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**AN IMMOBILIZED CELL BIOREACTOR  
FOR THE MALOLACTIC FERMENTATION OF WINE**

A THESIS PRESENTED IN PARTIAL FULFILMENT OF  
THE REQUIREMENTS FOR THE DEGREE OF  
MASTER OF TECHNOLOGY IN BIOTECHNOLOGY AT  
MASSEY UNIVERSITY

DENISE E. JANSSEN

1991

## ABSTRACT

Malolactic fermentation using immobilized cells of *Leuconostoc oenos* was investigated in order to improve this fermentation at an industrial-scale.

Three strains of bacteria were investigated in some detail, and one was chosen for further work. A satisfactory growth medium for the strain of bacteria used was found to be an apple juice broth. The effect, on both the growth and malic acid bioconversion for *Leuconostoc oenos* strain 1070, of having 6% v/v ethanol in the growth media was tested and found to cause a longer lag phase, and be slightly beneficial, respectively.

Oak chips were decided on as the immobilization media, in preference to bone char, and a synthetic, apple-juice based wine was used to determine operation parameters for a continuous culture bioreactor.

Temperature, pH, ethanol concentration, SO<sub>2</sub>, malic acid concentrations, anaerobic conditions and dilution rate were investigated and it was shown that lower malic acid concentrations, and also an interaction between low pH, high temperature and high ethanol concentration affected the malic acid bioconversion adversely. Increasing the dilution rate above 0.35 h<sup>-1</sup> caused a 30% drop in the bioconversion rate. The pH level had no effect on bioconversion if the temperature was kept at 21°C or lower. Decreasing the temperature, increasing the ethanol concentration above 10% v/v and increasing SO<sub>2</sub> levels all caused a slight drop in bioconversion rates while strict anaerobic growth and bioconversion conditions caused an increase. The bioconversion rates ranged between 20 and 100 mg malic acid consumed/100ml oak chips/hour.

An industrial prototype bioreactor was built and used at Villa Maria Wineries, Auckland, during the 1991 vintage and successfully processed 200 litres of Chardonnay-style wine in 2 days. The bioconversion rate was between 25 and 30 mg malic acid consumed/100ml oak chips/hour. Informal taste tests showed satisfactory malolactic characteristics in the treated wine.

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## 1.0 INTRODUCTION

Malolactic fermentation (MLF) entails the conversion of L-malic acid to L-lactic acid via decarboxylation using a suitable microorganism. The primary uses of MLF are to lower the acidity in high-acid wine, to give wine enhanced sensory characteristics, especially diacetyl or "buttery" notes, and to improve microbiological stability (Eggenberger, 1988).

Currently, in cool climate wine areas, this fermentation is used for most red wines and some white wines, especially Chardonnays. Normal industrial practice is to use batch fermentation, either initiated naturally by bacteria occurring in the winery or following inoculation with commercially available preparations. Length of fermentation varies greatly with wine type and temperature, and may vary from one week for some red wines to up to one year for a Chardonnay-style wine. Delayed onset of fermentation is seen by winemakers as the primary problem with malolactic fermentation in New Zealand (Pilone, 1988).

Immobilized cells offer several advantages for MLF of wine:

- faster fermentation
- continuous operation
- improved temperature control
- greater tolerance to wines which have high alcohol and sulphur dioxide concentrations and low pH
- better control over the timing and extent of deacidification
- absence of flavour effects caused by bacterial growth in the wine.

Potential disadvantages of the technique include:

- the possibility of microbial contamination of the reactors
- transfer of taints from the reactor to the wine
- loss of activity on prolonged operation
- leakage of cells or immobilization substrate into the wine

(Davis *et al.*, 1985).

For this method to be attractive in commercial practice it must be:

- cheap
- simple
- easily performed in an industrial situation
- not liable to cause oxidation of the wine
- robust
- not susceptible to contamination
- able to impart correct flavour changes to the wine
- must use commercially acceptable supports and organisms

Investigation into any immobilization method must include the effects of major variations in substrate and environmental parameters so that the bioreactor throughput to give effective treatment can be calculated. These parameters include: SO<sub>2</sub>, pH, ethanol concentration, temperature and flowrate.

This thesis deals with continuous malolactic fermentation of Chardonnay-style wines using immobilized cells, with the ultimate aim of developing and testing a prototype bioreactor in an industrial situation. Chardonnay-style wine was seen as providing the most difficult conditions for MLF and the style most in need of improved fermentation methodology.

## 1.1 Organism

Microorganisms used for wine deacidification include *Lactobacillus* spp., *Pediococcus* spp., *Leuconostoc* spp. and selected species of the yeast, *Schizosaccharomyces*. *Schizosaccharomyces* spp. deacidify wine but do not perform a true MLF as malic acid is first decarboxylated to pyruvic acid which is then further metabolized to ethanol. Most commercial cultures available for batch inoculation are strains of *Leuconostoc oenos* and strain selection is of critical importance because of a wide variation in growth and sensory characteristics (Davis *et al.*, 1985).

All the bacteria used are alcohol-tolerant but *Leuconostoc oenos* is preferred and is best able to tolerate low pH (Kelly *et al.*, 1989). It is also the only MLF organism isolated from low pH wine and is assumed to be the predominant organism for MLF in naturally inoculated wine.

*Lactobacillus* and *Pediococcus* spp. are often seen as producing inferior wines, and are also spoilage organisms as they can continue to grow after MLF has occurred (Wibowo *et al.*, 1988; Daeschel *et al.*, 1991).

Use of *Schizosaccharomyces pombe* has been limited as most strains will produce some off-flavours and off-aromas if they remain in contact with fermenting must for too long. In other cases, a decrease of varietal character and appearance of atypical aroma without any off-flavour has occurred (Gallander, 1977). The yeast, however, has attractive properties such as high acid and sulphur dioxide tolerance, good growth in must and wine, and easy cultivation and storage of strains (Joshi *et al.*, 1991). Research using an immobilized yeast shows some potential for this organism though further work is required (Magyar and Panyik, 1989).

A mutant of *Schizosaccharomyces malidevorans* which uses insignificant amounts of glucose has been used for MLF (Rodriguez and Thornton, 1988). This yeast removes the malic acid from the juice prior to the main wine fermentation and has provided

satisfactory results on a commercial scale. There is currently no commercial culture of this organism available.

Because it is the most common organism for MLF and is accepted internationally by winemakers, all experimental work in this thesis was conducted with selected strains of *Leuconostoc oenos*. A description of this bacterium is given below.

### 1.1.1 Morphology

The bacterium *Leuconostoc oenos* is a Gram-positive, non-sporeforming, non-motile, catalase negative, facultatively anaerobic rod which requires a rich medium for growth (Garvie, 1986; Kelly *et al.*, 1989). Cells are lenticular, 0.8 - 1.0  $\mu\text{m}$  long and 0.6 - 0.8  $\mu\text{m}$  wide, with most strains occurring in pairs or long chains. The latter strains tend to form sediments in broth culture. Colony morphology is divided into two types, rough and smooth, which equate to chain-forming and pair-forming, respectively. Rough strains grow well in wine but are prone to lysis by bacteriophage, while most smooth strains are resistant to bacteriophage but are not all able to grow in wine (Davis *et al.*, 1988).

### 1.1.2 Malolactic Fermentation

Because of the distinct need for a large number of *L. oenos* cells before malic acid disappearance is noted, two phases of the MLF process have been defined - growth and bioconversion.

With the notable exception of one group (Naouri *et al.*, 1989), most research into MLF using free cells of *Leuconostoc oenos* has been concerned with the growth of the organism, as this is the slowest phase of the fermentation. Once cell numbers have reached about  $10^6/\text{ml}$  malic acid consumption is seen to proceed relatively rapidly (King and Beelman, 1986). In Chardonnay-style wine the growth conditions include

low carbohydrate concentration, high alcohol concentration (up to 15% v/v), low pH (as low as pH 2.8) and often low temperature (8 - 20°C) (Anonymous, 1990). Malolactic fermentation in this style of wine particularly is thus a slow process due to the poor growth conditions for the bacterium. By pre-growing cells in favourable conditions and then immobilizing them for bioconversion in wine the fermentation can be accelerated (Gestrelus, 1982).

For *Leuconostoc oenos* there is no requirement for rapid cell growth to perform MLF (Lafon-Lafourcade, 1970). Consequently, deacidification can occur in the absence of any flavour modification that would normally occur as a result of cell growth. This may or may not be desired as MLF is used to impart flavour changes as well as to reduce acidity.

Malic acid bioconversion is growth-associated, but only a very low level of growth is needed. This is shown by the need to have viable cells for MLF (Henick-Kling *et al.*, 1989). Where any studies have been done concerning the bioconversion activity, ethanol has usually been left out of the reaction mixture. This is a major error as the alcohol concentration affects at least growth (Asmundson and Kelly, 1990) and the lipid concentration in the cell wall (Body *et al.*, 1991), and the rate-limiting step in bioconversion of malate is the rate of transport into the cell (Henick-Kling, 1988). Alcohol will therefore have an effect on the rate of MLF, especially at higher temperatures.

Ignoring this aspect, the current information shows that the bioconversion occurs at different optimum conditions to those for the maximum growth rate of the bacteria, i.e. 40°C/pH 3.5 - 4.0 *cf* 30 - 35°C/pH > 6.3 (Naouri *et al.*, 1989). Conditions for optimum production of the enzyme system during bacterial growth are different again, i.e. 21°C/pH 4.5, although this is very similar to conditions where maximum biomass of bacteria is obtained i.e. 20°C/pH 4.0 (Asmundson and Kelly, 1990). The total and specific malolactic bioconversion activity is greatest with cells in mid-log phase (Naouri *et al.*, 1989).

### 1.1.2.1 Effect of pH

The pH affects the ability of the organism to utilize sugars (Davis *et al.*, 1985). One study showed that 121 strains of *Leuconostoc oenos* were able to grow in the pH range 3.4 - 7.5 but at pH 3.0 only 31% of these strains showed measurable growth (Davis *et al.*, 1988). Increases in the rate of MLF and specific growth rate with increasing pH have been cited (Davis *et al.*, 1986), although some studies state that the optimum pH for malic acid bioconversion is lower than the optimum for growth i.e. pH 3.5 - 4.0 *cf* pH 5.0 - 6.0 (Rossi and Clementi, 1984; Naouri *et al.*, 1989).

The enzyme system is constitutive with bioconversion activity shown in the pH range 2.5 - 6.0 (Naouri *et al.*, 1989). The observed reduction of bioconversion ability on addition of cells to a low pH wine is related to the lowering of the internal pH. Cells grown on a low pH medium do not suffer this effect as they are already adapted (Henick-Kling, 1988).

### 1.1.2.2 Effect of Temperature

To prevent the loss of volatiles, winemakers prefer to carry out MLF at low temperatures (<20°C). Bacterial growth at 15°C is either very low or non-existent with the greatest specific growth rate in the range 30 - 35°C (Davis *et al.*, 1985; Kelly *et al.*, 1989; Naouri *et al.*, 1989). With increasing ethanol concentration, however, the growth rate decreases and the maximum occurs at a lower temperature, until at 14% v/v ethanol the optimum temperature is 18-20°C (Asmundson and Kelly, 1990). This agrees with other studies where an increase in wine temperature to 26 - 28°C has caused an increase in cell death rate (Lafon-Lafourcade *et al.*, 1983; Trueck and Hammes, 1989). MLF has been performed at 8°C and pH 3.34 though the fermentation was not complete after 12 months and no alcohol was present in the wine as it had been autoclaved (Henick-Kling *et al.*, 1989). Naouri *et al.* (1989) stated that the optimum temperature for carrying out the actual bioconversion was 40°C at pH 3.5, though this was in the absence of alcohol.

### 1.1.2.3 Effect of SO<sub>2</sub>

A concentration of SO<sub>2</sub> greater than 50 mg/l restricts growth of *L. oenos*, especially at low pH when the SO<sub>2</sub> is in the undisassociated, antimicrobial state (Davis *et al.*, 1986). However, MLF has occurred in wine with SO<sub>2</sub> concentrations of 100 - 160 mg/l and tolerance is strain dependent (Gestrelus, 1982; Wibowo *et al.*, 1985). Davis *et al.* (1988) using pH 4.5 and 0% ethanol, found that all of 121 strains grew at 64 mg/l total SO<sub>2</sub>, while at 96 and 128 mg/l, 46% and 11% grew, respectively.

Britz and Tracey (1990) indicated that, at 25°C and 7% v/v ethanol, 50 mg/l SO<sub>2</sub> stimulated growth whereas 25 mg/l did not. At 15°C, using 25 mg/l SO<sub>2</sub> and either 7 or 10% v/v ethanol, the growth rate was depressed, but increasing the ethanol to 13% v/v gave a dramatic positive effect. They repeated the experiment, and, finding similar results, could not offer an explanation for the stimulation under what is seemingly more adverse conditions.

While immobilized malolactic bioconversion has been carried out using wines containing SO<sub>2</sub>, no study has been found that investigated the effect of SO<sub>2</sub> on the bioconversion activity.

### 1.1.2.4 Effect of Ethanol

Of the 121 strains studied by Davis *et al.* (1988), all cultures grew in 10% v/v ethanol, with this figure dropping to 83 and 12 for growth in 12.5 and 15% v/v ethanol, respectively. Alcohol tolerance decreases as temperature increases and pH decreases (Wibowo *et al.*, 1985). Asmundson and Kelly (1990) have shown that, after an initial stimulation at low ethanol concentrations, the growth rate decreased linearly with increasing ethanol concentration for two strains studied, while for two others no stimulation was noted, although the growth yield for all strains was still 90% of maximum at concentrations up to 8% v/v. King and Beelman (1986) found that low amounts of ethanol (<2% v/v) stimulated growth whilst concentrations greater than 6% v/v inhibited growth. Britz and Tracey (1990) showed that the stimulation occurred

only at a higher temperature (25 *cf.* 15°C). Tracey and van Rooyen (1988) showed that the ethanol concentration significantly affected the utilization of sugars and L-malic acid. Whether the effect was positive or negative was strain dependent.

The effect of ethanol has been omitted in most bioconversion studies. No comparison of concentrations or any combined effects with temperature or pH have been noted.

#### 1.1.2.5 Effect of growth media on cell growth

Growth in broth culture is stimulated by tomato, grape or apple juice and also by anaerobic conditions; in addition there is strain-variable use of sugars (Garvie and Mabbitt, 1967; Beelman, 1982; Tracey and Britz, 1987; Champagne *et al.*, 1989; Kelly *et al.*, 1989). Malic acid up to a concentration of 1 g/l inhibits the specific growth rate by 75%, after which the inhibition remains constant with increasing malic acid concentration. Some strains, however, show a slight increase in rate between 1 and 5 g/l malic acid (McCord and Ryu, 1985; Kunkee, 1990).

Use of different strains of yeast for the ethanol fermentation in wine can affect the MLF. It is thought that this is because inhibitors (e.g. phenols) may be produced, or metabolites required by *Leuconostoc oenos* have been utilised (Fornachon, 1968; Davis *et al.*, 1988), although the possibility of bacteriophage causing "stuck" fermentations has also been highlighted (Patel, 1988). There may also be a direct antagonism between yeast and malolactic bacteria growing together as it has been suggested that the bacteria produce L-ornithine from L-arginine which inhibits or kills yeast cells (Beelman *et al.*, 1982). Later research has shown that the situation may be more complex than this (King and Beelman, 1986).

#### 1.1.2.6 Inoculation into wine

Addition of culture directly into wine results in sudden and rapid death of the cells, especially at low pH values (Wibowo *et al.*, 1985). Davis *et al.* (1985) suggested the use of half-strength grape juice containing 0.5% yeast extract (5g/l) and culture

conditions of pH 4.5 and 30°C as an intermediate step to reduce death rates. Preculturing using 40-80% wine has also been suggested and this resulted in increased growth but decreased malic acid degradation (Hayman and Monk, 1982). A 1:1 combination of wine and apple juice medium has been recommended as a preculture medium for *Leuconostoc oenos* inocula supplied to wineries for MLF by DSIR Fruit and Trees (Dr Rod Asmundson, personal communication).

Some wineries inoculate at the same time as, or just after, the yeast inoculation. This can cause problems as some strains of *Leuconostoc oenos* convert sugars to acetate, which is an off-flavour in wine (Tracey and van Rooyen, 1988).

## 1.2 Immobilization Methods

The numerous techniques for achieving immobilization can be categorized according to the physical process involved, namely: attachment, entrapment, aggregation or containment. The more widely used techniques fall mainly into the first two categories (Webb, 1989).

### 1.2.1 Attachment

Natural adhesion of cells to surfaces is a widespread phenomenon that provides one of the simplest techniques for cell immobilization. It is the method used in both of the oldest industrial immobilized cell systems - vinegar production and wastewater treatment. Current systems have preferred to use particulate solid supports, often materials such as sand or surface-active porous materials such as coke or wood chips (Vega *et al.*, 1988).

Attached cells often differ markedly in behaviour from floating cells, and growth rate,

cell wall structure and composition, immunogenicity, enzyme activities and sensitivity to antibacterial agents can be affected. Factors which affect adhesion of cells include: the type of support (particle size, porosity, charge, composition); microorganism (strain, age, surface charge); and environmental conditions (pH, temperature, liquid flowrate and ionic strength) (Gõdia *et al.*, 1987).

The specific mechanisms of adhesion differ depending on the bacteria and the particular surface. First, physical forces may be involved in the attraction or electrostatic repulsion of bacteria to or from surfaces, if both are charged or hydrophobic. Second, the charged nature of surfaces attracts soluble polymers to which bacteria are attracted. Bacteria become attached to these surface-bound nutrients via cellular appendages, such as pili and exopolysaccharides. Third, bacteria may be simply transported by gravitational forces to form sediments on surfaces. Fourth, Brownian motion brings bacteria into contact with surfaces, and fifth, various forms of chemical bonding, such as hydrogen bonding, attract and attach bacteria to surfaces (Costerton and Lappin-Scott, 1989).

Once bacteria reach and settle on a surface, attachment commences and a two-phase process, with a reversible and then an irreversible attachment phase, has been defined (Rutter, 1980). During the initial reversible phase, bacteria are held to the surface by weak attractions. Firmer binding occurs when physical and chemical forces combine to hold the bacterial cells irreversibly to the surface. Some of these forces include the production of exopolysaccharides by the attached cells and the formation of a biofilm matrix.

Cells which do not naturally adhere to surfaces can sometimes be encouraged to attach by chemical means such as cross-linking using glutaraldehyde, silanization to silica supports or by chelation to metal oxides. In such cases the strength of attachment is similar to that in natural adhesion (Webb, 1989).

Attached cells are in direct contact with the surrounding environment and hence subject to any forces of shear or attrition which may result from the relative motion

of particles and fluid. It is therefore likely that some cells will become detached and enter the bulk fluid phase. It is also difficult to control or even determine the depth of the attached biofilm. Nevertheless, the technique is cheap and simple and the art is to provide the correct surface in a suitable form for colonization by the desired organism or population of organisms to as high a density as is possible (Webb, 1989).

