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FLUORIDE INHIBITION OF WINE YEASTS

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF MASTERS IN SCIENCE IN
MICROBIOLOGY AT MASSEY UNIVERSITY.

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ABSTRACT

Stuck or slowed fermentations are costly in time and money to winemakers. There are many variables that can interrupt fermentation. One of the lesser known factors is the effect of fluoride on grape juice fermentations. Winemakers in California have had problems with slow or stuck fermentations with grapes that have been treated with the insecticide Cryolite, which contains fluoride.

A selection of 6 yeasts, 3 commercial strains and 3 natural strains, commonly associated with winemaking were used in this study. Preliminary experiments investigated a wide range of fluoride challenge with different pH and cell densities on solid and liquid media. The effectiveness of fluoride was compared between sodium fluoride and Cryolite, as the fluoride source. The effect of fluoride was more potent with sodium fluoride, as the fluoride source. The minimum inhibitory concentration of fluoride for the yeast strains was recorded. The most sensitive commercial yeast was *Saccharomyces cerevisiae* RS1, the most resistant commercial yeast was *Saccharomyces bayanus* RS2. The most sensitive yeast overall was *Hansenula saturnus* AWRI-354.

The next stage examined the effect of fluoride on the selected yeast in small scale grape juice fermentations. Within this investigation the effect of different media sources and heat treatments was included. Fluoride concentrations reflected levels of fluoride found in grape musts and wines.

During this study we found that the effect of fluoride on yeasts is increased with lower pH and lower cell densities. The effect of fluoride on yeast growth and fermentation was also strain dependent.

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INTRODUCTION

Grape juice is a challenging medium for microbes to grow in. It is sweet, acidic and sometimes contains added sulphur dioxide. Only microorganisms which can tolerate these restrictive conditions can grow. This group is comprised of yeasts and certain lactic and acetic acid bacteria.

The yeasts which grow readily in grape juice are the fermenting yeasts, mainly *Saccharomyces*, and these are added as pure culture. Other wild yeast such as *Kloeckera* species may also initiate fermentation.

Pure starter cultures are chosen for their supreme tolerance of the harsh environment of the grape juice medium. An effective fermentator i.e. a yeast which efficiently converts sugars to ethanol and carbon dioxide, but is tolerant to grape juice conditions, saves money and time. The quick and reliable completion of alcoholic fermentation allows the winemaker to improve productivity.

The use of viticultural antiparasitic products are essential to the success and economic yield of grape crops. These products are applied to all parts of the plant, whether intentionally or not where they adhere and are either gradually washed away by rain, eliminated by evaporation, or destroyed by the sun's rays. Residues from these antiparasitic products have important implications on the suitability for human consumption of the wine and table grapes produced, and also may affect the process of the winemaking.

Scientific knowledge in the latter part of the twentieth century has allowed winemakers greater control over biological pests. Cryolite (Kryolith; Ice spar; sodium aluminium fluoride, $(\text{AlF}_6\text{Na}_3)$) is a natural compound that is used as an insecticide against three main pests, that is; the Omnivorous Leaf Roller, Western Leaf Skeletoniser and Orange Tortrix. These insects can cause considerable damage to the vine, leaves and berries of the grape plant. The Omnivorous Leaf Roller attacks the berries. This damage attracts vinegar flies, and other secondary feeding insects, that carry spoilage organisms which can initiate bunch rot. The Orange Tortrix feed on berries and stems, causing berry drop and stem girdling. The Skeletoniser eats the lower epidermidis and green part of the leaf, interfering with photosynthesis.

There are several advantages of using Cryolite. It is a natural compound, and is found in large mineral deposits in Greenland and the Urals, but it can also be easily synthesized. The consequence of Cryolite's commonality is that it is cheap. It is also very effective on the targeted insects, and has no adverse effect on beneficial insects such as bees. A single application of Cryolite is typically used due to the long term residual effect.

Recent literature (Andris, 1990., Kunkee, 1991., Wahlstrom *et al* , 1991., Wahlstrom *et al*, 1996.) shows that vineyards using Cryolite, however, have had problems with slowed or stuck fermentations, whereby the alcoholic fermentation is slowed down below the normal rate or fermentation does not start at all. Cryolite is commonly used in California, more specifically Big

Valley and to a lesser extent Napa Valley. Residues of Cryolite have been detected in the grape must and in wines from California, at levels of 10mg/L. Other independent vineyards report levels at 15 to 20 mg/mL and at these levels objectionable flavours and smells are noted (Morenzoni,1991 personal communication).

Fluoride residues are also becoming undesirable in products due to their affect on ethyl carbamate production in wine. It is known that certain wines contain urea which is a precursor to the production of ethyl carbamate, a suspected carcinogen (Andris, 1990., Famuyiwa and Ough, 1991., Henschke and Jiranek, 1993., Kunkee, 1991., Ough *et al*, 1989., Trioli and Ough,1989). Recently the Japanese introduced an enzyme containing an acid urease which rapidly removes urea from wine therefore halting the production of ethyl carbamate. Researchers since then, have shown that fluoride inhibits the activity of this enzyme which reduces the likelihood for ethyl carbamate elimination (Andris, 1990., Famuyiwa and Ough, 1991).

The investigation of residues of fluoride in wine therefore, is important as these residues may have health implications to the consumer, as well as an organoleptic effect on the wine. It is also very significant with respect to the pecuniary effectiveness of the making of the product as stuck or delayed fermentations cost the winemaker both time and money.

The most significant source of fluoride in grape must appears to be the fluoride component of the Cryolite compound. The fluoride ion is apparently translocated into the grape berries and therefore into the grape juice (Kunkee, 1991). The antimicrobial action of fluoride is well documented in dental literature, for example, in the inhibition of streptococcal enolase in oral microflora (Belli *et al.*, 1995). The inhibition of yeast enolase by fluoride is also well recorded (Bunick and Kashket, 1982., Curran *et al*, 1994., Kaufmann and Bartholmes,1992., Lebioda *et al*, 1993). Fluoride acts via a number of different mechanisms against oral microflora (Kashket and Kashket, 1985., Marquis, 1995., Marquis, 1989., Marquis, 1977.,Villar-Palasi and Larner, 1970) but the effect of fluoride, by residual action on yeast during winemaking, is not well known.

