EFFECTS AND MANAGEMENT OF LACTOBACILLI IN YEAST-CATALYZED ETHANOL FERMENTATIONS

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ABSTRACT

This thesis focuses on the effects of lactobacilli and their end-products, lactic acid and acetic acid, on *Saccharomyces cerevisiae* growth and fermentation, and on antimicrobials used to manage such contaminants. To assess the effects of the bacteria, normal gravity (22 - 24 g/100 ml dissolved solids) wheat mashes inoculated with yeast at ~ 10^6 colony forming units (CFU)/ml were deliberately infected (coinoculated) with each of five industrially important strains of lactobacilli at ~ 10^5, ~ 10^6, ~ 10^7, ~ 10^8, and ~ 10^9 CFU/ml. Controls with yeast alone or with bacteria alone (~ 10^7 CFU/ml) were included. End-products, yeast growth and fermentation rates were monitored. Results indicated that production of lactic acid by lactobacilli and suspected competition of the bacteria with yeast cells for essential growth factors in the fermenting medium were the major reasons for reductions in yeast growth and decreases in final ethanol yield.

A chemically defined minimal medium was used to determine the effects of added acetic and lactic acid, and their mode of action on two strains of *S. cerevisiae*. The effects of these two acids on yeast intracellular pH (pH_i), plasma membrane H^+-ATPase activity and on the plasma membrane fatty acid composition were studied. It was found that the specific growth rates (μ) of the two yeast strains decreased exponentially ($R^2 > 0.9$) as the concentrations of acetic or lactic acid were increased. Acetic and lactic acids synergistically reduced the specific growth rate of yeast. Acetic acid caused the yeast cell to expend ATP to pump out excess protons that result from the passive diffusion of the acid into the cell at medium pH (pH_e) followed by its dissociation within the cell as a result of higher pH_i. Lactic acid (0.5 % w/v) caused intracellular acidification (which could lead to arrest in glycolytic flux) as a result of a significant decrease ($P = 0.05$) in the plasma membrane H^+-ATPase activity. Moreover, the plasma membrane fluidity was reduced due to decrease in unsaturated fatty acyl residues.

Among the antimicrobials studied, urea hydrogen peroxide (UHP) was superior compared to stabilized chlorine dioxide and nisin, but its bactericidal activity was greatly affected by the presence of particulate matter. When used near 30 mM (in unclarified mash), in addition to its bactericidal effect, UHP provided near optimum levels of assimilable nitrogen and oxygen that aided in vigorous yeast fermentation. This process was patented.

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"In most of mankind gratitude is merely a secret
hope for greater favours"
- Duc de la Rochefoucauld

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I Dedicate this thesis To

My Mom, Nagama Devi and
Dad, Varadarajulu

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

"But surely this is an old tale you tell, they say;
But surely this is a new tale you tell, others say.
Tell it once again, they say;
Or, do not tell it yet again, others say.
But I have heard all this before, say some;
Or, but this is not how it was before, say the rest."
(Naqshbandi recital, from *The Way of the Sufi*, by Idries Shah)

Ethanol is produced commercially by fermentation of cereal grains, molasses or other materials with high starch and/or sugar contents. The process involves conversion of sugars to alcohol and carbon-dioxide by the yeast, *Saccharomyces cerevisiae*. About 24 billion litres of ethanol are produced per year around the world (Dixon, 1999). This includes both potable alcohol (beer, wine, and distilled spirits) and non-potable (industrial and fuel) alcohol. The demand for ethanol, as a substitute for gasoline, is expected to increase due to concerns related to environmental impacts and global warming (in addition to the soaring gas prices) which calls for maximizing the existing production. According to the US Department of Energy Life-Cycle analysis, use of ethanol (as a fuel) would reduce green house gases by 99 % when compared to the gasoline that it replaces. Replacing the banned gasoline oxygenate MTBE (methyl tertiary butyl ether) with ethanol will also increase the demand.

$$H(C_6H_{10}O_5)_nOH \xrightarrow[\text{Water}]{\text{Amylolytic enzymes}} nC_6H_{12}O_6 \xrightarrow{\text{Yeast}} 2nCH_3CH_2OH + 2nCO_2\uparrow$$

Starch	Glucose	Ethanol	Carbon dioxide
901 kg	901 X 180/162 = 1000 kg	511 kg	489 kg

Theoretically, based on the equation above, 100 kg glucose when fermented completely should yield 51.1 kg ethanol. But this degree of stoichiometry cannot be achieved since the yeast uses some of the glucose for growth (biomass production). The conversion efficiency in most production plants, at best, is 90 - 93 %. Since growing

yeasts produce ethanol at a rate 33 times faster than non-growing cells (Kirsop, 1982), it is desirable to keep the yeast growing for as long as possible. Therefore, a slight reduction in ethanol yield caused by cell growth has to be tolerated.

A number of factors other than growth also contribute to the reduction in final ethanol yields and thereby lower the productivity of a plant. These include: (i) minor end-products of yeast metabolism (such as glycerol, higher alcohols, esters, organic acids and aldehydes), (ii) stuck and sluggish fermentations, (iii) losses during mashing and (iv) bacterial infections (Ingledew, 1999). These factors have to be reduced or eliminated in order to maximize both ethanol yield and profits.

Among the factors listed, bacterial infection (contamination) is a major cause of reduction in ethanol yield during the fermentation of starch-based feedstocks by *S. cerevisiae*. High numbers of bacterial contaminants interfere with yeast metabolism through competition for nutrients, through production of end-products (lactic acid and acetic acid) which inhibit yeast growth and metabolism, or through lowering the activity of enzymes such as glucoamylase. Fermentation patterns may also be altered (Ingledew, 1993). Lactic acid bacteria are the most troublesome bacterial contaminants encountered because of their ability to survive low pH, high alcohol, and high temperature. They grow rapidly under the exact conditions of the alcohol fermentation. Lactobacilli are of major concern to distilleries and fuel alcohol plants. In the literature, uncertainities exist regarding the effect of lactobacilli on ethanol yield. When bacterial contaminants are compared, the problem of the correlation of ethanol yield loss with the presence of lactic and/or acetic acid is complicated in part due to the fact that homofermentative lactic acid bacteria metabolize glucose to two molecules of lactic acid almost in stoichiometric quantities, whereas heterofermentative lactic acid bacteria produce lactic acid, CO_2, and ethanol as well as minor amounts of acetic acid and glycerol. Despite considerable research in the area, the effect of lactic acid bacteria on the rate and completion of yeast-catalyzed fermentations remains unclear (Huang *et al.*, 1996). At the time this work was started, any correlation between the extent of bacterial contamination and losses in ethanol yield had yet to be documented. In view of the narrow profit margins of fuel alcohol distillers, losses of even 1 % of total ethanol can play an important role in the financial success of the plant.

The management of bacterial contaminants is often achieved in industry by using single antibiotics like penicillin G, streptomycin, virginiamycin, and tetracycline (Day *et al.*, 1954; Aquarone, 1960) or mixtures of these compounds. However, antibiotics are expensive, and the concept of antibiotic use in an industrial process is in question in spite of the absence of antibiotic residues in spent grains subsequent to distillation. General misuse of antibiotics in society has contributed to a build-up of reservoirs of antibiotic-

resistant bacteria (Khachatourians, 1998), providing an incentive to scientists to examine other antimicrobial substances which are not antibiotics.

This project was therefore designed with the following objectives:

(i) to study the relationship between bacterial contamination of wheat mashes and the corresponding effects of these bacteria on yeast growth and ethanol yield;

(ii) to deduce the mechanism of action of lactic acid on yeast (*S. cerevisiae*) and to re-evaluate the mechanism of action of acetic acid on *S. cerevisiae* (since both these weak-acids are end-products of lactic acid bacterial fermentation that inhibit yeast growth and metabolism); and

(iii) to assess the effectiveness of antibiotics in the alcohol fermentation and identify a suitable antimicrobial that could be used (instead of antibiotics) to effectively manage the bacterial contaminants in fermentation mashes.

2. REVIEW OF LITERATURE

2.1 Importance of lactic acid bacteria in alcohol production

Lactic acid bacteria are the most troublesome group of contaminating bacteria found in breweries, distilleries and fuel alcohol plants. These bacteria are Gram-positive, rod-shaped, facultative anaerobes with a fermentative metabolism which produce high levels of acids as end-products. Depending on their metabolism, lactic acid bacteria are grouped as homofermentative (1 mole of hexose converted to 2 moles of lactic acid through the Embden - Meyerhof pathway) and heterofermentative (1 mole of hexose converted to ~1 mole CO_2, ~1 mole ethanol, ~1 mole of lactic acid with minor amounts of acetic acid and pyruvic acid through the 6 - phosphogluconate pathway) lactic acid bacteria (Kandler, 1983). Kandler also mentioned a group of lactic acid bacteria called facultative heterofermenters. This group of homofermentative bacteria possess an inducible phosphoketolase pathway with pentoses acting as inducers. In winemaking, the occurrence of the so-called 'ferocious' lactobacilli have been reported (Boulton *et al.*, 1996). These fast growing ferocious lactobacilli produce acetic acid (rather than lactic acid) in concentrations high enough to impede yeast growth and fermentation.

Lactic acid bacteria are relatively resistant to heat and can grow and metabolize at low pH. Some strains are known to grow rapidly in the fermentor after only 24 h of fermentation at which point the oxygen has been mostly depleted and the aerobic bacteria have died off (Wright, 1995). Chang *et al.* (1995) collected samples from a commercial ethanol production plant to enumerate bacterial contamination in each step of a starch-based ethanol production process. All bacterial isolates (that survived and propagated through the process) were identified as lactic acid bacteria, the major ones being *Lactobacillus fermentum*, *Lactobacillus salivarius* (heterofermentative) and *Lactobacillus casei* (facultatively heterofermentative). The lactic acid bacteria isolated from distillery fermentations are, as would be expected, particularly suited to the conditions existing in these fermentations (Bryan-Jones, 1975). Therefore, the enumeration of bacteria in many distilleries is often limited to the detection of "lactics" due to the fact that the aerobes and facultative anaerobes with little pH tolerance do not pose a serious threat to product quality or production efficiency.

2.2 Interactions between lactic acid bacteria and yeast

In nature, micro-organisms are rarely found in pure cultures but most of our understanding of microbial physiology is derived from studying pure cultures. There is a need for investigation of growth and metabolism of organisms growing in association with each other. Yeasts and lactic acid bacteria are often encountered together in natural ecosystems and may be in competition for the same substrates (Alexander, 1971). A thorough understanding of their interactions would help in achieving more controlled fermentations. Momose *et al.* (1969) observed that lactobacilli caused aggregation of yeast. They found that the aggregation resulted due to the electrostatic force between yeast and bacterial cell surfaces and was reversible on changing the pH. Challinor and Rose (1954) studied the interactions between a yeast (*S. cerevisiae*) and a bacterium (*Lactobacillus* sp.) grown together in a defined medium. They observed the yeast to have synthesized a missing substance essential for the growth of the *Lactobacillus* sp. Inhibitory and stimulatory interactions between champagne strains of *S. cerevisiae* and lactic acid bacteria were studied by Lemaresquier (1987). Among the lactic acid bacteria tested (*Pediococcus cerevisiae, Lactobacillus hilgardii, Lactobacillus plantarum, Lactobacillus brevis* and an unknown *Lactobacillus* sp.), he found that the growth of *L. plantarum* was stimulated by yeast, while the others were inhibited. The stimulation may be due to vitamins excreted by the yeast (Young *et al.*, 1956). Ethanol and SO_2 produced by the yeast in wine fermentation could also be responsible for inhibition of lactobacilli (King and Beelman, 1986), but Lemaresquier (1987) suggested that the inhibition may be due to the release of inhibitors (similar to antibiotics) by the yeast.

Yeast was shown to stimulate bacterial survival in a poor culture medium and, as a result, aid in an increase in the lactic acid production by 60 %, whereas the bacteria reduced the growth and alcoholic fermentation of yeasts by 65 % (Leroi and Pidoux, 1993a). In a complex medium with beet molasses, where sucrose is the most important carbohydrate, hydrolysis of sucrose by yeast seemed to be a mechanism of lactobacilli stimulation (Essia Ngang *et al.*, 1992). While characterizing the interactions between *L. hilgardii* and *Saccharomyces florentinus* isolated from sugary kefir grains, Leroi and Pidoux (1993b) showed that CO_2, pyruvate, propionate, acetate and succinate excreted by the yeast stimulated the growth of lactobacilli and, in turn, the production of lactic acid. They also suggested that the interactions differed greatly depending on the sugar source. deOliva-Neto and Yokoya (1997) confirmed amino acids as the main nutrients needed to stimulate the growth of the contaminant, *L. fermentum*, during an alcoholic fed-batch yeast fermentation. Lactobacilli have long been known to be fastidious - requiring a number of amino acids and vitamins for growth (Kandler and Weiss, 1986).

2.3 Lactic acid bacteria and loss in alcohol yield

Contaminating organisms may enter the fermentor in a number of ways. Cleaning and sanitation are not as rigorous in fuel alcohol plants as in breweries. Contaminants arise from tankage, transfer lines, heat exchangers, grains, active dry yeast, backset, or the yeast slurry inoculum if yeast cells are recycled (Reed and Nagodawithana, 1991). Microbial numbers can be significantly reduced by maintaining backset at temperature over 70° C prior to use, by keeping equipment clean and steamed thoroughly or chemically sanitized, by adding antibiotics such as penicillin or virginiamycin to fermentors, and by pasteurizing or chemically treating the substrate (Ralph, 1981). In spite of this, the problem of bacterial contamination still exists in the ethanol production industry.

Dolan (1976) predicted ethanol losses of 1 - 3 % which corresponded to bacterial contamination levels between 10^6 and 10^7, and 3 - 5 % losses with bacteria in the range between 10^7 and 10^8. He gave no specific figures or experimental detail. From studies in an industrial scale distillery it was suggested that when bacterial numbers exceeded 10^8 per ml, the spirit loss was approximately 5 per cent (Dolan, 1979; Barbour and Priest, 1988). However, Makanjuola *et al.* (1992) showed that a bacterial count of 4.5×10^8 at 30 h resulted in 17 per cent reduction of ethanol yield which was substantially higher than expected from Dolan's predictions. In a cell-recycled ethanol fermentation contaminated by *L. fermentum*, the ethanol productivity reduced from 9.00 $gL^{-1}h^{-1}$ (in the absence of *L. fermentum*) to 5.72 $gL^{-1}h^{-1}$ (Chang *et al.*, 1995). Alcoholic fermentation of high-test molasses and yeast viability were strongly inhibited by *L. fermentum* after a few cycles in a fed-batch process with cell recycling (deOliva-Neto and Yokoya, 1994).

Chin and Ingledew (1994) showed that four cycles of 50 % backsetting of wheat mashes highly contaminated with *L. fermentum* (up to 150 million cells per ml) did not affect alcohol production, although there was a slight reduction in yeast growth. They also indicated that gross contamination of dilute wheat mashes (14° Plato) with *Lactobacillus delbrueckii* (up to 6×10^8 cells per ml) did not reduce ethanol yield, but lactic acid produced by bacteria did accumulate to a high enough concentration to cause loss in yeast viability towards the end of the fourth and fifth sequential fermentation runs. It is known that the concentration of lactic acid in fermentation increases with bacterial numbers (Barbour and Priest, 1988). Lactic acid and other byproducts, especially acetic acid, are liberated into the medium by contaminating lactic bacteria and this leads to a decrease in ethanol production rates (Essia Ngang *et al.*, 1990). These authors also reported that the inhibition of yeast by these metabolites is further accentuated in the presence of viable lactobacilli. Lactic acid appears to be an important factor in inhibition of ethanol production

by yeast. The lowering of the medium pH due to lactate production might also inhibit the saccharification process (Makanjuola *et al.*, 1992; Ingledew, 1993). The excessive amounts of acetic acid produced by contaminating bacteria can slow alcoholic fermentations but does not solely account for the bacterial inhibition of yeast (Edwards *et al.*, 1999).

Makanjuola *et al.* (1992) also indicated that two strains of *L. plantarum* (33a and 33b) cause flocculation of yeast cells. These bacteria also caused a high level of inhibition of ethanol formation by the yeast. Therefore, they suggested that flocculation might be an important mechanism for inhibition. Stewart (1975) reported a mechanism called coflocculation in yeast. It refers to the situation where two non-flocculant yeast strains, when mixed in a fixed ratio exhibited flocculation. White and Kidney (1979) have shown such cosedimentation to occur between a strain of yeast and various species of beer spoilage bacteria including *Lactobacillus* spp.

According of Makanjuola *et al.* (1992), there is great difficulty in estimating the losses of ethanol due to bacterial contamination on an industrial scale. Bacterial counts seem unlikely to give a very reliable guide, since there is a wide variation in the inhibition caused by different strains. A better understanding of the mechanism by which ethanol production is inhibited might suggest improved methods for assessment. Huang *et al.* (1996) studied the relationship between sluggish fermentations and the antagonism of yeast by lactic acid bacteria. They reported that the alcoholic fermentation of Chardonnay grape juices by *S. cerevisiae* was inhibited by the three bacterial isolates, YH-15, YH-24 and YH-37.

2.4 Inhibition of microbial growth by weak organic acids

Organic acids are known to have both fungistatic and fungicidal effects which are maximal at low pH (Neal *et al.*, 1965). Benzoic and sorbic acids are very effective inhibitors of microbial growth and are intentionally added to many foods as preservatives (Dziezak, 1986). Acids such as acetic, propionic and lactic are often added to foods to prevent or delay the growth of pathogenic or spoilage bacteria (Dziezak, 1986; Podolak *et al.*, 1996). Lactic acid is the major metabolite of lactic acid bacteria, and may cause a significant pH change in the growth medium sufficient to antagonise many microorganisms. For example, acidification with lactic acid inhibits the growth of *Escherichia coli*, *Pseudomonas*, *Salmonella* and *Clostridium* spp. (Piard and Desmazeaud, 1991). A pH change in the medium resulting from the concentration of this weak acid is, however, not extensive because a large part of lactic acid does not dissociate at the pH value used (pK_a for lactic acid = 3.86). The extent of any pH change is also influenced by the medium composition, medium pH and the degree of buffering provided.

Early experiments by Levine and Fellers (1940) demonstrated that acetic acid was more lethal to microorganisms than hydrochloric acid or lactic acid. They concluded that this toxicity was not due to hydrogen ion concentration alone, but seemed to be a function of the concentration of undissociated molecules. With acetic acid in the medium, lowering the pH increased the inhibitory activity confirming that the undissociated molecule was the effective inhibitor. Thus, the inhibition by organic acids used as antimicrobial agents would increase with decreasing pH depending on their dissociation constants (Freese *et al.*, 1973). This implies that efficacy relies upon the undissociated form of the molecule which passively diffuses across the lipid bilayer of the cell membrane due to its high solubility in the phospholipid portion of the plasma membrane. The molecule then dissociates inside the cell - the extent of dissociation depending on the intracellular pH. The membrane is impermeable to the ionized (dissociated) acid (Hunter and Segel, 1973; Kashket, 1987). In case of *S. cerevisiae* IGC 4072, metabolizing aerobically, a mediated transport system behaving as an electroneutral proton symport for the anionic form of acetic acid has been reported, when this yeast was grown in a medium with acetic acid (Casal *et al.*, 1996). Cássio *et al.* (1987) reported an accumulative electroneutral proton-lactate symport with a proton-lactate stoichiometry of 1:1 for *S. cerevisiae* IGC 4072 grown aerobically in lactic acid medium. However, it cannot be generalized that only the undissociated form is actively antimicrobial, as Eklund (1983) has demonstrated cellular effects attributable to both dissociated and undissociated forms of sorbic acid above pH 6.0 in minimum inhibitory concentration experiments. He showed that the inhibitory action of undissociated acid was 10 - 600 times greater than that of dissociated acid. But the latter caused more than 50 % growth inhibition of *Bacillus subtilis, Staphylococcus aureus* and *E. coli* at pH levels above 6.0 (where more than 95 % would be present as sorbate anion).

Chung and Goepfert (1970) showed that *Salmonella* was inhibited by lactic acid at a pH value below 4.4 and by acetic acid at a pH below 5.4. Considering the respective pK_a values of these two acids (3.86 and 4.74), inhibition apparently occured when the non-ionized (undissociated) fraction reached about 20 % of the total acid concentration. Acetic acid ($pK_a = 4.74$) has between two and four times more molecules in the undissociated form over a pH range between 4.0 and 4.6 compared to lactic acid (Lindgren and Dobrogosz, 1990). The electronegative hydroxyl group of lactic acid pulls electrons from the carboxyl group and lowers the pK_a value to 3.86 (Russell and Diez-Gonzalez, 1998). Using *S. aureus* as a model strain, Daly *et al.* (1972) established that the inhibitory activities of both acetic and lactic acids increased as the pH decreased. Wong and Chen (1988) showed that the growth of *Bacillus cereus* was completely arrested at pH values of 6.1, 6.0 and 5.6 in the case of acetate, formate and lactate, respectively, and they found

that acetate was more inhibitory than the other acids tested. They also reported that spore germination was inhibited at the 50 % level at 0.1 mol/L of formate, lactate and acetate at pH 4.4, 4.3 and 4.2 respectively.

2.4.1 Importance of acetic and lactic acids in alcohol production by *Saccharomyces cerevisiae*

Acetic acid (a minor end product of heterofermentative lactic acid bacteria and wild yeasts, or a major end product synthesized by the aerobic bacterium, *Acetobacter*) and lactic acid are inhibitory to yeast growth at 0.5 - 9 g/L and 10 - 40 g/L respectively. An 80 per cent reduction in yeast cell mass occurs at 7.5 g/L acetic acid and 38 g/L lactic acid (Maiorella *et al.*, 1983). While studying the winemaking of musts at high osmotic strength by 15 strains of thermotolerant *S. cerevisiae*, Caridi *et al.* (1999) reported the production of 1.63 - 3.65 g acetic acid/L by the yeast in must with 40° Brix as a consequence of osmotic stress. This, of course had a clearly detrimental effect on the ethanol production. deOliva-Neto and Yokoya (1994) reported that there was a decrease in alcohol yield when lactic acid concentration exceeded 6.0 g/L, and at a concentration of above 4.8 g/L, lactic acid seriously interfered with yeast viability. Essia Ngang *et al.* (1989) examined the effect of lactic acid on *S. cerevisiae* growing on high gravity beet molasses. They observed that lactic acid reduced the specific growth rate of yeast by over 60 %, and the specific rate of alcohol production by 40 %. The overall alcohol yield was not affected by the presence of up to 30 g/L of lactic acid in a wort with 70 g/L of sugar. However, as the concentration of acid increased there was a reduction in the fermentation rate. They concluded that the toxicity of lactic acid depended on its concentration and was enhanced by an increase in osmotic stress. Reduced yields of ethanol, lower yeast crops, reduced carbohydrate utilization and an increase in acidity are all caused by the buildup of lactic acid in the fermentation medium (Makanjuola *et al.*, 1992; deOliva-Neto and Yokoya, 1994).

Pinto *et al.* (1989) reported that acetic acid was responsible for the death of *S. cerevisiae* both at high and at low temperatures. Such lipophilic weak acids at intermediate and lower temperatures induced a second type of cell death which appeared to be a consequence of intracellular acidification (Cardoso and Leão, 1992). In case of *Zygosaccharomyces bailii*, unlike *S. cerevisiae*, intracellular acidification induced by weak acids (acetic and other carboxylic acids) was less pronounced and did not have a significant role in cell death at an intermediate temperature of 25° C (Fernandes *et al.*, 1999).

Pampulha and Loureiro (1989) showed that acetic acid inhibited exponentially the fermentation in a respiratory deficient mutant of *S. cerevisiae* (IGC 3507-111). The presence of ethanol potentiates the inhibitory effect (of acetic acid) in a synergistic way and

narrows the temperature range of growth from 3-42° C to 19-26° C (Ramos and Madeira-Lopes, 1990). Rasmussen *et al.* (1995) reported that addition of acetic acid (4 g/L) midway through grape juice fermentations slowed the fermentations. However, it is unclear whether the amount of acetic acid produced by spoilage bacteria would inhibit yeast growth and cause stuck fermentations as observed by Huang *et al.* (1996) who reported that high levels of volatile acidity (3 g/L) produced by a bacterial isolate YH-15 were associated with stuck fermentations.

The effect of acetic acid in inducing slow/stuck fermentations was studied by Edwards *et al* (1999). They found that the inhibitory effect of acetic acid on *S. cerevisiae* was dependent on the strain (evident from the wide range of minimum inhibitory concentrations observed, 4.5 - 7.5 g/L, for the 11 strains studied).

2.4.2 Interactive effects of acetic and lactic acids on microorganisms

Adams and Hall (1988) investigated the individual and cumulative effects of lactic and acetic acids on *E. coli* and *Salmonella enteritidis*. They confirmed that the undissociated forms of these two acids are toxic for the bacteria. They attributed the higher toxicity of acetic acid to the fact that its pK_a is higher than that of lactic acid (and therefore at a given pH, more acetic acid is undissociated in the medium compared to lactic acid). The authors also showed that these two acids acted synergistically in weakly-buffered media; lactic acid decreased the pH, thereby increasing the toxicity of acetic acid. Lactic and acetic acids were shown to be slightly synergistic in their inhibitory interrelationship against *Salmonella typhimurium* (Rubin, 1978).

Moon (1983) studied the inhibition of the growth of acid-tolerant yeasts by acetate, lactate and propionate and found that propionate was more inhibitory than acetate or lactate. She also studied the inhibition of yeast growth by mixtures of acetic, lactic and propionic acids at one pH value and derived simple polynomial expressions linking growth rate with concentrations of the preservatives. Formulae included interactive terms that implied synergisms, although it was not stated whether differences were statistically significant. The results, however, suggested that the three acids used in combination were synergistically inhibitory to *Hansenula canadensis* and *Geotrichum candidum*. *Saccharomyces uvarum* was inhibited by synergistic mixtures of acetate/proprionate whereas *Endomycopsis burtonii* was inhibited by synergistic mixtures of acetate/lactate, acetate/propionate and lactate/propionate.

2.5 Mode of action of weak organic acids on microorganisms

The inhibitory activity of organic acids depend on the pH of the medium, the dissociation constant of the acids and their molar concentration. In solution, weak acids exist in a pH-dependent equilibrium between the dissociated and undissociated state. The effect of pH on weak acid dissociation is given by the Henderson-Hasselbalch equation:

$$pH = pK_a + \log\frac{[A^-]}{[HA]}$$

where A^- and HA are the dissociated and undissociated species, respectively. At neutral pH there is very little undissociated acid, but the concentration of the undissociated acid increases logarithmically as the pH declines. Since organic acids are generally more toxic to microorganisms at low pH, it had generally been assumed that the antimicrobial activity of these acids was caused by the undissociated molecules (Salmond *et al.*, 1984). The weak acids diffuse into the microbial cell until an equilibrium of the undissociated molecule is established across the membrane, and then dissociates in the cytoplasm in accordance with the intracellular pH (Fig. 2.1). The protons liberated this way will either be pumped out of the cell in exchange for a cation or will be absorbed by the buffering capacity of the cytoplasm (Booth and Kroll, 1989).

Inhibition of growth by weak acids has been proposed to be due to (i) membrane disruption (Freese *et al.*, 1973; Stratford and Anslow, 1998); (ii) inhibition of essential metabolic reactions by either a possible fall in internal pH as a result of uptake of the undissociated acid and its subsequent dissociation within the cells or as a direct inhibitory action of the acid on critical enzymes of the cell (Neal *et al.*, 1965); (iii) stress on intracellular pH homeostasis (Salmond *et al.*, 1984) and (iv) accumulation of toxic anions (Eklund, 1985). In yeasts, it has been proposed that weak acids could cause the induction of an energetically expensive stress response that attempts to restore homeostasis and results in the reduction of available energy pools (ATP) for growth and other metabolic functions (Bracey *et al.*, 1998), or could cause the uncoupling of transmembrane proton gradients from the energy-requiring processes, which are linked to the interactions of these weak acids with cell membranes (Cole and Keenan, 1987; Warth, 1977).

With the advent of the 'chemiosmotic hypothesis' (Mitchell, 1961) as an acceptable model for energy transduction events such as oxidative phosphorylation and active transport, studies were done to test weak acid preservatives as uncouplers.

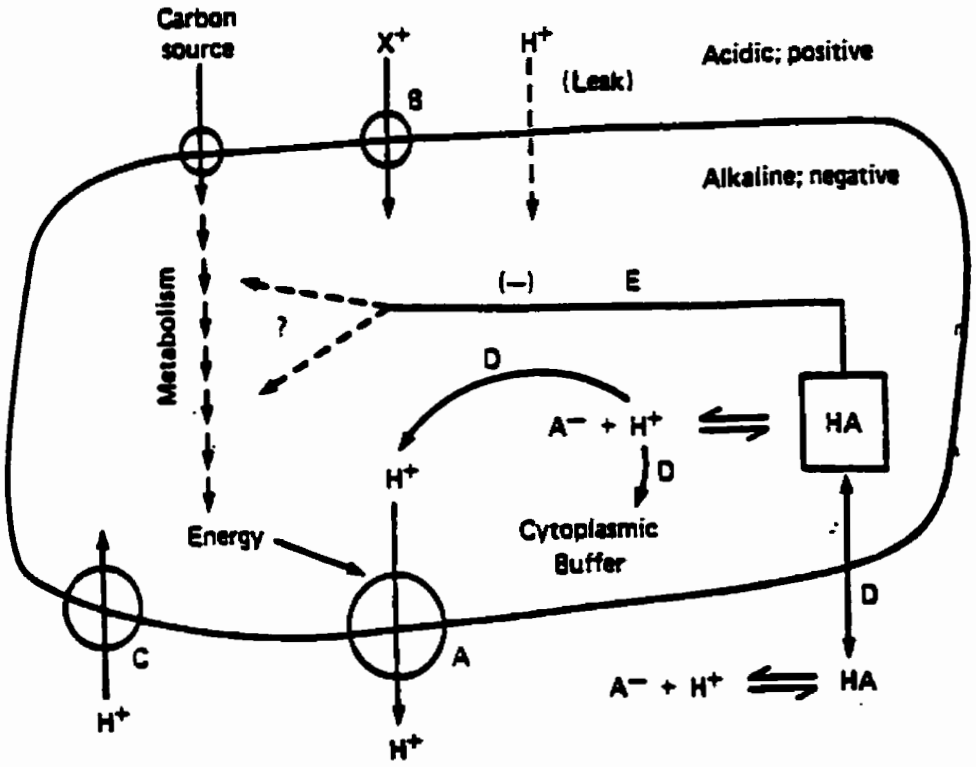


Figure 2.1 Proton movement in cells with respect to pH homeostasis (Booth and Kroll, 1989). (A) Proton efflux is driven by chemical energy provided either by ATP or by reducing power (in yeast, the plasma membrane H^+-ATPase works on proton efflux by using energy provided by ATP). (B) In order to achieve net alkalinisation of the cytoplasm a net electrogenic flux of cations into the cell must be active to dissipate the membrane potential generated by the proton pump. (C) At steady state, proton extrusion is balanced by a number of proton entry pathways which carry out useful work (e.g. ATP synthesis, transport and motility). (D) A weak acid will distribute across the membrane in accordance with the initial transmembrane pH gradient. Some of the protons liberated by the weak acid will be expelled by the proton pump but, once the capacity for proton extrusion is outstripped, internal pH will fall. (E) Any compound (e.g. a food preservative) which interferes directly with the generation of either ATP or reducing power will limit the capacity of the cell to regulate its internal pH.

Sheu and Freese (1972) determined that in bacteria, short chain fatty acids such as acetic acid reversibly react with the cell membrane and alter its structure. They postulated that by uncoupling the electron transport chain, the short chain fatty acids interfered with the regeneration of ATP or the transport of certain metabolites into the cells. However, there is no consistent evidence to suggest that weak acid food preservatives have any uncoupling activity (Booth and Kroll, 1989). In an attempt to compare the inhibitory action on *S. cerevisiae* of weak-acid preservatives, uncouplers and medium-chain fatty acids, Stratford and Anslow (1996) suggested that decanoic acid had a mode of action (inhibition of yeast growth being a consequence of rapid cell death) distinct from both weak-acid preservatives and uncouplers. They proposed the cell death to be due to membrane rupture and loss of cytoplasm.

Studies by Sheu *et al.* (1972) on the effects of acetate and other short-chain fatty acids on sugar and amino acid uptake by *B. subtilis* indicated that acetate uncouples the amino acid carrier protein from the cytochrome-linked electron transport chain and inhibits amino acid transport noncompetitively. Serine uptake by membrane vesicles of *B. subtilis* is inhibited when exposed to fatty acids. Using the same system, it was shown that active transport of amino acids such as L-leucine and L-malate was inhibited by fatty acids (Freese *et al.*, 1973).

In a later study, Sheu *et al.* (1975) recognized that if compounds that donate electrons (reducing) were no longer available to the cell as a result of transport inhibition by the fatty acids, oxygen consumption would be reduced. In order to understand these processes, the bacterial cell was converted into a sphaeroplast under isotonic conditions and these membrane vesicles were used to study uptake of a substrate against a gradient. Oxygen was consumed in the presence of membrane preparations and an energy source indicating that the inhibition of substrate transport is mainly due to an "uncoupling" of transport from ATP production via the electron transport chain (probably by elimination of a proton gradient). Therefore, it was postulated that lipophilic agents such as undissociated short-chain fatty acids would shuttle protons through the membrane until the proton motive force had been destroyed and transport thus eliminated (since active transport of a molecule would depend upon the proton gradient generated during the oxidation of a substrate).

Freese and Levine (1978) further postulated that the most effective antimicrobial agents would be lipophilic enough to attach to microbial membranes and yet be soluble in aqueous phase. This is because they can approach the membrane from the aqueous medium, and easily penetrate the membrane lipid bilayer without the need to expend

energy. Undissociated acids of short chain length can penetrate the cell more easily because they possess these characteristics (Doores, 1983). A variety of weak acids at or below their pK_a values are potent inhibitors of amino acid transport in *Penicillium chrysogenum*. The effective compounds include sorbate, benzoate and propionate (Hunter and Segel, 1973). Some acids will dissociate to give anions (e.g. lactate, citrate) which the cell can transport (Cássio *et al.*, 1987) and whose presence therefore does not inhibit energy-yielding metabolism. Other acids such as acetic and formic are not only proton conductors, but inhibitory concentrations of their anions may occur within the cell (Corlett Jr. and Brown, 1980).

Biological and chemical systems are dependent upon an interaction between acids and bases. The microbial cell normally maintains the intracellular pH within a narrow range (near neutrality) which fluctuates very little over wide changes in extracellular pH. Yeast cells are known to maintain their intracellular pH within a narrow physiological range (between about 5.0 and 6.5) despite large decreases in external pH (Imai and Ohno, 1995). An alteration of this causes destruction of the microbial cell (Doores, 1983).

2.5.1 Importance of intracellular pH (pH_i)

Maintenance of intracellular pH (in microorganisms) within a narrow physiological range is essential for the optimal activity of a number of critical metabolic processes (Busa and Nuccitelli, 1984). Changes in intracellular pH are believed to be important in controlling the cell cycle, and rates of DNA and RNA synthesis appear to increase with higher pH_i within the normal physiological range (Madshus, 1988). While studying the role of intracellular pH in the regulation of cation exchanges in yeast, Ryan and Ryan (1972) concluded that pH_i was a major factor in the control of the K^+-H^+ exchange system, and that a second system involved in K^+-K^+ or K^+-Na^+ exchange was directly influenced by changes in the intracellular pH. In addition, key enzymes of glycolysis and gluconeogenesis are believed to be regulated by pH_i. As key enzymes in glycolysis and gluconeogenesis are regulated by cascade reactions of c-AMP dependent protein kinases (e.g. phosphofructokinase, phosphorylase, fructose 1,6-bisphosphatase), c-AMP plays an important role in this regulation. Moreover, c-AMP is regulated by pH_i (Imai *et al.*, 1994). Intracellular pH is also being recognized as having a role in signalling. Acidification of cytoplasm can interfere with signal transduction (Lambert and Stratford, 1999). Furthermore, pH_i is also thought to regulate other cellular responses, including induction of heat shock protein synthesis and thermotolerance (Bracey *et al.*, 1998).

2.5.1.1 Methods to measure intracellular pH

A number of methods have been employed to estimate pH_i. The most common method used is the equilibrium distribution of radioactively labelled weak-acid across the plasma membrane (Kotyk, 1963). This method is based on the assumption that only undissociated form of the acid can diffuse through the cell membrane and not the anion. There should be no binding, metabolism or active transport of the probe molecule. Although widely used, this method has limited application as an *in vivo* method because measurements are made in buffers following centrifugation steps. Other difficulties with this method include inhibitory effects (of weak acids) on cell physiology, compartmentalization of weak acids within the cell, and the method's limited temporal resolution (Busa and Nuccitelli, 1984).

Another method to estimate pH_i is by using pH-sensitive microelectrodes. This method is destructive (Madshus, 1988) and is clearly not suitable for studying large populations of organisms.

Measurement of pH_i can also be done using ^{31}P-nuclear magnetic resonance spectroscopy. This method is based upon the fact that ^{31}P-NMR chemical shift of intracellular orthophosphate is a function of cell pH (Salhany *et al.*, 1975). This is a non-invasive method, but a universal problem with this technique is the need to use high cell densities. The technique also calls for expensive equipment that is complicated to use (Busa and Nuccitelli, 1984).

Many attempts have been made to use pH-dependent fluorescent probes, or dyes, to measure pH_i in yeast (Slavik, 1982). The pH dependent fluorescence forms the basis of the method. Using probes such as carboxyfluorescein diacetate (CFDA) (Imai *et al.*, 1994), fluorescent techniques have the potential advantage of rapid, inexpensive, non-invasive determination of intracellular pH.