### 1.2.2 Entrapment

Entrapment of cells can be performed on a microscopic level with microporous particles such as brick, ceramics, sintered glass or kieselguhr (pore entrapment), or on a macroscopic level with particles having relatively large pores (cage entrapment). Examples of the latter include knitted stainless steel wire crushed into spheres and reticulated polyurethane foams cut into cuboidal shapes (Webb, 1989).

The third, and most popular, method of entrapment is immobilization within a gel. The first matrix used was polyacrylamide, but the polymerization conditions required severely damaged the cells. Current interest is in natural polymers such as agar, kappa-carrageenan and calcium alginate (Gõdia *et al.*, 1987). The main drawbacks of these systems are the instability of calcium alginate against phosphates and the disruption of gel particles due to carbon dioxide evolution during fermentation.

### 1.2.3 Flocculation

By flocculating to form large aggregates, cells may become immobilized in the sense that it then becomes possible to retain them in continuously operated fermenters, e.g. packed- or fluidized-beds. This has been used for beer production using yeast, activated sludge wastewater treatment and fungal mycelia growth. Microbial species not normally regarded as flocculent can be induced to flocculate under certain conditions (Prince and Barford, 1982).

#### 1.2.4 Containment

Cells may be immobilized by containing them behind a barrier. This barrier is generally a semi-permeable membrane such as that used for microfiltration or ultrafiltration, and cells are retained while nutrients and products diffuse through the membrane. This method has been tried with mammalian-cell tissue culture to reduce the amount of downstream processing required (Ward *et al.*, 1990). Equipment is often expensive and high value products are usually involved in order to make the process economic.

### 1.3 Application of Cell Immobilization to MLF

#### 1.3.1 Methods Used for Immobilization

As far as can be discerned the only immobilization technique that has been applied to MLF is that of gel entrapment.

The concept was first introduced by Divies and Siess (1976) and Divies (1977), who immobilized *Lactobacillus casei* in a polyacrylamide gel and determined the L-malic acid-degrading activity in model systems.

Since then, *Leuconostoc oenos* has been immobilized in polyacrylamide gel (Lee and Pack, 1980; Rossi and Clementi, 1984), alginate gels (Spettoli *et al.*, 1982, 1984; Gestrelus, 1982; Cuénat and Villettaz, 1984; Trueck and Hammes, 1989; Naouri *et al.* 1991) and kappa-carrageenan (McCord and Ryu, 1985). Further work with kappa-carrageenan containing silica has shown improved operational stability (Spettoli *et al.*, 1987).

*Leuconostoc mesenteroides* has been immobilized in agar and alginate (Totsuka and Hara, 1981).

*Lactobacillus* spp. have been immobilized in kappa-carrageenan (McCord and Ryu, 1985; Naouri *et al.*, 1991) containing bentonite (Crapisi *et al.*, 1987) or silica (Spettoli *et al.*, 1987).

*Schizosaccharomyces pombe* has been immobilized in calcium alginate gel (Magyar and Panyik, 1989).

No reference has been found for the use of adhesion-immobilized cells for MLF, though the method is common in ethanol production research where supports have included wood chips, ceramic particles, silica gel, ion exchange resin, glass fibre, cotton fibres, synthetic sponge and untreated sugar cane bagasse (Gödia *et al.*, 1987). Adsorption of flor yeasts to oak chips for sherry production is currently in commercial use in Australia (Rankine, 1989). It is presumed that naturally occurring MLF is initiated by microorganisms adhering to surfaces within a winery as once a winery has conducted MLF it is very difficult to stop the fermentation happening in further vintages (Pilone *et al.*, 1974).

### 1.3.2 Effect of Immobilization on Bioconversion

Totsuka and Hara (1981) could perform MLF at higher ethanol and SO<sub>2</sub> concentrations, and at a lower pH, with polyacrylamide immobilized cells compared with free cells. Operation at high (15% v/v) ethanol (Spettoli *et al.*, 1982) and SO<sub>2</sub> (139 mg/l) concentrations (Cuénat and Villettaz, 1984) has been achieved with immobilized cells though MLF with free cells has also been achieved at these values (van der Westhuizen and Loos, 1981; Wibowo, *et al.*, 1985).

Table 1 gives a comparison of bioconversion activities reported in the literature and the conditions under which the assays were conducted. All the previous studies in the literature used a similar activity assay method as used in the batch experiments of this thesis, with the exception that ethanol was generally omitted and all the studies used different strains of the bacterium or different organisms. Where *L. oenos* cells were

in a continuous system (Spettoli *et al.*, 1984, 1987) the volume of the reactor was not given and so the activity could not be calculated.

**Table 1:** Specific activity of malolactic organisms as given in the literature

Specific Activity (mg malic acid/g biomass.h)	Assay conditions	State of cells	Reference
1290	pH 3.0, 25°C Batch	Free <i>L. oenos</i>	Henick-Kling, (1988)
2960	pH 4.5, 21°C Batch	Free <i>L. oenos</i>	Naouri <i>et al.</i> (1989)
2410	pH 4.3, 30°C Batch	Free <i>L. oenos</i>	McCord & Ryu (1985)
1340 - 1540	pH 4.3, 30°C Batch	Immobilized <i>L. oenos</i>	McCord & Ryu (1985)
7640 5100	pH 3.5, 20°C pH 3.4, 40°C Batch	Free <i>L. oenos</i>	Naouri <i>et al.</i> (1991)
6440 4020	pH 3.0, 20°C pH 3.4, 25°C Batch	Immobilized <i>L. oenos</i>	Naouri <i>et al.</i> (1991)
74	pH 3.3, 21°C wine, no SO <sub>2</sub> Batch	Free <i>L. oenos</i>	Trueck & Hammes (1989)
110	pH 3.3, 21°C wine, no SO <sub>2</sub> Batch	Immobilized <i>L. oenos</i>	Trueck & Hammes (1989)
76 (calculation based on wet weight of cells)	pH 3.15, 20°C 10% v/v ethanol 21ppm SO <sub>2</sub> Batch	Immobilized <i>L. oenos</i>	Spettoli <i>et al.</i> (1982)
200	pH 3.0, Temp not given Batch	Immobilized <i>L.</i> <i>oenos</i>	Rossi and Clementi, (1984)
10 (per 4x10 <sup>8</sup> cells)	pH not given 25°C, batch 50g/l glucose	Immobilized and free <i>Schiz. pombe</i>	Magyar and Panyik (1989)
1.2 (per 4x10 <sup>8</sup> cells)	pH not given 25°C, batch 30g/l glucose 8.8% v/v ethanol	Immobilized and free <i>Schiz. pombe</i>	Magyar and Panyik (1989)
70	pH 3.3, 18°C continuous	Immobilized <i>Lactobacillus</i>	Crapisi, <i>et al.</i> , (1987)

The three studies comparing free and immobilized *L. oenos* cells show that gel entrapment lowers the malolactic bioconversion activity of the bacteria and also lowers the optimum pH and temperature, although figures from Naouri *et al.* (1991) are contradictory. Trueck and Hammes (1989) found that Ca-alginate immobilized cells took 50% longer to degrade malic acid in wine compared with free cells and surmised that this may have been due to diffusion difficulties. No other studies directly compared immobilized and free cells of the same strain. Addition of alcohol appears to reduce the bioconversion activity by about 90%, and this includes the *Schizosaccharomyces pombe* cells which have a different enzyme system.

The half-life of gel-entrapped *Leuconostoc oenos* cells has been given as varying between 0.5 and 7 days (Spettoli *et al.*, 1982, 1987; Trueck and Hammes, 1989; Naouri *et al.* 1991) while a figure of 46 days has been cited for *Lactobacillus* (Crapisi *et al.*, 1987). Physical problems occur over time with disintegration of the gel caused by the presence of phosphate and CO<sub>2</sub>.

#### 1.4 Experimental outline

Since it is apparent that the best conditions for cell growth are different from those prevailing in wine (where MLF is required) the emphasis in this work was on separating the growth and bioconversion phases. This allows better growth of cells before MLF is performed.

The literature shows that continuous, immobilized malic acid bioconversion can be performed by *Leuconostoc oenos*. Bioconversion parameters, especially interactions between parameters, have remained largely unstudied and the processes do not seem to have been designed with industrial practice in mind.

While the gel entrapment method has worked at a research level, for a working winery

the method would require trained personnel and a considerable amount of time - both of which are at a premium during the vintage when MLF is performed. The simpler method of adhesion was therefore tested in the experiments described in the present thesis.

The basic objectives of the experiments in this thesis were:

- select a strain of *Leuconostoc oenos*, and a growth medium
- select an immobilization support
- determine bioconversion parameters of immobilized cells in a continuous bioreactor
- scale-up the bioreactor and use as a prototype in a winery

Strains of *L. oenos* , currently in use as commercial inocula for MLF, were available from DSIR Fruit and Trees, Palmerston North, and a growth medium for maximum enzyme production has been defined by Naouri *et al.* (1990).

Bonechar and oak chips were tried as immobilization supports as they are food compatible, relatively cheap, and easy to obtain. Oak chips were perceived to have the extra advantage of giving desirable flavours to the wine.

To use a continuous bioreactor in an industrial situation the optimum feed flowrate must be calculated, and to determine this flowrate, the effect on bioconversion of parameters such as pH, temperature, ethanol concentration and SO<sub>2</sub> were investigated. This gave an estimate of reactor size and flowrate for a prototype reactor that was successfully used at Villa Maria Wineries, Auckland, during the 1991 vintage.

## 2.0 METHODS AND MATERIALS

### 2.1 Organisms

*Leuconostoc oenos* strains 1070, 1041 and 1011 were obtained as freeze-dried cultures from Dr. Rod Asmundson (DSIR Fruit and Trees, Palmerston North) and were revived by growth in 50 ml aliquots of tomato juice broth for one week at 21°C. Tomato or apple juice broth agar (2% w/v) stab cultures from the 50 ml aliquots were used to maintain the organisms. Cultures were subcultured into new stab cultures every five months. Storage of the stab cultures was at 4°C.

All initial growth of organisms was at 21°C ( $\pm 0.5^\circ\text{C}$ ) and pH 4.5 in 100 ml Erlenmeyer flasks with Bellco metal caps. Cultures were not shaken.

The protocol for the preparation of bioreactor inoculum was:

- transfer a loop of cells from stab culture (new culture used for every experiment) into 100ml Apple Juice (AJ) broth and then grow to mid-log phase
- transfer 4x25ml from this culture into 4x75 or 4x100ml AJ broth and grow until mid-log phase
- inoculate bioreactors using one flask per bioreactor

### 2.2 Immobilization Media

French oak chips as normally used in wine production were obtained from Columbit Marketing, Auckland. They were 0.25-0.5mm thick, and were sieved for the other

two dimensions to a size range of 1 - 2mm.

Bone char was obtained from the N.Z. Sugar Co. Ltd (Auckland, NZ) and was sieved to remove fines of <1mm particle size.

Immobilization media was sterilized in 100ml Erlenmeyer flasks at 121°C for 15 minutes.

### 2.3 Growth and Bioconversion Media

Media and growth conditions were as defined by Naouri *et al.* (1989) for the optimum production of malolactic activity in the bacteria.

#### 2.3.1 Tomato Juice Broth (TJ broth)

Peptone	5	g/l	(NZ-Case, Sheffield, USA)
Yeast extract	12.5	g/l	(bioMérieux, Charbonnières les Bain, France)
Diammonium citrate	5	g/l	(BDH, Poole, UK)
Glucose	10	g/l	(BDH, Poole, UK)
DL-malic acid	10	g/l	(Sigma, St.Louis, USA)
Tomato juice	100	ml/l	(Clarified fresh tomato juice)

The medium was adjusted to pH 4.5 using HCl and was then sterilized at 121°C for 15 minutes.

### 2.3.2 Apple Juice Broth (AJ broth)

Peptone	5	g/l	(NZ-Case, Sheffield)
Yeast extract	12.5	g/l	(bioMérieux)
Diammonium citrate	5	g/l	(BDH)
Glucose	10	g/l	(BDH)
DL-malic acid	8	g/l	(Sigma)
Apple juice	200	ml/l	(Freshup Crisp, NZ Apple & Pear Board)

The medium was adjusted to pH 4.5 using NaOH and was then sterilized at 121°C for 15 minutes. Where the medium contained ethanol this was added aseptically after autoclaving and was stirred in using a magnetic flea with minimal aeration.

### 2.3.3 Apple Juice Wine (AJ wine)

Apple juice	200	ml/l	(Freshup Crisp)
DL-malic acid	8	g/l	(Sigma)
Tartaric acid	2.6	g/l	(BDH)
Ethanol	13%	v/v	

The medium was adjusted, using NaOH, to the pH desired for the experiment and was then sterilized at 121°C for 15 minutes. The ethanol was added aseptically after autoclaving and was stirred in using a magnetic flea with minimal aeration.

This synthetic wine was used for all experiments. In the Chardonnay/synthetic wine comparison (Section 3.5) the malic acid concentration was varied as stated. Total malic acid concentrations varied slightly with the batch of apple juice used and are noted for each experiment.

#### 2.3.4 Yeast Nitrogen Base Wine (YNB wine)

Bacto Yeast Nitrogen Base	6.7	g/l	(Difco, Michigan, USA)
DL-malic acid	2.8	g/l	(Sigma)
Tartaric acid	2.6	g/l	(BDH)
Ethanol	12.7%	v/v	

The medium was adjusted to pH 3.5 using NaOH and was then sterilized at 121°C for 15 minutes. The final pH was 3.6 and the L-malic acid concentration was 1.4 g/l.

### 2.4 Analytical Methods

#### 2.4.1 Growth

All growth was monitored by measuring absorbance at 600nm using a Pye SP6-550 spectrophotometer.

#### 2.4.2 Biomass

Biomass was determined by growing cells to various absorbances in 100ml of TJ broth and AJ broth, both with and without 6% v/v ethanol, and then centrifuging at 13,700g for 30 minutes in a Sorvall RC5C centrifuge. The supernatants were decanted and the cells were washed once in distilled water and recentrifuged. The pellets were resuspended in distilled water and then dried for 24 hours at 104°C. A curve of dry cell weight versus absorbance was used for biomass estimations in the specific activity measurements. Errors were estimated at  $\pm 10\%$  based on a larger sample (1 litre) that was dried for the batch bioconversion in AJ wine experiment (Section 4.1).

### 2.4.3 L-Malic Acid

Reagents:

Glycine buffer	(BDH)	0.1 mol/l	pH 10.0	...	1.53ml
L-glutamate	(Sigma)	0.5 mol/l	ca. pH 10.0	...	0.20ml
$\beta$ -NAD	(Sigma)	27 mmol/l		...	0.15ml
Aspartate aminotransferase	(Sigma)	2000 kU/l		...	0.01ml
L-malic dehydrogenase	(Sigma)	6000 kU/l		...	0.01ml

A 0.1 ml sample with a malate concentration diluted to between 0.02 and 0.05 g/l was mixed with the first three reagents above, and an absorbance at 340nm was taken using a Pye SP6-550 spectrophotometer. The enzymes were then added and a second absorbance reading taken 3-5 minutes later. The change in absorbance was used in a standard formula to obtain the malate concentration of the sample (Bergmeyer, 1985). L-malic acid (Sigma) was used as a standard.

Errors based on replicate samples were  $\pm 10\%$  for concentrations above 3g/l and  $\pm 5\%$  for concentrations under this figure. All assays were done in duplicate.

### 2.4.4 Malolactic Activity

A known weight of cells from 30ml of culture was suspended in 3 ml of 0.1M citrate buffer at pH 4.5, containing 10 g/l DL-malic acid. The buffer also contained ethanol (6% v/v) if alcohol was part of the initial growth conditions of the culture being tested. The malolactic activity was measured by using a series of duplicate assays of the L-malic acid remaining in the citrate buffer after a specified time period. The results were expressed as  $\mu$ moles L-malic acid degraded per hour per mg dry matter (specific activity) or  $\mu$ moles L-malic acid degraded per hour per litre culture suspension (total activity).

#### 2.4.5 Total Reducing Sugars

This was determined using the dinitrosalicylic acid (DNS) method (Miller, 1959).

The DNS reagent had the following composition:

Sodium hydroxide	10	g/l	(BDH)
Potassium sodium tartrate	182	g/l	(BDH)
Dinitrosalicylic acid	10	g/l	(BDH)
Phenol	2	g/l	(BDH)
Sodium sulphite	0.5	g/l	(BDH)

Samples were diluted to between 0 and 1 g/l sugar and then 1 ml of diluted sample, 1 ml of distilled water and 3 ml of DNS reagent were added to a test tube and boiled for 15 mins. After cooling for 20 minutes the absorbance at 575nm was read against a water blank. A standard curve for glucose was used for determining the reducing sugar concentrations.

#### 2.4.6 Plate Counts

The entire contents (chips and liquid) of a bioreactor were blended for one minute using a kitchen blender in an anaerobic cabinet. This liquid was then dilution plated onto Todd Hewitt agar (Difco) containing 100ml/l Freshup Crisp apple juice. Plates were incubated anaerobically at 30°C for 11 days after which colonies were counted.

Errors exist in this method as no duplicates were done although an average of the dilution plates was carried out. Also, the effectiveness of blending to remove the cells off the chips and break up the cell chains was not checked.

### 2.4.7 Ethanol

The ethanol concentration in all AJ wine feeds was checked by gas chromatography using a Shimadzu GC-4 (Kyoto, Japan) with a Porapak Q column. The column temperature was 200°C, with a carrier gas (nitrogen) flow rate of 30 ml/min. Isopropanol was used as an internal standard.

## 2.5 Immobilized Cell Reactor

### 2.5.1 Equipment

Figure 1 shows the equipment used for immobilized cell continuous culture as set up for an experiment. The bioreactor columns were cylindrical perspex with water jackets for temperature control. Dimensions were 40mm internal diameter by 202mm height with flat screw-on ends giving a total volume of 254 ml. Water from constant-temperature water baths was pumped through the water jackets at a rate so that the outlet temperature was no more than 0.5°C lower than the inlet temperature.

The wine inlets and outlets were via nipples in the ends with flat discs of stainless steel mesh containing the immobilization media. Tubing for the inlet went from a feed flask via a Masterflex peristaltic pump (Cole-Parmer, Chicago, USA) to the bioreactor, while the overflow line was silicone tubing attached to the top nipple, and fed into an open Erlenmeyer flask. Inoculum and sterilized immobilization media were poured in by unscrewing the tops of the reactors.

Up to four bioreactors were used in parallel and feed was pumped into the bottom of the bioreactor using a multihead Masterflex peristaltic pump with variable speed drive. This meant the flowrate for each of the four bioreactors was the same but different feed media could be used for each bioreactor.