The purpose of this research project was to investigate the effect, if any, the fluoride ion has on yeast involved in the process of winemaking. In this context different yeast, both commercial starter cultures and wild yeasts, were used to determine this effect. The range of fluoride was broad and included concentrations similar to fluoride levels found in grape must and wines as previously reported (Andris, 1990., Kunkee, 1991., Morenzoni, 1991, personal communication, Wahlstrom *et al* , 1991., Wahlstrom *et al*, 1996.). The conditions were intially optimal for the effect of fluoride to be demonstrated. Experimental parameters then focused on the reconstruction of "real world" conditions. From there a clearer idea of the effect of fluoride on yeast may be obtained.

1. LITERATURE REVIEW

1.1 WINEMAKING

For several thousand years fermentations have spontaneously occurred producing wines of variable quality. Some wineries today still produce wines by natural fermentation, preferring and revering the traditional methods over and above modern winemaking protocols. There is much debate on the pros and cons of traditional winemaking, with proponents for it extolling the organoleptic quality of their wine and opponents preferring the advantage of reproducibility using starter cultures, amongst other benefits. Some authors, however, have found no significant differences whatsoever (Reed and Nagodawithana, 1991). Indeed, the inoculating of must with a starter culture does not guarantee the immediate and continued dominance of that particular yeast or strains of yeast over the natural flora.

Modern winemaking describes the combined process of the growth of the grape vine, with the perfect variety and berry production for the style of wine, the manipulation of alcoholic fermentation and the management of the raw wine into the final product that matches the market it was made for. This is the process of vinification and oenology.

1.1.1 Vinification and Oenology

The increased interest in vinification and oenological research during the last ten years has undoubtedly arisen from the pressure for the wine industry to produce good quality wine for reasonable prices. The average consumption of wine per capita has declined over the past two decades (with some exceptions) and this drop in consumption has led to the 'wine lake' in Europe and other wine regions. Consumers are now more aware of the variety in quality and prices, and pressure is brought to bear on the winemaker to provide for this demanding market. The market demands the advancement of technology in wine production to increase the proportions of the highest quality wines and to produce, reliably, wines of an established standard at minimum cost. This has led to an increased interest in wine-making research.

1.1.2 Viticulture

The use of antiparasitic products is an important part of the viticultural care imparted on the varieties of *Vitis vinifera* grape plants, which are commonly used to produce grapes for winemaking. As well as the use of pesticides to combat pathogenic fungi, insects and other pests, the vineyard is fertilised, irrigated, pruned, trellised etc. to allow the grapes to grow to their best quality.

1.1.3 The Process of Winemaking

For white wines the grape berry is considered at its ideal ripeness, for harvesting, between 18-20° Brix, and for red wine 18-25° Brix (Rankine, 1989). As well as the sugar composition of the grape berry, acid levels are important and are composed mainly of L-malic and D-tartartic acids. At maturity the levels of acid decreases to 5-8 g/L by respiration of the malic acid through the grape tissues (Rankine, 1989).

Once the grapes are collected, whether by hand or mechanically, they are destemmed and crushed. In white wine production the initial free-flow juice is used for premium wine, such as champagne, and the juice pressed in succession is used for lower quality wines.

Sulphur dioxide may be added either during or after the crush, at levels of 50-100 mg/L total to prevent oxidation of the grape must (browning) and also to halt the growth of wild yeast and bacteria. The process of white winemaking differs from red wine in that the skins are removed after gentle pressing. The juice may be clarified by settling for 24 hours or by centrifugation.

After clarification of the white wine juice yeast is added, usually as selected starter cultures of *Saccharomyces cerevisiae*. These cultures have been favoured for their ability to ferment energetically, tolerance of the harsh environment, endurance from the added sulphur dioxide, and are chosen to compliment the style of wine.

Musts undergoing fermentation are temperature controlled and the fermentation temperature of the white wine is lower than that of red. In red winemaking the juice remains in contact with the skins to help extract flavour and colour. The cap (the skins and pulp), which settles on top of the must, is plunged through the juice either by hand, or by pumping the juice over the cap to maximise colour and tannin extraction. This also helps to provide an even fermentation.

Upon the completion of alcoholic fermentation, the new wine is racked off into another vessel, leaving behind the settled yeast and solids (lees). Some wines undergo malolactic fermentation for flavour and deacidification, these are typically red wines and some white wines, more often from cool climate regions that produce high acid/ low pH juices. Malolactic fermentation is assisted by extended lees contact or later, by the addition of a selected bacterial culture. The wines may have further racking, filtration or fining followed by ageing and blending depending on the type of wine to be made.

The last step is the bottling of the wine once it has been stabilised, and this is usually done by centrifugation or further racking. White wines are bottled under sterile conditions while red wines are not. The robustness of the reds is due to tannin extracts and other chemical constituents which confer a large degree of microbial and chemical stability.

1.2 MICROBIOLOGY OF WINEMAKING

The microbiology of winemaking has developed from the pioneering studies of Louis Pasteur, over 130 years ago (Amerine *et al*, 1980). Pasteur demonstrated that yeasts were responsible for the alcoholic fermentation of the grape juice into wine, and other microbes such as bacteria, could grow in this harsh environment causing its spoilage. The microbial ecology of winemaking is complex, involving yeast, fungi and bacteria; therefore the quality of wine can be influenced by a myriad of factors, some of which are well described, other factors are not known.

1.2.1 Yeast

The yeasts represent a unique group of fungi characterised by vegetative growth that is predominantly unicellular, and by the formation of sexual states which are not enclosed in fruiting bodies. *S. cerevisiae* has been recognised as an Ascomycete for well over a century, but it was not until later that some yeasts were thought to be Basidiomycete.

1.2.2 Yeast involved with Winemaking

Yeasts are the primary microorganisms of winemaking. The term wine yeast describes, broadly, any yeast involved in must fermentation but more specifically it describes strains grown in pure culture for use in winery fermentations. Such strains are almost always part of the species *Saccharomyces cerevisiae*.

The quality of the wine is influenced by three key yeast fermentation mechanisms. Yeast conduct the alcoholic fermentation reducing berry sugars to ethanol and carbon dioxide, at the same time producing small quantities of other compounds (e.g. higher alcohols, organic acids, esters, aldehydes, ketones, sulphur and flavours). Secondly yeast can have a detrimental effect on the quality of the wine, if the wrong species grows in the grape juice, or if it causes a secondary growth in the finished wine. Lastly, flavour may be changed from cell constituents after the yeast has autolysed, also providing a food source for spoilage or malolactic bacteria to grow. The interruption of the primary alcoholic fermentation has serious consequences for the winemaker both in quality and cost.