2.5.1.2 Effects of weak acids on the intracellular pH of yeasts

According to Kashket (1987), the intracellular pH of micro-organisms is usually more basic than the external medium, and it favors the ionization of the weak acid molecules (the undissociated form that diffuses through the cell membrane). This causes an acidification of the cytoplasm, and also results in inhibitions, especially of enzymes, by excesses of salt. He also reported that the organic acids and alcohols produced could act as protonophores in the membrane and accentuate the entry of protons.

The effect of an inorganic acid (phosphoric acid) and a weak organic acid (acetic acid) on the internal acidification of yeast cells was studied by Neal *et al.* (1965). They

concluded that acetic acid acidified the cell interior more effectively than phosphoric acid. At pH 4.0, formic, acetic and butyric acids all inhibited glycolysis in intact cells (Neal *et al.*, 1965). Studies on the mechanism of the antifungal action of benzoic acid at low external pH levels by Krebs *et al.* (1983) suggest that the toxic action of benzoic acid in cells of *S. cerevisiae* is mainly caused by the dissociation of the undissociated acid in the cytoplasm as a consequence of an internal pH that was greater than the pK_a of the acid. Therefore, the internal pH could decrease to values in the range at which phosphofructokinase is sensitive. The subsequent inhibition of glycolysis (due to loss in phosphofructokinase activity) could cause a fall in the concentration of ATP, which could restrict growth.

Pampulha and Loureiro-Dias (1989) studied the combined effect of acetic acid, extracellular pH and ethanol on the intracellular pH of fermenting yeast as determined by using the distribution of [1-^{14}C] propionic acid to measure pH_i. They reported that the internal pH did not depend on the concentration of total external acetic acid but only on the concentration of the undissociated form of the acid. Further, they found that ethanol accentuated the effect of acetic acid both with respect to inhibition of fermentation and internal acidification. Ionization constants (K_a) of weak acids are known to change with the nature of solvents (alcohols). Acetic acid in 10 % methanol has a pK_a of 4.904 at 25°C (Robinson and Stokes, 1968). This suggests that ethanol could increase the pK_a of acetic acid which would therefore result in higher amounts of undissociated acid (at a given pH) in the medium that can diffuse into the cell. This may be one of the reasons for an increased effect of acetic acid on internal acidification in the presence of ethanol.

Guldfeldt and Arneborg (1998) have described the effects of acetic acid and extracellular pH (pH_{ex}) on the intracellular pH (pH_i) of non-fermenting individual *S. cerevisiae* cells by using fluorescent microscopy and a perfusion system. These authors concluded that the decreases in pH_i at pH_{ex} 5.6 and 6.5 were significantly smaller than the decreases in pH_i at pH_{ex} 3.5 and 4.5, indicating that the decreases in pH_i were dependent on pH_{ex}. In *Z. bailii*, weak acids and hydrogen ions in different concentrations affected the intracellular pH value significantly. The lowest pH_i value measured was not at the most extreme, but at intermediate conditions of inhibition (Cole and Keenan, 1987).

2.5.1.3 Regulation of intracellular pH

The mechanisms involved in the regulation of intracellular pH are reviewed in detail by Madshus (1988). These include: Na^+/H^+ antiport, anion antiport, Na^+/HCO_3^- symport and H^+-translocating ATPases.

In yeasts, a proton-translocating H^+-ATPase, present in the plasma membrane is involved in the regulation of intracellular pH. In *S. cerevisiae*, the H^+-ATPase is most abundant, constituting over 20 % of the total membrane protein. The membrane H^+-ATPase couples ATP hydrolysis to the expulsion of protons, generating a proton gradient across the membrane (Serrano, 1984). This transmembrane proton gradient is essential for active transport of nutrients (such as maltose and amino acids) and thus growth (Eddy, 1982). Significantly, the plasma membrane H^+-ATPase has been shown to be rate limiting and essential for growth (Serrano *et al.*, 1986). Considering the high energy investment resulting from the proton pump working at its maximum capacity (under special conditions), usually ATPase activity is maintained at much lower values (Serrano, 1984). For example, when cells of *S. cerevisiae* were incubated in vivo with glucose, the plasma membrane H^+-ATPase activity increased as much as 10-fold (Serrano, 1983) which could be a consequence of intracellular acidification caused by glucose (Ramos *et al.*, 1989).

When weak-acid preservatives are present in the medium, the cellular response to inhibition may involve removal of preservatives by an efflux pump (Warth, 1989), although evidence for this is disputed (Cole and Keenan, 1987). Of greater importance is the plasma membrane H^+-ATPase (if the principal inhibitory effect of weak-acids is to reduce pH_i). The membrane H^+-ATPase has been shown to be involved in weak-acid resistance (Cole and Keenan, 1987) although, its role remains questionable given that if pH_i were raised by proton pumping, further weak-acid molecules would penetrate the cell and reacidify the cytoplasm. The plasma membrane H^+-ATPase is indeed increased during growth under acidic conditions (Eraso and Gancedo, 1987). These facts suggest that intracellular pH and plasma membrane H^+-ATPase play and important role in yeast physiology.

The changes in the activity of the plasma membrane H^+-ATPase could be due to the alteration in the membrane lipid composition, since it is known that changes in the lipid composition can significantly alter the activity of proteins in the plasma membrane (van der Rest *et al.*, 1995). The physical state of the bilayer including surface charge, density and fluidity can influence enzyme activity in a number of ways (Gennis, 1989). Plasma membrane H^+-ATPase has an absolute requirement for lipids in order to function (Serrano *et al.*, 1988) and the enzyme activity is a function of the type of lipid incorporated.

2.5.2 Mode of action of acetic acid and lactic acid on *S. cerevisiae*

Pampulha and Loureiro-Dias (1990) have studied two possible inhibition mechanisms in *S. cerevisiae*: either acidification of the cytoplasm or the action of acetic acid

directly on transport or enzymatic activities. These authors reported that enolase was the most affected enzyme by acetic acid, and that this resulted in alteration of glycolysis.

The maximum specific growth rate and yield coefficient of a respiratory deficient mutant of *S. cerevisiae* decreased whereas the specific rate of glucose consumption increased in the presence of acetic acid in batch cultures (Pampulha and Loureiro-Dias, 2000). They also observed a decrease in cell yield from 14 to 4 g biomass/mole ATP when acetic acid concentration increased from 0 to 170 mM. The authors concluded that about 1 mole ATP is consumed for every mole of acetic acid diffusing into the cells.

Not much research has gone into understanding the mode of action of lactic acid on yeast. Being a short chain acid, lactic acid should be expected to act in a manner similar to acetic acid (Eklund, 1989). However, studies on the mode of action of acetic acid and lactic acid on yeasts indicate that they may not act in the same manner on the cell, as Maiorella *et al.*, (1983) reported that acetic acid interference with yeast metabolism resulted in an increase in ATP requirement for cell maintenance whereas "the exact mechanism of lactic acid inhibition is probably different than that of acetic acid". Data for the action of acetic, lactic and propionic acids on yeasts showed growth inhibition different from that predicted on the basis of dissociation constants and also indicated that these acids may not act in the same manner (Moon, 1983).

2.6 Management of bacterial contaminants in alcohol production

The methods used in the alcohol fermentation industry to control contaminant bacteria include stringent cleaning and sanitation, acid-washing of yeast destined for reuse (in case of breweries), adjustment of mash pH, and the use of antibiotics during fermentation. The method(s) used depends to a large degree on the end use of the alcohol.

Penicillin G is the most common antibiotic and has been used in the alcohol production industry during fermentation since the '50s to control bacterial contaminants (Aquarone, 1960). Day *et al.* (1954) have studied different antibiotics as contamination control agents in grain alcohol fermentations. They found penicillin to be more effective in inhibiting bacterial growth than other antibiotics such as aureomycin, bacitracin, chloromycetin and terramycin. Tyrothricin, streptomycin and polymixin were found to be ineffective. Penicillin G, the first β-lactam antibiotic discovered, is primarily active against Gram-positive bacteria. Its target is the transpeptidation reaction involved in the cross-linking step of peptidoglycan biosynthesis in the Gram-positive bacteria. This antibiotic does not inhibit yeast activity even when present at concentrations as high as 500,000 units/L (Aquarone, 1960).

Kheirolomoom *et al.* (1999) analyzed the stability of penicillin G as a function of temperature, pH, and the combined effects of pH and temperature; and derived a second-order polynomial model for penicillin G decomposition reaction rate constant. The authors reported that penicillin G was more stable at the pH range 5.0 - 8.0 and lower temperatures (temperature range used: 0-52°C). It was less stable at acidic pH than basic pH values. They also found that the stability of penicillin G decreased with increase in temperature for all pH values (pH range tested: 1.8-10.0).

The emergence of resistant microflora (to penicillin G) and the instability of penicillin G at acidic pH values have resulted in the use of other antibiotics such as penicillin V, monensin, tetracycline (Aquarone, 1960) and virginiamycin (Hynes *et al.*, 1997) either individually or as mixtures. Virginiamycin, since is more stable in acidic pH (unlike penicillin G), was investigated for its effectiveness on the contaminants in alcohol production (Hynes *et al.*, 1997). The authors concluded that the use of virginiamycin reduced potential ethanol yield losses of up to 11 % of the produced ethanol. They also found that the effectiveness of virginiamycin varied among the strains tested, and was reduced over prolonged incubation in wheat mash (especially in the absence of yeast).

Stroppa *et al.* (2000) studied the effects of the antibiotics penicillin and monensin on contaminants of alcohol-fermentations. They observed a decline in viable cell numbers of all the strains tested in the presence of either antibiotic (although the reduction of *L. plantarum* was in the magnitude of 1 or 2 logs in 24 - 48 h), at all the three concentrations applied (1.0, 2.0, 4.0 ppm and 1.5, 3.0, 6.0 ppm of penicillin and monensin, respectively). These authors proposed the use of these two antibiotics in combination since penicillin was most effective over short contact times, and monensin more efficient with prolonged contact.

The concept of antibiotic use in an industrial process is in question in spite of absence of antibiotic residues in the spent grains subsequent to distillation. The emergence of antibiotic-resistant bacteria as a result of antibiotic misuse (Khachatourians, 1998) is now becoming a serious threat in human health. This provides an incentive to examine other antimicrobials (to control bacterial contaminants in alcohol production) which are not antibiotics.

2.6.1 Chlorine dioxide (ClO_2)

Chlorine dioxide has been used for decades in the disinfection of drinking water (Johnson and Kunz, 1998). ClO_2 is more potent than chlorine as a disinfectant. It lacks flavor profile, odors and corrosiveness typical of chlorination methods (Johnson, 1997). It acts as an oxidizing agent. Chlorine dioxide has potential antimicrobial properties and is

sometimes used to sanitize equipment in breweries. A 50 - 100 ppm solution of ClO_2 is used for post-rinse sanitation (Johnson, 1997). Johnson and Kunz (1998) reported the use of chlorine dioxide as a yeast-washing agent. They observed that ClO_2 cleared the two yeast slurries tested of bacteria even at the 13 ppm level, without decreasing the viability of yeast. These properties of chlorine dioxide provide a chance to evaluate its use to control contaminating bacteria during the fermentation of grain mashes.

2.6.2 Nisin

Nisin is a polypeptide bacteriocin (lantibiotic) of 34 amino acids produced by *Lactococcus lactis* subsp. *lactis*. It has an amphipathic character. The N-terminal part of the nisin molecule contains relatively large number of hydrophobic residues, whereas the C-terminal part is hydrophilic. Nisin is cationic due to the presence of three lysine residues and one (in nisin Z) or two (in nisin A) histidine residues, and the absence of glutamate and aspartate. The nisin molecule exhibits greatest stability under acid conditions (Hurst, 1981).

Since its discovery about 70 years ago, nisin has proven to be an effective inhibitor of a broad spectrum of Gram-positive bacteria (Delves-Broughton, 1990). It also inhibits the outgrowth of spores of bacilli and clostridia (Hurst, 1981). Even the Gram-negative bacterium *E. coli* becomes sensitive to nisin when its outer membrane is made permeable by osmotic shock. The inhibition of other Gram-negative bacteria using nisin can be achieved by the addition of an agent which modifies and chelates the outer membrane, such as Ethylenediaminetetraacetic acid (EDTA) (Dielbandhoesing *et al.*, 1998).

The primary target of nisin in sensitive bacteria is the energy-transducing cytoplasmic membrane. Addition of nisin results in an efflux of essential small cytoplasmic components (amino acids, monovalent cations, ATP), disruption of proton motive force and cessation of biosynthesis (Bruno *et al.*, 1992). However, nisin does not inhibit yeasts or filamentous fungi (Delves-Broughton, 1990). These organisms have a rigid cell wall, a complex structure consisting of glucan cross-linked with chitin and cell wall proteins. In *S. cerevisiae*, the cell wall protein 2 (Cwp2p) plays a prominent role in the protection of cells against antimicrobial peptides such as nisin (Dielbandhoesing *et al.*, 1998).

Nisin has therefore been tried, and shown to have potential in controlling spoilage lactic bacteria in beer (Ogden, 1986; Ogden and Waites, 1986; and Ogden *et al.*, 1988), and wine (Radler, 1990a,b). In the brewing industry, nisin finds its application also in washing pitching yeast as an alternative method to acid washing (which affects yeast viability) (Ogden, 1987). It has also been indicated by these researchers that the yeasts are

completely unaffected. This suggests the possibility of introducing nisin during the fermentation to control bacterial contaminants.

2.6.3 Hydrogen peroxide/urea hydrogen peroxide

The physiological differences between yeast and lactobacilli suggest the use of hydrogen peroxide to manage these bacteria in mashes used for alcoholic fermentations. Literature concerning the antibacterial effects of hydrogen peroxide covers a period of over 100 years. Lactobacilli lack the enzyme catalase which decomposes hydrogen peroxide and therefore they are unable to eliminate its toxic effect. In an attempt to evaluate sulfite and hydrogen peroxide as bacterial-contamination control agents, Chang *et al.* (1997) have reported that the viability of *L. fermentum* could be selectively controlled by hydrogen peroxide at concentrations of 1 to 10 mM in a cell-recycled ethanol fermentation process.

For maximal bactericidal activity, hydrogen peroxide should be electrolytically pure and allowed to come into contact with only stainless steel or other corrosion-resistant materials (Luck, 1956). At higher temperatures, bactericidal efficiency of hydrogen peroxide increases (Amin and Olson, 1967). The stability of the compound also decreases as pH increases (Luck, 1956). Moreover, in contact with organic matter, hydrogen peroxide breaks down into nascent oxygen and water. To avoid the problem of instability, Banerjee (1947) prepared an adduct of hydrogen peroxide and urea in his laboratory and claimed that this compound, urea hydrogen peroxide, was perfectly stable in dry state at ordinary temperatures. Urea hydrogen peroxide has since been used as an antiseptic for topical application on wounds, and against gingivitis and dental plaque (Zinner *et al.*, 1978; Etemadzadeh, 1991).

3. MATERIALS AND METHODS

3.1 Studies on the effects of lactobacilli and their endproducts, lactic acid and acetic acid, on yeast growth and alcohol production

3.1.1 Organisms used

3.1.1.1 Bacteria

Twelve species of lactobacilli were screened for growth rates in deMan, Rogosa, Sharpe (MRS) broth (Unipath, Nepean, ON, Canada) as described by Casey and Ingledew (1981) at 30° C, and for alcohol tolerance. Five species capable of extensive growth in wheat mash within 36 h and tolerance to greater than 10 % (v/v) ethanol were selected for further study so that information gained would be of use to the alcohol industry. Two species were obtained from Centro de Technologia Copersucar, Piracicaba, SP, Brazil and were tentatively identified to species and numbered biotype by API 50 CHL test kits (bioMérieux, Montreal, PQ, Canada) as *L. plantarum* 1 and *Lactobacillus paracasei* ssp. *paracasei* 2 (called *L. paracasei* hereafter). Two other strains, *Lactobacillus rhamnosus* (ATCC 15280) and *L. fermentum* (ATCC 14931), were obtained from the American Type Culture Collection, Rockville, MD. The fifth strain was an industrial isolate labelled Cargill # 3 from Cargill Corn Milling (Eddyville, IA). An API 50 CHL test kit for *Lactobacillus* identified this latter strain as *L. paracasei* ssp. *paracasei* 2 but it differed in microscopic and colony morphology when compared to the *L. paracasei* strain obtained from Copersucar. Therefore, for the purpose of this work, it was designated as *Lactobacillus* # 3. All lactobacilli used are homofermentative except for *L. fermentum*.

3.1.1.2 Yeast

Two strains of *S. cerevisiae* were used in this study. One was an isolate purified from an industrial preparation of active dry yeast marketed to fuel alcohol plants and potable alcohol distilleries by Alltech Inc. (Nicholasville, KY), and the other, an alcohol tolerant, temperature tolerant yeast used for ethanol production from sugarcane or sorghum (ATCC 26602, American Type Culture Collection, Rockville, MD) which was isolated from a sugar refinery in England. (Two yeast strains, both of commercial significance, were used in order to ensure that the lineage of the selected strains and any resultant genetic

diversity would not influence the physiological findings and events described in this thesis).

3.1.2 Composition of the medium used to study the effects of acetic and lactic acids on yeast

A chemically defined (minimal) mineral salts medium with glucose (2 % w/v) and vitamins was used (Thomas *et al.*, 1998). The final concentrations of ingredients in the medium were : (mmoles/L) $(NH_4)_2SO_4$, 37.85; K_2HPO_4, 0.86; KH_2PO_4, 6.83; $MgSO_4$, 2.03; NaCl, 2.05; and (µmoles/L) H_3BO_3, 24; $MnSO_4$, 20; Na_2MoO_4, 1.5; $CuSO_4$, 10; $CoCl_2$, 1.5; $ZnSO_4$, 100; KI, 1.8; $FeCl_3$, 100; $CaCl_2$, 82; and (µg/L) biotin, 200; calcium pantothenate, 2000; folic acid, 20; myo-inositol, 10,000; niacin, 400; pyridoxine HCl, 400; riboflavin, 200; thiamine HCl, 200. The vitamin solution was prepared as a 1,000 fold concentrated stock and kept frozen at -20° C. When needed, an aliquot was thawed and filter-sterilized (0.2-µm membrane filter), and the required amount was added to the medium.

3.1.3 Preparation of bacterial inocula

Lactobacilli were grown in 250 ml screw-capped side-arm Erlenmeyer flasks containing 50 ml of MRS broth. Then, 4 ml of late log phase culture were transferred to 1 L screw-capped flasks containing 200 ml of MRS broth. The headspace of each flask was flushed with filter-sterilized CO_2 gas (0.22 µm membrane filter) and incubated at 30° C in a # G25 Controlled Environmental shaker (New Brunswick Scientific Co. Inc., Edison, NJ) at 150 rpm. Growth of these organisms was measured by a Klett-Summerson colorimeter (Klett Mfg. Co., New York, NY) with a number 66 red filter (420 - 660 nm) and the time for growth to early stationary phase was determined. A relationship between Klett units and the number of colony forming units (CFU) /ml in mid-log phase cultures was established for each strain.

Bacterial cells (1,000 ml) were aseptically harvested by centrifugation at 10,200 × g for 15 min at 4° C (Sorvall RC 5C centrifuge; GSA rotor, Sorvall Instruments, Division of Du Pont, Newtown, CT). The pellet was washed twice with sterile 0.1 % w/v peptone water (Difco laboratories, Detroit, MI) and the cells were then resuspended in 50 ml of sterile 0.1 % w/v peptone water and chilled in ice until dispensed into fermentors.

3.1.3.1 Inoculation of bacteria to wheat mashes

Appropriate volumes of the 20 fold concentrated cell suspension were added to 500 ml quantities of mash in fermentors to give final viable bacterial cell numbers of ~10^5, 10^6,

10^7, 10^8 and 10^9 cells (CFU) /ml. Volumes of inoculation were always made equal with sterile 0.1 % w/v peptone water to keep dilution of nutrients the same. In control mash fermentations, bacteria were not inoculated, but yeast were added at ~10^6 viable cells/ml. Growth and metabolism of bacteria in the absence of yeast were studied by inoculating mashes with ~10^7 CFU/ml.

3.1.4 Preparation of yeast inoculum for fermentations

Eleven grams of *S. cerevisiae* active dry yeast (ADY) ("Allyeast Superstart", Alltech Inc., Nicholasville, KY) were dispersed into 99 ml of pre-warmed (38° C), sterile, 0.1 % (w /v) peptone water and incubated at 38° C for 20 min with periodic shaking. Aliquots (0.25 ml) of this suspension were added to each fermentor to obtain ~10^6 viable yeast cells /ml.

3.1.5 Mashing of wheat and fermentation

Commercial red spring wheat bought from a local supplier was ground at setting # 5 on a S 500 Disk Mill (Glen Mills Inc., Clifton, NJ). For mashing, 19 L of distilled water containing 1 mM $CaCl_2 \cdot 2H_2O$ was warmed to 60° C in a jacketed steam kettle. Seven kg of ground wheat was slowly added followed by 35 ml of high temperature (HT) α - amylase (Alltech Inc.). After 5 min, the temperature was raised to 90 - 95° C and held for 45 min with stirring to gelatinize starch. It was then cooled to 80° C by passing cold water through the jacket of the kettle and a second 35 ml dose of HT α - amylase was added. The mash was held for 30 min at this temperature to complete liquefaction of the gelatinized starch. The mash was strained under aseptic conditions through a sterile stainless steel food grade sieve (1.5 mm pore diameter), distributed into sterile bottles and frozen at -40° C. Three days prior to fermentation, the mash was thawed and 500 ml quantities were aseptically transferred to sterile, jacketed 1 L Celstir bioreactors (fermentors) (Wheaton Instruments, Millville, NJ). Diethyl pyrocarbonate (DEPC) (Sigma Chemical Co., St. Louis, MO) was then added at a concentration of 0.01 % v/v to sterilize the mash. The fermentors were cooled immediately to 4° C by connecting them to a refrigerated water bath circulator and then stored at this temperature for 48 h.

The fermentors were connected to a water bath circulator maintained a 30° C and stirred magnetically (IKA-Labortechnik, Staufen, Germany). One ml of filter-sterilized urea was added to each of the fermentor to give a final concentration of 8 mM (Jones and Ingledew, 1994). Saccharification of dextrins to glucose was carried out by adding 0.8 ml glucoamylase (Allcoholase II, Alltech Inc.) per fermentor, 30 min before inoculation with yeast. Just prior to yeast inoculation, fermentors were contaminated (inoculated) with

bacteria at the levels mentioned above. The temperature was maintained at 30° C throughout the fermentation. Samples were withdrawn for analysis from each fermentor every 6 h for the first 24 h and then at 36, 48 and 72 h.

3.1.5.1 Assay methods

3.1.5.1.1 Viable counts of bacteria and yeast

Viable cell counts were monitored by the membrane filtration technique (Ingledew *et al.*, 1980). For enumeration of yeast cells, the membranes were incubated aerobically at 30° C on the surface of YPD plates (yeast extract, 10 g/L; peptone, 10 g/L; dextrose, 20 g/L; and agar, 15 g/L) supplemented with 0.005 % (w/v) gentamycin and 0.01 % (w/v) oxytetracycline (Sigma Chemical Co.) to suppress the growth of bacteria. The plating was done in triplicate for each dilution used.

Viable counts of bacteria on membrane-filtered samples were obtained by placing the filters on plates of MRS agar containing 0.001 % (w/v) of cycloheximide (Sigma Chemical Co.) to inhibit the growth of yeast, and incubating in a CO_2 incubator (National Appliance Co., Portland, OR) at 30° C after 2 cycles of evacuating and refilling with commercial-grade (>99.5 percent) CO_2. The results were expressed as colony forming units (CFU) /ml.

3.1.5.1.2 Determination of dissolved solids

Portions of samples were centrifuged at 10,300 × g for 30 min and the supernatants were collected and stored at -20° C until analysed. Total dissolved solids in these supernatants were determined by measuring the specific gravity at 20° C with a DMA45 density meter (Anton Parr KG, Graz, Austria). The readings were converted to grams of dissolved solids per 100 ml.

3.1.5.1.3 HPLC analysis

Ethanol and lactic acid were determined by High Performance Liquid Chromatographic (HPLC) analysis. A 5 µl aliquot from a suitably diluted supernatant fraction of fermentation sample was analysed using a FAM-PAK™ column which analyses sugars, alcohols and organic acids (Waters Chromatographic Division, Milford, MA) maintained at 65° C. Orthophosphoric acid (1.5 mM) was used as the mobile phase at a flow rate of 1 ml/min. Methanol was used as the internal standard. In another portion of the study dealing with the effects of acetic and lactic acids on yeast, a HPX-87H column (Bio-Rad laboratories Ltd., Mississauga, Ontario, Canada) which analyses sugars, alcohols, and organic acids maintained at 40° C was used. Sulphuric acid (5 mM) was used as the mobile phase at a flow rate of 0.7 ml/min. Boric acid (2 % w/v) was used as

the internal standard. The components were detected with a differential refractometer (Model 410, Waters Chromatographic Division). The data was processed using the Maxima 810 computer program (Waters Chromatographic Division).

3.1.6 Growth conditions for studying the effects of acetic acid and lactic acid on yeast

Growth was measured turbidometrically using a Klett-Summerson colorimeter equipped with a number 66 red filter (420 to 660 nm). Calibration curves of Klett units plotted against cell number and cell mass were constructed. Starter cultures were grown with shaking (100 rpm) (Model G25 Controlled Environmental Shaker) at 30° C for 24 h, in 50 ml of minimal medium (pH 4.5) in 250 ml Erlenmeyer flasks. Then, ~ 2×10^7 cells of the Alltech yeast strain and ~ 4.5×10^7 cells of *S. cerevisiae* ATCC 26602, respectively, were inoculated into experimental flasks and grown at 30° C with shaking (100 rpm). The flasks used were 250 ml screw-capped, side-arm, Erlenmeyers with 50 ml medium and a range of concentrations of the acids, (0, 0.1, 0.2, 0.3, 0.4 and 0.5 % w/v for acetic acid and 0, 0.2, 0.4, 0.6, 0.8, and 1.0 % w/v for lactic acid). Experiments were done in duplicate. The specific growth rates (μ in h^{-1}) and lag times (h) were calculated for both yeast strains at various concentrations of both acetic and lactic acids.

3.1.6.1 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of each acid for both yeast strains was determined. For this work, MIC was defined as the lowest chosen concentration of the acid that inhibited yeast growth for a period of at least 72 h. The concentrations of acetic and lactic acids tested were 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.9 % w/v; and 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 % w/v respectively. Each concentration was tested in duplicate.

3.1.7 Determination of fermentation rates at various concentrations of acetic and lactic acids

The yeast strains were grown in minimal medium with 2 % w/v glucose and different concentrations of the acids (0, 0.1, 0.2 and 0.3 % w/v acetic acid; and 0, 0.2, 0.4, and 0.6 % w/v lactic acid). Samples were withdrawn at 3, 6, 9, 12, 15.5, 20 and 24 h, filtered through a 0.45 µm filter, and analysed for glucose consumed and ethanol produced using HPLC.

3.1.8 Experimental design for the evaluation of the interactions between acetic and lactic acid

The experiment was planned and conducted using response surface central composite design (Cochran and Cox, 1957) for two variables at 5 levels (Table 3.1a & 3.1b). The maximum concentrations of acetic acid and lactic acid selected were based on the criteria that they should not completely inhibit the metabolic activity of the yeasts studied. Two replicate experiments were conducted. There were 13 treatment combinations of the two acids, including five centre points. The growth of the yeasts was monitored as a measure of turbidity in each of the 13 experimental flasks for 24 h at 3 h intervals. The specific growth rates were calculated from the exponential phases of growth.

3.1.8.1 Statistical analysis of data

Data was analysed using the General Linear Model of SAS® (SAS Institute, 1988). Estimates for the linear, quadratic and interaction effects of each acid (i.e. acetic and lactic acid) were developed which fit the following equation :

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \epsilon$$

where y is the specific growth rate (μ) at a certain level of acetic and lactic acid, x_1 is the concentration of acetic acid, x_2 is the concentration of lactic acid, β_n is the parameter estimates, β_0 is the estimate for the y-intercept, β_1 is the estimate for the linear effect of acetic acid concentration, β_2 is the estimate for the linear effect of lactic acid concentration, β_{11} is the estimate for the quadratic effect of acetic acid concentration, β_{22} is the estimate for the quadratic effect of lactic acid concentration, β_{12} is the estimate for the interactive effect between acetic acid and lactic acid, and ϵ is the error term.

3.1.9 Measurement of Intracellular pH

The intracellular pH method (Imai et al., 1994) was followed with slight modifications. This is a truly non-invasive *in vivo* fluorometric method the modification of which avoids many centrifugation steps and resuspensions in buffers. In this method, yeast cell suspensions are treated with esterified 5(6)-carboxyfluorescein {5(6)-carboxyfluorescein diacetate} which is not fluorescent by itself. This compound once inside the yeast cells is hydrolyzed by esterases in the cytosol to yield fluorescent 5(6)-carboxyfluorescein. The intracellular pH of yeast was determined using a calibration curve which showed a linear relationship between pH and fluorescence intensity.

Table 3.1a Levels of acetic acid and lactic acid corresponding to coded values as designated by the central composite design (Alltech strain)

Independent Variable	Code level				
	- 1.414	- 1	0	+ 1	+ 1.414
Acetic acid (% w/v)	0	0.037	0.125	0.213	0.25
Lactic acid (% w/v)	0	0.07	0.25	0.43	0.5

Table 3.1b Levels of acetic acid and lactic acid corresponding to coded values as designated by the central composite design (ATCC 26602)

Independent Variable	Code level				
	- 1.414	- 1	0	+ 1	+ 1.414
Acetic acid (% w/v)	0	0.051	0.175	0.299	0.35
Lactic acid (% w/v)	0	0.102	0.35	0.598	0.7

The cells grown with various concentrations of acetic or lactic acid in the medium were harvested (3,000 × g for 5 min at 20° C) in the mid-exponential phase. Appropriate amounts of the cultures were harvested to yield 1-ml pellets of yeast. Pellets were washed twice with sterile double distilled water at room temperature (20° C) and resuspended in double distilled water and the volume was adjusted to 3.0 ml. Then, 0.3 ml of 5(6)-carboxyfluorescein diacetate (10 mM dimethyl sulfoxide solution) was added to the yeast suspension. The solution was immediately shaken vigorously for 1 min and kept on ice. After 15 min, it was mixed again and allowed to stand for another 15 min. Yeast loaded with 5(6)-carboxyfluorescein was washed three times with sterile double distilled water. The resulting pellet was resuspended in fresh double distilled water and the volume made up to 2.0 ml. An aliquot of the suspension (0.3 ml) was added to 5.0 ml of double distilled water. (This can be kept in an ice bath for about 1-2 h after centrifugation). The suspension was transferred to a quartz cuvette and the fluorescence intensity measured using a fluorescence spectrophotometer (model F-2000; Hitachi, Ltd. Tokyo, Japan) at an emission wavelength of 518 nm and excitation wavelengths of 441 and 488 nm. After measuring the fluorescence intensity at both the excitation wavelengths, the cells were removed and the fluorescence of water was measured (background fluorescence) at both the wavelengths and was subtracted. This experiment was repeated three times for both the strains at each concentration of acetic or lactic acid.

3.1.9.1 Intracellular pH estimation

A calibration curve was made by using 5(6)-carboxyfluorescein in the indicated pH buffer (50 mM citrate disodium hydrogen phosphate buffer; pH 6.4, 6.2, 6.0, 5.8, 5.6, 5.4, 5.2, 5.0 and 4.8). It was constructed by plotting the ratio of fluorescent intensities (emission wavelength 518 nm) at excitation wavelengths of 488 and 441 nm as a function of pH (Fig. 3.1). Intracellular pH was calculated using this calibration curve.

3.1.10 Activity of plasma membrane ATPase

To study the effect of acetic and lactic acids on the plasma membrane ATPase of *S. cerevisiae*, the activity of the ATPase was determined in the total membrane fraction prepared from cells grown in the presence of increasing concentrations of acetic or lactic acid and harvested (3,000 × g for 5 min at 4° C) in the mid-exponential phase according to the method of Rosa & Sa-correia (1991).

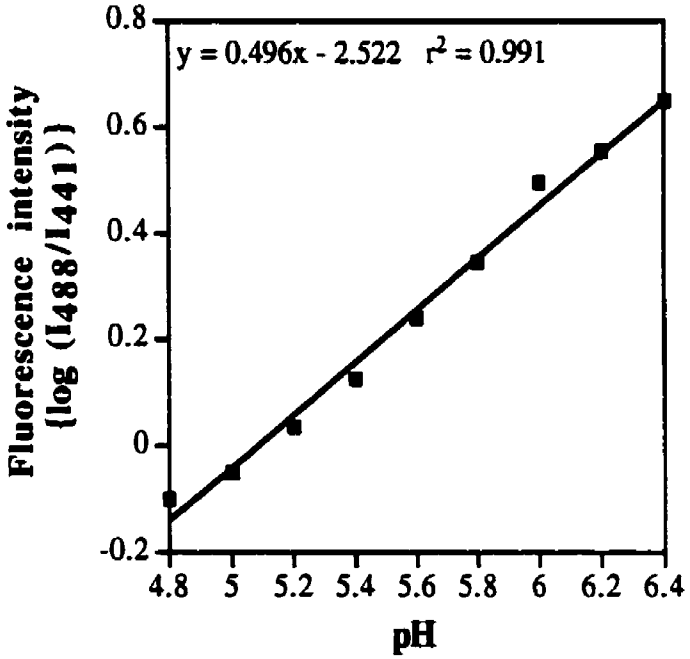


Figure 3.1 Relationship between pH and the logarithmic ratio of fluorescence intensities measured at 518 nm after excitation at 441 and 488 nm of 5(6)-carboxyfluorescein in the indicated pH buffer.

3.1.10.1 Preparation of the total membrane fraction

The cell suspensions, in aliquots of 2 ml, were disintegrated with 1.5 ml of glass beads (Sigma; 0.5 mm diameter) by mixing with a vortex mixer for 1 min and placing on ice for 1 min (repeated eight times). The homogenates were diluted with 5 ml of a medium containing 0.33 M sucrose, 0.1 M Tris (adjusted to pH 8.0 with HCl), 5 mM EDTA, and 2 mM dithiothreitol. Supernatants collected by centrifugation at 4° C for 3 min at 1,000 × g, were recentrifuged for 45 min at 40,000 × g (Sorvall SS-34 rotor) at 4° C. The total membrane fraction was resuspended in a medium containing 20 % glycerol, 10 mM Tris (adjusted to pH 7.5 with HCl), 0.1 mM EDTA, and 0.1 mM dithiothreitol. Protein concentrations of the membrane fractions were determined using the Bio-Rad protein assay kit II (Bio-Rad laboratories Ltd.).

3.1.10.2 ATPase assay

The ATPase activity of each total membrane fraction was determined in the assay medium containing 50 mM 2-(N-morpholino) ethanesulfonic acid (MES) (pH 5.7, adjusted with NaOH), 10 mM $MgSO_4$, 50 mM KCl, 5 mM sodium azide (to inhibit mitochondrial ATPase), 0.2 mM ammonium molybdate (to inhibit acid phosphatases) and 100 mM KNO_3 (to inhibit vacuolar ATPase). Under these conditions, the observed ATPase activity is reported to be predominantly that of plasma membrane origin (Rosa & Sa-correia, 1991). After 5 min of thermostabilization of the assay mixture at 30° C, the reaction was started by the addition of a concentrated solution of ATP (final concentration 10 mM). After 5 min, the reaction was stopped by the addition of 0.5 ml of trichloroacetic acid (10 % w/v) at 4° C to 0.5 ml of the reaction mixture. The membranes were then separated by centrifugation (10,000 × g for 5 min at room temperature). The concentration of inorganic phosphate (P_i) liberated was determined using an inorganic phosphorus estimation kit (Sigma). The ATPase activity measured was expressed in µmol P_i released/min/mg protein.

3.1.11 Lipid extraction

Lipids from yeast cells were extracted according to the method used by Kemp *et al.* (1975). Yeast cells were harvested by centrifugation and washed three times with deionized water. The washed cells were resuspended in 1 ml of deionized water. Ten ml of chloroform : methanol : 5 M HCl (5:6:1 by volume) containing 50 mg of butylated hydroxy toluene (BHT) per litre were added to 1 ml of cell suspension. After 20 min at room temperature, 22 ml of deionized water were added and mixed thoroughly. The lower chloroform-rich phase was then taken to dryness in a rotary evaporator. The lipids were hydrolysed by refluxing for 1 h with 40 ml of 6 % w/v KOH in 95 % v/v ethanol. After cooling, an equal amount of deionized water was added. The mixture was extracted three times with half a volume of petroleum ether to remove unsaponified material. The mixture was then acidified with HCl (to pH 2.0) to liberate free fatty acids and extracted three times with petroleum ether. These extracts were pooled together and taken to dryness in a rotary evaporator. The lipid sample was dissolved in 2 ml of hexane.

3.1.11.1 Preparation and analysis of fatty acid methyl esters

Lipid extracts were transferred to test tubes (100 mm × 10 mm) with teflon-lined screw caps. One-ml solutions of heptadecanoic acid in n-hexane (10.88 mg/ml) were added as the internal standard. Hexane was evaporated off by heating the tubes under nitrogen gas on a heating block (80° C). The tubes were cooled to room temperature, and 2 ml of methanolysis reagent (sulphuric acid : methanol : toluene; 1:20:10 by volume) was added and capped tightly. The tubes were then heated at 100° C on a heating block for 30 min and cooled to room temperature. To this, 2 ml of deionized water was added followed by 2 ml of n-hexane. The contents of the tube were mixed thoroughly. The organic layer was removed using a Pasteur pipette and dried over anhydrous sodium sulphate (2.0 g). Aliquots of 0.5 ml were dispensed into autosampler vials, sealed and stored at -70° C. The fatty acid methyl esters were analyzed by gas chromatography (Hewlett Packard 5890 series II plus) under the following conditions : injection temperature 250° C, oven temperature 170° C, detector temperature 300° C; carrier gas (H_2) 30 ml min^{-1}, air 400 ml min^{-1}, nitrogen gas (AuxGas) 30 ml min^{-1}; and a methylphenyl silicone fused silica capillary column (25 mm × 0.2 mm; HP19091B-102).