**Figure 1:** Immobilized cell bioreactors



### 2.5.2 Retention Times

The volume of immobilization media (including interstitial spaces) used for all experiments was 100ml (20g, 55°C air-dried weight for oak chips; 75g for bone char). The void volumes of 80ml for oak chips and 70ml for bone char were used for calculating the dilution rates/retention times for all experiments. There was 154ml of headspace above the chips, but this was ignored as the dilution rate ( $D$ ) was always above the washout figure for the free bacteria ( $D_{crit}=0.03$  for AJ broth: Kelly *et al.*, 1989) and therefore the headspace was spent pure medium with a few displaced cells.

For all experiments, at least five retention times were allowed before data were collected to ensure that all growth medium had been removed from the reactors. Steady state was considered to have been reached if the malic acid concentrations in the effluent were constant  $\pm 20\%$  for at least five retention times.

### 2.5.3 Sterilization of Reactors

Before use, the bioreactor columns were sterilized by pumping through a 2% v/v formalin solution using the peristaltic pump system. The formalin was then removed using sterile water.

### 2.5.4 Inoculation and Cell Growth

After initial growth in flasks as stated, the inocula, as well as the sterilized immobilization media, were aseptically poured into the reactor. Growth medium was then pumped in intermittently for several days. Details of this growth phase for each experiment are given in Table 2.

### 2.5.5 Bioconversion Calculations

The malic acid bioconversion rate was calculated by the following equation:

$$\text{Rate} = (\text{influent malic acid concentration} - \text{effluent malic acid concentration}) \\ * \text{flowrate} / \text{volume of immobilization medium}$$

**Table 2:** Conditions for initial growth of cells in bioreactors

	<u>Section Number (See Key)</u>							
	3.5	3.4	4.3	4.4	4.5	4.2	4.6	4.7
<u>Inoculant</u>								
Culture age	7 day	Stab	5 day	7 day	11 day	9 day	8 day	4 day
Into AJB (%ethanol)	4x75ml	3x100ml	4x100ml (6% v/v)	4x75ml (6% v/v)	4x75ml	4x100ml (6% v/v)	4x75ml	4x100ml
Abs. 600nm after	0.487-0.531 3 days	0.380-0.395 8 days	0.410 5 days	0.319-0.326 4 days	0.345-0.414 5 days	0.390-0.420 5 days	0.354-0.391 3 days	0.500 7 days
<u>Reactors:</u>								
Inoculated Day 1	1600	1615	1630 <sup>@</sup>	1630	1600	1115	1700	1130
Day 2 (See NOTES)	0820/0915;0.48 1600/1625;0.48	0850/1120;0.14 1430/1530;0.14	0830/1530;0.20	1515/1530;2.4	1330/1600;0.42	1530/1700;0.20	2245/2300;3.0	1030/1300;0.26 1500/1620;0.20
Day 3	0835/0855;0.48 1322/1555;0.14	1620/1655;0.14	0830/1320;0.20	0945/0953;3.75 1608/1618;3.75	0900/1145;0.33	0830/1150;0.20	1255/1325;0.45	0830;0.20 1400/1600;0.17
Day 4	0820;0.14	1400/1430;0.48	0830;0.20 1000(9%) 1450;0.17	0825/0910;0.20 1445/1545;0.20	1430/1610;0.33	0830/1145;0.20	0822/0927;0.23 1600/1630;0.33	0820;0.17 1300(9%) 1615;0.16
Day 5	1615(9%)	0815/1330;0.14	0815(12%) 1645;0.26 wine feed	0815;0.20 1630(9%)	1610/1925;0.33	1135/1155;3.00	0815;0.23	1615(13%)
Day 6	1150(13%)	0814/1500;0.14 1600/1635;0.48		1115;0.37 1630(13%)	0815/1610;0.33		0845;(9%) 1545;0.16	1200;0.16 wine feed *
Day 7		0815/0950;1.67 1130;0.26 1530;0.14		0830;0.20 0930;0.37	0855;0.33 1345;0.23 (9%)	820;0.33 0845(9%) 1600;0.2 (13%)	0900;0.23 (13%) 1630;0.23 wine feed	
Day 8	1600;0.37 wine feed	1100(9%) 1630(13%)		1040;0.37 wine feed	0900;0.28 1100;0.23 (13%)			
Day 9		1415;0.37 wine feed #			1400;0.28 wine feed	0845;0.20 wine feed		

**KEY**

Section number 3.5: Wine/synthetic wine comparison  
 3.4: Oak chips/bonechar comparison  
 4.3: Anaerobic/non-anaerobic comparison  
 4.4: Temperature/pH comparison  
 4.5: pH/ethanol comparison  
 4.2: Chardonnay/apple juice wine recycle comparison  
 4.6: Malic acid/flowrate comparison 1  
 4.7: Malic acid/flowrate comparison 2

**NOTES**

All times are given using the 24 hour clock and all ethanol concentrations are % v/v.

On Day 1 after inoculation the columns were completely filled with AJ Broth (6% v/v ethanol) and the temperature was set at 21 ± 0.5°C.

From Day 2 onwards, the times show pump on/pump off; and associated dilution rate (h<sup>-1</sup>)

Where no time off is shown, the pump has been left on at the stated dilution rate. Final figures in each column give the time and dilution rate at the start of the wine feed.

Where the ethanol concentration of the medium was increased, a time is given along with the new concentration in brackets.

@ Growth temperature dropped to 9°C overnight. Increased to 21°C on Day 2

# Contaminated by yeast

\* Growth temperature found to be 13°C

### 3.0 PRELIMINARY SCREENING EXPERIMENTS

#### 3.1 Introduction

Before the main experiments using immobilized *L. oenos* could be performed, several screening experiments were carried out to determine the strain of the organism to use and to select the growth, bioconversion and immobilization media.

#### 3.2 Effect of addition of DL- or L- Malic Acid on growth/bioconversion

##### 3.2.1 Objective

Although *L. oenos* does not utilise D-malic acid, it was necessary to test whether the addition of this isomer to media had an effect on the organism.

##### 3.2.2 Experimental Conditions

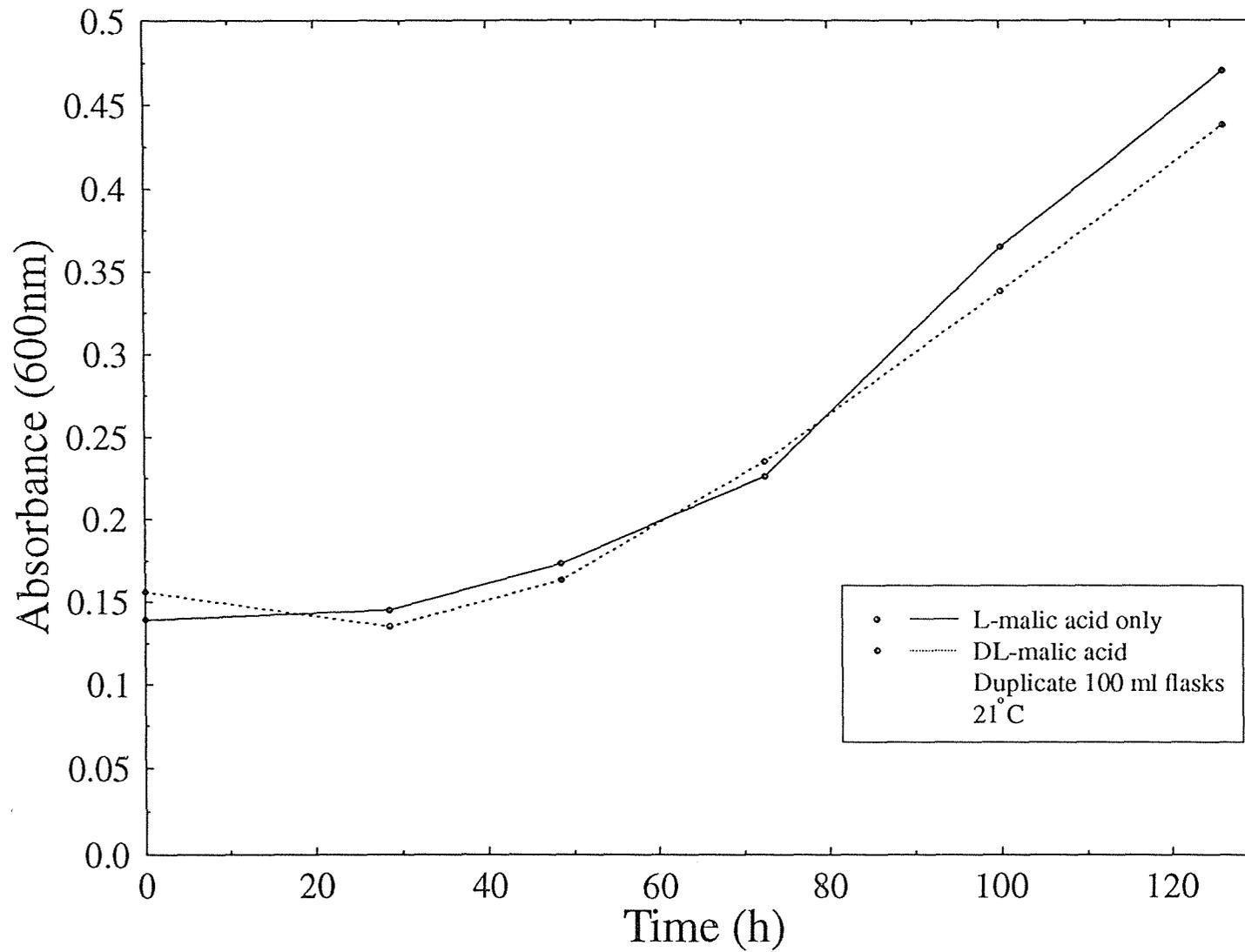
A 50ml culture of *L. oenos* 1070 from an agar stab was grown to mid-log phase and 10ml of this culture were used to inoculate 100ml of AJ broth. This 100ml culture was grown to late-log phase and 4 x 25ml aliquots were inoculated into 4 x 100ml AJ broth. Two flasks contained DL-malic acid and the other two L-malic acid with a concentration of 4 g/l L-malic acid in all flasks. The pH was 4.5 and the growth temperature was 21°C. Gram stains at the end of the experiment showed pure cultures of small, Gram positive, lenticular rods.

### 3.2.3 Results and Conclusions

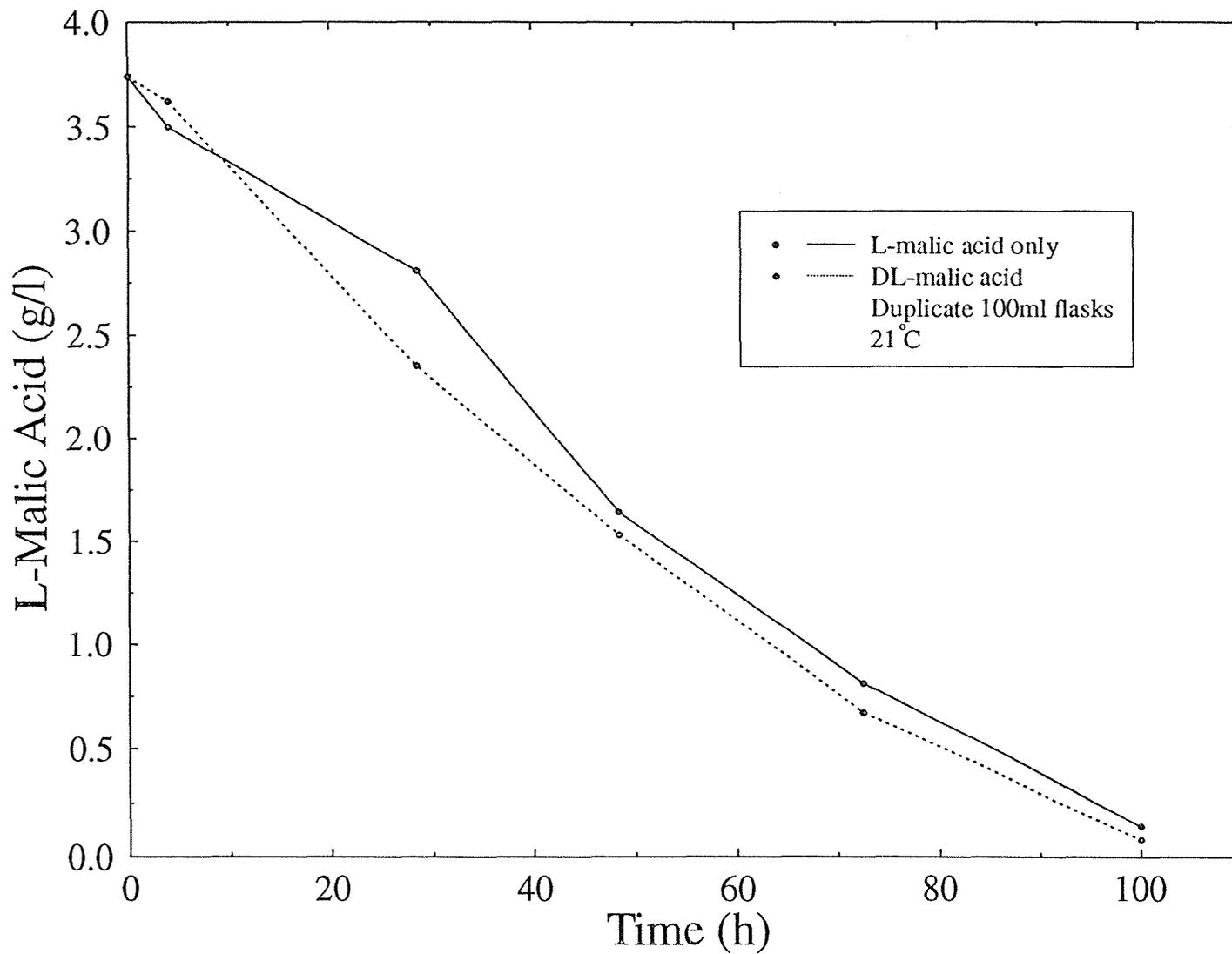
Results for the growth and malic acid bioconversion are shown in Figures 2 and 3. No difference was noted between the DL- and L- malic acid broths in either growth or L-malic acid bioconversion and so it was assumed that D-malic acid had no effect on the fermentation.

Henceforth all experiments were carried out using the cheaper DL-malic acid as a substrate. All malic acid concentrations are reported as the L- isomer.

**Figure 2:** Growth of *L. oenos* strain 1070 in DL- and L-malic acid broth



**Figure 3:** L-malic acid bioconversion by *L. oenos* in DL- and L-malic acid broth



### 3.3 Selection of Bacterial Strain and Growth Medium

#### 3.3.1 Objectives

The growth medium and a strain of *L. oenos* to use were required to be defined. Naouri *et al.* (1989) had performed a factorial experiment to determine the optimal growth conditions for enzyme production, and the medium composition they defined was now tested with each of the strains used in the commercially-available DSIR inoculant. A comparison using apple juice instead of tomato juice was also conducted to decide if apple juice could be substituted for tomato juice as it is easier to procure and is used for other DSIR research with *L. oenos*. Maximum growth rate and activity were the basis for deciding the strain and medium for future use.

A trial in which alcohol (6% v/v) was present in the growth medium was also conducted to check whether this caused any difference in activity, and if adapting the bacteria to alcohol at an early stage was feasible.

#### 3.3.2 Experimental Conditions

The inoculum for growth and activity studies on strain 1070 was an eight-day old stationary phase culture in TJ broth whereas the inocula for strains 1041 and 1011 were ten-day old cultures in mid-log phase, also in TJ broth.

Duplicate AJ and TJ broths were made up in 100ml Erlenmeyer flasks with and without 6% ethanol (v/v), and inoculated with 6ml of the TJ broth inoculum. Malolactic activity assays were conducted at 113, 141 and 147 hours after inoculation for strains 1041, 1011 and 1070, respectively.

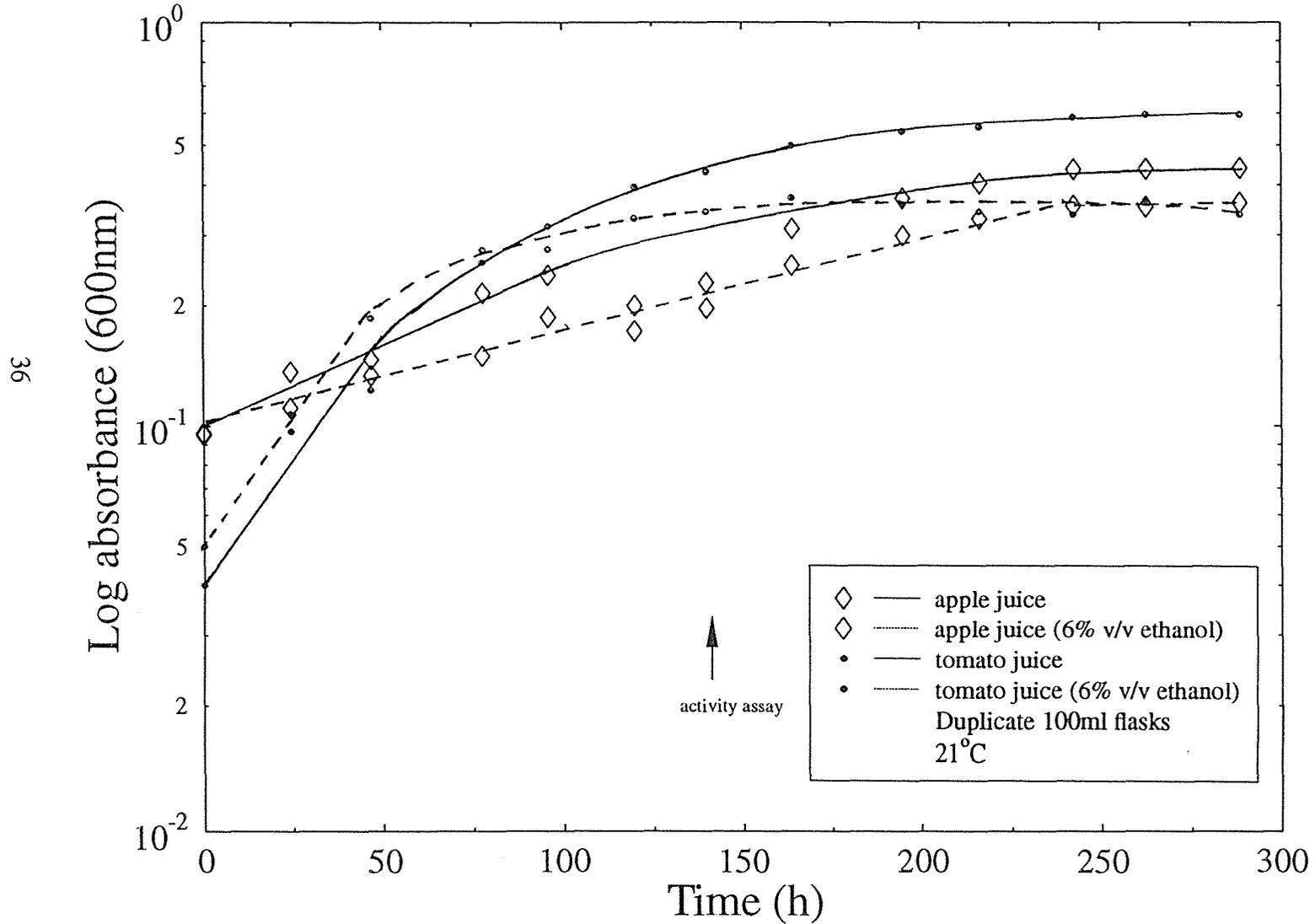
### 3.3.3 Results and Discussion

Specific growth rates and activity values are shown in Table 3 and graphs showing growth in Figures 4 and 5 and 6.

