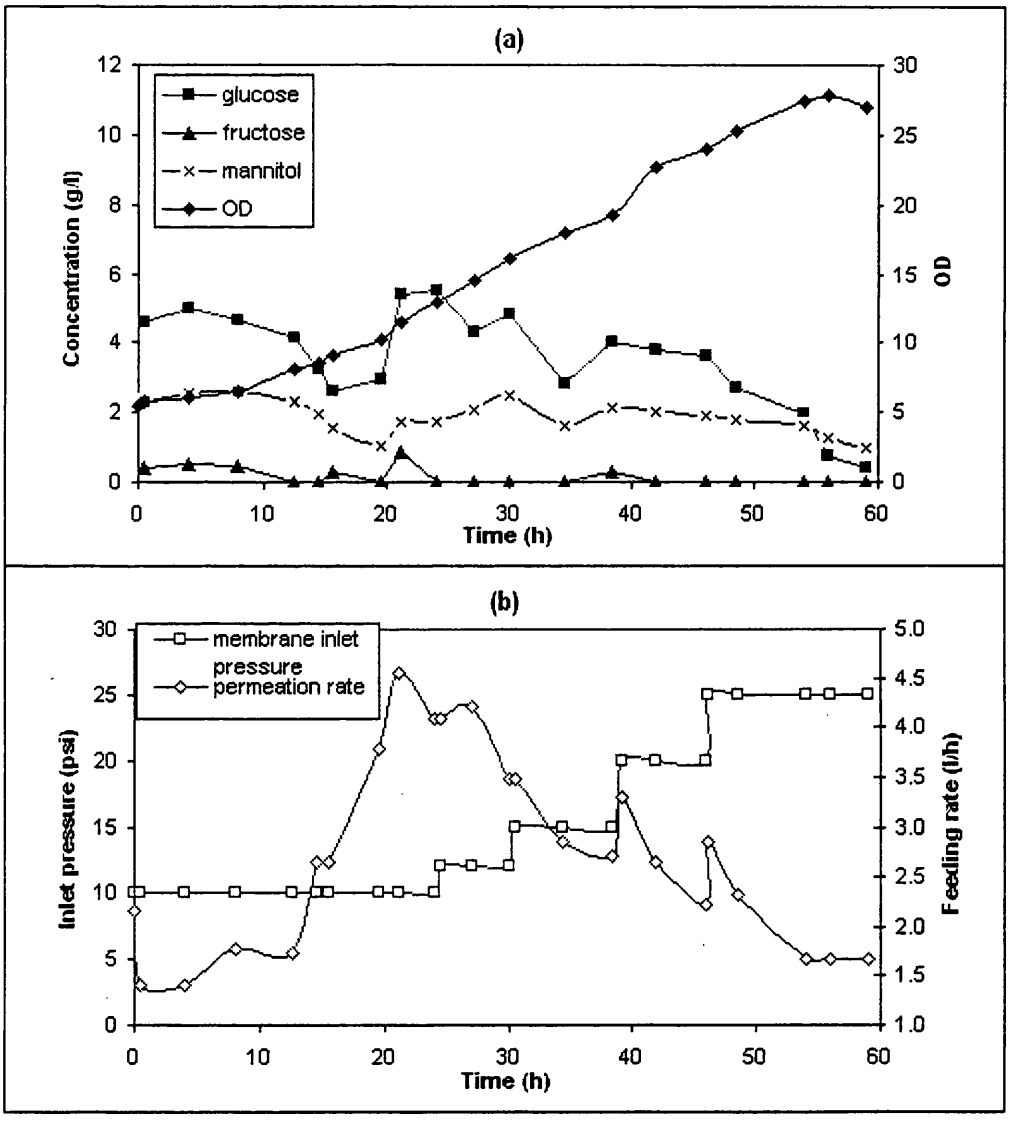


Figure 5-13 Time-courses of glucose and fructose and the change in feeding rate and membrane inlet pressure during cultivation of *O. oeni* in the MBR with complete cell recycle
Temperature 25°C, pH = 4.5, feeding medium glucose 12 g/l, fructose 8 g/l

amount of glucose and fructose are supplied and that it would not be significantly inhibited by the formation of lactic acid as this was removed from the MBR.

5.3.5.2 Relationship between permeation rate and membrane inlet pressure

In order to provide enough nutrients for the growth of *O. oeni* while not to waste fresh growth medium, it was necessary to control the feeding rate (via the permeate flow rate). By adjusting membrane inlet pressure (MIP) (pressure gauge P_1 in Figure 5-8) or adjusting the speed of peristaltic pump G (Figure 5-8), a membrane permeate rate could be controlled, and as a consequence the feeding rate was also controlled via the level sensor. The MIP was initially set at 10 psi and the speed of pump G was used to control the permeate rate.

Figure 5-13 (b) above shows the permeate profile and MIP. The permeate rate (feeding rate) was controlled up to 4.6 l/h at 21 h under the constant MIP of 10 psi. However, at high cell biomass concentration the permeation rate was limited by MIP rather than the permeate pump rate (peristaltic pump G) and so declined to 4 l/h at 24 h. The permeation rate then began to decline more significantly as the membrane became more fouled as the cell concentration increased. If the MIP was then increased, the permeation flow rate was temporarily improved, but quite rapidly, as time progress, the flow rate declined further. For example, at a cell concentration of dry cell weight (DCW) 11 g/l (OD 25) a flow rate of 2.8 l/h was possible at 25 psi, but at the end of the experiment, only 1.7 l/h was obtained. It is typical that fouling increased with increasing MIP.

Figure 5-13 (a & b) also shows the relationship between cell concentration (OD) and various parameters, such as the feeding rate, membrane inlet pressure, sugar concentration and end product mannitol. This plot helps to understand the sophisticated relationships between the membrane performance and operating conditions in the MBR and to recognize the growth behavior of *O. oeni* on glucose and fructose mixture medium during the progress of the cultures with complete cell recycle. The levels of glucose, ultimately limit the growth of bacteria and the high cell concentration fouls the membrane to such an extent so that the culture becomes substrate limited. Indeed, at the end of the fermentation

the cell concentration declines implying the glucose feed rate cannot maintain the maintenance requirement of the cells.

5.3.5.3 Product formation

Product formation was monitored and the fermentation products are shown in Figure 5-14. Although lactic acid concentration increased with the growth of cells, the final concentration was below 6.0 g/l and so indicated that a large amount of lactic acid had been removed from the fermentor during the progress of cultivation of *O. oeni.* Acetic acid concentration was between 0.5 and 1.0 g/l, ethanol concentration remained between 0.2 and 0.6 g/l.

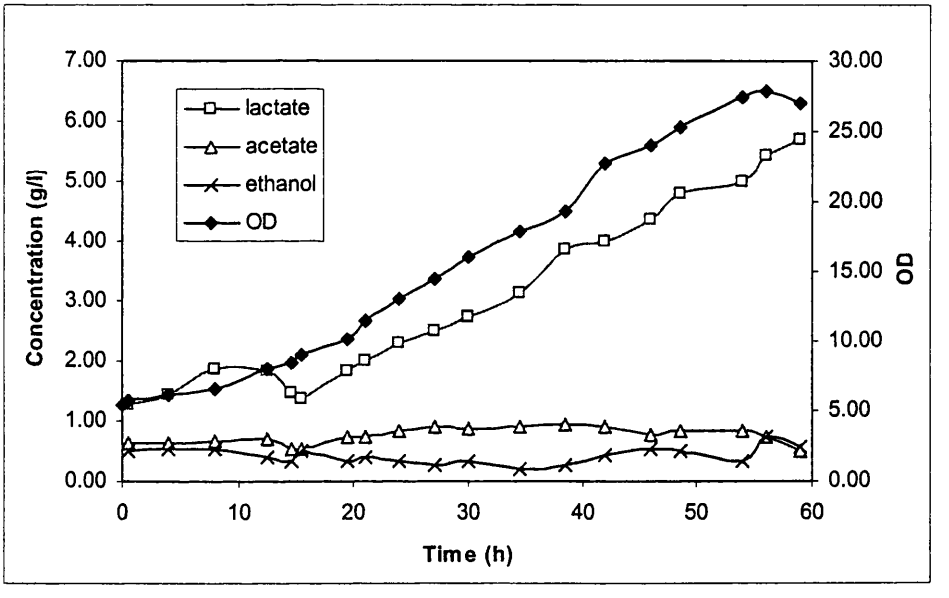


Figure 5-14 Time-courses of products produced in cultivation of *O.oeni* in MBR with complete cell recycle

It is interesting to note that only lactate increases during the fermentation while acetate, ethanol and mannitol did not. This suggests that more fermentation is directed to lactate formation with time as the cell concentration increases. However, if fermentation balance is carried out at the end of the fermentation considerable end products were not accounted for.

5.3.6 MBR culture using 10 g/l glucose: 10 g/l fructose

This experiment was performed as above but with 10 g/l glucose and 10 g/l fructose. The cells were used for the maturation of 'green cider' in later section (section 5.5.5).

5.3.6.1 Cell growth and substrate uptake

The results of this experiment are shown in Figure 5-15 and 5-16. For cell growth there was a lag phase of 10 hours. After this, growth increased in log fashion until about 37 hours where the cell concentration was about 17 of OD. After this time until the end of the culture the growth was linear with a final OD of 25 being achieved. The specific growth rate μ obtained by line regression was 0.044 h^{-1}, higher than 0.035 h^{-1} and 0.033 h^{-1} obtained previously (Fig. 5-12 and Fig.5-10, respectively). This indicates that the ratio of glucose to fructose had an effect on the growth rate of *O. oeni*, as was also demonstrated in the batch culture in test tubes (section 3.2.6). As shown in Figure 5-15 and 5-16, it is possible to prolong the logarithmic growth period and to achieve high cell productivity providing the supplement of nutrients is not limited. The cells grew logarithmically from OD 6.23 at 13 h to 17.4 at 37.5 h (DCW 2.74 to 7.64 g/l) with a cell productivity 0.20 g cell/l/h during the log growth period. A cell productivity of 0.18 g cell/l/h was obtained in the duration of whole 50 h of cultivation. This high density cell of *O. oeni* was used for the investigation into rapid maturation of 'green cider' (see later in section 5.5.5).

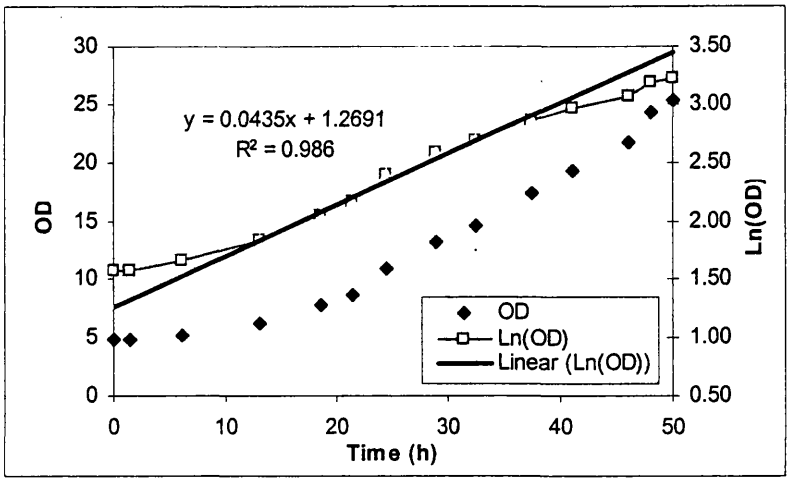


Figure 5-15 Growth curve of *O. oeni* in the MBR with complete cell recycle
Feeding medium: Glucose 10 g/l, fructose 10 g/l

It should be noted that the maximum specific growth rate was found to be much lower than that previously observed in 2 liter batch culture with pH control, 0.044 h^{-1} instead of 0.066 h^{-1} at the 1:1 ratio of glucose to fructose, probably as a result of mechanical stresses and the possibility the ingress of air into the MBR equipment. Similar results were observed in the other cultures of *O. oeni*. A similar phenomenon occurred in the high-density cultivation of *Lactococcus cremoris* (Bibal *et al.* 1991).

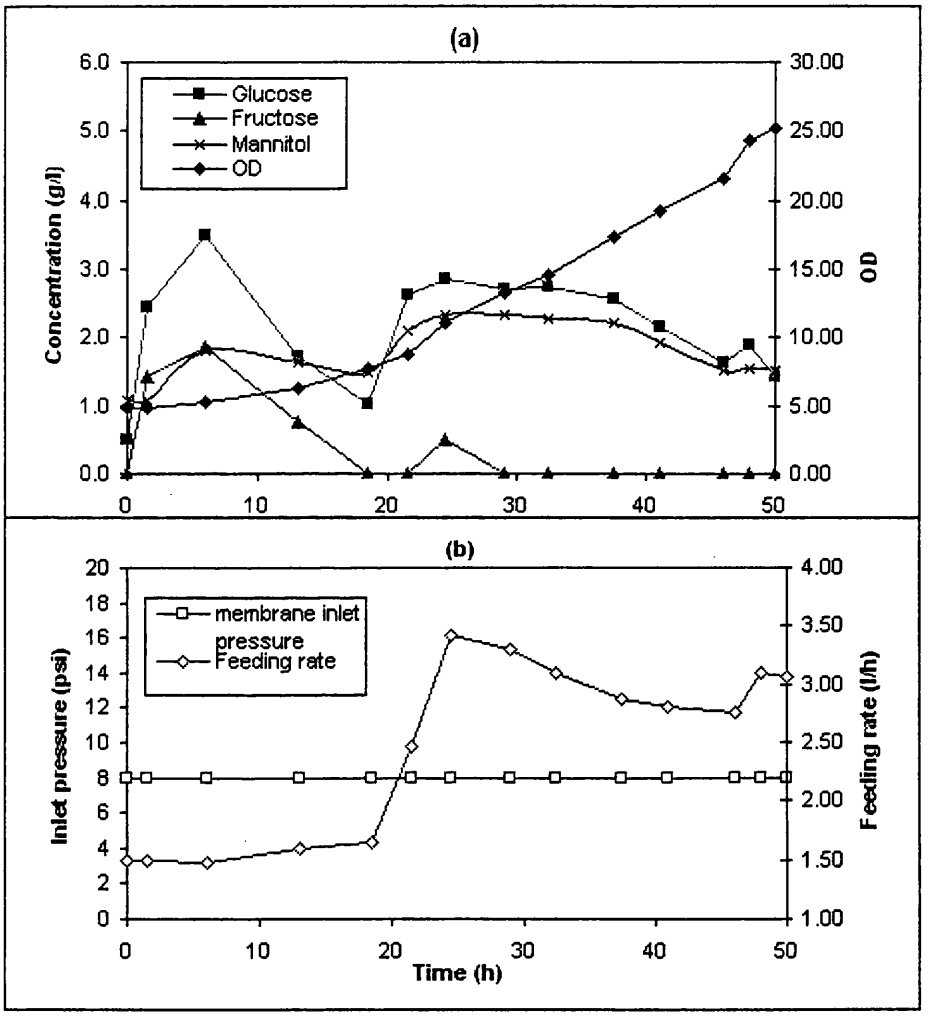


Figure 5-16 Time-courses of glucose, fructose and mannitol and the change in feeding rate and membrane inlet pressure during cultivation of *O. oeni* in the MBR with complete cell recycle
Temperature 25°C, pH = 4.5, feeding medium glucose 10 g/l, fructose 10 g/l

Substrate uptake during the cultivation was monitored and is shown above in Figure 5-16 (a). The glucose concentration was maintained in the range of 1.5 to 3.0 g/l as a whole and it should be noted that below 1.5 g/l glucose was observed at 50 h. Fructose was still

present in the medium culture up to 18 hours, which implies that the slow growth of *O. oeni* during this period. Mannitol concentration produced maintained in 1.5-2.5 g/l.

5.3.6.2 Relationship between permeation rate and membrane inlet pressure

The relationship between permeation rate and MIP can be also investigated and the results shown in Figure 5.16 (b) above. Considering the membrane fouling that had formed in the previous experiment (in 12 g/l-8 g/l glucose-fructose), the MIP was constantly set at 8 psi (Pressure gauge P_1) during the whole process of the cultivation (Fig. 5-16 b). The feed rate was regulated by peristaltic pump G, increasing stepwise the speed so as to increase the permeation rate. In this run, the feed rate increased stepwise to a maximal feed rate of 3.42 l/h at 24.5 h, the feed rate then decreased gradually to 2.76 l/h at 46 h due to the membrane fouling, followed by a slight improvement of feed rate by the speed of pump G. An OD 25.3 at 50 h, over 3.0 l/h of feed rate was still achieved, nearly 2 times higher than the final flow rate of 1.7 l/h at the end of the fermentation at 25 psi (Fig. 5-13 b). Furthermore, the permeation rate gradually declined, unlike the rapid decreases in the previous experiment (Fig.5-13 b). It is suggested that the low MIP could reduce the membrane fouling by only adjusting peristaltic pump to achieve high feed rate. This avoids the fouling problem associated with the high MIP.

5.3.6.3 Product formation

Product formation was also monitored. Lactic acid concentration was below 6.0 g/l during the 50 h of cultivation and acetic acid concentration was in a range of 0.6-1.7 g/l. Lactic acid and acetic acid concentration went up with the cell growth during a first period of 18 h. After 30 h the proportion of lactic acid increased with the growth of *O. oeni* and this may possibly be explained by the cell growth and the decline of the feed rate while acetic acid concentration did not go up under these conditions, and basically kept the concentration of 1.6-1.7 g/l in this period of 30- 50 h (Figure 5-17).

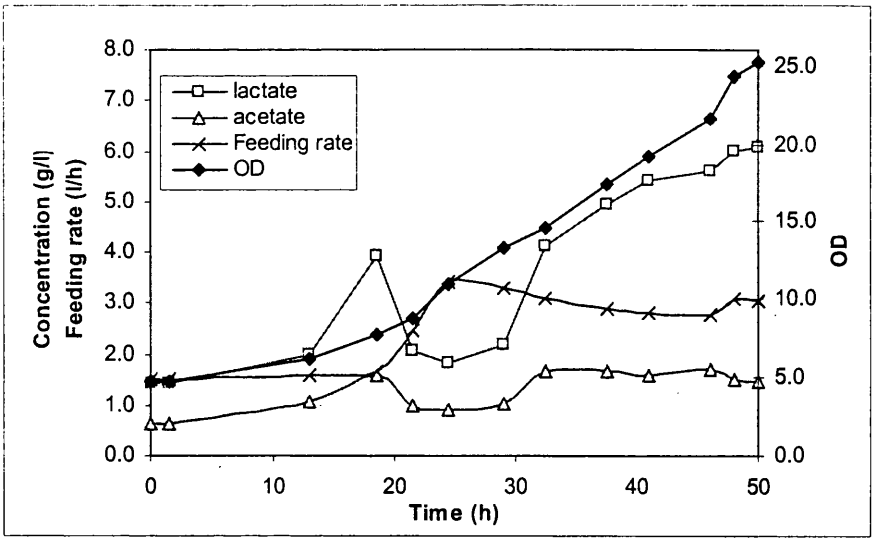


Figure 5-17 Time-courses of product formation during the *O. oeni* cultivation in the MBR with complete cell recycle

5.4 High Concentration Cultivation of *L. brevis* in MBR

L. brevis is also able to transform the flavour profile of cider. The growth of *L. brevis* was investigated in the MBR so as to ascertain its performance and compare this with *O. oeni*. The culture was monitored by measuring the cell density (OD, dry weight), substrate and product concentration in samples from the culture; both the feeding rate and membrane inlet pressure were monitored as well.

5.4.1 Materials and Methods

Two experiments were carried out to investigate *L. brevis* behavior in the MBR with complete cell recycle. The second was a repeat of the first. The MBR was set up as described in Chapter 2 section 2.13.

The growth medium and the feeding medium composition are shown in Table 5.3. The pH of the medium was adjusted to 5.5, autoclaved at 121°C for 15 or 25 minutes. The medium was used in 200 ml, 2000 ml and 20 liter batch sub-cultures for the inoculum. The *L. brevis* was grown on Z broth containing 10 g/l glucose without pH control. The

culture of 20 litre was finally used to inoculate the MBR culture. The feeding of the MBR was begun after inoculation with full strength feeding medium (Table 5.3).

Table 5.3 Medium for inoculum and growth of *L. brevis* in MBR

Composition	Concentration (g/l)	
	growth medium	feeding medium
Yeast extract	10	20
KH_2PO_4	6	6
NH_4Cl	2	2
Trace element	20 (ml/l)	20 (ml/l)
Glucose	10	20
pH	5.5	5.5

5.4.2 MBR culture of *L. brevis*

Cell growth The results of the first experiment are shown in Figure 5-18, 5-19 and 5-20. The growth curve of *L. brevis* obtained in the MBR with complete cell recycle was plotted in Figure 5-18. For the initial period of 7 h, the cells grew slowly from the beginning of OD 6.0, and then grew up to OD 21.5 as a log phase culture until 23 hours with a specific growth rate of 0.065 h^{-1} and the cell productivity of 0.405 g cell/l/h. After this, the culture growth rate decelerated until the end of the experiment (48 h), OD up to 37.8, corresponding to 17.5 g/l DCW. The overall cell productivity was 0.307 g cell/l/h during the 48 h of cultivation. It was also noted that the specific growth rate was much lower than 0.109 h^{-1} achieved in the batch culture, probably as a result of mechanical stresses and environmental conditions in the MBR.

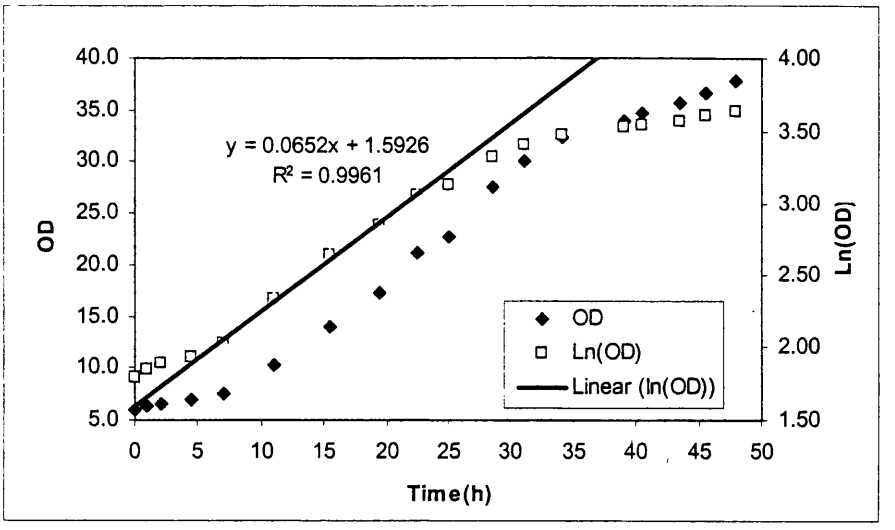


Figure 5-18 Growth curve of *L. brevis* in the MBR with complete cell recycle
Glucose 20 g/l pH=5.5 and temperature 27°C

Substrate uptake The substrate uptake was observed by measuring the residual glucose concentration (Fig.5-19). The glucose concentration was over 4 g/l in the initial 10 h, and then maintained a level between 2 g/l and 4 g/l up to 23 h sufficient to supply the nutrient requirement for growth maintaining high specific growth rate of 0.065 h^{-1}. After 23 h, glucose reduced gradually as cell concentration increased and feed rate decreased as a result of the limit of filtration capacity (see below Fig. 5-20).

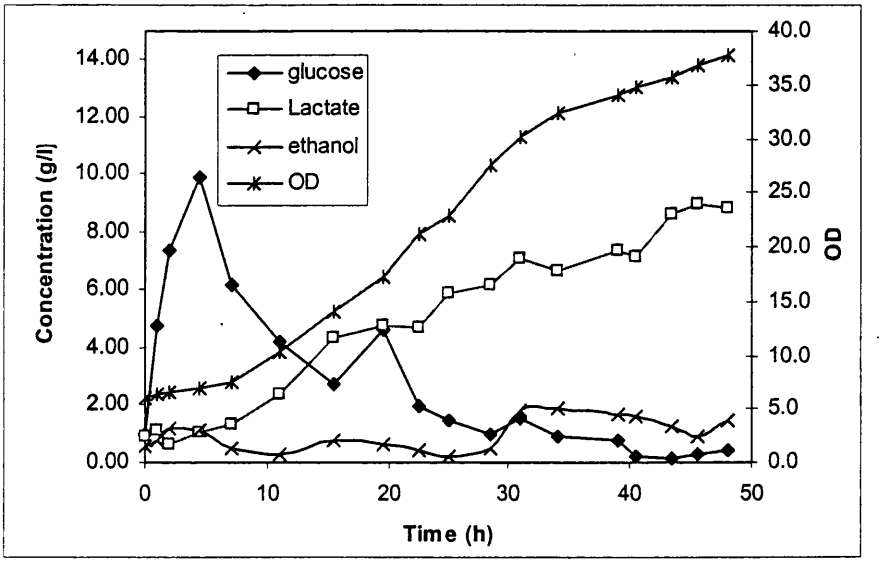


Figure 5-19 Time-courses of biomass, glucose and product in the MBR with complete cell recycle of *L. brevis* Temperature 25°C, pH = 5.5; feeding medium glucose 20 g/l

Product formation The lactic acid concentration increased as cell concentration increased in Fig.5-19 above. It went up steadily as the glucose was fully utilized. Ethanol levels increased throughout the culture but never rose above 2 g/l. Acetic acid concentration was below 0.2 g/l from start to 10 h and then could not be detected after this (no show of data).

Membrane inlet pressure The membrane inlet pressure (MIP) (Figure 5-20) was initially set at 10 psi until to 28.5 h. At this point the permeation rate began to fall and the MIP was increased to 15 psi to improve the permeation rate. Attempts at back-flushing (10minutes at 5 psi, not shown in Figure 5-20), successively set at 11 psi, at 31 – 33 h the permeation rate increased but rapidly fell considerable. These were largely unsuccessful. A subsequent increase in MIP had little or no effect on the permeation rate as the cell biomass increased.

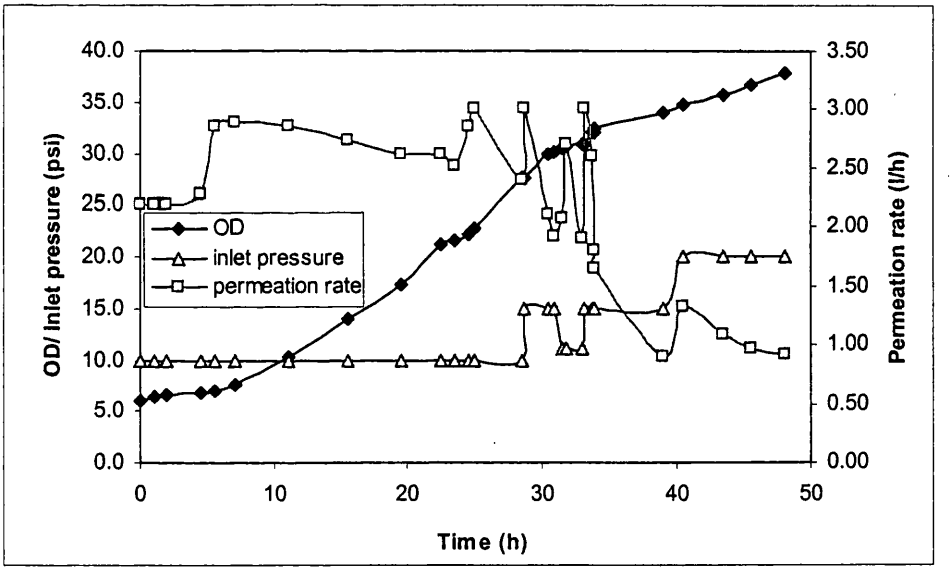


Figure 5-20 Cell concentration, membrane permeation rate, membrane inlet pressure of the MBR as a function of time during the growth of *L. brevis*

Feed rates Feed rates (permeation rates) were adjusted as shown in Figure 5-20. The increase in feed rate was achieved by adjusting peristaltic pump (G) at the constant MIP. It was apparent that a feed rate of 2.6 l/h was obtained at 11 psi by back-flushing for 15 minutes, unfortunately, such a feed rate declined quickly to 1.8 l/h, so increasing MIP to enhance the feed rate, 3.3 l/h, after a quick increase of it for a short period, and decreased quickly down to 1.7 l/h and gradually declined to 0.9 l/h at 39 h. This again is a classic

observation made by many workers (Belfort *et al.*1994; Beaubien *et al.* 1996). Therefore, once a deposit layer had been formed at the membrane surface, it was difficult to recover the permeation rate in practical application. It would seem that the increasing MIP was unlikely to improve the feed rate successfully; rather a better cross-flow regime within the module would be required.