1.2.3 Natural Yeasts

Natural yeasts may be best described as yeast found on grapes and in the must that contribute to the fermentation. Differences in grape maturity, climate and viticultural practises probably allow for the large variation of yeast cell counts on grapes. For example Reed and Nagodawithana (1991) have reported 5×10^4 - 1×10^5 cells per millilitre of must (or per berry). There is, however, a great deal of similarity between species found on grapes and in must, regardless of where they are distributed in vineyards around the world. Most of the yeast cells found on grapes belong to the genera *Kloeckera*, *Hanseniaspora* and to a lesser extent, *Metschnikowia* and *Candida*.

Natural, spontaneous fermentations are usually initiated by *Kloeckera apiculata*, a weak fermentor, which is succeeded by more alcohol-tolerant yeast during the main fermentation. Towards the end of fermentation the true wine yeasts such as *Saccharomyces cerevisiae* dominate (Reed and Nagodawithana, 1991). As grapes are transported from vine to vat there is a considerable increase in cell count.

1.2.4 Selected Pure Culture Yeast and Active Dry Wine Yeast.

During the nineteenth century the use of selected yeast cultures for the initiation of fermentation for brewer's worts and for bread doughs was common practise as these substrates do not ferment spontaneously due to their higher pH (Reed and Nagodawithana, 1991).

In the wine industry inoculation with selected strains of wine yeasts was not practised to any extent until the twentieth century. In the 1950's, the practise of using selected wine yeasts for inoculation was very common in the United States,

Australia, New Zealand, and South Africa. It was also practised in Europe, although the practice was not always admitted. In the 1960's, active dry wine yeast (WADY) was introduced in the United States and its use spread quickly to Australia, New Zealand, and South Africa. Its use in Germany, Italy, and France is now common, and it is being used in other wine-producing countries of Europe and South America (Reed and Nagodawithana, 1991).

Yeast strains are maintained in culture collections by wine research institutes and often these strains are duplicated within and between institutes. These cultures are identified by institute name and number but may also be called by commercial trade name such as Epernay, Prise de Mousse etc. Often individual wineries or wine companies isolate and maintain their own favourite yeast.

1.2.5 Description of Species

The *Saccharomyces* yeast are the strongest fermenters. Cells propagate vegetatively by multilateral budding. Sporulation can be induced on acetate agar.

Saccaromyces cerevisiae is the species par excellence for wine fermentations. The cells are usually spheroid, ovoid, ellipsoid, or elongated with a cell size of 3-7 by 1-12 micrometers. The species may produce up to 18-20% ethanol by volume. *S.bayanus* (now *S. cerevisiae*) also produces high concentrations of ethanol. It is often a more flocculent yeast.

Kloeckera apiculata is apiculate, and sometimes round to oval, and elongate. The cell size is quite small, 1.5-5.0 by 2.5-11.5 micrometers. The yeast is an imperfect form of *Hanseniaspora uvarum*. It produces 5-6% ethanol but tolerates higher concentrations. It occurs abundantly in musts and generally dominates the very early phase of the wine fermentation. *Kloeckera* and *Hanseniaspora* species produce relatively high concentrations of acetic acid, ethyl acetate, mannitol, sorbitol, and ribitol.

1.2.6 Malolactic Fermentation and Lactic Acid Bacteria

The biological deacidification of wines is conducted by lactic acid bacteria (LAB) as an enzymatic reaction following their growth in the wine. Malic acid is converted to lactic acid reducing the acidity of the wine, imparting flavour modifications and microbial stability. This is often described as malolactic fermentation (MLF) but it is not a true fermentation. This process is an integral

part of winemaking in cool climate regions and provides potential for spoilage problems.

The major species of LAB associated with wine are *Leucosnostoc oenos*, *Pediococcus damnosus*, *Pd. parvulus* and several species of *Lactobacillus* (Wibowo *et al.*, 1985).

MLF may be initiated by the addition of starter cultures or by natural fermentation. The pH of the wine is the main factor determining which species of LAB will develop. Wines below pH 3.5 do not support the growth of pediococci or lactobacilli, and *Leucosnostoc oenos* predominates.

1.3 FACTORS AFFECTING YEAST GROWTH DURING FERMENTATION

Table 1 Factors Affecting Yeast Growth During Alcoholic Fermentation

Factor
Inoculation of grape juice
Temperature of fermentation
Addition of sulphur dioxide
Composition of the grape juice
Settling and clarification of the juice
Interaction with other microorganisms

There are many variables associated with the successful fermentation of grape juice as outlined in Table 1. If one or more of these factors are not correct, the quality of the wine may be affected to its detriment.

Without a doubt, the availability of commercial selected yeasts for starter cultures, is one of the most important technological advances in winemaking. The intention of this practise is to be able to consistently produce good quality wines with a reliable and rapid fermentation, which means that any uncertainties previously associated with uncontrolled fermentations are avoided. Although it is expected that these starter cultures dominate the indigenous yeasts, studies show this may not be necessarily true as other factors can affect the fermentation process (Fleet, 1990). These factors are briefly described.

1.3.1 Temperature of Fermentation

Temperature affects the growth rate of yeasts and this response may vary with the different species, changing the ecology of the fermentation. Studies by Heard and Fleet, (1985) have shown that temperatures below 20°C substantially increase the growth of the non-*Saccharomyces* yeast altering the balance of the fermentation.

1.3.2 Sulphur Dioxide

Sulphur dioxide is added to the grape juice to control oxidation reactions and restrict indigenous microflora. In traditional wine making it is assumed that the sulphur dioxide will inhibit the indigenous non-saccharomyces species, allowing the dominance of *S.cerevisiae*. In wines produced by inoculation it is assumed that the indigenous species of both non-saccharomyces and indigenous *S.cerevisiae*, will be repressed, allowing the starter culture to excel. There are, however, very few studies to confirm this, indeed studies by Heard and Fleet (1985) show that indigenous species grow in must containing the recommended dose of sulphur dioxide (50-100 mg/L).

1.3.3 Composition of the Juice

The rate and completeness of fermentation as well as the concentration of many aroma and flavour constituents is influenced by the composition of the grape juice. Sugar concentration, acid content, supplementation with nitrogenous and vitamin nutrients, concentration of dissolved oxygen and concentration of soluble solids, all contribute to how well the yeast will ferment.