**Table 3:** Tomato juice/apple juice comparison

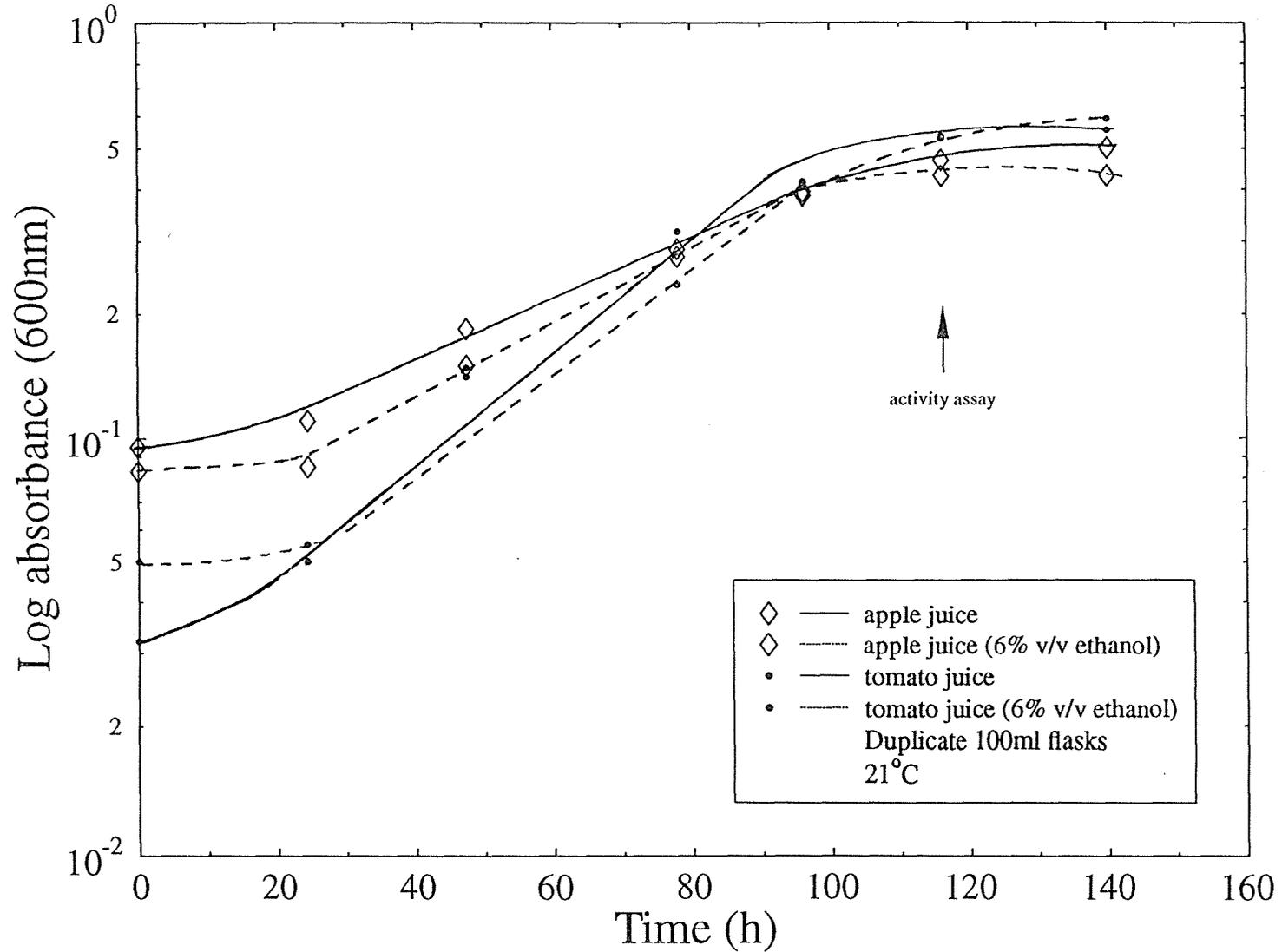
Strain	Medium	Specific Growth Rate ( $h^{-1}$ ) between (h)	Total Activity (mg malic acid/100ml culture/h)	Specific Activity (mg malic acid/g biomass/h)
1011	TJ broth	0.03 0-78	4.2	228
1011	TJ broth (6% v/v ethanol)	0.02 0-47	7.4	335
1011	AJ broth	0.01 0-96	1.6	268
1011	AJ broth (6% v/v ethanol)	0.01 0-242	2.3	322
1041	TJ broth	0.03 24.5-96	9.4	416
1041	TJ broth (6% v/v ethanol)	0.03 24.5-96	9.5	523
1041	AJ broth	0.02 24.5-96	3.9	322
1041	AJ broth (6% v/v ethanol)	0.02 24.5-96	8.4	697
1070	TJ broth	0.03 24-80	15.7	644
1070	TJ broth (6% v/v ethanol)	0.03 48-98	15.5	630
1070	AJ broth	0.02 72-104	11.6	577
1070	AJ broth (6% v/v ethanol)	0.02 72-104	11.9	590

**Figure 4:** Growth of *L. oenos* strain 1011 in various media

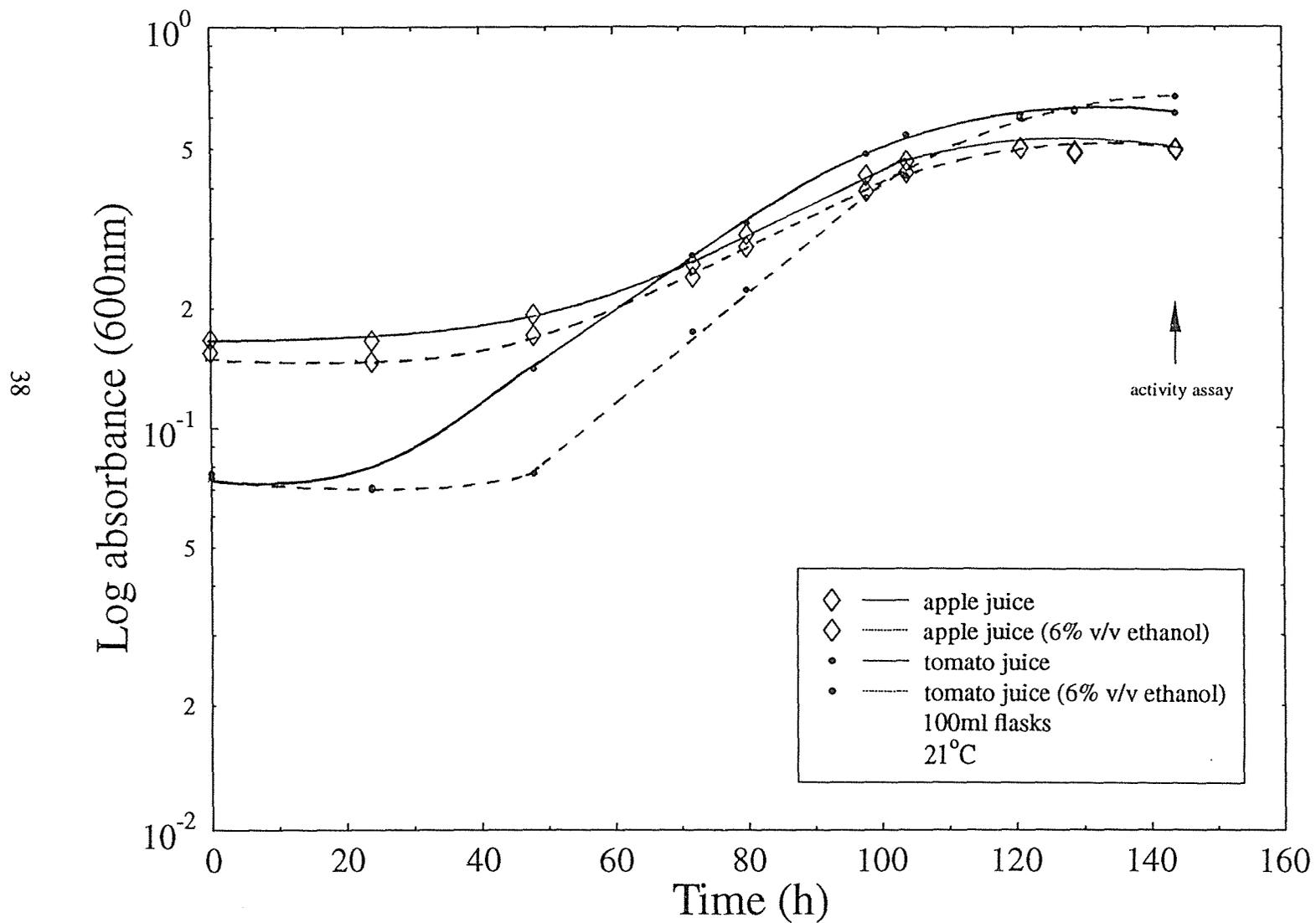


**Figure 5:** Growth of *L. oenos* strain 1041 in various media

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**Figure 6:** Growth of *L. oenos* strain 1070 in various media



These experiments confirm results reported in the literature that there is strain variability with *L. oenos*. All three strains were isolated from New Zealand Chardonnay wines with high alcohol concentrations and have shown good growth in this wine style. The growth rates are, therefore, quite similar but the differing effect of the ethanol concentration on the enzyme activity of each strain is shown with these results.

There was a slight difference in growth rate between tomato and apple juice for all strains, with tomato juice providing higher values. Addition of alcohol made little difference to the specific growth rates. The rates are low but are comparable with data from Kelly *et al.* (1989) where growth rates for various strains ranged from 0.010 to 0.033 h<sup>-1</sup> using an apple juice medium at a temperature of 21°C.

The final absorbance was slightly greater in all cases for the tomato juice cultures compared with the apple juice. The 6% v/v alcohol cultures showed a longer lag phase and except for strain 1070, a lower apparent biomass concentration. A possible reason for the lower final absorbance is that alcohol induces chain formation in the bacteria. Therefore, there may be the same number of cells in the alcohol cultures but chain formation is causing less light scattering compared with dispersed cells.

Strain 1011 showed very slow growth; only the culture with tomato juice, but without alcohol, achieved an absorbance similar to that of the other two strains.

For strains 1011 and 1041 the addition of alcohol gave increased total and specific activity, but this did not occur with strain 1070, which may be due to the cells being in stationary phase. This alcohol effect on bioconversion is similar to the effect on growth, where a slight amount of alcohol stimulates some strains. Tomato juice gave higher total activity for all the strains but the specific activities for both media were similar. The activity for strain 1070 is likely to be a conservative figure as nearly all the L-malic acid was utilised after two hours when the second assay was done.

Naouri *et al.* (1989) have shown that activity is greatest in mid to late log phase.

Thus for strain 1041 the figure given should be a maximum but for strain 1011 the mid point was difficult to determine and with strain 1070 the culture was in stationary phase. Hence, it is possible that higher activities could be observed for strains 1011 and 1070.

The strains studied showed little difference in specific activity between cultures with and without 6% v/v alcohol but were a factor of 10 lower than figures given by Henick-Kling (1988) and Naouri *et al.* (1989, 1991). This may be due to the strain variability which is common in *L. oenos* (Davis *et al.*, 1985), or because the literature figures are derived from cultures that were not selected for alcohol resistance and therefore have different cell membrane characteristics which permit higher bioconversion. The picture is reversed when comparing the preliminary screening results with the literature figures for media containing ethanol, as the former gave a 6- to 7-fold higher bioconversion rate. This may indicate strain variability again, or may arise because the thesis results were obtained with media containing only 6% v/v alcohol while all the literature figures used a higher concentration where the inhibition would be greater.

Fruit juice is required for growth as shown by Garvie and Mabbitt (1967), with tomato juice producing the best results. They also showed that apple juice supported growth and this was confirmed by the present experiment.

#### 3.3.4 Conclusions

The experiment showed that *L. oenos* could be grown on AJ broth instead of TJ broth without a large decrease in specific growth rate or bioconversion activity. Strain 1070 gave the highest total and specific malolactic activity of the three strains tested. Addition of 6% v/v ethanol to the cultures caused a longer lag phase for growth and either no effect on, or increased, bioconversion activity. Apple juice was the preferred medium for the experiments because of ease of use, availability and repeatable composition. For all further work, strain 1070 was used and AJ broth, either with or without 6% v/v ethanol, was used as the growth medium.

### 3.4 Comparison of Immobilization Media

#### 3.4.1 Objectives

Two supports were tested to identify a suitable adhesion support matrix for the cells. Bonechar has been extensively used by Massey University researchers for the acetone/butanol/ethanol fermentation and is easily and cheaply obtained (Qureshi and Maddox, 1990). Oak chips were of particular interest as Chardonnay wine is normally held in oak barrels for several months as part of the ageing process and thus any oak flavours leached out of the chips should be a positive rather than negative factor.

#### 3.4.2 Experimental Conditions

A control experiment was conducted to determine whether oak chips removed malic acid from the growth media. A 1 litre Erlenmeyer flask was filled with 900 ml of AJ broth (pH 3.6) and 20 g of autoclaved oak chips. After taking an initial sample of the broth, the chips were added, the flask was incubated at 30°C on a rotary shaker for three days and then another broth sample was taken.

For the comparison of bonechar and oak chips under continuous conditions, four bioreactor columns were sterilized and two were filled with 75g (100ml) of autoclaved bonechar and two with 20g (100ml) of autoclaved French oak chips. Initial biomass growth within the bioreactors was as given in Table 2.

The AJ wine composition included 4.5 g/l L-malic acid with a final pH of 3.45 after autoclaving and alcohol addition. The bioreactors were held at 21°C and the dilution rate was 0.53h<sup>-1</sup> for the bonechar and 0.46h<sup>-1</sup> for the oak chips.

### 3.4.3 Results and Discussion

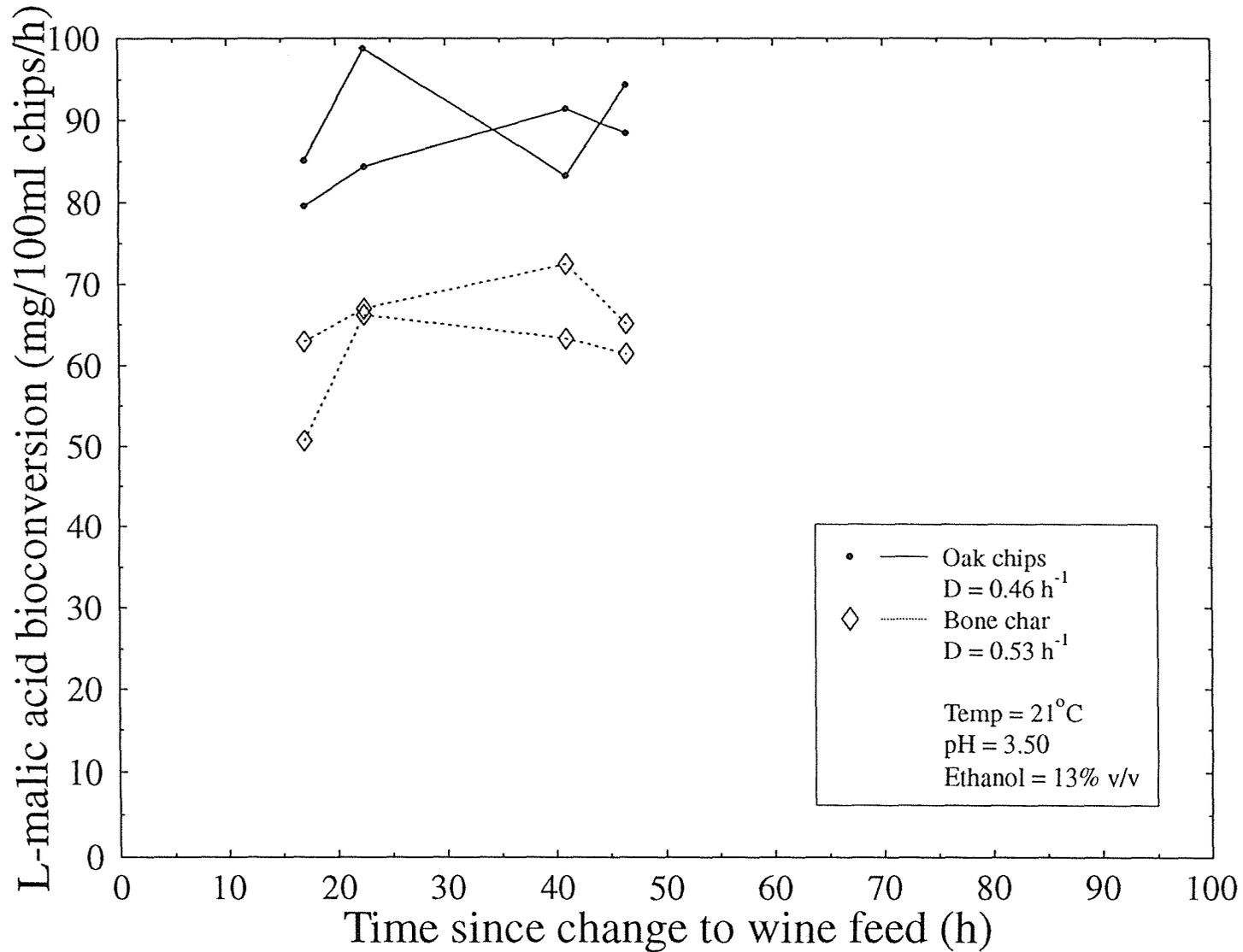
The control experiment showed no malic acid was removed by the chips over three days. Results for the comparison between bonechar and oakchips are shown in Figure 7. At the end of the experiment all four bioreactors were found to be contaminated with a budding yeast which was subsequently isolated and shown using a batch trial to not degrade malic acid. Tentative identification based on spore formation was *Torulopsis*. Any effect this yeast had would have been consistent for both media and so the contamination was not considered to have affected the experiment.

Both immobilization media retained cells above the washout rate for *L. oenos* but the oak chips gave an increased removal rate compared with the bonechar. This is likely to be due to the increased surface area of the chips providing more space to adsorb or trap cells.