5.4.3 Repeat MBR culture of *L. brevis*.

This experiment was a repeat of above run and this batch of *L. brevis* was used for the maturation of artificial 'green cider' in later section (section 5.5.4). The results of this experiment are shown in Figure 5-21 and 5-22.

Cells growth The cells were grew up from an initial inoculum concentration step with an OD of 7.12 to a final OD 27 during 27.5 h of cultivation (Fig.5-21). The Overall cell productivity was 0.335 g cell/l/h. A cell productivity of 0.449 g cell/l/h was obtained in the duration of log growth phase from 8 h to 23 h and the specific growth rate was 0.067 h^{-1}, a doubling time 10.3 h.

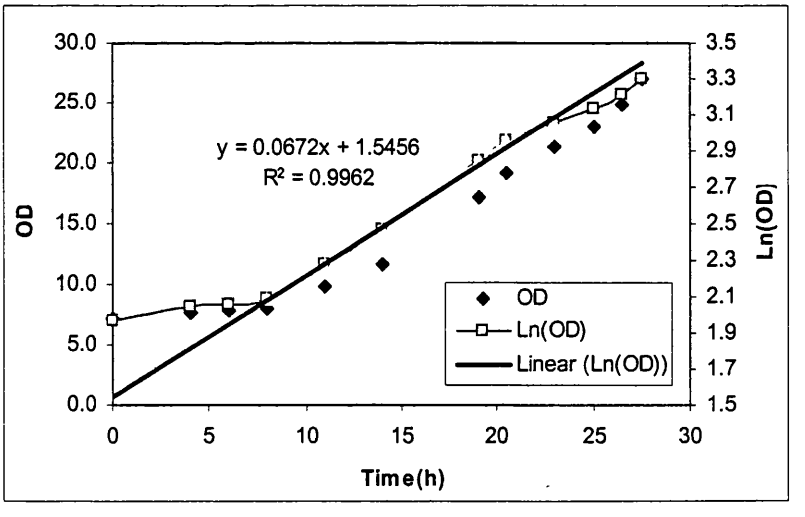


Figure 5-21 Growth curve of *L. brevis* in the MBR with complete cell recycle

Substrate uptake and product formation Substrate concentration and product formation of *L. brevis* are shown in Figure 5-22 (a). Glucose concentration increased at

the beginning of cultivation but then decreased as the cell grew. Glucose concentration was below 1.7 g/l after 14 h; finally it was almost completely consumed. Lactic acid concentration had little variation during the first eight hours, and then increased with the increase in biomass and decrease in glucose. Acetic acid was not found throughout the cultivation. Ethanol was not determined in the experiment, but must be produced, as lactate only accounts for about 50% of the carbon recovered. In fact, it was demonstrated in Chapter 4 (section 4.5.4), 1 mol glucose produced 0.63 -0.46 mol ethanol in the range of dilution rate 0.010-0.089 h^{-1}.

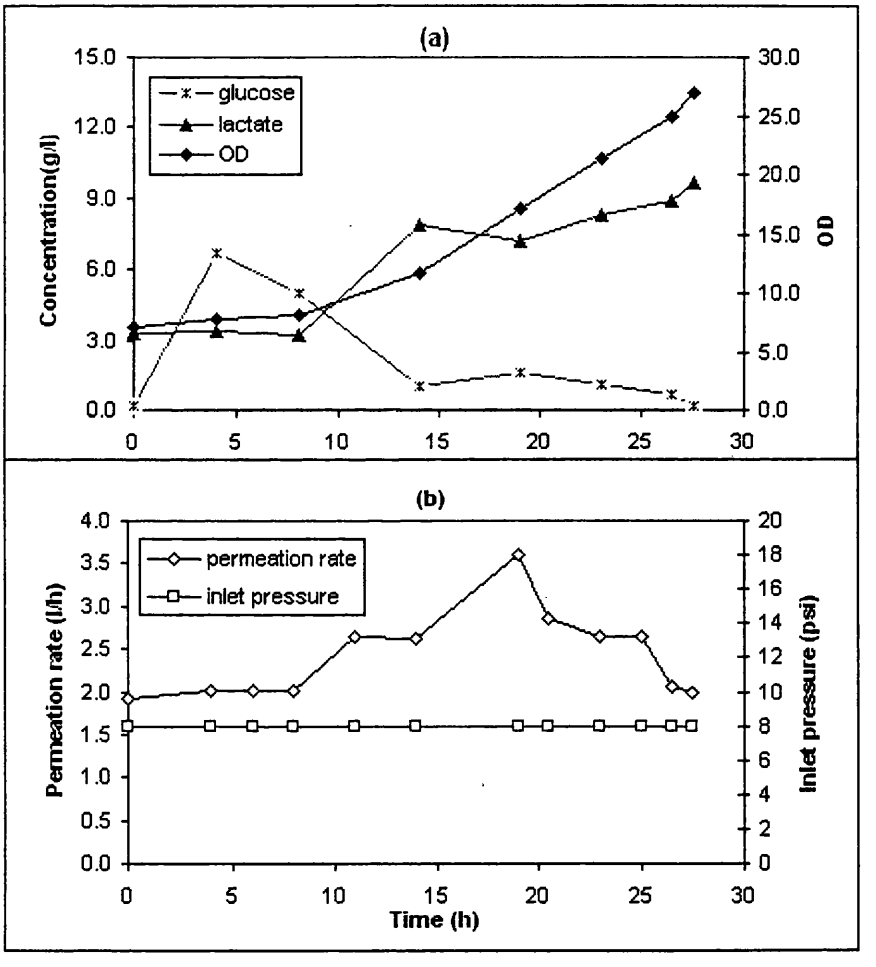


Figure 5-22 Cultivation of *L. brevis* in an MBR with complete cell recycle
Temperature 25°C, pH = 5.5; feeding medium glucose 20 g/l

Membrane inlet pressure and permeation rate With the experience of the previous run (section 5.4.2) the initial MIP was set at 8 psi in this run and maintained throughout the run (Fig.5-22 b). The permeation rate was controlled by the speed of pump, as also

shown in Figure 5-22 (b). The stepwise increase in feed rate supplied efficient nutrients for the growth of *L. brevis*. The permeation rate declined after about 20 h of operating period, a similar phenomenon occurred in above experiments for the cultures of *O. oeni* and *L. brevis*.

Figure 5-22 (a & b) shows that the permeation rate reduced and ultimately limited the performance of the MBR. The permeation rates are significantly reduced by increased cell concentration (about 22 OD) after 20 hours of fermentation. Log growth could be maintained up to about 23 hours, after which nutrients became limited as indicated by low glucose concentration. Increased membrane area would be required to produce significantly higher cell concentration. This experiment however, does demonstrate that it is possible to produce higher cell concentration for further experiments including maturation of cider. The level of cells was at least a factor of 10 higher than in standard batch cultivation.

5.5 Rapid Maturation of Artificial 'Green Cider'

The process of cider maturation, in which microbes are used to turn the acidic raw cider into flavorsome smooth drink, is traditionally time-consuming and expensive, as maturation can take up to several months. During the process of maturation, the main process is malolactic fermentation (MLF). In addition, some LAB also catalyse a series of reactions on tannin-derived compounds to produce subtle, but important, flavor changes associated with high quality cider (section 1.4.1 Chapter 1). These reactions take place in a highly alcoholic and highly acidic environment that is unfavorable environment for the growth of LAB. However, biotransformation may aid the survival of the LAB in the extreme environment by supplying maintenance energy and reducing acidity. The approach here was therefore to propagate the cells in a more favorable environment first, before introducing them to the cider for transformation so avoiding slow growth and poor yield encountered when the starter cultures are used to inoculate cider. Previous work has shown that this was possible (section 3.7 Chapter 3). During the rapid maturation process, the propagated culture needs only to survive rather than produce good growth and as such a highly active maturation system is possible using this two stage strategy.

Bioreactors consisting of high densities of *O. oeni* cells immobilized in beads of polyacrylamide, alginate or κ-carrageenan have been investigated as a technology for conducting continuous, rapid MLF in wines and cider (Divies 1993, Herrero *et al.* 2001). In this technology, the cells act as a biocatalyst, and because of their high concentration, rapidly convert substrate to product (Spettoli *et al.* 1984, McCord & Ryu 1985, Naouri *et al.* 1989). MLF conducted by high densities of *O. oeni* in wine was performed under non-proliferating conditions in batch culture at various initial pH values, malic acid concentrations and cell densities (Maicas *et al.* 2000). A membrane bioreactor (MBR) is based on the containment of high densities of microbial cells between membranes (Mehaia *et al.* 1990, Paterson *et al.* 1989). A cell-recycle membrane bioreactor was applied to conduct continuous malolactic fermentation (Gao *et al.* 1995) in wine, more than 95% degradation of malic acid in wine was achieved during the period of 56 h. However, the experiment was that a suspension of cells of *O. oeni* was only 300ml in 500ml conical flask as a bioreactor and the filtration unit was fitted with a membrane module of 0.024m^2 surface area. Moreover, these cells were cultured first in several 500ml of conical flasks and harvested by centrifugation, and after washing were transferred to a 500ml flask bioreactor for conducting MLF in wine. The reactor was used continuously for eight hours during the day, after which it was stored overnight at 4°C and used again the next day. It was seen that this small volume (300ml) process was complicated and included cultivation, cell washing and concentration by centrifugation and finally, a cell transfer step to the maturation vessel. This process also had a semi-continuous operation in maturation process which would undoubtedly lead to variable product.

MBR was also used to try and speed up the process of cider maturation by concentrating cells (*L. brevis* X_2) that were cultured first in 2 × 20 liter batch cultures in Centre for Complex Fluids Process Research at University of Wales Swansea (Donovan 2000). In contrast to Donovan's work, the main difference in these present studies is the use of the MBR system to provide intensive cell propagation prior to cider maturation simplifying the overall process considerable. The MBR was used to propagate high density of cells in complete cell recycle mode as described above (section 5.4). After propagation the MBR operation was shifted directly to continuously feed in with 'green cider' for maturation. The total volume of suspension cells is 6 liter, 20 times larger as the volume of flask and

membrane area 0.13m^2, 5.5 times as larger as compared to the experiment of Gao *et al.* (1995). In addition, the maturation process did not have to stop during whole operation over 220 h. High densities of cells of two strains, *O. oeni* and *L. brevis*, were then used to conduct MLF in artificial 'green cider'. The biodegradation of malic acid was used to evaluate the effectiveness of the process at several different conditions in a cell-recycle membrane bioreactor system.

5.5.1 Materials and methods

Preparation of cells of *O. oeni* and *L. brevis* The cells of *O. oeni*, OD 25.3, were propagated first in MBR (see section 5.3.6). The cells of *L. brevis* OD 27, were propagated in MBR as well (section 5.4.3).

Artificial 'green cider' Artificial 'green cider' (AGC) was composed of three parts, A, B and C and is shown Table 5.4. The prepared 'green cider' was contained in several glass carboys. The AGC was prepared as follows: Part A was dissolved in 18.4 liter of distilled water in a 24 liter carboy, the volume of distilled water used was depended on the amount of ethanol used; part B, glucose was dissolved in 0.5 liter water. This level of glucose (0.2 g/l) was a similar concentration to that corresponding to the level found in the green cider in the plant at Bulmers. Both A and B were autoclaved at 121 °C for 20 mins. After cooling, part B was added in above carboy to mix with part A. 1.1 l of 95% ethanol (part C) was also added in the carboy to mix with part A, ethanol concentration was 5% (v/v) ethanol of 20 l AGC. 1.7 l and 2.5 l ethanol corresponding to 8% and 12% ethanol in 20 l of AGC were also used as required.

5.5.2 Equipment and conditions

Experimental set-up is shown as Figure 5-8. Temperature was set at 25°C, membrane inlet pressure was set at 7 psi, and the pH of medium was set 3.8. High density cells of *O. onei* or *L. brevis* were recycled continuously and maintained in the MBR system. Artificial 'green cider' was feed in through silicone rubber pipe connected to the bottle of 20 l fluids. The feed rate (flow rate) was adjusted indirectly by peristaltic pump G controlling the membrane permeate rate.

Table 5.4 Composition of artificial 'green cider'

Composition		Concentration	Amounts weighed in 20 liter of AGC
Part A	yeast extract KH_2PO_4 Malic acid [a] trace element pH autoclaved at 121°C for 20 mins	0.2 g/l 0.3 g/l 2.0 g/l 1 ml/l ~3.8	4.0 g 6.0 g 40 g 20 ml
Part B	Glucose autoclaved at 121°C for 20 mins	0.2 g/l	4.0 g in 0.5 liter water
Part C	Ethanol [b] (v/v, %)	5 8 10 12	1.1 l 1.7 l 2.1 l 2.5 l

a. if malic acid is 5 g/l, 100 g malic acid will be weighed in 20 liter AGC instead of 40 g malic acid.
b. ethanol concentrations (5%, 8%, 10%, 12%) were used in different experiments.

5.5.3 Analytical methods

The cell density was measured from 3ml samples withdrawn through sample port from the fermentor, 1 ml out of the 3 ml was removed and diluted to 20 or 30 times in a 50 ml beaker and the OD was measured at 660nm (1cm lightpath after suspended cell diluted).

Samples (10 ml) from permeate were taken periodically and collected in 25 ml glass bottles and analyzed for organic acids. The samples were either analyzed immediately by HPLC or stored in -20°C freezer until analysis.

5.5.4 Maturation of artificial 'green cider' by *L. brevis*

5.5.4.1 Biotransformation of 'green cider' at different flow rates

The first experiment was to study malic acid degradation in AGC. The MBR system shown in Figure 5-8 and reaction suspensions containing OD 27 of *L. brevis* as described as section 5.4.3 were used in this work.

The degradation of malic acid was monitored and is shown in Figure 5-23 when AGC was passed continuously through the bioreactor at different flow rates. The malic acid

degradation was greater than 90% when the flow rate of AGC maintained at 0.6 l/h during the first 30 h, approximate 10 hours of residence time in the reactor to obtain 90% or more malic acid degradation. At the flow rate of 0.9 l/h, about 84% of the malic acid in the AGC was degraded at 30-60 h, the residence time was 6.7 h. With a further increase in flow rate 1.8 l/h (3.3 h residence time) the malic acid degradation declined to produce 70-74% removal of malic acid. About 36% degradation of malic acid was measured at 3.0 l/h (2 hours residence time). From this situation the flow rate was reduced to 0.9 l/h again, and malic acid degradation increased to 70%. This compares to the 84% of degradation at a same flow rate during the operation time of 30-60 h, malic acid degradation percentage decreased after the continuous operation for 100 h. The residual malic acid concentration is also shown in Figure 5-23. As expected, residual malic acid increased as the flow rate of AGC increased during the whole process of continuous operation.

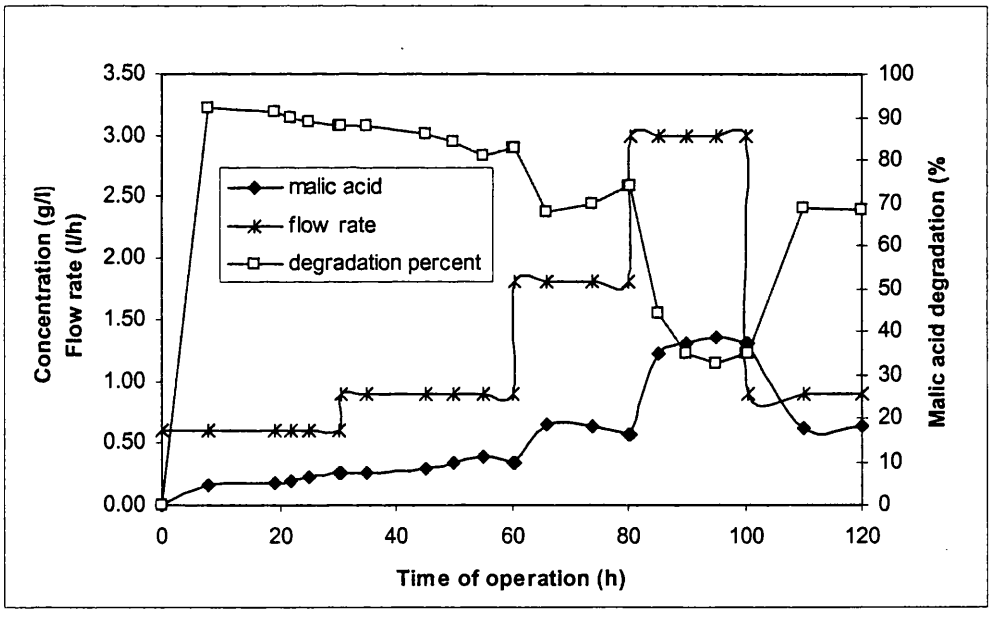


Figure 5-23 Time-courses of malic acid degradation, concentration and flow rate in the MBR charged with high density (initial OD 27) of cells of *L. breivs*

5.5.4.2 The change in cell density and pH

The cell density and pH were also monitored during the operation and as shown in Figure 5-24 below, the flow rate and membrane inlet pressure (MIP) are shown as well.

The change of OD During the first eight hours of continuous operation, the high density (OD) of *L. brevis* declined quickly from OD 27 to 22 possibly due to the change of environment of cells from growth medium to AGC. There was then a steady decline in the culture to an OD of 19 at the end of the fermentation. This indicates that the stability of high density of *L. brevis* could maintain for over 120 h under the conditions tested.

The change of pH The initial pH of the AGC was 3.85 and this rose to over 4 after 19 h of the continuous operation and then gradually rose further to pH 4.2 at 35 h and flow rate 0.9 l/h. The pH was maintained at this value up to 80 h. A subsequent slight decline (to pH 4.0) was then observed with the increase in the flow rate of 3.0 l/h, finally a further increase in pH, to 4.2, was observed as the flow rate reduced to 0.9 l/h. The change in pH was associated with the change in both the degradation rate of malic acid and the flow rate of AGC passed continuously through the cell-recycle bioreactor charged with cells of *L. brevis*.

Flow rates and membrane inlet pressure The change in flow rate of AGC passing continuously through the reactor was recorded by controlling the peristaltic pump G when the MIP was set at low pressure of 7 psi.

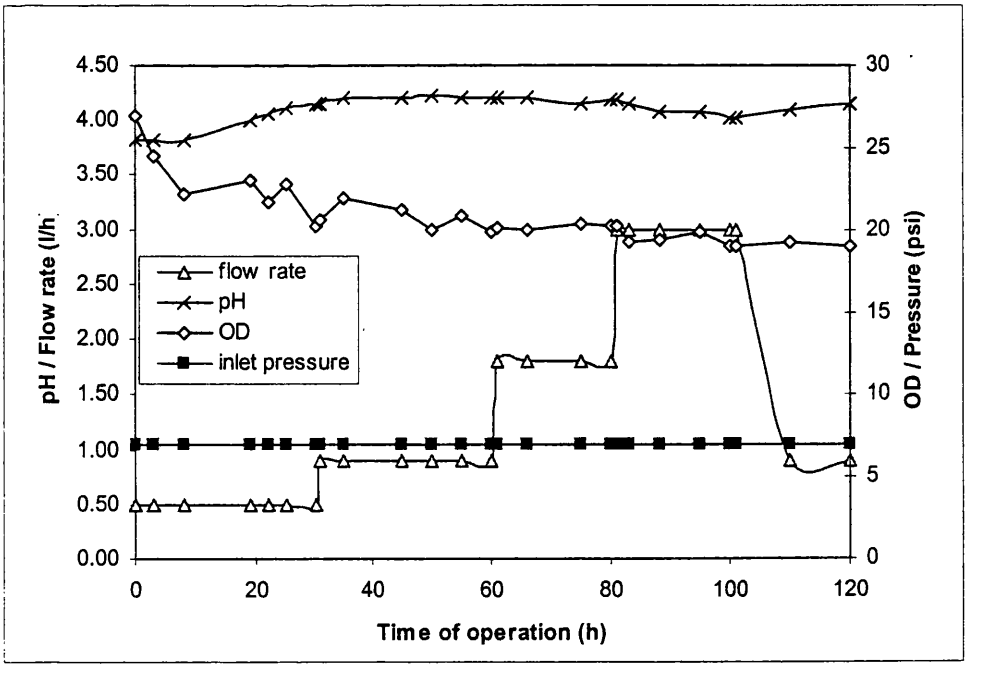


Figure 5-24 pH and OD of reaction suspensions (*L. brevis*) at different flow rates

5.5.4.3 Malate degradation rates with *L. brevis*

Faster flow rates gave a low residence time in the MBR and as a consequence the malic acid concentration increased, however, the calculated specific rate of malic acid degradation (g malate/h/g DCW) showed a different pattern (Figure 5-25). When the flow rate increased from 0.6 to 0.9 l/h, the specific rate of malate degradation also increased from 0.020 to 0.029 g malate/h/g DCW. At flow rate of 1.8 l/h a specific rate of malate degradation 0.050 g malate/h/g DCW was obtained. The further increase of flow rate resulted in the decline in the specific rate of malate degradation, 0.044 g/malate/h/g DCW at 3.0 l/h. So the increase in flow rate does not lead to the loss in an ability to degrade malic acid. A maximum degradation rate of malic acid was achieved at 1.8 l/h (residence time 3.3 h) of AGC. A comparison of flow rate 0.9 l/h at 30 h and the same rate at 100 h shows slight loss of degradation, i.e. a specific rate of malate degradation 0.026 g malate/h/g DCW at 100 h, compared to the specific rate of 0.029 g malate/h/g DCW at the same flow rate during the first 30 h. This is about 10% loss in ability to degrade malic acid.

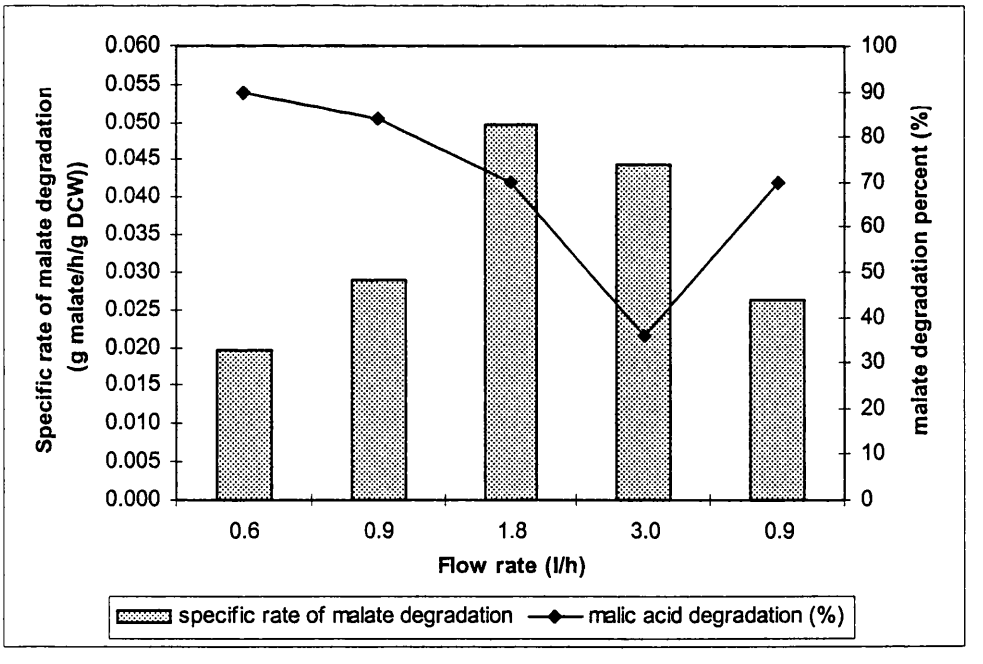


Figure 5-25 Specific rates of malate degradation and malate degradation percentage at different flow rates in the MBR charged with high density of cells of *L. brevis*

Table 5.6 shows the changes in volumetric rate of malate degradation, specific rate of malate degradation, degradation percent, and residual malate concentration and residence time of malic acid at different flow rates.

Table 5.5 Malic acid degradation by *L. brevis* at different flow rate*

Flow rate (l/h)	Volumetric rate of malate degradation (g/l/h) [a]	Specific rate of malate degradation (g malate/h/g DCW) [b]	Degradation percentage (%) [c]	Residual malate concentration (g/l)	Residence time (h)
0.6	0.18	0.020	90	0.20	10
0.9	0.25	0.029	84	0.32	6.7
1.8	0.42	0.050	70	0.60	3.3
3.0	0.36	0.044	36	1.28	2.0
0.9	0.21	0.026	70	0.60	6.7

* average data.
a. calculated by formula: (flow rate× malate concentration× degradation percentage)/total volume of bioreactor = (flow rate× 2 g/l× degradation percentage)/6 l.
b. Calculated by volumetric rate/dry cell weight (DCW).
c. (malate consumed/total malate) × 100%= (2 g/l – residual malate)/2 g/l × 100%.

The data in the Table 5.5 is plotted as a function of residence time. A model equation $y = a + bx + cx^2$ was fitted to the data where y is specific rate of malate degradation (g malate/h/g DCW), x is residual malate (g/l), constants a, b and c are:

$$a = -0.0013\ g\ h^{-1}g^{-1},\ b = 0.1193\ l\ g^{-1}h^{-1},\ \text{and}\ c = -0.0648\ l^2\ g^{-2}\ h^{-1}$$

Figure 5-26 (a) shows a relationship between the specific rate of malate degradation at which the malate was transformed and the concentration of residual malate in the product stream, which was closely related to the flow rate through the MBR. This model also shows that the transformation of malic acid is related to malic acid concentration and shows evidence of substrate inhibition. Once the concentration of residual malate in the system increased to some extent, then the specific rate of malate degradation decreased due to malate substrate inhibition. The maximum malate concentration calculated from this polynomial was 0.921 g/l.

A double reciprocal plot of the system is shown below in Figure 5-26 (b) and also implies some inhibitions. The linear regression gives a straight line with intercept of 4.5902, this is a reciprocal of specific rate of malate degradation, by which the possible maximum

specific rate of malate degradation was estimated. The calculated data (1/4.5902) was 0.218 g malate/h/g DCW. This means that without malate substrate inhibition the possible maximum specific rate of malate degradation was 0.218 g malate/h/g DCW under these conditions tested.

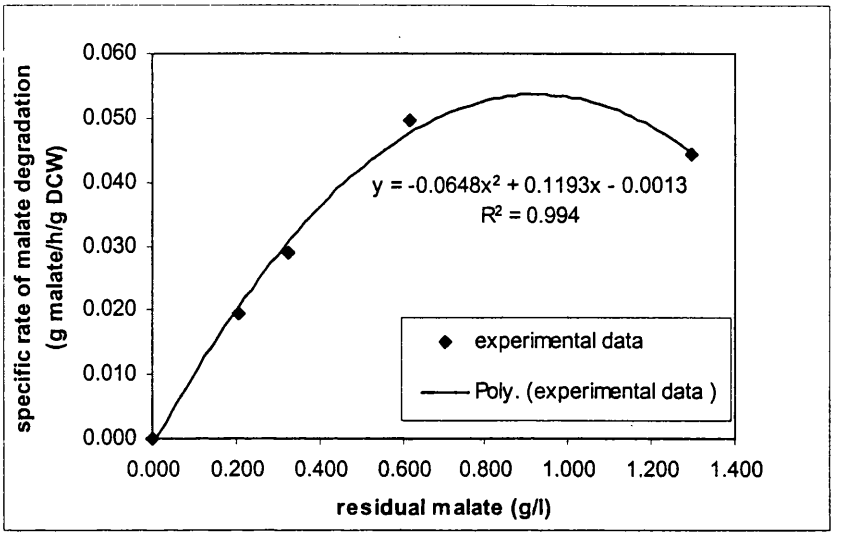


Figure 5-26 (a) The specific rate of malate transformation versus the residual malate in MBR charged with high density cells of *L. brevis* and polynomial fit
Each data point is an average taken from the primary data Figure 5.23 and 5.25

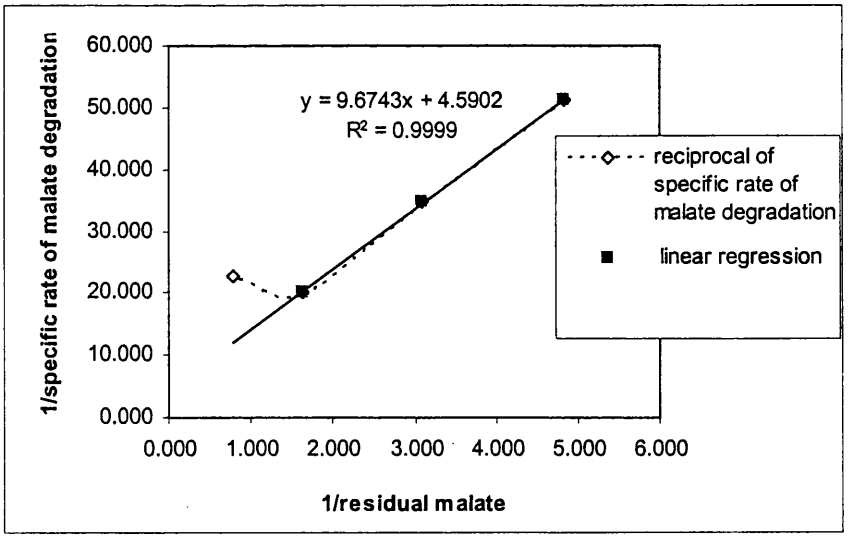


Figure 5-26 (b) A double reciprocal plot of specific rate of malate degradation and residual malate in the MBR charged with high concentration of *L. brevis*

5.5.5 Maturation of artificial 'green cider' by *O. oeni*

This next experiment was to compare performance of malate degradation using *O. oeni*. It involved four stages:

(1) The effect of different flow rates in the period of 0-115h
AGC contained malic acid 2 g/l, ethanol 5%
Flow rate: 0.5 l/h (0-33h) 0.9 l/h (33-60h) 1.9 l/h (60-82h) 2.8 l/h (82-105h) 0.9 l/h (105-115h)

(2) The effect of malic acid concentration in the period of 115-140h
AGC contained malic acid 5 g/l, ethanol 5%, flow rate 0.9 l/h

(3) The transition period of 140-160h
AGC contained malic acid 2 g/l, ethanol 5%, flow rate 0.5 l/h

(4) The effect of ethanol concentration in the period of 160-218h
AGC malic acid 2 g/l, flow rate 0.9 l/h
Ethanol 8% (160-184h) 12% (184-204h) 10% (204-218h)

5.5.5.1 Biotransformation of malic acid at different flow rates

The MBR system shown in Figure 5-8 and reaction suspensions containing OD 25.3 of *O. oeni* (section 5.3.6) were used. The investigation into the degradation of malic acid in AGC passed continuously through the bioreactor was carried out.