3.2 Management of lactobacilli in yeast-catalyzed alcohol production

3.2.1 Penicillin G

3.2.1.1 Assessment of the bactericidal effect of penicillin G in wheat mash

Normal gravity (20 - 21 g /100 ml dissolved solids) wheat mash (pH 5.5) was prepared and distributed into ten, 1 L jacketed glass fermentors in 500 ml quantities. The fermentors were connected through a circulating waterbath which was maintained at a temperature of 30° C throughout the experiment. Filter sterilized urea was added to the mashes so that the final concentration of urea was 8 mM. Glucoamylase (Allcoholase II) (0.4 ml) was added to all the fermentors for saccharification. The lactobacilli included in the study were *L. plantarum*, *L. paracasei*, *Lactobacillus* #3, *L. rhamnosus*, and *L. fermentum*. Penicillin G at a concentration of 1.5 mg/L (2,475 units/L) was added to each treatment. All treatments were studied in duplicate.

Bacteria were grown in MRS broth, harvested by centrifugation (10,200 × g for 15 min) at 4° C and resuspended in sterile 0.1 % w/v peptone water and stored in ice until used. The mashes were deliberately "infected" with the respective bacteria by inoculating appropriate quantities of the cell suspensions to the respective treatments so that they would have an initial bacterial load of approximately 10^7 CFU/ml. Samples were withdrawn from all the fermentors at 0, 12, 24, 36, 48 and 72 h to study the survival of these bacteria by enumerating the colony forming units using membrane filtration.

3.2.1.2 Use of penicillin G in the fermentation of wheat mash

Penicillin G is not stable at acidic pH values at 5 and lower. In order to to see if penicillin G performed similarly at both the pH conditions (4.5 and 5.6), a set of experiments were carried out using normal gravity wheat mash (pH 5.6), and wheat mash with its pH adjusted to 4.5 using 1 N sulfuric acid. The mashes were prepared and distributed into eight, 1 L jacketed glass fermentors in 500 ml quantities. Filter sterilized urea was added to the mashes so that the final concentration of urea was 8 mM. The treatments included a control with no bacteria and no penicillin G, a control with no bacteria but with penicillin G at 1.5 mg/L, bacteria inoculated at ~ 10^7 CFU/ml mash and penicillin G added at 1.5 mg/L, and bacteria inoculated at ~ 10^7 CFU/ml mash (no penicillin G). All treatments had yeast inoculated at approximately 10^6 CFU/ ml.

L. paracasei was grown in MRS broth, harvested by centrifugation (10,200 × g for 15 min) at 4° C and resuspended in sterile 0.1% w/v peptone water (slurry was stored in ice). Glucoamylase (Allcoholase II) (0.4 ml) was added to all the fermentors for

saccharification. The mashes to be contaminated with bacteria were inoculated with *L. paracasei* to give approximately 10^7 CFU/ml. Penicillin G (1.5 mg/L) was added to treatments where needed. The fermentors were inoculated with yeast (ADY) at approximately 10^6 CFU/ml, 30 min after the addition of glucoamylase and the fermentations were carried out at 30° C. Then, samples were withdrawn from all the fermentors at 0, 12, 24, 36, 48 and 72 h; and analysed for viable numbers of bacteria and yeast (by membrane filtration), dissolved solids (using the DMA45 digital density meter), ethanol and lactic acid (using HPLC).

3.2.2 Stabilized chlorine dioxide

Sanitech (product of Alltech Inc., Nicholasville, KY) releases chlorine dioxide under acidic conditions which has potent antimicrobial properties. It acts as an oxidizing agent. This is used for sanitizing equipment in breweries.

3.2.2.1 Determination of the bactericidal effect of chlorine dioxide

The bactericidal effectiveness of chlorine dioxide was tested against five lactobacilli species which are resistant to low pH and to alcohol concentrations of 10 % v/v or more (*L. plantarum, L. paracasei, Lactobacillus* # 3, *L. rhamnosus* and *L. fermentum*). This study was carried out at various pH levels to find if the effect of chlorine dioxide was affected by the pH of the media. It has been recommended (by the manufacturer) that chlorine dioxide is more effective when used at pH 4 - 5. Since the mash pH is about 5.5, the range of pH levels studied were 4.0, 4.5, 5.0, 5.5 and 6.0. (The mash pH is taken into consideration because the ultimate objective was to see the effect of chlorine dioxide on contaminant bacteria when added directly to fermentation mashes).

Fifty ml quantities of MRS broth in 250 ml screw-capped, side-arm Erlenmeyer flasks were used in this study. After adjusting the pH with 0.1 N HCl, the media were sterilized at 121° C for 20 min. The media were inoculated with 1 ml of the bacterial culture grown to late log phase (~ 10^9 CFU/ml). Chlorine dioxide at 100 ppm (as recommended by the manufacturer) was added to the flasks. Controls (no chlorine dioxide) were also included at all the pH levels. All the treatments were done in duplicate. The flasks were incubated at 30° C in an incubator-shaker (150 rpm) and the growth was monitored over time using the Klett-Summerson colorimeter (as a measure of turbidity) at 420 - 660 nm. Later, similar set of experiments were carried out at pH 5.5 with lower concentrations of chlorine dioxide (0, 40, 50, 60, 70, 80, and 90 ppm) against all the five lactobacilli at 30° C since it was found that chlorine dioxide at 100 ppm greatly affected the growth rate of yeast.

3.2.2.2 Determination of the dose of chlorine dioxide to be used in fermentation mashes

Fifty ml quantities of normal gravity wheat mash were distributed into sterile, 250 ml screw-capped Erlenmeyer flasks. A commercial isolate of *L. paracasei* was used since this bacterium is well adapted to fermentation conditions and tolerant to higher concentrations of ethanol. The bacteria was grown in MRS broth at 30° C, harvested by centrifugation at 10,300 × g for 15 min at 4° C and resuspended in sterile 0.1 % w/v peptone water. The slurry was stored in ice. Appropriate quantities of the bacterial suspension were added to the mashes so that the bacterial numbers were approximately 10^7 CFU/mL. Five different doses of chlorine dioxide (0 ppm - control, 100, 200, 300 and 400 ppm were tested in triplicate (it has been claimed by the manufacturer that 100 - 300 ppm is effective in killing bacteria). The flasks were then placed in an incubator shaker at 30° C; 150 rpm. After 48 h, samples were withdrawn from the flasks, centrifuged at 10,200 × g for 30 min and the supernatant was analysed for lactic acid by HPLC. It was established from initial studies that there was a linear relationship between final lactic acid concentration and initial viable bacterial cell numbers.

3.2.2.3 To study the effect of chlorine dioxide on yeast growth

Yeast extract-peptone-dextrose (YPD) broth was prepared. The pH was adjusted to 5.5 using 0.1 N HCl. This acidified broth was dispersed in 50 ml quantities into 250 ml side-arm, screw-capped Erlenmeyer flasks and sterilized at 121°C for 20 min. These flasks were then inoculated with 1 ml of *S. cerevisiae* cells grown to late log phase (~ 10^8 CFU/ml) at 30° C. Chlorine dioxide at 100 ppm was added to the flasks. Control with no chlorine dioxide was also included. The treatments were done in duplicate. The flasks were incubated at 30° C in an incubator-shaker (150 rpm) and the growth of yeast was monitored over time by the Klett-Summerson colorimeter (as a measure of turbidity) at 420 - 660 nm. Later, the same experiment was carried out using lower concentrations of chlorine dioxide (0 ppm - control, 50, 60, 70, 80 and 90 ppm) (since it was found that chlorine dioxide at 100 ppm greatly affected the growth rate of yeast). All treatments were duplicated.

3.2.3 Nisin

3.2.3.1 Determination of the bactericidal effect of nisin

The bactericidal effectiveness of Nisaplin® (nisin at 1000 units per mg produced by Aplin & Barrett, UK) was tested against five species of *Lactobacillus* which are all resistant

to low pH and to alcohol concentrations of 10 % v/v or more (*L. plantarum, L. paracasei, Lactobacillus* # 3, *L. rhamnosus* and *L. fermentum*).

Fifty ml quantities of MRS broth were dispensed into screw-capped, side-arm Erlenmeyer flasks (250 ml). These flasks were inoculated with 1ml of bacterial culture (grown to late log phase; ~ 10^9 CFU/ml). Nisin (a gift from Aplin & Barrett Ltd., Dorset, UK) was dissolved in 0.02 N HCl and filter sterilized using 0.45 µm pore diameter membranes. Appropriate amounts of this solution were added to the media as required. The concentrations of nisin tested were 50, 60, 70, 80, 90 and 100 mg/L because the manufacturer recommends the use of 50 - 100 mg/L in fermentations. All the treatments for all five lactobacilli were carried out in duplicate. The flasks were flushed with sterile CO_2 gas (passed through a 0.22 µm membrane filter), incubated at 30° C in an incubator-shaker (150 rpm) and the growth was monitored by measuring turbidity using a Klett-Summerson colorimeter with red filter.

Nisaplin® was tested against the test organism *Micrococcus luteus* (ATCC 4698) to check the effectiveness of the preparation. The growth of the organism in Bond's broth (peptone, 10 g/L; beef extract, 3 g/L; yeast extract, 1.5 g/L; sodium chloride, 3 g/L; and sucrose, 1.0 g/L; pH adjusted to 5.5 with 0.1 N HCl) in the presence of nisin at 50 mg/L was monitored for 72 h in at 30° C.

3.2.4 Using urea hydrogen peroxide and hydrogen peroxide

3.2.4.1 Determination of the most suitable concentration

Normal gravity wheat mash made as mentioned in section 3.1.5 (with particulates left in mash), was distributed into sterile, 250 ml screw-capped Erlenmeyer flasks at 50 ml/flask. For this particular set of experiments, *L. paracasei* was used since it is well adapted to fermentation conditions and tolerant to higher concentrations of ethanol. Appropriate quantities of the bacterial suspension (prepared as described above) were added to the mashes so that the bacterial numbers approximated 10^7 CFU/ml. Six different concentrations (2.1, 5.4, 10.7, 21.3, 32.1, and 42.6 mM) of urea hydrogen peroxide (Sigma Chemical Co.) were tested in triplicate. A 40 % w/v solution of urea hydrogen peroxide was made in deionized water, filter-sterilized through a 0.22-µm-pore size membrane filter and dispensed. The inoculated flasks were then incubated at 30° C in an orbital shaker (150 rpm). After 48 h, samples were withdrawn from the flasks, centrifuged at 10,200 × g for 30 min and the supernatants were analysed for lactic acid. It was established from initial studies that there was a linear relationship between final lactic acid concentration and initial viable bacterial cell numbers.

3.2.4.2 Determination of the bactericidal effect of urea hydrogen peroxide

Unclarified wheat mash was distributed in 500 ml quantities into ten, jacketed, sterile glass fermentors. The mashes were inoculated with *L. plantarum, L. paracasei, L. rhamnosus, L. fermentum* and *Lactobacillus* # 3 at approximately 10^7 CFU/ml followed by the addition of a solution of 40 % w/v urea hydrogen peroxide (a volume to give a final concentration of 32 mM) immediately after the 0 h sample was withdrawn. All tests were done in duplicate. Samples were withdrawn at 0, 2, and 4 h and analysed in triplicate for viable bacterial numbers (CFU/ml) by the membrane filtration technique.

3.2.4.3 Use of urea hydrogen peroxide in batch fermentation of unclarified wheat mash

Wheat mash was prepared and distributed into 1 L jacketed glass fermentors in 500 ml quantities. The fermentors were connected through a circulating waterbath maintained at 30° C throughout the fermentation and stirred magnetically (IKA-Labortechnik). The treatments included: control with no bacteria but yeast and 30 mM urea added; yeast coinoculated with bacteria and 30 mM urea added; no bacteria but yeast and 30 mM urea hydrogen peroxide added; yeast coinoculated with bacteria and 30 mM urea hydrogen peroxide added; no bacteria but yeast and 30 mM urea and 30 mM hydrogen peroxide (BDH Chemicals Inc., Toronto, Ontario, Canada) added separately; yeast coinoculated with bacteria and 30 mM urea and 30 mM hydrogen peroxide added separately (Table 3.2). *L. paracasei* (~10^7 CFU/ml) was used to infect the mash where required. Samples were withdrawn from infected fermentors for determination of initial viable numbers of bacteria. After 90 min, 0.4 ml glucoamylase (Allcoholase II, Alltech Inc.) was added to all the fermentors to saccharify the liquefied mash. Thirty min after the addition of glucoamylase, yeast was inoculated into all fermentors at approximately 10^6 CFU/ml. This allowed a preincubation period of 2 h for the urea hydrogen peroxide and hydrogen peroxide before yeast inoculation. Samples were withdrawn immediately after yeast inoculation at 0 h, and at 12 h, 24 h, 36 h, 48 h and 72 h for analysis.

Table 3.2 Treatment details for evaluating the use of urea hydrogen peroxide in batch fermentation of unclarified wheat mash.

Treatment	Yeast	Bacteria	Urea *	Hydrogen peroxide *	Urea hydrogen peroxide *
1	+	-	+	-	-
2	+	+	+	-	-
3	+	-	-	-	+
4	+	+	-	-	+
5	+	-	+	+	-
6	+	+	+	+	-

* A concentration of 30 mM.

3.2.4.4 Comparison of two other nitrogenous sources (diammonium hydrogen phosphate, ammonium dihydrogen phosphate) along with urea in combination with hydrogen peroxide in batch fermentation of unclarified wheat mash

Diammonium hydrogen phosphate (DAP) and ammonium dihydrogen phosphate were compared to urea in combination with hydrogen peroxide (H_2O_2) to find if they had the same effect. The treatments included control with yeast, 30 mM urea and no bacteria; yeast, 30 mM urea and 30 mM H_2O_2; yeast, *L. paracasei*, 30 mM urea and 30 mM H_2O_2; yeast, 30 mM H_2O_2 and 60 mM $(NH_4)H_2PO_4$; yeast, *L. paracasei*, 30 mM H_2O_2 and 60 mM $(NH_4)H_2PO_4$; yeast, 30 mM H_2O_2 and 30 mM DAP; and yeast, *L. paracasei*, 30 mM H_2O_2 and 30 mM DAP. All the treatments were done in duplicate. Yeast was inoculated at ~10^6 CFU/ml and *L. paracasei* was inoculated at ~10^7 CFU/ml. The fermentation was carried out exactly as mentioned previously with urea hydrogen peroxide (3.4.4.3).

3.2.4.5 Evaluation of the bactericidal effectiveness of urea hydrogen peroxide in the presence of mash particles

Liquefied wheat mash (α-amylase treated) was filtered through Whatman no. 1 filter paper and the insoluble mash solids were collected, washed three times with sterile distilled water and refiltered. Collected solids were spread on stainless steel trays and frozen at -40° C. Trays were placed in a tray dryer (Labconco Corporation, Kansas City, MO) and lyophilized for 48 h. Once the particles were dry, the lumps were broken and powdered with a mortar and pestle and stored at room temperature.

The experiment was done in 250 ml screw-capped, side-arm flasks with 50 ml of MRS broth in each. The treatments included the use of urea hydrogen peroxide at two different doses (2 mM and 42.6 mM) in the presence and absence of wheat mash particles (10 % w/v). The doses were chosen based on (i) the observations made by Anders *et al.* (1970) that > 1.5 mM of H_2O_2 would induce cell death of lactic acid bacteria and (ii) that 42.6 mM was the maximum concentration of urea hydrogen peroxide tested to manage lactic acid bacteria in grain mashes (although 30 mM was found to be quite effective). All treatments had *L. paracasei* inoculated at ~ 10^7 CFU/ml. All treatments were done in duplicate. In treatments where clear media were used, the growth of the organism was measured by optical density (Klett units) using a Klett-Summerson colorimeter. In the presence of particles, samples were withdrawn at 0, 2, 4, 6, 12 and 24 h and assessed (in triplicate) using the membrane filtration technique for bacterial viable counts (CFU/ml).

3.2.4.6 Decomposition of hydrogen peroxide and urea hydrogen peroxide in wheat mash

Normal gravity wheat mash was prepared and distributed into two, 1 L jacketed glass fermentors in 400 ml quantities. The fermentors were connected through a circulating waterbath maintained at 30° C and magnetically stirred. Hydrogen peroxide at 40 mM was added to one fermentor while urea hydrogen peroxide at 40 mM was added to the other. This experiment was repeated three times (triplicate). Samples were withdrawn at 0.5, 1, 1.5, 2, 3 and 5 h after addition of the bactericidal agents and analysed for the presence of hydrogen peroxide by fluorometry. The method involved (i) hydrolysis of stable reagent dichlorofluorescein diacetate (LDADCF) by sodium hydroxide to the less stable non-fluorescent compound L-dichlorofluorescein (LDCF) and (ii) subsequent oxidation of L-dichlorofluorescein (LDCF) and measurement of the formed fluorescent compound dichlorofluorescein (DCF) by the horseradish peroxidase (HRP)-catalyzed reaction with hydrogen peroxide (Keston and Brandt, 1965). Fluorescence was measured using the primary filter 405 and secondary filter 2A-12 (corresponding to 468 nm excitation wavelength and 519 nm emission wavelength) using a fluorometer (Model 111, GK Turner Associates, Palo Alto, CA). Concentrations of hydrogen peroxide were calculated from the standard curve prepared by using different known concentrations of hydrogen peroxide.

3.2.4.7 Enzyme Assays

3.2.4.7.1 Preparation of cell extracts

Cells of lactobacilli were grown, harvested, washed and resuspended at (~ 1.5×10^{10} cells/ml) in 40 mM potassium phosphate buffer (pH 7.2). The cell suspensions were passed 3 times through a French pressure cell (American Instrument Co. Inc., Silverspring, MD) at 20,000 lb/in^2 (1,410 kg/cm^2). Cell debris was removed from each extract by centrifugation at 10,300 × g for 30 min. The supernatants (cell-free extracts) were used as the enzyme source. The entire procedure was carried out at 4° C. The enzyme assays were done without delay, and for the estimation of total protein cell extracts were stored at 4° C for no longer than 24 h.

3.2.4.7.2 Specific activity of NADH peroxidase

Oxidation of NADH (Sigma Chemical Co.) by hydrogen peroxide at 30° C was followed spectrophotometrically at 340 nm (Chang *et al.*, 1997). The minor amount of NADH oxidizing activity (in the absence of H_2O_2) was subtracted. The reaction mixture (1 ml) contained 40 mM potassium phosphate buffer (pH 7.2), 0.2 mM EDTA, 0.17 mM NADH, 0.02 mM FAD, cell extract (0.05 ml) and 1.3 mM H_2O_2. The reaction was

initiated by adding H_2O_2. All solutions used in the assay were flushed with oxygen-free nitrogen gas for about 10 min. Total protein in the cell extract was measured using the Bio-Rad protein assay kit II (Bio-Rad laboratories). The specific activities presented are means of three separate assays using a different cell extract for each assay.

4. RESULTS

"Every novel idea in science passes through three stages.
First people say it isn't true. Then they say its true but
not important. And finally they say it's true and important,
but not new."

(Anonymous)

4.1 Effects of lactobacilli and their endproducts, lactic acid and acetic acid, on yeast growth and alcohol production

4.1.1 Screening of lactobacilli based on their growth rates and alcohol tolerance

Twelve strains of lactobacilli (Table 4.1) from the culture collection of Dr. W.M. Ingledew, Dept. of Applied Microbiology and Food Science, University of Saskatchewan, were screened on the basis of growth rates and alcohol tolerance. One ml of a 24 h culture of each strain was transferred to 50 ml MRS broth in 250 ml screw-capped, side-arm Erlenmeyer flasks. The flasks were flushed with sterile CO_2 gas (passed through a 0.22 µm membrane filter) and incubated at 30° C with shaking (150 rpm). The growth was monitored at two hour intervals up to 24 h using a Klett-Summerson colorimeter (equipped with filter # 66). The experiments were done in duplicate. Growth curves were obtained for each strain. The generation times in the logarithmic phase of growth (Table 4.1) were then calculated using the formula :

$$n = \frac{\log K_t - \log K_0}{\log 2}$$

where, K_0 is Klett reading at 0 time,

K_t is the Klett reading after a time interval of 't', and

n is the number of generations.

$$\text{Generation time (g) (in h)} = \frac{t}{n}$$

Seven strains with low generation times (faster growth rates) were selected and tested for alcohol tolerance by growing them in the presence of 5, 10 and 15 % v/v ethanol. Suitable controls were also included. Five strains with fast growth rates and capability of growth in media containing 10 % v/v or more of ethanol (Fig. 4.1) were finally selected for further studies (as mentioned in the Materials and Methods).

Table 4.1 Generation times (in the logarithmic phase of growth) of selected strains of lactobacilli in MRS broth at 30° C

Strain[a]	Generation time (h)
Lactobacillus fermentum	1.74*
Lactobacillus plantarum 1	1.80*
Cargill # 2 (*Lactobacillus* # 2)	2.62*
Cargill # 1 (*Lactobacillus* # 1)	2.68*
Cargill # 3 (*Lactobacillus* # 3)	2.74*
Lactobacillus paracasei	2.88*
Lactobacillus rhamnosus	3.37*
Lactobacillus delbreuckii[b]	7.81
Lactobacillus fructivorans	9.70
Lactobacillus homohiochi	13.23
Lactobacillus delbreuckii (ATCC 9649)	n/d[c]
Cargill # 4 (*Lactobacillus* # 4)	n/d[c]

[a] Obtained from the culture collection of Dr. W.M. Ingledew, Dept. of Applied Microbiology and Food Science, University of Saskatchewan, Canada.

[b] Miller Brewing Co., Milwaukee, WI.

[c] not determined. These strains grew extremely slowly and hence it was difficult to determine the generation time.

* The strains selected for study of alcohol tolerance.

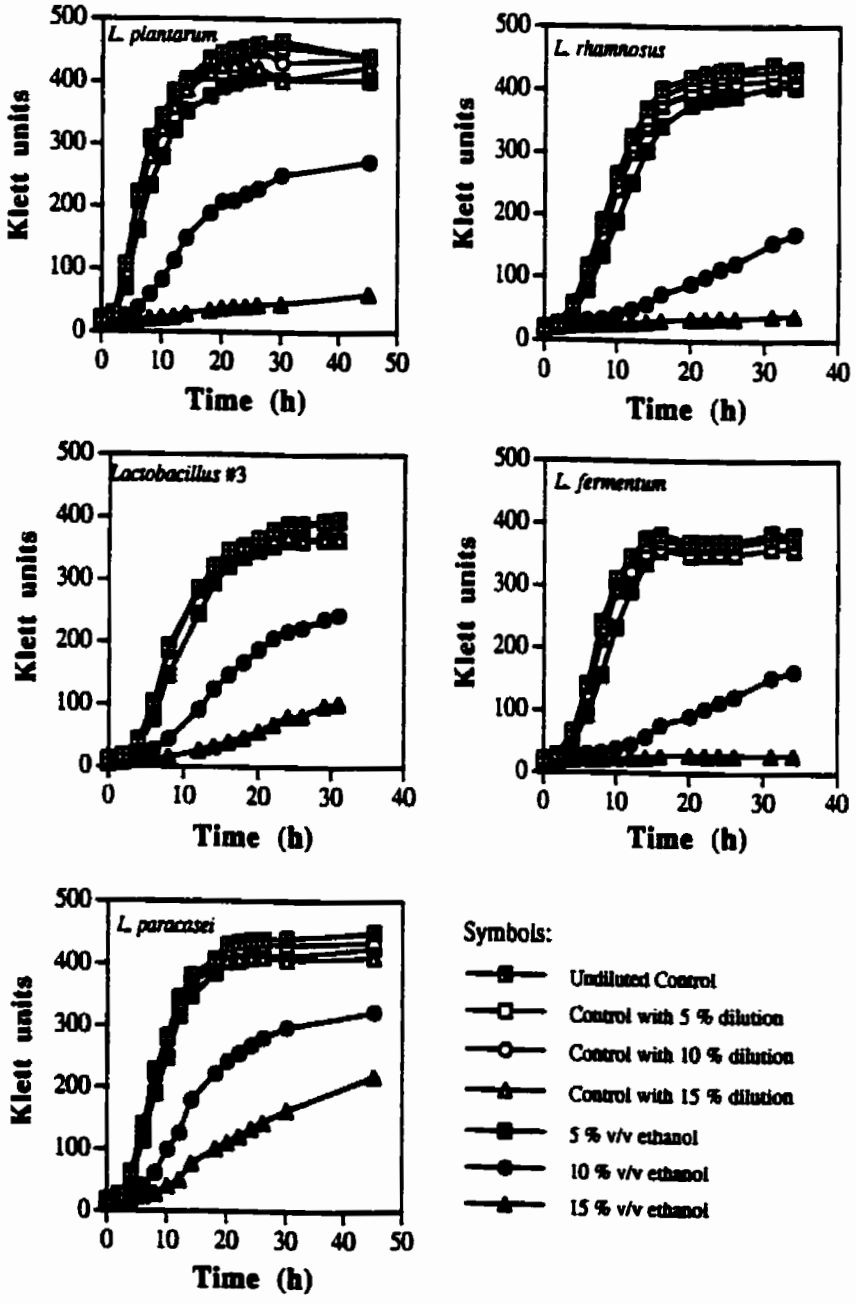


Figure 4.1 Growth of different lactobacilli in MRS broth in the presence and absence of 5, 10, and 15 % v/v ethanol at 30° C. In the controls with 5, 10, and 15 % dilutions, the media were diluted with the corresponding amounts of sterile deionized water.

Among the 7 strains as indicated in Table 4.1, five strains were capable of growth at 10 % v/v ethanol (Fig. 4.1), but two isolates obtained from Cargill Corn Milling, IA; Cargill #1 (*Lactobacillus* # 1) and Cargill #2 (*Lactobacillus* # 2) grew at an extremely slow rate with 5 % v/v ethanol and did not grow with 10 % v/v ethanol in the medium.

4.1.2 Effects of lactobacilli on yeast growth and alcohol production

4.1.2.1 Fermentation rates

The disappearance of dissolved solids from mash during yeast fermentation is a measure of conversion of glucose to alcohol. Specific gravity is a measure of dissolved solids in the mash. This value decreases as sugar is converted to ethanol (density 0.789) and CO_2 gas (which leaves the fermentor). Only small differences in fermentation rates were observed between yeast fermentations containing lactobacilli and the controls with no bacterial inoculation (Fig. 4.2). When the initial bacterial numbers were high, only a slight change in the rate of carbohydrate utilization was observed, but all the fermentations completed to constant specific gravity. The bacteria appeared not to consume more than 1 g per 100 ml of mash dissolved solids (fermentable carbohydrates) for growth and metabolism as determined by specific gravity (Fig. 4.2). However, the assessment of specific gravity as a measure of sugar fermentation is not valid in the case of bacterial fermentation because one mole of glucose is either converted to 2 moles of lactic acid (homofermentative strains) or to 1 mole of lactic acid, one mole of ethanol and one mole of CO_2 gas (heterofermentative strains). Little or less weight loss would take place, therefore specific gravity measurements would not change as much as in a yeast fermentation. Since the lactic acid made by *L. plantarum* was 1 % w/v, the organism must have used at least 1 g of sugar/100 ml mash (*L. plantarum* being an homofermentative strain). Similar trends were observed in the experiments done with *L. paracasei*, *Lactobacillus* # 3, *L. rhamnosus* and *L. fermentum*.

4.1.2.2 Growth of lactobacilli in yeast-catalyzed fermentations

The growth of the 5 different lactobacilli inoculated in wheat mash at various levels was followed (Fig. 4.3). Bacteria growing in the absence of yeast remained viable while in the presence of yeast, they died off towards the end of fermentation. Moreover, the death rate of these bacteria increased with increases in the concentration of lactic acid in the presence of ethanol.

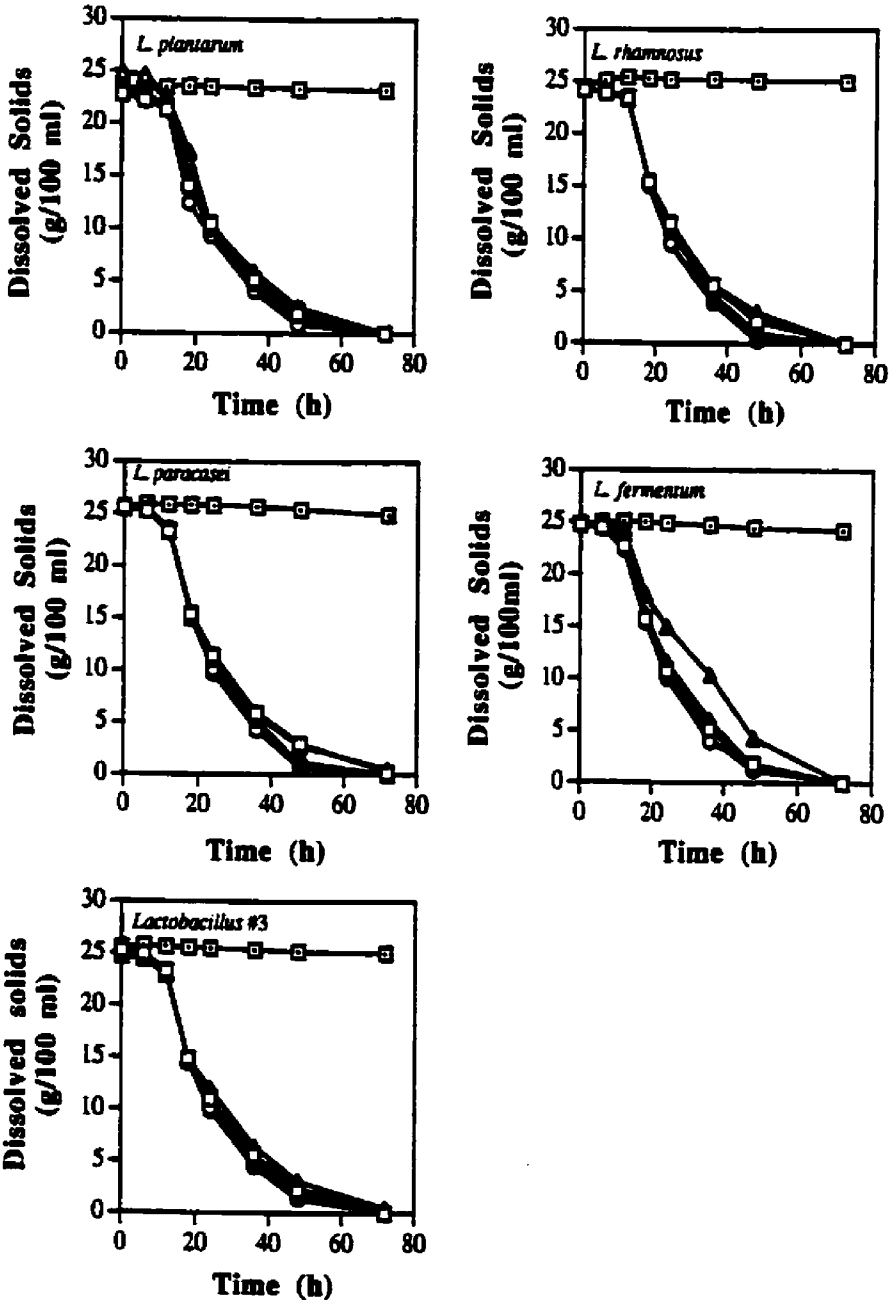


Figure 4.2 Concentration of dissolved solids over time in a wheat mash fermentation at 30° C inoculated at various cell concentrations with different lactobacilli. The mashes were inoculated with yeast at approximately 10^6 CFU/mL. Symbols : ❑, control (no bacterial inoculation); ■, ~10^5 CFU of bacteria/ml; ○, ~10^6 CFU of bacteria/ml; ●, ~10^7 CFU of bacteria/ml; Δ, ~10^8 CFU of bacteria/ml; ▲, ~10^9 CFU of bacteria/ml; and ❑, ~10^7 CFU of bacteria/ml (no yeast inoculation).

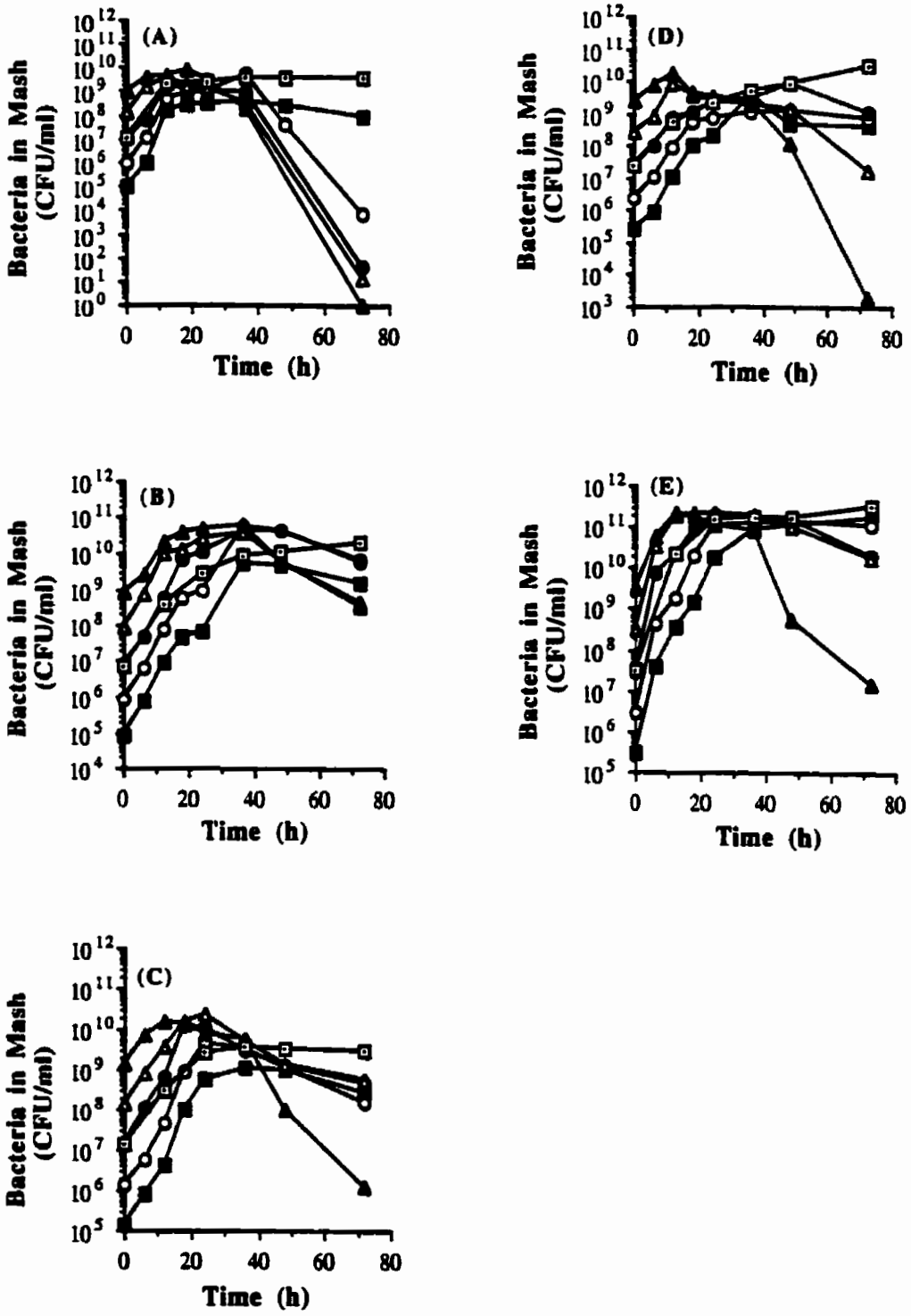


Figure 4.3 Growth of (A) *L. plantarum*, (B) *L. paracasei*, (C) *Lactobacillus* # 3, (D) *L. rhamnosus* and (E) *L. fermentum*, inoculated at various levels in wheat mash fermented at 30° C. The mashes contained yeast at approximately 10^6 CFU/ml. Symbols : bacteria inoculated at approximately : ~10^5 CFU/ml (■); ~10^6 CFU/ml (○); ~10^7 CFU/ml (●); ~10^8 CFU/ml (Δ); ~10^9 CFU/ml (▲); and ~10^7 CFU/ml (no yeast inoculation) (❑).

This suggests that ethanol and lactic acid act synergistically to kill these bacteria and that the toxicity of ethanol is enhanced by the decrease in pH caused by the lactic acid in the medium. Based on the results shown in Figure 4.3, the homofermentative strains *L. paracasei* and *Lactobacillus* # 3 appear to be relatively more tolerant to ethanol than *L. rhamnosus* and *L. plantarum*. *L. fermentum*, a heterofermentative strain, produced only 0.5 % w/v lactic acid while *Lactobacillus* # 3 produced 1.59 % w/v (Fig. 4.4). As the inhibitory effect of ethanol is accentuated by the low pH caused by lactic acid, *Lactobacillus* # 3 died faster than *L. fermentum* while *L. paracasei* is relatively more tolerant to ethanol even at higher lactic acid concentrations in the medium.