1.3.4 Juice Settling and Clarification

In white wine making, some form of clarification treatment usually takes place to reduce suspended solids, and to help reduce the indigenous microbiological population. This may be done by allowing the juice to settle overnight or centrifuging the must. Bentonite, a clay compound, may be added to remove excess proteinaceous material. Clarification of the must in white wine making may lead to problems during fermentation. Clarification removes nutrients and oxygen contained in particulate matter. Nutrients such as ergosterol and unsaturated fatty acids require oxygen and may be limiting factors to fermentation under anaerobic conditions (Larue *et al.*, 1980). Particulate matter is also required as attachment sites for yeasts aiding flocculation and as attachment sites for CO₂. A mixing

action is provided by the bubbles of the CO₂, which can aid in the diffusion of sugars to yeast cells and ensures an even fermentation (Dittrich, 1987., Thorton, 1990).

1.3.5 Interaction of Yeasts With Other Microorganisms

Products of other microbes (e.g. fungi and Lactic Acid Bacteria (LAB)) which may be present in must/wine may also affect the yeast and inhibit alcoholic fermentation. For example, grapes which have supported the growth of the mould *Botrytis cinerea* have been reported to contain antifungal substances (Ribéreau-Gayon, 1985).

LAB have also been reported to inhibit yeast alcoholic fermentation (Biodron, 1969). This may be due to LAB utilization of compounds required by the yeast such as copper, or due to the production of acetic acid, lactic acid or yeast-inhibiting ornithine (from the degradation of arginine) by LAB strains. Certain LAB strains are also able to produce "bacteriocins". These are similar to yeast-produced killer toxins as they are proteinaceous, and are usually inhibitory only to other closely related LAB strains (Tagg *et al.*, 1976). However, some bacteriocins (e.g nisin) have a much wider spectrum of action and are able to inhibit bacteria of other genera (Hurst, 1983). Recently a broad spectrum bacteriocin produced by *Lactobacillus reuteri*, "reuterin", has been reported, and this is able to inhibit yeast and other fungi (Axelsson *et al.*, 1989; Talarico and Dobrogosz, 1989).

1.3.6 Killer Yeasts

Killer yeasts were first discovered in 1965 and many yeasts, including *S.cerevisiae* strains, have the ability to produce proteinaceous or glyco-protein toxins called 'killer toxins'. These are generally only effective against sensitive yeasts of the same or similar species. Killer activity is known to occur in wine vinifications (Barre, 1978., Heard and Fleet, 1987a). Dried yeast starter cultures with killer properties are available commercially (Shimizu *et al.*, 1985) and killer yeasts have been known to cause stuck fermentations (van Vuuren and Wingfield, 1986).

1.3.7 Slowed or Stuck Fermentations

An occasional but serious problem in winemaking is the premature cessation of yeast growth and alcoholic fermentation, giving a wine with residual, unfermented sugars and less than the expected concentration of ethanol (Ribéreau-Gayon, 1986., Reed and Nagodowithana, 1988). Such fermentations are commonly referred to as being "stuck". Factors considered to cause this problem include: excessive clarification of the juice; fermentation temperature being too high; juice deficiency in nutrients or factors required for maximum yeast growth and survival; presence of fungicidal or pesticidal residues; and influences of acetic-acid bacteria. Recently, researchers at Boreaux have proposed that medium chain-length fatty acids such as decanoic and octanoic acids have a key role in causing stuck fermentations. These acids accumulate during fermentation and, above certain concentrations, become toxic to the growth and survival of *S.cerevisiae* (Lafon-Lafourcade *et al.*, 1984). *Saccharomyces cerevisiae* is considered to be the main producer of these acids but their production by indigenous, non-*Saccharomyces* wine yeast has not been investigated and requires study. Removal of these acids from wines by adsorption with yeast cell-wall hulls (yeast ghosts) overcomes stuck fermentations (Lafon-Lafourcade *et al.* 1984; Ribéreau-Gayon, 1986). Moreover, juices supplemented with yeast hulls exhibit more complete fermentation and a reduced tendency to become stuck (Munoz and Ingledew, 1989., Edwards *et al.*, 1990). Adsorption of toxic acids may not be the only mechanism by which these hulls prevent stuck fermentations. According to Munoz and Ingledew (1989), these hulls may also serve as a source of sterols, which are essential for sustained growth of yeasts under conditions of limited oxygen.

1.4 SLOWED OR STUCK FERMENTATIONS BY PESTICIDE RESIDUE

Pesticides are frequently used on grapes to control pathogenic fungi, insects and other pests, both in the vineyard and during postharvest storage. The interaction of pesticides with alcoholic fermentation of grape must largely depend upon the type of formulation, frequency, method of application, climatic conditions and type of yeast used for fermentation. The treatment of grapes with pesticides may introduce fermentation inhibitors into the must.

Conner (1983) tested 14 fungicides, 7 insecticides, and 4 herbicides, and listed the lowest concentrations at which they were inhibitory to yeast growth. Only one of

the insecticides, an organochlorine insecticide, dicofol, was inhibitory at a level of 5mg/L. None of the herbicides were particularly inhibitory. Four of the fungicides were inhibitory below 10mg/L. Kundu *et al* (1981) documented that 60% of the fungitoxicants applied to grapes have been found to be transferred to must and that red wines contained more toxicant residue than white wines. This is a reflection of the pulp fermentation process used for red wine making.

The effect of pesticide residue accumulation and subsequent transfer to the must has important implications. Firstly, the pesticide residue affects yeast growth and/or fermentation ability. Secondly the wine produced is destined for human consumption, and the pesticide residues may alter the organoleptic properties of the wine and also cause concern for the health of the consumers directly or indirectly.

1.4.1 Stuck or Slowed Fermentation in the Presence of Cryolite

Cryolite is an insecticide that is used in California, more specifically Big Valley and to a much lesser extent Napa Valley, where many wineries are located.

Wineries using grapes from vineyards which have been treated with Cryolite spray programs have experienced fermentation problems. Cryolite residue has been detected in the grape must and in wines from California at levels of 10ppm. Stuck or slowed fermentations have been noted in wineries and during University experimental wine fermentations (Kunkee, 1991., Morenzoni, 1991, personal communication). The fluoride ion from Cryolite is apparently translocated into the grape berries from there into the juice. The 1991 University studies found that very few milligrammes of sodium fluoride per litre inhibited wine fermentations, to varying degrees, depending upon the concentration of fluoride and upon the kind of grape juice itself (Kunkee, 1991). Another report noted fluoride levels of 15 to 20 mg/L in wine and at these levels objectionable flavours and smells were noted (Morenzoni, 1991, personal communication).