The MBR time-courses is shown in Figure 5-27. Malic acid degradation was greater than 92% when a flow rate of AGC maintained at 0.5 l/h over 30 h. As the flow rate increased to 0.9 l/h the malic acid degradation was about 86% in the following period of 30 h. With a flow rate 1.9 l/h, approximate 61% malic acid was degraded in the period of 61- 82 h. A flow rate of 2.8 l/h only permitted the degradation of 41% of the initial level of malic acid over 82-104 h. As the flow rate declined to 0.9 l/h again, average 80% of degradation still occurred, compared to the 86% of degradation during the operation time of 32-60 h, malic acid degradation percentage decreased after the continuous operation for 100 h. The residual malic acid concentration is also shown in Figure 5-27. As expected, a residual malic acid increased as the flow rate of AGC increased and so the final malate concentration was a function of residence time.

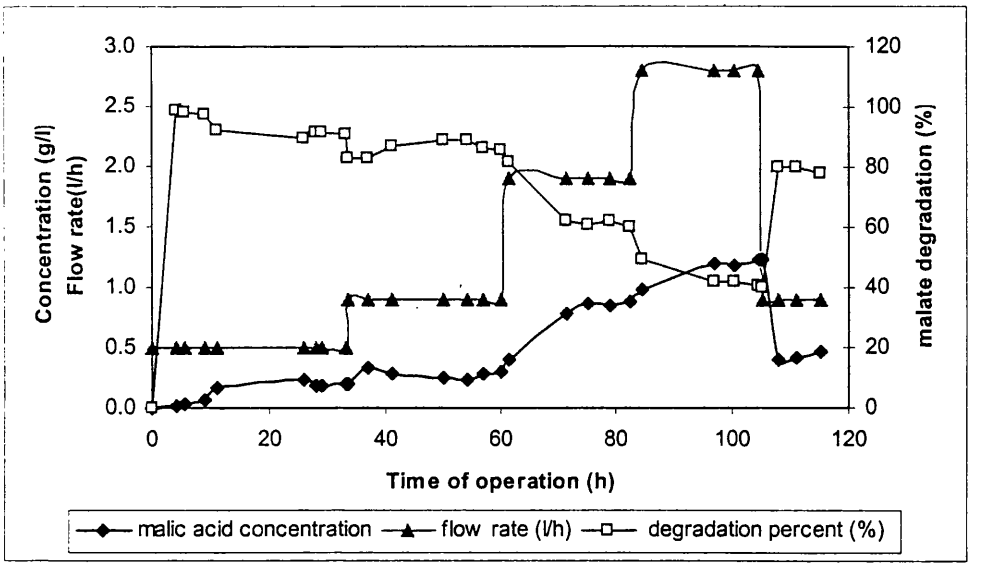


Figure 5-27 Time-courses of malic acid degradation, malate concentration and flow rates in the MBR charged with high density (initial OD 25.3) of cells of *O. oeni*

5.5.5.2 The change in cell density and pH during the first period 115 hours

The cell density and pH were also monitored during the operation in the same run. Figure 5-28 shows these data and the flow rates.

The change in the flow rate of AGC passing continuously through the reactor was recorded by adjusting the peristaltic pump G while the MIP was constantly set at 7 psi. During the first 10 h, the OD decreased rapidly from 25 to 18 possibly due to the change in environment of cells from growth medium to the AGC, and then OD decreased very slowly over the rest of the experiment. The results show that a stabe high density of *O. oeni* could be maintained at this environment. The initial pH of AGC fed in was 3.75 and the pH maintained 3.8-3.86 during this period of 115 h.

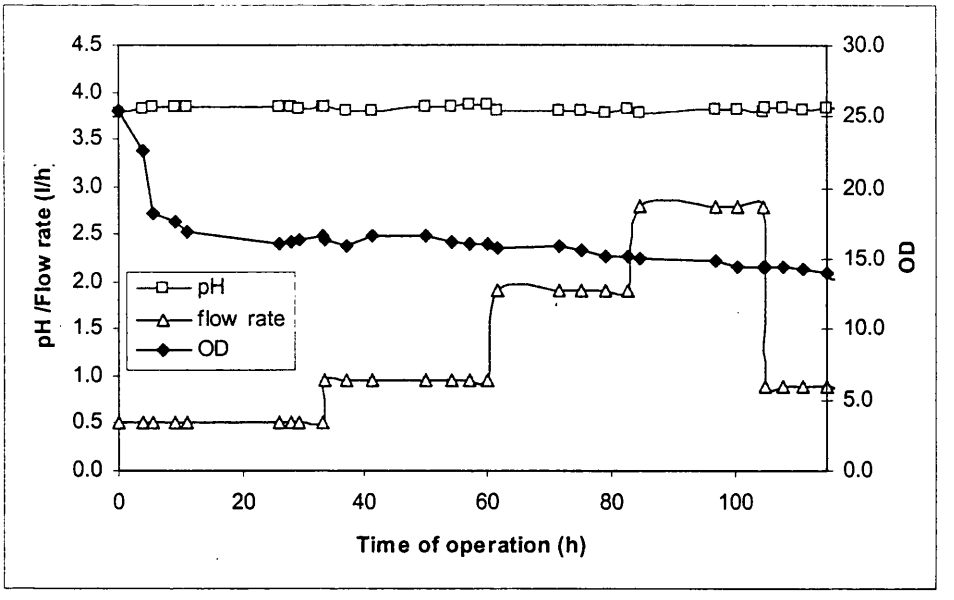


Figure 5-28 pH and OD of reaction suspensions (*O. oeni*) in the MBR at different flow rates

5.5.5.3 Malate degradation rates with *O. oeni*

Although the proportion of malic acid degradation declined as the increase of flow rate passed continuously through the cell-recycle bioreactor charged with cells of *O. oeni*, the specific rate of malic acid degradation increased (Figure 5-29). Almost the same specific rate of degradation malic acid in AGC was recorded at the flow rate of 1.9 and 2.8 l/h. After continuous operation of 100 h the specific rate of degradation still remained 0.037 g malate/h/g DCW at 0.9 l/h and so these cells showed the same activity to degrade malic acid compared with that during the period of 33-60 h. It indicates that the malolactic activity of the MBR could be stable over long period of time under these conditions. Table 5.6 shows the data recorded in this study for the volumetric rate of malate degradation, specific rate of malate degradation, degradation percent, and residual malate concentration and residence time of malic acid in the MBR at different flow rates. A comparison of specific rate of malate degradation by *O. oeni* (Table 5.6) to that by *L. brevis* (Table 5.5) shows that *O. oeni* has greater ability to transform malate than *L. brevis* at same conditions and the specific rate of malate transformation was maintained at a high level at a high flow rate, i.e. 0.058 g malate/h/g DCW at 2.8 l/h (residence time 2.1 h) for *O. oeni* and 0.044 at 3.0 l/g (2 h residence time) for *L. brevis* and so this suggests that *O. oeni* is around 25% more effective at malate transformation.

As far as the author knows, these are the first data that compares the specific malate transforming ability of these bacteria.

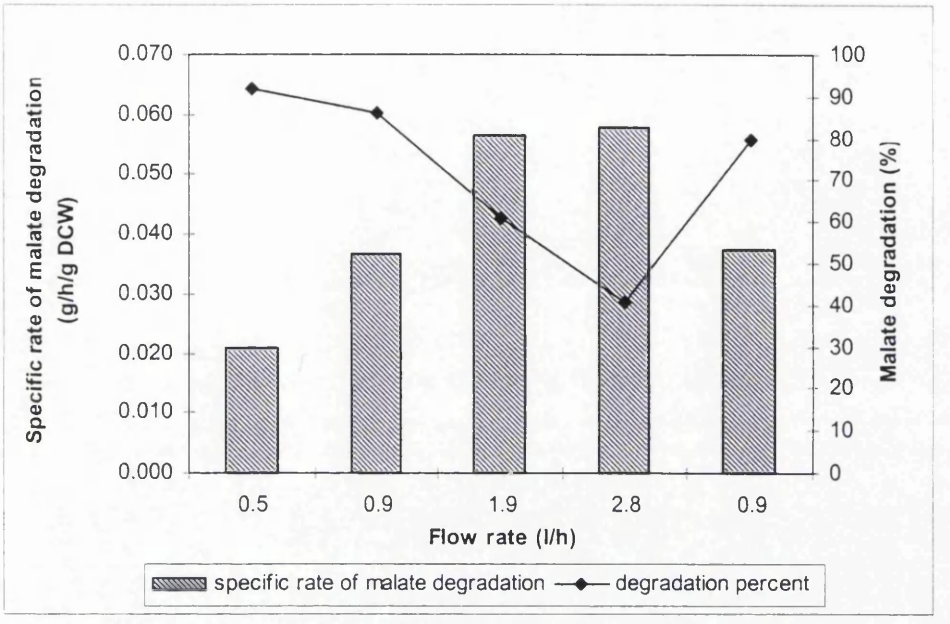


Figure 5-29 Malic acid degradation at different flow rates in the MBR charged with high density of cells (initial OD 25.3) of *O. oeni*

Table 5.6 Malic acid degradation with *O. oeni* at different flow rates*

Flow rate (l/h)	Rate of malate degradation (g/l/h) [a]	Specific rate of malate degradation (g malate/h/g DCW)[b]	Degradation percent (%) [c]	Residual malate concentration (g/l)	Residence time (h)
0.5	0.15	0.021	92	0.16	12
0.9	0.26	0.037	86	0.28	6.7
1.9	0.39	0.056	61	0.78	3.2
2.8	0.38	0.058	41	1.18	2.1
0.9	0.24	0.037	80	0.40	6.7

* Average data.

a. Calculated by formula: (flow rate× malate concentration× degradation percentage)/total volume of bioreactor = (flow rate× 2 g/l× degradation percentage)/6 l.

b. Calculated by volumetric rate/dry cell weight (DCW).

c. (malate consumed/total malate) × 100%

A model equation $y = a + bx + cx^2$ was fitted to the data where y is specific rate of malate degradation (g malate/h/g DCW), x is residual malate (g/l), constants a, b and c are:

$a = 0.001\ g\ h^{-1}g^{-1}$, $b = 0.1273\ l\ g^{-1}h^{-1}$, $c = -0.067\ l^2\ g^{-2}\ h^{-1}$

Figure 5-30 shows a relationship between the specific rate of malate degradation at which the malate was transformed and the residual malate concentration in the product stream.

This model shows that transformation of malic acid is related to malic acid concentration and exhibits malate substrate inhibition. As the residual malate concentration in the system increased to 0.95 g/l, which was calculated from this regression polynomial, the specific rate of malate degradation would have been reached a peak and then would be decreased.

A plot of reciprocal of specific rate of malate degradation versus reciprocal of residual malate concentration is shown below in Figure 5-30 (b). A straight line with intercept 5.215 was obtained by linear regression and the possible maximum specific rate of malate degradation 0.192 g malate/h/g DCW was calculated by the data (1/5.215).

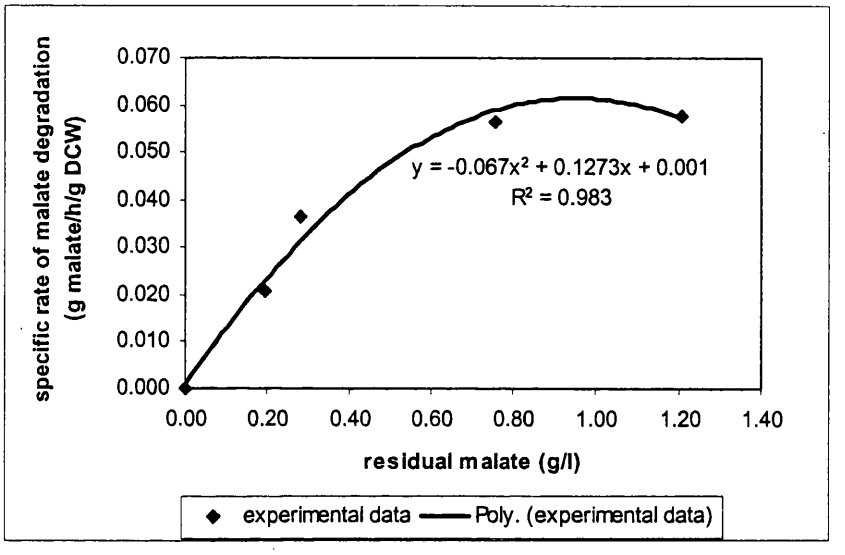


Figure 5-30 (a) The specific rate of malate transformation versus the residual malate in the MBR charged with *O. oeni* (initial OD 25.3)

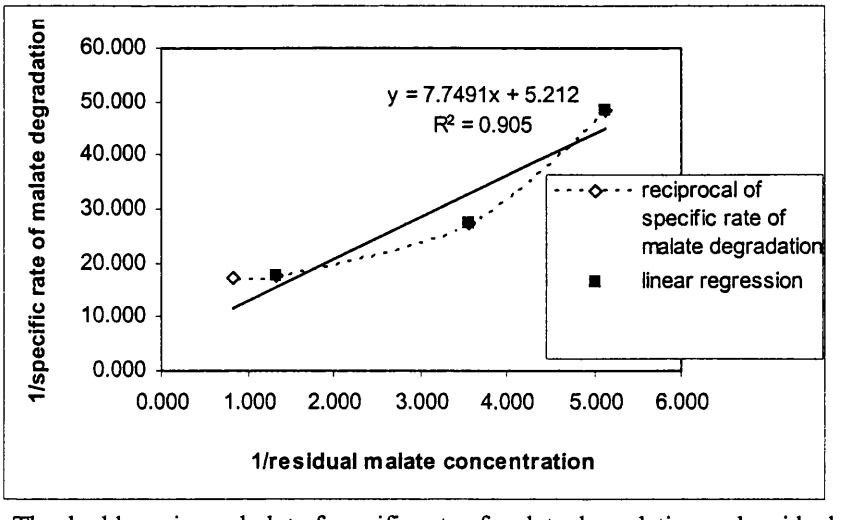


Figure 5-30 (b) The double reciprocal plot of specific rate of malate degradation and residual malate in the MBR charged with high concentration of *O. oeni*

5.5.5.4 The effect of malic acid concentration on malic acid degradation with *O. oeni* in the MBR

After AGC (containing 2 g/l malic acid and 5% ethanol) passed continuously through the cell-recycle reactor charged with cells of *O. oeni* for 115 h, An AGC containing 5 g/l malic acid and 5% ethanol was used and a flow rate of 0.9 l/h was maintained for 25 h. The data gathered during this period is shown below (Table 5.7). Although degradation percent of malic acid was higher at 2 g/l malic acid than at 5 g/l at a same flow rate, a specific rate of malate degradation 0.037 g malate/h/g DCW only halved 0.071 g malate/h/g DCW recorded at 5 g/l malic acid. This implies that the activity to degrade malic acid did not decrease with the increase in initial malic acid concentration of AGC under the conditions. Continuous malolactic fermentation in red wine was conducted by using *O. oeni* in a bioreactor system and the maximum malic acid degradation (92-95%) was reached at a dilution rate of 0.016 h^{-1} and 3.9-4.0 g/l malic acid (Maicas[1] *et al.* 1999). The calculated volumetric rate of malic acid degradation is 0.061 g/l/h in the bioreactor system, which is much lower than the data obtained in my work, i.e. with 0.43 g/l/h of rate of malic acid degradation at dilution rate 0.15 h^{-1} (0.9 l/h) and 5 g/l malate.

Table 5.7 Malic acid degradation at 2 g/l and 5g/l malic acid*

Malic acid concentration (g/l)	Flow rate (l/h)	Volumetric rate of malate degradation (g/l/h) [a]	Specific rate of malate degradation (g/h/g DCW) [b]	Residual malate concentration (g/l)	Degradation percent (%) [c]
2	0.9	0.24	0.037	0.40	80%
2	1.9	0.39	0.056	0.80	60%
5	0.9	0.43	0.071	2.15	57%

* average data.

a. Calculated by formula: (flow rate× malate concentration× degradation percentage)/total volume of bioreactor

b. Calculated by volumetric rate/DCW

c. (malate consumed/total malate) × 100%

5.5.5.5 The effect of ethanol concentration on malic acid degradation with *O. oeni* in the MBR

After 140 h operation, an AGC (2 g/l malate, 5% ethanol) was again passed through the reactor and the flow rate was reduced to 0.5 l/h for 19 h and the malic acid concentration was reduced from 2.15 to 0.68 g/l, as shown in Figure 5-31. At 159 h, AGC with 2 g/l

malic acid and 8% ethanol was fed into the reactor and the flow rate increased to 1.0 l/h. During the 31 h of 159-190 h, average 51% malic acid was degraded at 8% ethanol, residual malate was about 0.96 g/l, and then an AGC containing 12% ethanol passed continuously through the reactor during the 190-204 h, the degradation percent declined rapidly to 18% at 204 h. When shifted to AGC with 10% ethanol, the degradation increased to 32%, with 1.3 g/l residual malic acid over the 204-218h period. This result indicates that the high concentration of ethanol significantly inhibited the activity of *O. oeni* to degrade malic acid.

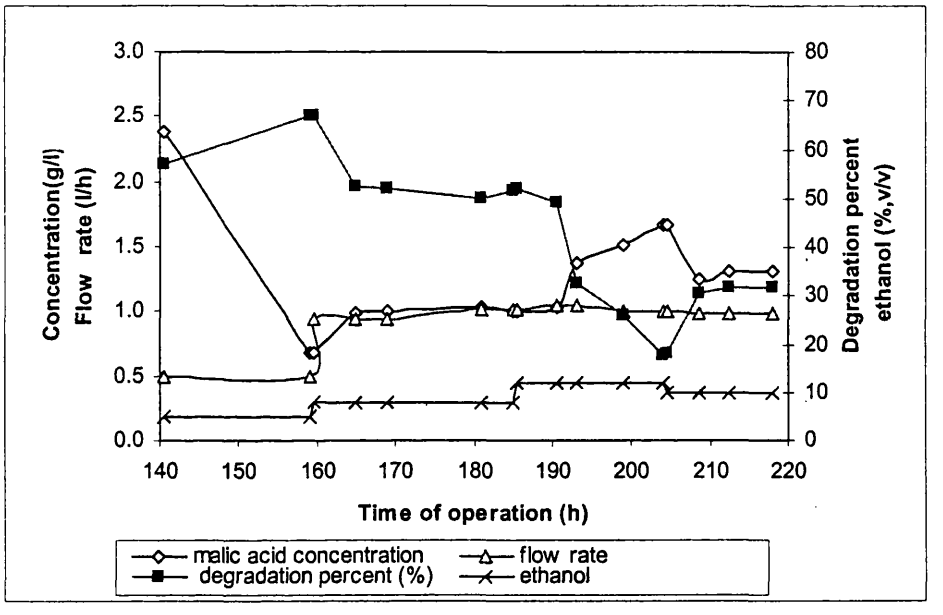


Figure 5-31 Malic acid degradation at different ethanol concentration in the MBR charged with high density of cells of *O. oeni*

Figure 5-32 shows the relationship between the specific rate of malate degradation and ethanol concentration. The specific rate of malic acid degradation increased from 0.019 g malate/h/g DCW at 5% ethanol and feed rate 0.5 l/h to 0.028 g malate/h/g DCW at 8% ethanol and 1.0 l/h. when the ethanol further increased to 12% the specific rate of degradation declined faster, i.e. a specific rate of transformation 0.028 g malate/h/g DCW at 190 h dropped to 0.011 g malate/h/g DCW at 204 h for 14 h (Figure 5-32). After shifted to 10% ethanol AGC, the specific rate of malic acid transformation with 0.020 g malate/h/g DCW at 210 h was obtained. The lower the specific rate of malate degradation is, the higher the ethanol concentration. The experiment data are shown in Table 5.8.

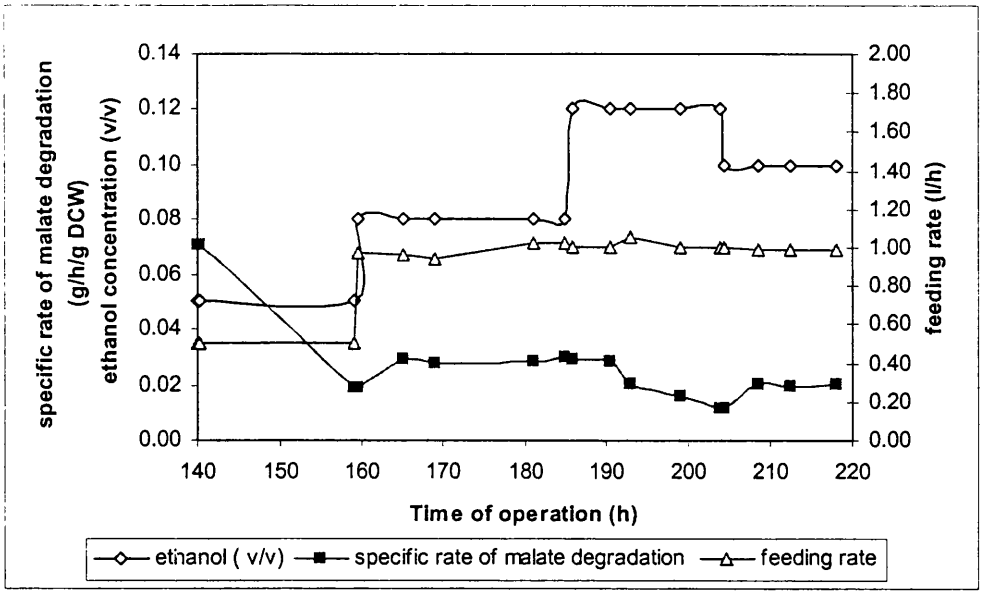


Figure 5-32 The specific rate of malate degradation at different ethanol concentrations in the MBR charged with high density of cells of *O. oeni*

Table 5.8 Malic acid degradation at different ethanol concentrations*

ethanol concentration (%, v/v)	Flow rate (l/h)	Specific rate of malate degradation (g/h/g DCW)	Residual malate concentration (g/l)	Degradation Percent (%)
5	0.5	0.019	0.66	67
5+	0.9	0.037	0.40	80
8	1.0	0.028	0.98	51
10	1.0	0.020	1.36	32
12	1.0	drop	increase	drop

* Average data; the data is based on the calculated method as shown in Table 5.6.
\+ data from Table 5.6, obtained in the period of 105-115 h.

5.5.5.6 The change in cell density and pH during the whole operational period

In the first period of 10 h, OD dropped dramatically from 25 to 18, and then slightly decreased as the process continued. OD was still 12 after continuously operating up to 220 h. The pH of the system was based on a range of 3.80-3.90 during the whole operation despite the fluctuating pH (Figure 5-33). In the interest of the comparison of relationships between varied factors, the changes in malic acid concentration, degradation percent (%) and flow rates at different conditions in the MBR charged with high density of cells of *O. oeni* are shown below in Figure 5-34.

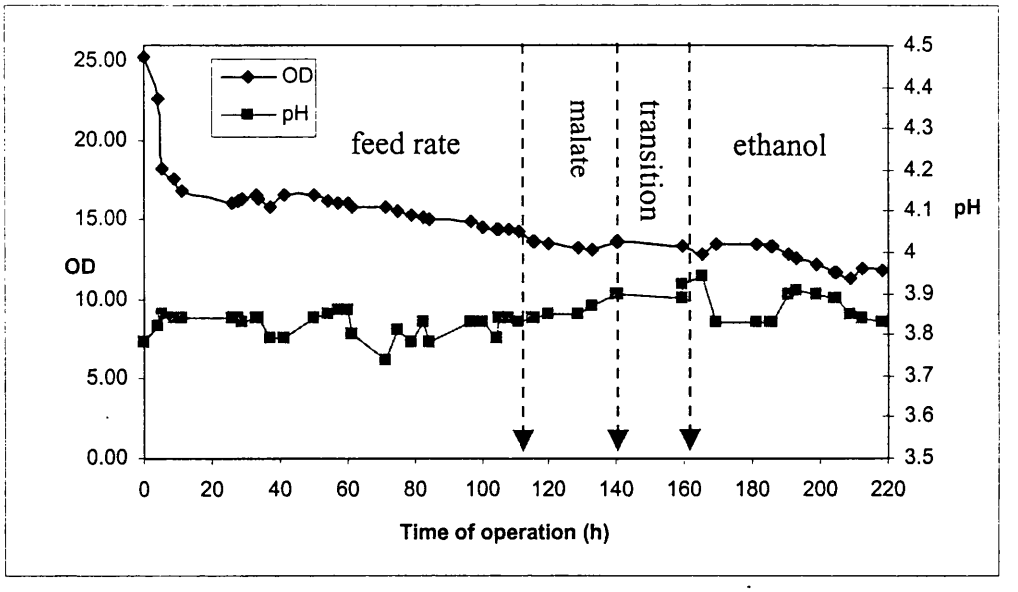


Figure 5-33 The changes in pH and cell density (OD) in MBR charged with high density of cells of *O. oeni*

Note: 0-115 h, malic acid 2 g/l, 5% ethanol, different flow rates (0.5l/h, 0.9l/h, 1.9l/h, 2.8l/h, 0.9l/h);

115-140 h, 5 g/l malic acid, flow rate 0.9 l/h, 5% ethanol;

140-159 h, flow rate 0.5 l/h, malic acid 2 g/l, 5% ethanol;

159-218 h, flow rate 1.0 l/h, malic acid 2 g/l, different ethanol concentrations (8%, 12%, 10%).