4.1.2.3 Effect of lactobacilli on growth and metabolism of yeast

As the inoculum size of the different lactobacilli was increased, growth rates of yeast decreased. With 10^5 CFU/ml of *L. plantarum* in the medium, the specific growth rate was 0.42 h^{-1}, while the specific growth rate decreased to 0.36 h^{-1} when the bacterial inoculum size was increased to 10^9 CFU/ml. Similar values were observed with the other four strains. Decreases in maximum yeast growth (Fig. 4.5), final ethanol concentration (Table 4.2) and in final pH of the medium also were seen. During fermentation, the concentrations of lactic acid achieved increased as the levels of bacteria inoculated increased (Fig. 4.4). In all experiments, total yeast cell numbers reached a maximum at 24 h of fermentation and then started to decrease (Fig. 4.5).

The effects of each *Lactobacillus* sp. at the 5 different inoculation levels on the maximum ethanol produced (% v/v) by yeast cells were studied in separate sets of experiments (Table 4.2). Single mashes were used for each experiment because between experiments, there are minor changes in the dissolved solids content of the mashes that would lead to the variations in final ethanol concentration produced by yeast in the absence of bacteria. Yeast-catalyzed fermentations completed within 48 h. Therefore, even when bacteria are present in high numbers, they must increase in biomass quickly in order to create enough metabolic potential to compete with yeast for sugar and create ethanol yield-reducing levels of lactic acid prior to termination of fermentation.

The increases in lactic acid with the 5 bacterial strains correlated with the decreases in the final ethanol concentrations (correlation coefficients of 0.92 - 0.99) and the viable yeast numbers. The metabolic end product of these bacteria, lactic acid, inhibits yeast growth and metabolism and is the major cause for the decrease in ethanol yield in this study.

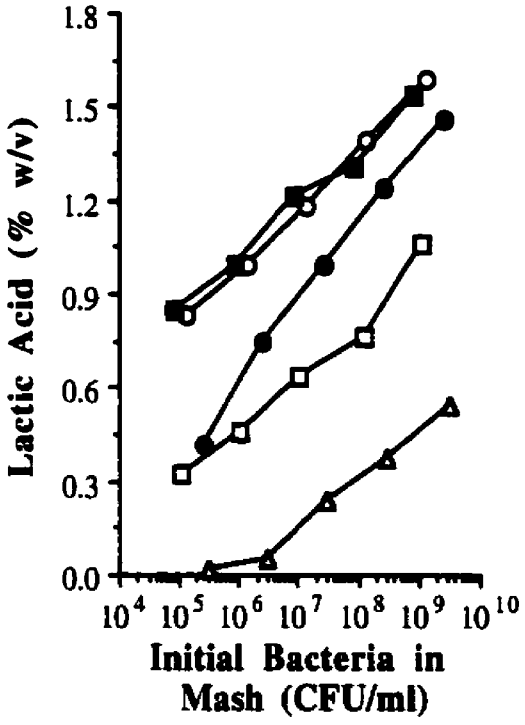


Figure 4.4 Effect of the initial numbers of lactobacilli on the final lactic acid concentration. The mashes contained yeast at approximately 10^6 CFU/ml. Symbols : ❑, *L. plantarum*; ■, *L. paracasei*; ○, *Lactobacillus* # 3; ●, *L. rhamnosus* ; and Δ, *L. fermentum*.

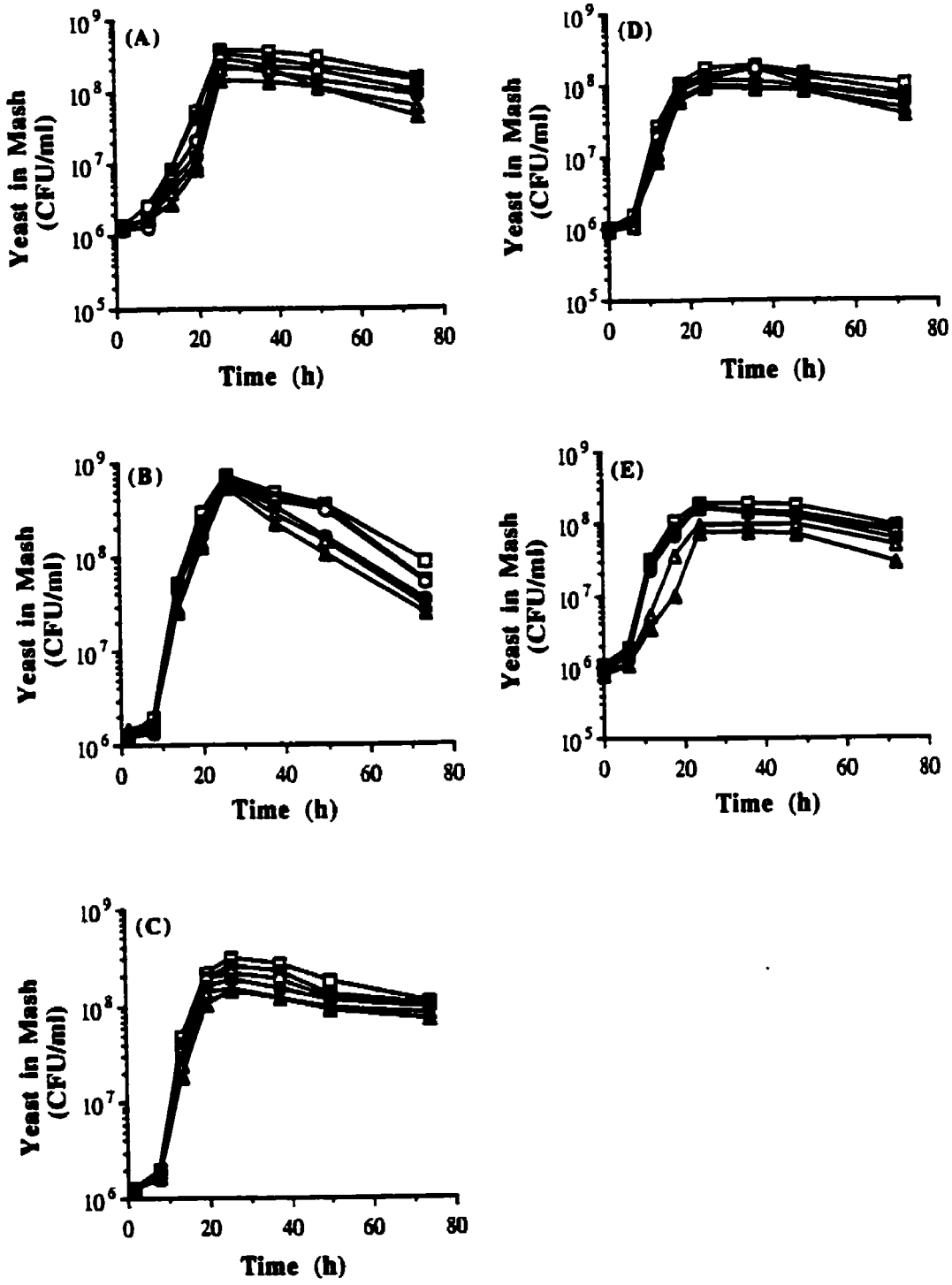


Figure 4.5 Growth of yeast in fermenting wheat mash at 30° C coinoculated with (A) *L. plantarum*, (B) *L. paracasei*, (C) *Lactobacillus* # 3, (D) *L. rhamnosus* and (E) *L. fermentum* at various levels. The mashes contained yeast at approximately 10^6 CFU/ml. Symbols : □, control (no bacterial inoculation); ■, ~10^5 CFU of bacteria/ml; ○, ~10^6 CFU of bacteria/ml; ●, ~10^7 CFU of bacteria/ml; Δ, ~10^8 CFU of bacteria/ml; and ▲, ~10^9 CFU of bacteria/ml.

Table 4.2 Maximum concentration of ethanol produced after fermentation of normal gravity (22 - 24° P) wheat mash at 30° C for 72 h by yeast coinoculated with lactobacilli at various levels[a]

Approx. numbers of bacteria inoculated (CFU/ml)	Maximum ethanol produced (% v/v)[b]				
	L. plantarum	*L. paracasei*	*Lactobacillus* #3	*L. rhamnosus*	*L. fermentum*
None (Control)	12.71	12.46	12.24	12.71	13.14
10^5	12.55	12.20	11.99	12.50	13.04
10^6	12.40	11.99	11.77	12.32	12.90
10^7	12.31	11.80	11.60	12.20	12.82
10^8	12.19	11.65	11.44	12.07	12.75
10^9	11.99	11.51	11.30	11.86	12.63

[a] Mashes were inoculated with approximately 10^6 CFU of yeast cells/ml and the bacterial numbers indicated.

[b] All assays were done in duplicate using HPLC analysis. Variations in ethanol (in duplicate assays) were in all cases less than 0.04 % v/v.

In the case of *L. fermentum*, a heterofermentative organism, the final lactic acid concentrations were not as high as those observed with the homofermentative strains (Fig. 4.4). Yet, the percent reduction in ethanol was similar (about 2 %) when mash was inoculated at approximately 10^6 CFU/ml with the homofermentative organisms (*L. plantarum* and *L. rhamnosus*). This may be partly due to the production of 0.03 - 0.05 % w/v of acetic acid by *L. fermentum* towards the end of fermentation - the actual amount depending on the inoculation level.

To alleviate concerns regarding the pH effect of lactic acid on saccharification of dextrins to glucose, experiments were conducted to demonstrate activities of glucoamylase over a range of pH conditions. Glucoamylase retained 91 % of its activity at pH 4.0, and 70 % at pH 3.0 (Fig. 4.6). The pH never dropped below 3.9 in any of the fermentations reported here.

Results obtained in this study show that an initial bacterial contamination of mash of approximately 10^6 CFU/ml led to as much as 3.8 % reduction in ethanol yield (Table 4.2). Higher levels (10^9 CFU/ml) led to more than 7 % losses in ethanol. Final lactic acid concentrations and decreases in ethanol yields correlated directly with the initial numbers of viable bacteria in the mash (Fig. 4.4 and 4.7).

4.1.3 Effects of lactic acid and acetic acid (end-products of lactobacilli metabolism) on yeast growth and fermentation

4.1.3.1 Inhibition of yeast growth by acetic and lactic acids

The specific growth rates (μ) of the two yeast strains (Alltech strain and ATCC 26602) decreased exponentially while lag times increased exponentially as the concentration of the acids in the medium were increased (Fig. 4.8 and 4.9). (Specific growth rate, $\mu = \frac{0.693}{\text{Doubling time}}$). The minimum inhibitory concentration (MIC) for acetic acid was 0.6 % w/v (100 mM) and that for lactic acid was 2.5 % w/v (278 mM) for both yeast strains tested (i.e. these were the concentrations at which absolutely no growth of the yeast strains was observed for at least 72 h after inoculation). Concurrently, it was found that acetic acid at concentrations of 0.05 - 0.1 % w/v and lactic acid at concentrations of 0.2 - 0.8 % w/v begin to stress the yeast as seen by reduced growth rates, and decreased rates of glucose consumption and ethanol production in minimal medium with glucose (2 % w/v) as the carbon source. Acetic acid is inhibitory to yeast at a much lower concentration than is lactic acid.

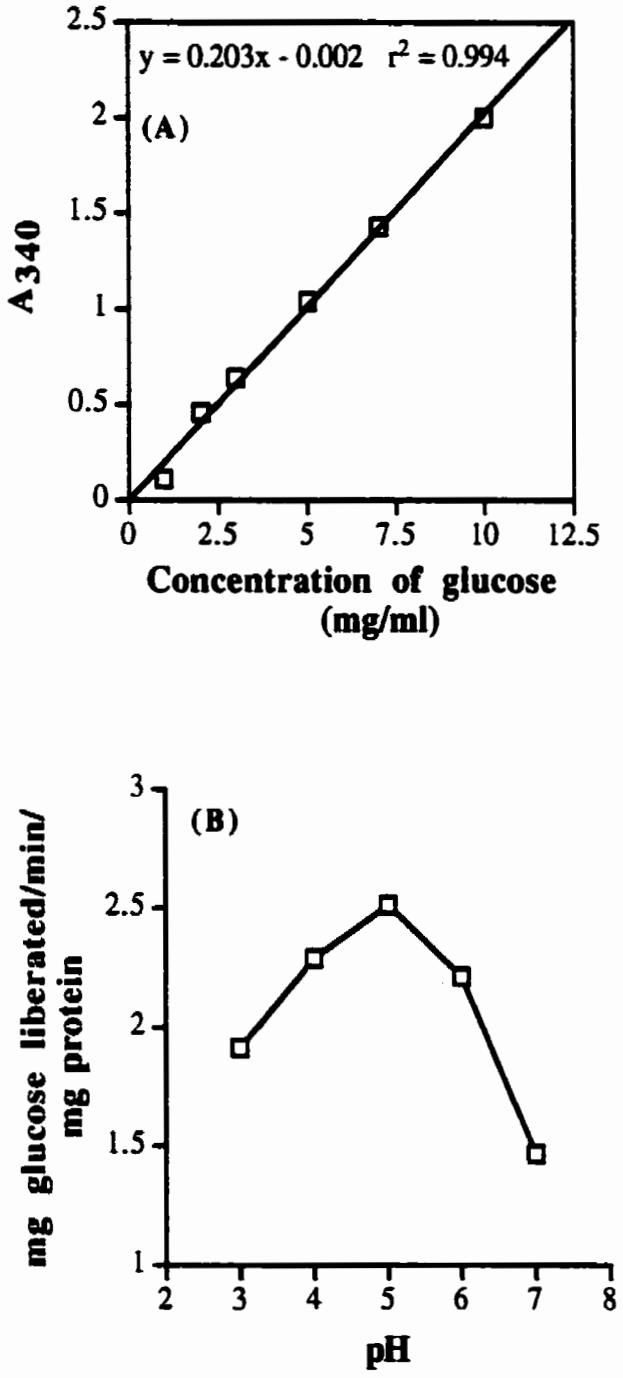


Figure 4.6 (A) Standard curve to determine the concentration of glucose using the Glucose (hexokinase) kit (Sigma Chemical Co.). (B) The specific activity of Allcoholase II (glucoamylase) enzyme (Alltech Inc.) at various pH levels. Substrate : 0.5 % w/v Dextrin solution, Buffer : 0.1M Citrate - 0.2 M Na_2HPO_4, Temperature of the assay = 30° C. Protein content of the enzyme preparation was 125 mg/ml (Lowry *et al.*, 1951).

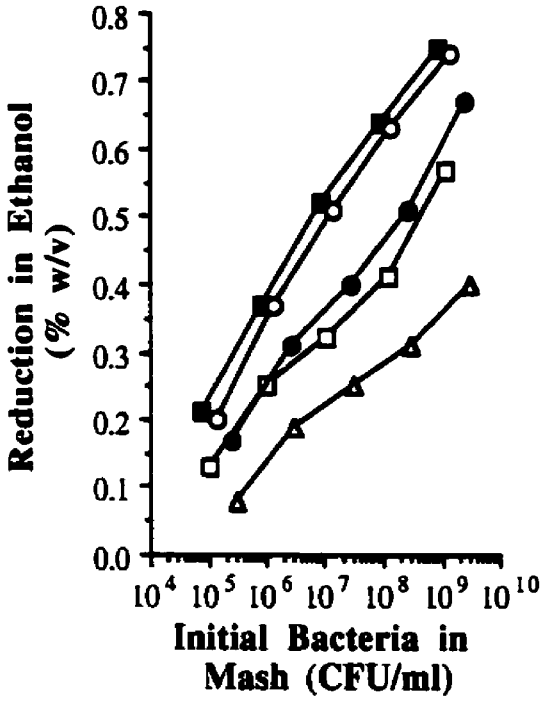


Figure 4.7 Effect of the initial numbers of lactobacilli on the reduction in final ethanol concentration compared to the control with no bacterial inoculation. The final ethanol concentrations (Table 4.2) were approximately 10 % w/v (12.7 % v/v), so the overall yield loss of produced ethanol ranged from 0.7 to 7.5 %. The mashes contained yeast at approximately 10^6 CFU/ml. Symbols : □, *L. plantarum*; ■, *L. paracasei*; ○, *Lactobacillus* # 3; ●, *L. rhamnosus* ; and Δ, *L. fermentum*.

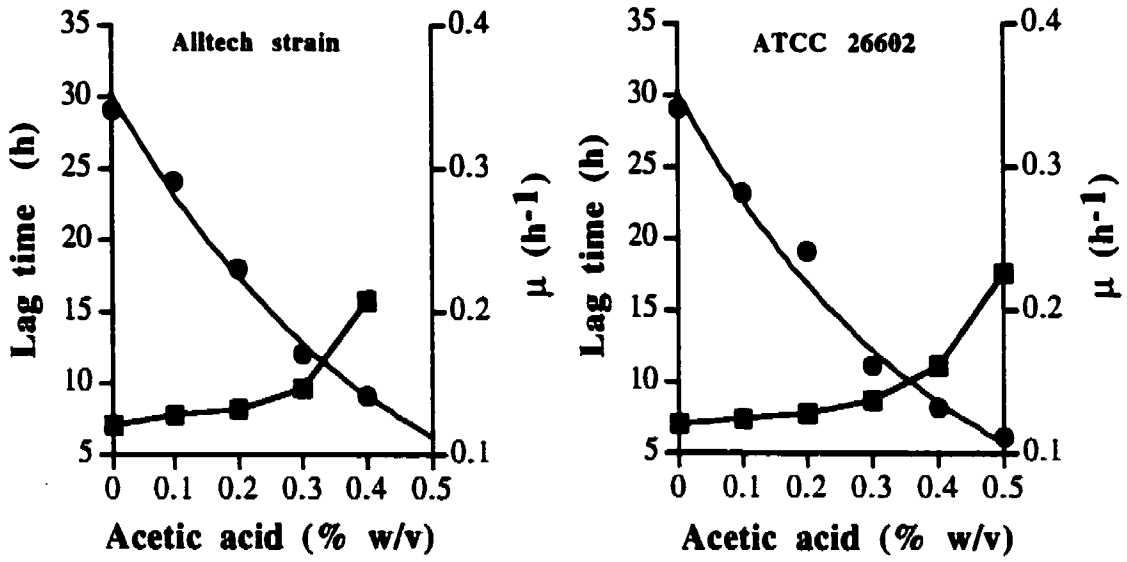


Figure 4.8 Effect of acetic acid on specific growth rates (●); and lag times (■) of two strains of *S. cerevisiae* in minimal medium at 30° C.

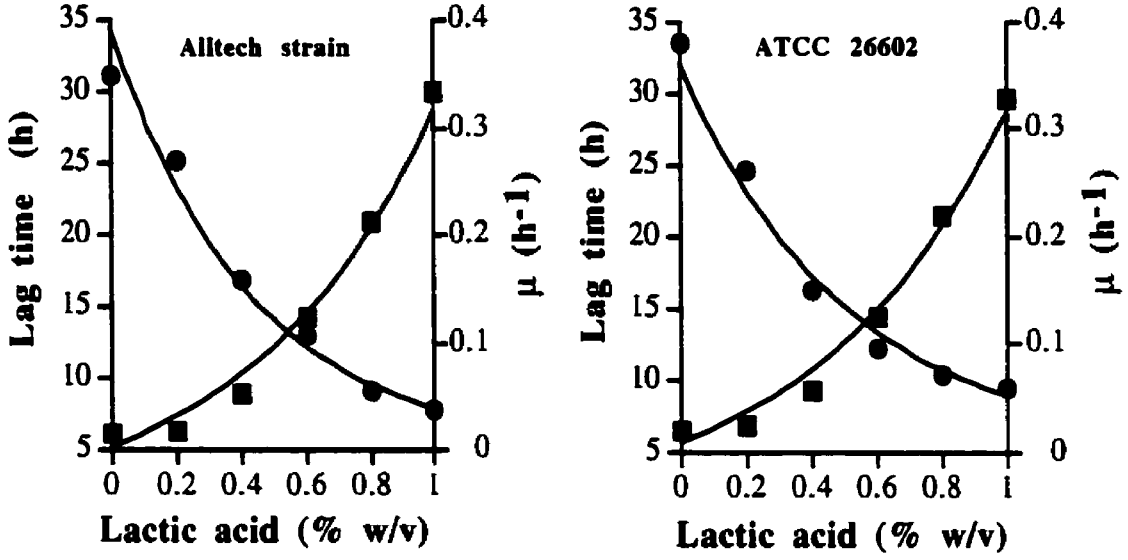


Figure 4.9 Effect of lactic acid on specific growth rates (●); and lag times (■) of two strains of *S. cerevisiae* in minimal medium at 30° C.

4.1.3.2 Effects of acetic and lactic acids on fermentation rates

There was a reduction in the rates of glucose consumption and ethanol production as the concentration of acetic acid increased in the media (Fig. 4.10). Production of biomass decreased with increasing concentrations of acetic and lactic acids (Table 4.3). It was necessary to show that the decreases in biomass observed in the presence of the acids were not just due to the lowering of pH of the medium (when the acids were added). To verify this, the pH of the medium was adjusted to 2.6 or 3.0 with 1 N HCl and the yeast were grown at 30° C. The total biomass produced after 24 h of growth was 1.3818 mg/ml and 1.4 mg/ml, for the Alltech strain and for ATCC 26602 respectively when the initial media pH was 2.6. Biomass values were 2.1168 mg/ml and 1.833 mg/ml for the Alltech strain and for ATCC 26602, respectively, when the initial media pH was 3.0. Values for dry weight when acetic or lactic acids were added (Table 4.3) were more than 8 fold less than when the medium was adjusted to the same pH values with HCl, but without organic acids. Therefore, it can be concluded that the reduction of total biomass of both yeast strains (Table 4.3) was due to the presence of acetic or lactic acid which at low pH values (2.64 or 3.19, Table 4.4) exist predominantly in the undissociated form. Even though biomass production decreased with increasing concentrations of acetic acid in the medium, all of the glucose was consumed and the same levels of maximum ethanol were produced in 24 h by both yeast strains (Fig. 4.10).

Lactic acid appears to have a different effect than acetic acid in both yeast strains (Fig. 4.11). While an increased acetic acid concentration delayed both the utilization of glucose and production of ethanol, lactic acid at a relatively low concentration (0.6 % w/v) shut down glucose utilization and ethanol synthesis in the minimal medium. A level of 0.8 % w/v lactic acid is an industrially relevant concentration and is easily produced through the action of lactic acid bacterial contaminants in fermentation.

4.1.3.3 Interaction of acetic and lactic acid on the inhibition of yeast growth

Tables 4.5a and 4.5b show the analyses of variance (ANOVA) for the two independent variables (acetic acid and lactic acid) for the Alltech and ATCC 26602 strains. Several criteria such as R^2 values, coefficient of variation (CV) and model significance were used to judge the adequacy of the models. For a good fit of any model, R^2 should be

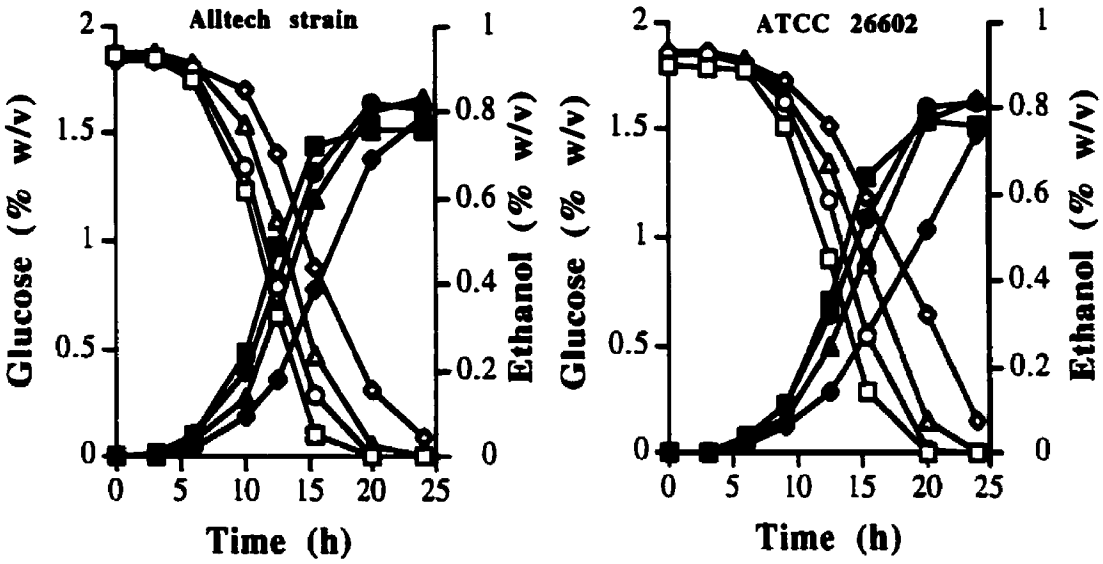


Figure 4.10 Glucose depletion (open symbols) and ethanol production (closed symbols) by *S. cerevisiae* in minimal medium at 30° C in the presence of increasing concentrations of acetic acid. Symbols : □, ■, 0 % w/v (control); ○, ●, 0.1 % w/v (17 mM); Δ, ▲, 0.2 % w/v (33 mM); and ◊, ◆, 0.3 % w/v (50 mM).

Table 4.3 Maximum yeast cell mass (mg/ml dry weight) obtained in minimal medium with various concentrations of acetic and lactic acids in 24 h at 30° C.

Acid	Concentration (% w/v)	Dry weight (mg/ml)	
		Alltech strain	ATCC 26602
Control (no acid)	0	2.1952	1.8224
Acetic	0.1 (17 mM)	2.0776	1.5664
	0.2 (33 mM)	1.7248	1.3338
	0.3 (50 mM)	1.4014	1.0998
Lactic	0.2 (22 mM)	1.8228	1.4430
	0.4 (44 mM)	1.1270	0.6630
	0.6 (66 mM)	0.0918	0.1794

Table 4.4 Percentages of undissociated acid and anions of acetic and lactic acids in minimal medium at pH values attained corresponding to the various acid concentrations.

Acid	Concn. (mM)	pH[a]	Undissociated acid (%)[b]	Anion (%)[b]	mole concn. of undissociated acid (mM)
Acetic	17	3.48	94.63	5.37	16.08
	33	3.31	96.37	3.63	31.80
	50	3.19	97.25	2.75	48.63
Lactic	22	2.95	87.68	12.32	19.29
	44	2.76	92.06	7.94	40.48
	66	2.64	93.97	6.03	62.98

[a] Values are mean of duplicate samples

[b] Values were calculated using the Henderson-Hasselbach equation ($pH = pKa + \log([A^-]/[HA])$) and pK_a values of acetic (4.74) and lactic acid (3.86)

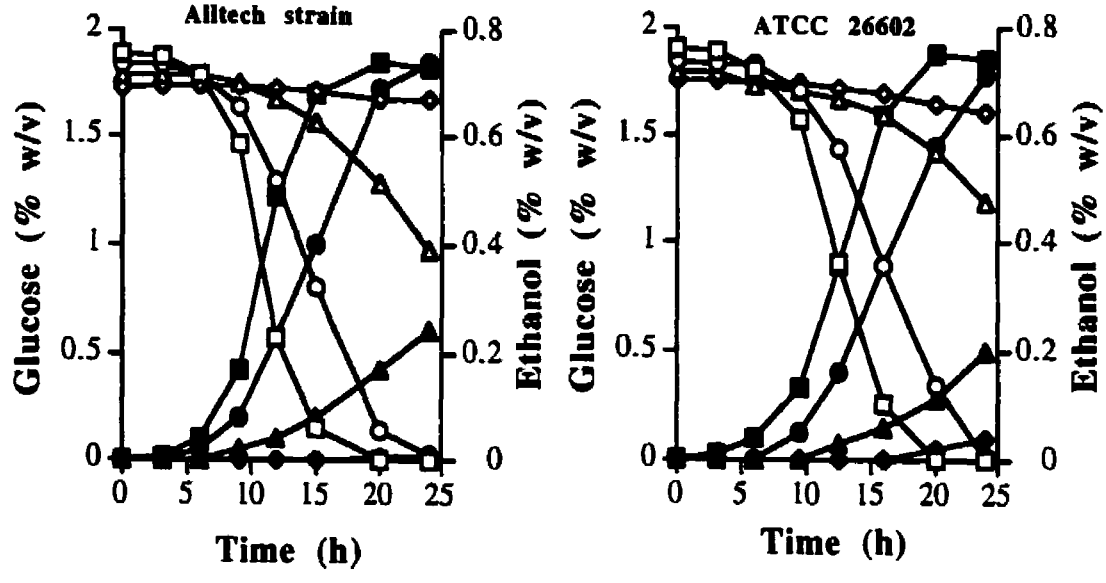


Figure 4.11 Glucose depletion (open symbols) and ethanol production (closed symbols) by *S. cerevisiae* in minimal medium at 30° C in the presence of increasing concentrations of lactic acid. Symbols : □, ■, 0 % w/v (control); ○, ●, 0.2 % w/v (22 mM); Δ, ▲, 0.4 % w/v (44 mM); and ◊, ◆, 0.6 % w/v (66 mM).

Table 4.5a : Computer-generated[1] analysis of variance for specific growth rates of *S. cerevisiae* (Alltech strain)

Source	DF	Type III SS	Mean square	F value	Prob
Trial	1	0.0000087	0.0000087	0.29	0.5961
Acetic	1	0.0011091	0.0011091	37.25	0.0001
Lactic	1	0.0002367	0.0002367	7.95	0.0110
Acetic * Acetic	1	0.0000211	0.0000211	0.71	0.4098
Lactic * Lactic	1	0.0035682	0.0035682	119.85	0.0001
Acetic * Lactic	1	0.0005281	0.0005281	17.74	0.0005

[1] (SAS/STAT®, SAS Institute, 1988)

Table 4.5b : Computer-generated[1] analysis of variance for specific growth rates of *S. cerevisiae* (ATCC 26602)

Source	DF	Type III SS	Mean square	F value	Prob
Trial	1	0.0000203	0.0000203	0.10	0.7513
Acetic	1	0.0029339	0.0029339	14.91	0.0011
Lactic	1	0.0031810	0.0031810	16.17	0.0007
Acetic * Acetic	1	0.0001114	0.0001114	4.57	0.0001
Lactic * Lactic	1	0.0006541	0.0006541	3.33	0.0001
Acetic * Lactic	1	0.0007031	0.0007031	3.57	0.0011

[1] (SAS/STAT®, SAS Institute, 1988)

at least 80 %, CV should not exceed 10 % and model significance (P value) should be less than 0.05 (Wang *et al.*, 1999). The models developed in this study were highly adequate, since the levels of R^2, CV and model significance agreed to the above criteria for a good fit (Table 4.6).

Analysis of variance for the Alltech strain (Table 4.5a) and for ATCC 26602 (Table 4.5b) was done to determine the statistical significance of linear, quadratic and interactive effects of acetic and lactic acids on the specific growth rates. Experiments conducted at different times yielded similar results. There were no significant differences observed between the trials ($P = 0.5961$ for the Alltech strain and $P = 0.7513$ for ATCC 26602). All the other effects (linear, quadratic and interaction) of acetic and lactic acids were highly significant ($P \leq 0.001$). The linear effect of lactic acid is still significant for the Alltech strain since $P = 0.011$, but the quadratic effect of acetic acid is not statistically significant ($P = 0.4098$) indicating statistical insignificance of the curvature produced by acetic acid (Table 4.5a). A significant interaction of acetic acid and lactic acid is observed in the reduction of the specific growth rate of both strains of *S. cerevisiae* ($P \leq 0.001$). This indicates synergism. Figure 4.12 indicates the influence of acetic acid and lactic acid on the specific growth rate (μ) of *S. cerevisiae*. These acids when present together in the medium exert a higher inhibitory effect (due to synergy) on the specific growth rate of yeast than when each acid was present alone. When there is 0.5 % w/v lactic acid present in the media, the presence of even 0.04 % w/v acetic acid (which does not cause a significant change in yeast growth rate when present by itself) causes a significant reduction in the growth rate of *S. cerevisiae* ($P \leq 0.001$) (Fig. 4.12).

4.1.4 Mode of action of acetic acid and lactic acid on *S. cerevisiae*

4.1.4.1 Effects of acetic and lactic acid on the intracellular pH (pH_i) of *S. cerevisiae*

Extracellular pH (pH_e) at the time of harvest (i.e. during the mid-exponential phase when 0.5 to 0.7 % w/v glucose was left in the medium with acetic acid and 0.6 to 1.0 % w/v glucose was left in the medium with various concentrations of lactic acid) are given in Table 4.7. Using these pH values and the pK_a values of acetic and lactic acids, the molar concentrations of the undissociated acids in the medium was calculated based on the Henderson-Hasselbach relationship ($pH = pK_a + \log [A^-]/[HA]$) (Table 4.7).

Table 4.6 Models for the response variable (specific growth rate) obtained from the general linear models procedure for the two strains of *S. cerevisiae*.

Variable and Source	df	Sum of squares	F value	P > F
(1) Alltech pure strain				
Model	6	0.1097708	614.47	0.0001
Error	19	0.0005657		
Corrected total	25	0.1103365		

$R^2 = 0.9948$

Coefficient of variation (CV) = 2.895 %

Coefficients for response surface model

Specific growth rate

$$y = 0.306 + (0.001/2) - 0.354x_1 - 0.081x_2 + 0.158x_1^2 - 0.508x_2^2 - 0.513x_1x_2$$

(2) ATCC 26602				
Model	6	0.0866123	73.38	0.0001
Error	19	0.0037370		
Corrected total	25	0.0903500		

$R^2 = 0.9586$

Coefficient of variation (CV) = 9.625 %

Coefficients for response surface model

Specific growth rate

$$y = 0.26 + (0.002/2) - 0.409x_1 - 0.214x_2 + 0.184x_1^2 + 0.112x_2^2 - 0.302x_1x_2$$

y = Specific growth rate (μ)

x_1 and x_2 : concentrations of acetic and lactic acids respectively.

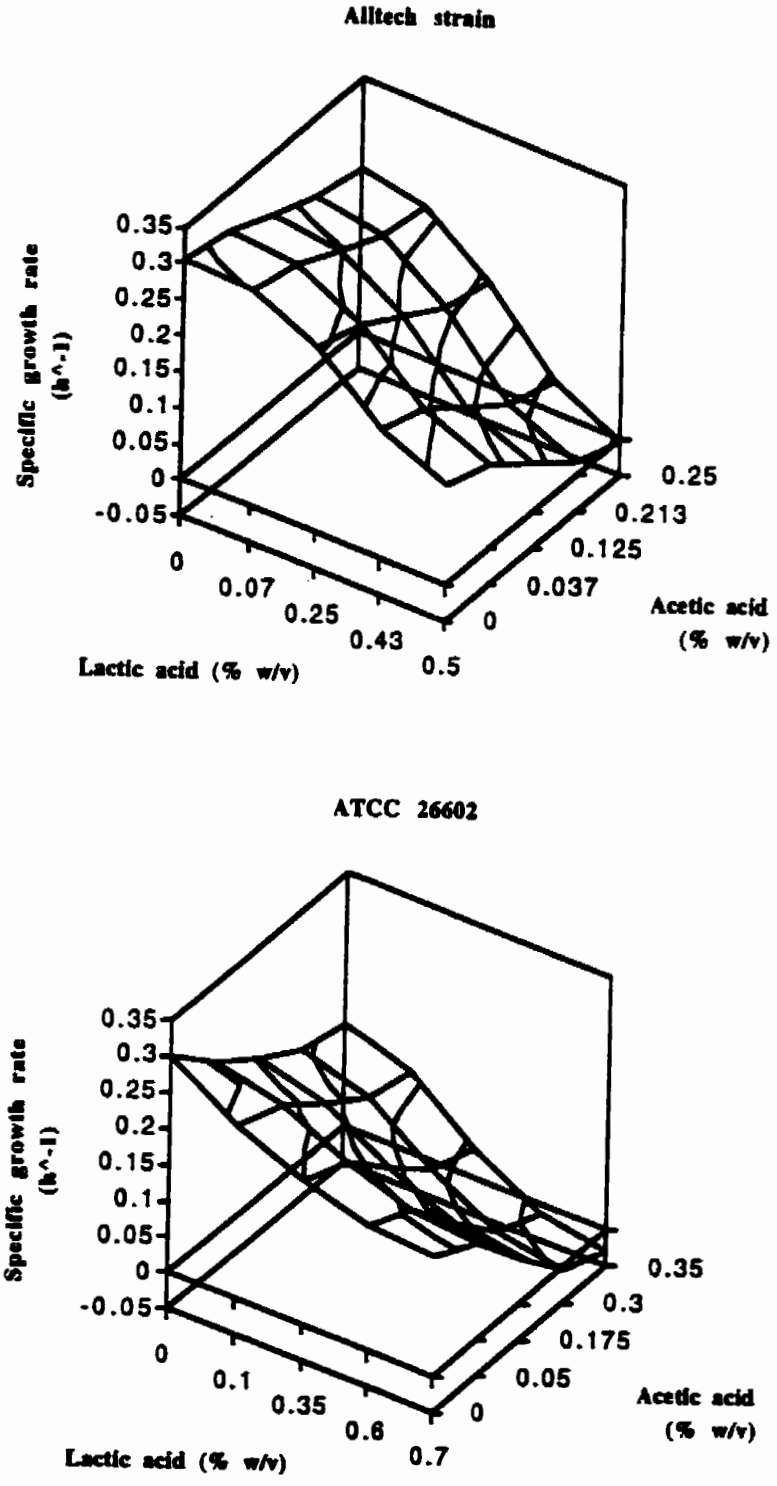


Figure 4.12 Influence of acetic acid and lactic acid on the specific growth rates (μ) of *S. cerevisiae* in minimal medium at 30° C. A negative (synergistic) interaction between the compounds is shown.

Table 4.7 The pH of the medium (pH_e) at the time of harvest of the cells of both strains of yeast and the corresponding molar concentration of undissociated acid as the concentrations of acetic and lactic acids were varied.