The use of cryolite was being banned by some wineries for two reasons. Firstly, in 1989, the European wine advisory body, the Organisation International de Vin (OIV), had reduced the existing limit of 3mg/L fluoride in wines imported to their countries to 1mg/L, with the stipulation that research be performed before they reinstate the 3mg/L limit. In 1991, after further review, the OIV reestablished the 3mg/L standard for fluoride in wines. A four-year study, completed in 1993, has shown that the application of the insecticide Kryocide, and its organic counterpart,

Cryolite, can significantly influence the levels of fluoride in red and white wines (Ostrom and Petrucci, 1994)

The second reason for not using any product that has residual fluoride in it is its effect on ethyl carbamate production. Ethyl carbamate, a carcinogen in sake and wine, was shown to be a natural byproduct of fermentation. When it was found in large quantities by Canadian researchers in dessert wines and sherries, the problem became recognised worldwide in all fermented beverages (Trioli and Ough, 1989). These products contain urea which is a precursor to the production of ethyl carbamate. In 1988 Kobashi *et al* demonstrated the removal of urea from alcoholic beverages with an acid urease. Research has shown that fluoride acts as an irreversible inhibitor of acid urease (Andris, 1990., Famuyiwa and Ough, 1991) allowing the production of this carcinogenic byproduct.

1.5 CRYOLITE (CRYOLITE 96/KRYOLITH)

Cryolite is a natural mineral containing up to 98% AlF_6Na_3 and it is found in large deposits in Greenland and the Urals. It was first used as an insecticide by Marcovitch and Stanley in 1929. Cryolite (Kryocide®) is a preferred insecticide as it is a natural compound, it is cheap and it targets specific insects, being less damaging to desired insects, such as bees, than other available insecticides. It is now prepared synthetically by the aluminium industry (Kryolite) and is used for the control of many insects, as listed below.

Cryolite has been used for the control of the pickle worm (*Diaphania nitidalis*) on cucurbits; the Mexican bean beetle (*Epilachna varivestis*) in snap beans; orange tortrix (*Argyrotaenia citrana*), pyroderces (*Pyroderces rileyi*), holcocera (*Holcocera iceryaeella*), and playnota (*Platynota stultana*) on citrus; the cotton bollworm (*Heliothis armigera*), boil weevil (*Anthonomus grandis*) and the rapid plant bug (*Adelphocoris rapidus*) on cotton; tomato hornworm (*Protoperce quinquemaculata*) on tobacco; and tomato fruit worms (*Heliothis armigara*) on tomatoes. Cryolite has been used also in investigations on control of the grape leaf folder (*Desmia funeralis*) in California and for evaluation of spray programs for control of the grape-berry moth (*Polychrosis viteana*) (Archer and Gauer, 1979).

A single application of Cryolite (as a stomach or contact insecticide) is usually effective in the control of lepidopterous pests of grapevines, if the application is

properly timed and sprayed onto the most highly infested part of the canopy. It has long been popular with grape growers because of its long residual activity.

1.5.1 Insects Targeted

There are three insects specifically targeted by vineyard spray programmes. They are the Omnivorous Leaf Roller, the Orange Tortrix and the Western Grapeleaf Skeletonizer.

1.5.1a) Omnivorous Leaf Roller

The omnivorous leaf roller, *Platynota stultana* Walsongham, was only recently found to be infesting California vineyards. It has become a serious pest of grapes in the San Joaquin Valley. First reported on grapes in 1963, the omnivorous leaf roller seriously damaged the crop in 1970; in some valley vineyards up to 50 % of the crop was lost (Winkler *et al*, 1974).

The pest overwinters as 3rd, 4th, and 5th instars. These overwintering insects occupy nests in unpicked clusters on the vines, in decayed bunches on the ground, in dried grapes "mummies" and in dried grape leaves under the vines. In early spring, as temperatures rise, the overwintering larvae pupate inside their webbed nests. Depending upon the temperature, the first moth flight may occur from early to late March. These adults deposit overlapping eggs in masses on newly emerged grape leaves and shoots or surrounding weeds to give rise to overlapping generations. The eggs hatch in about seven days and the young, yellowish -white or creamy larvae move vigorously or hang from the foliage by a silken thread. During this period they are spread easily by wind to other vines. Young larvae move to protected places, such as the rolls made by the grape leaf folder or where two leaves are touching to make their nests by webbing and folding the leaves. Later in the season, many larvae make nests inside the clusters. The life cycle of the insect, under San Joaquin valley temperatures, is completed in 30 to 45 days. (Winkler *et al*, 1974).

Damage - The leaf-tying, folding, webbing and feeding of larvae can cause much injury to grape foliage. Newly hatched larvae often feed on new growth and terminal shoots to retard shoot elongation. Significant damage to berries does not occur until mid-summer. Fruit damage is two-fold. Early season feeding causes cavities and scars on the berries. Later season feeding by larvae breaks the skin of the berry and allows yeast and fungi to initiate bunch rot. The rot then spreads

through the clusters often resulting in its entire destruction. Later infestations can cause defoliation followed by sunburn injury to the fruit.

Control - In the early years of the development of this insect in California vineyards, sprays of Cryolite or standard lead arsenate were used for control. Today growers are urged to employ microbial insecticide, *Bacillus thuringiensis*. Effective control is obtained by this means when careful applications are made to obtain coverage.

1.5.1b) Orange Tortrix

Although the Orange Tortrix, *Argyrotaenia citrana* Fernald, has been known to be present in California since 1885, economic damage to grapes was not reported until 1968. At that time it was found in young vineyard plantings in a newly developing area in the Salinas Valley in Monterey County (Winkler *et al*, 1974). Morphologically it is identical to the Apple Skinworm, a pest of deciduous fruits, ornamentals, and weeds in several coastal counties of both northern and southern California. It is not an economic pest in the warm, dry interior valleys of the state.

The Orange Tortrix moth can be recognised by the brownish or buff colour with a "saddle" of a darker shade across the folded wings. When at rest, the folded wings flare out at the tip like a bill. The female is somewhat larger and more robust than the male. The eggs of the Orange Tortrix are laid on the smooth surfaces of the vines' leaves, shoots, or berries. The oval flat cream-coloured eggs are deposited in several egg masses. When full grown a larvae is about one-half inch long, usually straw-coloured but may be greenish, dark grey or smoky-coloured.

In the Salinas Valley, the insect can be found in various stages of development in the vineyards throughout the year. Larvae may be found on any part of the vine, but tend to congregate in certain areas on the vine accordingly; in early spring they may be found in or on swollen buds; as shoots develop they feed on the developing leaves and tips; then, on the leaves which they web together; and, still later, the larvae are found in clusters where they web berries together to form nests deep within the cluster.