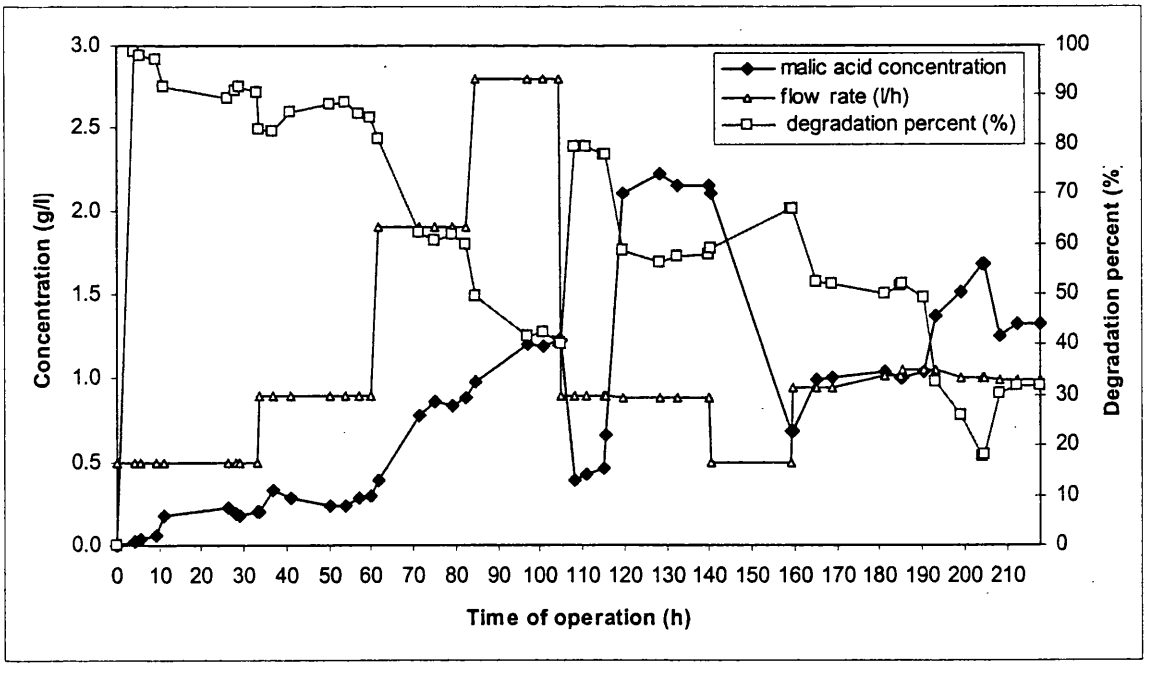


Figure 5-34 The changes in malic acid concentration and degradation (%) at different conditions in the MBR charged with high density (initial OD 25.3) of cells of *O. oeni*
(see note as Figure 5-33)

5.6 Conclusion

Membrane bioreactors combine selective mass transport with chemical reactions and the selective removal of products from the reaction site and increase the conversion of product-inhibited or thermodynamically unfavourable reactions. Membrane bioreactors using biological catalysts can be used in production, processing and treatment operations. In this research work, an MBR system (Fig.2-8 & 5-8) was used for the high density cell cultivation of LAB and the cider maturation. This work using an MBR includes three aspects:

- Cross-flow microfiltration process within the MBR to allow all product separation
- Propagation of *L. brevis* and *O. oeni* in the MBR by removal of toxic materials
- Biotransformation of malic acid using the propagated cells to allow intensive contact for product formation and product removal

5.6.1 Cross-flow microfiltration process

Fermentation broths are very complex solutions and therefore, it is very difficult to generalize about their behavior in a filtration system. In the first part of this chapter the behavior of the microfiltration was investigated (sections 5.2.2 to 5.2.4). The flux with pure water increased linearly with the increase in the membrane inlet pressure (MIP), whereas non-linear increase was observed as the MIP increased in a range of pH 3.0-7.0 of autoclaved growth medium. The flux with medium containing cells increased when the MIP increased, but further increase in MIP did not lead to further increase in the flux beyond 20 psi (a limiting flux). This indicates that medium containing cells caused membrane fouling due to the formation of a cell cake on the membrane surface. It is well known (Belfort *et al.* 1994) that a high MIP readily causes the membrane fouling, especially when a high cell density is present where serious membrane fouling and even complete membrane clogging resulted in the termination of the experiment. In addition, the cell concentration had little influence on the flux after the OD in the MBR had reached 2.5 at 20 psi, and the flux did not change significantly until the cell concentration OD was over 20. This conclusion was made in the trials described in section 5.3 and 5.4. The outcome of the experiment showed that the pH of medium containing cells had an influence on the flux under the conditions tested, the flux increased with the increase of

pH possibly due to changes in the physiology and surface chemistry of cells and that of the membrane. This evidence supported the fact that the membrane was readily cleaned at pH 12-13. General conclusions about how pH will affect the flux are difficult to make due to the complexity of a fermentation broth and the chemistry of membrane material. Fouling mechanisms also were discussed (sections 5.2.1 & 5.2.5) and explanations of the observation phenomena can be made. The effects of these parameters on the flux have to be determined for each specific fermentation broth and for membrane materials. To reach the best performance during micro-filtration and to ensure longer operating time, a great attentioner has to be paid to: the control of pH; the choice of operating pressure; and permeate flow rate. During LAB microfiltration, reversible fouling dominates the separation and the operating conditions must be carefully selected and controlled so as to avoid fouling and extend the operating time to improve membrane productivity. The main conclusion was that a low MIP should be chosen in the application of the MBR, especially in high density of cell cultivation. This reduces fouling to a minimum.

The heart of the MBR is the membrane module as this determines the major cost associated with the MBR. The size and the longevity (membrane productivity) are a critical limitation to the MBR design. It was therefore important to study the influence of media and cell variables on the MBR performance. These observations are similar to those made by other workers (Nagata *et al.* 1989; Chang *et al.* 1991; Persson *et al.* 2001; Ripperger & Altmann 2002). For example, Ripperger & Altmann (2002) described the reversibility and irreversibility of the particle deposition on the membrane surface. The filtrate flow increased at low transmembrane pressures. At higher pressures only a very small increase of the filtrate flow could be observed, while the layer height increased linearly with the transmembrane pressure. During the decrease of the pressure, the layer height did not change significantly, the filtration rate declined linearly to the transmembrane pressure.

5.6.2 The MBR as a cell propagation system

Membrane bioreactors were adopted for the high density cell cultivation as a result of the many perceived advantages. The principal advantages here were to maintain sufficient nutrients and remove inhibitory end product and complete retention of active cells giving

very active intensive system. A high cell density of *O. oeni* or *L. brevis* is crucial for the creation of a faster maturation process by managing and intensifying the growth and metabolism of lactic acid bacteria. The main challenge was how to propagate high densities of cells *O. oeni* or *L. brevis* and to maintain high cell viability for further biotransformation. An MBR system with complete cell-recycle using cross-flow filtration was employed to investigate the high-density cell cultivation of *O. oeni* and *L. brevis* (section 5.3 & 5.4). The maximum OD 28 for *O. oeni* and OD 37 for *L. brevis* were achieved in the MBR culture, 31 fold and 25 fold respectively higher than cell concentration obtained in batch culture (Chapter 4, section 4.3 & 4.5). High volumetric cell productivities were obtained in the MBR cell recycle systems, e.g. volumetric productivity for *O. oeni* 180 mg/l/h during all culture period in the MBR, 23 folds and 10 folds higher, respectively compared with 7.9 mg/l/h in batch culture and maximum value 17.6 mg/l/h in continuous culture at pH 4.5 (section 4.4.3.5). The higher the cell mass, the higher the cell productivity obtained in the MBR.

These results obtained in the MBR system showed the performance of the MBR system was better than that of batch and continuous culture system. The details would have been discussed in section 6.2.2.2 Chapter 6.

Membrane fouling is a major limitation of the MBR system especially in high density cultivation. In the above experiments it was observed that the decline in growth rate of cells was mainly due to the nutrient limitation resulting from the decrease in the permeation rate. A basic investigation into the understanding of the relationship between the permeation rate and membrane fouling was carried out. The low MIP set initially has been proven to be an effective solution which can reduce the membrane fouling and prolong membrane operation time (section 5.3.5 & 5.3.6). For example, during the culture process of *O. oeni*, with OD 25 and operation time around 50 h, a feeding rate 2 l/h was achieved when MIP increased from initial 10 psi to 25 psi (Fig. 5-13), whereas 3 l/h obtained at constant MIP 8 psi throughout the whole process if an initial MIP 8 psi was set (Fig. 5-16 b). The approach to adjusting the speed of peristaltic pump to obtain high permeation rate can substantially reduce the membrane fouling. The other reason that caused decline in the permeation rate perhaps is the longer operation time, e.g. more than

25 h. In addition, it should be noted that the specific growth rate reduced in the MBR culture compared with batch culture probably as a result of mechanical stresses. So it is still a major challenge for us as to how to minimize the membrane fouling and to enhance the growth rate in the future work. Alternatively, the membrane area should be substantially increased to cope with the reduced flux. With commercial membranes becoming cheaper this option is not as impractical as it once was.

5.6.3 Biotransformation of malic acid in the MBR

High-density cells of *O. oeni* or *L. brevis* can act as a very active biocatalyst and, in the absence of growth, rapidly degrade L-malic acid to L-lactic acid in artificial 'green cider' (AGC) in the MBR. This property was used to evaluate an MBR system for the continuous, rapid biotransformation of malic acid. In these studies, the membrane bioreactor with cross-flow microfiltration was explored for MLF in AGC for over 5 days (*L. brevis*) and 9 days (*O. oeni*).

At initial OD 27 of *L. brevis*, specific rate of malic acid degradation in AGC with 2 g/l malic acid and 5% ethanol was in the range of 0.020–0.044 g malate/h/g DCW at the residence time 10- 2.0 h, corresponding to malate degradation percent 90%-36%. The malolactic activity of *L. brevis* was evaluated by specific rate of malate degradation with slight decrease from 0.029 to 0.026 g malate/h/g DCW at residence time 6.7 h for over 110 h operation.

The increase in flow rate resulted in the decrease in degradation percent but did not reduce the specific rate degradation. The specific rate of malate degradation in the same components of AGC was in the range of 0.021-0.058 g malate/h/g DCW at the residence time 12-2.1 h, corresponding to malate degradation percent 92%-41%. The specific rate of malate degradation was maintained 0.037 g malate/h/g DCW at residence time 6.7 h for 120 h operation. This result indicates that no loss in malolactic activity was observed and the system would run for substantially longer periods.

For comparison in this study, the higher specific rate of malate transformation with 0.071 g malate/h/g DCW was obtained at 5g/l malic acid compared to 0.037 g malate/h/g DCW

obtained at 2 g/l malic acid in AGC at the same residence time 6.7 h. The increase in ethanol concentration resulted in the decline in specific rate of malate degradation by *O. oeni* at same malic acid concentration and same residence time (Table 5.8). High ethanol concentration with 12% (v/v) was able to drastically reduce the malic acid degradation percent and degradation rate, even led to lost ability to degrade malic acid.

A 300 ml cell-recycle membrane bioreactor system with a flow rate 360 ml/h red wine was run for 56 h, after this the MLF activity was lost rapidly (Gao & Fleet 1995). The current performance of all known published systems is reviewed in Table 6.3 Chapter 6. Table 5.9 below represents the rate of malate degradation with high concentration of malolactic bacteria in different bioreactor systems for malic acid transformation in wine and cider. Obviously, the rates of malate degradation in continuous model systems (CSTR, FBR and MBR-CCR) are greater than that in batch model systems (screw tubes or shake flask) and this work also shows bigger rates of malate degradation than others. It should be noted that it is very difficult to compare performance with that of other workers due to multifactor inhibition i.e. malate, pH, temperature and ethanol concentration (Chapter 3).

Table 5.9 Comparison of malic acid degradation with high concentrations of malolactic bacteria in different bioreactors

Reactor [a] (sample volume)	Malolactic Bacteria	Malate (g/l)	Rate of malate Degradation [c] (g/h)	Reference
Screw tubes (10 ml)	*O. oeni*	1.0-6.0	0.002-0.010	Gao and Fleet (1994)
Screw tubes (10 ml)	*O. oeni*	3.5-7.0	0.0002-0.0034/day	Maicas et al. (2000)
Shake flask (0.1 l)	*O. oeni*	3.5	0.007	Maicas et al. (2001)
Shake flask (5 ml)	*O. oeni*	2.3-4.5	0.048-0.096	Rossi & Clementi(1984)
Tubular glass reactor (18ml)	*O. oeni*	6.7	0.050-0.065	Durieux et al. (2000)
FBR (0.99 l)	*Sac bayanus and O. oeni* [b]	5.2	0.61-1.80	Nedovic et al. (2000)
CSTR (80 ml)	*O. oeni*	4.0	0.14	Janssen et al. (1993)
FBR (1.4 l)	*O. oeni*	5.5	0.33-0.66	Naouri et al. (1991)
FBR (1.4 l)	*Lactobacillus sp*	5.5	0.33-0.66	Naouri et al. (1991)
MBR-CCR (0.3 l)	*O. oeni*	4.6	0.38-1.56	Gao and Fleet (1995)
MBR-CCR (6 l)	*O. oeni*	2	0.9-2.4	Section 5.5.5 in Chapter 5
		5	2.6	
MBR-CCR (6 l)	*L. brevis*	2	1.0-2.5	Section 5.5.5 Chapter 5

a. CSTR-continuous stirred tank reactor; FBR-fluidized bed reactor; MBR-CCR-membrane bioreactor with complete cell recycle.
b. *Saccharomyces bayanus* and *Oenococcus oeni*
c. calculated by "malate concentration (g/l) × malate degradation (%)×feed rate (l/h)" for continuous model.

These experiments demonstrated that this reactor design was practical and a feasible arrangement and could be used for extended periods not only without decline in flow rate due to membrane fouling, but also with the maintenance in malolactic activity for 120 h when the artificial 'green cider' passed continuously through the cell-recycle reactor charged with cells of *O. oeni*. The decrease in OD and increase in pH were observed during the operating period. It is almost certain that the enhanced degradation of malic acid would occur if cell density can be increased further. In addition, the influences of pH and temperature on the degradation of malic acid need to be investigated further. Simultaneously, the use of membrane bioreactor technology for the continuous, rapid degradation of malic acid in cider should appeal to the cider company, as it offers the potential for more reliable and predictable control over the MLF in cider. It is believed that the performance of membrane bioreactor applied to the process of cider maturation or wine maturation could be more successful with further detailed research.

Chapter 6 Discussion and Conclusion

6.1 Introduction

This project set out to compare and contrast the growth and performance of MLF bacteria *O. oeni* and *L. brevis* especially in the context of their use in alcoholic beverages. The results of this study show that two LAB, *O. oeni* and *L. brevis*, could be grown in batch, continuous cultures and in membrane bioreactors (MBR). Using these organisms, a novel membrane bioreactor system for the rapid maturation of cider was developed. The MBR system was run successfully for periods up to 9 days and with correct management could be run for much longer periods. The control of the maturation process could lead to the production of a cider of consistent flavor, thus eliminating the need for the costly art of blending and vast storage facilities previously regarded as indispensable for the production of a quality product. This technology offers cidermaking a modern and flexible process for cider maturation. This work also opens a way for the development of other maturation systems, from rice wines, alcoholic fruit juices, and soy sauce. This process can also be used to produce flavor concentrates that require biotransformation for flavor development.

The major challenge of this research work was to develop a process and find equipment that could withstand harsh conditions with a highly alcoholic (up to 10% w/v) and highly acidic (pH 3.5) environment. Through the comparison of the two organisms, the overall aim to create a faster maturation process by managing and intensifying the growth and metabolism of lactic acid bacteria has been achieved. Meanwhile, more work was required to test its viability in the production environment.

In the context of the study four main areas of work were planned and the results can be summarized and discussed below.

6.2 Growth of LAB and maturation system

To achieve rapid and efficient maturation a number of studies were carried out to determine the most appropriate organisms and conditions. A prerequisite for maturation is the production of large quantities of LAB. The first stage was therefore to study the growth medium and the growth. Two organisms were then compared to see which the most appropriate organism was for maturation. Having produced the organism the maturation process was then investigated in the MBR and appropriate operational conditions for maturation were determined.

6.2.1 Creation of a simplified medium for the successful growth of LAB *O. oeni* and *L. brevis*.

A medium, Zhang's broth (section 2.1 Chapter 2), was successfully formulated. This medium allowed the studies on the physiology and growth of LAB in relation to the conditions found in the "maturation environment" (Chapter 3). The medium is simple and safe without nitrogen materials derived from meat extracts. A new safe production medium compatible with cider or inocula has been produced where yeast extracts can act as suitable nitrogen source. The experimental results of growth of *O. oeni* and *L. brevis* show only small difference (Table 6.1) on Zhang's broth when compared to the growth on Donovan's broth (section 3.3 & 3.4) which contained bacteriological peptone and meat extracts.

Table 6.1 A comparison of the growth rate of two strains on varied nitrogen sources [a]

	MRS	Donovan's broth	Zhang's broth
O. oeni	$0.035h^{-1}$ (19.8 h)	$0.069\ h^{-1}$ (10.0 h)	$0.063\ h^{-1}$ (11.0 h)
L. brevis	$0.088\ h^{-1}$ (7.9 h) [b]	$0.080\ h^{-1}$ (8.7 h) [b]	$0.080\ h^{-1}$ (8.7 h)

a. the composition of three broths see Table 2.1 (MRS), Table 2.2 (Donovan's broth) and Table 2.4 (Zhang's broth); *O. oeni* on a mixture of 5 g/l glucose-5 g/l fructose, *L. brevis* on with 5 g/l glucose.
b. the data derived from Donovan (2000).

A mixture of glucose-fructose as carbon source and energy source was preferable for the growth of *O. oeni* (section 3.4 Chapter 3). The ratio 1:1 (5 g/l:5g/l) of glucose to fructose gave a reasonable specific growth rate ($0.063\ h^{-1}$) and cell yield as measured by optical

density (OD_{max} 1.4) and therefore was employed as carbon sources in the 2 liter batch and continuous culture of *O. oeni* with pH control. *L. brevis* grew well in Donovan's broth with glucose or fructose, but grew better in the presence of fructose than glucose as reported in Chapter 4 (Donovan 2000). It is quite clear from the results that the metabolism of glucose and glucose-fructose mixtures is different in the two organisms used in this study.

6.2.2 Comparisons of growth of *O. oeni* and *L. brevis*

6.2.2.1 Growth in cider environment

(1) Ethanol inhibits the growth of *O. oeni* and *L. brevis*, but both LAB are able to grow in high alcohol concentration (Table 3.30 & section 3.5 Chapter 3). Both LAB studied were shown to be highly adapted to cider environment (7-10%, v/v). This gives the LAB a natural advantage for the growth within cider environment (ethanol up to 10%). A significant difference is that *O. oeni* exhibits greater ethanol tolerance than *L. brevis* either in presence of glucose or fructose at 10-12% (v/v) ethanol. Moreover, the composition of media (various sugars) has an influence on the ethanol tolerance of the LAB. The ethanol tolerance of *O. oeni* was improved, as measured by specific growth rate when a mixture of sugars (glucose-fructose, glucose-sucrose, and fructose-sucrose) was used, as compared with glucose, fructose or sucrose alone (Table 3.30 & Tables 3.19, 3.20, 3.21 in section 3.5). This is a novel observation and has significance in maturation processes. A 1:1 mixture of glucose-fructose is optimal and gave growth even up to 12% (v/v) ethanol.

(2) The growth of two LAB strains was observed in a temperature range 10 to 35°C or 36°C in the absence of ethanol. The growth rate of both increased as temperature increased below optimum temperature, and then decreased as temperature increased above. The optimum temperature for the maximum growth rate of *L. brevis* in the absence of ethanol is 36°C and is reduced to 28°C in the presence of 5% (v/v) ethanol (Donovan 2000), whereas the maximum growth rate of *O. oeni* is obtained at 25°C, which is independent of the presence of ethanol. Greater levels of inhibition in both LAB were observed at high

temperature than at low temperature in the presence of ethanol (Table 3.22 & 3.23). These results indicate that both LAB are more sensitive to ethanol at high temperature.

(3) The biotransformation ability of LAB *L. brevis* and *O. oeni* to metabolize organic acids was improved by small additions of glucose. The results show that there is considerable scope for manipulating the medium to enhance the maturation capacity of the cells. For example, the specific growth rate of *O. oeni* was stimulated by up to 175% by an addition of 0.2 g/l (~1mM) glucose in the presence of malate, and up to 233% in the presence of citrate (Table 3.27). Similarly, OD_{max} of *O. oeni* also increased from 0.1 to 0.26 (by up to 260%). As for the growth of *L. brevis*, OD_{max} increased from 0.20 to 0.46 (by up to 230%) in media containing 0.2 g/l glucose alone and 0.2g/l glucose-5g/l malate mixture (Donovan 2000). Although the specific growth rate was not reported, growth curves showed that the growth rate was increased by an addition of 1mM glucose in the presence of malate. The results provide good evidence that the maturation process of cider containing about 1mM glucose found in 'green cider' can be stimulated in an MBR in the presence of high cell concentrations and at low pH (section 5.5 Chapter 5).

(4) The growth rate of *L. brevis* was influenced by apple phenolics in the presence of glucose. For example, chlorogenic acid, quinic acid, shikimic acid and gallic acid all affected the growth of *L. brevis*. Additions of shikimic acid stimulated growth rate by up to 330%, while caffeic acid inhibited by up to 83% and ferulic acid 71%. In addition, these apple phenolics showed little influence on the growth rate of *O. oeni* apart from the inhibition of coumaric acid and caffeic acid in the presence of glucose (Table 3.29). However, OD_{max} decreased up to 5-10% in all cases. These results suggest that the *O. oeni* may have a small impact on flavor associated with these compounds.

6.2.2.2 Growth in different culture systems

As far as the culture of LAB, there are no published reports of continuous cultures or high density cell cultures as found in an MBR for either LAB *O. oeni* or *L. brevis*. The results of *O. oeni* and *L. brevis* propagation in batch, continuous and MBR systems are summarized as follows.

The growth cultures of the strains of *O. oeni* and *L. brevis* used in this project were developed progressively from initial growth investigation in test tube cultures, as described in Chapter 3. These results were then employed as a basis for the scaling up in 2 litre batch cultures with pH control. The control of pH can enhance the growth rate of both strains, particularly the growth rate of *L. brevis* increased significantly from 0.080 h^{-1} without pH control to 0.110 h^{-1} with pH control. In comparison, the growth rate of *O. oeni* did not increase significantly from 0.063 to 0.066 h^{-1} (Table 6.2). This provides evidence that *O. oeni* has significantly more tolerance to end products, such as lactate and acetate, and more suitable for low pH conditions compared to *L. brevis*. However, more rapid growth and higher biomass yield of *L. brevis* were observed as compared to *O. oeni* under optimal growth conditions. Both strains were grown in continuous cultures at a variety of dilution rates at constant optimal pH based on the batch fermentations. The precise growth parameters obtained in chemostat reveal the characteristics of growth of both strains. *L. brevis* X_2 showed higher values $Y_{x/s}^{max}$, Q_x^{max} and Y_{ATP}^{max} than *O. oeni*.

With increasing end-product concentration, it is unavoidable that the fermentation becomes inhibited. The MBR has provided a solution to the problem of end product inhibition, while providing sufficient nutrients for the growth of high density cells. The MBR system (Figure 2-8 & 5-8) used in this study is able to produce high density cells up to 12 g/l of dry weight of *O. oeni* and is over 32 times higher than batch (0.357 g/l of dry weight) and continuous culture (a maximum 0.37 g/l). Similarly, with *L. brevis* a 17.5 g/l dry weight was achieved in the MBR, over 27 times higher than batch (0.638 g/l) and 16 times higher than continuous fermentation (a maximum 1.08 g/l). The volumetric productivities were 7.9 mg/l/h and 17.6 mg/l/h in batch and continuous culture respectively while in the MBR 180 mg/l/h is 23 times and 10 times higher over batch and continuous cultures respectively. Also, cell volumetric productivity of *L. brevis* is over a 13-fold and 3-fold in the MBR compared to that in batch and continuous fermentation. Furthermore, it is clear that end product like lactic acid were removed through membrane filter. With propagation of *O. oeni* of 12 g/l of cell dry weight in the MBR, lactic acid concentrations in the range of 1.1-6.0 g/l were determined. This compares to 2.23 g/l and 4.28-6.89 g/l of lactic acid in batch and continuous cultivation respectively where much

lower cell concentrations were produced. Similar results were also observed for the culture of *L. brevis* in the three different fermentation systems (Table 6.2).

Table 6.2 Comparison of growth and productivity in different fermentation systems*

Fermentation	Parameters	*O. oeni*[a]	*L. brevis*[b]	section
Batch Culture	μ_{max} (in test tubes) (h^{-1})	0.063	0.080	a. section 4.3.1, Chapter 4; b. section 4.5.1, Chapter 4.
	μ_{max} (h^{-1})	0.066	0.110	
	Dry-weight (g/l)	0.357	0.638	
	$Y_{x/s}$ (g cell/mol sugar)	7.4	11.5	
	Q_x (mg cell/l/h)	7.9	22.8	
	Lactic acid (g/l)	2.23	NE	
Continuous Culture	D (μ) (h^{-1})	0.007-0052	0.010-0.089	a. section 4.4.3 & 4.4.4 Chapter 4; b. section 4.5.3 & 4.5.4 Chapter 4.
	D_{crit} (h^{-1})	0.065	NE	
	Dry-weight (g/l)	0.31-0.37	0.94-1.08	
	Q_x^{max} (mg/l/h)	17.6	93.2	
	$Y_{x/s}^{max}$ (g cell/mol sugar)	7.93	22.3	
	m_s (mmol sugar/g cell/h)	0.45	0.21	
	Y_{ATP}^{max}(g cell/mol ATP)	8.03	24.7	
	m_{ATP} (mol ATP/g cell/h)	0.0006	0.0004	
	Lactic acid (g/l)	4.28-6.89	8.29-10.1	
Culture in MBR	D (h^{-1})	0.25-0.58	0.32-0.60	a. section 5.3 Chapter 5 b. section 5.4 Chapter 5
	μ_{max} (h^{-1})	0.044	0.065	
	Dry-weight (g/l)	12	17.5	
	Q_x (mg/l/h)	180 (0-50h)	307 (0-48h)	
	Lactic acid (g/l)	1.1-6.0	3.2-9.6	

*** Conditions**: pH = 4.5, T = 25°C, 5g/l glucose-5 g/l fructose in batch and continuous culture, 10 g/l glucose- 10 g/l fructose in the MBR for *O. oeni*.
pH = 5.5, T = 27°C, 10 g/l glucose in batch and continuous culture, 20 g/l glucose in the MBR for *L. brevis*.
all experiments were carried out with pH-control unless otherwise mentioned.
NE: not examined.

Symbols: μ_{max} - maximum specific growth rate; D - dilution rate; D_{crit} – critical dilution rate; $Y_{x/s}$ – biomass yield/growth yield; Q_x –volumetric cell productivity; $Y_{x/s}^{max}$ – maximum biomass yield/growth yield; m_s - maintenance coefficient; Q_x^{max} – maximum volumetric cell productivity; Y_{ATP}^{max} – maximum ATP yield; m_{ATP} –energy coefficient.

Previous work on the growth of *O. oeni* in batch culture with the mixture of glucose-fructose as substrate has been reported. Maicas[2] et al. (1999) found both the highest biomass yield of *O. oeni* ($Y_{x/s}$ = 6.9 g cell/mol sugar) and the maximum dry weight (0.22 g/l) when a mixture of 10 g/l glucose-6 g/l fructose was added to the culture medium and the pH was controlled at 4.8. Also, the biomass yield of *O. oeni* ($Y_{x/s}$ = 7.1 g cell/mol sugar) and dry weight (0.92 g/l) were observed when a mixture of glucose-fructose (12 g/l: 12 g/l) were added to the culture medium with initial pH 5.0 (Salou *et al.* 1994). The growth of *L. brevis* with pH control in batch culture has not been described previously.

6.2.3 High productivity in MBR systems – cell production

Successful propagations of the LAB *O. oeni* and *L. brevis* in the MBR system with complete cell recycle were achieved (Table 6.2 above and sections 5.3 & 5.4 in Chapter 5). The performance of the MBR system used is better than those of batch and continuous culture systems as shown in Table 6.2. High cell concentration cultivation of *O. oeni* and *L. brevis* in the MBR achieved higher cell concentration and much larger volumetric productivity and lower concentration of end product in the MBR. These results provide good prospects for operating the MBR as a large scale for all propagation of LAB. This is also a crucial factor to allow a successful maturation process. When 'green cider' is continuously fed in the MBR, high cell concentrations will allow rapid throughput (i.e. short residence time).

The vitality and productivity of the process is dependent on the operability of the membrane in relation to fouling, cell concentration and the flux of the membrane. A number of conclusions from this work could be made:

(1) The membrane performance (the flux of membrane) was influenced by many factors and these included: pH of growth medium, the interaction of the medium, cells and the membrane, the cell concentration and membrane inlet pressure and so indirectly, the forces associated with cell fouling of the membrane (section 5.2 Chapter 5).

(2) The MBR operation conditions can affect membrane performance. Low membrane inlet pressure reduced dramatically the fouling of membrane and prolonged the operating time of the system especially in high cell concentration cultivation. For example, the permeation rate of membrane finally reached under the same condition is larger when inlet pressure was set initially at 8 psi than when it was set at 10 psi (section 5.3.5 & 5.3.6; section 5.4.1 & 5.4.2).

(3) A limitation of MBR is membrane fouling caused by the formation of cake and concentration polarization.

(4) Another limitation of MBR with complete cell recycle includes possible shear stress on cells entering the filtration unit and pumps. This may possibly result in a considerable slower specific growth rate in the MBR than in batch and continuous cultures.