Acid	Concn. (% w/v)	pH_e[b]	% undissociated acid[c]	Molar concn. of undissociated acid (mM)
Acetic	0.05 (8.3)[a]	2.48	99.45	8.25
	0.10 (16.7)	2.54	99.37	16.60
	0.15 (25.0)	2.57	99.32	24.83
	0.20 (33.3)	2.59	99.29	33.06
	0.25 (41.7)	2.61	99.26	41.39
Lactic	0.1 (11.1)	2.38	96.69	10.73
	0.2 (22.2)	2.42	96.37	21.39
	0.3 (33.4)	2.48	95.83	32.01
	0.4 (44.5)	2.52	95.43	42.47
	0.5 (55.6)	2.56	94.99	52.81

[a] Numbers in parentheses indicate concentration in mmoles/L

[b] Values are the averages of two separate observations that did not differ by > 0.02 units

[c] Calculated based on Henderson-Hasselbach equation ($pH = pK_a + \log [A^-]/[HA]$) where $A = CH_3COO^-$ or $CH_3CHOHCOO^-$

The intracellular pH values (pH_i) of both yeast strains at different concentrations of acetic acid in the medium are shown in Table 4.8 and Fig. 4.13. Tables and figures are provided for experiments using acetic and lactic acids in order to indicate both statistical significance of data and the trends in pH_i values and in plasma membrane H^+-ATPase activities. The significance of differences among treatments was tested by Duncan's multiple range test. In case of the Alltech yeast, the intracellular pH did not differ significantly from the control with no acetic acid ($P = 0.05$) until acetic acid reached 0.15 % w/v. Moreover the intracellular pH values were not significantly different from one another in the various treatments with different concentrations of acetic acid (Table 4.8).

No significant differences ($P = 0.05$) were observed in the pH_i of *S. cerevisiae* (ATCC 26602) as the concentration of acetic acid increased in the medium (Table 4.8) although there was an increase in the molar concentration of the undissociated acid; (Table 4.7). A small decrease in the intracellular pH was observed as the concentrations of lactic acid in the medium increased, although at lower concentrations of lactic acid (up to 0.2 % w/v) the changes in pH_i were not significant ($P = 0.05$). At 0.5 % w/v (55 mM), the pH_i decreased significantly by 0.6 units in both yeasts (Table 4.9 and Fig. 4.14).

4.1.4.2 Effects of acetic and lactic acid on the plasma membrane H^+-ATPase activity of *S. cerevisiae*

The effects of acetic acid and lactic acid on the activity of the plasma membrane H^+-ATPase (which is involved in the regulation of intracellular pH in yeast) were studied *in vivo* in mid-exponential phase cells of *S. cerevisiae* (Alltech strain and ATCC 26602). The results are shown in Tables 4.10 and 4.11 (Figs 4.15 and 4.16). The data were subjected to Duncan's multiple range test. The activity of the plasma membrane ATPase increased significantly with increasing concentrations of acetic acid (Table 4.10; Fig. 4.15) for both strains of yeast studied. The relationship between increases in the activities of ATPase and increases in the concentrations of acetic acid appeared to be linear up to 0.25 % w/v (42 mM) acetic acid in the medium (the maximum concentration used in the study) for both the strains. When the acetic acid concentration was 0.25 % w/v, a 58 % increase in plasma membrane ATPase was observed in Alltech strain and a 55 % increase was shown by ATCC 26602 (Table 4.10). This appears to be the reason why there is no significant changes in pH_i of these two yeast strains in the presence of increasing concentrations of acetic acid in the medium (Fig. 4.13).

Table 4.8 Intracellular pH values (pH_i) of *S. cerevisiae* as concentrations of acetic acid in the medium were varied.

Acetic acid (% w/v)	Intracellular pH (pH_i)*	
	Alltech strain	ATCC 26602
0 (control)	5.41[a]	5.16[a]
0.05	5.34[ab]	5.18[a]
0.10	5.24[ab]	5.08[a]
0.15	5.24[ab]	5.06[a]
0.20	5.22[b]	5.10[a]
0.25	5.18[b]	5.13[a]

* Values are means of three separate experiments
The letters in superscripts indicate the levels of significance (P = 0.05) between treatments and **not** between the strains (based on Duncan's multiple range test). Means with the same letter are not significantly different.

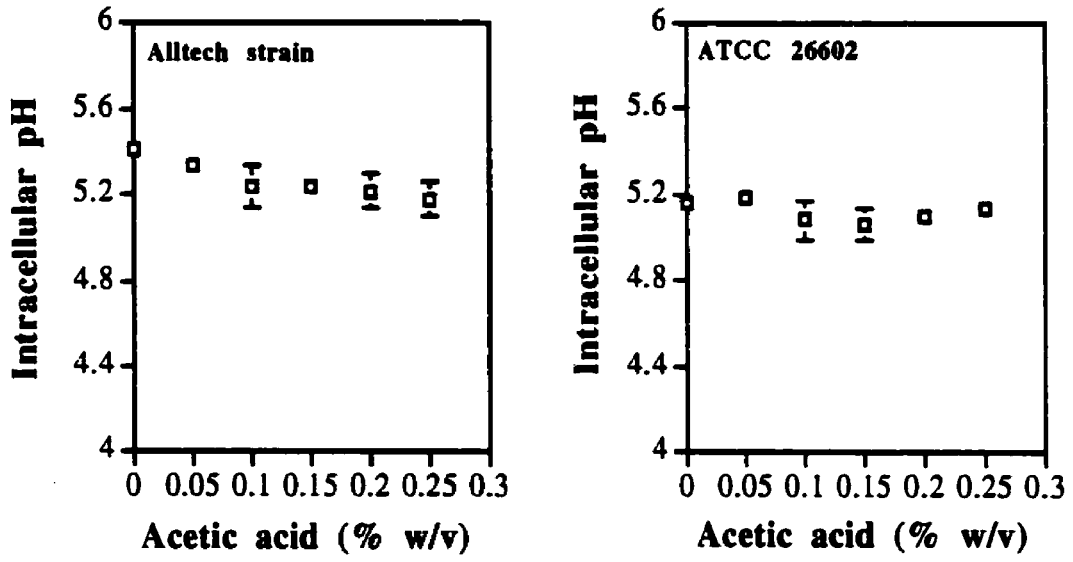


Figure 4.13 Intracellular pH (pH_i) of *S. cerevisiae* at various concentrations of acetic acid in the medium. Values are means of three separate experiments. Error bars indicate ± standard deviation.

Table 4.9 Intracellular pH values (pH_i) of *S. cerevisiae* as concentrations of lactic acid were varied in the medium.

Lactic acid (% w/v)	Intracellular pH (pH_i)*	
	Alltech strain	ATCC 26602
0 (control)	5.41^{a}	5.16^{a}
0.1	5.24^{ab}	5.08^{ab}
0.2	5.22^{bc}	5.10^{ab}
0.3	5.10^{cd}	4.97^{b}
0.4	4.99^{d}	4.79^{c}
0.5	4.80^{e}	4.60^{d}

* Values are means of three separate experiments
The letters in superscripts indicate the levels of significance (P = 0.05) between treatments and **not** between the strains (based on Duncan's multiple range test). Means with the same letter are not significantly different.

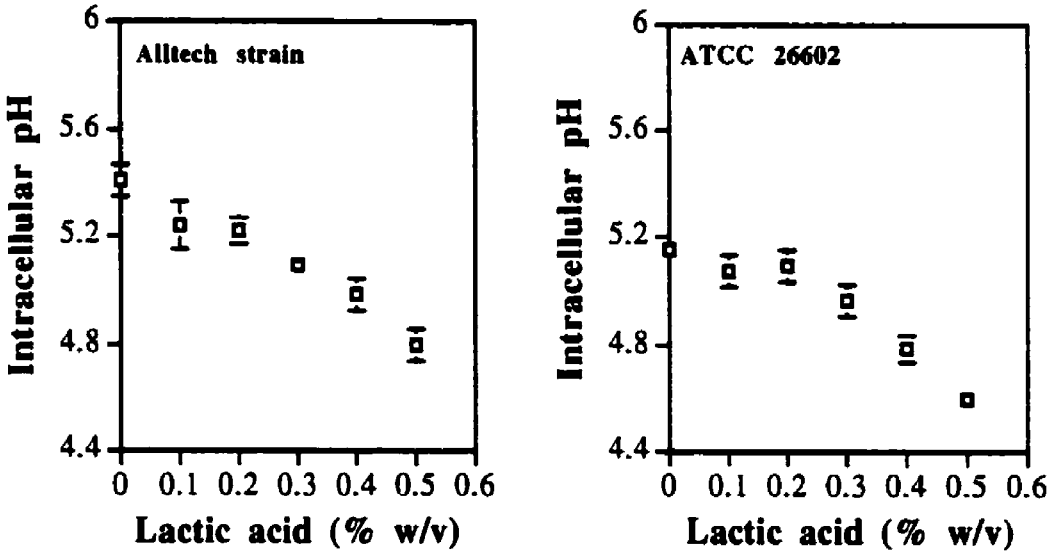


Figure 4.14 Intracellular pH (pH_i) of *S. cerevisiae* as lactic acid concentrations were varied in the medium. Values are means of three separate experiments. Error bars indicate ± standard deviation.

Table 4.10 Plasma membrane H^+-ATPase activity of two strains of *S. cerevisiae* in the presence of various concentrations of acetic acid.

Acetic acid (% w/v)	ATPase activity (μmol P_i released/min/mg protein)*	
	Alltech strain	ATCC 26602
0 (control)	2.304[a]	2.341[a]
0.05	2.475[a]	2.309[a]
0.10	2.938[b]	2.688[b]
0.15	3.172[bc]	2.921[c]
0.20	3.389[cd]	3.329[d]
0.25	3.638[d]	3.621[e]

* Values are means of three separate assays done at different times
The letters in superscripts indicate the levels of significance (P = 0.05) between treatments and **not** between the strains (based on Duncan's multiple range test). Means with the same letter are not significantly different.

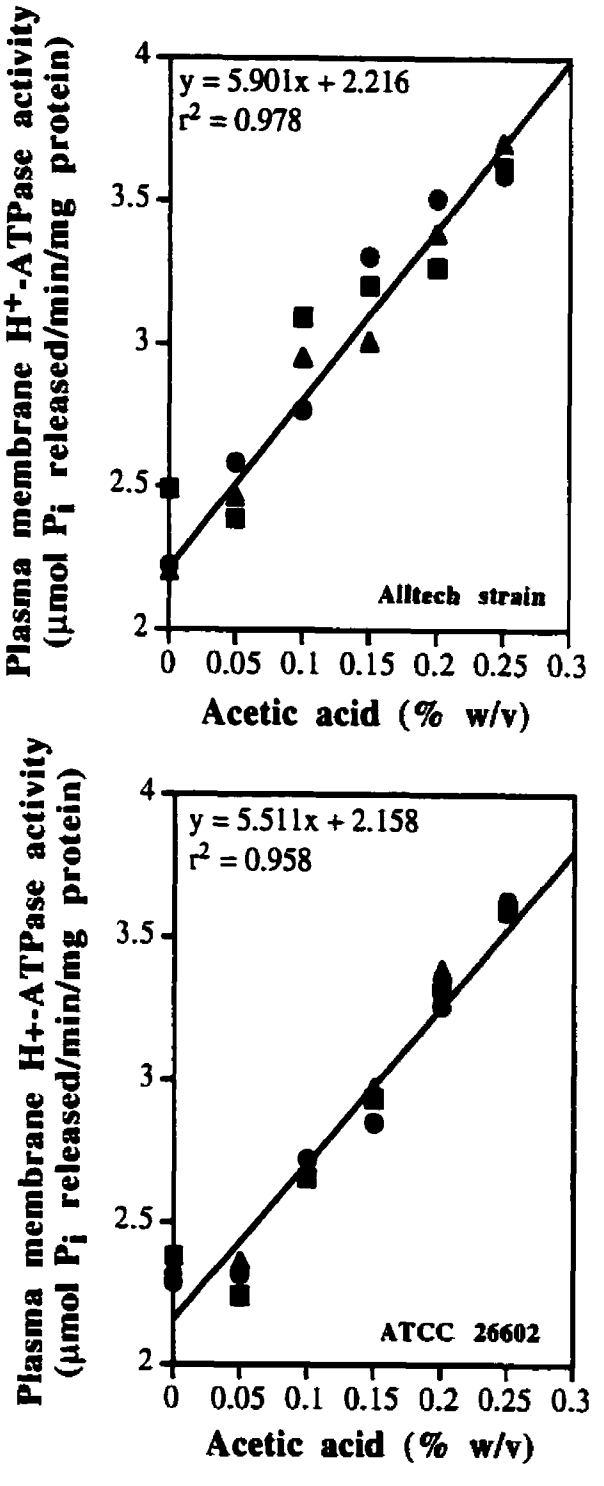


Figure 4.15 Plasma membrane H^+-ATPase activity (µmol P_i released/min/mg protein) of *S. cerevisiae* (mid-exponential phase cells) in the presence of various concentrations of acetic acid in minimal medium at 30° C. The symbols indicate values from separate experiments.

Table 4.11 Plasma membrane H^+-ATPase activity of two strains of *S. cerevisiae* in the presence of various concentrations of lactic acid.

	ATPase activity (μmol P_i released/min/mg protein)*	
Lactic acid (% w/v)	Alltech strain	ATCC 26602
0 (control)	2.304^{a}	2.341^{a}
0.1	2.212^{ab}	2.250^{ab}
0.2	2.144^{ab}	2.244^{b}
0.3	2.268^{a}	2.307^{a}
0.4	2.092^{b}	2.063^{c}
0.5	1.639^{c}	1.646^{d}

* Values are means of three separate assays done at different times
The letters in superscripts indicate the levels of significance (P = 0.05) between treatments and **not** between the strains (based on Duncan's multiple range test). Means with the same letter are not significantly different.

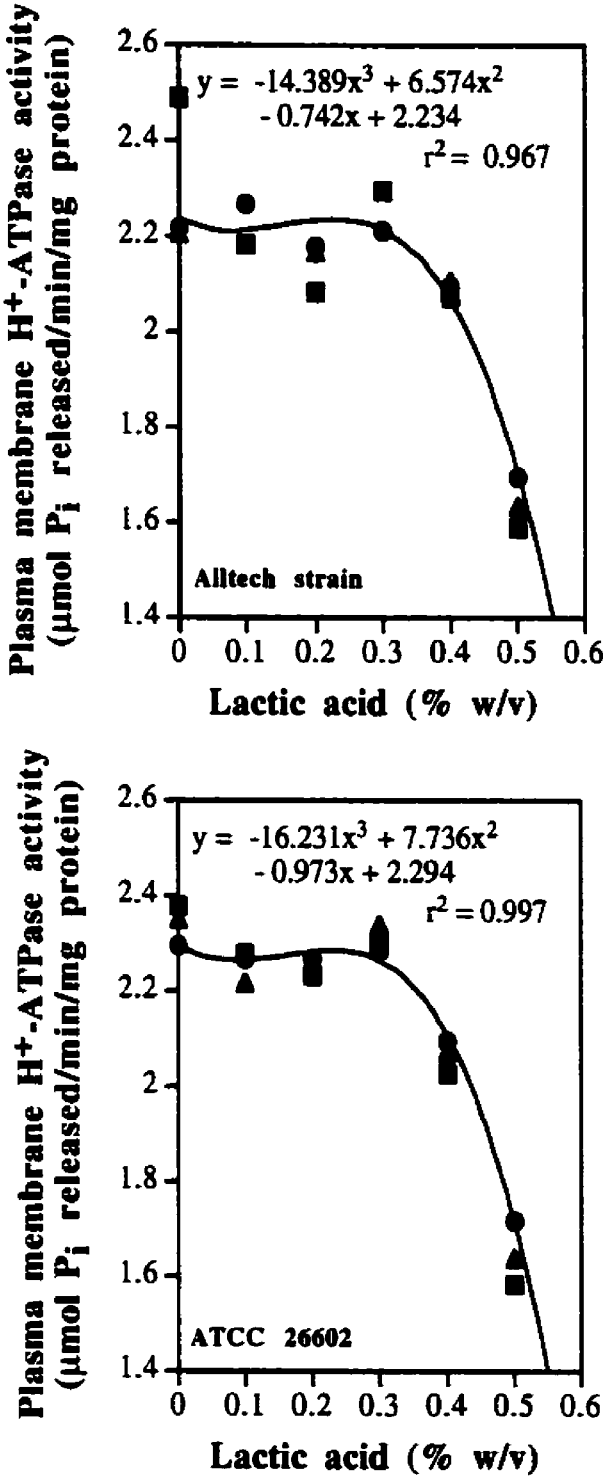


Figure 4.16 Plasma membrane H+-ATPase activity (µmol P_i released/min/mg protein) of *S. cerevisiae* (mid-exponential phase cells) in the presence of various concentrations of lactic acid in minimal medium at 30° C. The symbols indicate values from separate experiments.

Lactic acid appears to act differently from acetic acid. The H^+-ATPase activity decreased significantly at higher concentrations (0.4 - 0.5 % w/v) of lactic acid in the medium (Table 4.11; Fig. 4.16). No significant differences (P = 0.05) were observed in the H^+-ATPase activity up to a concentration of 0.3 % w/v lactic acid (Table 4.11). The relationship was not linear, instead fitting best as a 3rd order polynomial. The decrease in the H^+-ATPase activity corresponds to the decrease in pH_i observed at higher concentrations (0.4 - 0.5 % w/v) of lactic acid in the medium (Fig. 4.14).

4.1.4.3 Effects of acetic and lactic acid on the plasma membrane fatty acid composition of *S. cerevisiae*

The lipid composition of the cell membranes of both yeast strains grown to the mid-exponential phase of growth in the presence of 0.5 % w/v lactic acid and 0.25 % w/v acetic acid (concentrations which significantly affected the plasma membrane H^+-ATPase activity) were determined and compared to the control with no acetic acid or lactic acid added to the medium. The results are shown in Table 4.12.

The fatty acid composition of both yeast strains was affected when yeast cells were grown with lactic acid (0.5 % w/v). In the presence of lactic acid, the proportion of unsaturated fatty acids (palmitoleic acid, C16:1; and oleic acid, C18:1) decreased considerably (47 % and 29 % for Alltech yeast; and 33 % and 19 % for ATCC yeast) compared to the control without any acid present in the medium (Table 4.12). At the same time, an increase in the corresponding saturated fatty acyl residues (palmitic acid, C16:0; and stearic acid, C18:0) was observed. These changes were not observed to the same extent when the cells were grown with 0.25 % w/v acetic acid (Table 4.12).

Table 4.12 Effect of acetic acid (0.25 % w/v) and lactic acid (0.5 % w/v) on fatty acid composition of *S. cerevisiae.*

Fatty acid	Percentage of total fatty acids*					
	Alltech strain			ATCC 26602		
	Control	Acetic acid	Lactic acid	Control	Acetic acid	Lactic acid
C14:0	4.1±0.1	3.3±0.0	3.5±0.0	3.5±0.1	3.4±0.4	3.7±0.3
C16:0	31.4±0.9	37.2±0.5	43.8±0.8	27.9±0.3	28.9±0.5	39.4±1.9
C16:1	25.6±0.5	23.6±1.6	13.5±0.6	34.8±0.5	34.1±0.7	23.4±0.6
C18:0	13.8±0.2	14.7±0.3	21.5±0.5	14.9±0.3	13.1±0.6	17.6±1.0
C18:1	25.2±0.6	21.2±0.9	17.8±0.3	18.9±0.5	20.4±0.4	15.4±0.4

* Values are means of two separate experiments ± the standard deviation

4.2 Management of lactobacilli in yeast-catalyzed ethanol production

4.2.1 Bactericidal effect of the antibiotic penicillin G in wheat mash

The results show that the bactericidal effect of penicillin G varies between different species of lactobacilli. *L. fermentum*, *L. paracasei* and *Lactobacillus* #3 appear to be more sensitive to the dose of penicillin G used (1.5 mg/L) compared to *L. plantarum* and *L. rhamnosus* (which appears to be the least sensitive organism of all tested; Fig. 4.17).

4.2.1.1 Bactericidal effect of penicillin G when used in the fermentation of wheat mash

There were no major differences in the fermentation rates of mashes adjusted to initial pH values of 4.5 and 5.6. Fermentation in all the treatments came to an end by 48 h after yeast inoculation (Fig. 4.18). Less than 0.1 % fermentable sugars remained at the end of the fermentation in each case.

The growth of *L. paracasei* (no antibiotic) was quite different in mashes adjusted to pH 4.5 and 5.6. The bacteria did not grow as well at pH 4.5 as they did at pH 5.6 (Fig. 4.19). Killing of the organism at pH 4.5 in the presence of penicillin G was less extensive (Fig. 4.19). This shows that the bactericidal activity of penicillin is not as good at pH 4.5 as it is at pH 5.6. This confirms that the stability of penicillin drops as the pH becomes acidic (and would be lost at pH values of 4.0 or below). Nevertheless, penicillin still resulted in a 4 log reduction of the bacterium at pH 4.5 (5 logs in 48 h in pH 5.6 mash).

Multiplication of *S. cerevisiae* is also affected at the low initial mash pH of 4.5 when compared to a normal mash at pH 5.6. The maximum viable cell numbers attained was higher when the initial mash pH was 5.6 (2.37×10^8) compared to 1.80×10^8 when mash initial pH was 4.5 (Fig. 4.20). When the initial mash pH was 4.5, the bacteria also did not grow as well (Fig. 4.19). This led to reduced lactic acid production (0.57 % w/v) (Fig. 4.21) and the yeast cells in the treatment with *L. paracasei* and (no penicillin G) did not loose viability as much as they did in the same treatment where the initial mash pH was 5.6 (Fig. 4.20). In this case, the bacteria grew well and a higher concentration of lactic acid was produced (0.99 % w/v, after 72 h). This suggests that the effects caused by this weak organic acid, lactic acid, are more lethal to yeast than those caused by a strong inorganic acid.

The maximum ethanol produced (% v/v) was higher when the initial mash pH was 5.6 (which reached a pH of 4.6, after 72 h) than when it was 4.5 (which reached a pH of 3.6 after 72 h). When penicillin G was added, there was no loss in ethanol observed at either initial pH conditions (Fig. 4.22).

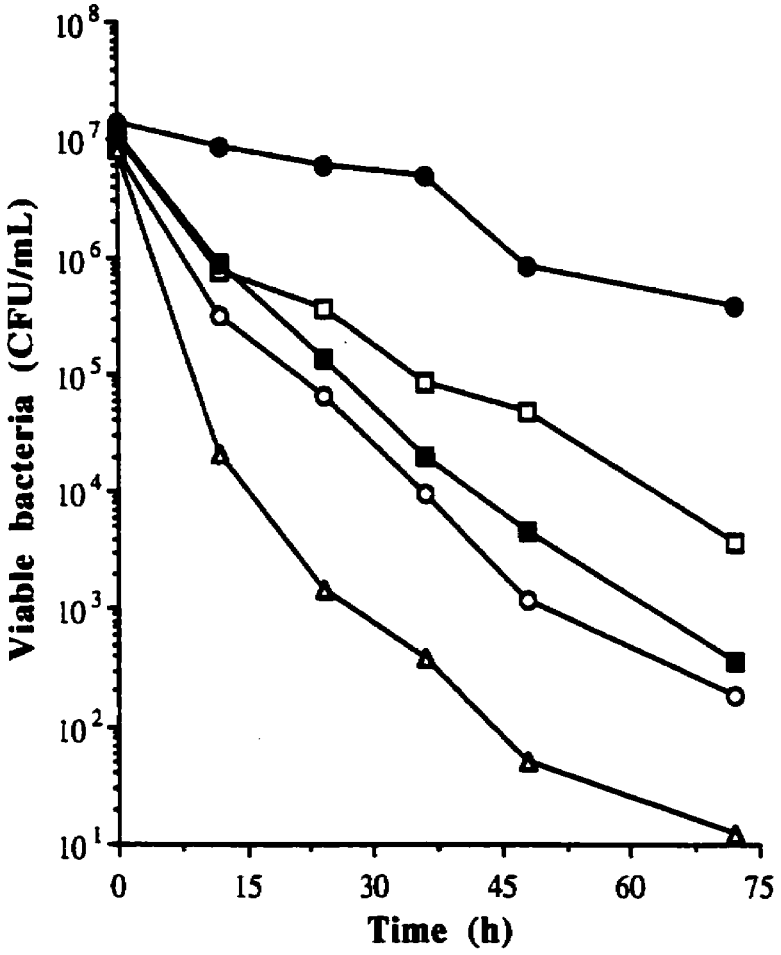


Figure 4.17 Survival of various lactobacilli (inoculated at ~ 10^7 CFU/ml) in the presence of penicillin G (1.5 mg/L or 2,475 units/L) in wheat mash (pH 5.5) at 30° C. Symbols : ❑, *L. plantarum*; ■, *L. paracasei*; ○, *Lactobacillus* #3; ●, *L. rhamnosus*; and Δ, *L. fermentum*.

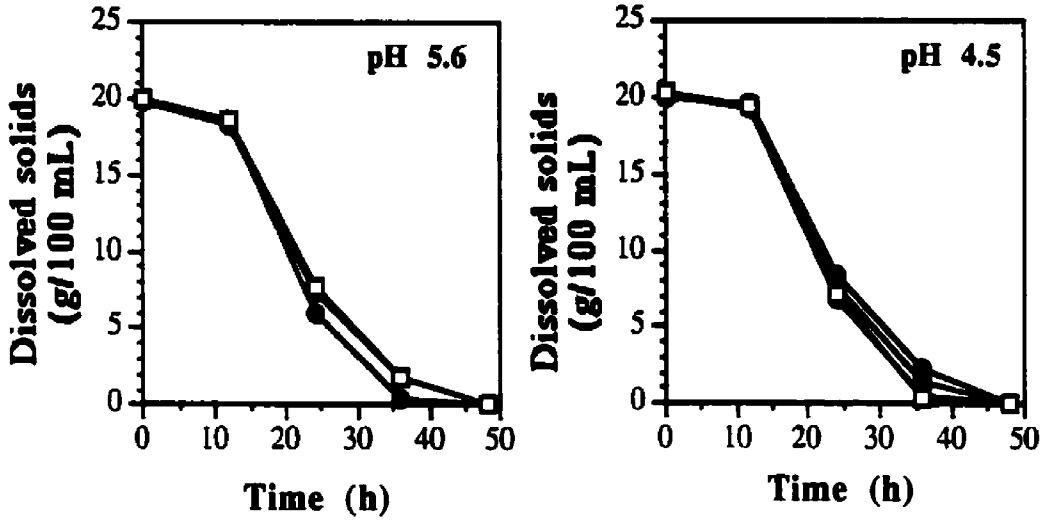


Figure 4.18 Concentrations of dissolved solids during the fermentation of wheat mash (pH 5.6 and 4.5) by yeast at 30° C. Symbols : □, Control (no bacteria and no penicillin G); ■, Penicillin G at 1.5 mg/L (2,475 units/L); ○, *L. paracasei* infected (inoculated) at ~ 10^7 CFU/ml + penicillin G; ●, *L. paracasei* infected (inoculated) at ~ 10^7 CFU/ml (no penicillin G). All treatments were inoculated with yeast at ~ 10^6 CFU/mL.

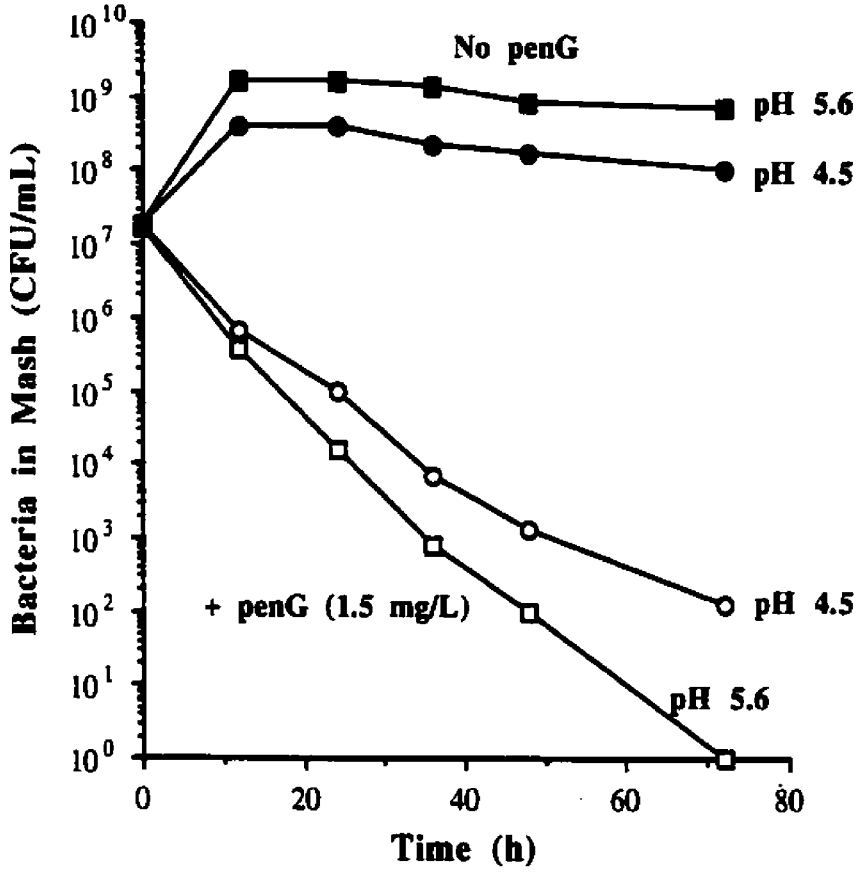


Figure 4.19 Viable *L. paracasei* in fermenting wheat mash (pH 5.6 and 4.5) at 30° C in the presence and absence of penicillin G.

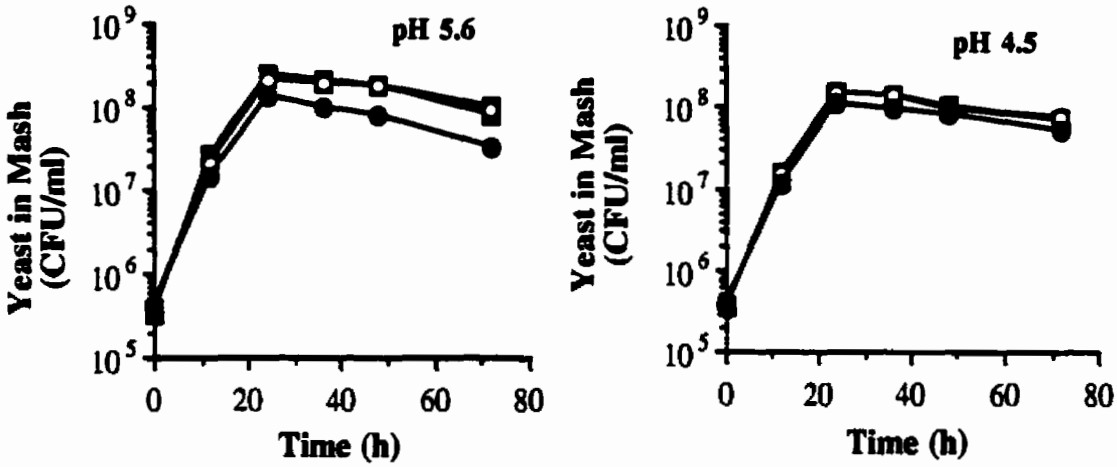


Figure 4.20 Growth of yeast during the fermentation of wheat mash (pH 5.6 and 4.5) at 30° C. Symbols : ❑, Control (no bacteria and no penicillin G); ■, Penicillin G at 1.5 mg/L (2,475 units/L); ○, *L. paracasei* infected (inoculated) at ~ 10^7 CFU/ml + penicillin G; ●, *L. paracasei* infected (inoculated) at ~ 10^7 CFU/ml (no penicillin). All treatments were inoculated with yeast at ~ 10^6 CFU/ml.

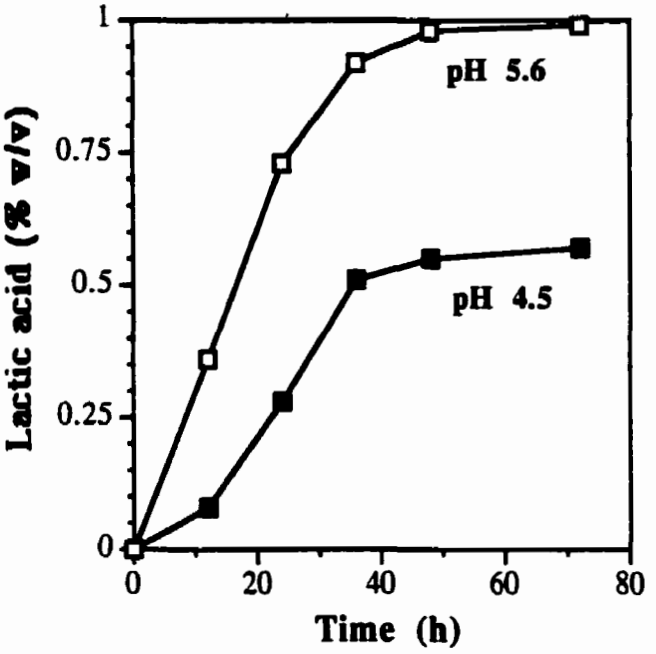


Figure 4.21 Concentration of lactic acid produced by *L. paracasei* in the absence of penicillin G in fermenting wheat mash (pH 5.6 and 4.5) at 30° C. Both treatments were inoculated with yeast at ~ 10^6 CFU/ml.

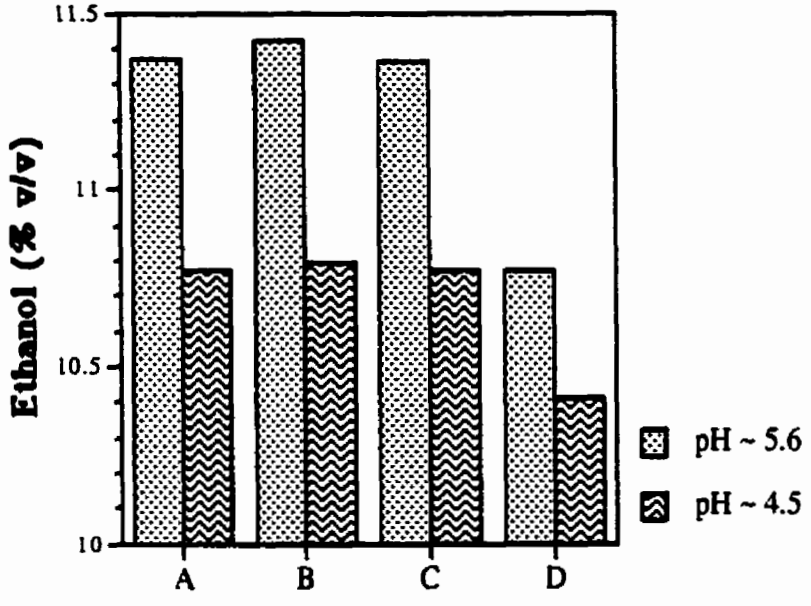


Figure 4.22 Concentration of ethanol produced after fermentation of normal-gravity wheat mash (pH 5.6 and 4.5) at 30° C for 72 h by yeast. (A) Yeast alone; (B) Yeast + penicillin G; (C) Yeast + *L. paracasei* + penicillin G; (D) Yeast + *L. paracasei*. Yeast was inoculated at ~ 10^6 CFU/ml and *L. paracasei* was inoculated at ~ 10^7 CFU/ml.

In the treatment where the mash was inoculated (contaminated) with the bacteria and penicillin G was not added, a 5.27 % yield loss of ethanol was recorded at pH 5.6 and a 3.34 % yield loss was recorded at pH 4.5 (Fig. 4.22). The results show that penicillin G at a dose of 1.5 mg/L, a concentration used by industry, effectively controls *L. paracasei.*

4.2.2 Bactericidal effect of stabilized chlorine dioxide

From the results, it is obvious that chlorine dioxide (ClO_2) demonstrates a bactericidal effect against all the five species tested at mash pH 5.5 and lower. Only *L. plantarum* and *L. fermentum* grew (at pH 6.0) while no growth was observed with other species even at pH 6.0 for up to 30 h (Fig. 4.23).

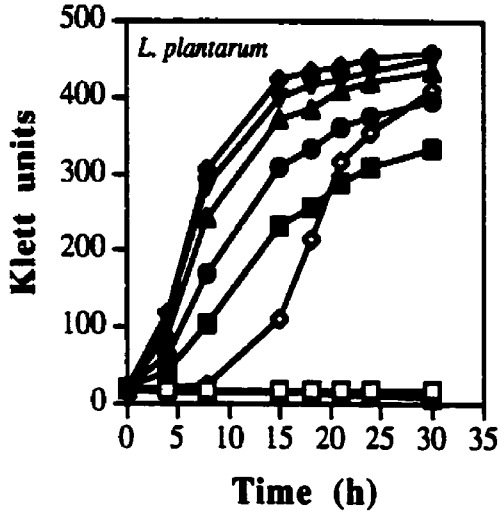
4.2.2.1 Dosage of chlorine dioxide to be used in fermentation mashes

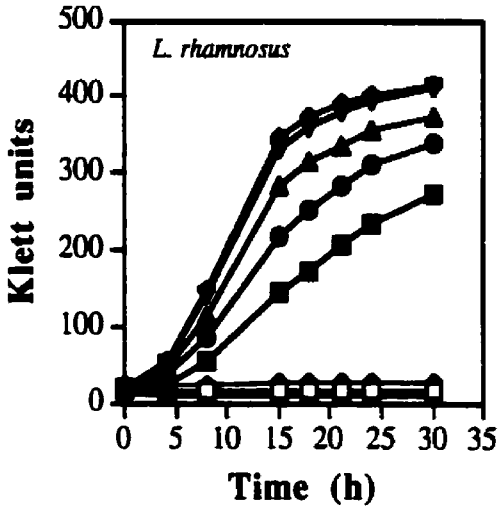
An experiment was conducted to determine if a higher dose of chlorine dioxide needs to be applied directly to the fermentation mash containing particulate materials. It is known that 100 ppm chlorine dioxide is sufficient to kill bacteria in clear media. From the results (Table 4.13), 100 ppm chlorine dioxide, the lowest concentration tested, was chosen as an effective dose.