During the dormant season larvae may be found in the cracks of the bark on canes and arms. They also may overwinter on grasses or weeds in the vineyards, or in the mummified clusters left on the vines.

There are usually from five to seven generations per year. In the Salinas Valley about 59 days are required from egg to adult.

Damage - In addition to contamination of the clusters, the Orange Tortrix larvae feed on the berries and stems within the cluster. Secondary infections by spoilage micro-organisms then cause decay of the fruit.

Feeding on the framework of the clusters causes berry drop, and in some cases, when the rachis is cut or girdled causes portions of the cluster to dry up.

Control - The Orange Tortrix in the Salinas Valley vineyards was found by Kido *et al* (1971) (Winkler *et al*, 1974) to be parasitised by an ichneumonid wasp, a braconid wasp, and a tachinid fly. Green lacewing larvae also feed on the larvae and eggs. Although the ultimate effects of the predators on the Orange Tortrix populations in the vineyard has not, as yet, been determined, serious considerations are being given to their biology in designing control programmes for this pest.

Chemical control, using the usual stomach or contact insecticides, such as standard lead arsenate, Cryolite or Carbryl, is effective, but the best control programs for growers in the Salinas Valley is still under intensive study.

1.5.1c) Western Grapeleaf Skeletonizer

The western grapeleaf skeletonizer, *Harrisina brillians* B. and McD., described by Lange (1944), (Winkler *et al*, 1974) is native to Mexico and the states of Arizona, New Mexico and Texas. It was first found in California in 1941, in San Diego County. It overwinters in the pupal stage in cocoons in fallen leaves and trash. The moths emerge in late May.

The adult is a metallic-looking bluish-black or greenish-black moth with a wing expanse of about 1 inch. Its eggs, in cluster or masses of a few or as many as a hundred, are usually deposited on the lower surface of the leaves. The larvae are yellow, with two transverse purplish bands and several narrow black bands; they feed on wild and cultivated grapes and Virginia creeper, much the same as does the Eastern species, *Harrisina americana* Meréville. Each body segment has four tufts of black spines. The spines are poisonous and, if touched, produce welts similar

to those produced by nettles. The mature larvae are about a half an inch long. They spin a silken cocoon in which to pupate. After emerging from the cocoon, the moths mate, and eggs are laid.

Damage - The young larvae, starting from the mass of eggs from which they hatch, feed side-by-side, soldier-like, retreating as they feed by eating out the parenchyma, or soft leaf tissue. The skeleton framework (veins) and upper epidermis of the leaf are left intact by the young larvae, but the full-grown larvae consume all of the leaf tissue except for the larger veins. It has been observed that the larvae from a single egg mass are capable of defoliating an entire vine. Detection is not easy until damage to the vine is already serious.

Control - Chemical insecticides such as Carbyl and Cryolite are quite effective against this pest. However, timing applications as broods emerge is essential.

1.6 FLUORIDE SUSPECTED

Fluoride is suspected as the active agent in sodium aluminium fluoride as it is commonly used as an antibacterial agent for the control of dental caries. The action of fluoride against oral bacteria is by affecting the Streptococcal ATP production through the inhibition of streptococcal enolase, halting the process of glycolysis (Figure 1.6).

1.6.1 Use of Fluoride in Dental Care

Antimicrobial agents are used extensively in toothpastes and mouthrinses to help maintain dental plaque at levels compatible with oral health. Although they are often selected in the basis of a broad spectrum of antimicrobial activity, they frequently function in the mouth at sub-lethal concentrations. At such levels, these agents can interfere with carbohydrate and nitrogen metabolism.

Fluoride is one of the most widely used inhibitors in dentistry as it can affect sugar transport by the PEP-PTS system of oral bacteria. While the primary caries-preventive action of fluoride is due to its effect on increasing the acid resistance of enamel, fluoride at low concentrations is known to decrease the rate of sugar uptake and acid production by plaque bacteria. Fluoride has also been reported to

reduce extrapolsaccharide production, and to inhibit the synthesis, but not the breakdown of intracellular polysaccharides. Fluoride inhibits enolase, which converts 2-phosphoglycerate to phosphoenolpyruvate. This leads to glycolysis being inhibited directly and sugar transport being affected indirectly by a reduction in the availability of PEP for the PTS. Fluoride also inhibits ATP-synthase and so interferes with the control of intracellular pH. Fluoride can also inhibit metallo-enzymes, eg. phosphatases and phosphorylases, as well as oxidative enzymes such as catalase and peroxidase and this may interfere with the redox balance within plaques (Marquis, 1977, 1989,1995).

The fluoride sensitivity of metabolism is pH-dependant with the greatest inhibition occurring under acidic conditions. This is because at low pH fluoride exists as H^+F^- which is lipophilic and more easily able to penetrate bacterial cell membranes. The intracellular pH is relatively alkaline and therefore once inside the cell H^+F^- dissociates and F^- inhibits various enzymes as described above, while protons acidify the cytoplasm and so tend to reduce:

1. the activity of glycolytic enzymes, that have a pH optimum around neutrality.
2. the transmembrane pH gradient (and hence PMF driven uptake and secretion processes).
3. the aciduricity of cells, for example, by interfering with the membrane-bound ATP synthase, which helps regulate the intracellular pH.