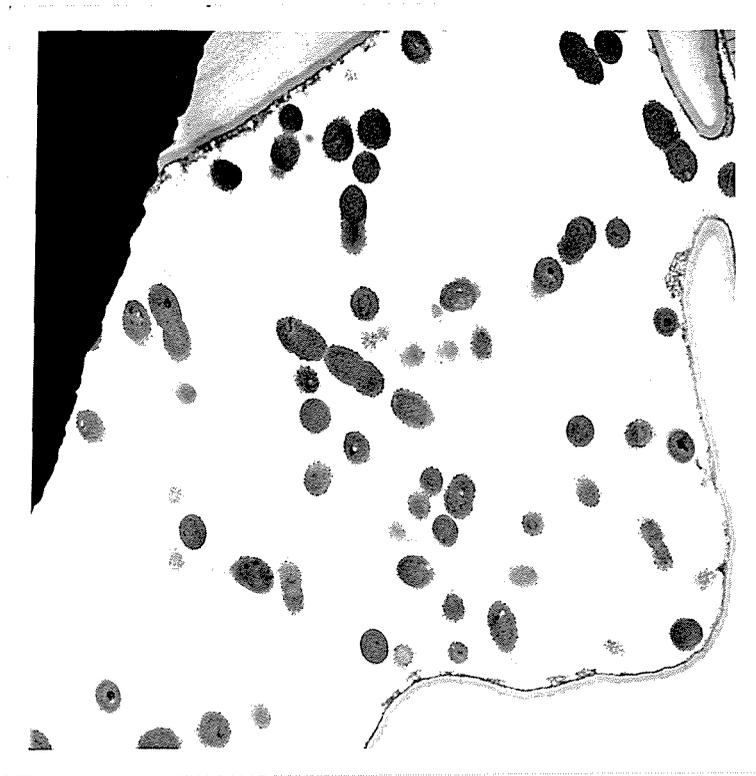
The bonechar bioreactors showed crystallization of tartrate after 41 hours of operation, and consistent decolorization of the AJ wine feed occurred. The tartrate crystallization was probably caused by small particles of bonechar forming crystal nuclei. Wine is normally cold stabilised to remove tartrate crystals just before bottling (Rankine, 1989) and so this crystallization is not necessarily an undesirable effect.

Scanning (SEM) and transmission (TEM) electron micrographs gave no conclusive evidence of the method of cell retention on the oak chips. Figure 8 shows a TEM of cells in a wood chip pore, and no linking material is visible between the chip and the cells. The pore was open ended and so the cells may be simply forced into the space by liquid flow. SEM, as in Figure 9, showed cells, often in pairs, seemingly attached to the surface of the chip, and also a white globular material. The latter may have been either a polysaccharide that was associated with the cells but had been affected by the EM fixing method, or possibly it was a product of the chips themselves. No control TEM of wood chips without cells was done to resolve this question. The cells, therefore, seem to be trapped in wood pores, adsorbed to the outside of the chips and possibly trapped by an extracellular polysaccharide.

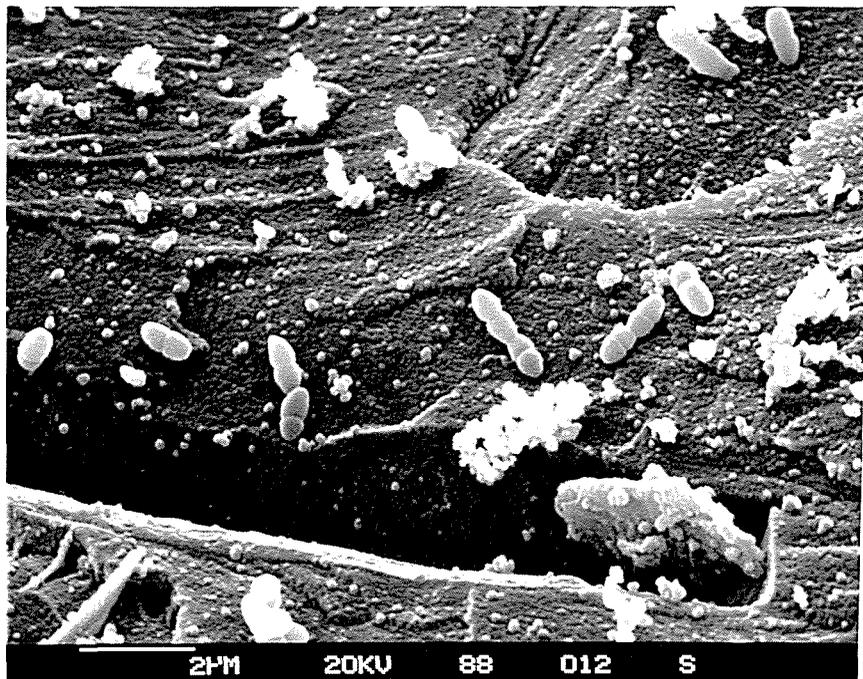
**Figure 7:** Comparison of immobilization media



**Figure 8:** Transmission electron micrograph of oak chip (11,200x)



**Figure 9:** Scanning electron micrograph of oak chip cross-section (7600x)



### 3.4.4 Conclusion

The decolorization was considered to be incompatible with winemaking practice and for this reason the bonechar was not used for further experiments. All subsequent work used oak chips as the immobilization medium as cells were retained above the washout rate for the bacteria, a higher removal rate was obtained compared with bonechar and no negative effects were noted.

## 3.5 Comparison of Real Wine and Synthetic Wine

### 3.5.1 Objectives

Two synthetic wines were used in comparison with a genuine Chardonnay to test whether a synthetic wine could be used for the experiments. Using real wine for the experiments posed several problems:

- availability: the requirement for un sulphited wine which has not undergone MLF is fulfilled only once during the vintage. This is in late April/early May. The large volume required was difficult to acquire, transport and store.
- variability: wine is a very complex liquid. Every vintage produces a different product as does every winery. The levels of all the constituents can vary greatly from wine to wine and thus any results could not be standardised over time.
- bacteriophage: this problem could be removed by autoclaving provided the alcohol were replaced. However, wines produced from heat-treated juice have been found to be less suitable for bacterial growth than those without

heat treatment (Henick-Kling *et al.*, 1989). This heat treatment effect may also apply to synthetic wine.

Apple juice wine was selected as the compromise between real wine and a truly synthetic wine as juice would still be variable, though not to the same extent as real wine since no yeast or fermentation factors are involved. In addition a synthetic wine using a Yeast Nitrogen Base (Difco) was evaluated as a completely synthetic medium without the presence of any juice product.

An average German white wine composition (Deutsche Forschungsanstalt für Lebensmittelchemie, 1981) was used as the standard to calculate ingredients in the synthetic wines. An average apple juice composition table (Deutsche Forschungsanstalt für Lebensmittelchemie, 1981) was used to define the apple juice. The main difference between real wine and AJ wine is the higher level of sugars in the latter.

### 3.5.2 Experimental Conditions

The true wine was a Montana Marlborough Chardonnay 1990 that had just finished yeast fermentation and had not been inoculated for malolactic fermentation nor had any sulphur dioxide been added after the yeast fermentation. The wine was centrifuged to remove the majority of the lees and stored at 4°C for 4 weeks prior to being used. Alcohol content was 12.7% v/v, Brix -1.3°, total SO<sub>2</sub> 67ppm with no free SO<sub>2</sub>, total acidity 9.9 g/l, volatile acidity 0.66 g/l (composition determined by Montana Wines Ltd.), malic acid 5.25 g/l and pH 3.42.

Apple juice wine was as given in the Methods and Materials except that the malic acid added was 1.5g/l and the ethanol concentration was 12.7% v/v. Total L-malic acid concentration was 2.0g/l and the pH was 3.63. YNB wine was as given in Materials and Methods.

Growth of the organism in the bioreactors was as given in Table 2. True wine, AJ wine and YNB wine were then each fed into the bioreactors at a dilution rate of  $0.46\text{h}^{-1}$ . The pH, total reducing sugars and malic acid concentration in the bioreactor effluents were monitored.

After 55 hours of operation, the feed streams for the AJ wine and YNB wine were exchanged. At 120 hours, 2 g/l glucose was added to the YNB bioreactor feed in an attempt to revive the cells.

At 122 hours the bioreactor feeds were turned off for 16.5 hours. After the feeds were turned back on the bioreactors were not sampled for 22 hours. This adjustment time was to allow a return to steady state.

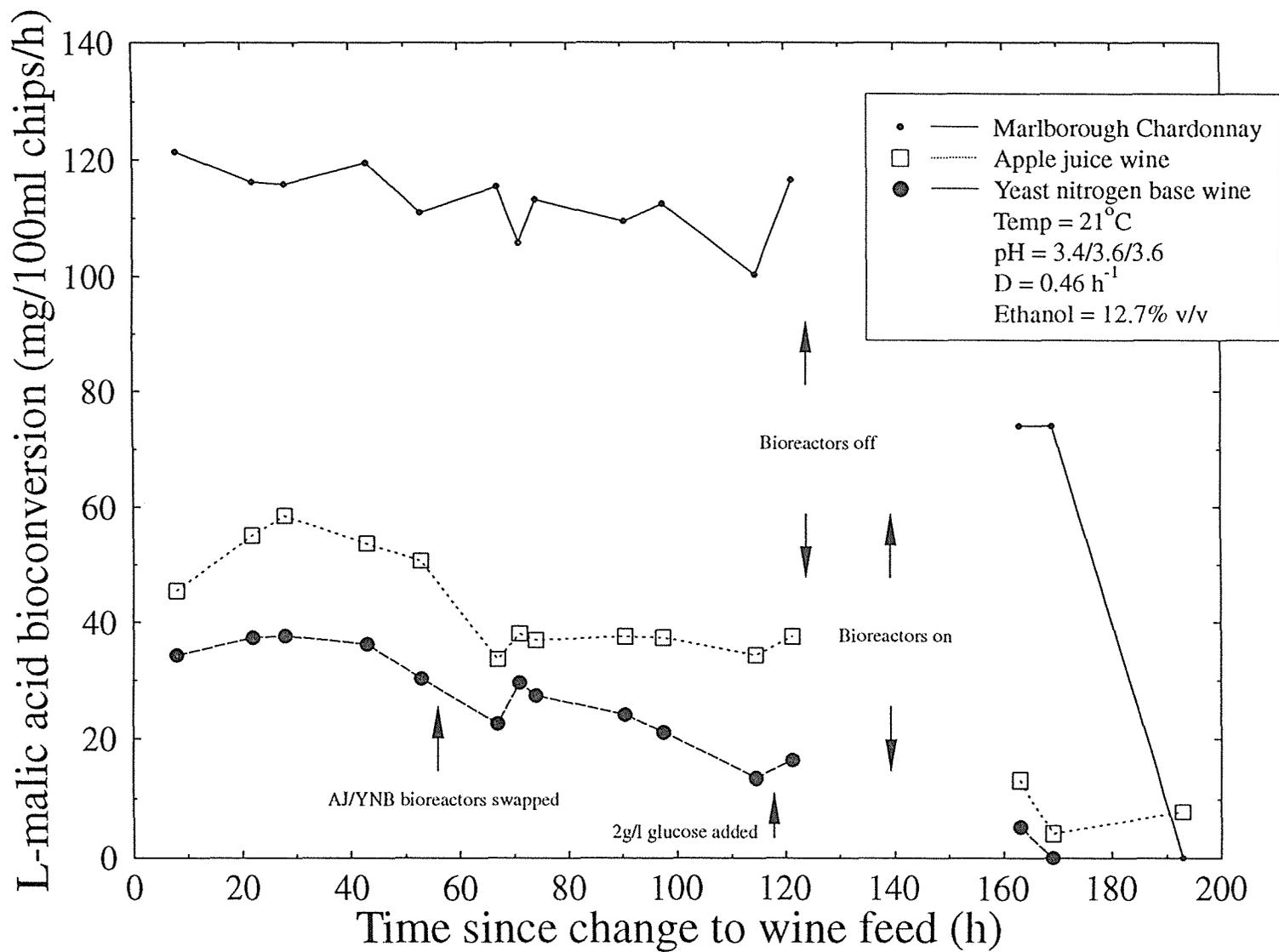
### 3.5.3 Results and Discussion

Phase contrast microscopy showed pure cultures of chain-forming, lenticular rods (consistent with *L. oenos*) in both the growth and wine feed phases but no bacterial growth was visible on the chips at any stage of the experiment. Reducing sugar analysis using the DNS assay showed no utilisation of sugars by the bacteria over time. The malic acid bioconversion rates of the bioreactors are shown in Figure 10.

There was a difference in pH between the synthetic and real wines but later work showed that between pH 3.4 and 3.6 there should be no difference in the effect of this on the bioreactors.

The reactor being fed with YNB wine showed steady loss of bioconversion ability even with the sugar added, whereas the reactors being fed with wine and AJ wine stabilised after about 60 hours and consistent malic acid bioconversion was maintained for 50 hours. However, both these bioreactors rapidly lost bioconversion ability after the feed had been turned off for 16.5 hours, and did not regain it though the AJ wine still showed some residual activity after 170 hours.

**Figure 10:** Comparison of real and synthetic wines



The lower bioconversion values of the AJ wine compared with the real wine may be a combination of several causes including:

- yeast factors in the Chardonnay
- heat treatment (autoclaving)
- higher malic acid concentration in the Chardonnay

Some evidence is provided by later experiments to show that the last reason may have been the cause although the heat treatment effect and yeast factors were not investigated. The ultimate aim of the experiments was to produce an industrial bioreactor, and so provided that the real wine performs with a consistent difference to the AJ wine, the bioconversion parameters for a prototype reactor can still be estimated, although the design will be conservative.

The DNS assay showed that the extra sugars in the AJ wine were not utilised and are therefore unlikely to be a problem in the AJ wine composition.

The Chardonnay contained 65ppm bound SO<sub>2</sub> (no free SO<sub>2</sub>) and bioconversion still occurred at a much higher rate than in the AJ wine. Aldehydes strongly bind SO<sub>2</sub> and are prevalent in wine because of the yeast fermentation. This may be the reason for the ability of the bacteria to have high bioconversion rates under apparently adverse conditions.

#### 3.5.4 Conclusions

The results for the YNB wine confirm the need for a juice product in the medium, as the bioreactor did convert malic acid but at a low, and continually declining, bioconversion rate.

With the possible exception of SO<sub>2</sub> addition, the AJ wine was a satisfactory alternative for Chardonnay wine although the real wine had a higher bioconversion rate.

### 3.6 Overall Conclusions

Results from the preliminary screening experiments show that *L. oenos* strain 1070 gave the highest total and specific activity and this strain was therefore chosen for further experiments. Results also showed that D-malic acid had no effect on the fermentation, apple juice could be used in preference to tomato juice in growth studies and addition of 6% v/v ethanol to the growth medium, while giving a longer lag phase for growth, gave either a zero or positive influence on bioconversion. Apple juice wine was a satisfactory alternative to genuine Chardonnay for bioconversion trials and oak chips were a preferable immobilization medium compared to bonechar.

## 4.0 BIOCONVERSION PARAMETERS

### 4.1 Activity of Free Cells in Batch Culture using AJ Wine

#### 4.1.1 Objective

It was of interest to know the bioconversion activity of free cells in batch culture using AJ wine as the assay medium. This gives a comparative figure for later work with the immobilized cells under continuous conditions and also with the previously published data (which generally had different assay conditions).

#### 4.1.2 Experimental Conditions

*L. oenos* 1070 was grown in 4 litres of AJ broth (10% v/v ethanol) at 21°C using a 7 litre Microferm (New Brunswick) fermenter with no stirring. When the absorbance was 0.575 (late log phase), the culture was centrifuged aseptically at 13,700g in 1 litre quantities using a Sorvall RC5C centrifuge. Cells were then washed in sterile water and recentrifuged. Pellets from 2 x 1 litre volumes were used for biomass measurements while pellets from the other 2 x 1 litre volumes were placed in 2 x 1 litre of AJ wine at 15°C, pH 3.45, 3.95 g/l malic acid and 10% v/v ethanol. These flasks were swirled every hour to keep cells in suspension and malic acid bioconversion was monitored.

#### 4.1.3 Results and Discussion

The data for malic acid bioconversion, when analyzed using linear regression, showed the activity of the cells was 850 mg malic acid degraded/g biomass/h (17 mg malic

acid/100ml culture/h) with a correlation coefficient of -0.89. This was higher than any of the figures in the existing literature which had alcohol in the assay medium but lower than all the other figures (See Table 1). Variation between strains may account for this increased activity or more likely the growth in alcohol adapted the bacteria to this environment. None of the bioconversion studies in the literature mentioned culturing the bacteria in the presence of alcohol.

The batch bioconversion in AJ wine gave 30% higher rates than the activity assays of strain 1070 using the citrate medium (Section 2.4.4), despite more stressed conditions, i.e. lower pH, more ethanol and lower temperature. Other than experimental error due to not enough points in the data (Section 3.3.3) and medium differences, this may be due to the difference between log and stationary phase cells. Naouri *et al.* (1989) showed that a 55-70% drop in specific activity, and a 10-40% drop in total activity, occurred between mid- and late-log phase cells. The pH/ethanol/temperature interaction may also have a stimulating effect on the bioconversion at the particular levels of each factor used in the experiments.

## 4.2 Comparison of Chardonnay and AJ wines, and effect of recycling feed

### 4.2.1 Objectives

The previous experiment with a real wine (Section 3.5) showed a very high bioconversion activity for the bioreactor treating the Chardonnay compared with the bioreactor treating the AJ wine. Hence a repeat experiment was conducted. The wine was as previously described but stored for 10 months at 4°C. Recycling of the effluent through the bioreactor to further reduce the malic acid concentration was also studied to determine whether the malic acid concentration affects the bioconversion.

### 4.2.2 Experimental Conditions

Bioreactors were prepared as described in Table 2. AJ wine was initially fed into the four bioreactors under conditions of pH 3.40,  $D = 0.25 \text{ h}^{-1}$ , 5.06 g/l L-malic acid and temp. = 21°C. After 97 hours two bioreactors were changed to Chardonnay wine feed to check for any stimulation of the bioreactor activity by the real wine. At the same time as the switch to Chardonnay, the other two bioreactors were fed with their effluents which had been previously collected. To prevent MLF occurring, these effluents had been chilled to 4°C immediately on leaving the bioreactors and were kept at this temperature when being used as a feed source. No attempt was made to filter the liquid before recycling. One recycle bioreactor had a tubing fault and the results have been discarded.