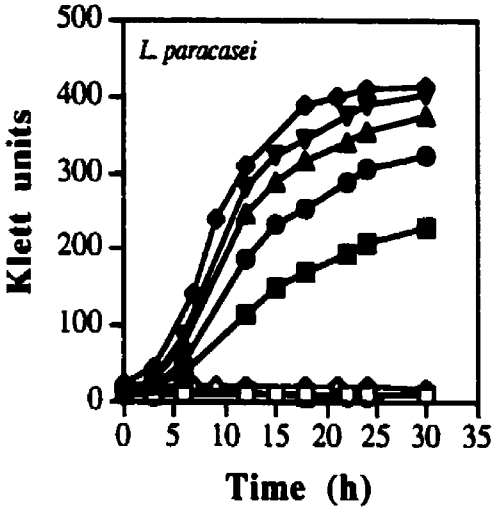
4.2.2.2 Effect of stabilized chlorine dioxide on yeast growth

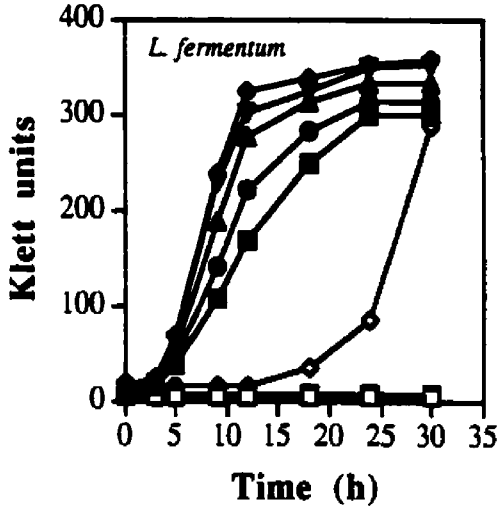
The growth rate of yeast was reduced considerably in the presence of chlorine dioxide (100 ppm) (Fig. 4.24). Then, a similar experiment in duplicate was carried out using lower concentrations (0 ppm - control, 50, 60, 70, 80 and 90 ppm). The results (Fig. 4.25) show that a concentration of 50 ppm ClO_2 led to a slight reduction of yeast growth rate, but the reduction was not extensive enough to negate 50 ppm as a suitable concentration for industrial application.

The pattern of growth of yeast in Figure 4.24 suggested a period of incubation of media with ClO_2 prior to yeast inoculation that could lead to the dissipation of the compound and allow growth of yeast at a normal rate. These experiments were done by designating different periods of preincubation of the media (YPD broth at pH 5.5) with 100 ppm of ClO_2 before yeast inoculation (0 - control, 1, 2, 3, 4, 5, 6 and 12 h). All treatments were done in duplicate. Yeast inoculation and monitoring of its growth were done as mentioned in the Materials and Methods. Results (Fig. 4.26a and 4.26b) show that even a preincubation of the media with ClO_2 for a period of 12 h was not sufficient to match the growth rate of yeast with that in the absence of ClO_2. In all the experiments, the pattern of curves appeared to be the same both in the presence and the absence of ClO_2.









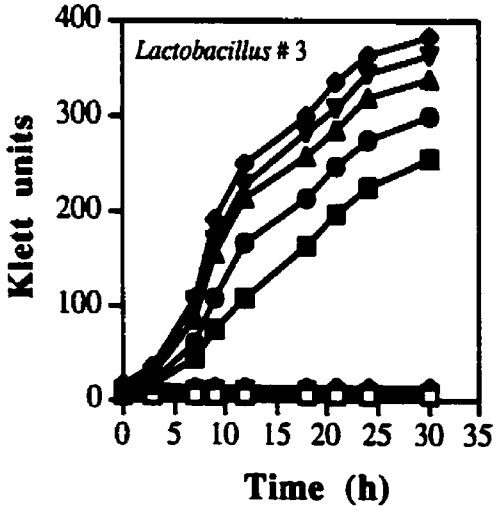


Figure 4.23 Effect of stabilized chlorine dioxide at 100 ppm on the growth of various lactobacilli in MRS broth at 30° C at different pH levels. ■, pH 4.0; ●, pH 4.5; ▲, pH 5.0; ▼, pH 5.5; and ◆, pH 6.0. All the filled symbols indicate 0 ppm chlorine dioxide controls while the presence of chlorine dioxide is indicated by open symbols.

Table 4.13 Concentration of lactic acid produced by *L. paracasei* inoculated at approximately 10^7 CFU/ml to 50 ml of wheat mash at 30° C (after 48 h) in the presence of stabilized chlorine dioxide at various levels.

Chlorine dioxide (ppm)	Lactic Acid (% w/v)[1]	Standard Deviation (σ)
Control (0)	1.10	0.03
100	0.03	0.01
200	0.00	0.00
300	0.00	0.00
400	0.00	0.00

[1] Average of triplicate samples.

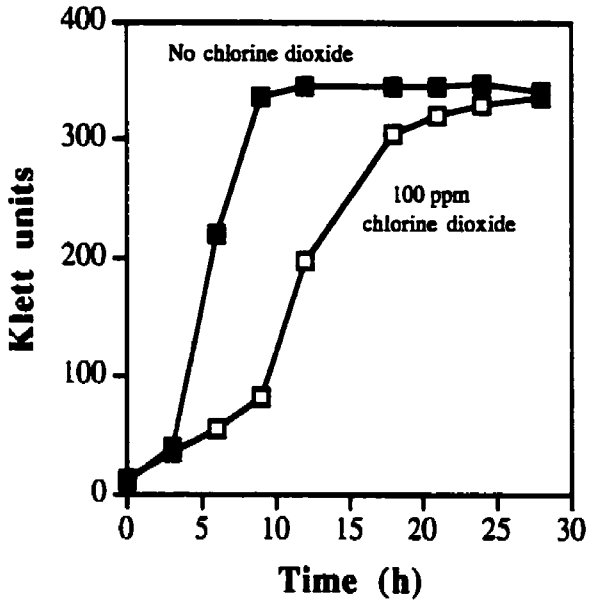


Figure 4.24 Effect of stabilized chlorine dioxide on the growth of *S. cerevisiae* (Alltech strain) in yeast extract-peptone-dextrose (YPD) broth of pH 5.5 at 30° C.

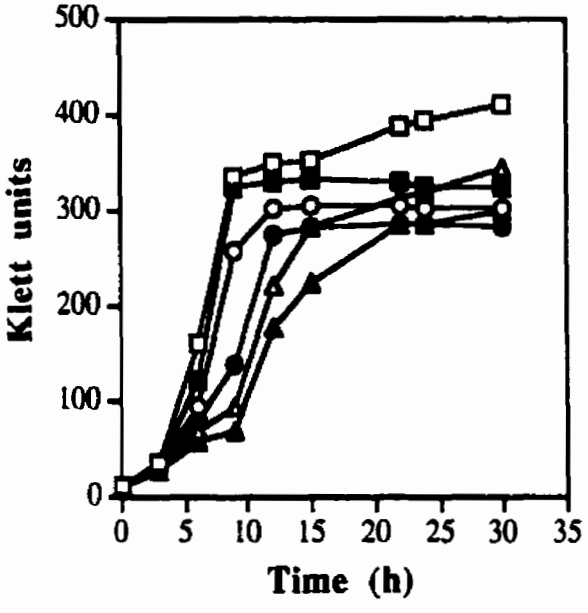


Figure 4.25 Growth of *S. cerevisiae* (Alltech strain) in YPD broth (pH 5.5) at 30° C as affected by various concentrations of stabilized chlorine dioxide. ❑, 0 ppm (control); ■, 50 ppm; ○, 60 ppm; ●, 70 ppm; Δ, 80 ppm; and ▲, 90 ppm.

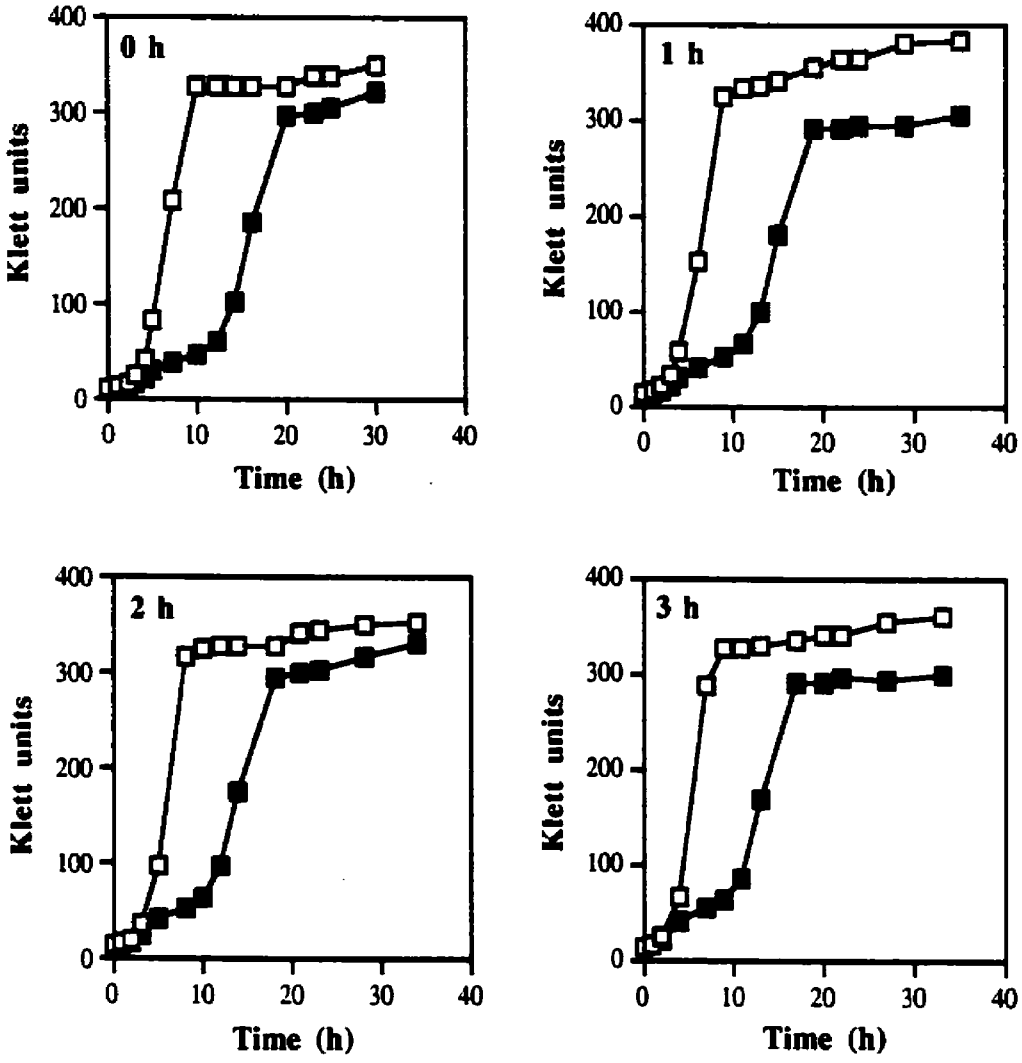


Figure 4.26a Effect of stabilized chlorine dioxide at 100 ppm on the growth of *S. cerevisiae* (Alltech strain) in YPD broth (pH 5.5) at 30° C. The media were preincubated with chlorine dioxide before yeast inoculation for various time intervals as indicated. Symbols : ❑ , chlorine dioxide at 0 ppm (control); and ■ , 100 ppm.

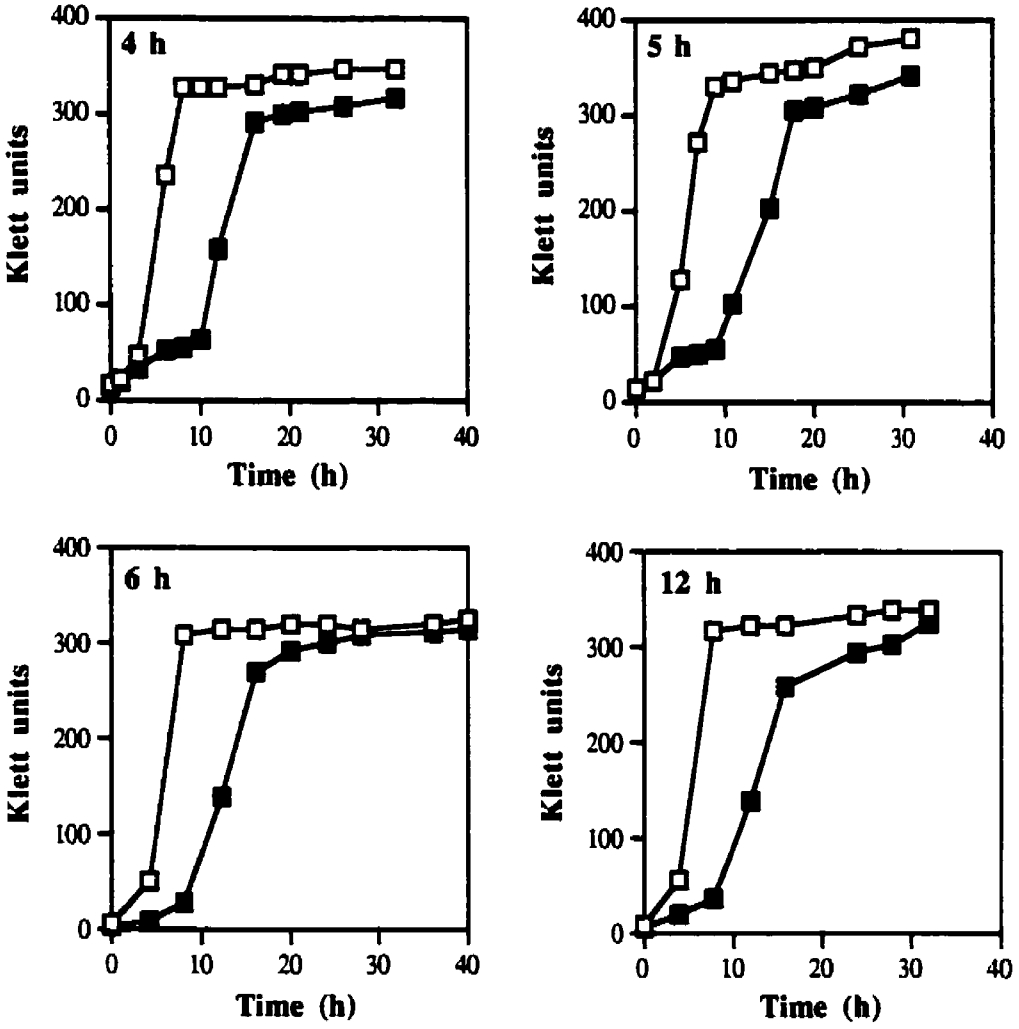


Figure 4.26b Effect of stabilized chlorine dioxide at 100 ppm on the growth of *S. cerevisiae* (Alltech strain) in YPD broth (pH 5.5) at 30° C. The media were preincubated with chlorine dioxide before yeast inoculation for various time intervals as indicated. Symbols : ❑ , chlorine dioxide at 0 ppm (control); and ■ , 100 ppm.

The results (Fig. 4.26a and 4.26b) imply that chlorine dioxide remains stable in the medium for at least 12 h in the absence of yeast. A preincubation period of the media with ClO_2 for over 12 h is not likely to be adopted commercially by the ethanol industry. The pattern of growth observed in Figures 4.26a and 4.26b suggest the possibility of induction of resistance in *S. cerevisiae* to chlorine dioxide in the initial 8 - 10 h of growth in the presence of ClO_2 (100 ppm).

To study if resistance to chlorine dioxide was induced in *S. cerevisiae*, the cells were grown in YPD broth (pH 5.5) to exponential phase in the presence of 100 ppm ClO_2 and these cells were inoculated into fresh media (YPD broth) both in the presence and absence of ClO_2 (100 ppm) and the growth was monitored (Fig. 4.27). The same pattern was still observed (Fig. 4.27) indicating that there is no development of resistance to chlorine dioxide in *S. cerevisiae*. The organism adapts to the presence of ClO_2 during the initial 8 - 10 h of growth, but resumes its normal rate of growth after the long lag.

4.2.2.3 Bactericidal effect of chlorine dioxide at lower concentrations

Concentrations of ClO_2 lower than 100 ppm were tested to find the minimum concentration of ClO_2 that would inhibit the growth of *L. paracasei* (since at a concentration of 50 ppm the growth of yeast is unaffected as seen in Figure 4.25). The result (Fig. 4.28) showed that the minimum inhibitory concentration of ClO_2 for *L. paracasei* was 90 ppm. At 80 ppm, there was a slight increase in cell density after 30 h (4 Klett units). Chlorine dioxide at a concentration of 90 ppm would be the least concentration that could be used for effective control of this one strain of lactobacilli. One manufacturer has indicated that 100 ppm is required to achieve a 99.99 % kill of both *Pseudomonas aeruginosa* and *Streptococcus* spp. The work reported here has been conducted with organisms more relevant to the ethanol fermentation industry.

The bactericidal effects of ClO_2 at concentrations ranging from 0 - 90 ppm were then tested against the other four strains of lactobacilli. Different lactobacilli differed in their ability to tolerate stabilized chlorine dioxide (Fig. 4.29). *L. plantarum* grew well even at 80 ppm ClO_2, while *Lactobacillus* #3 grew well at 70 ppm within 24 h. These concentrations are inhibitory to culture yeast (*Saccharomyces cerevisiae*). Therefore, stabilized chlorine dioxide (ClO_2) is not suitable to control lactobacilli during alcoholic fermentation of grain mashes. The concentrations found to inhibit all possible lactic bacteria (at least 80 ppm) would affect yeast growth and reduce its vitality and rate of fermentation.

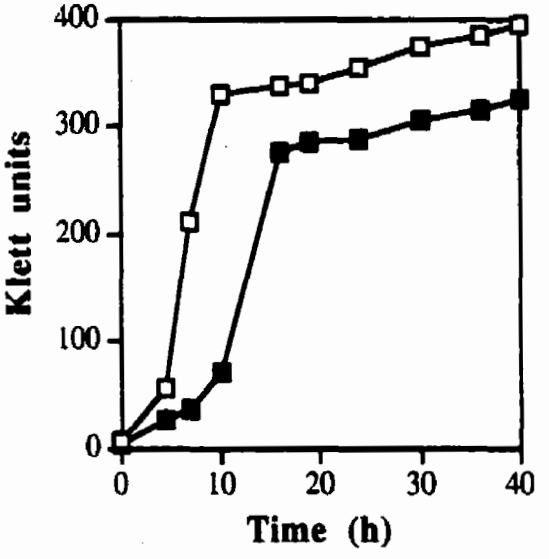


Figure 4.27 Effect of stabilized chlorine dioxide on the growth of *S. cerevisiae* (Alltech strain; pre-grown to log phase in YPD broth (pH 5.5) with 100 ppm chlorine dioxide at 30° C) in the presence of 100 ppm chlorine dioxide in YPD broth (pH 5.5) at 30° C. Symbols : □, No chlorine dioxide (control); and ■, chlorine dioxide at 100 ppm.

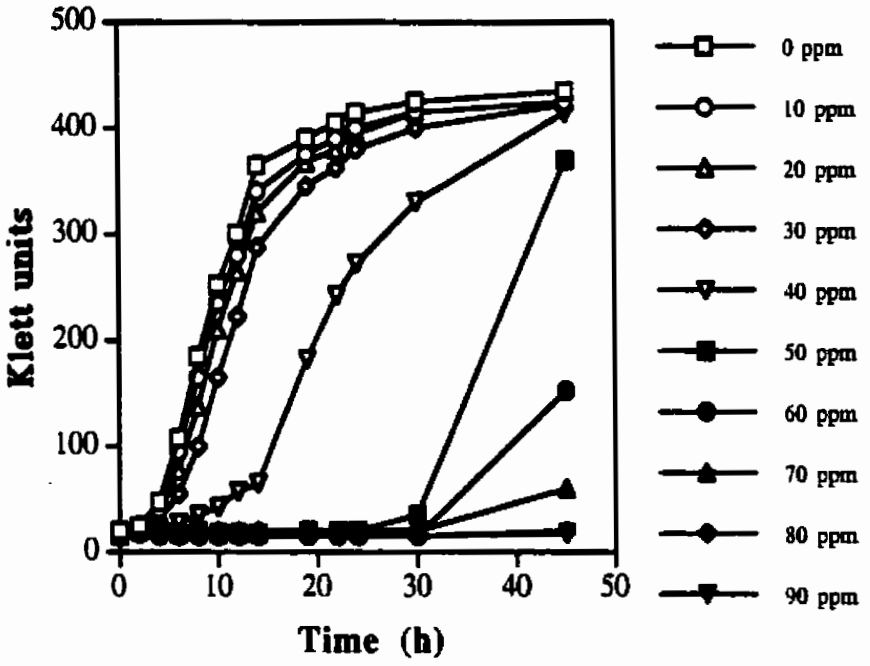


Figure 4.28 Growth of *L. paracasei* in the presence of various concentrations of stabilized chlorine dioxide in MRS broth (pH 5.5) at 30° C.

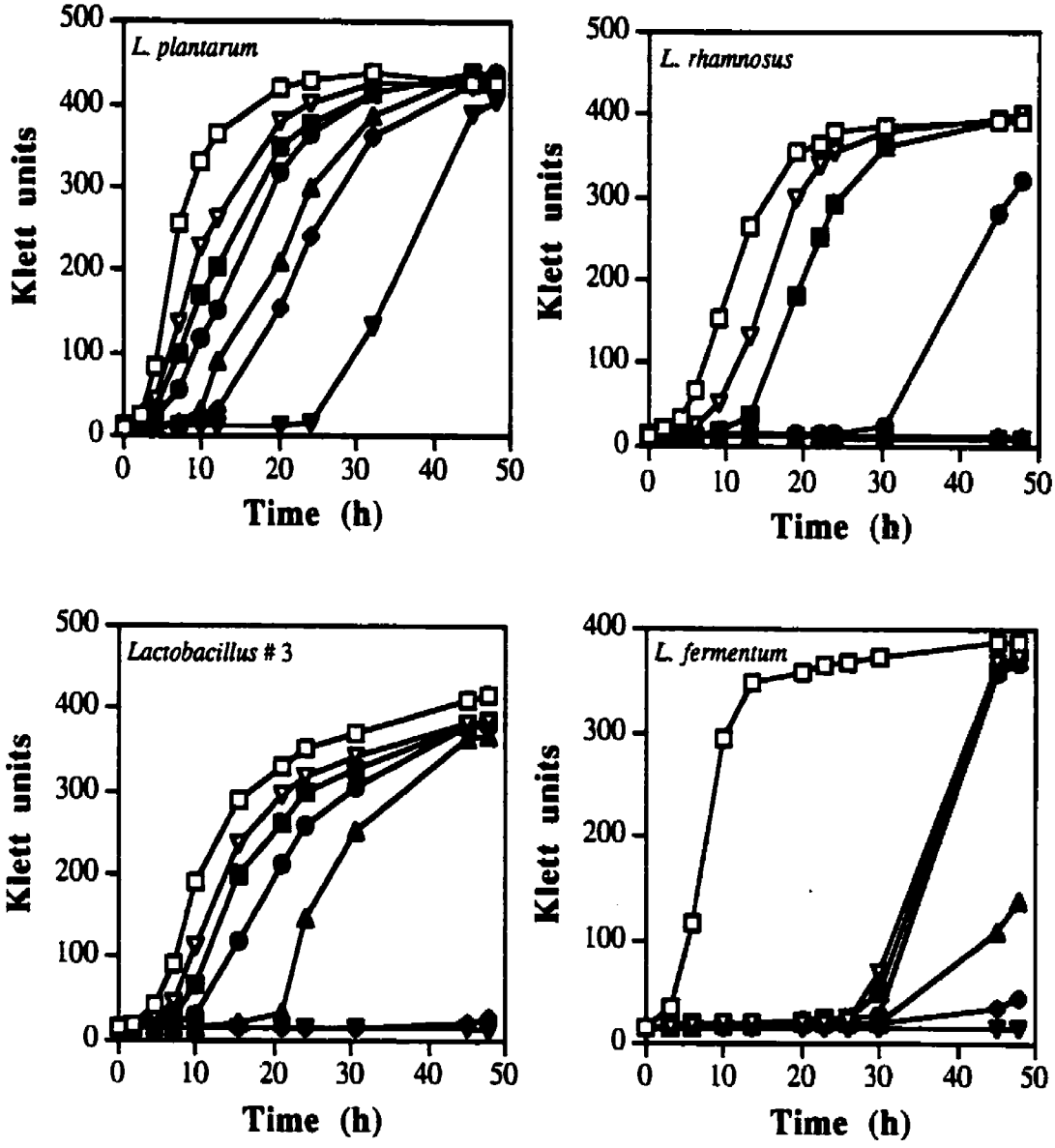


Figure 4.29 Growth of various lactobacilli in the presence of different concentrations of stabilized chlorine dioxide in MRS broth (pH 5.5) at 30° C. Symbols are: □, 0 ppm (control); ▽, 40 ppm; ■, 50 ppm; ●, 60 ppm; ▲, 70 ppm; ◆, 80 ppm; and ▼, 90 ppm.

4.2.3 Bactericidal effect of nisin

The effectiveness of nisin varies with each organism studied (Fig. 4.30). Nisin even at a concentration of 100 mg/L did not affect the growth of *L. paracasei* and *Lactobacillus* #3. In the case of *L. plantarum*, the addition of nisin delayed growth for about 12 h even at 50 mg/L. However, growth resumed at close to the previous rate and *L. rhamnosus* showed no growth until 30 h (even at 50 mg/L) after which growth resumed at all the concentrations of nisin used. *L. fermentum* was totally inhibited by nisin at 50 mg/L for up to 48 h (Fig. 4.30).

Nisaplin® (the nisin preparation used) was also tested against the test organism *M. luteus* (ATCC 4698). There was absolutely no growth of the organism in the presence of nisin at 50 mg/L for 72 h in Bond's broth at 30° C. This indicated that the preparation of nisin was effective.

4.2.4 Bactericidal concentrations of urea hydrogen peroxide

Urea hydrogen peroxide at a concentration of 32 mM reduced the final lactic acid produced by *L. paracasei* in unclarified wheat mash from 1.14 % w/v (in the control) to 0 % w/v (Table 4.14). Urea hydrogen peroxide in mash breaks down into urea and hydrogen peroxide. If yeast was added at the beginning or immediately after the addition of urea hydrogen peroxide, the hydrogen peroxide would be decomposed into water and molecular oxygen through the action of catalase (present in yeast) resulting in the loss of the bactericidal effect of hydrogen peroxide on contaminating lactobacilli. Therefore, it was necessary to preincubate the mash with urea hydrogen peroxide for a specified period prior to yeast inoculation. This may be done during saccharification of the mash or post-saccharification (in the fermentor), but must be done prior to yeast addition. For maximal bactericidal action on high levels of contaminating bacteria, a preincubation period for 2 h with urea hydrogen peroxide was required to kill up to 5 logs of bacteria and to reduce any chance of growth of these bacteria in new mash (Fig. 4.31).

4.2.4.1 Bactericidal effect of urea hydrogen peroxide

Application of urea hydrogen peroxide resulted in four to five log reductions in viable cell numbers of all five industrially important isolates of lactobacilli studied in 2 h (Table 4.15). The results indicate that urea hydrogen peroxide is effective in preventing the growth of bacterial contaminants which cause reduction in ethanol yield.

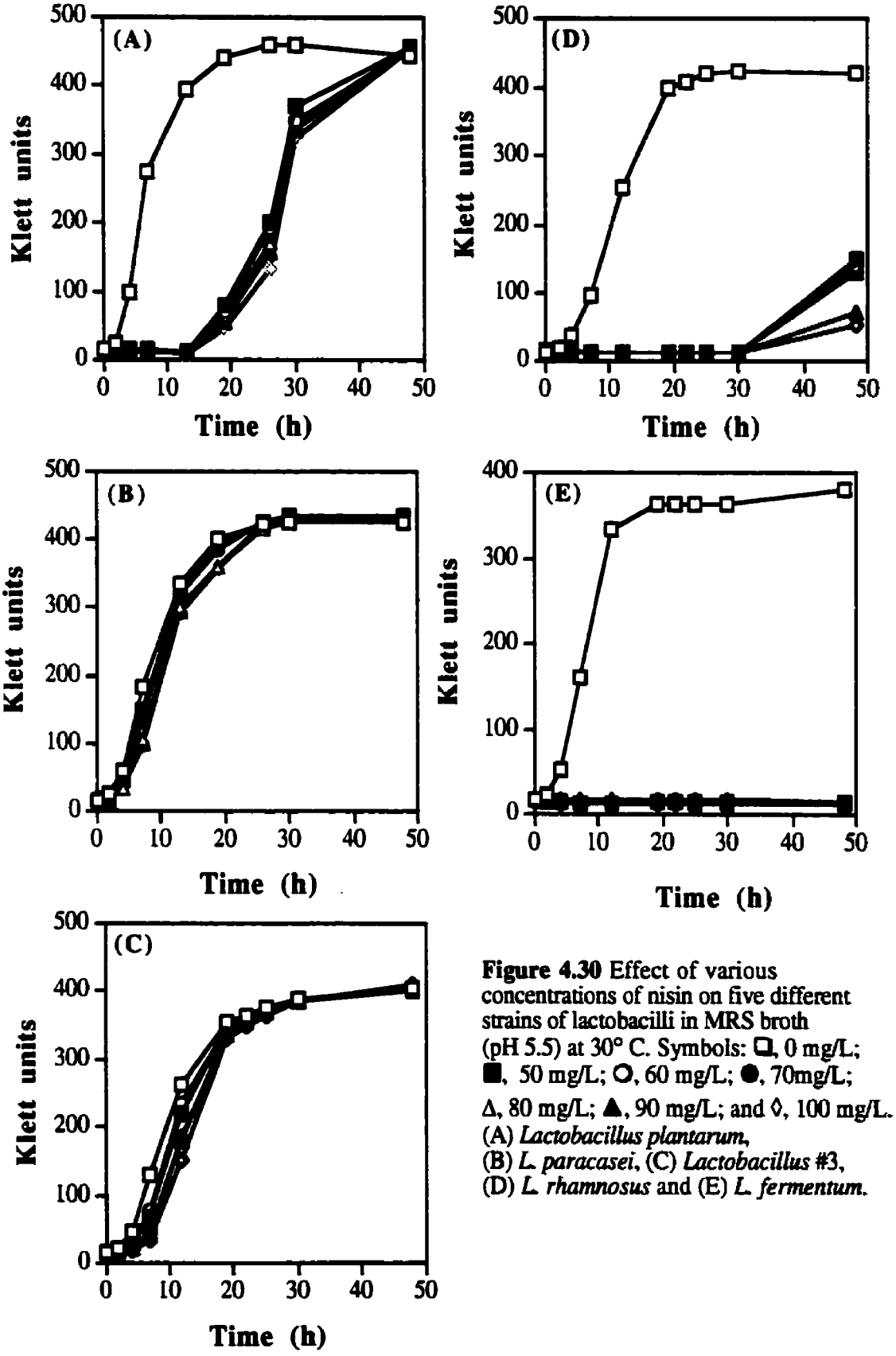


Figure 4.30 Effect of various concentrations of nisin on five different strains of lactobacilli in MRS broth (pH 5.5) at 30° C. Symbols: □, 0 mg/L; ■, 50 mg/L; ○, 60 mg/L; ●, 70mg/L; Δ, 80 mg/L; ▲, 90 mg/L; and ◊, 100 mg/L. (A) *Lactobacillus plantarum*, (B) *L. paracasei*, (C) *Lactobacillus* #3, (D) *L. rhamnosus* and (E) *L. fermentum*.

Table 4.14 The 48 h concentration of lactic acid produced by *L. paracasei* inoculated at ~ 10^7 CFU/ml into unclarified wheat mash at 30° C in the presence of urea hydrogen peroxide at various levels.

Urea hydrogen peroxide (mmoles/L)	Lactic Acid (% w/v)[1]
0	1.14 ± 0.03
2.1	1.10 ± 0.01
5.4	1.05 ± 0.01
10.7	1.01 ± 0.03
21.3	0.57 ± 0.03
32.1	0.00 ± 0.00
42.6	0.00 ± 0.00

[1] Mean of triplicate samples ± standard deviation

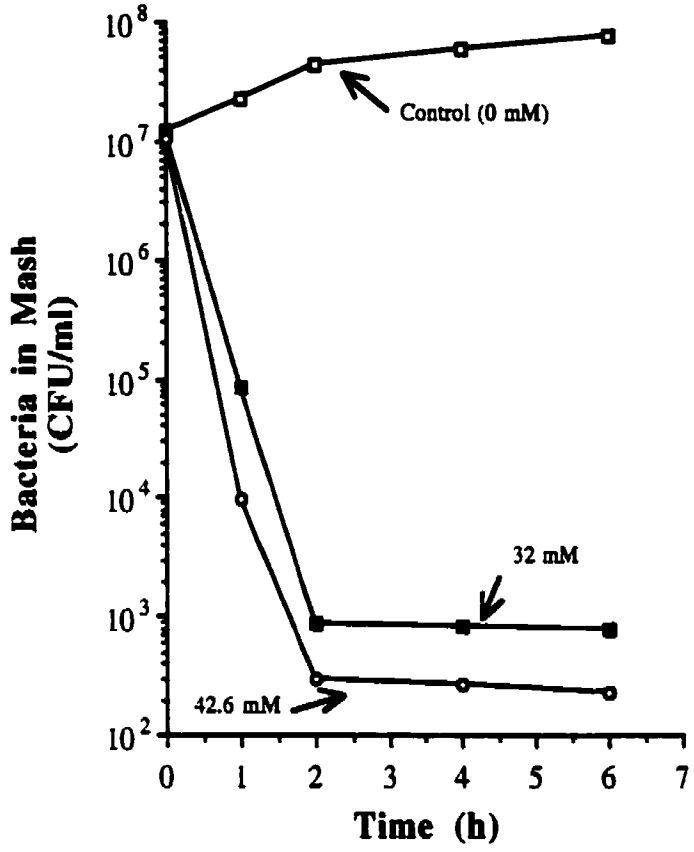


Figure 4.31 Viable numbers of *L. paracasei* in unclarified wheat mash (no added yeast) with added urea hydrogen peroxide over a 6 h incubation at 30° C.

Table 4.15 The survival of various lactobacilli in unclarified wheat mash at 30° C in the presence of urea hydrogen peroxide at 32.1 mmoles/L (the numbers are expressed in CFU/ml*).

Time (h)	*L. plantarum*	*L. paracasei*	*Lactobacillus* # 3	*L. rhamnosus*	*L. fermentum*
0	1.09×10^7	1.15×10^7	0.88×10^7	0.85×10^7	0.83×10^7
2	5.67×10^2	8.67×10^2	7.67×10^2	3.00×10^2	7.50×10^2
4	5.83×10^2	8.33×10^2	7.67×10^2	3.33×10^2	7.00×10^2

* All values are the mean of duplicate samples. Plating was done in triplicate.

Further work (on UHP) indicated that application of 2 mM urea hydrogen peroxide was sufficient to kill Gram-positive sugar and brewing contaminants *Pediococcus damnosus* (ATCC 29358), *Pediococcus* spp. (BSO 77) and the Gram-negative contaminant, *Zymomonas anaerobia* in MRS broth when inoculated at ~ 10^7 CFU/ml. Similarly, Block (1991) has reported that hydrogen peroxide is also lethal to other bacterial species like *S. aureus*, *E. coli*, *Streptococcus* spp. and spore forming *Bacillus* spp.

The bactericidal effectiveness of urea hydrogen peroxide did not differ significantly when added as a powder or in the form of a filter-sterilized 40 % (w/v) solution in deionized water (Fig. 4.32).

Batch fermentations of unclarified wheat mash contaminated with *L. paracasei* at ~10^7 CFU/ml were carried out in the presence and absence of urea hydrogen peroxide. The details of the treatments are given in the Materials and Methods section. The fermentations were complete (less than 0.05 g/100 ml dissolved solids remaining) in 36 h in all the treatments. Viable yeast counts reached a maximum during the first 24 h. Compared to the controls, the yeast viable numbers were higher in treatments with urea hydrogen peroxide and with hydrogen peroxide. The viable number of yeast cells were the lowest in samples treated with bacteria, but where no agents were added (Fig. 4.33).

The viable bacterial numbers in mash dropped significantly from ~10^7 CFU/ml to ~2×10^2 CFU/ml in the first 2 h when treated with urea hydrogen peroxide or hydrogen peroxide. Once the yeast was inoculated, remaining viable bacteria were able to reinitiate growth (Fig. 4.34) because the yeast rapidly decomposed the residual hydrogen peroxide in the medium using catalase enzyme. Even though the fermentation was effectively over at 36 h, the bacteria continued to grow (Fig. 4.34) - presumably on substrates not used by yeast and on lytic products from yeast. At 36 h, however, the bacterial numbers were too low to have caused significant reductions of ethanol yield; control of the fermentation at the critical time was effected by this practice.