1.6.2 Fluoride Action Against Yeast

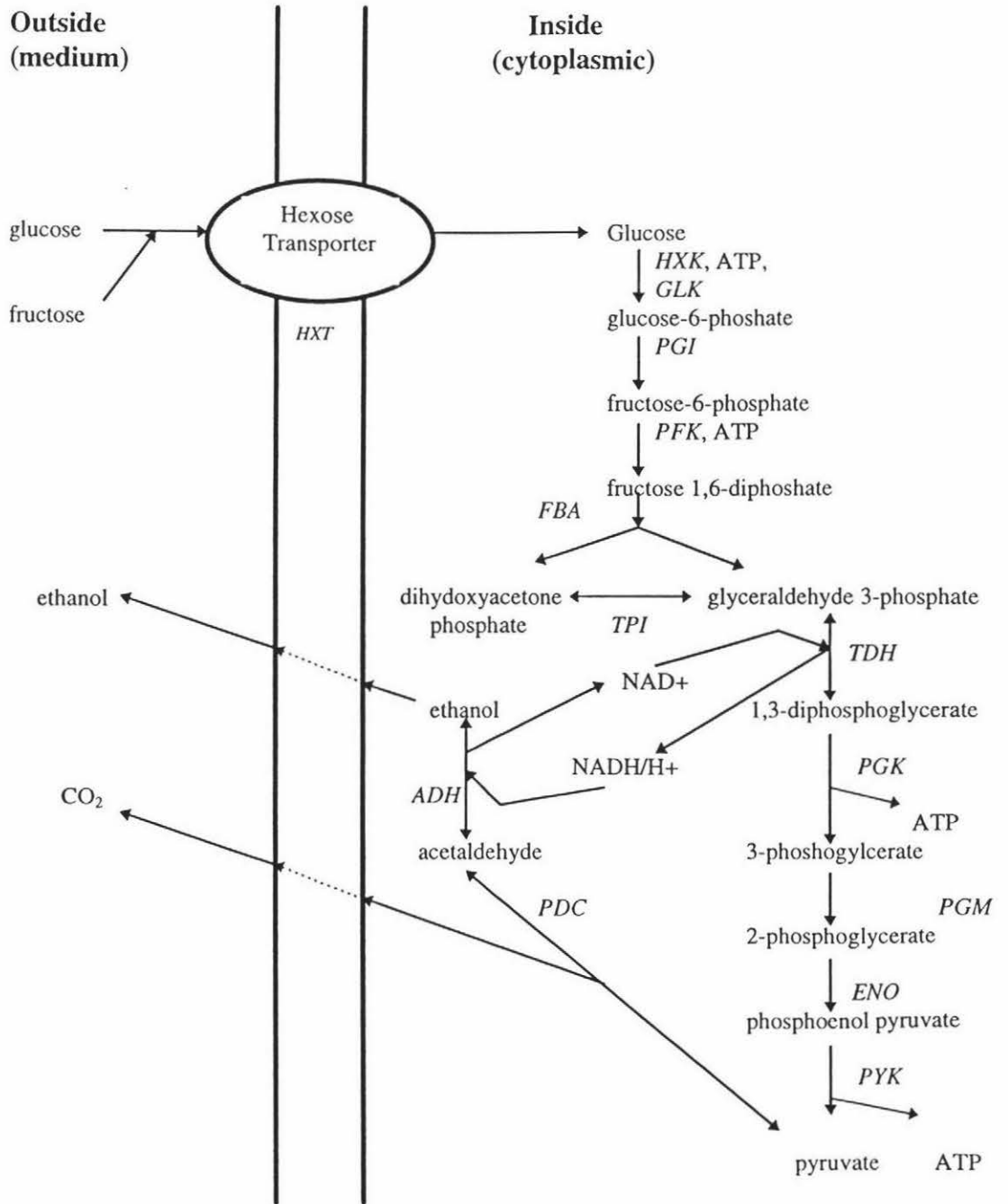
The antifungal action of fluoride against yeast is not fully known. It is possible that fermentation may be affected via the inhibition of yeast enolase. It is not clear if the effect of fluoride is fungicidal or fungistatic. It may be fungicidal to a sensitive proportion of the population thus lowering the numbers of competent fermenting cells. Fluoride sensitivity may be related to the yeast strain, yeast population, pH and a number of other variables including the type of wine that is to be made.

There have been recently a number of studies investigating wine yeast sensitivity to fluoride, the affect on fermentation by fluoride and also the level of fluoride in grape must and wines from vineyards using Cryolite (Ostrom and Petrucci, 1994;

Wahlstrom et al, 1994; Wahlstrom et al, 1991; Wahlstrom et al, 1996; Kunkee, 1991). Previous research suggests that high levels of fluoride adversely affects fermentation rate (Wahlstrom et al, 1991), and differences in fermentation rate upon fluoride challenge, are reflected in relative yeast populations densities (Wahlstrom et al, 1996).

Figure 1.6. Enzymatic Steps of the Glycolytic Pathway.

Key: HXT (hexose transporter), HXK (hexokinase), GLK (glucokinase), PGI (phosphoglucose isomerase), PFK (phosphofructokinase), FBA (aldolase), TPI (triosephosphate isomerase), TDH (glyceraldehyde -3-phosphate dehydrogenase), PGK (phosphoglycerate kinase), PGM (phosphoglycerate mutase), ENO (enolase), PYK (pyruvate kinase), PDC (pyruvate decarboxylase), ADH (Alcohol dehydrogenase).



2. PRELIMINARY EXPERIMENTS

2.1 INTRODUCTION

The initial aim of this research was to investigate the effect, if any, of the fluoride ion has on yeast involved in the winemaking. Initially, the optimal conditions for fluoride toxicity were determined. In later experiments more realistic fermentation conditions were utilised. The fluoride source used was a commercial preparation of Sodium Fluoride as there is reportedly little dissociation of the fluoride ion from Na_3AlF_6 (Cryolite) in water solutions (Morenzoni, 1991, personal communication). A pure chemical compound would therefore allow for greater dissociation of fluoride and so more optimal conditions for fluoride toxicity. YM agar was used in preference to grape juice as this media is less complex and the constituents of the agar media are more clearly identified. Colony morphology is also visible on agar plates, allowing for immediate identification in change of colony characteristics. The sensitivity parameters are crude but can be refined as the guidelines are identified.

Several yeast involved in wine fermentations were grown on YM agar to determine the effect of fluoride on cell growth. The influence of fluoride was examined at a range of concentrations, pH levels and yeast cell concentrations. In order for the results to be meaningful and relevant to the wine industry the pH of the media was reduced to 3.3 and cell counts for some parts of the experiment were equivalent to wine conditions. The results are reported in this section.

Agar sets poorly at low pH when normal amounts of agar are used, hence it was also necessary to determine a method of making YM agar pH 3.3. Two techniques were investigated in order to find a successful procedure to screen the selected yeast. The results are also reported in this section.

2.2 MATERIALS AND METHODS

2.2.1 Yeast Strains Used

Wine yeast strains used in this study represent yeasts which are typically involved with grape fermentation, either as starter cultures or as natural microflora. These belong to the Food and Fermentation laboratory culture collection of the Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand. Their original sources and descriptions are listed in Table 2.1. The yeast used in these experiments were:-

- Red Star™ Montrachet *Saccharomyces cerevisiae* RS1
- Red Star™ Cuvee *Saccharomyces bayanus* RS2
- Red Star™ Prise de Mousse *Saccharomyces bayanus* RS10
- Lalvin™ Prise de Mousse *Saccharomyces bayanus* EC1118
- *Brettanomyces species* UCD-615
- *Hansenula saturnus* AWRI-354
- *Kloeckera apiculata* SR50

These commercial strains were chosen for this study because they are well known and extensively used throughout the world. The wild yeast strains; *Brettanomyces* sp., *Hansenula saturnus* and *Kloeckera apiculata* are also commonly associated with winemaking. Whether their activity should be considered as beneficial, detrimental or indifferent depends on a variety of issues, but the reality is that they are commonly associated with winemaking regardless of what steps are taken to deter their growth.