### 4.2.3 Results

The results, shown in Figure 11, demonstrate that AJ wine can be deacidified at a similar rate to real wine. The higher bioconversion rate in the previous experiment may have been due to the increased malic acid concentration in the Chardonnay

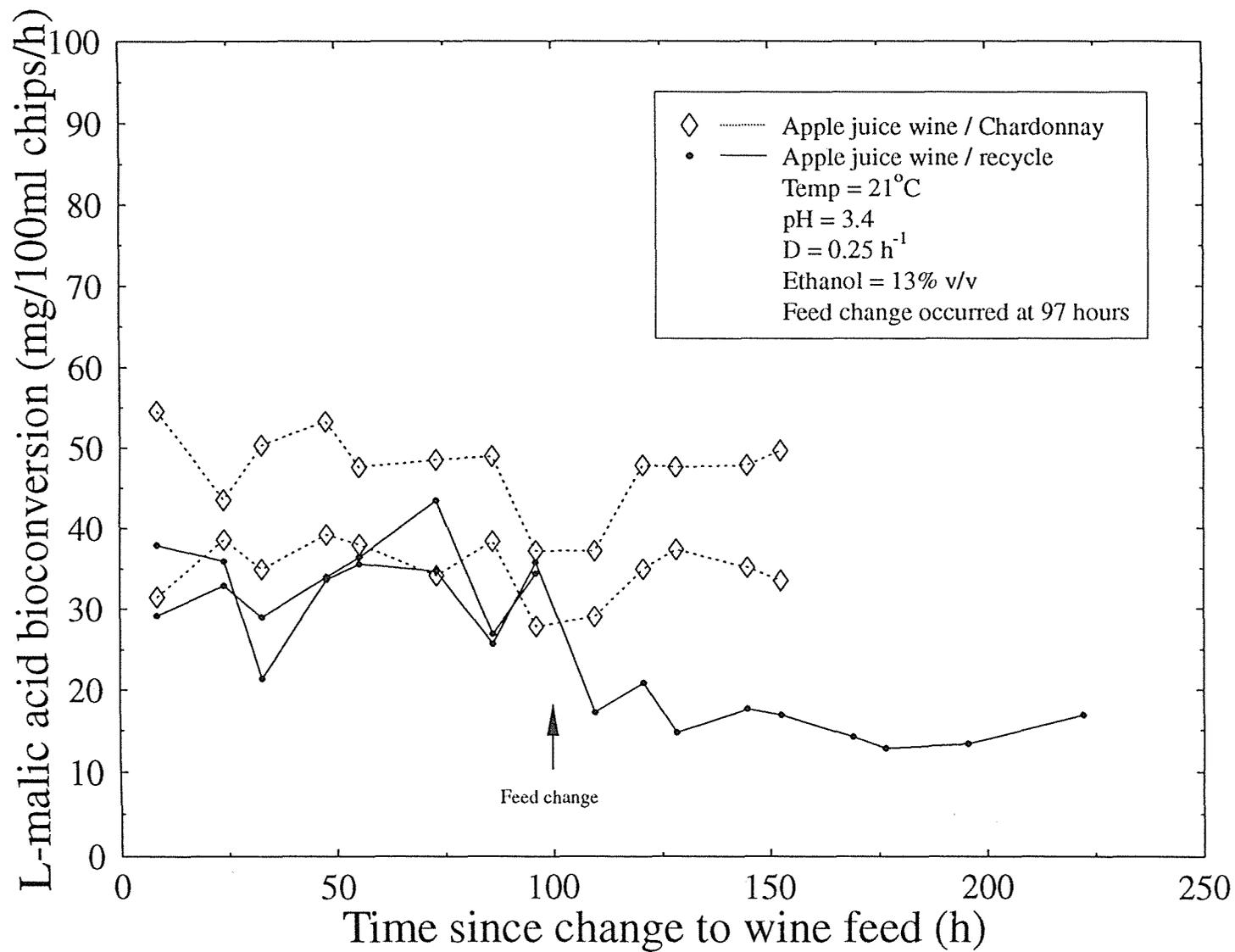
compared with the AJ wine, greater biomass in the reactor or the age effect, as some reduction in activity when using AJ wine was observed after 90 hours in the initial experiment (Section 3.5) and also in later experiments (Section 4.4).

This experiment gives an indication of variance between the bioreactors as four replicates exist until 96 hours. A one-way analysis of variance showed that, at  $P=0.05$ , the steady-state mean malic acid utilization rates of the four bioreactors between 24 and 96 hours were not the same. The graph shows that while three of the bioreactors are similar, the bioconversion in the fourth is consistently about 10 mg malic acid/100ml chips/h higher. A bacterial count of the bioreactors would probably have resolved this problem as the activity would be per g biomass (i.e. specific) rather than per volume (i.e. total), and could thus be directly compared. Therefore, in any comparison between bioreactors, caution must be exercised as a "rogue" bioreactor would negate any effects of the parameters under study. However the possible existence of rogue bioreactors is ignored for most of the runs where results appeared reasonable, else all comparisons would have no significance.

The activity of the bioreactors was reduced with the use of the recycle feed. This is further evidence that malic acid concentrations matter, although the difference between the feed levels was only 1.72 g/l (initial feed = 5.06 *cf.* recycle = 3.34). Other factors possibly affecting the bioreactors at this stage would be reactor age/run time, and bacteriophage as the recycle feed was not stored in a sterile container.

**Figure 11:** Comparison of Chardonnay and AJ wines, and effect of recycling feed

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### 4.3 Comparison of Non-anaerobic and Anaerobic Conditions

#### 4.3.1 Objectives

Growth of *L. oenos* is known to be stimulated by anaerobic conditions. In preparing the media some aeration occurred during addition of alcohol and while changing feed flasks. Hence this experiment was performed to test whether this aeration affected the performance of the bioreactors.

#### 4.3.2 Experimental Conditions

All feed media for the "anaerobic" bioreactors (including growth media) were given a carbon dioxide headspace after autoclaving by bubbling the gas through the media for 20 minutes (this had no effect on the pH). Deaerated alcohol was then added. Before inoculation with the chips and organism, all the pipework and bioreactors were filled with nitrogen gas. Bioreactor growth conditions were as defined in Table 2.

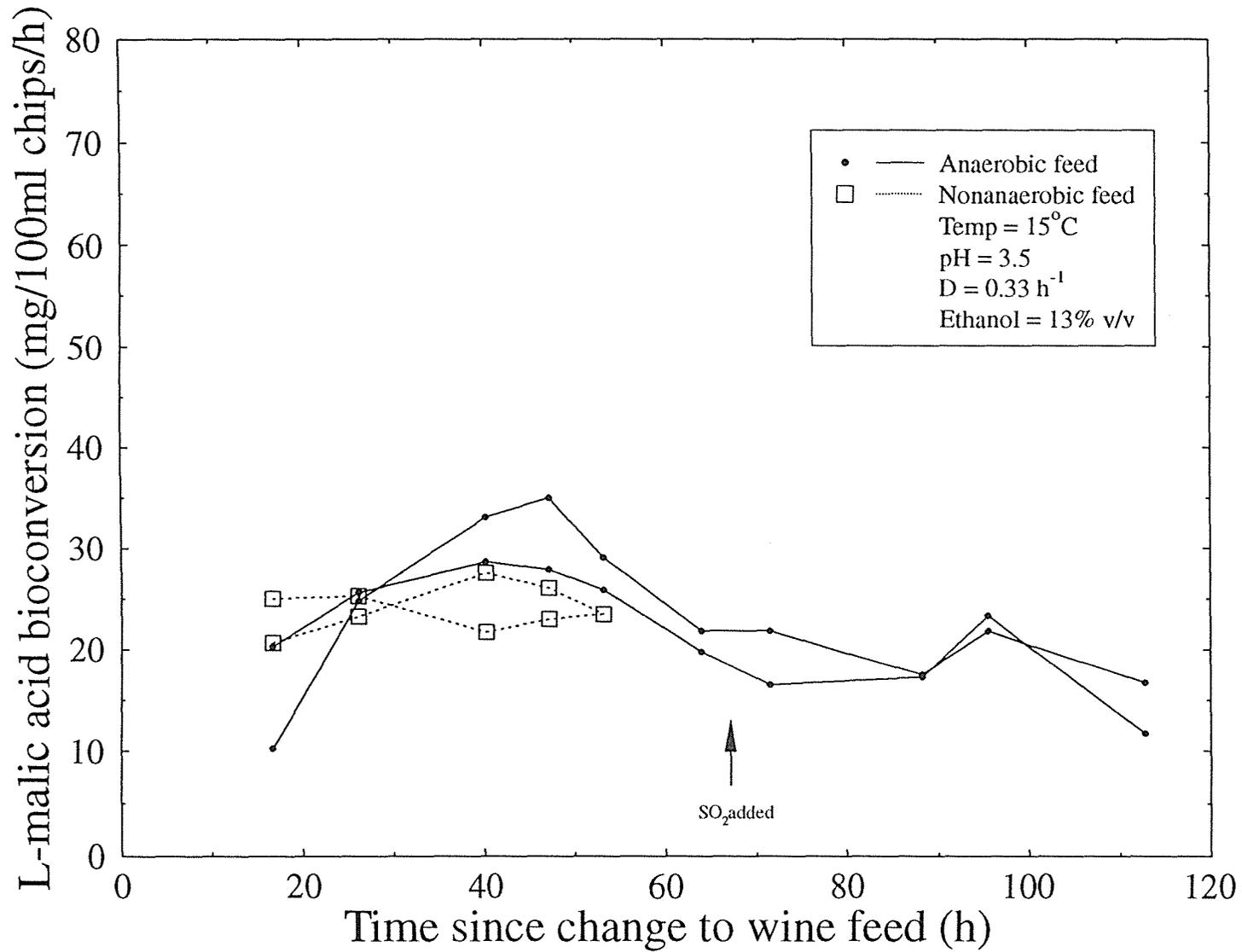
The AJ wine feeds were pumped into the bioreactors at pH 3.45, 15°C, 4.00 g/l L-malic acid and  $D = 0.33 \text{ h}^{-1}$ . At 65 hours, 6ppm  $\text{SO}_2$  (bound), in the form of sodium metabisulphite, was added to the wine feed to test for any change in activity in the presence of this antimicrobial antioxidant.

#### 4.3.3 Results

The results of the experiment are given in Figure 12, and show that bioconversion rates for all the bioreactors were low, most likely due to the shortened growth conditions providing fewer cells. Addition of the sulphur dioxide did not cause a reduction in the bioconversion activity. While there seems to be a higher activity with the anaerobic bioreactors, because of the low overall bioconversion rates little significance can be given to the difference.

**Figure 12:** Comparison of non-anaerobic and anaerobic conditions

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#### 4.4. Effects of Temperature and pH

##### 4.4.1 Objectives

Possibly the two most important factors affecting the bioconversion performance of a bioreactor used for MLF will be temperature and pH. Temperature can often be controlled to some extent although a figure below 20°C would be normal winemaking practice. The pH varies with the season as the malic acid concentration is determined by the amount of sunlight received by the grapes.

To study the effect on bioconversion, three bioreactors were used, each at a different pH value, and the temperature was varied. A fourth bioreactor maintained a centre-point pH and temperature, and was used to monitor the effect of time. This was a sequential experiment and the last bioreactor was necessary so that the variation in bioconversion over time could be considered when comparing activity at the different temperatures.

##### 4.4.2 Experimental Conditions

The columns were filled with 20g of autoclaved oak chips and initial growth was as defined in Table 2. For the three varying bioreactors, AJ wine at three different pH values (3.15, 3.45, 3.75) was used and the temperature was held at 27°C for 72 hours. After this the temperature was kept at 21°C until 142 hours when it was reduced to 15°C between 142 and 270 hours, and then further decreased to 9°C between 270 and 334 hours. For the control bioreactor the wine feed was kept at pH 3.45 and 21°C for the entire experiment. Dilution rate for all bioreactors was  $0.46\text{h}^{-1}$  and the malic acid concentration was 3.97g/l.

#### 4.4.3 Results

Phase contrast microscopy of effluents showed yeast contamination of the control bioreactor after 223 hours. However, cell numbers were not high as no growth was visible to the naked eye, and no excess gas production occurred.

##### 4.4.3.1 pH

The results in Figure 13 show that for all temperatures there was negligible difference in bioreactor performance between pH 3.45 and pH 3.75. The bioconversion may thus be compared with the literature pH profiles for growth as this pH range is at the optimum pH for growth. The interaction of high temperature (27°C) and low pH caused the bioreactor operated at pH 3.15 to have a lower, and finally non-functioning, bioconversion activity. This effect also corresponds with results for growth at these values (Wibowo *et al.*, 1985) and shows the need to consider interactions as well as individual parameters.

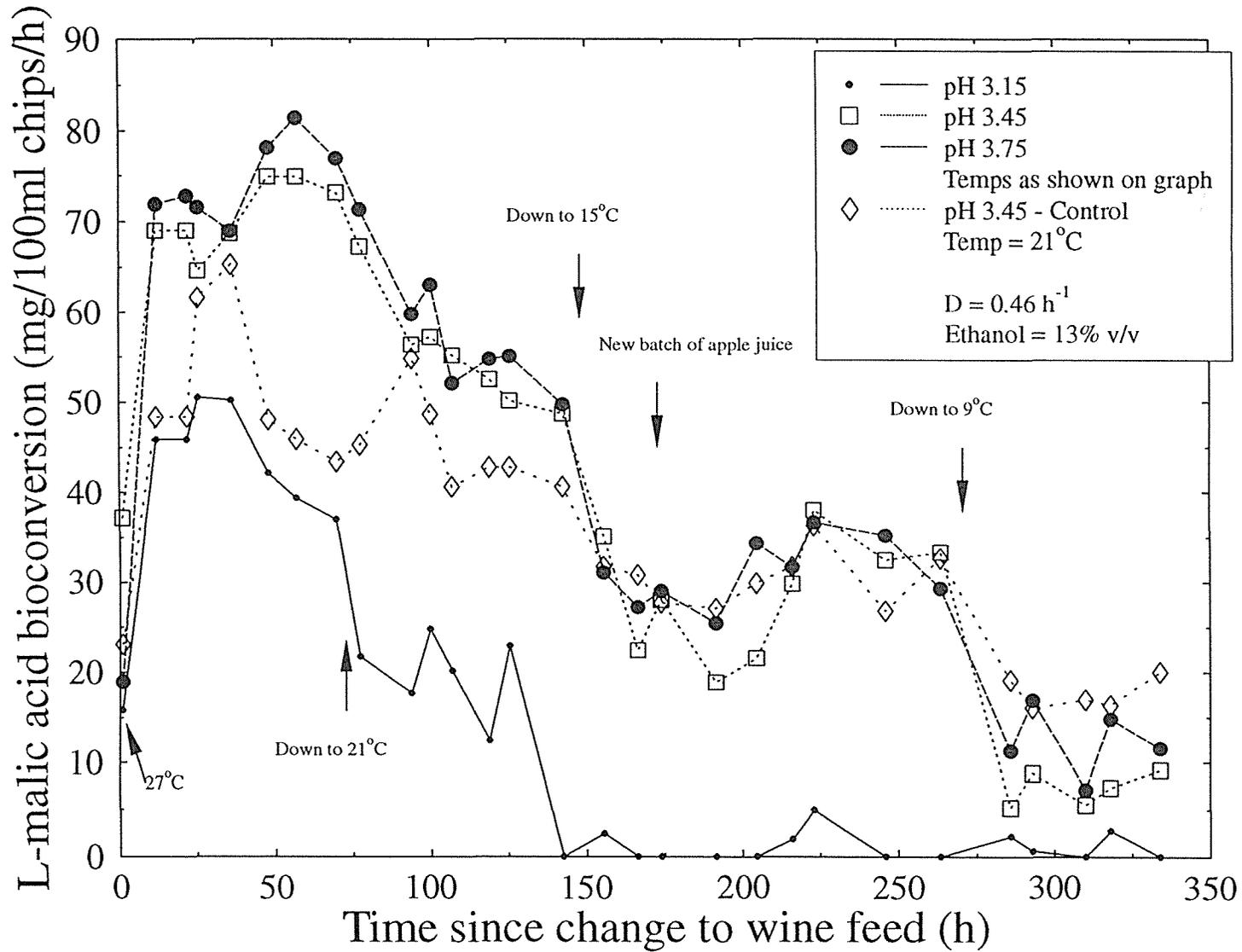
##### 4.4.3.2 Temperature

The higher temperature (27°C) seems to have increased the biomass and/or cell productivity at the higher pH values, because when reduced to 21°C, the bioreactors displayed a higher activity than the control bioreactor which had exactly the same conditions at this stage.

When time is accounted for by using the control bioreactor, the reduction in activity due to the temperature decrease is twice as much between 27°C and 21°C as it is between the next two temperature decreases. That is, there is a sharp drop and then a consistent diminishing of the bioconversion as the temperature is reduced.

**Figure 13:** Effect of temperature and pH on bioconversion

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#### 4.4.3.3 Time

The activity over time reduced to 80% of the initial bioconversion value up till 140 hours of operation and then a further, more rapid decline to about 60% of the initial value was noted. After 280 hours a decline to 40% of the initial figure occurred.

#### 4.4.4 Conclusions

Bioconversion activity at pH 3.15 was lower than at pH 3.45 and 3.75. A high temperature (27°C) combined with a low pH (3.15) wine feed caused loss of activity in the bioreactor while this high temperature gave increased activity at higher pH values. A decline of up to 60% of the activity occurs after 280 hours of operation.

### 4.5 Effects of pH and ethanol

#### 4.5.1 Objectives

All the experiments had presumed a Chardonnay-style wine with a high alcohol content as a worst-case scenario. This experiment was used to determine whether a lower alcohol concentration would enhance the bioconversion rate. The comparison was combined with a pH difference of pH 3.15 and pH 3.45 to clarify whether bioconversion would be stable at pH 3.15 if a lower temperature (15°C) were used and also to examine the pH/ethanol interaction. Sulphur dioxide was also added at the end of the experiment to a higher concentration than previously used to test whether the bioconversion would be affected.

#### 4.5.2 Experimental Conditions

Four bioreactor columns were sterilized and filled with 20g of autoclaved French oak chips and initial growth was as defined in Table 2.

AJ wine was then fed into the bioreactors at a dilution rate of  $0.31 \text{ h}^{-1}$  and the temperature was held at  $15^{\circ}\text{C}$ . The AJ wine feed contained  $4.05\text{g/l}$  L-malic acid, while the ethanol concentration and pH in each bioreactor were as shown in Table 4. Sulphur dioxide was added into the wine feed to a level of 9ppm (bound) after 99 hours of reactor operation.