The lactic acid concentration in the medium where urea hydrogen peroxide was used was as low as that in the treatment with yeast alone with no bacteria (~0.03 % w/v) (Fig. 4.35). In the treatment where hydrogen peroxide alone was used, 0.05 % (w/v) lactic acid was detected. But, in the medium where neither bactericidal agent was used, 0.9 % (w/v) lactic acid was found at the time when the ethanol production was maximal in all treatments. This level (0.9 % w/v) severely affected yeast viability (Fig. 4.33).

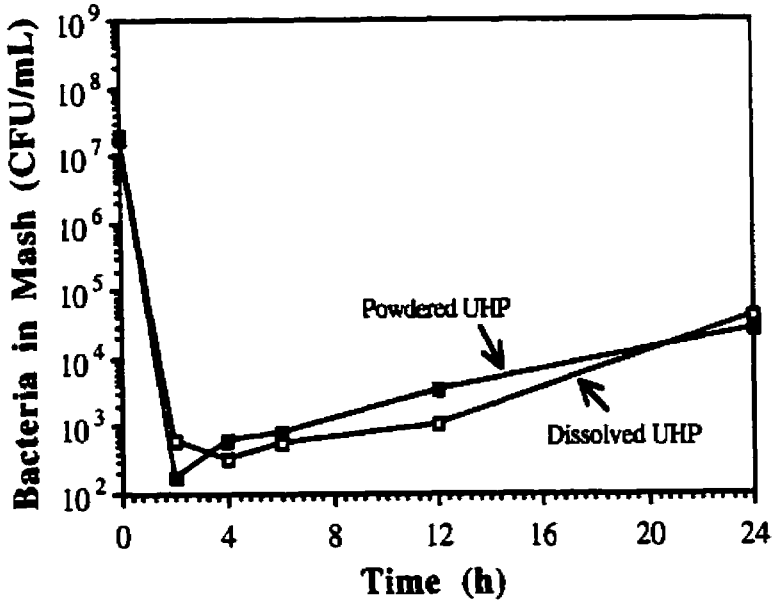


Figure 4.32 Survival of *L. paracasei* in wheat mash at 30° C in the presence of urea hydrogen peroxide. A 40 % w/v solution of urea hydrogen peroxide was made in sterile deionized water.

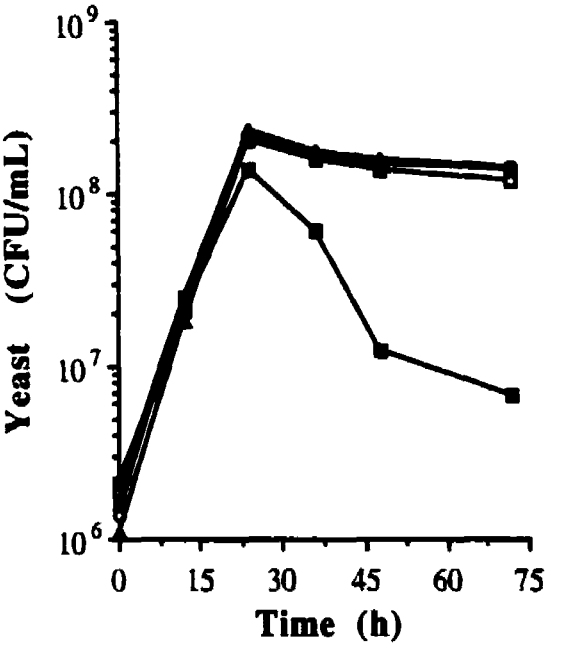


Figure 4.33 Growth of yeast during the fermentation of wheat mash at 30° C. Symbols : ❑, Control (yeast + 30 mM urea, no bacteria); ■, yeast + *L. paracasei* + 30 mM urea (no antimicrobial agent); ○, yeast + urea hydrogen peroxide at 30 mM; ●, yeast + *L. paracasei* + urea hydrogen peroxide at 30 mM; Δ, yeast + 30 mM H_2O_2 + 30 mM urea (added separately); ▲, yeast + *L. paracasei* + 30 mM H_2O_2 + 30 mM urea (added separately). In all cases, yeast was inoculated at ~10^6 CFU/ml. Where added, *L. paracasei* was inoculated at ~10^7 CFU/ml.

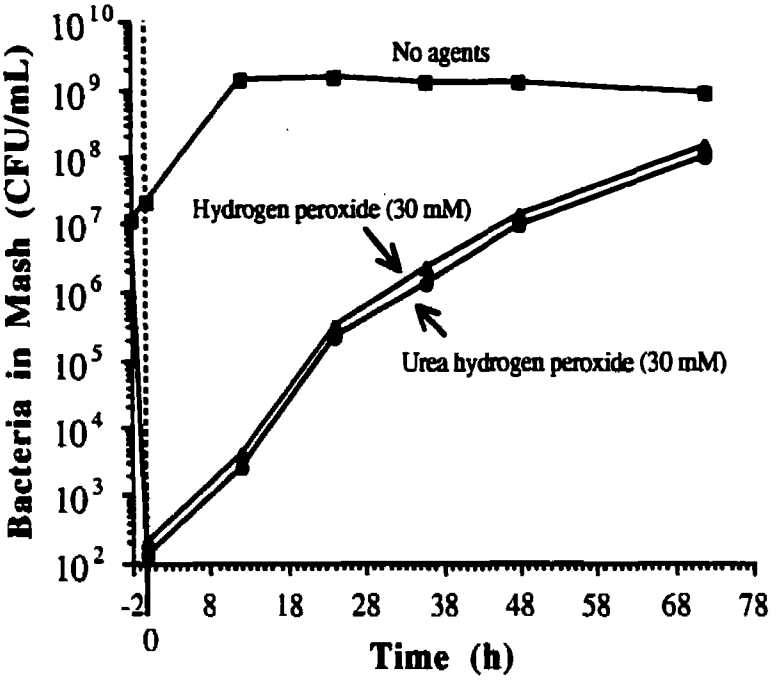


Figure 4.34 Growth of *L. paracasei* in fermenting wheat mash at 30° C in the presence and absence of hydrogen peroxide or urea hydrogen peroxide. All treatments had yeast inoculated at ~ 10^6 CFU/ml (at time 0 h). Urea hydrogen peroxide (30 mM) yields 30 mmoles/L of H_2O_2 and 30 mmoles/L urea.

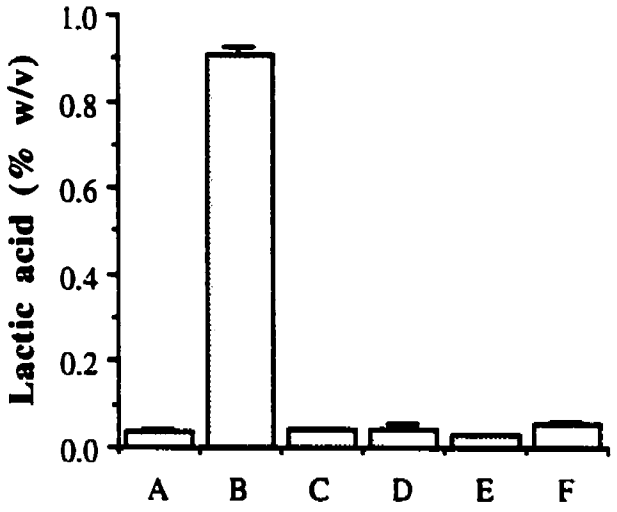


Figure 4.35 Concentration of lactic acid produced (after 36 h) by *L. paracasei* in the presence and absence of urea hydrogen peroxide or hydrogen peroxide in fermenting wheat mash at 30° C. **A**, Control (yeast + 30 mM urea, no bacteria); **B**, yeast + *L. paracasei* + 30 mM urea (no antimicrobial agent); **C**, yeast + urea hydrogen peroxide at 30 mM; **D**, yeast + *L. paracasei* + urea hydrogen peroxide at 30 mM; **E**, yeast + 30 mM H_2O_2 + 30 mM urea (added separately); **F**, yeast + *L. paracasei* + 30 mM H_2O_2 + 30 mM urea (added separately). In all cases, yeast was inoculated at ~10^6 CFU/ml. Where added, *L. paracasei* was inoculated at ~10^7 CFU/ml. Error bars indicate mean of duplicate values ± standard deviation.

The maximum concentrations of ethanol produced in all the treated fermentors were not significantly different from each other, but in the fermentor that had neither urea hydrogen peroxide nor hydrogen peroxide to kill the *L. paracasei*, there was a 5.84 % reduction in overall ethanol yield compared to the control with yeast alone and no agents added (Fig. 4.36).

4.2.4.2 Comparison of two other nitrogenous sources along with urea in combination with hydrogen peroxide in batch fermentation of unclarified wheat mash

Diammonium hydrogen phosphate (called DAP in the fermentation industry) and ammonium dihydrogen phosphate were compared to urea in combination with hydrogen peroxide to find if this combination had a similar effect as did urea hydrogen peroxide. The treatment details are outlined in the Materials and Methods section.

Fermentations were complete (less than 0.05 g/100 ml dissolved solids remained) in 36 h in treatments 1 through 3 where urea was used. This was because of the increased availability of assimilable nitrogen. As grain mashes are deficient in usable nitrogen, yeast growth and fermentation rate benefit from the added nitrogen source (urea). But in treatments 4 through 7 where either $(NH_4)H_2PO_4$ or $(NH_4)_2HPO_4$ was used, the fermentation was not complete even after 72 h (stuck), leaving ~ 0.5 g dissolved solids/100 ml. The dissolved solids remaining were verified by HPLC analysis to be mainly glucose.

The viable bacterial numbers in mash dropped significantly from ~10^7 CFU/ml to ~2×10^2 CFU/ml and ~ 8×10^2 CFU/ml (where DAP was used as nitrogen source) in the 2 h pre-incubation due to the presence of hydrogen peroxide in the absence of yeast. Once the yeast was inoculated, surviving lactic bacteria started to grow because the yeast again decomposed the remaining hydrogen peroxide in the medium using the enzyme catalase. In the treatments where $(NH_4)H_2PO_4$ and $(NH_4)_2HPO_4$ were used, the growth rates of bacteria initially were higher compared to that in which urea was used, but the onset of a progressive death of these bacteria was observed after 36 h when $(NH_4)H_2PO_4$ was used and after 48 h when DAP was used (Fig. 4.37). This might be attributed to the pH of the medium (the pH was 3.8 - 3.9) and/or the presence of phosphate anion. It can even be speculated that these organisms after a stress due to hydrogen peroxide are not able to adapt to a second stress (acidity). Nutrients may be limiting as well by this time.

Numbers of viable yeast cells reached a maximum during the first 24 h. The maximum viable numbers attained in the various treatments are shown in Fig. 4.38.

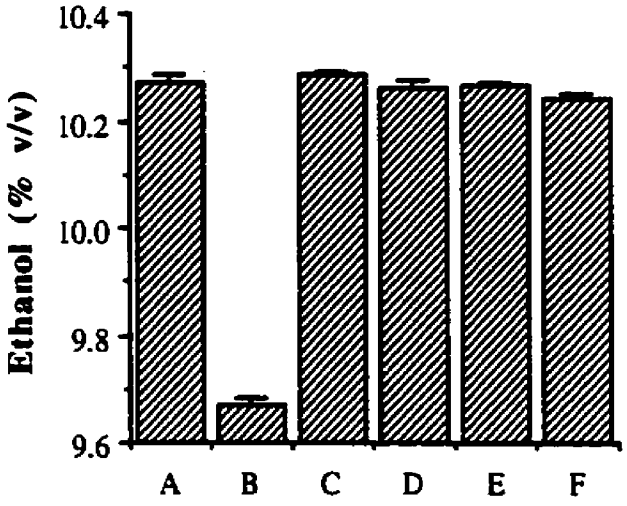


Figure 4.36 Concentration of ethanol after 36 h of fermentation of wheat mash by *S. cerevisiae* at 30° C. **A**, Control (yeast + 30 mM urea, no bacteria); **B**, yeast + *L. paracasei* + 30 mM urea (no antimicrobial agent); **C**, yeast + urea hydrogen peroxide at 30 mM; **D**, yeast + *L. paracasei* + urea hydrogen peroxide at 30 mM; **E**, yeast + 30 mM H_2O_2 + 30 mM urea (added separately); **F**, yeast + *L. paracasei* + 30 mM H_2O_2 + 30 mM urea (added separately). In all cases, yeast was inoculated at ~10^6 CFU/ml. Where added, *L. paracasei* was inoculated at ~10^7 CFU/ml. Error bars indicate mean of duplicate values ± standard deviation.

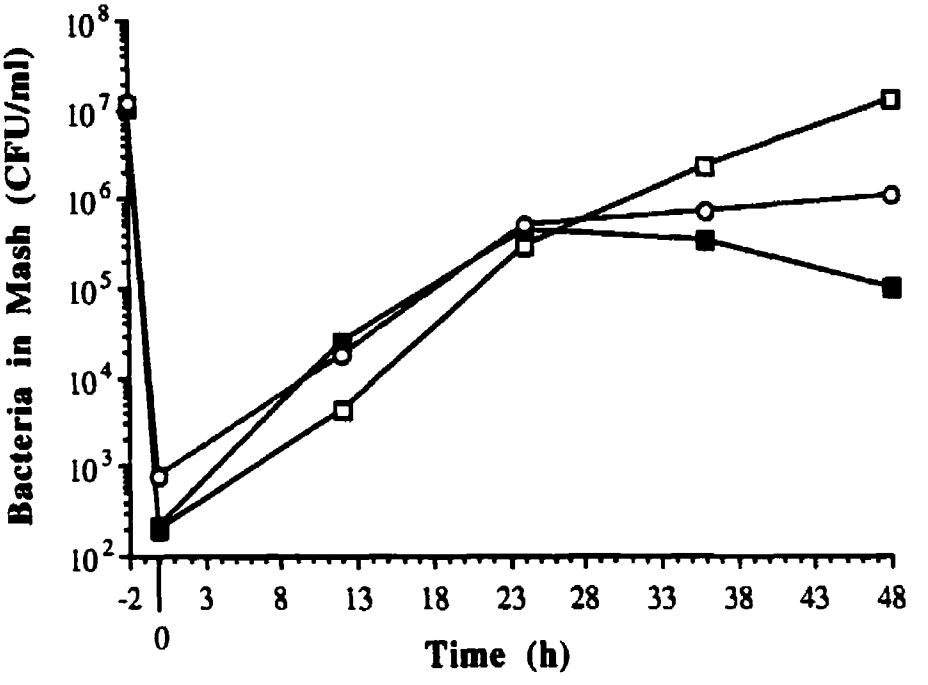


Figure 4.37 Growth of *L. paracasei* in fermenting wheat mash at 30° C in the presence of hydrogen peroxide at 30 mM with different nitrogen sources (□, urea; ■, ammonium dihydrogen phosphate; and ○, diammonium hydrogen phosphate). All treatments had yeast inoculated at ~ 10^6 CFU/ml (at time 0 h). Approx. 10^7 bacteria/ml had been inoculated at time - 2 h. Added ammonium dihydrogen phosphate (60 mM) and DAP (30 mM) yield the same amount of usable nitrogen as that yielded by 30 mM urea.

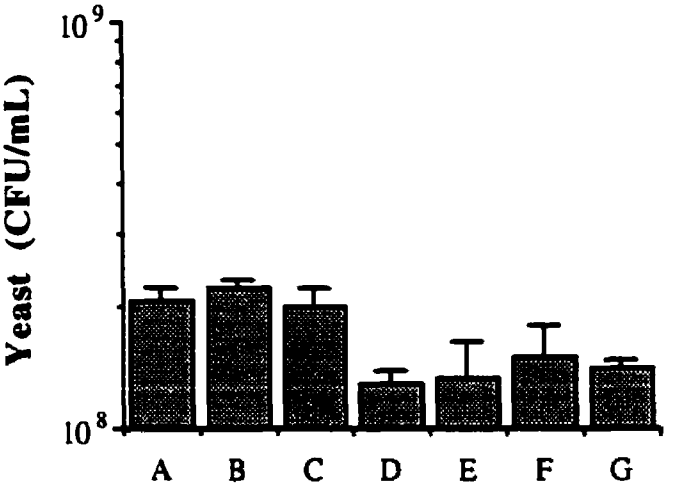


Figure 4.38 Maximum viable cells of yeast during the fermentation of wheat mash at 30° C. **A**, Control (Yeast + 30 mM urea, no bacteria); **B**, Yeast + 30 mM urea + 30 mM H_2O_2; **C**, Yeast + *L. paracasei* + 30 mM urea + 30 mM H_2O_2; **D**, Yeast + 30 mM H_2O_2 + 60 mM $(NH_4)H_2PO_4$; **E**, Yeast + *L. paracasei* + 30 mM H_2O_2 + 60 mM $(NH_4)H_2PO_4$; **F**, Yeast + 30 mM H_2O_2 + 30 mM $(NH_4)_2HPO_4$; and **G** , Yeast + *L. paracasei* + 30 mM H_2O_2 + 30 mM $(NH_4)_2HPO_4$. Yeast inoculated at ~10^6 CFU/ml; *L. paracasei* inoculated at ~10^7 CFU/ml. Error bars indicate ± standard deviation of the mean of duplicate samples.

In treatments where $(NH_4)H_2PO_4$ or DAP was used, the maximum cell numbers attained were less (ca. 140 million cells/ml) compared to the control and the treatments where urea was used (ca. 210 million cells/ml) as the nitrogen source (either in the presence or absence of H_2O_2). This might have been due to the decrease in pH as a result of NH_4^+ removal from the media by yeast (leaving phosphate anion behind). The pH dropped to 3.8 in DAP-fortified fermentation whereas it was approximately 4.5 in the control and in treatments with urea as the nitrogen source. When urea is used as a nitrogen source, no anion remains in the ferment in contrast to the likely situation with increased levels of DAP.

In all treatments, the majority of ethanol production was achieved in 36 h. The maximum ethanol concentrations produced are shown in Figure 4.39. A 2.0 - 2.2 % reduction in overall ethanol yield was observed where $(NH_4)H_2PO_4$ was used as nitrogen source in the absence and presence of bacteria respectively (compared to the control with yeast alone and no agents added). A 1.6 - 1.9 % overall yield reduction was observed where DAP was used as a nitrogen source in the absence and presence of bacteria respectively (Fig. 4.39). Urea appears to be a better source of nitrogen for this fermentation compared to ammonium dihydrogen phosphate and diammonium phosphate (DAP) when used in combination with hydrogen peroxide at 30 mmoles/L. Interestingly, the availability of urea hydrogen peroxide (in a solid and stable form) makes it a good choice for use in the production of fuel and industrial ethanol.

4.2.4.3 Decomposition and bactericidal effectiveness of urea hydrogen peroxide in the presence of particulate matter

Hydrogen peroxide (released from the breakdown of urea hydrogen peroxide in the medium) is presumed to be quickly decomposed in the presence of particulate materials. Results have indicated that a dose of 2 mmoles/L of urea hydrogen peroxide is enough to kill *L. paracasei* in a clear medium (MRS broth), whereas a much higher dose is needed in the presence of grain particles (Table 4.16). A similar experiment was conducted with clarified wheat mash obtained by removing mash particles by filtering mash through Whatman no. 4 filter paper followed by a filtration using diatomaceous earth. The clarified mash was then passed through a 0.45 μ Versaflow capsule (Gelman Sciences Ltd.) and used. It was then found that urea hydrogen peroxide at a dose as low as 2 mmoles/L killed *L. paracasei* when inoculated at ~10^7 CFU/ml. There was absolutely no bacterial growth observed for up to 48 h after addition of urea hydrogen peroxide.

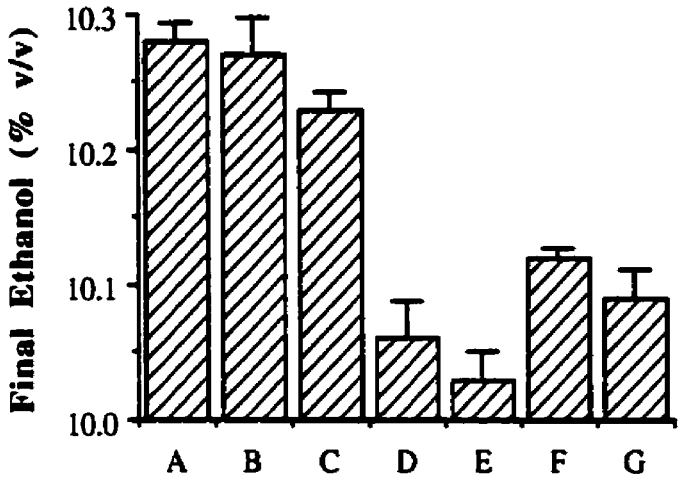


Figure 4.39 Final ethanol concentration after 48 h of fermentation of wheat mash at 30° C. **A**, Control (Yeast + 30 mM urea, no bacteria); **B**, Yeast + 30 mM urea + 30 mM H_2O_2; **C**, Yeast + *L. paracasei* + 30 mM urea + 30 mM H_2O_2; **D**, Yeast + 30 mM H_2O_2 + 60 mM $(NH_4)H_2PO_4$; **E**, Yeast + *L. paracasei* + 30 mM H_2O_2 + 60 mM $(NH_4)H_2PO_4$; **F**, Yeast + 30 mM H_2O_2 + 30 mM $(NH_4)_2HPO_4$; and **G** , Yeast + *L. paracasei* + 30 mM H_2O_2 + 30 mM $(NH_4)_2HPO_4$. Yeast inoculated at ~10^6 CFU/ml; *L. paracasei* inoculated at ~10^7 CFU/ml. Error bars indicate ± standard deviation of the mean of duplicate samples.

Table 4.16 Survival of *L. paracasei* in the presence and absence of particulate matter at two different doses of urea hydrogen peroxide.

Time (h)	MRS broth		MRS broth + 10 % (w/v) wheat mash particles	
	2 mmoles/L	42.6 mmoles/L	2mmoles/L	42.6 mmoles/L
0	1.07×10^7	1.02×10^7	1.06×10^7	1.08×10^7
2	2.92×10^6	$< 10^2$	1.83×10^6	3.21×10^4
4	$< 10^2$	-	2.47×10^6	4.17×10^2
6	-	-	2.82×10^6	5.67×10^2
12	-	-	9.15×10^7	7.83×10^2
24	-	-	8.57×10^8	1.27×10^3

Values are the mean of duplicate samples. The numbers are expressed as CFU/ml. A value of $< 10^2$ indicates that no colonies were seen on plates. A dash indicates that the Klett value remained at 0 indicating no regrowth of the bacteria (since media was clear, growth was measured by optical density when MRS broth was used).

A similar experiment was also done using wet miller's corn mash received from Williams Ethanol Services Inc. (Pekin, IL) which had approximately 1 % w/v particulate material. It was found that 5 mM urea hydrogen peroxide reduced the numbers of *L. paracasei* by 6 logs in two hours. At a higher concentration of 6 mM, the bacteria were killed totally in an hour (Fig. 4.40). It was previously found that urea hydrogen peroxide in wheat mash at a concentration of 40 mM was totally decomposed in 5 h. No significant differences were observed in the decomposition of hydrogen peroxide, whether added as H_2O_2 or as urea hydrogen peroxide (Fig. 4.41).

4.2.4.4 NADH peroxidase activity

Lactic acid bacteria lack the enzyme catalase, but hydrogen peroxide when present in sublethal amounts, is decomposed by the enzyme NADH peroxidase. To determine that the activity of NADH peroxidase was lost in the absence of H_2O_2, the NADH peroxidase activities of all five selected industrially-important lactobacilli were assayed. Assays were performed on cultures grown in MRS broth at 30° C in screw-capped Erlenmeyer flasks flushed with sterile CO_2 gas, on cultures grown in MRS broth with H_2O_2 at a sublethal level (0.75 mM) and on cultures transferred to fresh MRS broth without H_2O_2 from those grown in the presence of 0.75 mM H_2O_2. The final set of experiments were done to see if the activity is reduced in the absence of hydrogen peroxide. The data was subjected to Duncan's multiple range test (SAS Institute, Cary, NC). A significant increase ($P = 0.05$) in NADH peroxidase activity was observed with all five lactobacilli studied, when grown in the presence of a sublethal concentration (0.75 mM) of H_2O_2 (Table 4.17). On transferring back to fresh MRS broth without H_2O_2 (for ~ 18 h), there was a significant loss ($P = 0.05$) in the specific activity. The data however suggest that if a high enough concentration of H_2O_2 was applied, the organisms would be unable to adapt quickly enough to enzymatically degrade the antimicrobial at a meaningful rate, and all contaminants regardless of their ability to make catalase would be killed. The metabolic capability of these organisms for H_2O_2 degradation would not be high enough to avoid H_2O_2-induced death.

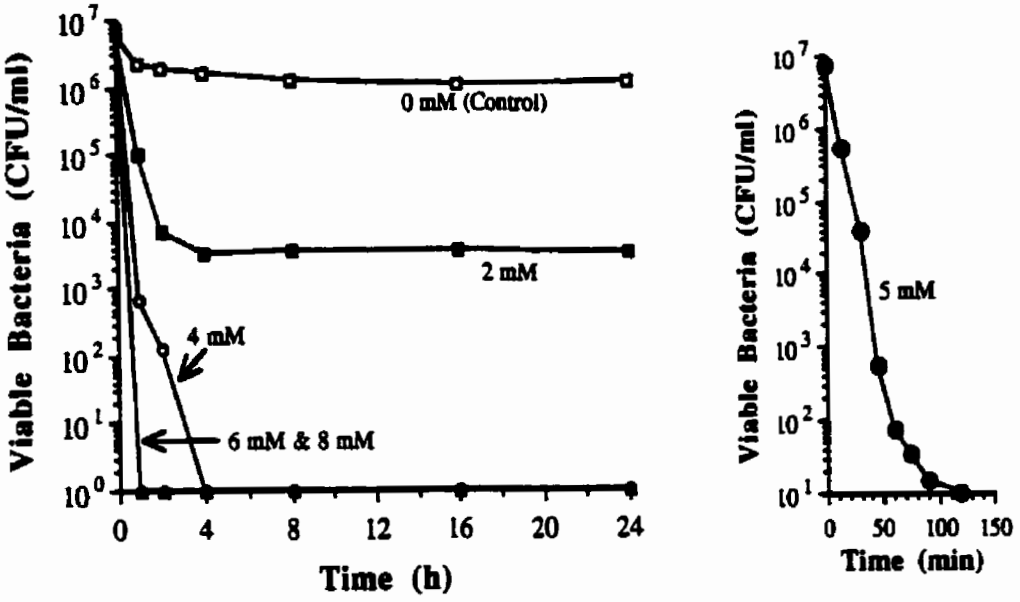


Figure 4.40 Survival of *L. paracasei* (inoculated at ~10^7 CFU/ml) in wet miller's corn mash in the presence of various concentrations of urea hydrogen peroxide at 30° C. Values are the means of two separate samples. Plating (by membrane filtration) was done in duplicate.

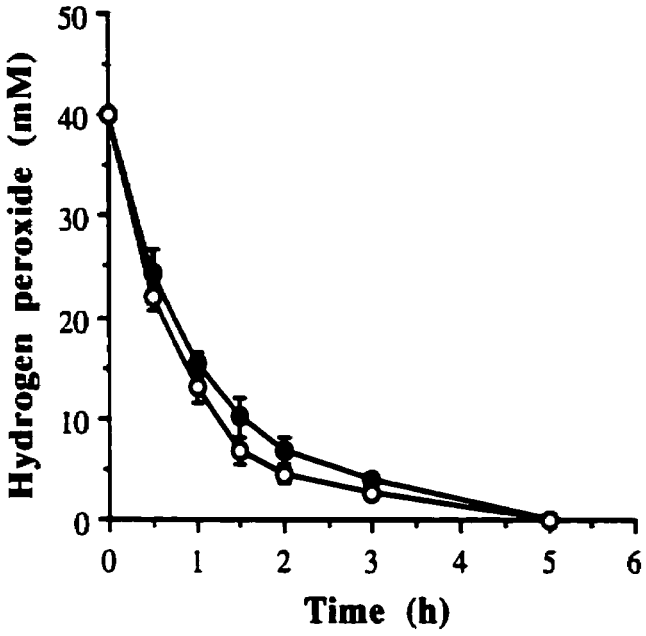


Figure 4.41 Decomposition of hydrogen peroxide when applied as H_2O_2 (O) or in the form of urea hydrogen peroxide (●) in normal gravity wheat mash at 30° C. The error bars indicate ± standard deviation obtained from triplicate analyses.

Table 4.17 NADH peroxidase activity in various lactobacilli grown in MRS broth at 30° C in the presence and absence of hydrogen peroxide (0.75 mmoles/L)

Organism	Specific activity (µmol NADH oxidized/min/mg total protein)*		
	No hydrogen peroxide (control)	Hydrogen peroxide (0.75 mM) added	No hydrogen peroxide[1]
L. plantarum	0.143 ± 0.012^{c}	0.233 ± 0.031^{a}	0.159 ± 0.018^{b}
L. paracasei	0.093 ± 0.020^{c}	0.241 ± 0.023^{a}	0.119 ± 0.031^{b}
Lactobacillus # 3	0.217 ± 0.012^{c}	0.324 ± 0.021^{a}	0.215 ± 0.030^{b}
L. rhamnosus	0.051 ± 0.029^{c}	0.347 ± 0.018^{a}	0.104 ± 0.007^{b}
L. fermentum	0.062 ± 0.003^{c}	0.149 ± 0.021^{a}	0.052 ± 0.009^{b}

[1] In this case, cells were grown in MRS broth containing 0.75 mM H_2O_2 until late log phase and then transferred to fresh media for ~ 18 h to stationary phase without hydrogen peroxide.

* The values given are the mean of three separate analyses ± the standard deviation. The letters in superscripts indicate the levels of significance (P = 0.05) between treatments and **not** between organisms (based on Duncan's multiple range test). Means with the same letter are not significantly different.

5. DISCUSSION

"The trouble with simple things is that one must
understand them very well."
(Anonymous)

5.1 Effects of lactobacilli, lactic acid and acetic acid on yeast growth and alcohol production

The occurrence of bacterial contamination is unavoidable in an industrial scale production of ethanol from starch or sugar-based substrates. As mentioned by Makanjuola *et al.* (1992), studies on the direct effects of contamination are not easily carried out. The differences between and within batches of raw materials will produce differences in chemical composition and measured fermentation parameters. Raw materials themselves may harbour contaminating bacteria which during fermentation may compete with yeasts for growth promoting nutrients. In this study , the effects of five contaminating lactobacilli were studied in conditions where all factors were controlled.

The differences observed in fermentation rates between the treatments containing *L. plantarum* and the controls with no bacterial inoculation were minimal (Fig. 4.2). Although a slight change in the rate of sugar utilization was observed when initial bacterial numbers were high, all fermentations completed (to constant specific gravity). This suggests that coflocculation (Makanjuola *et al.*, 1992) as a reason for incomplete utilization of carbohydrates and loss of ethanol yield due to high levels of bacterial contamination was not a factor here. When coflocculation exists, yeast are unable to utilize all the fermentable carbohydrates in the mash which results in a "stuck" fermentation, residual unfermented sugars, and a concomitant overall loss (up to 17 %) of ethanol (Makanjuola *et al.*, 1992). In the present study, less than 0.1 % fermentable sugars remained at the end of these fermentations, and this obviously ruled out coflocculation.

The bacteria when inoculated alone at ~ 10^7 CFU/ml did not consume more than 1 g/100 ml of mash dissolved solids (Fig. 4.2) for their growth and metabolism. This implies that in most cases, the homofermentative lactobacilli make less than 1 % w/v lactic acid under these conditions and that some growth factor other than sugar limits further growth of lactic acid bacteria in mash. It follows that most glucose would be available for conversion to ethanol by yeast as suggested by Chin and Ingledew (1994). However,

when two organisms grow together in the same medium, there is always a competition between them for certain nutrients. These bacteria anaerobically metabolise glucose to lactic acid to derive energy for growth and cell maintenance, and it is the production of lactic acid which leads to a reduction in overall ethanol yield (since every mole of lactic acid formed is at the expense of production of one mole of ethanol).

Since they did not die off as fast as the other organisms, *L. paracasei* and *Lactobacillus* #3 appear to be the most tolerant to ethanol of the five strains of lactobacilli studied (Fig. 4.3). These two strains, both recent industrial isolates, may be particularly suited to the stressful conditions in an alcohol fermentation. This supports the finding of Bryan-Jones (1975). When the bacteria were grown without yeast in the mash, their growth rates were initially slightly lower than that observed when they were grown in the presence of yeast. This might be attributed to the fact that these bacteria benefit from undetermined growth factors excreted by the yeast during its growth (Young *et al.*, 1956; Kandler and Weiss, 1986; Lemaresquier, 1987).

In contaminated yeast fermentations, the final lactic acid concentration measured at 72 h is proportional to the number of new bacterial cells produced. Once the maximum population is achieved, it is followed by the death of a proportion of the cells while the survivors still metabolise glucose for cell maintenance. Therefore, when high numbers of cells are produced, higher rates of lactic acid production occur. Under anaerobic conditions, these bacteria derive energy by fermenting carbohydrates to lactic acid (homofermentative strains), or to a mixture of end products such as ethanol, CO_2, lactic acid and minor end products like acetic acid (heterofermentative strains).

Decreases in maximum yeast growth, final ethanol concentration, and in final pH of the medium also were seen as the inoculum of lactobacilli increased. This is in agreement with the results obtained by Makanjuola *et al.* (1992). During fermentation, the concentrations of lactic acid achieved increased as the levels of bacteria inoculated increased. This was also observed by other authors (Barbour and Priest, 1988; Makanjuola *et al.*, 1992). In fact, both final lactic acid concentrations and decreases in ethanol yields at end fermentation were directly correlated to initial numbers of viable bacteria in mash. This is the first time that a linear relationship between these parameters has been reported. Likewise, it is seen that a relationship exists between the metabolic pathways (hetero vs homofermentation), growth rates and lactic acid concentrations produced by these bacteria. Growth of the heterofermentative strain as indirectly measured by optical density in MRS broth was less than that of homofermentative strains which, via glycolysis, produce twice the amount of ATP per molecule of glucose fermented. This agrees with the theory on the relationship of ATP to biomass in anaerobically grown

bacteria; and twice the weight of lactic acid is produced by homofermentative lactic acid bacteria than heterofermentative strains (Gottschalk, 1979).

As little as 1 % decrease in overall ethanol yield is highly significant to distillers of fuel alcohol since their profit margins are very narrow (Makanjuola *et al.*, 1992). In large plants with outputs of 400 - 1100 million L ethanol/yr, such a loss would reduce income by $ 1 - 3 million annually. In the study reported here, decreases of over 2 % in overall ethanol yield were observed with *L. paracasei* and *Lactobacillus* # 3, even when these bacteria were inoculated into mash at low levels of 10^5 CFU/mL. This may be because they did not die off towards end fermentation as did the other strains (Fig. 4.3). These observations are in contrast to those reported by Dolan (1979) and Barbour and Priest (1988) for industrial scale fermentations. Makanjuola *et al.* (1992) have reported a substantial (21 %) reduction in ethanol yield when high numbers (1.6×10^8 CFU/ml) of contaminant bacteria were present in the mash.

In the case of *L. fermentum*, a heterofermentative organism, the reduction in ethanol production was comparable (when inoculated at ~ 10^6 CFU/ml) with homofermentative organisms (*L. plantarum* and *L. rhamnosus*), although the final lactic acid concentration was not as high as those achieved by homofermentative strains. This could be due to the production of 0.03 to 0.05 % w/v acetic acid by *L. fermentum* (the actual amount depended on the inoculation level). Acetic acid is more toxic to yeast than lactic acid since its pK_a value is higher than that of lactic acid. It is the undissociated form of the organic acid that is responsible for antimicrobial activity (Baird-Parker, 1980). A higher concentration of the undissociated form of acetic acid exists at the pH values of mash fermentation (pH 5.0 - 5.5). The two acids have been shown to have a synergistic negative effect on yeast growth and metabolism (Moon, 1983). It has also been reported that ethanol accentuates the inhibitory effect of acetic acid on fermentation by yeast (Pampulha and Loureiro-Dias, 1989). The presence of lactic acid and acetic acid along with ethanol would act synergistically on yeast growth and metabolism resulting in a loss in overall ethanol yield. It has been reported that acetic acid, in its undissociated form, causes an increased expenditure of energy (ATP) for cell maintenance (Maiorella *et al.*, 1983). However, data for the action of acetic acid and lactic acid on yeasts show growth inhibition different from that predictable on the basis of dissociation constants, indicating that these acids may not act in the same manner on the yeast cell (Moon, 1983).

Lactobacilli are extremely fastidious. They require a variety of growth factors like nucleotides, amino acids and vitamins (Kandler and Weiss, 1986). Biotin and vitamin B_{12} are required by a few strains (Koser, 1968; Kandler and Weiss, 1986). Biotin is also an essential growth factor for *Saccharomyces cerevisiae* (Koser, 1968). Therefore, these

bacteria, when present in high numbers, could quickly scavenge from the medium large amounts of the essential growth factors required by the yeast. Since the growth rates of these selected lactobacilli are faster than yeast (*S. cerevisiae*) and lactic acid bacteria found in breweries and the food industry, removal of essential growth factors could result in reduction in yeast growth rate and catalytic activity. This would reduce the final ethanol yield. Although the growth rates of the selected lactobacilli are faster than other lactic acid bacteria, these bacteria could not be grouped as 'ferocious' lactobacilli (Boulton *et al.*, 1996) that commonly occur in winemaking, since they did not produce large amounts of acetic acid rather than lactic acid. Due to the relative sizes of yeast and lactobacilli, it is likely however that 50 - 100 fold more lactobacilli than yeast would be needed before a real competitive effect would be seen.

It has been reported that there can be a significant loss in glucoamylase activity when the pH of mash undergoing simultaneous saccharification and fermentation falls below 3.5 (Chin and Ingledew, 1994). The lowest pH observed in the present study was 3.9. The glucoamylase (Allcoholase II) at pH 4.0 retained 91 percent of its activity. Therefore, the reduction of pH in the medium due to lactic acid production was not a factor likely to affect the saccharification process in this work.