The experimental results reveal that the limitation of the growth of LAB is mainly due to the insufficient nutrients at high cell concentration owing to feeding rate decline (membrane fouling). There may be two strategies for reaching high cell concentration: (1) increasing the concentration of feeding growth medium without the change membrane of this MBR system. This seems a simple and suitable method for the cultivation of high cell concentration; (2) adding membrane area to the cycling loop. For example, doubling the membrane area or managing membrane to reduce fouling by cleaning. This method increases costs and complexity of the MBR system but may be a better solution for increasing feed rates (i.e. membrane permeation rate and so membrane productivity).

6.2.4 High productivity in MBR systems – malolactic fermentation

The malic acid transformation period can be shortened significantly by the two LAB. With retention time of 6 hours or less within the MBR, a controlled system (section 5.5 Chapter 5) can be compared to a conventional batch fermentation of a typical 20 - 40 day. The MBR system has been run successfully for a period up to over 9 days and with correct management could be run for much longer periods. Malic acid in artificial 'green cider' under various conditions such as feeding rate, malic acid concentration and ethanol concentration in the MBR system was degraded efficiently. The following conclusions were made from this work:

- An optimal feeding rate for malic acid degradation was about 2 l/h (retention time 3 h) for *O. oeni* and *L. brevis*;

- Higher malic acid concentration did not reduce the ability of *O. oeni* to degrade malic acid. For example, specific rate of malic acid transformation, in terms of g malate/h/g DCW, 0.071 at 5g/l malate and 0.037 at 2 g/l when feeding rate 0.9 l/h (6.7h);

- The MBR system maintained a high activity to degrade malate in 5-10% (v/v) ethanol. Ethanol inhibited the ability of *O. oeni* to degrade malate. The specific rate of malic acid transformation declined with the increase of ethanol concentration, and dropped rapidly in the presence of 12% (v/v) ethanol.

6.2.4.1 Summary of strategies of MLF induced by malolactic bacteria

The strategies involved in the MLF are shown below in Figure 6-1 during the maturation process and generally included three methods: spontaneous MLF, starter cultures and high cell concentration.

(1) As previously described in Chapter 1, spontaneous MLF is the most unreliable and time-consuming and is not used in most commercial cider and wine manufacturing.

(2) Malolactic starter cultures are being widely employed to induce MLF all over the world. The main limitation in utilizing these bacteria is the relatively low cell concentration obtained by traditional fermentation methods. Moreover, the growth of LAB occurs under unflavourable environment such as low concentration of growth nutrients, high acidity and high ethanol concentration, and several weeks or months are normally required to obtain an appropriate number of cells able to degrade the malic acid efficiently present in wines/ciders. MLF depends upon the growth of LAB in the wine as a batch culture and is strongly influenced by environmental conditions, so that the process is often delayed; even failure to induce MLF is not unusual. These problems have driven the search for alternative technologies that enable more rapid and reliable MLF to take place.

(3) The induction of MLF with high concentration of cells enable better control of the process as well as guaranteeing that it takes place at the required time during the wine production (Gao & Fleet 1994; Formisyn *et al.* 1997; Maicas[1] *et al.* 1999). Moreover, the use of a reactor presents numerous advantages to a traditional deacidification. For example, starter cultures can be reused, the production of secondary products can be controlled to some extent, and the fermentation can be induced and halted at the moment desired by the winemaker or cidermaker. The sensory quality of the products is not adversely affected by this technology. This behavior is commercially important because a low concentration of such metabolites like as diacetyl and acetoin is a quality factor in alcoholic beverages – wine, cider and beer.

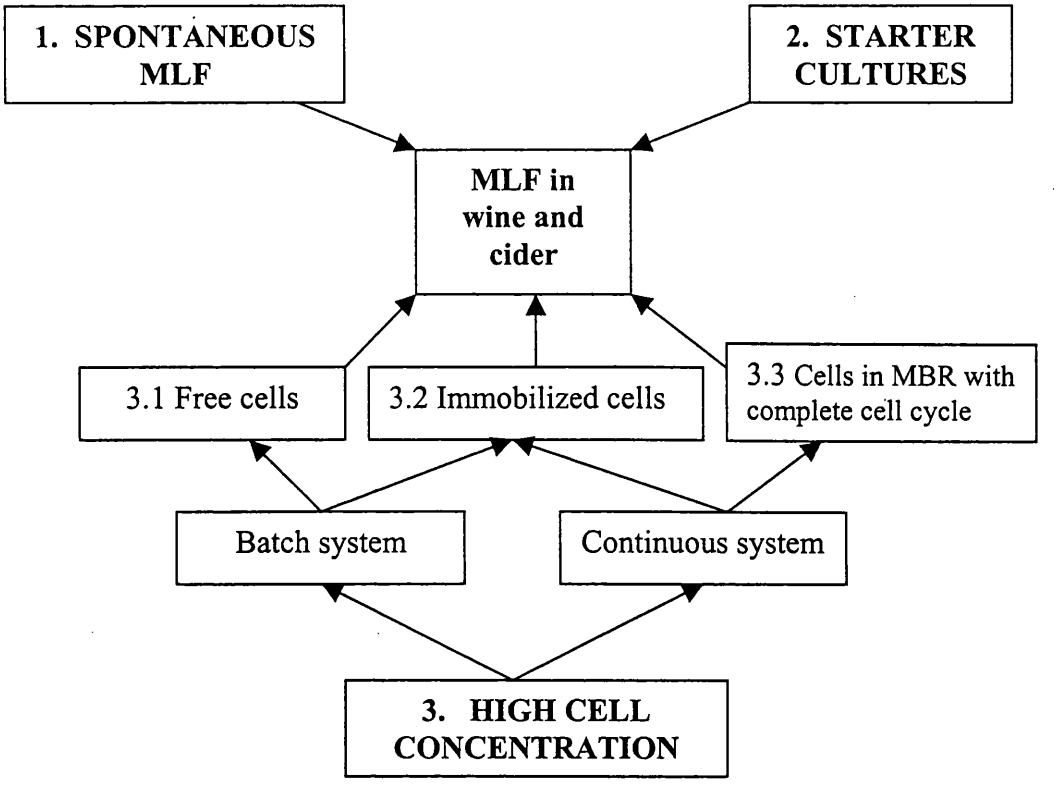


Figure 6-1 Summary of the strategies of MLF induced by malolactic acid bacteria in wine and cider

6.2.4.2 MLF in MBR systems with high cell concentration

Cell density (10^6 CFU/ml) was considered enough to induce MLF in wine (Firme *et al.*1994), but a different observation that concentrations of 10^8 CFU/ml or less gave little or no detectable degradation and higher concentrations of 10^9 CFU/ml gave approximately 90% degradation of malic acid was also reported (Gao & Fleet 1994). Higher cell density can further enhance the MLF process. To date MLF induced by high cell concentration includes three main methods as described above in Figure 6-1:

(1) Free cells of high density inoculated directly as batch model for maturation (in screw tubes or shake flasks);

(2) Immobilized cells as batch (in flasks) or continuous model (continuous stirred tank reactor (CSTR) or fluidized bed reactor (FBR));

(3) High concentration cells in MBR with complete cell recycle (MBR-CCR).

The third strategy in the MBR with complete cell recycle (MBR-CCR) is most attractive and has following benefits:

(1) no end-product inhibition due to very low sugar concentration;
(2) avoidance of slow growth and poor yields encountered when starter cultures are used to inoculate cider due to the separation of cell propagation and maturation process;
(3) reduction of risk of undesirable flavour formation such as acetic acid, diacetyl ;
(4) flexible operation and control for desirable product by control of residence time.

There is only one report of such a system. A 300 ml cell-recycle membrane bioreactor system has been applied to MLF in red wine. This MBR system with a flow rate 360 ml/h was run for 56 h, and then the MLF activity was lost rapidly (Gao & Fleet 1995). Other strategies for controlling continuous fermentations have been explored either by the utilization of free cells in CSTR (continuous stirred tank reactor) or by the use of immobilization of cells in CSTR and FBR (fluidized bed reactor). Table 6.3 below reviews the current performance of all known systems.

6.2.5 Application and perspectives

The results of this project show that the new process can lead to the intensive production of the two strains to commercially useful amounts for the cider maturation process and that the bioprocess engineering can make the operating/cidermaking processes more profitable and reliable and are potential one of the most exciting developments in the cider processing industry to date. Industrial implications of this technology are widespread within the cider industry. It is also believed that the method could be put to use elsewhere, for example in rice wines, soy sauce, starter cultures for dairy and vegetable fermentations.

This project represents a significant implication to product innovation via enhanced maturation. The membrane bioreactor technology can produce and utilize the intensified cultures of LAB for the production of inoculants and the rapid maturation of cider. The MBR gives better manipulation of LAB in the production environment and provides considerable scope for the enhancement of the maturation processes. The major benefits

Table 6.3 Examples of alternative methods studied to induce malolactic fermentation of wine and cider in bioreactors with high concentration of LAB*

Bacteria or enzyme	Immobilization Matrix	Reactor (Sample volume)	L-malic acid (g/l)	Bioconversion [a] (%)	Retention time (h)	Operation period (h)	Cell concentration	Source material	references
Oenococcus oeni	- [d]	Screw tubes (10 ml)	1.0-6.0	82-96	6	-	10^9 (cfu/ml)	Synthetic medium	Gao and Fleet (1994)
Oenococcus oeni	-	Screw tubes (10 ml)	3.5-7.0	14-100	500	-	10^6-10^8	Red wine	Maicas et al. (2000)
Oenococcus oeni	-	Shake flask (0.1 l)	3.5	40-45	24	100	2×10^9	Red wine	Maicas et al. (2001)
Oenococcus oeni	polyacrylamide	Shake flask (5 ml)	2.3-4.5	71	10 min	-	13% wet wt/v	Synthetic medium	Rossi and Clementi(1984)
Oenococcus oeni	κ-Carrageenan	Shake flask (5 ml)	5.0	100	84	-	10^9 - 10^{10}	Synthetic medium	McCord and Ryu (1985)
Oenococcus oeni	cellulose sponge	Shake flask (0.1 l)	3.5	50	24	100	2×10^9	Red wine	Maicas et al. (2001)
Oenococcus oeni	Ca-alginate	sleeved reactor (2 l)	4.88	99	NR	-	NR	Cider	Cabranes et al. (1998)
Oenococcus oeni	Ca-alginate	Erlenmeyer flask (0.1 l)	3.2	100	7 days	-	5×10^7	Cider	Herrero et al. (2001)
Oenococcus oeni	-	Erlenmeyer flask (0.1 l)	3.2	100	7 days	-	10^7	Cider	Herrero et al. (2001)
Lactobacillus sp.	calcium alginate	FBR (1.4 l)	5.5	100-50 [c]	11.7	72	NR	Synthetic medium	Naouri et al. (1991)
Oenococcus oeni	calcium alginate	FBR (1.4 l)	5.5	100-50 [c]	11.7	96	NR	Synthetic medium	Naouri et al.(1991)
Lactobacillus sp.	calcium alginate	CSTR (NR)	8.98	69-75	2	16 days [b]	7×10^{10}	Wine	Crapisi et al. (1987)
Oenococcus oenii	calcium alginate	CSTR (NR)	8.98	62-71	2	10 days [b]	8×10^{10}	Wine	Crapisi et al. (1987)
Oenococcus oeni	κ-Carrageenan	MBR-CCR (0.3 l)	0.8-4.6	25-100	0.9	125	10^{10}	Wine	Gao and Fleet (1995)
Oenococcus oeni	-	CSTR (0.2 l)	4.2	92-95	62.5	300-500	3×10^7-10^8	Wine	Maicas et al. (1999)
Oenococcus oeni	κ-Carrageenan	CSTR (NR)	2.27	77	0.64 vol/h	2-4 days	NR	Wine	Spettoli et al. (1987)
Lactobacillus sp.	κ-Carrageenan	CSTR (NR)	2.27	85	0.64 vol/h	8 days	NR	Wine	Spettoli et al. (1987)
Oenococcus oeni	oak chips	CSTR (80 ml)	8.0 (DL)	98.7	2.2	11 days [b]		Synthetic medium	Janssen et al. (1993)
Saccharomyces cerevisiae and *Oenococcus oeni*	Ca-alginate	Erlenmeyer flask (0.1 l)	3.6	99	9 days	-	10^7	Cider	Herrero[3] et al. (1999)
Saccharomyces bayanus and *Oenococcus oeni*	Ca-alginate	FBR (0.99 l)	5.2	70-95	2-8	NR	10^9	Cider	Nedovic et al. (2000)
Oenococcus oeni	LentiKats	Tubular glass reactor (18ml)	6.7	75-98	0.55	NR	6.5×10^8	Cider	Durieux et al. (2000)
Oenococcus oeni	-	MBR-CCR (6 l)	2 or 5	92-41	2-12	220	10^{11}	Artificial cider	Section 5.5.5 Chapter 5
Lactobacillus brevis	-	MBR-CCR (6 l)	2	90-36	2-10	120	10^{11}	Artificial cider	Section 5.5.4 Chapter 5

* Figures in parentheses represent working volumes (CSTR continuous stirred tank reactor, MBR-CCR membrane bioreactor with complete cell recycle, FBR fluidized bed reactor, NR no reported data).
a. Amount of L-malic acid consumed / initial amount of L-malic acid. b. half-life of a bioreactor containing immobilized cells. c. relative percent of malic acid consumed compared to the maximum degradation.
d. "- " free cells in reactors.

are the safe and rapid growth and utilization of high concentrations of LAB to improve the quality, efficiency (increase speed) and reliability (reproducibility) of processing. Processing using this method, potentially also makes cideramaking more flexible and responsive to a fluctuating market, and allows substantial cost savings related to shortened maturation period and reduced product storage. It can lead to the development of new flavor types using specific strains of LAB, resulting in new products. It may also be possible to produce a flavor concentrate that can be blended with bland ciders to produce a traditional English bittersweet cider, either in UK or overseas where there is an absence of traditional cider apple.

6.3 Future work

The main challenges of the utilization of the MBR for the growth and biotransformation reactions lie in the efficient use of the system and overcoming the expense of membranes by enhancing reactor productivity. There are various strategies which may be considered for intensifying the biotransformation capacity of the membrane system. The basic principle is to get the best organisms in the best environment to give the best product. The fermentation strategies can be closely considered for the most efficient and economic integration of the MBR system into the high density cell cultivation and cider making process.

The further development of the present work should be carried out in the control system of the MBR and optimization of malolactic fermentation conditions for the high density cell cultivation and cider making process in practical situation (i.e. the factory).

6.3.1 Modification of control systems of the MBR

The development of optimal operation conditions for an MBR is dependent on the ability to measure various parameters and have the appropriate control systems in place in order to implement the desired strategies. First, a pressure gauge should be set at the outlet of membrane for the measurement of membrane pressure drop. In addition, a recycle flow rate of fluid (medium containing cells) should be recorded not only to know a relationship

between pressures and recycle flow rate but also to understand the influence of recycle flow rate on the growth and viability of cells in the MBR system. Third, the flux of membrane should be measured automatically. Finally, a temperature control system should be adjusted flexibly. The temperature was pre-set at 25°C in this MBR system used. The growth of both *O. oeni* and *L. brevis* and the cider maturation by both strains were carried out at 25°C, which is at least not an optimal temperature for the growth of *L. brevis*.

6.3.2 Optimization of malolactic fermentation

6.3.2.1 Cell concentration and malolactic activity

The results have shown that the high density cell concentration can speed up malic acid degradation. Then, is a better performance obtained from a higher cell concentration in the MBR system? What is the optimal cell concentration for the transformation of malic acid in the MBR system? It was known that there are the important interactions of bacteria-bacteria and bacteria- membrane. The environment conditions could cause change in cell size and cell shape. Moreover, the growth of cells was influenced by shear stress in the MBR. Do these factors influence the viability and vitality of cells in the MBR system, especially with high cell density? These questions need to be answered.

6.3.2.2 Temperature and pH

It was known that temperature strongly affects the development of malolactic fermentation and lactic acid bacteria metabolism, so the investigation that aims to determine the metabolism of organic acids during malolactic fermentation should be performed. In addition, the effect of pH on the malic acid degradation has been described in section 1.2.2 & 1.3.1 Chapter 1. Previous work (Salou *et al.* 1991) has shown that the maximum specific growth rate of *O. oeni* and the maximum malolactic activity were achieved at different pH value; however, the effect of pH on malolactic fermentation with high density of non-proliferating cells has not been investigated. Hence, it is crucial to study the influence of both pH and temperature on the malolactic fermentation in the presence of high density of non-proliferating cells loading in the MBR system with complete cell recycle in the future work.

6.3.3 Model development and validation

It will be important to obtain numerical models for the prediction of MBR operation and control strategies. The experimental work detailed in this work has provided the raw data and a preliminary study. The process control and membrane selection represent a complex set of interactions involving the physiological performance of the microbes, environmental conditions and the type and configuration of the membrane used. The reactor model should be based on the prediction of permeate flux and kinetics of cell growth in relation to end-product inhibitions, cell concentration, reactor conditions as well as the concentration and flow rate of the feed medium. The models should allow the optimization of the process and cost, membrane performance and reactor productivity.

6.3.4 Practical application of the technology

Both *O. oeni* and *L. brevis* used in this study not only show the strong ability to adapt well cider environment and malolactic activity, but exhibit the stability in the ability to degrade malic acid in MBR system when the artificial 'green cider' pass continuously through the MBR under various conditions tested. It should be noted however, that although artificial 'green cider' has similar components with real cider and environment, it does not contain minor components found in yeast fermented cider. It is therefore necessary to investigate further the malolactic activity and the ability to degrade malic acid of both strains, as well as the enhancement to the cider flavour and 'mouth feel' during the controlled maturation process of a real yeast fermented cider.

Further work may also include the enzymatic engineering, genetic modification and metabolic engineering to produce 'super maturation bacteria' which may fill all requirements for optimization of the biotransformation for the particular environment, stability, vitality, low nutritional requirements, production of desirable flavours. This would present many marketing and ethical problems to allow such a development.

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Appendix A

Abbreviations and symbols

Abbreviations

AGC	artificial 'green cider'
ATP	adenosine triphosphate
°C	degrees centigrade
CFU	colony forming units
CSTR	continuous stirred tank reactor
DCW	dry cell weight
FBR	fluidized bed reactor
GC	gas chromatography
h	hour
HPLC	high performance liquid chromatography
kg	kilogram
l	litre
LAB	lactic acid bacteria
L. brevis	*Lactobacillus brevis*
MBR	membrane bioreactor
MBR-CCR	membrane bioreactor with complete cell recycle
MIP	membrane inlet pressure
mg	milligram
ml	milliliter
MLF	malolactic acid fermentation
ML	malolactic
mM	millimolar
MRS	de Man Rogosa Sharpe medium
mol	mole
NAD	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
nm	nanometre
OD	optical density at 660nm
OD_{max}	maximum optical density at 660nm
O. oeni	*Oenococcus oeni* (*Leuconostoc oenos*)
psi	pounds per square inch
T	temperature
TMP	transmembrane pressure
UV	ultraviolet light
µm	micrometer
v/v	concentration volume per volume
w/w	concentration weight per weight
YE	yeast extract
%	percentage

Symbols

μ	specific growth rate	(h^{-1})
μ_{max}	maximum specific growth rate	(h^{-1})
D	dilution rate	(h^{-1})
D_{crit}	critical dilution rate	(h^{-1})
F	feeding rate	(l/h)
K_s	substrate saturation constant	(g/l)
m_{ATP}	energy coefficient	(mol ATP/g cell/h)
m_s	maintenance coefficient	(g sugar/g cell/h) or (mmol/g/h)
P	pressure	(psi)
Q_s	specific rate of substrate uptake	(g sugar/g cell/h)
Q_p	specific rate of product formation	(g/g/h)
Q_x	volumetric biomass productivity	(g/l/h)
q_s	specific rate of substrate uptake	(mol sugar/g cell/h)
q_{ATP}	specific rate of ATP utilization	(mol ATP/g cell/h)
S	substrate concentration	(g/l)
S_i	inlet substrate concentration	(g/l)
t_d	doubling time	(h)
V	culture volume	(l)
x	steady-state cell concentration	(g/l)
Y_{ATP}	ATP yield of biomass	(g cell/mol ATP)
Y_{ATP}^{max}	maximum ATP yield	(g cell/mol ATP)
$Y_{p/s}$	product yield	(g/g) or (mol/mol)
$Y_{p/x}$	product yield	(g/g) or (mol/g)
$Y_{x/s}$	observed yield/biomass yield	(g cell/g sugar) or (g/mol)
$Y_{x/s}^{max}$	theoretical biomass yield/maximum biomass yield	(g/g) or (g/mol)

Nb. Other abbreviations and symbols are defined in the text.

Appendix B

The samples of linear regression analysis for the calculation of maximum specific growth rate (μ_{max})

The optical density (OD) was measured at 660nm during the growth of *O.oeni* on 5g/l glucose. The duplicate data are shown in Table B and the linear regressions are shown in Fig. B below.

Table B the experimental data of OD and ln(OD) in batch culture of *O. oeni* in test tubes

Time(h)	OD	ln(OD)	ln(OD)	Time(h)	OD	ln(OD)	ln(OD)
0	0.241	-1.423		0	0.3	-1.204	
6	0.262	-1.339		6	0.321	-1.136	
33	0.341	-1.076	-1.076	33	0.4	-0.916	-0.916
40	0.382	-0.962	-0.926	40	0.448	-0.803	-0.803
44	0.411	-0.889	-0.889	44	0.471	-0.753	-0.753
67	0.499	-0.695	-0.695	67	0.556	-0.587	-0.587
77.5	0.617	-0.483	-0.483	77.5	0.68	-0.386	-0.386
91.5	0.861	-0.150	-0.15	91.5	0.89	-0.117	-0.117
96.5	0.956	-0.045	-0.045	96.5	0.986	-0.014	-0.014

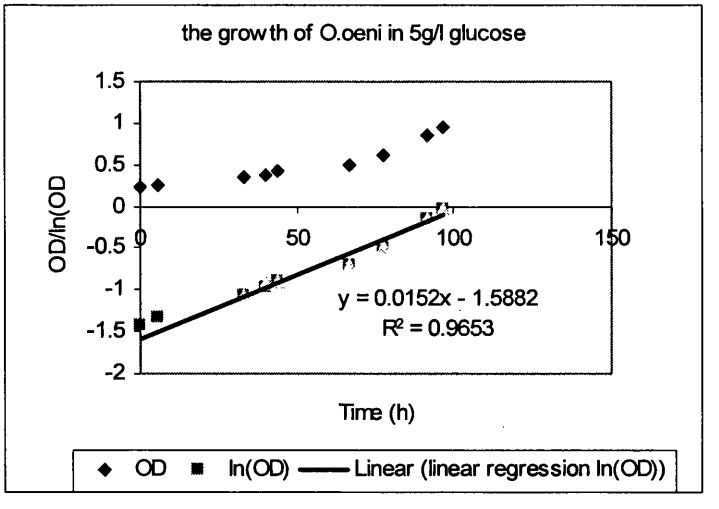


Figure B-1 the graphical estimation of the growth of *O.oeni* in 5g/l glucose (tube 1)

From Fig. B-1 above it was known that the maximum specific growth rate was 0.015 h^{-1} in the first test tube culture. Similarly, the maximum specific growth rate with 0.013 h^{-1} was obtained in the second tube from Fig. B-2 below. So the average value of the two tube cultures is 0.014 h^{-1}, which is expressed by the μ_{max}. $\mu_{max} = 0.014\ h^{-1}$.

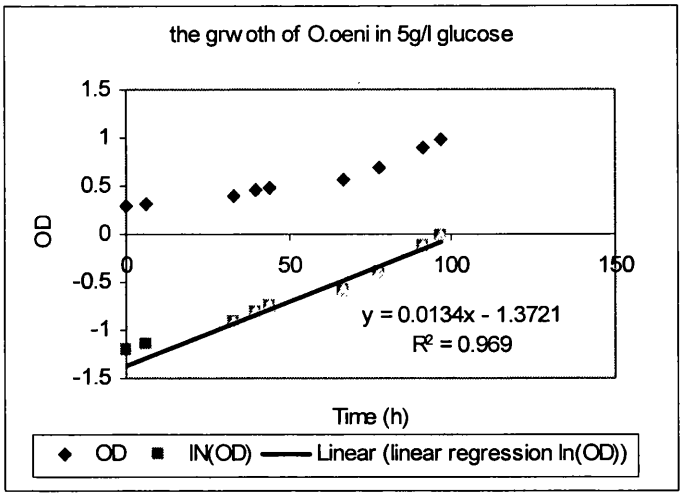


Figure B-2 the graphical estimation of the growth of *O.oeni* in 5g/l glucose (tube 2)

As for the calculation of maximum specific growth rate on other conditions, the same method was used. The raw data (OD at 660nm) and the graphical estimation were omitted.

Appendix C

Calibration of the spectrophotometer: relationship between the calculated OD and measured OD of *O.oeni*

Because the spectrophotometer accurately measures over a small range it is necessary to formulate graphically a relationship between calculated optical density (OD) and measured optical density so that biomass estimation may be made for measured values of optical density outside of the linear response of the apparatus. The data collected from the spectrophotometer (Pye Unicam SP8-400 UV/VIS Spectrophotometer) is presented in Table C and Figure C-1 below.

Table C Calculated and measured OD readings over different dilution ratios

dilution ratio	1/dilution ratio	measured OD	calculated OD
	0	0	0
50 fold	0.020	0.278	0.277
40 fold	0.025	0.346	0.346
30 fold	0.033	0.463	0.457
20 fold	0.050	0.655	0.692
15 fold	0.067	0.904	0.927
12 fold	0.083	1.106	1.149
10 fold	0.100	1.295	1.384
8 fold	0.125	1.575	1.730
5 fold	0.200	2.195	2.768
1 fold	1.000		13.84

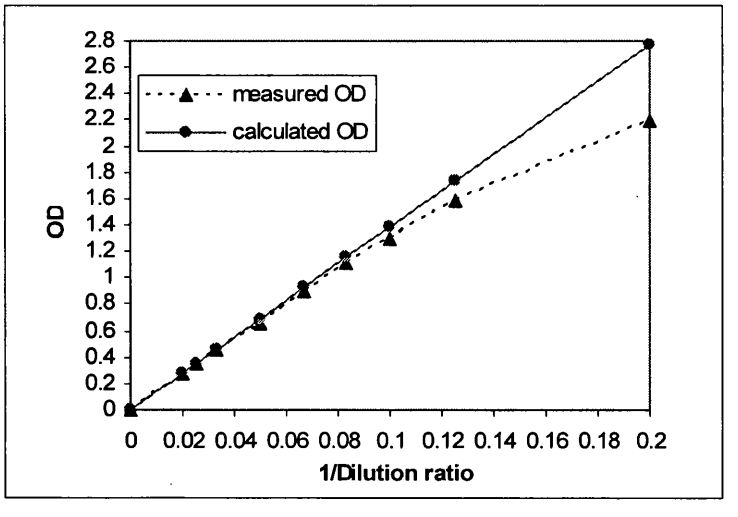


Figure c-1 Calculated and measured OD in 1ml cuvette (1cm light path at 660nm) vs dilution ratio of *O.oeni* cell suspension

Figure shows that the optical density of the culture was linear to the amount of growth provided the OD of the culture did not exceed 1 with 1-cm light path at 660nm. If values above 1 were obtained, then they were diluted so avoiding non linear response at high concentration.

A plot of calculated OD against measured OD shows that the mathematical relationship between the two values. A polynomial 2nd order curve fits the data and is presented in Figure C-2.

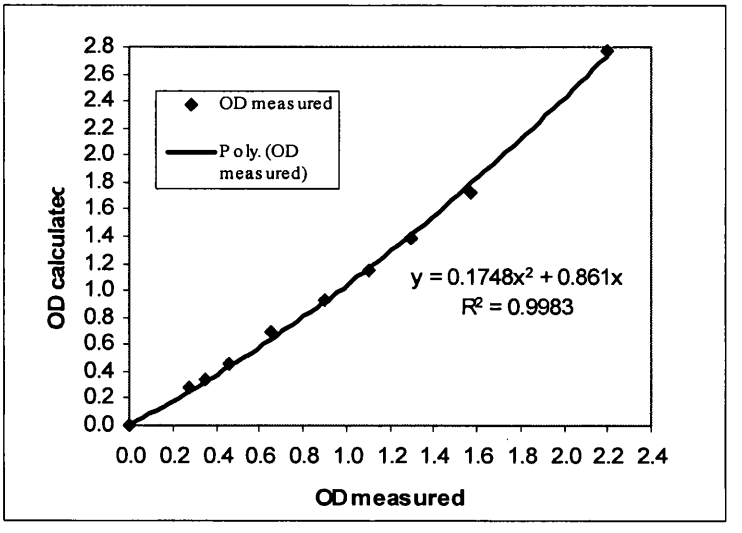


Figure c-2 Relationship between calculated and measured OD of *O.oeni* at 660nm

Figure c-2 shows that there is a linear response up until a reading of about 1 optical density unit, after this the polynomial expression ($y = 0.1748x^2 + 0.861x$) describes the relationship between calculated and measured values of optical density.