#### 4.5.3 Results

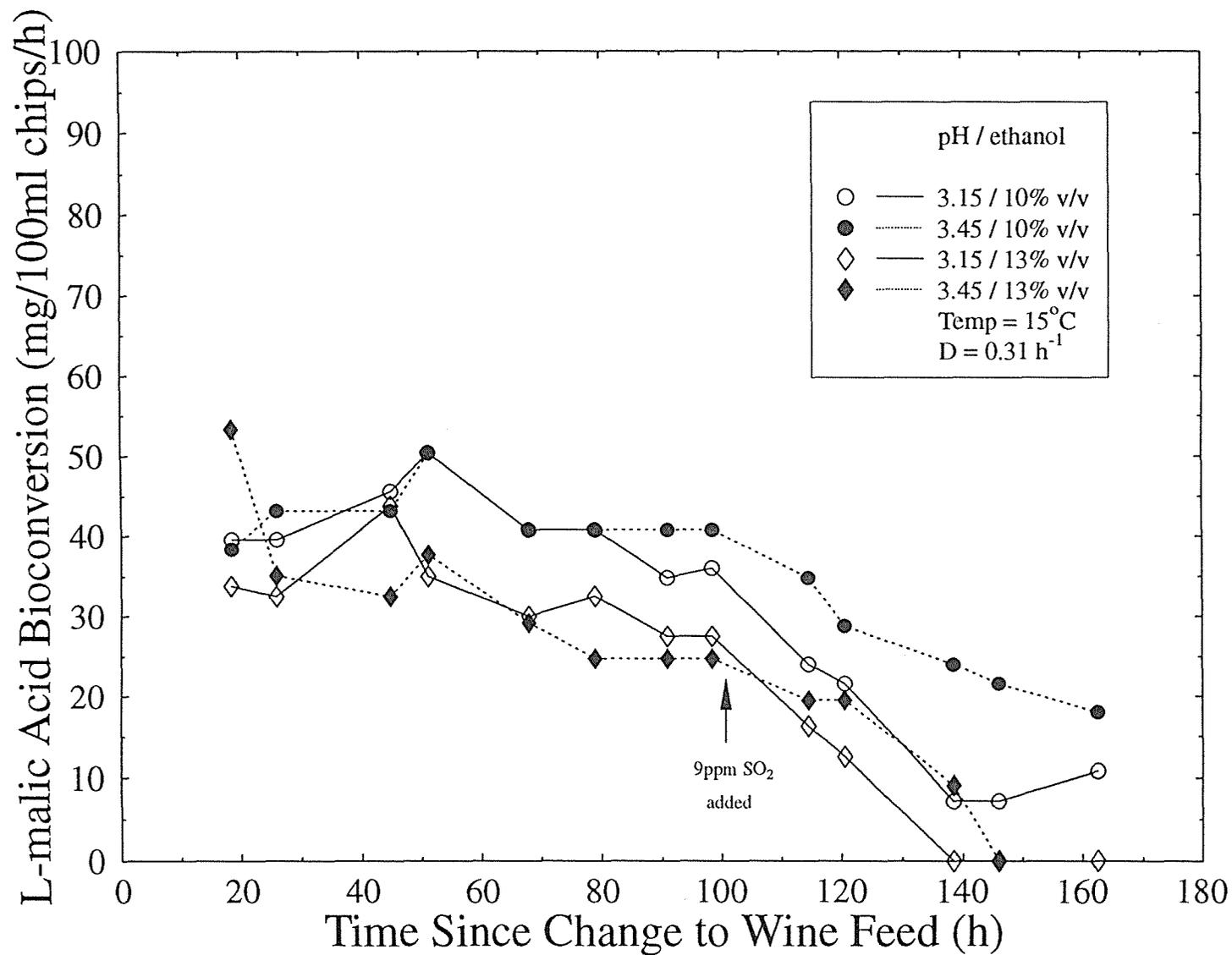
Bioconversion activities of the bioreactors are shown in Figure 14 and the steady state activity from 26 to 98.5 hours, together with the wine feed parameters, in Table 4.

**Table 4:** Bioconversion activity at different pH and ethanol concentrations

pH	Ethanol Concentration	Bioconversion rate (mg malic acid/ 100ml chips/h)
3.14	10% v/v	43
3.53	10% v/v	44
3.14	13% v/v	35
3.53	13% v/v	32

The lower ethanol concentration allowed a higher bioconversion rate at both pH values. Since all the bioreactors experienced the same growth conditions, i.e. were adapted before the wine feed to AJ broth containing 13% v/v ethanol, an increased number of cells in the growth phase was not the reason for the increased bioconversion.

**Figure 14:** Effect of pH and ethanol on bioconversion



Results from this experiment also show that the bioreactors can be operated at pH 3.15 and a steady state was achieved until 80 hours. This was in contrast to the pH/temperature experiment (Figure 13) where the bioreactor at pH 3.15 showed a steady decline after being operated at 27°C until 72 hours. This confirmed previous research (Wibowo *et al.*, 1985) which showed that bacterial growth at high temperature and ethanol concentration combined with low pH led to death of the organism, i.e. pH has minimal effect on bioconversion unless the temperature is above 15°C.

The addition of SO<sub>2</sub> caused a decrease in bioconversion and this was more noticeable at the lower pH value and higher ethanol concentrations. At 160 hours the bioreactors with 10% v/v ethanol were still functioning and possibly achieving a steady state while the combination of SO<sub>2</sub> and 13% v/v ethanol had stopped all bioconversion in the other two bioreactors.

#### 4.6 Effect of malic acid concentration and wine flowrate: Experiment 1

##### 4.6.1 Objectives

An experiment was conducted to compare the relative effects of the malic acid concentration in the feed wine and the effect of an increase in flowrate (dilution rate). Different malic acid concentrations are thought to affect growth of *L. oenos* (McCord and Ryu, 1985) and the real/synthetic wine and recycle experiments (Sections 3.5 and 4.2) indicated that bioconversion might be similarly affected. Increasing flowrates would have the effect, assuming plug flow, of increasing the malic acid concentration as well as providing increased physical shear to remove organisms. Plug flow was assumed from visual observation of the horizontal line of colour change between growth and bioconversion media in the reactor headspace.

#### 4.6.2 Experimental Conditions

Four bioreactor columns were sterilized and filled with 20g of autoclaved French oak chips and initial growth was as defined in Table 2.

Apple juice wine was fed into the bioreactors at a dilution rate of  $0.29 \text{ h}^{-1}$  and the temperature was held at  $15^{\circ}\text{C}$ . Flowrate was increased twice after steady states had been reached. Malic acid concentrations in the feed and the time of flowrate increases were as shown in Table 5 below.

**Table 5:** Wine feed conditions: Malic/flowrate comparison 1

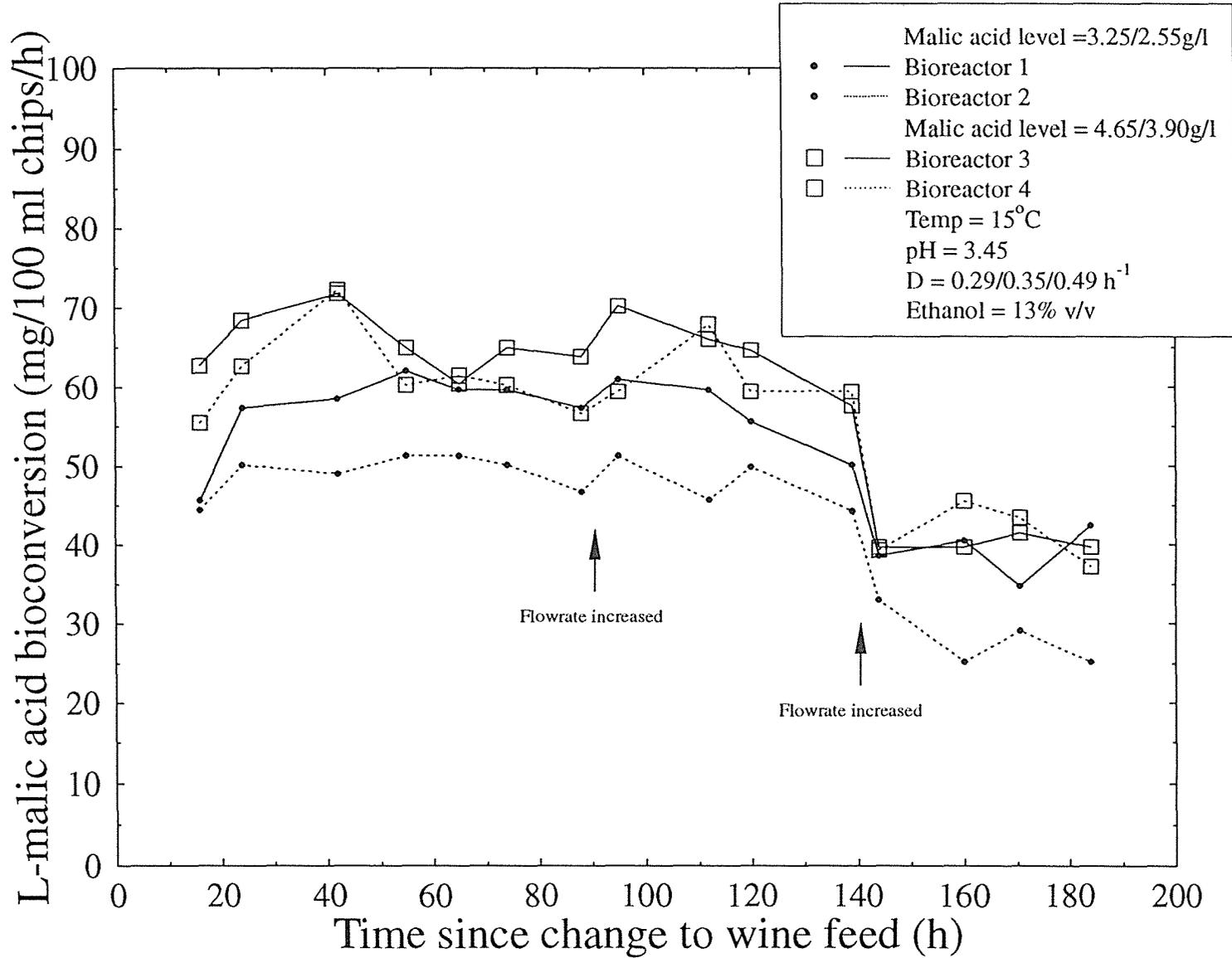
	<b>Bioreactors 1 and 2</b>	<b>Bioreactors 3 and 4</b>
<b>Malic acid</b>		
0-139 h	3.25g/l	4.65g/l
139-184 h	2.55g/l	3.90g/l
<b>Dilution rate</b>		
0-88.5 h	$0.29 \text{ h}^{-1}$	
88.5-139 h	$0.35 \text{ h}^{-1}$	
139-184 h	$0.49 \text{ h}^{-1}$	

#### 4.6.3 Results

Malic acid bioconversion rates over time for each bioreactor are given in Figure 15. All the bioreactors achieved steady state for 63 hours at  $D = 0.29 \text{ h}^{-1}$  (18 retention times) and there was no activity change when the flowrate was increased to  $D = 0.35 \text{ h}^{-1}$  for the next 51 hours (18 retention times). Any effect of increased malic acid contact by the cells, caused by the flow increase was either negligible or offset by washoff of cells. The increase to  $D = 0.49 \text{ h}^{-1}$ , however, caused a 30% drop in the

**Figure 15:** Effect of malic acid concentration and flowrate on bioconversion: Experiment 1

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rate, though a steady state was achieved at this lower rate. This second increase may have caused a physical washing off of the cells from the chips rather than a growth rate change as the flowrate change was from  $10 \times \mu_{\max}$  to  $15 \times \mu_{\max}$ , i.e. both figures well above the washout rate.

Two sets of duplicates exist in this experiment but the two malic acid concentrations were not as different as desired. The results therefore may be interpreted as two sets of duplicates or possibly four replicates. Only one of the lower malic acid concentration bioreactors gave lower values for bioconversion during the first 139h, and it gave a similar response to the other three after the step-down in malic acid concentration. Given the observed variation in the Chardonnay/recycle experiment (Section 4.2), the amount of variation is probably not due to malic acid concentrations as much as the amount of cells in the bioreactor, i.e. this experiment may include a "rogue" bioreactor, and so a comparison between bioreactors has not been done.

Plate counts were carried out at the end of the experiment and the results are given in Table 6. For bioreactor 2, which was the possible "rogue" bioreactor, the methodology was different as the wine was drained out and the chips were blended in sterile water.

**Table 6:** Relationship between cell counts and bioconversion rates

Bioreactor number	Bioconversion rate between 144-184 h (mg malic acid/100ml chips/h)	Cell numbers (per ml)
1	40	$3 \times 10^7$
2	28	$6 \times 10^7$
3	41	$8 \times 10^7$
4	41	$10 \times 10^7$

Assuming that the cell numbers for bioreactor 2 would be even greater if the bacteria in the wine had been included in the count as with the other bioreactors, the results do not correlate to the bioconversion activity. Bioreactor 2 shows the lowest activity, but not the lowest number of cells, although the error in the cell count may be quite large as duplicate dilution series were not done.

#### 4.7 Effects of malic acid concentration and wine flowrate: Experiment 2

##### 4.7.1 Objective

A repeat experiment was set up to investigate a wider range of malic acid concentrations and flowrates.

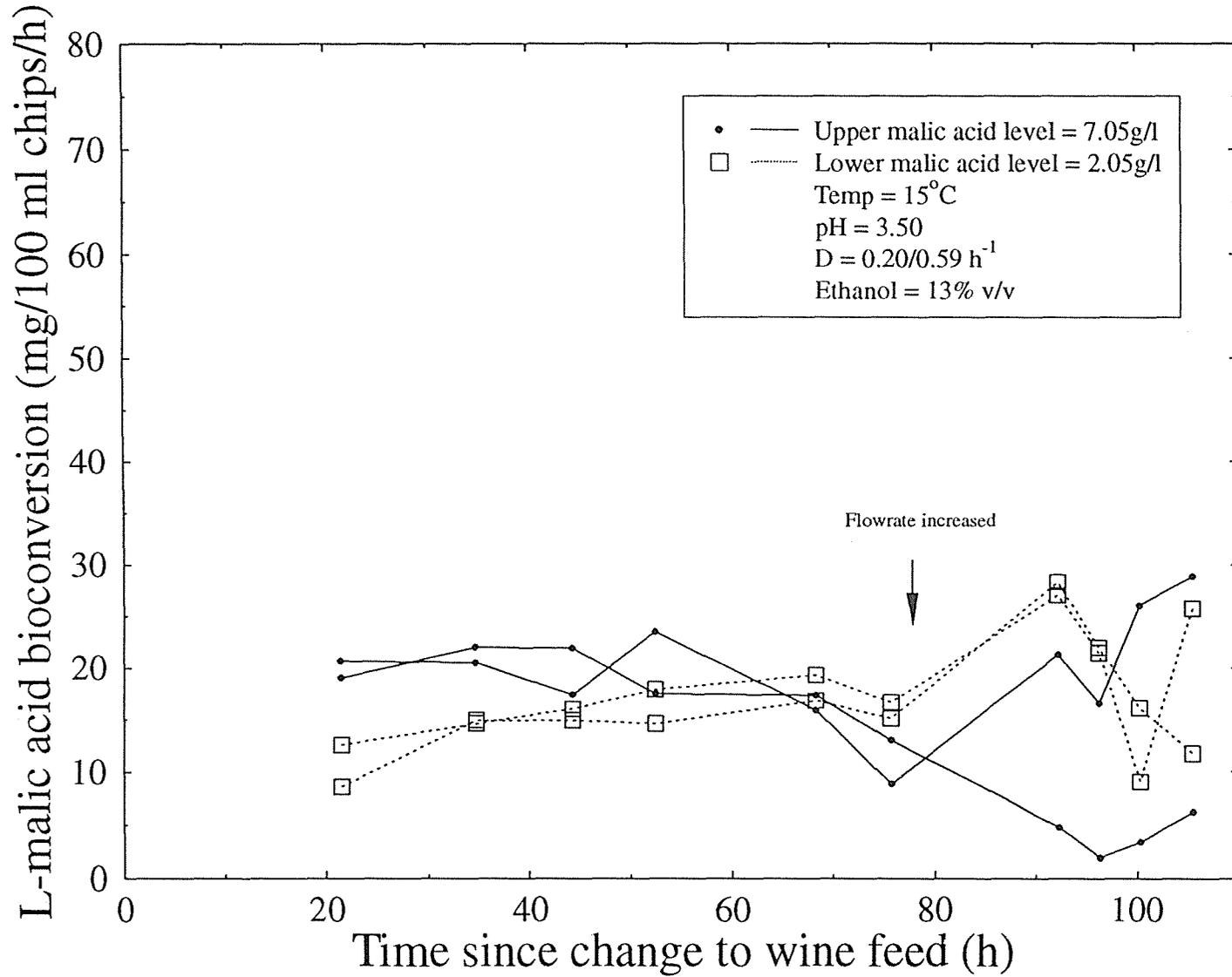
##### 4.7.2 Experimental Conditions

Four bioreactor columns were sterilized and filled with 20g of autoclaved French oak chips and initial growth was as defined in Table 2. Apple juice wine was fed into the bioreactors at a dilution rate of  $0.20 \text{ h}^{-1}$  at a temperature of  $15^\circ\text{C}$ . The dilution rate was increased after 77.5 hours to  $0.59 \text{ h}^{-1}$ , while the malic acid concentrations in the feed wines were  $2.05 \text{ g/l}$  and  $7.05 \text{ g/l}$ .

##### 4.7.3 Results

Malic acid bioconversion rates over time are given in Figure 16 and are very low compared with other experiments. The growth period for these bioreactors was shorter than for other runs, and the growth temperature was  $13^\circ\text{C}$  for possibly all of the growth period. These are the most likely reasons for the very low bioconversion rates achieved by these bioreactors.

**Figure 16:** Effect of malic acid concentration and flowrate on bioconversion: Experiment 2



The difference between the rates achieved is within the variability of the assay and bioreactors and so very little significance can be attached to the results in this experiment, except that it shows that the growth conditions are an important part of the final efficiency of the bioreactors.

#### 4.8 Overall Discussion

Results from the temperature/pH and pH/ethanol experiments (Sections 4.4 and 4.5) suggest no variation in bioconversion over the pH range of 3.15 - 3.75 unless the temperature is 21°C or higher. At higher temperatures the lower pH values cause a drop in rate. These experiments also show that 10 and 13% v/v ethanol in the wine have no interaction with the pH unless SO<sub>2</sub> is added, when the more adverse conditions cause a decrease in bioconversion. While this effect for growth has been documented in the literature (Wibowo *et al.*, 1985), the interaction of pH, temperature and ethanol has not previously been investigated for bioconversion.

Lowering the temperature caused a decrease in bioconversion although this decrease can be offset by the growth conditions of the bioreactor, e.g. the Chardonnay/recycle experiment (Section 4.2) was at 21°C while the first malic acid/flowrate experiment (Section 4.6) was conducted with other parameters the same but at 15°C, yet the latter gave a malic acid bioconversion rate almost twice as high (30 mg malic acid/100ml chips/h). This result, coupled with the second malic/flowrate experiment (Section 4.7), show that the initial growth of cells in the bioreactors is an important factor yet to be studied.

The growth rate for free cells is virtually nil at 15°C, and at 18°C is only 25% of that achieved at 21°C (Kelly *et al.* 1989). The effect of growth should therefore be minimal during bioconversion unless attached cells have different characteristics to

free cells. The dilution rates for the continuous experiments were at least ten times the maximum expected growth rate at the temperatures used.

In contrast to the literature results for gel-entrapped cells, where immobilization decreased the bioconversion rate, nearly all the results for adhesion-immobilized continuous culture in AJ wine gave figures at least double the batch rate and, although the cell numbers may not be directly comparable, implies that attachment positively affects the bioconversion rate of the cells. Calculating on volume rather than cell weight, the activity for the batch culture (Section 4.1) was 17 mg/100ml culture/h (at 21°C) and for the same conditions, except at 15°C, the pH/ethanol experiment (Section 4.5) gave a continuous activity of 40 mg/100ml chips/h, i.e. immobilized cells gave a higher bioconversion under more adverse conditions. The batch system cells were grown and tested with media containing 10% v/v ethanol and so there ought to have been no alcohol shock to the cells although an oxygen stress may have occurred with centrifuging as this was not done anaerobically.