The results obtained from the studies on the effects of lactobacilli on yeast growth and fermentation indicate that apart from the diversion of small amounts (less than 1 % w/v) of fermentable sugar for growth of these bacteria, the production and effects of lactic acid and a suspected competition by these bacteria with yeast for essential nutrients (unstudied) are the important reasons for the reduction in yeast growth, metabolism and ultimately the final ethanol yield.

The endproducts of metabolism by lactobacilli, lactic acid and acetic acid, are inhibitory to yeast growth. The effects of these two weak acids on yeast growth and fermentation and their modes of action on yeast were studied at 30° C using a chemically defined medium with glucose (2 % w/v) as the carbon source. Chemically defined media was used because the growth of yeast is always faster in complex than in minimal media. Moreover, the presence of components such as yeast extract in yeast extract-peptone-dextrose (YEPD) broth offers some protection against stress conditions. It is difficult (and in some cases impossible) to quantitate the uptake of substrates in complex media and to study the effects of stress conditions. Use of chemically defined media overcomes many of the limitations of complex media, although growth rates are reduced and are not representative of industrial fermentations.

Minimum inhibitory concentrations (MIC) of acetic acid and lactic acid for both yeast strains were 100 mM and 278 mM, respectively. Stratford and Anslow (1998) have

reported a concentration of 90 mM acetic acid to be the MIC for *S. cerevisiae* X2180-1B. Similar values for acetic acid were reported by Maiorella *et al.* (1983). Acetic acid is inhibitory to yeast at a much lower concentration than lactic acid. At a given acidic pH (because of the higher pK_a value of acetic acid), there is more undissociated acetic acid present than would be found with an equal concentration of lactic acid (Lindgren and Dobrogosz, 1990). The undissociated forms of these acids being uncharged and lipophilic in nature, diffuse into yeast cells through the cell membrane. Once inside, these acids because of the higher intracellular pH, dissociate producing hydrogen ions and thereby cause changes in yeast metabolic activity (Hunter and Segel, 1973; Kashket, 1987).

Even in the presence of increasing concentrations of acetic acid in the medium, all of the glucose was still consumed and the same levels of maximum ethanol were produced in 24 h by both yeast strains studied (Fig. 4.10) - although a decrease was observed in total yeast biomass. This can be explained by the classic weak acid theory that undissociated molecules freely diffuse through the cell membrane and dissociate in the cytoplasm due to the higher intracellular pH, thereby acidifying the cytoplasm. The cell, however, tries to maintain its internal pH homeostasis by pumping out the excess protons via the H^+ translocating plasma membrane ATPase which utilizes ATP for its activity. The interference of acetic acid therefore results in an increased ATP requirement for cell maintenance (Maiorella *et al.*, 1983). In other words, the ATP required for production of cell mass is channelled for maintenance of pH homeostasis inside the cell rather than for growth. This causes a reduction in the total biomass produced. According to van der Rest *et al.* (1995), ATPase activity is estimated to consume 10 - 15 % of the ATP produced during yeast growth and has a reaction stoichiometry of one proton extruded per molecule of ATP hydrolyzed.

The inhibitory activity of acetic and lactic acid in the medium is determined by the pH of the medium, the dissociation constants of the acids and by their molar concentrations. These values are given in Table 4.4. Taking this into consideration, different effects are observed for glucose uptake and ethanol production in the two yeast strains as caused by acetic and lactic acids, although both the acids have similar molar concentrations of undissociated acid in the medium. Studies on the mode of action of these acids indicate that they may not act in the same manner on the cell. For example, Maiorella *et al.* (1983) reported that acetic acid interference with yeast metabolism resulted in an increase in ATP requirement for cell maintenance whereas the mechanism of lactic acid inhibition was probably different. Data for the action of acetic, lactic and propionic acids on yeasts showed growth inhibition different from that predictable on the basis of

dissociation constants indicating that these acids may not act in the same manner (Moon, 1983).

Higher maximum concentrations of acetic acid and lactic acid were chosen for strain ATCC 26602 (Table 3.1b) because this strain was capable of growth at higher concentrations of both these acids compared to the Alltech strain (although the MIC of both the acids for both strains were similar). If disproportionate inhibitory concentrations of the two acids are used, the ratios will shift to one end of the spectrum, thus appearing to be additive (Rubin, 1978). This is probably why it has been reported that acetic acid and lactic acid when present together exert an additive inhibitory effect on *Salmonella gallinarum* (Sorells and Speck, 1970).

It is difficult to demonstrate that two or more agents act synergistically or antagonistically on the specific growth rate of a culture. Only by very careful experimental design can such synergy be assessed. Response surface central composite design is one way of detecting interactions between two or more agents. However, when concentrations of weak acids are set at particular values, the proportions of dissociated and undissociated weak acid at any given pH will vary depending upon the dissociation constant of the acid. In this study, the interactive effect of acetic acid and lactic acid on the specific growth rate of *S. cerevisiae* was evaluated based on the concentrations of these acids (at particular values) in the medium (i.e. fluctuations in the molecular species weren't taken into consideration). The results obtained indicate the presence of a significant interaction between acetic acid and lactic acid in the reduction of the specific growth rates of both strains of *S. cerevisiae* ($P \leq 0.001$) indicating synergy (Fig. 4.12). This provides the explanation to a phenomenon noted in the fuel alcohol industry that small concentrations of acetic acid (i.e. ~ 0.05 % w/v) have an enormous inhibitory effect on fermentation of mashes which already have significant levels of lactic acid produced by contaminating lactic acid bacteria. The industrial practices of backsetting (containing lactic acid) and the use of process condensate (containing acetic acid) exacerbate this problem (Ingledew, 1993).

5.1.1 Mechanism of action of acetic and lactic acid on *S. cerevisiae*

Weak organic acids such as acetic ($pK_a = 4.74$) and lactic ($pK_a = 3.86$) acids inhibit the growth of both bacteria and fungi. In solution, weak acids exist in a pH-dependent equilibrium between the dissociated and undissociated state. The inhibition effected by weak acids is enhanced by low pH because this favours the undissociated state of the molecule. Uncharged (undissociated) molecules are freely permeable across the plasma

membrane. The weak undissociated acids diffuse into the cell until equilibrium is reached in accordance with the pH gradient across the membrane (see Fig. 5.1 and 5.2 for diagramatic representations at pH 2.5 and 4.0). Once inside the cell, due to a higher intracellular pH (pH_i), the undissociated molecule will at least in part dissociate resulting in the release of charged anions and protons and a reduction in cytoplasmic pH. As a result, a number of studies have implied that inhibition of yeast growth could be due to disruption of pH_i homeostasis by factors such as low pH (O'Hara *et al.*, 1989), ethanol (Cartwright *et al.*, 1986) and weak acids (Salmond *et al.*, 1984). Moreover, many studies have suggested that the reduction in pH_i is the principle inhibitory action of weak acids (Salmond *et al.*, 1984; Cole & Keenan, 1987; Booth & Kroll, 1989).

In this study, however, no significant intracellular acidification was observed in the presence of increasing concentrations of acetic acid compared to the control with no acetic acid in the medium (Fig. 4.13). The maximum concentration of acetic acid used in the study was 41.7 mM. Fernandes *et al.* (1999) have reported that concentrations above 200 mM acetic acid began to induce a demonstrable intracellular acidification in *S. cerevisiae* . Maintenance of pH_i homeostasis can be energetically expensive, resulting in the membrane H^+-ATPase consuming between 40 - 60 % of total cellular ATP (Holyoak *et al.*, 1996). Therefore, the maintenance of pH_i homeostasis in the presence of weak acids (acetic acid) may deplete cellular ATP levels significantly. Such a depletion of ATP restricts growth. That explains why the production of maximum biomass decreased as the concentrations of acetic acid in the medium increased (Table 4.3), although all the glucose was depleted from the medium. Reduction in growth of *S. cerevisiae* by acetic acid is due to the increased energy that the cell uses to pump out the excess protons using H^+-ATPase of the plasma membrane in order to maintain the pH_i homeostasis of the cell. This is evident from the increase in the plasma membrane H^+-ATPase activity with increasing concentrations of acetic acid (Fig. 4.15). Maiorella *et al.* (1983) has reported that the presence of acetic acid in the medium causes an increased expenditure of energy in the form of ATP for cell maintenance in *S. cerevisiae*.

Lactic acid, on the other hand, when present at higher concentrations ($\geq$ 0.4 % w/v) in the medium reduced the intracellular pH significantly (Fig. 4.14). It is generally accepted that low cytoplasmic pH is detrimental to the microbial cell. Changes in intracellular pH are believed to be important in controlling the cell cycle, and rates of DNA and RNA synthesis appear to increase with higher pH_i within the normal physiological range (Madshus, 1988). In addition, key enzymes of glycolysis and gluconeogenesis are believed to be regulated by pH_i. As key enzymes in glycolysis and gluconeogenesis are

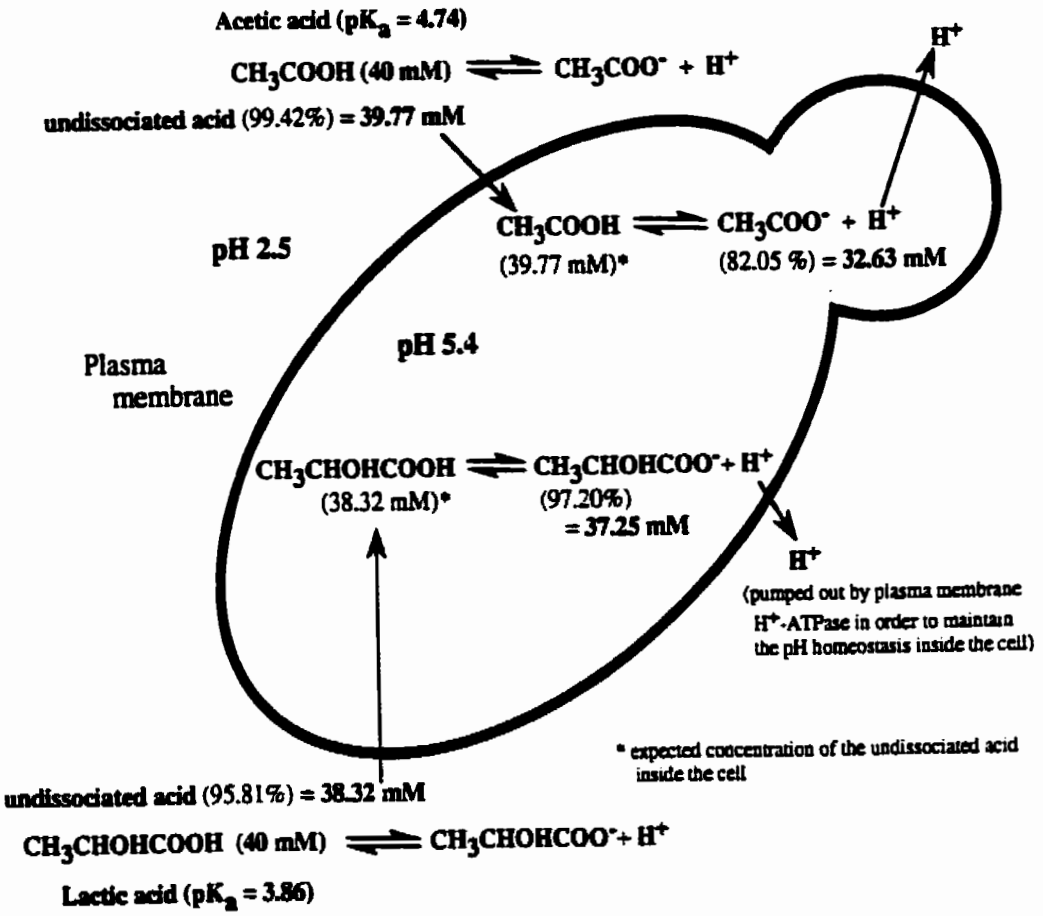


Figure 5.1 An illustration of the concentrations of anions and undissociated acids that would be present in the medium and inside the cell (based on the pH_{out} and pH_{in}) when an equal concentration (40 mM) of acetic acid or lactic acid is present in the medium (pH 2.5). The concentrations were calculated based on the Henderson-Hasselbach equation. A sample calculation is shown in appendix I. (The concentrations do not remain steady since there is a constant pumping out of the excess protons (by H^+-ATPase) to raise the pH_i resulting in further penetration of weak acid molecules into the cell that reacidify the cytoplasm).

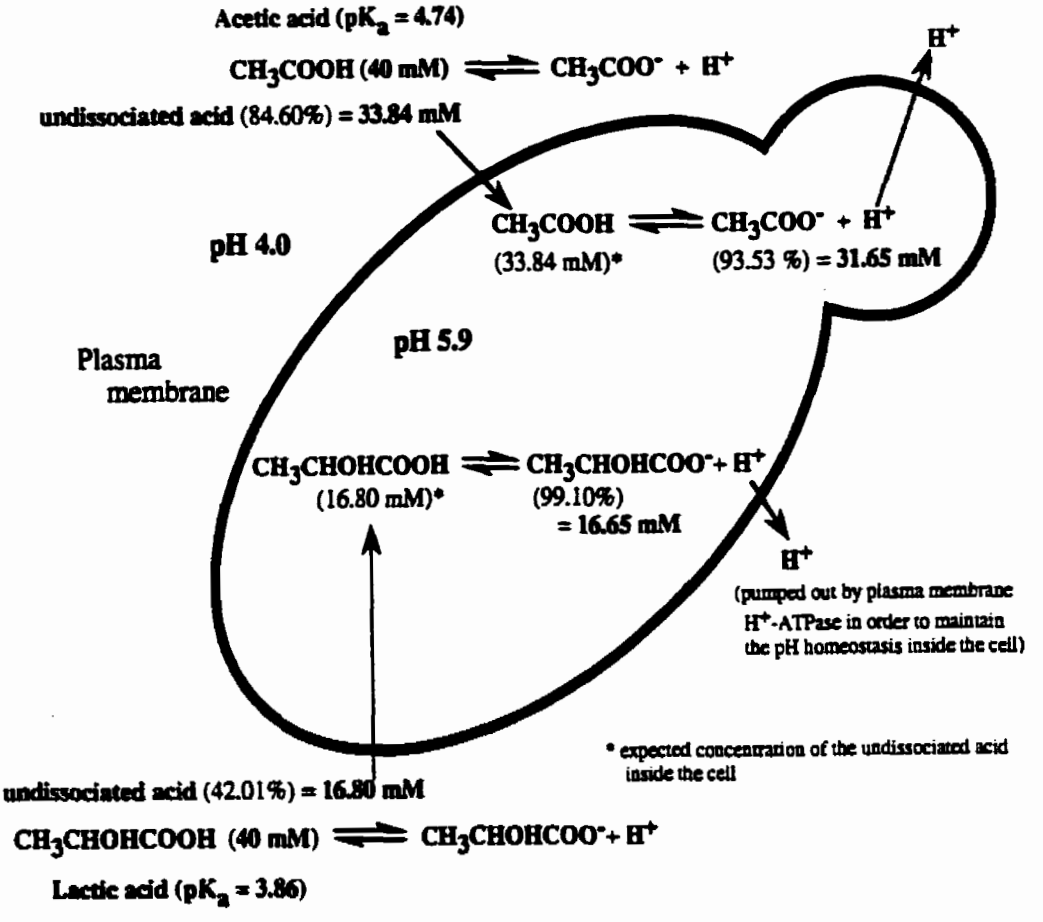


Figure 5.2 An illustration of the concentrations of anions and undissociated acids that would be present in the medium and inside the cell (based on the pH_{out} and pH_{in}) when an equal concentration (40 mM) of acetic acid or lactic acid is present in the medium (pH 4.0). The concentrations were calculated based on the Henderson-Hasselbach equation.

regulated by cascade reactions of c-AMP dependent protein kinases (e.g. phosphofructokinase, phosphorylase, fructose 1,6-bisphosphatase), the c-AMP plays an important role in these regulations. Moreover, c-AMP can be regulated by pH_i (Imai *et al.*, 1994). When weak-acid preservatives are present in the medium, the plasma membrane H^+-ATPase is of greater importance (in yeasts) if the principal inhibitory effect of the weak-acids is to reduce pH_i. The membrane H^+-ATPase couples ATP hydrolysis to the expulsion of protons, generating a proton gradient (Serrano, 1984) (Fig. 5.3). This transmembrane proton gradient is essential for active transport of nutrients and thus growth (Eddy, 1982).

The change in the intracellular pH observed at higher concentrations of lactic acid could therefore lead to disruptions in the transmembrane proton gradient (since there is a decrease in the activity of the plasma membrane H^+-ATPase), thereby causing an inhibition of nutrient uptake. The internal pH values could decrease to values in the range at which phosphofructokinase is sensitive (Krebs *et al.*, 1983) subsequently causing inhibition of glycolysis that leads to a fall in the concentration of ATP in the cell ultimately resulting in restriction of growth. Intracellular acidification (Fig. 4.14) and a probable disruption of transmembrane proton gradient caused by the reduction in membrane H^+-ATPase activity (Fig. 4.16) could be the reasons for the cessation of alcohol production in the presence of lactic acid (0.6 % w/v) in the medium (Fig. 4.11).

The changes in the activity of the plasma membrane H^+-ATPase could be due to the alteration of plasma membrane lipid composition. Lipids are essential for the catalytic activity of many membrane-bound enzymes and changes in the lipid composition can significantly alter the activity of proteins in the plasma membrane (van der Rest *et al.*, 1995). For this reason, the fatty acid composition of the cell membrane of both yeast strains (Alltech and ATCC 26602; in the mid-exponential phase of growth) in the presence of 0.5 % w/v lactic acid and 0.25 % w/v acetic acid (concentrations which significantly affected the membrane H^+-ATPase activity) was determined and compared to the control with no acetic acid or lactic acid added to the medium.

The fatty acid composition of both *S. cerevisiae* strains was altered by the presence of lactic acid (0.5 % w/v) in the medium. There was a significant decrease in unsaturated fatty acid residues (Table 4.12). Lipids can potentially activate plasma membrane H^+-ATPase that has an absolute requirement for the lipids in order to function (Serrano *et al.*, 1988), and that enzyme activity is a function of the type of lipid incorporated. Modification in the fatty acid composition likely will cause changes to the plasma membrane fluidity. Unsaturated fatty acids play an important role in keeping cell membrane fluidity and

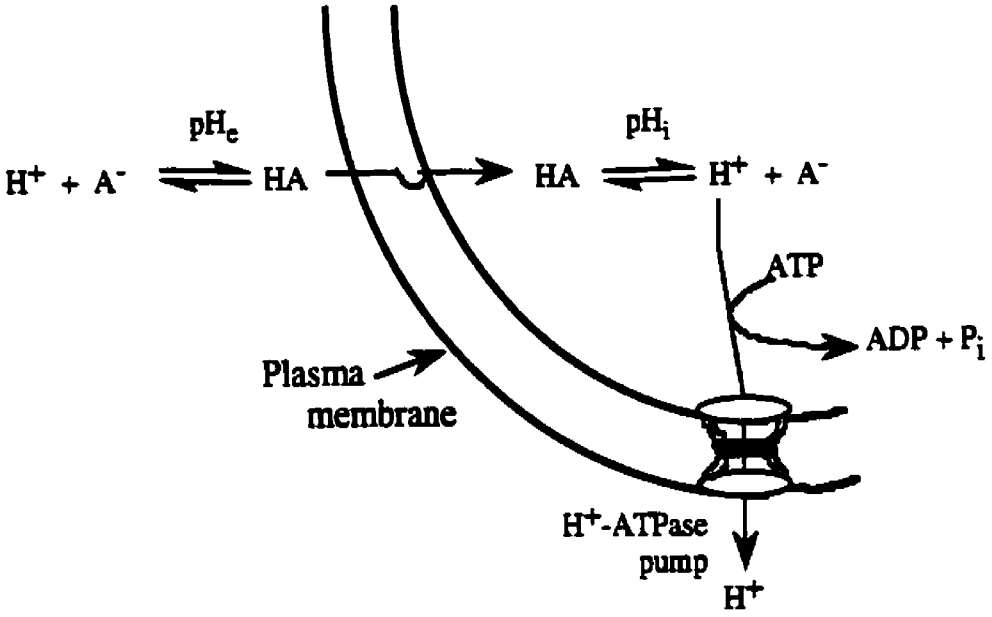


Figure 5.3 Media and cytoplasmic weak-acid/anion equilibria. Undissociated acid (HA) freely diffuses through the plasma membrane. Inside the cell, HA dissociates to H^+ and A^-. Charged anions (A^-) and protons (H^+) are retained within the cell; cytoplasmic protons are expelled by the membrane bound H^+-ATPase. This may result in further change in dissociation of acetic or lactic acid in the medium. (Adapted from Lambert and Stratford, 1999).

function. An increase in unsaturated fatty acyl residues results in an increase in membrane fluidity; and the presence of increased unsaturated residues increases the stability of membrane-bound enzymes (Thomas *et al.*, 1978). This is probably why a significant decrease in the plasma membrane H^+-ATPase activity was observed when the cells were grown in the presence of 0.5 % w/v lactic acid (Fig. 4.16). Thus, the presence of lactic acid at higher concentrations affects the plasma membrane fluidity which results in decreasing the stability and activity of H^+-ATPase. This leads to the inability of the yeast cell to maintain its internal pH, the disruption of proton gradient across the membrane finally leading to the disruption of many physiological functions essential for cell growth. The results from this work on the differences in intracellular pH values, plasma membrane H^+-ATPase activity and the membrane fatty acid composition indicate that although both lactic acid and acetic acid are short chain weak acids, the mode of inhibition of yeast by both these acids is not similar.

5.2 Management of lactobacilli in yeast-catalyzed ethanol production

Management of lactobacilli in the industry is usually achieved with the use of antibiotics either as single antibiotics or as a mixture of antibiotics. Penicillin G is the predominantly used antibiotic. Due to the emerging problem of antibiotic resistance, it was decided to evaluate a few non-antibiotic antimicrobials such as stabilized chlorine dioxide and hydrogen peroxide/urea hydrogen peroxide, and nisin for use to control lactobacilli during fermentation.

Stabilized chlorine dioxide does not appear to be very effective against the selected commercial strains of lactobacilli with fast growth rates and high ethanol tolerance, since the concentrations required to control these bacteria are also inhibitory to the culture yeast resulting in poor fermentation performance by the yeast.

The chemical reaction as to how chlorine dioxide is generated from the stabilized form is as follows:

$$5\ NaClO_2 + 4\ H^+ \rightarrow 4\ ClO_2\ (gas) + 5\ Na^+ + Cl^- + 2\ H_2O$$

The acidification of sodium chlorite, ($NaClO_2$) generates chlorine dioxide (ClO_2). Once the gas is gone it leaves behind two by-products, salt and water. At higher temperatures, the solution destabilizes by faster release of ClO_2 gas. This results in less effective gas and a more corrosive solution. For best results, chlorine dioxide has to be used at temperatures less than 27° C (Johnson, 1997). Unfortunately, this eliminates the use of stabilized ClO_2 in saccharification tanks where the temperature is maintained at 60° C. However, chlorine dioxide can be used to sanitize equipment such as heat exchangers, surge tanks, and fermentors.

With respect to nisin, its addition to the medium at 50 mg/L or 100 mg/L delayed the growth of some lactobacilli (*L. plantarum* and *L. rhamnosus*). Different strains of lactobacilli showed differences in their sensitivities to nisin (Fig. 4.30). The lactobacilli that resumed growth after several hours may have developed resistance induced by the presence of nisin in the medium. During the induction with nisin, the cells produce an anionic, phosphate-containing polysaccharide with subunits of rhamnose and galactose and this will protect sensitive cells against the bactericidal action of nisin (Breuer and Radler, 1996).

It has been reported by Faia and Radler (1990) that *L. casei* (now called *L. paracasei*) is very resistant to nisin and would be killed only at extremely high concentrations of nisin (>2,000 units/ml). Application of nisin at these concentrations would be far too expensive for use in the fuel alcohol industry. Ogden *et al.* (1988) have already indicated that at the price of commercial grade nisin (Nisaplin) of ~ £ 186/Kg (10^9 units), its addition at 100 units/ml to fermentations or beer would be too expensive. Therefore, nisin is not a good choice as an antimicrobial to be used in fermentations for fuel ethanol production.

Urea hydrogen peroxide (which eliminates the risk of emergence of antibiotic-resistant microorganisms) appears to be an eco-friendly agent to effectively manage lactic acid bacteria and other bacterial contaminants encountered during the production of industrial or fuel ethanol.

Batch fermentations were complete by 36 h in all cases in the experiments done to study the effect of urea hydrogen peroxide on mashes deliberately contaminated with *L. paracasei*. This was due to the increased availability of assimilable nitrogen under all conditions. As grain mashes in general are deficient in usable nitrogen (Ingledew, 1999), both yeast growth and fermentation rate benefit from the added urea whether it is added as free urea or as urea hydrogen peroxide. In samples with bacteria but without the agents, the yeast viability decreased significantly towards the end of fermentation. This is likely due to the competition for nutrients by the bacteria as well as the production of lactic acid which at levels of 0.8 % w/v begin to stress the yeast. In experiments done to compare diammonium hydrogen phosphate (DAP), and ammonium dihydrogen phosphate with urea (all added to provide the yeast with equal amounts of nitrogen) along with hydrogen peroxide, urea appeared to be a better source of nitrogen in combination with hydrogen peroxide at 30 mM. Interestingly, the availability of urea hydrogen peroxide (in a solid and stable form) makes this compound a good choice for use in the production of industrial or fuel ethanol. The use of urea hydrogen peroxide for fuel alcohol production by

fermentation has now been patented both in the USA (Ingledew *et al.*, 1999) and in Canada (Ingledew *et al.*, 2000).

Hydrogen peroxide is a natural product of the action of some flavoprotein oxidases of lactic acid bacteria with oxygen and it may accumulate in "aerobic" cultures of many strains. In lactococci sensitive to hydrogen peroxide, pre-exposure to a sublethal concentration of the compound allowed the organism to grow in the presence of a lethal concentration of hydrogen peroxide (Condon, 1987). Condon (1987) observed a simultaneous induction of NADH peroxidase and, to a lesser extent, of NADH oxidase. Since lactic acid bacteria lack catalase (due to their inability to synthesize hemoporphyrins), they use NADH peroxidase to rid themselves of hydrogen peroxide when it is present at sublethal levels (Anders *et al.*, 1970; Piard and Desmazeaud, 1991). NADH peroxidase catalyzes the following reaction.

$$NADH + H^+ + H_2O_2 \rightarrow 2H_2O + NAD$$

As the activity of NADH peroxidase is rapidly lost when the organism grows in the absence of H_2O_2 (Table 4.17), the risk of resistant mutants (organisms that would constitutively express high levels of NADH peroxidase) is reduced.

Urea hydrogen peroxide also offers the advantages of providing yeast with a nitrogen source (in the form of urea) and a supply of oxygen (from the breakdown of hydrogen peroxide into water and oxygen) - in addition to its bactericidal activity against lactic bacteria and other microbial contaminants. "Stuck" or sluggish fermentations, common in the alcohol industry, are caused by inadequate levels of yeast nutrients that lead to a cessation of yeast growth with a concomitant reduction in ethanol yield (Ingledew, 1995). Two such nutrients usually deficient in fermentation mashes are usable (assimilable) nitrogen and oxygen. Use of urea hydrogen peroxide serves to prevent "sluggish" or "stuck" fermentations that would lead to reduction in alcohol yields.

Yeasts used in alcohol production are not proteolytic and can use only low molecular weight nitrogenous compounds such as ammonium ion, urea, amino acids or dipeptides (Ingledew, 1993; Patterson and Ingledew, 1999). Urea and liquid ammonia are commonly used in the fuel alcohol industry as inexpensive sources of nitrogen for yeast (Ingledew, 1995); diammonium phosphate is often added to must in wine making. In addition to a source of usable nitrogen, oxygen is required in small quantities for the synthesis of unsaturated fatty acids and sterols which are both essential components of the yeast cell membrane (Andreasen and Stier, 1954). Unfortunately, oxygen is not available at optimal levels due to industrial practices and its lower solubility in mashes (Ingledew, 1995). Deficiencies of oxygen and usable nitrogen also affect the ethanol tolerance of

yeast. Judicious use of nutrients can lead to the production of more than 23 % v/v ethanol by commercial yeast strains in batch fermentation (Thomas *et al.*, 1993).

Urea hydrogen peroxide leaves no residues when added to the fermentation medium. The pH of the mash is not affected as it would be if ammonium salts were employed, nor are there residues in the whole or thin stillage. Moreover, urea hydrogen peroxide at the dose recommended can also eliminate a wide variety of contaminating bacteria that are present in low levels in the mash, and eliminate the use of yeast foods (N-containing), oxygen, and antibiotics.

Urea hydrogen peroxide proves to be an ideal additive for use in the production of industrial or fuel ethanol. A dose of 2 mmoles/L, urea hydrogen peroxide or hydrogen peroxide can be used as a disinfectant only in mashes free of particulate materials. Supplementing the mash with yeast nutrients would still be required. For mashes with particulates, ~ 32 mmoles of urea hydrogen peroxide (or hydrogen peroxide)/L is required to disinfect the mash. At this concentration, urea hydrogen peroxide provides all of the usable nitrogen and oxygen needed to ensure a predictable, trouble-free fermentation. In contrast, hydrogen peroxide at 32 mM would only serve as a disinfectant.

Urea hydrogen peroxide is available at this time only as a specialty (pharmaceutical grade) chemical. At present UHP is available at $ 70 (US)/kg. At least one producer estimated $ 2 - 6 (US)/kg for a demand only 10-50 times current sales of this chemical. It remains to be seen if cost of production of UHP can be reduced further so that it would be utilizable in the fuel alcohol industry. Cost projections should include considerations on elimination of the use of antibiotics and the replacement of part or all yeast foods (including oxygen) by UHP.

6. CONCLUSIONS

1. Linear relationships exist between the initial numbers of viable bacteria in mash and both decreases in overall ethanol yield and final lactic acid produced.

2. A decrease in the overall ethanol yield of approximately 2 % was observed in wheat mash fermenting at 30° C, if *L. plantarum, L. rhamnosus, and L. fermentum* were inoculated at ~10^6 CFU/ml. Smaller initial numbers (only 10^5 CFU/ml) of *L. paracasei* or *Lactobacillus* #3 were sufficient to cause decreases of more than 2 % in overall ethanol yields. Such effects may have been due to the higher ethanol tolerance of the latter two bacteria, the more rapid adaptation (shorter lag phase) of these two organisms to fermentation conditions, and/or to their more rapid growth and metabolism.

3. The addition of ~10^9 CFU of bacteria/ml in mash causes 3.8 to 7.6 % reductions in overall ethanol yields depending on the strain.

4. Lactic acid produced by lactobacilli is the major factor responsible for reductions in yeast growth and final ethanol yield (apart from a suspected competition for essential growth factors between yeast and lactobacilli).

5. The minimum inhibitory concentration (MIC) of acetic acid for yeast growth in a defined medium (with 2 % w/v glucose as the carbon source) is 0.6 % w/v (100 mM), and that of lactic acid is 2.5 % w/v (278 mM) for the two strains of yeast studied (Alltech strain and ATCC 26602). However, acetic acid at concentrations as low as 0.05 - 0.1 % w/v and lactic acid at concentrations of 0.2 - 0.8 % w/v are able to stress the yeast as seen by reduced growth rates, and decreased rates of glucose consumption and ethanol production.

6. Acetic acid and lactic acid, end-products of lactic acid bacterial fermentation, when present in the medium synergistically inhibit yeast.

7. Reductions in yeast growth caused by acetic acid are due to the increased expenditure of energy required by the cell to pump out the excess protons using plasma membrane H^+-ATPase in order to maintain the pH_i homeostasis of the cell.

8. Lactic acid, on the other hand, when present at higher concentrations in the medium reduces the activity of the plasma membrane H^+-ATPase significantly, and this results in intracellular acidification which in turn could affect critical metabolic reactions in the cell. The mechanism may involve disruption of the transmembrane proton gradient.

9. The plasma membrane lipid composition is altered in the presence of 0.5 % w/v lactic acid. There is a decrease in the proportion of unsaturated fatty acids (C16:1, palmitoleic acid and C18:1, oleic acid) which leads to a decrease in the plasma membrane fluidity. This subsequently affects the stability of membrane bound proteins.

10. Urea hydrogen peroxide (UHP) appears to be an ideal additive for controlling lactobacilli in ethanol fermentations. Stabilized chlorine dioxide and nisin do not appear to be good choices for use against lactobacilli during the fermentation of starch or sugar-based mashes. Penicillin and virginiamycin (reported by Hynes *et al.*, 1997) are both useful antibiotics for this industry.

11. Urea hydrogen peroxide also provides nitrogen and oxygen, the two essential nutrients required for better fermentation performance by the yeast. At present, cost may be the only impediment to the use of UHP in the fuel alcohol industry.

12. The bactericidal activity of urea hydrogen peroxide/hydrogen peroxide is affected significantly by the presence of particulates in the mash. In clarified mashes (similar to the corn wet milling industry), only 2 mM urea hydrogen peroxide is required for antibacterial activity, whereas in mashes with particulates (as in dry milling of corn or wheat) $\geq$ 30 mM urea hydrogen peroxide is required. In the latter case, sufficient amounts of nutrients are provided by UHP for yeast growth.

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APPENDIX I

Sample calculations showing the percentages of anion and the undissociated acid calculated for acetic acid and lactic acid at a known pH (ca. 2.5) using the Henderson-Hasselbach equation, $pH = pK_a + \log \frac{[A^-]}{[HA]}$

For lactic acid ($pK_a = 3.86$) ;	For acetic acid ($pK_a = 4.74$) ;
$pH - pK_a = \log \frac{[A^-]}{[HA]}$	$pH - pK_a = \log \frac{[A^-]}{[HA]}$
$2.50 - 3.86 = \log \frac{CH_3CHOHCOO^-}{CH_3CHOHCOOH}$	$2.50 - 4.74 = \log \frac{CH_3COO^-}{CH_3COOH}$
$-1.36 = \log \frac{CH_3CHOHCOO^-}{CH_3CHOHCOOH}$	$-2.24 = \log \frac{CH_3COO^-}{CH_3COOH}$
i.e. $\frac{CH_3CHOHCOO^-}{CH_3CHOHCOOH} = 0.0437$	i.e. $\frac{CH_3COO^-}{CH_3COOH} = 0.0058$
$= \frac{CH_3CHOHCOO^-}{CH_3CHOHCOOH} = \frac{4.19\%}{95.81\%}$	$= \frac{CH_3COO^-}{CH_3COOH} = \frac{0.58\%}{99.42\%}$

Note : At a given acidic pH, there is more acetic acid in the undissociated (uncharged) form compared to lactic acid because of the higher pK_a value for acetic acid.

APPENDIX II

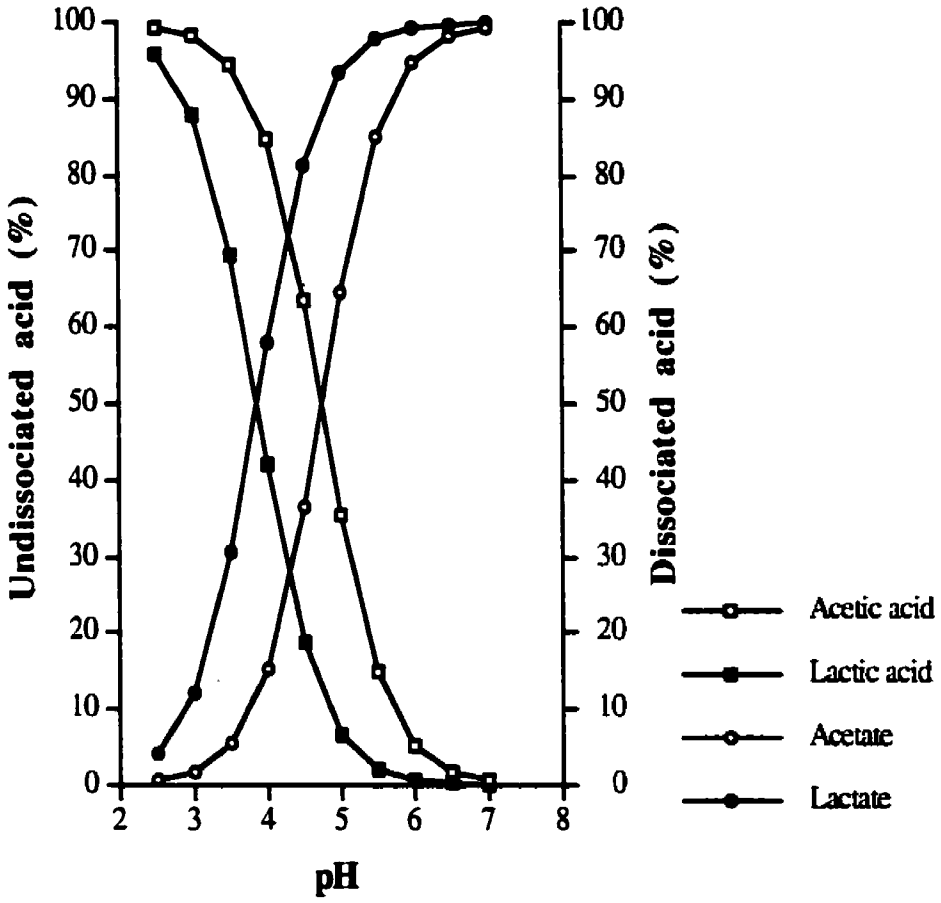

Figure A Percentages of undissociated and dissociated forms of acetic and lactic acids at various pH levels of the medium (calculated based on the Henderson-Hasselbach relationship).