2nd order equation for a polynomial is $y = 0.1748x^2 + 0.861x$

where y is calculated optical density

x is measured optical density

Appendix D

Calibration curves for the carbohydrate and sugar alcohols (glucose, fructose, mannitol, erythritol and glycerol) assayed during this study

Figure D- 1, 2, 3, 4 & 5 give the calibration curves required for the quantification of these compounds which were detected by HPLC with ED40 Electrochemical Detector, in Pulsed Amperometry mode (DIONEX).

$y_{(glucose)} = 6 \times 10^{-7}x$ (mM)

$y_{(fructose)} = 3 \times 10^{-6}x$ (mM)

$y_{(mannitol)} = 7 \times 10^{-7}x$ (mM)

$y_{(glycerol)} = 7 \times 10^{-7}x$ (mM)

$y_{(erythritol)} = 1 \times 10^{-7}x$ (mM)

where x is peak area (mV sec)

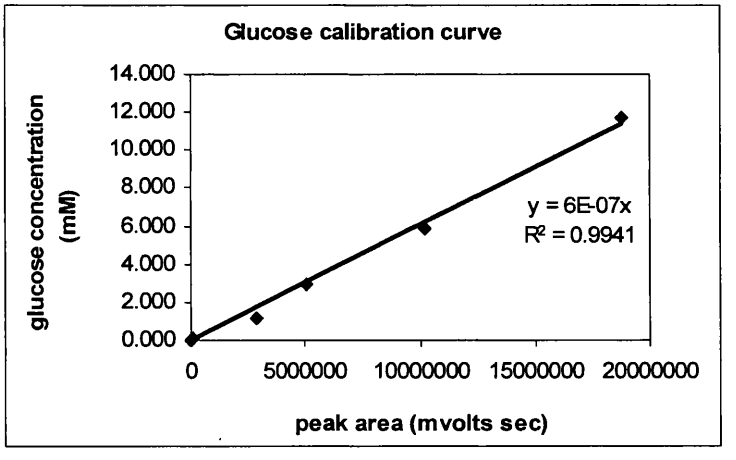


Figure D-1 Calibration curve for the calculation of glucose concentration

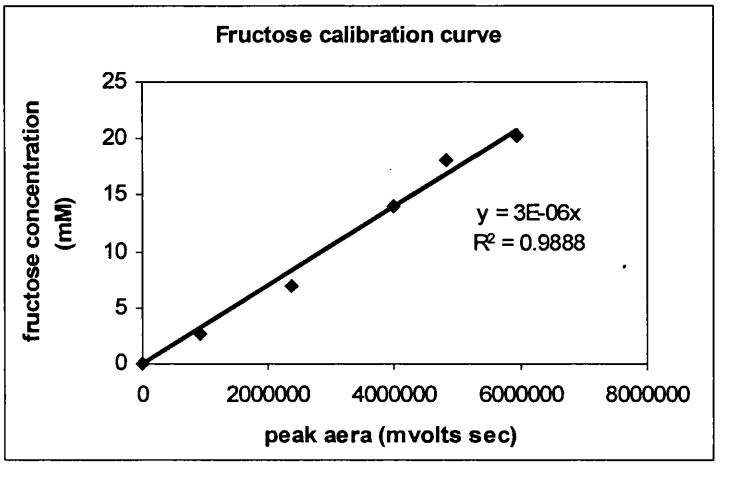


Figure D-2 Calibration curve for the calculation of fructose concentration

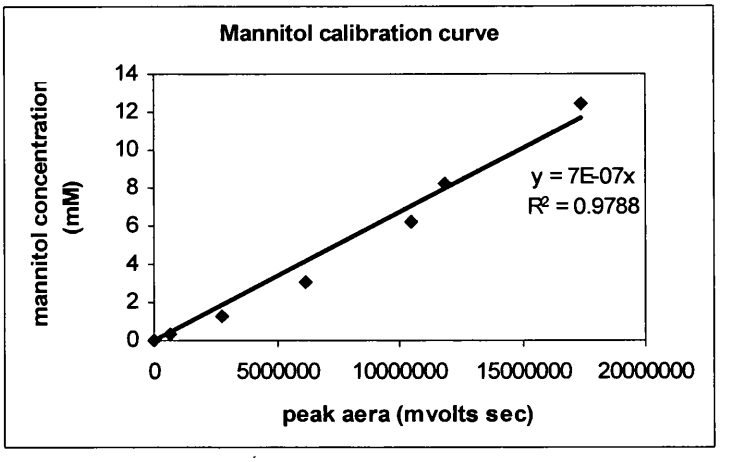


Figure D-3 Calibration curve for the calculation of glucose concentration

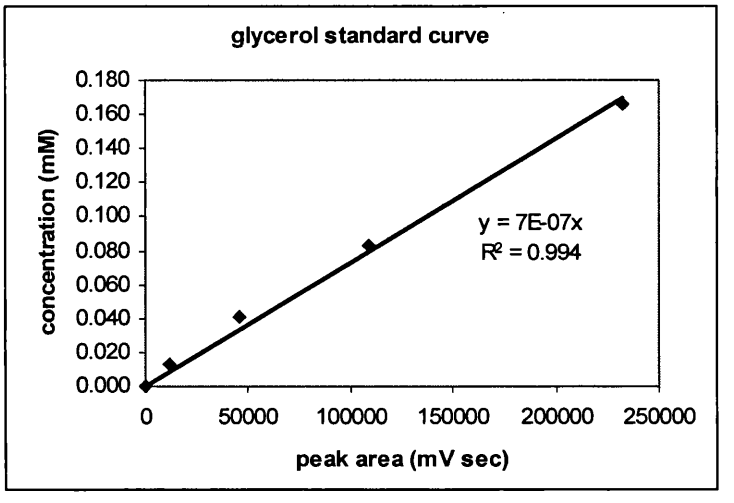


Figure D-4 Calibration curve for the calculation of glycerol concentration

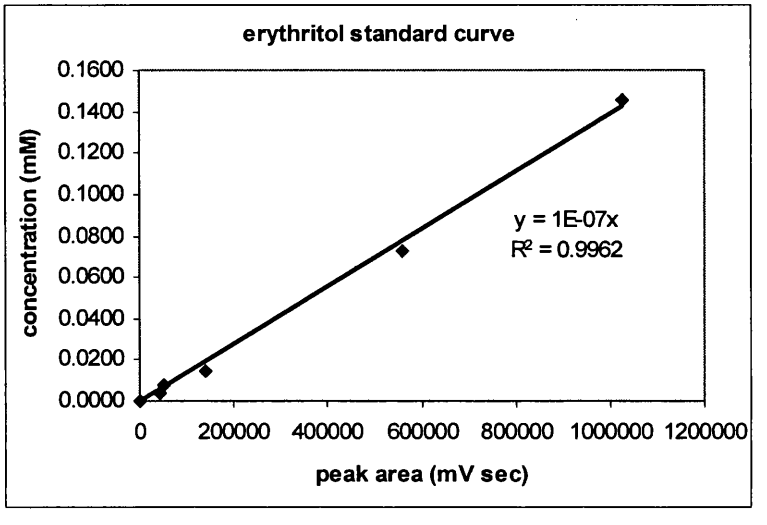


Figure D-5 Calibration curve for the calculation of erythritol concentration

Appendix E

Calibration curves for the organic acids (lactic acid, acetic acid and malic acid) assayed during this study

Figure E-1, 2 & 3 give the calibration curves required for the quantification of these compounds which were detected by HPLC with ED40 Electrochemical Detector, in conductivity mode (DIONEX).

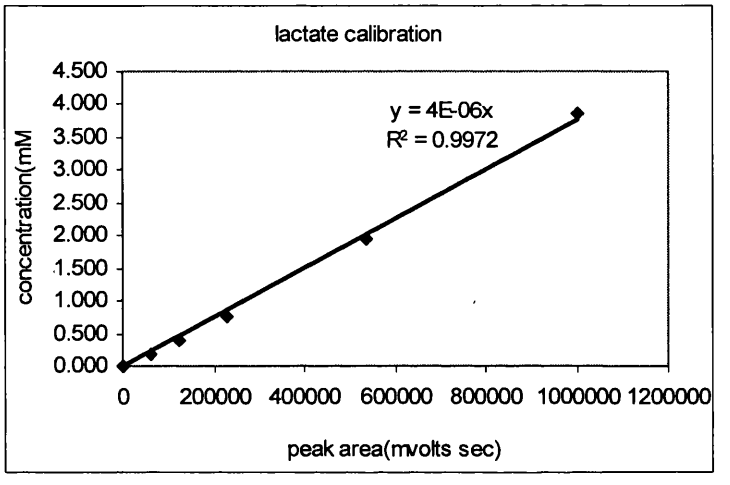


Figure E-1 Calibration curve for the calculation of lactic acid concentration

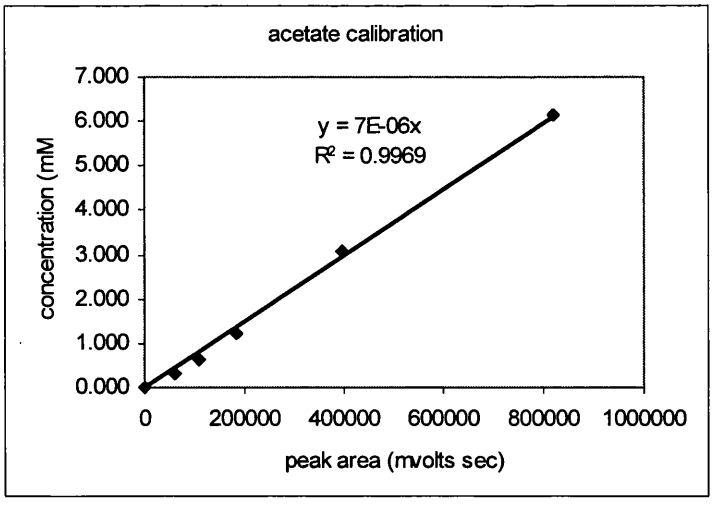


Figure E-2 Calibration curve for the calculation of acetic acid concentration

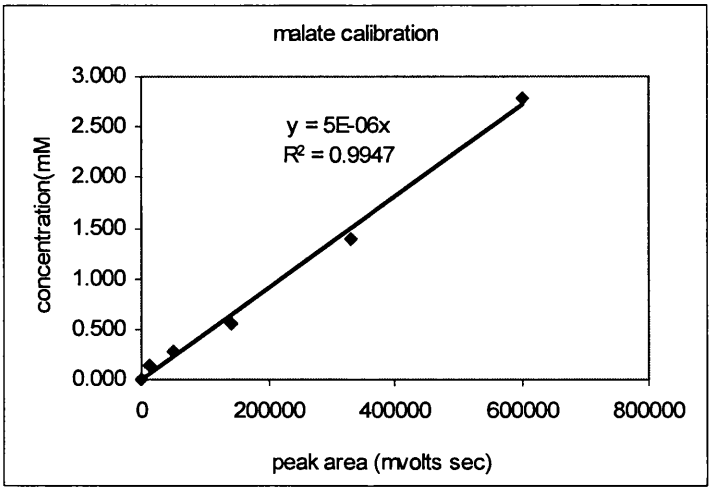


Figure E-3 Calibration curve for the calculation of malic acid concentration

$y_{(lactic\ acid)} = 4 \times 10^{-6}x$

$y_{(acetic\ acid)} = 7 \times 10^{-6}x$

$y_{(malic\ acid)} = 5 \times 10^{-6}x$

where y is the lactic, acetic or malic acid concentration (mM)

x is the peak area (mvolts sec)

Appendix F

Calibration curve for ethanol estimation in this study

Figure F gives the calibration curve required for the quantification of ethanol which was detected by GC with FID (VARAIN).

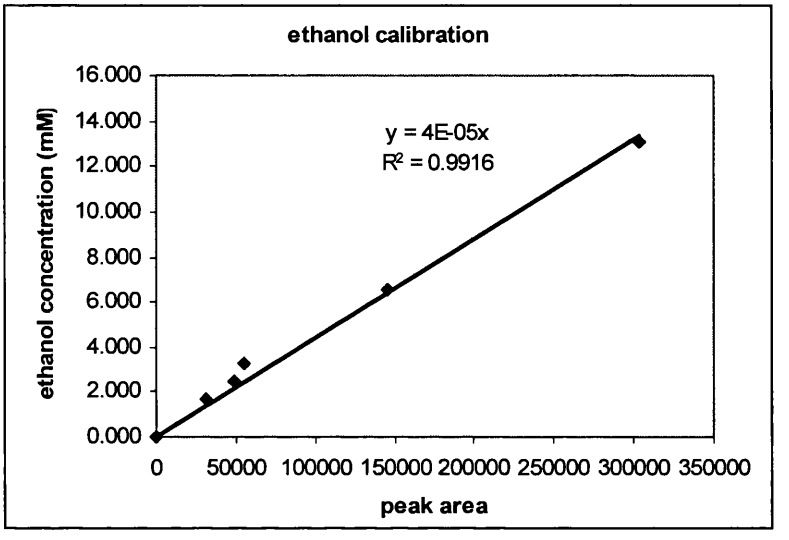


Figure F Calibration curve for the calculation of ethanol concentration

y (ethanol)= $4 \times 10^{-5}x$

where y is ethanol concentration (mM)

x is peak area (mV sec)

Appendix G

Arrhenius plots for the calculation of activation energy (E) of *O. oeni*

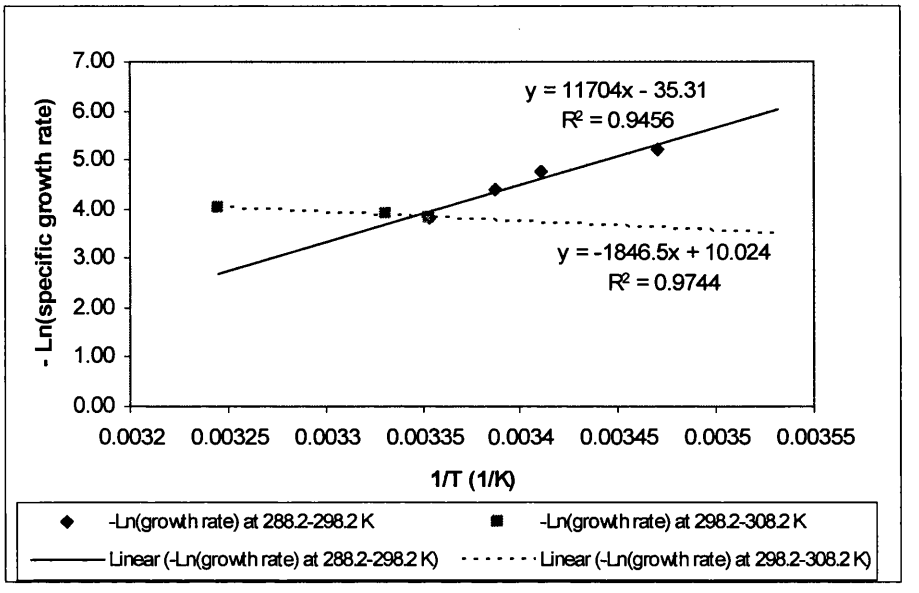


Figure G-1 -Ln (μ) vs. 1/T (K^{-1}) and linear regression in two temperature ranges 288.2-298.2K (15-25°C) and 298.2-308.2K (25-35°C) in the absence of ethanol in batch culture of *O. oeni*

The linear regression equations of two phases in the absence of ethanol:

$\ln\mu_{01} = -11704/T + 35.31 \quad (288.2K \leq T < 298.2K)$

$\ln\mu_{02} = 1846.5/T - 10.024 \quad (298.2K < T \leq 308.2K)$

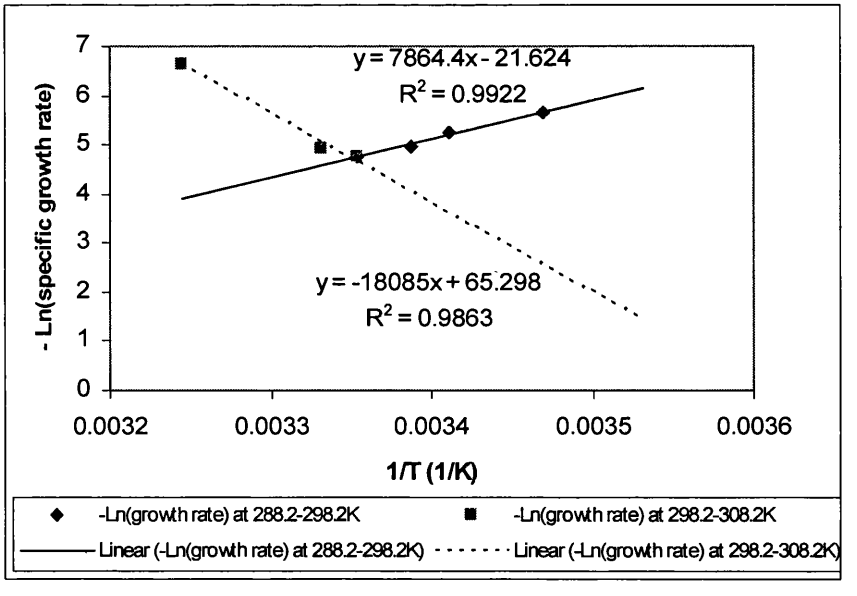


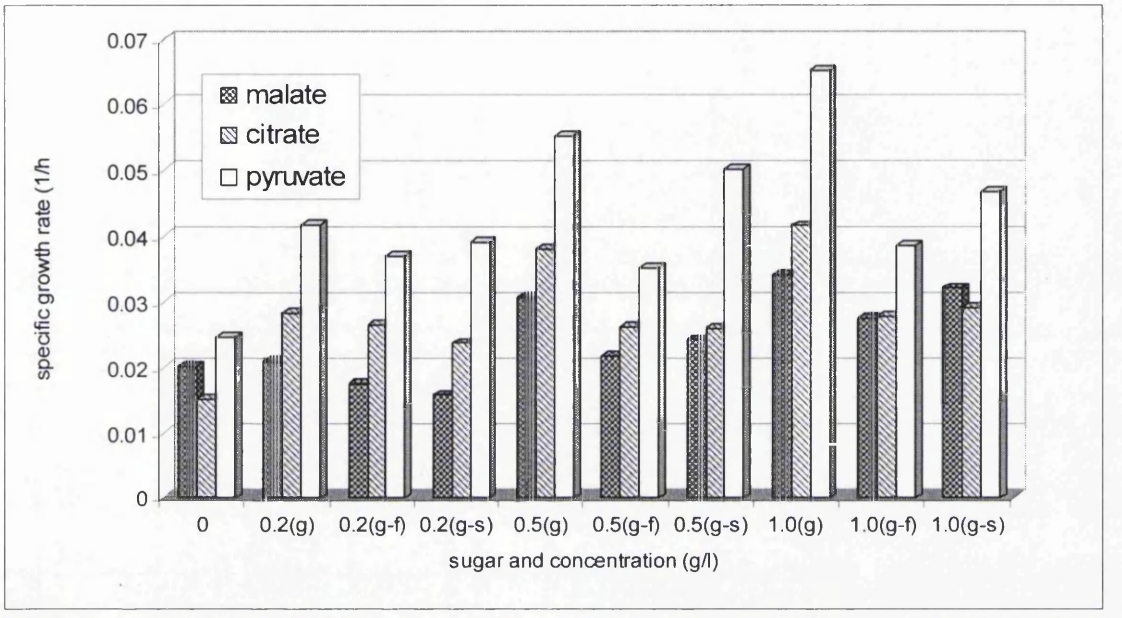
Figure G-2 -Ln (μ) vs. 1/T (K^{-1}) and linear regression in two temperature ranges 288.2-298.2K (15-25°C) and 298.2-308.2K (25-35°C) in the presence of 6% (v/v) ethanol in batch culture of *O. oeni*

The linear regression equations: $\ln\mu_{61} = -7864.4/T + 21.624 \quad (288.2K \leq T < 298.2K)$

$\ln\mu_{62} = 18085/T - 65.298 \quad (298.2K < T \leq 308.2K)$

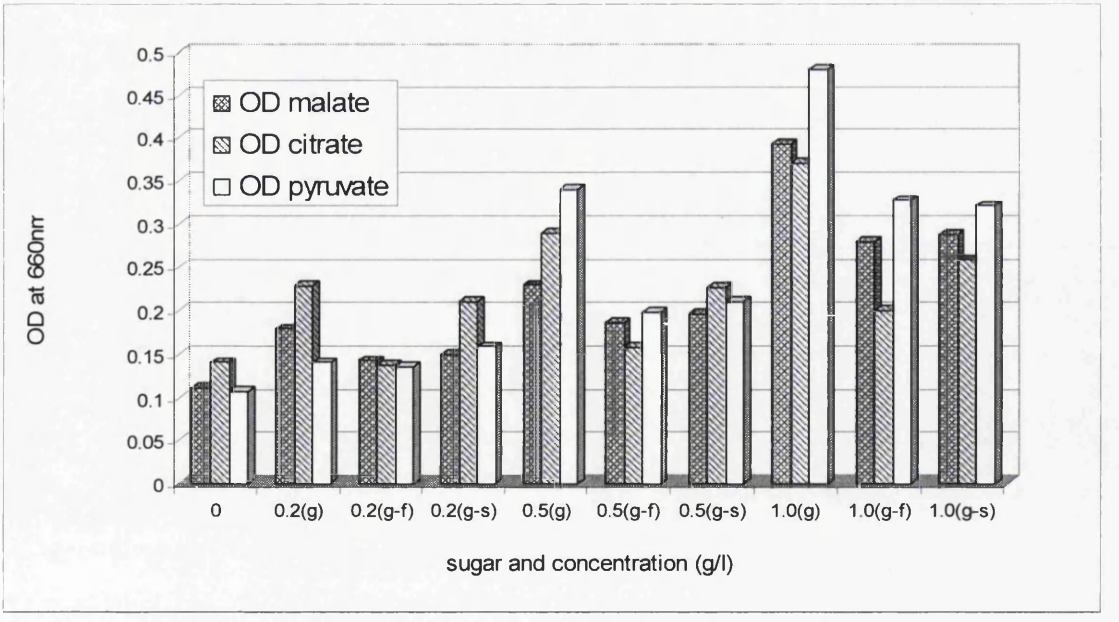
Appendix H

The influence of organic acids on the growth of *O. oeni* in the presence of low sugar concentrations



Symbols: g - glucose, f - fructose, s – sucrose. 0.2g/l, 0.5g/l and 1.0g/l glucose; 0.2g/l, 0.5g/l and 1.0g/l mixture (1:1, w/w) of glucose-fructose; 0.2g/l, 0.5g/l and 1.0g/l mixture (1:1, w/w) of glucose-sucrose.

Figure H-1 the influence of organic acids on specific growth rates of *O. oeni* with different sugars and different sugar concentrations



Symbols: g - glucose, f - fructose, s – sucrose. 0.2g/l, 0.5g/l and 1.0g/l glucose; 0.2g/l, 0.5g/l and 1.0g/l mixture (1:1, w/w) of glucose-fructose; 0.2g/l, 0.5g/l and 1.0g/l mixture (1:1, w/w) of glucose-sucrose.

Figure H-2 the influence of organic acids on optical density (OD) at 660nm with different sugars and different sugar concentrations

Appendix I

The influence of phenolic acids on the growth of *O. oeni* in different sugars

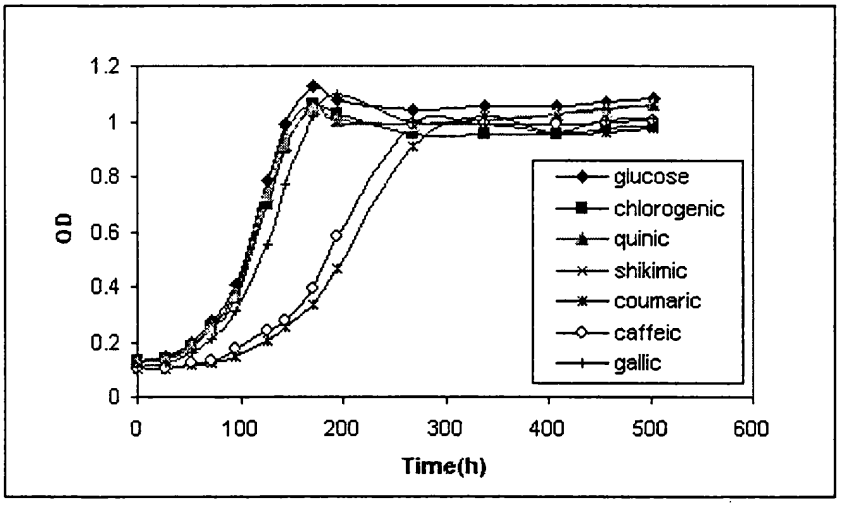


Fig.I-1 in 5g/l glucose

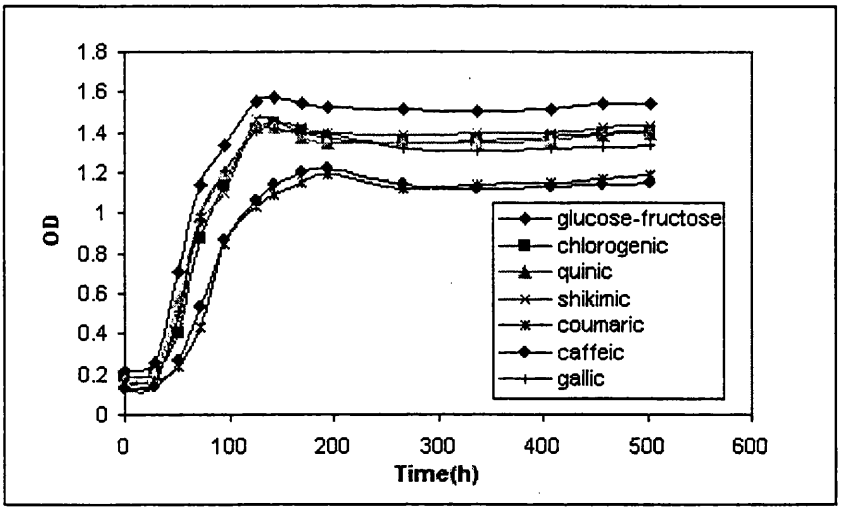


Fig.I-2 in 5g/l glucose-5g/l fructose

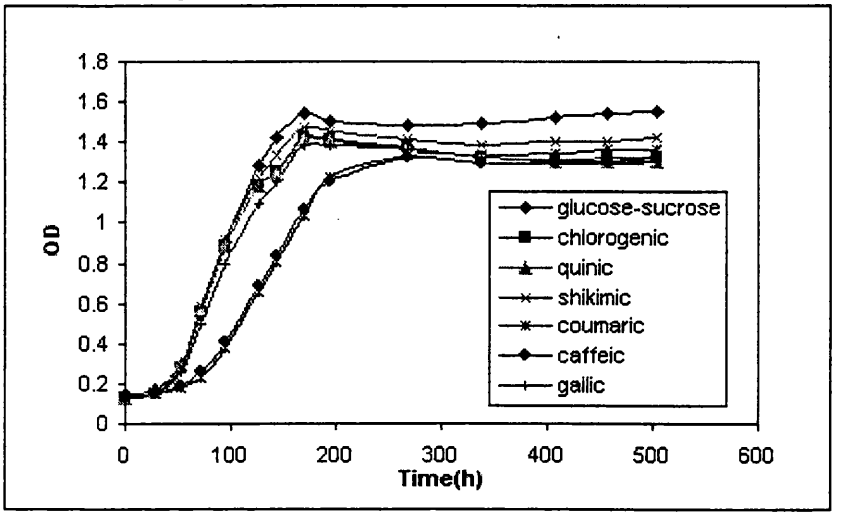


Fig.I-3 in 5g/l glucose-5g/l sucrose

Appendix J

The calculation of growth yield/observed biomass yield ($Y_{x/s}$) and specific rate of sugar uptake (Q_s) at different pH values

pH	3.5	4.0	4.5	5.0	6.0
Maximum OD	0.791	0.828	0.887	0.849	0.518
OD_0	0.077	0.115	0.077	0.089	0.124
Dry weight (g/l)	0.314	0.314	0.357	0.335	0.174
Time (h) Δt at maximum OD	96	49	45	45	85
$[glucose]_0$ $S_{0(glu)}$ (g/l)	3.963	3.690	4.181	3.844	3.765
$[fructose]_0$ $S_{0(fru)}$ (g/l)	4.793	4.311	4.690	4.878	4.374
[glucose] $S_{(glu)}$ residual(g/l)	0.522	0	0.379	0	0.423
[fructose] $S_{(fru)}$ residual(g/l)	0.342	0.224	0.251	0.724	0.587
$S_{0(glu)}+ S_{0(fru)}$ (g/l)	8.756	8.001	8.871	8.722	8.139
[glucose]+[fructose] $S_{(glu)}+S_{(fru)}$ (g/l)	0.864	0.224	0.630	0.724	1.010
$\Delta S = (S_{0(glu)}+ S_{0(fru)})- (S_{(glu)}+S_{(fru)})$	7.891	7.777	8.241	7.998	7.129
$Y_{x/s}$ (g cell/g sugar consumed)	0.040	0.040	0.043	0.042	0.024
Q_s (g sugar/g cell/h) Specific rate of sugar uptake	0.26	0.51	0.51	0.53	0.48
Q_x(mg cell/g sugar/h) Specific rate of cell production	0.41	0.82	0.96	0.93	0.29

Notes: glu-glucose, fru-fructose;
S_0- initial concentration, S-residual concentration.
OD_0-initial OD, OD-maximum OD at 660nm in 1 cm light-path.