The main emphasis of research in the literature is the effect on growth of varying parameters. This thesis has shown that the effects on immobilized bioconversion of these parameters are different to the effects on growth. However, because of the strain variability of *L. oenos*, the growth characteristics of strain 1070 itself under varying parameters would have to be ascertained rather than using literature figures to directly compare growth with immobilized bioconversion. Apart from the strain variability, comparison of bioconversion with growth parameters as given in the literature is difficult as ethanol has not generally been added to growth media. Ethanol concentrations over 10% v/v are a definite stress to the cells and have interactions with pH, temperature and SO<sub>2</sub> as shown with the pH/ethanol experiment (Section 4.5) of this thesis. The literature also does not mention bacteriophage affecting the fermentation and this may be a factor, especially where real wine is used. No account was made for bacteriophage affecting the growth/bioconversion in any of the literature. Strain 1070 as used in this thesis has been screened and does not harbour any phage (Dr. M. Huang, personal communication, 1991).

The lack of ethanol in growth/assay media of previous studies is a grave error if the information is to have any practical use for the wine industry. Alcohol has a large effect and, especially when considered with pH and temperature, the optimum bioconversion parameters would likely be similar to growth in the presence of alcohol and fall in a much narrower range than the literature currently suggests. It is suggested that an optimum bioconversion temperature of 40°C as given by Naouri *et al.* (1989) would be impossible if 13% v/v alcohol were in the media, as the cells would die off rapidly.

The treatment/use of real wine may stimulate the bacteria as the bioreactors maintained a steady state after 120 hours in the Chardonnay/synthetic wine experiment (Section 3.5), whereas the control bioreactor in the temperature/pH experiment (Section 4.4) gave a 20% drop by this stage. The Chardonnay/recycle experiment (Section 4.2) also showed continued steady state bioconversion when fed the real wine at a similar stage (96 - 150 hours), which again suggests stimulation by the genuine wine.

As stated in Section 1.1.2.5, growth is initially inhibited by up to 1 g/l malic acid and then slightly restimulated with increasing malic acid concentrations. There is no information in the literature about the effect of malic acid levels on bioconversion, but the malic acid/flowrate, Chardonnay/synthetic and recycle experiments (Sections 4.6, 3.5 and 4.2) all imply that an increase in malic acid concentration in the feed increases the bioconversion rate. The range of concentrations in these experiments was 2 - 5 g/l, which is the stimulation range given in the literature for growth. A much larger concentration difference (2 - 7 g/l) was tested in the second malic acid/flowrate comparison (Section 4.7) but indefinite results occurred, presumably due to inadequate growth time of the bioreactors, and so no information is available for the wider range of concentrations.

Because of the inability to correlate the bioreactor experiments without cell counts, a larger factorial experiment studying immobilized bioconversion is suggested to gauge further interaction of parameters. Britz and Tracey (1990) conducted three

factor factorial experiments on growth of the organism which showed intriguing results for the interaction of temperature, ethanol concentration and SO<sub>2</sub> addition (See Section 1.1.2.3) and this thesis has shown that at least the temperature/pH interaction matters for bioconversion.

#### 4.9 Conclusions

For a large-scale reactor the important parameters were determined to be:

minimum length for growth phase of 7-8 days

wine flowrate

bioconversion temperature

To prevent wash-off of cells the dilution rate should not be above  $D=0.35 \text{ h}^{-1}$ , although steady state bioconversion can be achieved at rates up to  $0.5 \text{ h}^{-1}$ . Provided the ambient winery temperature is 21°C or below, the pH and ethanol effects would be minimal and no temperature control would be required, although a reduction in temperature causes a reduction in bioconversion rate.

## 5.0 INDUSTRIAL PROTOTYPE TRIAL

### 5.1 Objectives

The aim of this thesis was to produce a prototype bioreactor to perform MLF in a winery. Villa Maria Wineries, Auckland, kindly consented to an experiment using Chardonnay from the 1991 vintage. The small-scale reactors were scaled up to a version which could ferment a barrel of wine (225l) within 2 days. Growth conditions were as similar as possible to the best achieved with the small bioreactors and the required wine flowrate was estimated from the bioconversion parameter experiments.

### 5.2 Methods

The 10 litre bioreactor as shown in Figure 17 was a direct scale-up of the smaller bioreactor with a diameter of 140mm and height of 655mm. The reactor was of perspex with flat ends glued at the bottom and sealed with a rubber O-ring and clamps at the top. Stainless steel mesh held the chips in at both ends. The peristaltic pump was a Watson-Marlow 501U with variable speed drive.

Feed and effluent containers were plastic 100 litre barrels with dry ice added to prevent oxidation. The feed barrel was filled by gravity from the main 20,000 litre tank. Inflow to the bioreactor was *via* a single piece of peristaltic pump tubing anchored at the bottom of the feed barrel. The overflow was another single piece of tubing from the top of the bioreactor feeding down to the bottom of the effluent barrel.

The growth in the bioreactor and wine feed conditions were as given in Table 7. The growth temperature was regulated by keeping the surrounding air temperature at 21°C for the first four days and thereafter the bioreactor was at the ambient winery temperature.

The wine used in the experiment was still on the lees and no attempt was made to remove the yeast. A buildup of yeast cells was noted in the bioreactor after 12 hours of operation.

**Figure 17:** Prototype bioreactor



**Table 7:** Pilot-scale trial using prototype bioreactor

Day one	5x50ml from stab of 27/3/91
Day four	1430: Inoculated 5x1 litre AJB (6% v/v ethanol)
Day nine	0830: Absorbance of inocula (600nm): 0.39, 0.51, 0.46, 0.49, 0.46 Gram stains OK. 1200: Inoculated 10 l bioreactor. 1.88kg chips, 21°C. Filled reactor with media (AJ Broth, 6% v/v ethanol).
Day 10	1200: Feed on 1.4 l/h for 2.5 h
Day 11	0900: Feed on 3.7 l/h for 1.75 h 1500: Feed on 3.6 l/h for 1.8 h
Day 12	1420: Feed on 1.5 l/h for 6.6 h
Day 13	1830: Feed on 1.3 l/h for 3 h
Day 14	Transport to Villa Maria Wineries, Auckland Gas production forced out most of medium. Auckland temperature in range 11 - 17°C. Average daytime temperature 15°C. 1645: Filled bioreactor with 9% v/v ethanol feed and continued feed until 3 l of effluent.
Day 15	0930: Feed on 1.8 l/h for 5.5 h 1500: Change to 13% v/v alcohol feed at 2 l/h for 0.3 h
Day 16	0850: Feed on 1.8 l/h 1600: Change to Chardonnay feed - pH 3.58, total acidity 8.25 g/l, SO <sub>2</sub> 9ppm free/63ppm bound, 4.8 g/l L-malic acid. 1715: Started filling effluent barrel
Day 17	0900: Peristaltic pump tubing split previous night and wine siphoning on to floor. 40 l in effluent barrel 1030: Sample of initial feed and effluent barrel 1515: Sample of effluent from bioreactor 1530: Tasting of initial wine and effluent 1645: Sample of effluent from bioreactor 1700: 70 l in effluent barrel - returned to bulk tank. Covers on barrels to prevent oxidation.
Day 18	0915: Sample of effluent from bioreactor. 90 l in effluent barrel 0930: Tasting of initial wine and effluent. Flowrate 5.1 l/h 1315: Sample of effluent from bioreactor 1615: Sample of effluent from bioreactor Bioreactor off and contents tipped into vessel 5009 (Chardonnay undergoing MLF)

### 5.3 Results

Figure 18 gives the malic acid bioconversion rate for the bioreactor and shows a steady state was achieved for 25 hours. The bulk effluent is a sample from the effluent barrel and indicates a higher apparent bioconversion rate because additional degradation of malic acid occurred with cells washed off the bioreactor overnight. The 10 litre prototype bioreactor gave the desired malolactic characteristics to the wine at a treatment rate of 100 litres per day for two days. This was despite the bioreactor being off for at least 6 hours overnight due to an equipment fault. Whereas all other feed parameters were in the average range for laboratory experiments, the dilution rate was higher than the maximum used previously ( $0.64 \text{ h}^{-1}$  cf.  $0.59 \text{ h}^{-1}$ ) and free  $\text{SO}_2$  was present at a low level. The bioconversion rate was at the low end of the range achieved with apple juice wine. This may be due to the low temperature of the growth conditions, presence of free  $\text{SO}_2$  or the high flowrate shearing bacteria off the oak chips, all of which result in a lower number of viable cells.

The tasting done was informal and was carried out by Villa Maria staff: Nigel Davies, Philip Baertschi and Kirsten Munro. It was a comparison between "before" and "after" the bioreactor MLF and comments were: "after MLF gave oak flavour, less acid, diacetyl, slightly oxidised." The pH of the initial wine was 3.58 and the effluent, 3.68. The tasting on the second day gave: After MLF: "less acid, MLF characters, less fruit, buttery, wood, not oxidised." The pH, however, was 3.62 for the initial wine (the pH probe was not calibrated) compared with 3.64 for the effluent. This wine was at a higher initial pH than is normal for a Villa Maria Chardonnay. HPLC analysis showed no variation in the acids (tartaric, malic, lactic, succinic) between the two days to account for the lack of pH change on day two. Acetic acid was not estimated.

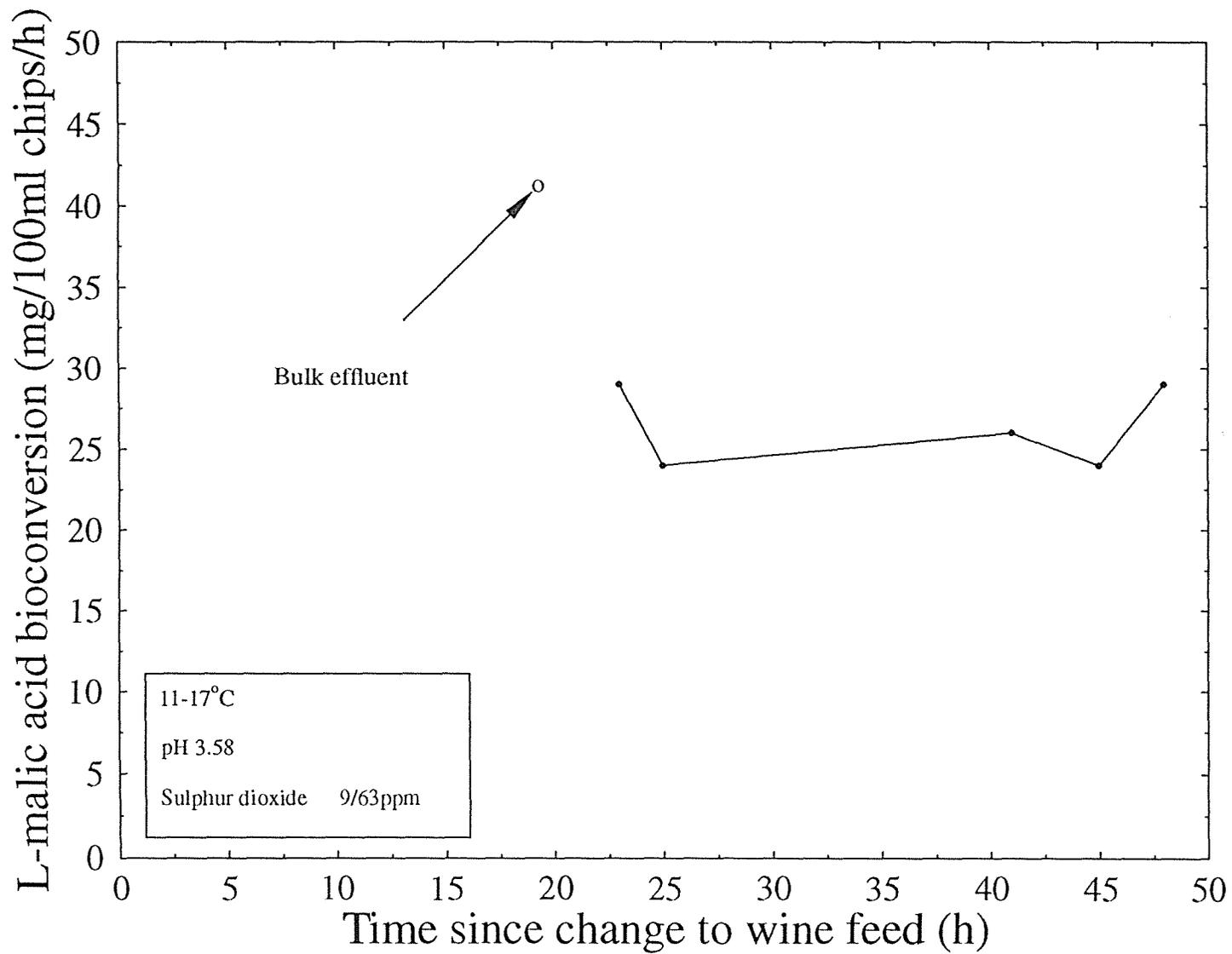
Only 0.5 g/l of malic acid was removed from a total of 4.8 g/l but the overall flavour of the wine was considered to be that expected of a wine having undergone MLF.

This agrees with research by Rodriguez *et al.* (1988), where interaction of the lees with wine and wood at warm temperatures (18-20°C), without any malic acid removal, produced a "complexity" in the wine equivalent to MLF in oak barrels.

The bioreactor had no flow for at least 6 hours but continued to perform bioconversion at a similar rate when restarted. This is in contrast to the Chardonnay/synthetic wine experiment (Section 3.5) where turning off the flow caused a rapid loss of bioconversion in the bioreactor. The length of time may be a factor as the cells depleted all the available malic acid in the small-scale experiment and the stressed condition caused by the high ethanol concentration probably resulted in death of the bacteria.

**Figure 18:** Pilot-scale trial using prototype bioreactor

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## 6.0 GENERAL DISCUSSION

### 6.1 Growth

Of the factors not studied in these experiments, the most important was the growth conditions of the bioreactors. The anaerobic/non-anaerobic and malic/flowrate comparison 2 experiments (Sections 4.3 and 4.7) suggest that a shortened total growth time (See Table 2) reduced the bioconversion by 50-60%. Several experiments have the same operating conditions but the bioconversion rates of the bioreactors varied widely. All the bioreactors had slightly different growth conditions, and as cell numbers were difficult to obtain without destroying the bioreactors, no specific activity could be measured and thus the experiments could not be compared directly.

The length of the continuous feeding phase for growth media, the dilution rate of this feed and the time allowed for adaptation to higher ethanol concentrations seems to have little effect on the bioconversion rates achieved by the reactors. The highest rates were achieved with the oak chip/bonechar comparison (Section 3.4) and these reactors had 6.5 days growth with intermittent feed and then a rapid increase in ethanol concentration with continuous feed. The real/synthetic wine comparison (Section 3.5), which had a high bioconversion for the Chardonnay feed, had 3 days intermittent feed and then a slow alcohol adaptation at a low and continuous dilution rate for four days. In flask culture at 21°C, AJ broth with a 10% inoculum takes 3-5 days to reach mid-log phase (where the highest bioconversion occurs). The effect of "rogue" columns, as in the malic acid/flowrate comparison (Section 4.6), shows that even with the same growth conditions some bioreactors can produce either more cells, or more efficient cells.

The effects of storage of the stab cultures and inoculum development have not been investigated and are areas where practical benefit could be gained from research into

adaptation to alcohol and effect on bioconversion. The yeast used for the alcoholic fermentation is known to affect the growth of *L. oenos* (Fornachon, 1968) but no work has been done to determine whether the yeast also affects the bioconversion activity of the cells. Further work is also required to determine a simplified optimum growth regime for an industrial-scale bioreactor.

## 6.2 Prototype Trial

Continuous MLF of a Chardonnay-style wine using oak chip immobilized *Leuconostoc oenos* was successful on an industrial-prototype scale, although flavour rather than acidity changes occurred in the wine to achieve the desired characteristics. The use of chips to give oak flavour is currently used by some wineries instead of barrels because of the high cost and the immobilized process combines oak flavouring with the MLF.

It has been shown that wine can be chemically deacidified and still provide the equivalent MLF characteristics of the bacterial method (Castino *et al.*, 1975). Chemical deacidification is therefore a competing process if no oak flavour is required but the method requires technical expertise and may also be expensive compared with using bacteria.

For a large-scale (20,000l) batch, the use of a 225l oak barrel is envisaged rather than a perspex column, and a simplified growth regime would be necessary for an industrial situation. Only low temperature steam sterilizing of barrel and chips could be carried out, but if a large inoculum is used the *L. oenos* should overgrow other yeast or bacteria. For an alcohol adaptation stage, 50% grape juice/50% wine could be used as both of these liquids are readily available in a winery during vintage.

It has been shown in these experiments that at least 3-4 days of steady state bioconversion can regularly be achieved by the bioreactors on AJ wine and 60% of the maximum bioconversion rate still occurs after 10 days of operation. These results should only be improved if real wine is used and the reactors should therefore be stable for the 10-14 days required to process a large-scale batch using the barrel reactor, assuming a 225l barrel and  $0.4 \text{ h}^{-1}$  dilution rate.

Of the advantages offered by immobilized cells as listed in the introduction to this thesis, the only factor not improved by this simple bioreactor would be temperature control. However, the bulk wine tank could be heated/chilled relatively cheaply for 10-14 days compared with trying to keep the temperature controlled for several weeks/months. Cells are also released into the wine, but this is not seen as a problem as the MLF occurs before centrifugation to remove the yeast cells and all the organisms will thus be removed in one operation. In any event, MLF as currently used depends upon addition of free bacteria to the wine.

The adhesion-immobilized process for MLF is effective and implementation of the practice in wineries will be dependent on simplifying the method as much as possible and overcoming the natural conservatism of winemakers.

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## ACKNOWLEDGEMENTS

Many people have suffered this thesis.

I would especially like to thank John Mawson and Ian Maddox for their time, ideas and tolerance. Special thanks are also due to Rod Asmundson for the original idea and Mike Boland for the impetus to get it all going. Ming Huang always smiles when you ask him for *L. oenos* cultures or information. All the staff at the Biochemical Processing Centre, DSIR have had some input - Laurie Kennedy, Marie Simpson and Mike Frude have pushed buttons or inoculated cultures at odd hours of the weekend when I've been out of town and Laurie has proofread the manuscript "a couple" of times. Doug and Crunch at the EM unit, DSIR did their usual efficient job with microscopy. Lindsay Alexander, Massey University, supplied a computer screen at a vital moment during writeup.

Win Currie at Montana Wineries very kindly supplied 10 litres of Marlborough Chardonnay for the experiments. Nigel Davies and the staff at Villa Maria supported this work with wine, time and equipment and thus provided the final chapter and proof that the system would work.