Dry weight $\Delta X = 0.44 \times$ (maximum OD – initial OD) (g cell/l)

Sugars consumed ΔS = glucose consumed + fructose consumed (g/l)

Observed biomass yield $Y_{x/s} = \Delta X / \Delta S$ (g cell/g sugar consumed)

Specific rate of sugar uptake $Q_s = \Delta X / \Delta S / \Delta t$ (g cell/g sugar consumed/h)

Appendix K

A relationship between μ_{max} and pH in 2 liter batch cultures of *O. oeni* with pH control

A relationship between μ_{max} and pH was obtained below by polynomial regression (Figure J):

$y = 0.0089x^3 - 0.1504x^2 + 0.8155x - 1.3696$

where y is specific growth rate (h^{-1})
x is pH of medium

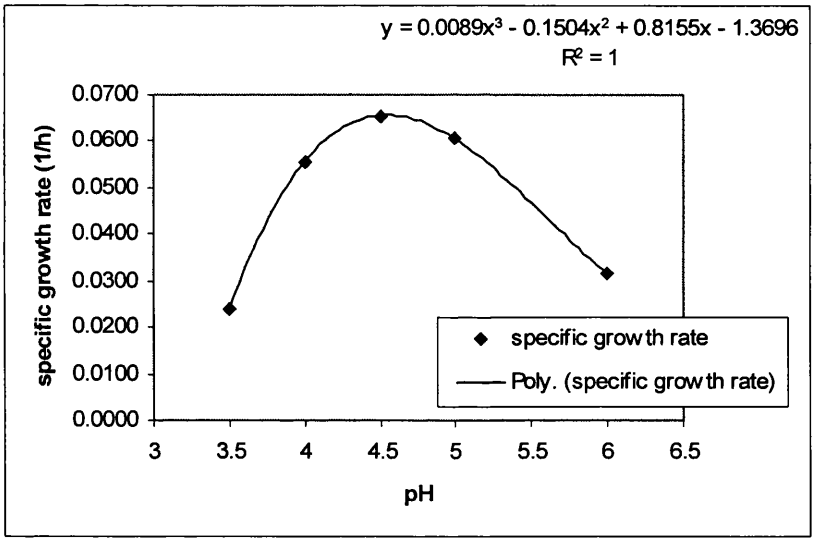


Figure J The specific growth rate of *O. oeni* in 2 liter batch cultures at various pH values

Appendix L

The time-courses of product formation (lactate, acetate, ethanol, glycerol, erythritol) during the fermentation of glucose-fructose (5g/l:5g/l) mixture by *O. oeni* at various pH values

The time-courses of product formation (lactate, acetate, ethanol, glycerol, erythritol) are shown below in Figure L. (mannitol is shown in Fig.4-1).

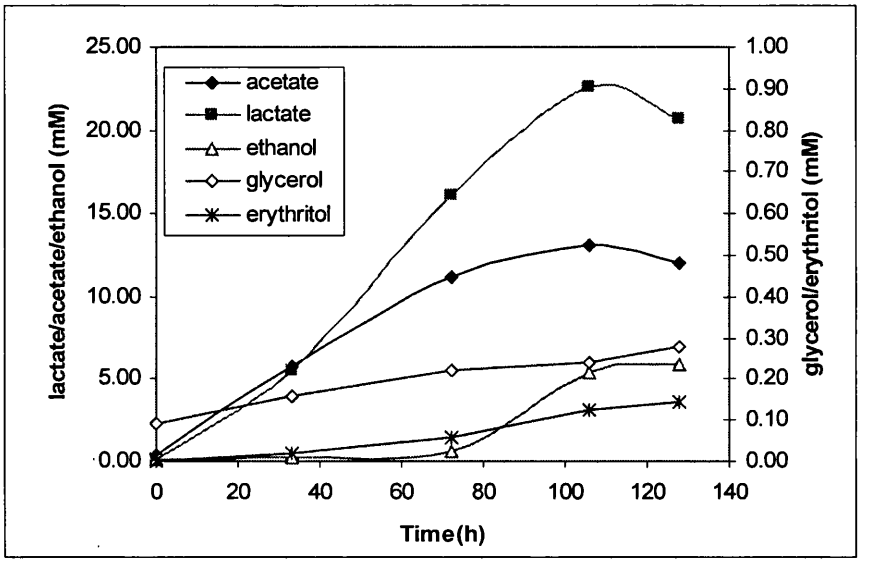


Figure K-1 Product formation at pH = 3.5

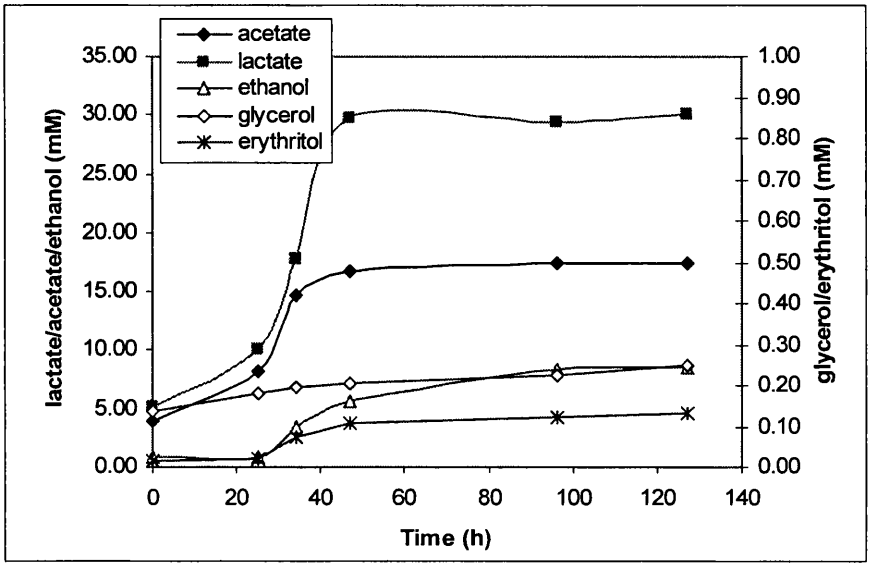


Figure K-2 Product formation at pH = 4.0

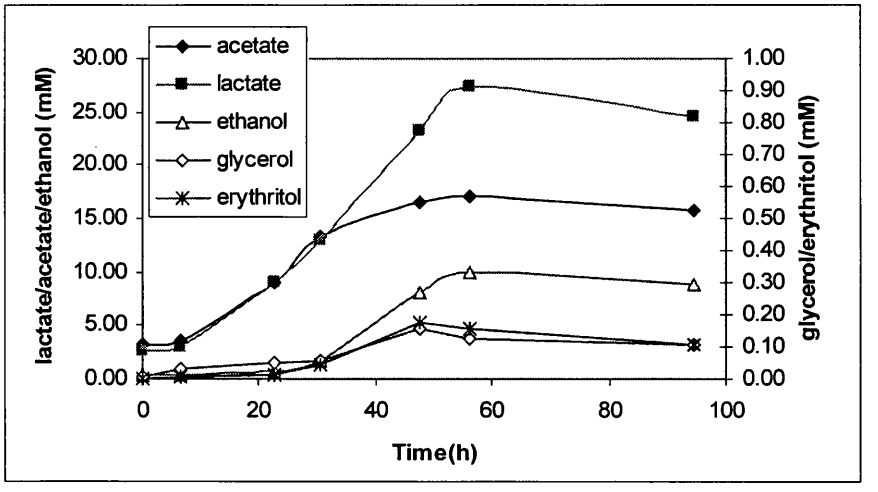


Figure K-3 Product formation at pH = 4.5

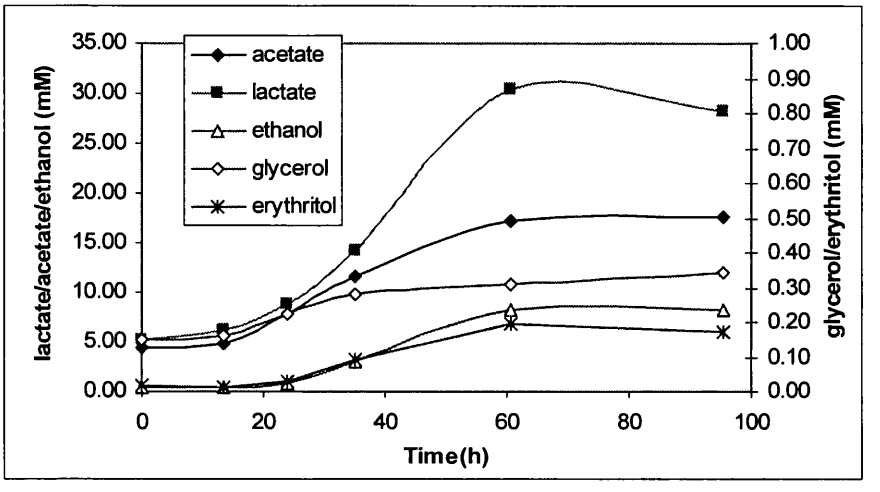


Figure K-4 Product formation at pH = 5.0

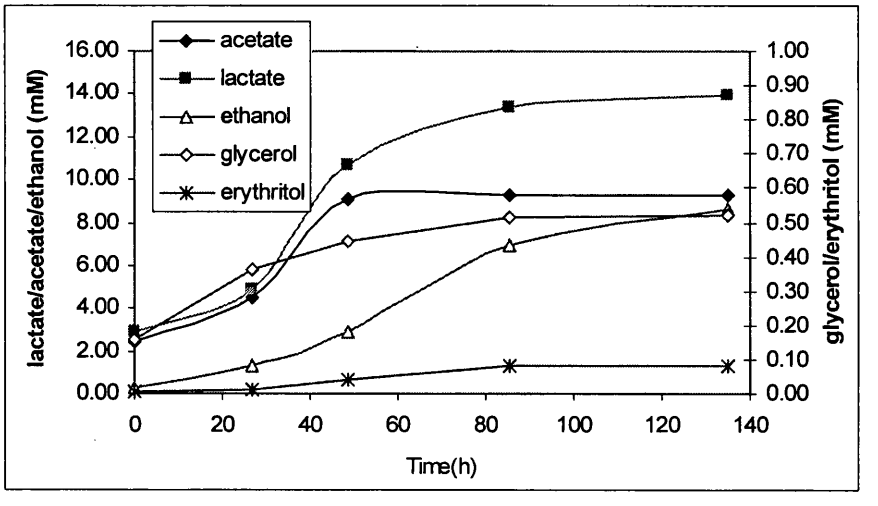


Figure K-5 Product formation at pH = 6.0

Appendix M

The calculation of product yield during the fermentation of 5g/l glucose-5g/l fructose by O. oeni with pH control

pH	3.5	4	4.5	5	6	
lactate	22.56	29.32	27.28	30.39	13.42	maximum lactate concentration (mM)
0 h (mM)	0.12	5.11	2.56	5.11	2.92	initial lactate concentration (mM)
acetate	13.13	17.49	17.09	17.2	9.24	
0 h	0.31	3.86	3.23	4.47	2.47	
ethanol	5.84	8.48	9.97	8.25	8.64	
0 h	0.07	0.9	0.46	0.44	0.27	
glycerol	0.24	0.23	0.12	0.31	0.51	
0 h	0.09	0.13	0.01	0.15	0.16	
erythritol	0.13	0.12	0.16	0.19	0.08	
0 h	0	0.01	0	0.02	0.01	
products formed (mM)						
lactate	22.44	24.21	24.72	25.28	10.5	
acetate	12.82	13.63	13.86	12.73	6.77	
ethanol	5.77	7.58	9.51	7.81	8.37	
glycerol	0.15	0.1	0.11	0.16	0.35	
erythritol	0.13	0.11	0.16	0.17	0.07	
products formed (g/l)						
lactate	2.020	2.179	2.225	2.275	0.945	
acetate	0.769	0.818	0.832	0.764	0.406	
ethanol	0.265	0.349	0.437	0.359	0.385	
glycerol	0.014	0.009	0.010	0.015	0.032	
erythritol	0.016	0.013	0.020	0.021	0.009	
time achieved maximum product concentration* (h)						
time	106	96.5	56	65.5	85.5	
the total sugar of glucose and fructose consumed (g/l)						
sugar	8.689	7.777	8.619	8.313	7.127	
dry weight formed (g cell/l)						
dry weight	0.314	0.314	0.357	0.335	0.174	

(* maximum ethanol pH3.5, 5.84g/l at 128h; pH4.0, 8.48g/l at 127h; pH6.0, 8.64g/l at 135h).

Yp/s (g product/g sugar)					
lactate	0.232	0.280	0.258	0.274	0.133
acetate	0.089	0.105	0.096	0.092	0.057
ethanol	0.031	0.045	0.051	0.043	0.054
glycerol	0.002	0.001	0.001	0.002	0.005
erythritol	0.002	0.002	0.002	0.002	0.001
Yp/x (g product/g cell)					
lactate	6.43	6.94	6.23	6.79	5.43
acetate	2.45	2.60	2.33	2.28	2.33
ethanol	0.85	1.11	1.23	1.07	2.21
glycerol	0.04	0.03	0.03	0.04	0.19
erythritol	0.05	0.04	0.05	0.06	0.05
volumetric product productivity (mg/l/h)					
lactate	19.1	22.6	39.7	34.7	11.1
acetate	7.3	8.5	14.9	11.7	4.8
ethanol	2.5	3.6	7.8	5.5	4.5
glycerol	0.1	0.1	0.2	0.2	0.4
erythritol	0.1	0.1	0.3	0.3	0.1

Appendix N

stoichiometric data, carbon recovery, energetic calculations at different pH values

concentration of sugars and products measered (all concentrations in terms of mM)

	initial glucose	final glucose	initial fructose	final fructose	final mannitol	initial mannitol	final ethanol	initial ethanol
3.5	21.99	0	26.60	0.37	21.11	0.30	5.84	0.07
4.0	20.48	0	23.92	1.24	19.76	2.57	8.48	0.54
4.5	23.20	0	26.03	1.39	21.69	1.46	9.97	0.46
5.0	21.33	0	27.08	2.28	24.09	3.83	8.25	0.44
6.0	20.90	2.35	24.27	3.26	20.77	3.79	8.64	0.27
	acetate		lactate		glycerol		erythritol	
3.5	13.13	0.31	22.56	0.12	0.24	0.09	0.13	0.00
4.0	17.49	3.86	29.32	5.11	0.23	0.13	0.12	0.01
4.5	17.09	3.23	27.28	2.56	0.12	0.01	0.16	0.00
5.0	17.2	4.47	30.39	5.11	0.31	0.15	0.19	0.02
6.0	9.24	2.47	13.42	2.92	0.51	0.16	0.08	0.01

concentrations of sugar consumed and products formed (mM)

	glucose	fructose	mannitol	acetate	lactate	ethanol	glycerol	erythritol
3.5	21.99	26.23	20.81	12.82	22.44	5.77	0.148	0.13
4.0	20.48	22.68	17.19	13.63	24.21	7.94	0.10	0.11
4.5	23.20	24.63	20.23	13.86	24.72	9.51	0.11	0.16
5.0	21.33	24.80	20.25	12.73	25.28	7.81	0.16	0.17
6.0	18.54	21.01	16.98	6.77	10.5	8.37	0.35	0.07

the global stoichiometry (in mol)

	glucose	fructose	mannitol	acetate	lactate	ethanol	glycerol	erythritol
3.5	1	1.19	0.95	0.58	1.02	0.26	0.007	0.006
4.0	1	1.11	0.84	0.67	1.18	0.39	0.005	0.005
4.5	1	1.06	0.87	0.60	1.07	0.41	0.005	0.007
5.0	1	1.16	0.95	0.60	1.19	0.37	0.008	0.008
6.0	1	1.13	0.92	0.37	0.57	0.45	0.019	0.004

	total sugar consumed (mM)	**central sugar (mM)**	**fructose not formed into mannitol (mM)**
	g+f	g+f-m	f-m
3.5	48.22	27.41	5.42
4.0	43.16	25.97	5.49
4.5	47.83	27.60	4.40
5.0	46.13	25.88	4.55
6.0	39.55	22.57	4.03

g-glucose, f-fructose, m-mannitol

	percentage of mannitol to fructose (%)	**dry weight g/l**	**C * (mol) in cells formed**
	m/f		
3.5	79	0.314	0.0131
4.0	76	0.314	0.0131
4.5	82	0.357	0.0149
5.0	82	0.335	0.0140
6.0	81	0.174	0.0073

* Assuming carbon content in cells account for 50% of dry cell weight

The overall stoichiometry for per mol central sugar (in mol)

pH	central sugar	acetate	lactate	ethanol	glycerol	erythritol	CO2 *
3.5	1.00	0.47	0.82	0.21	0.005	0.005	0.68
4.0	1.00	0.52	0.93	0.31	0.004	0.004	0.84
4.5	1.00	0.50	0.90	0.34	0.004	0.006	0.85
5.0	1.00	0.49	0.98	0.30	0.006	0.007	0.80
6.0	1.00	0.30	0.47	0.37	0.016	0.003	0.68

carbon recovery (%)

	C-acetate	C-lactate	C-ethanol	C-glycerol	C-erythritol	C-CO2	C in cells	C%
3.5	0.94	2.46	0.42	0.016	0.019	0.68	0.48	83
4.0	1.05	2.80	0.61	0.012	0.017	0.84	0.51	97
4.5	1.00	2.69	0.69	0.012	0.023	0.85	0.54	97
5.0	0.98	2.93	0.60	0.019	0.027	0.80	0.54	98
6.0	0.60	1.40	0.74	0.047	0.012	0.68	0.32	63

* CO2 was supposed to be the addition of amount of acetate and ethanol

energetic calculations

	mol ATP/mol central sugar* (acetate+lactate)	g biomass/mol central sugar	g biomass/mol ATP	g biomass/mol sugar
3.5	1.29	11.5	8.9	7.17
4.0	1.46	12.1	8.3	7.28
4.5	1.40	12.9	9.2	7.61
5.0	1.47	12.9	8.8	7.55
6.0	0.77	7.69	10.1	4.40

* central sugar refers to glucose and fructose not converted into mannitol

Appendix O

The calculation of biomass yield ($Y_{x/s}$) during the fermentation of different concentrations (1:1, w/w) of glucose-fructose mixture by *O. oeni*

Concentration* (g/l) [glucose]+[fructose]	3	9	18	36	90
Maximum OD	0.292	0.887	0.906	0.942	0.596
Initial OD_0	0.048	0.077	0.102	0.100	0.075
Cell dry weight (g/l) $\Delta X=0.44*(OD_{max}-OD_0)$	0.107	0.356	0.354	0.370	0.229
S_0 (glucose, g/l)	1.334	4.181	8.793	17.620	44.481
S_0 (fructose, g/l)	1.353	4.690	9.011	17.972	45.230
S (glucose, g/l)	0	0	0	0	15.016
S (fructose, g/l)	0.120	0.084	0	0	0
S_0 (g/l)	2.687	8.871	17.804	35.592	89.710
S (g/l)	0.120	0.084	0.000	0.000	15.016
$-\Delta S= -(S_0-S)$ (g/l)	2.567	8.787	17.804	35.592	74.694
$Y_{x/s}$ (g/g)	0.042	0.041	0.020	0.010	0.003

* The rough value of addition of glucose and fructose concentration, and the ratio of glucose-fructose was around 1:1.

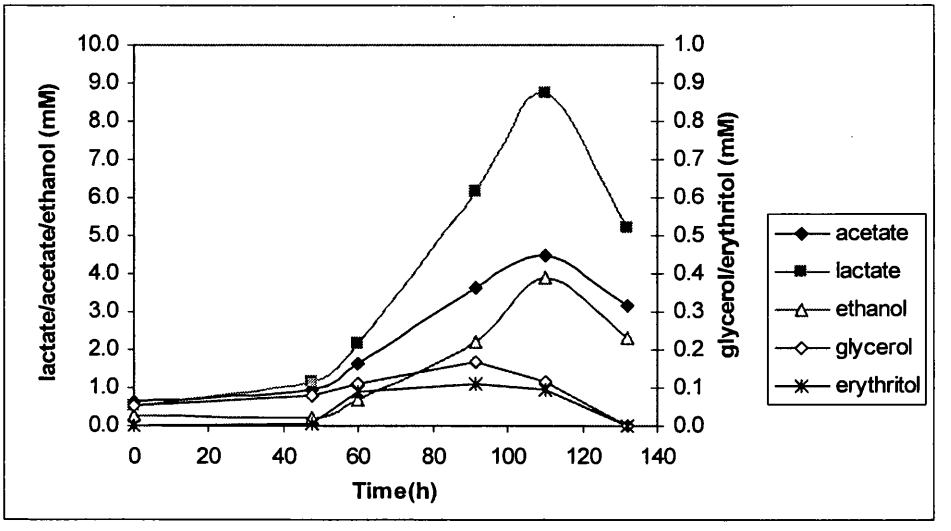
Notes: glu-glucose, fru-fructose;

S_0- initial concentration, S-residual concentration.

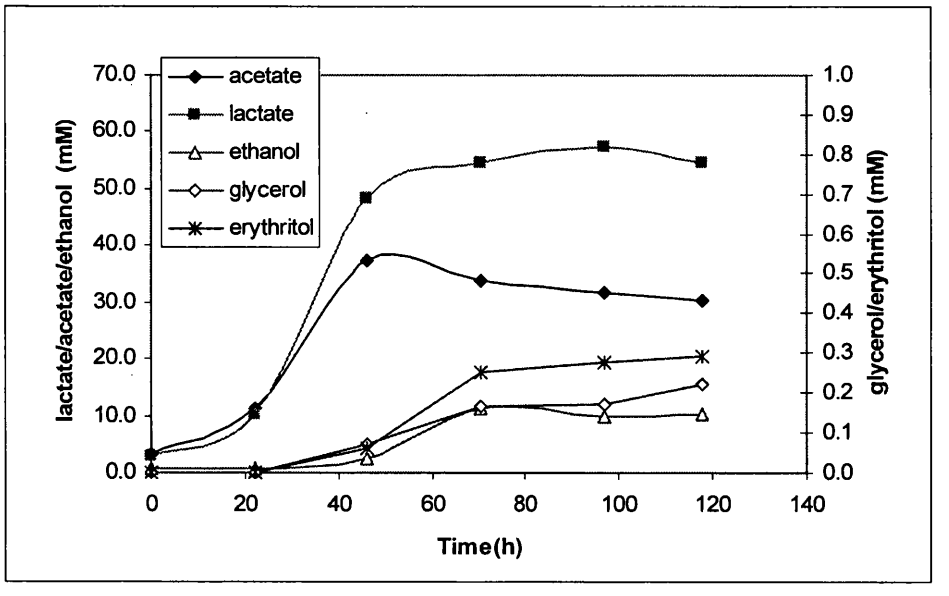
OD_0-initial OD, OD-maximum OD at 660nm in 1 cm light-path.

Appendix P

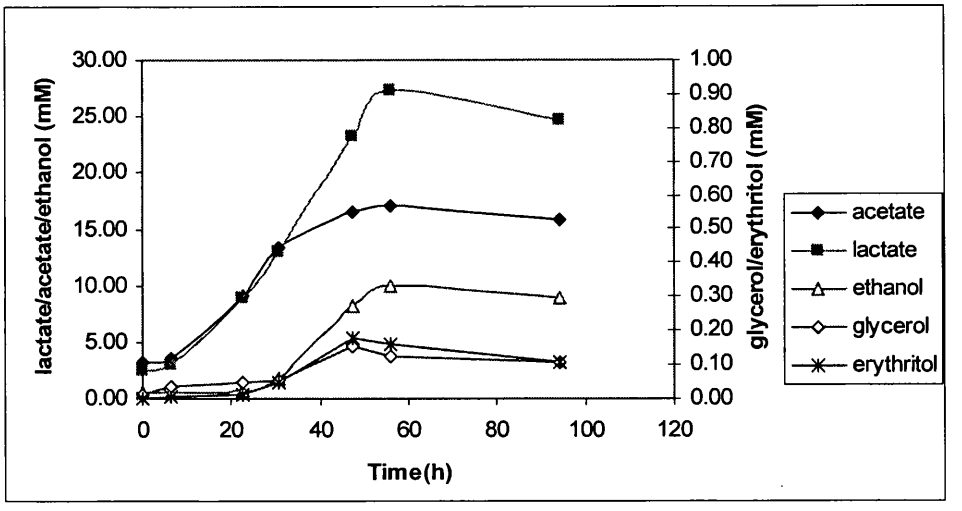
The time-courses of product formation during the fermentation of different concentrations (1:1 w/w) of glucose-fructose mixture by *O. oeni*



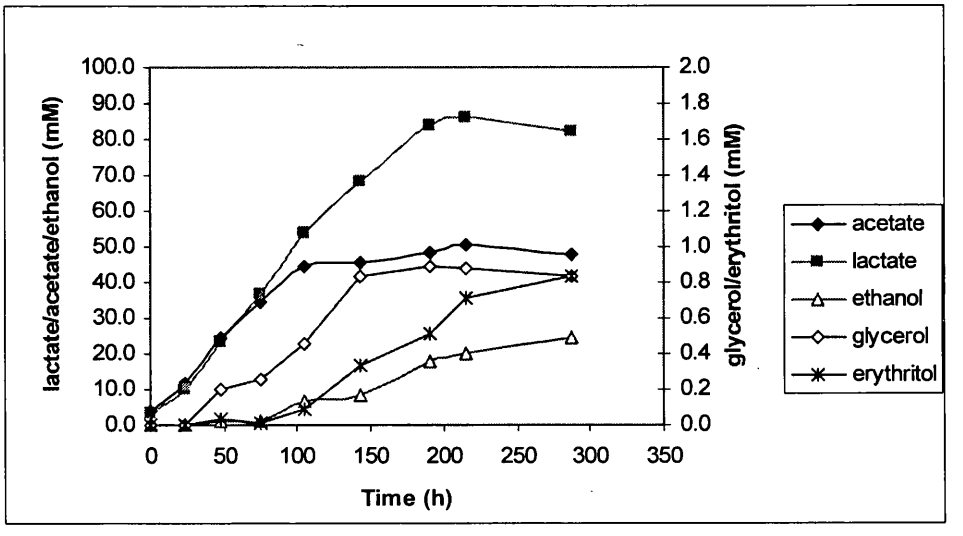
(a) $[glucose]_0$ = 7.4 mM, $[fructose]_0$ = 7.5 mM



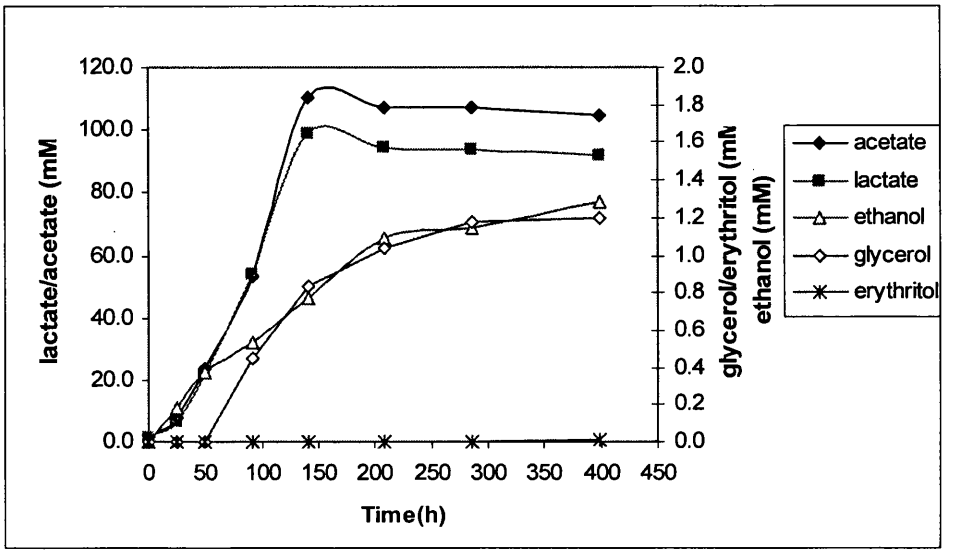
(b) glucose = 48.8 mM = 8.8 g/l, fructose = 50.0 mM = 9.0 g/l



(c) $[glucose]_0 = 23.2$ mM, $[fructose]_0 = 26.0$ mM



(d) $[glucose]_0 = 97.8$ mM, $[fructose]_0 = 99.8$ mM



(e) $[glucose]_0 = 246.8$ mM, $[fructose]_0 = 251.0$ mM

Appendix Q

The calculation of substrate uptake, product formation, growth parameters, cell productivity, fermentation balance (stoichiometry), carbon recovery and energetics during the fermentation of different concentrations of glucose-fructose mixture by *O. oeni*

concentration (mM)	1.5-1.5g/l	4.5-4.5g/l	9-9g/l	18-18g/l	45-45g/l
Glucose (mM) 0 hour	7.40	23.2	48.8	97.78	246.84
Final	0	0	7.66	12.14	91.43
Fructose 0 h	7.51	26.03	50.05	99.83	251
Final	0.76	1.39	0	0	0
Mannitol Final	4.84	21.69	31.31	71.9	118.54
0 h	0.19	1.46	2.97	4.37	2.81
Lactate Final	8.74	27.28	57.17	86.13	91.51
0 h	0.66	2.56	2.71	3.35	1.46
Acetate Final	4.46	17.09	33.66	50.82	104.54
0 h	0.5	3.23	3.26	3.85	1.02
Ethanol Final	3.88	9.97	11.4	20.1	1.29
0 h	0.23	0.46	0.55	0	0
Glycerol Final	0.167	0.12	0.167	0.883	1.195
0 h	0.053	0.01	0	0	0
Erythritol Final	0.11	0.16	0.252	0.708	0.008
0 h	0	0	0	0	0
dry weight (g/l) Final	0.129	0.391	0.399	0.372	0.263
0 h	0.021	0.034	0.045	0.044	0.033
sugar consumed (mM)					
glucose	7.40	23.20	41.14	85.64	155.4
fructose	6.75	24.64	50.05	99.83	251.0
glucose+fructose (g/l)	2.55	8.62	16.43	33.42	73.24
products formed (mM)					
mannitol	4.65	20.23	28.34	67.53	115.7
lactate	8.08	24.72	54.46	82.78	90.05
acetate	3.96	13.86	30.4	46.97	103.5
ethanol	3.65	9.51	10.85	20.1	1.29
glycerol	0.11	0.11	0.17	0.88	1.20
erythritol	0.11	0.16	0.25	0.71	0.008
products formed (g/l)					
mannitol	0.847	3.686	5.164	12.304	21.081
lactate	0.727	2.225	4.901	7.450	8.105
acetate	0.238	0.832	1.824	2.818	6.211
ethanol	0.168	0.437	0.499	0.925	0.059
glycerol	0.010	0.010	0.015	0.081	0.110
erythritol	0.013	0.020	0.031	0.086	0.001
dry weight (g cell/l)	0.108	0.357	0.354	0.328	0.230
Fermentation time (h)	110	45	71	215	398
Yx/s (g cell/g sugar)	0.042	0.041	0.022	0.010	0.003
Yx/s (g cell/mol sugar)	7.63	7.46	3.88	1.77	0.57

Qs (g sugar/g cell/h)	0.21	0.54	0.65	0.47	0.80
cell productivity					
(mg cell/l/h)	0.98	7.93	4.99	1.53	0.58
Yp/s (g product/g sugar)					
mannitol	0.332	0.428	0.314	0.368	0.288
lactate	0.285	0.258	0.298	0.223	0.111
acetate	0.093	0.096	0.111	0.084	0.085
ethanol	0.066	0.051	0.030	0.028	0.001
glycerol	0.004	0.001	0.001	0.002	0.002
erythritol	0.005	0.002	0.002	0.003	0.000
the stoichiometry for per mol glucose (in mol)					
glucose	1.00	1.00	1.00	1.00	1.00
fructose	0.91	1.06	1.22	1.17	1.62
mannitol	0.63	0.87	0.69	0.79	0.74
lactate	1.09	1.07	1.32	0.97	0.58
acetate	0.54	0.60	0.74	0.55	0.67
ethanol	0.49	0.41	0.26	0.23	0.01
glycerol	0.015	0.005	0.004	0.010	0.008
erythritol	0.015	0.007	0.006	0.008	0.0001
the conversion percentage of fructose into mannitol	69	82	57	68	46
Fructose not converted to mannitol relative to per glucose (mol)	0.28	0.19	0.53	0.38	0.87
central sugar (in mol)					
glucose+fructose	1.28	1.19	1.53	1.38	1.87
the stoichiometry for per mol central sugar (in mol)					
central sugar	1.00	1.00	1.00	1.00	1.00
lactate	0.85	0.90	0.87	0.70	0.31
acetate	0.42	0.50	0.48	0.40	0.36
ethanol	0.39	0.34	0.17	0.17	0.004
glycerol	0.012	0.004	0.003	0.007	0.004
erythritol	0.012	0.006	0.004	0.006	0.000
CO2	0.81	0.84	0.65	0.57	0.364
C in cells	0.47	0.54	0.23	0.12	0.03
carbon recovery (%)	92	97	80	66	34
energetic calculations					
mol ATP/mol central sugar	1.27	1.40	1.35	1.10	0.67
g biomass/mol central sugar	11.4	12.9	5.6	2.8	0.8
g biomass/mol ATP	8.9	9.3	4.2	2.5	1.2
g biomass/mol sugar	7.6	7.6	3.9	1.8	0.6

Appendix R

The biomass amounts obtained at varied dilution rate during the continuous culture of *O. oeni* in the mixture of glucose- fructose

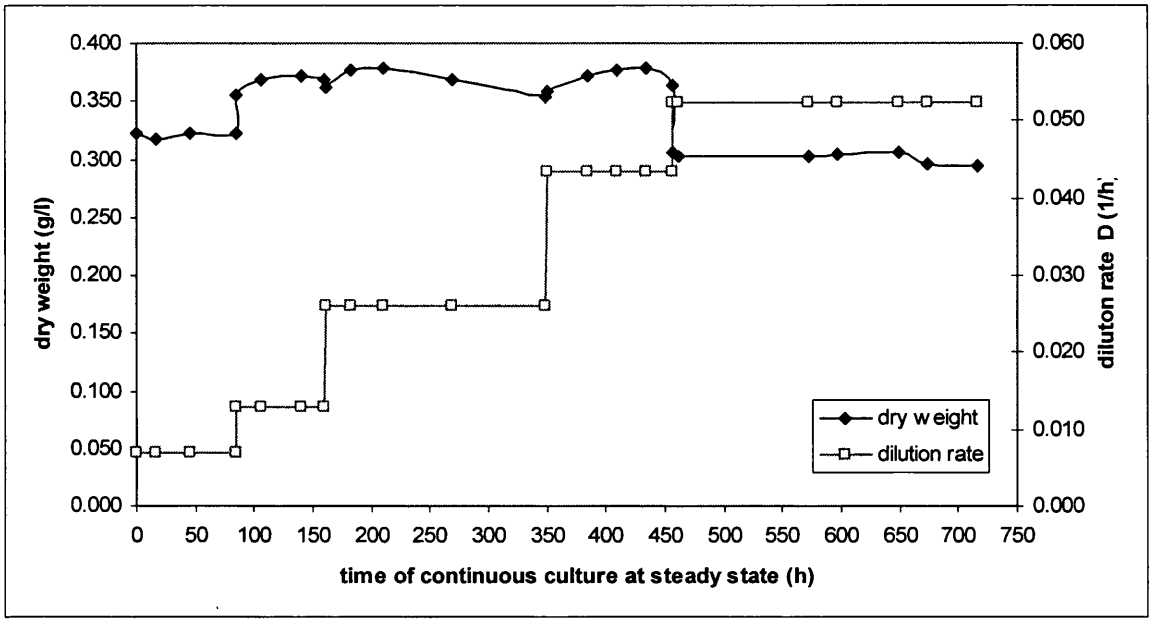


Figure 4 – 9 Representative results of continuous culture of *O. oeni* at steady state at various dilution rates in a chemostat

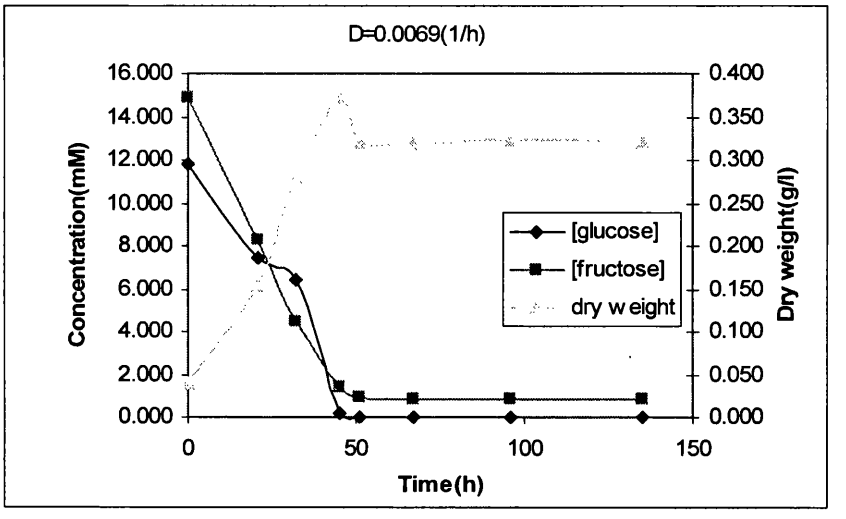
Feed medium including: Z broth with glucose 5 g/l, fructose 6 g/l,

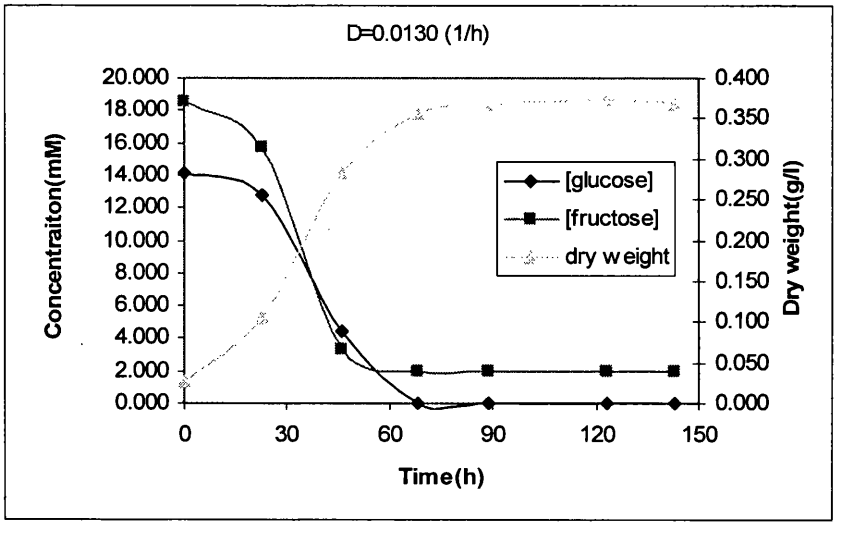
The culture in a reactor with 2 litre and conditions: pH = 4.5, T = 25°C.

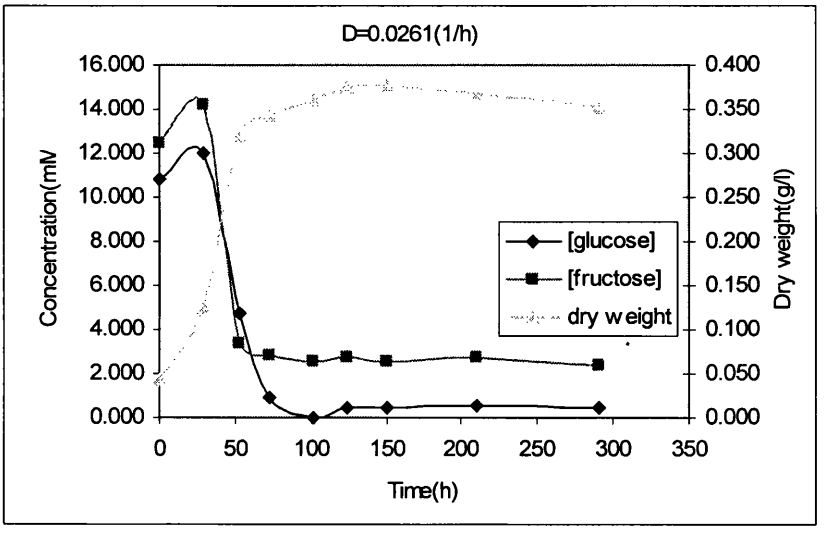
Appendix S

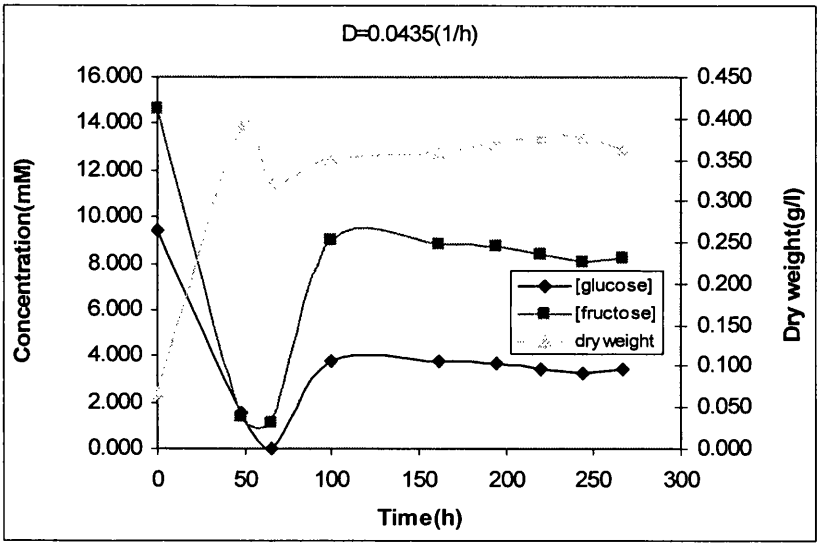
The time-courses of cell growth and substrate consumption (glucose and fructose) during the continuous culture of *O. oeni*

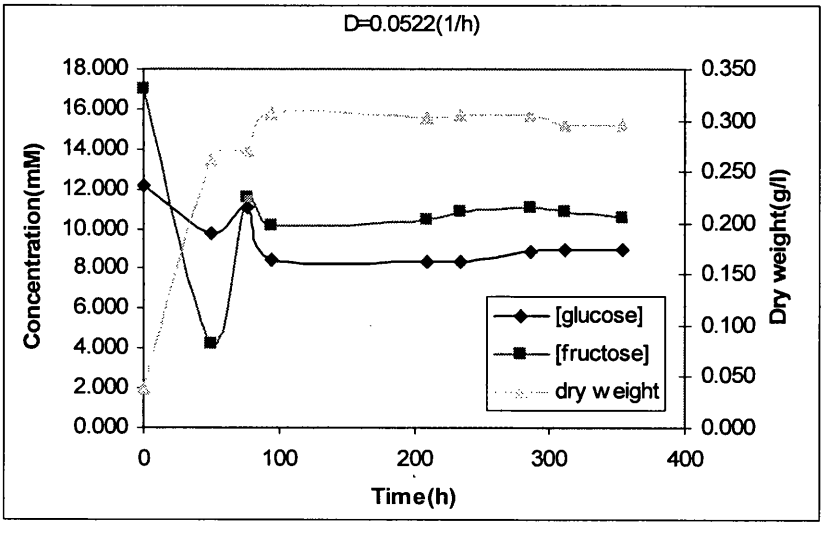
The fermentation was carried out in a reactor with working volume of 1.15 litres. Feed medium contained 5g/l glucose-5g/l fructose, temperature 25°C and pH was controlled at 4.5 by 1M NaOH.






D=0.0261(1/h)
Concentration(mM
Dry weight(g/l)
Time(h)
[glucose]
[fructose]
dry weight


D=0.0435(1/h)
Concentration(mM)
Dry weight(g/l)
Time(h)
[glucose]
[fructose]
dry weight


D=0.0522(1/h)
Concentration(mM)
Dry weight(g/l)
Time(h)
[glucose]
[fructose]
dry weight

Appendix T

Graphical determination of the substrate saturation constant K_s and maximum specific rate μ_{max}

A plot of S/D vs S was pictured by equation (4.23) $S/D = S/\mu_{max} + K_s/\mu_{max}$, and a straight line with a slope $1/\mu_{max}$ and intercept K_s/μ_{max} was obtained.

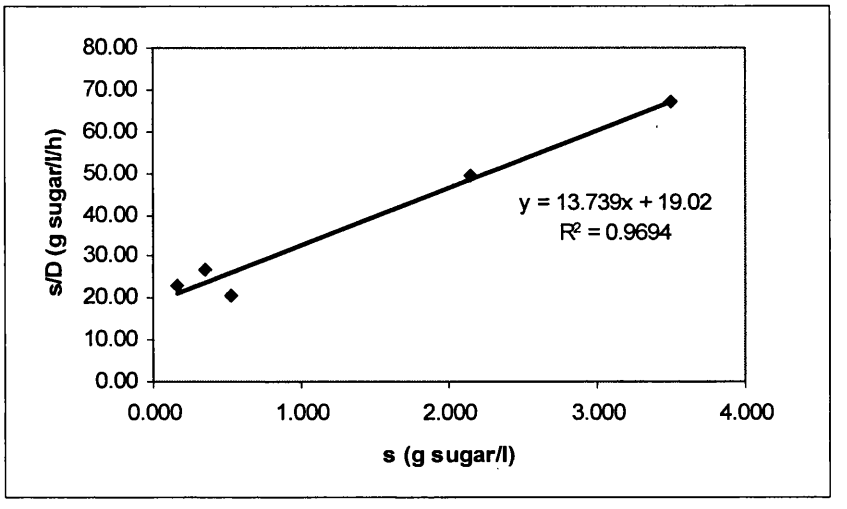


Figure S Graphical determination of the K_s and μ_{max} using data from chemostat culture of *O. onei* glucose 5g/l, fructose 6g/l, T =25°C pH = 4.5

$1/\mu_{max} = 13.739$

$K_s/\mu_{max} = 19.02$

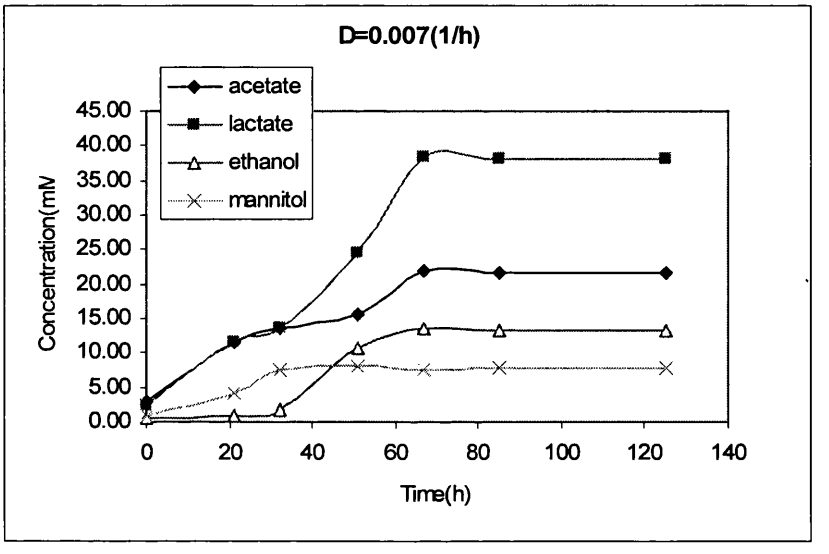
$\mu_{max} = 0.073\ (h^{-1})$

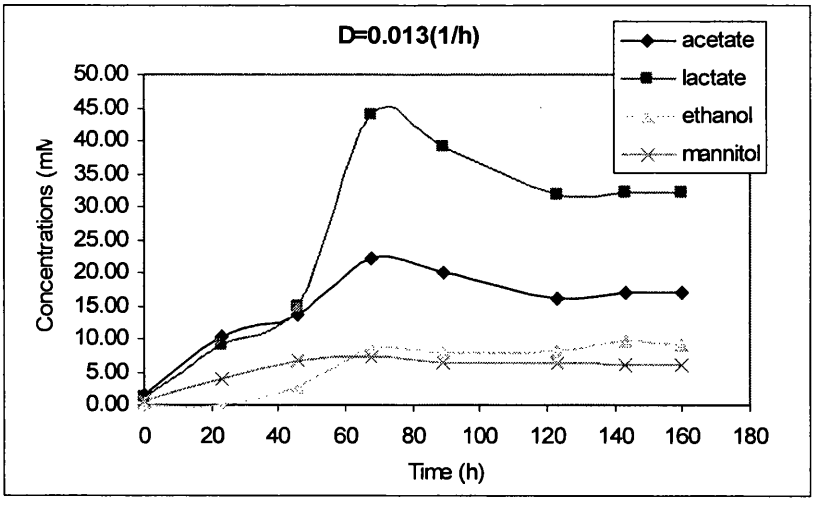
$K_s = 1.384\ (g/l)$

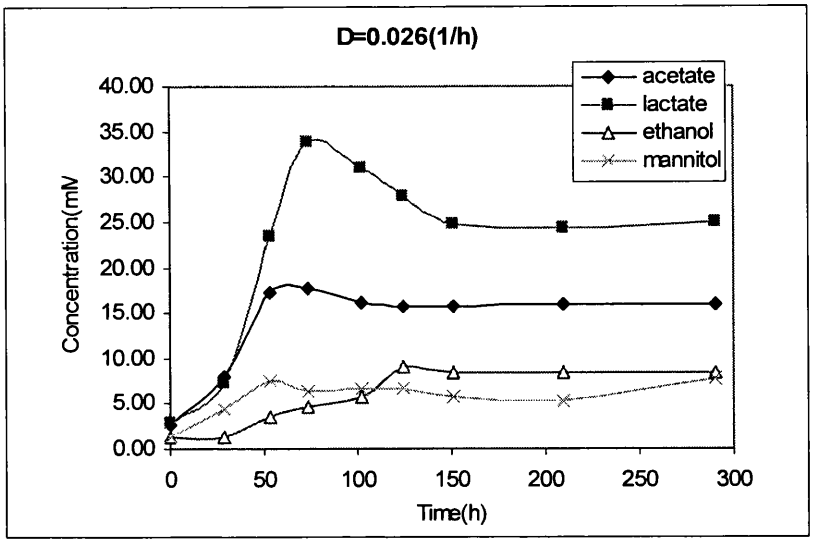
Appendix U

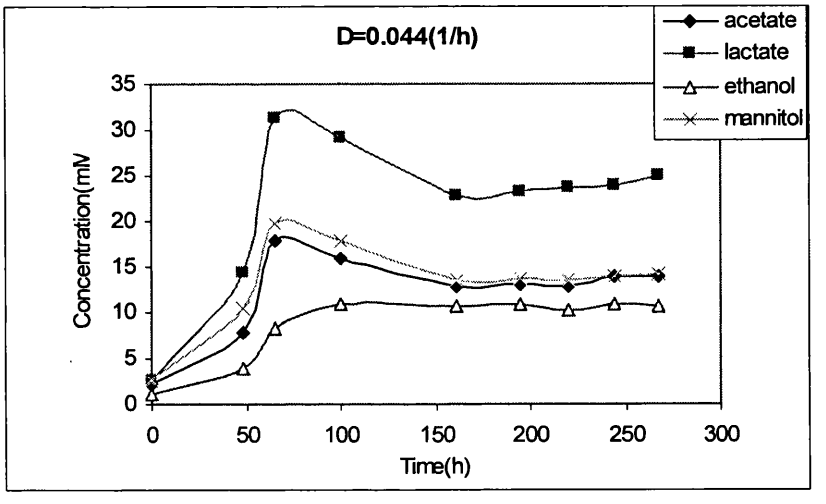
The time-courses of product formation during continuous culture of *O. oeni* on glucose-fructose mixture

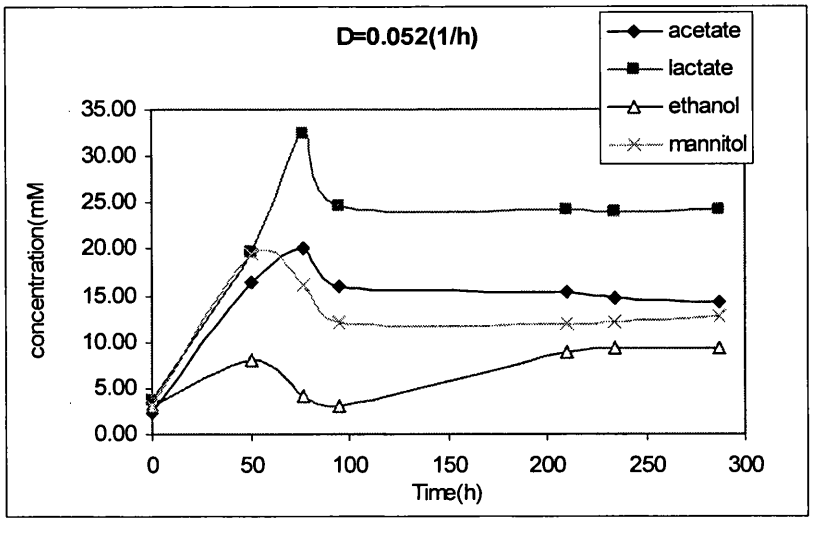
Feed medium contained 5g/l glucose-5g/l fructose, temperature 25°C and pH 4.5. The time-courses of mannitol, lactate, acetate and ethanol were shown in Fig.U below and final concentrations of end products were obtained at various dilution rates tested.






D=0.026(1/h)
acetate
lactate
ethanol
mannitol
Concentration(mM
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0
50
100
150
200
250
300
Time(h)


D=0.044(1/h)
acetate
lactate
ethanol
mannitol
Concentration(mM
35
30
25
20
15
10
5
0
0
50
100
150
200
250
300
Time(h)


D=0.052(1/h)
acetate
lactate
ethanol
mannitol
concentration(mM
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0
50
100
150
200
250
300
Time(h)

Appendix V

The calculation of substrate uptake, product formation, volumetric cell productivity, overall stichiometry, carbon recovery and energetics during continuous cultures of *L. brevis* in chemostat at temperature 27°C and pH 5.5

D	0.010	0.030	0.052	0.070	0.089
Initial glucose (g/l)	10 g/l				
glucose consumed					
g/l	9.504	9.234	9.171	9.213	8.788
mM	52.74	51.24	50.89	51.13	48.77
residual glucose (g/l)	0.214	0.484	0.547	0.506	0.93
dry-weight (g/l)	0.944	0.975	0.982	1.079	1.051
lactate (mM)	56.06	54.66	50.99	47.65	46.03
ethanol(mM)	28.87	28.30	28.64	23.47	20.61
Yx/s=dry weight/glucose consumed (g cell/g glucose)					
	0.099	0.106	0.107	0.117	0.120
Yx/s(g cell/mol glucose)					
	17.8	19.1	19.3	21.1	21.6
qs=D/Yx/s (g glucose/g cell/h)					
	0.101	0.284	0.486	0.598	0.744
Qx= D*x (mg cell/l/h)					
	9.44	29.3	51.1	75.5	93.5
Overall stoichiometry for per glucose and carbon recovery (%)					
Dilution rate	0.010	0.030	0.052	0.070	0.089
glucose	1.00	1.00	1.00	1.00	1.00
lactate (mM)	1.06	1.07	1.00	0.93	0.94
ethanol(mM)	0.55	0.55	0.56	0.46	0.42
CO2*	0.55	0.55	0.56	0.46	0.42
C** in cells	0.75	0.79	0.8	0.88	0.9
C(%)	93	94	92	84	83
* assuming CO2 is equal to the amount of ethanol.					
**based on an assumption that the 50% dry weight is carbon.					
ATP produced per glucose (mol ATP/mol glucose)					
	1.06	1.07	1.00	0.93	0.94
Y_{ATP}(g cell/mol ATP)					
	16.8	17.8	19.3	22.6	22.8
q_{ATP} = D/Y_{ATP}(mol ATP/g cell/h)					
	0.0006	0.0017	0.0027	0.0031	0.0039