2.2.2 Culture Media

YM broth was prepared by dissolving 21 g of commercial YM broth (Difco) in 1L of DI water. The pH was approximately 6.2 ± 0.2 and the media consisted of the following (per litre): Yeast extract, 3g., malt extract, 3g., peptone, 5g, and dextrose, 10g. The pH was adjusted as required with 50% H₃PO₄ or 30% KOH. Aliquots (10mL) were dispensed into capped test-tubes and sterilised at 15lb for 15 minutes.

YM agar was prepared from YM broth by the addition of 15g of agar per litre, well mixed and the pH altered to 6.8 or 4.2 by 50% H₃PO₄ or 30% KOH. The agar

was sterilised and the molten agar cooled to 45-50°C, then the desired amount of NaFl stock solution added aseptically. The plates were poured (*ca* 25mL) into sterile plastic Petri dishes, and cooled for 1 hour. Plates were stored in plastic Petri dish sleeves at 4°C.

2.2.3 Methods for Making YM Agar at pH 3.3

Agar made at a low pH will hydrolyse in the autoclave. Two methods of agar preparation at low pH were investigated. The first was the addition of mineral acid to cooled media (45°C) after autoclaving. The pH of the agar was then checked with litmus paper. The second method was to prepare and autoclave the agar and the fluoride solutions separately, before cooling to 65°C, and pouring into sterile petri-dishes. The agar pH was checked by litmus paper. The latter method was utilised because it showed consistent pH values with fewer manipulations after autoclaving and the agar produced a firm surface (data not shown).

2.2.4 YM Agar pH 3.3

YM agar pH 3.3 was prepared with:-

Solution I, 100mL lots of YM broth pH to 3.3 with 50% H₃PO₄. Sterilised and held at 65°C in a waterbath.

Solution II, 100mL of water-agar at 4g/100mL, sterilised and held at 65°C in a waterbath.

To Solution I the required amount of NaFl stock was added. Solution I was then added to Solution II. This was mixed and hand poured into sterile Petri dishes. Final amount of agar per litre was 20g.

2.2.5 Maintenance and Preparation of Yeast Cultures

All strains were routinely cultured in YM broth incubated at 30°C. Strains were stored at 4°C on YM agar slopes. The yeast strains were subcultured from slopes to 10mL of sterile YM broth (pH 6.8) and incubated at 30°C. Strains were routinely checked for morphological characteristics and for colony characteristics. This was done by streaking the yeast out on a YM agar plate isolating single colonies, examining the plate for colony morphology then picking a colony and

examining under phase contrast microscope. To avoid subculturing a mutant, the inoculum was never taken from a single colony. The yeast cultures for experimentation were subcultured from plates into 10mL of sterile YM broth and grown at 30°C for 2 days. The cultures were diluted 1/100 in sterile 0.1% peptone water as appropriate (see section 2.2.6).

For experiments requiring enumeration of colonies per millilitre (CFU/mL) the cultures were counted in a cytometer under Zeiss phase contrast. If dilutions were required they were made with sterile 0.1% peptone water.

2.2.6 Reagents

Sodium Fluoride Stock Solution

Sodium Fluoride stock solution was prepared to a concentration of 1.0g/100mL sterile water (10mg/mL).

Peptone Water 0.1%

Peptone water for dilutions was prepared by adding 1 g of peptone (Difco) to 1L of DI water. This was stirred to dissolve the peptone, dispensed (4.5mL) into test-tubes and sterilised by autoclaving at 121°C for 15 minutes.

2.3 GROWTH STUDIES

2.3.1 Experiment Procedure

Each yeast broth culture was spotted (10µL) on duplicate YM plates at the appropriate NaFl concentration as shown in Figure 2.2. These were incubated at 30°C for appropriate time periods. The plates were compared to positive controls (i.e YM agar plates with no added fluoride) and scored on the basis of colony growth compared to the control.

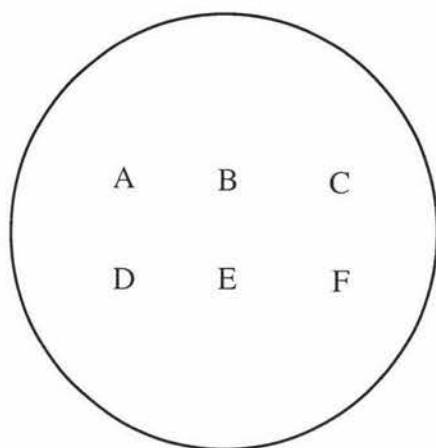


Figure 2.2 Layout of Plating Pattern

NB: Letters represent different strains of yeast.

2.4 RESULTS AND DISCUSSION

The effect of various concentrations of fluoride on yeast cells was influenced by culture density and pH. Tables 2.4.1 and 2.4.2 show that at pH 6.8 full growth of all four commercial strains was obtained regardless of the amount of NaF in the agar plates. Wild yeast AWRI-354 showed some sensitivity at 45 mg/L of fluoride ion. UCD-615 did not grow well after incubating for 48 hours and needed a longer incubation time.

As shown in Table 2.4.2 RS1 was the only commercial yeast affected by addition of the fluoride ion (271mg/L). AWRI-354 was the most sensitive yeast. When yeast cell concentration and pH were reduced all yeast strains were adversely affected by fluoride ions. AWRI-354 appeared to be the most sensitive yeast, with RS1 the most sensitive commercial yeast.

The effect of fluoride on yeast appeared to be influenced by both pH and cell numbers. Incubation time may also be important. The results from Table 2.4.4 indicate that in some cases fluoride sensitivity was partially overcome with longer

incubation time. This may reflect an inhibition of cell metabolism rather than a true reduction in cell numbers.

The combined effect of a lower pH (pH 3.3) and reduced cell numbers summarised is in Table 2.4.5, while the same cell counts at pH 6.8 are summarised in Table 2.4.6. (Note that the commercial yeast EC1118 was exchanged for SR50 - *Kloeckera apiculata*). This experiment reiterated that the two most sensitive yeast are RS1 and AWRI-354, both showing complete killing at all cell dilutions when exposed to 45mg/L and above. All yeasts used appeared to be more sensitive at pH 3.3 than at pH 6.8 and this sensitivity was increased as the initial cell numbers were reduced.

These results indicate that the pH of the media has considerable influence on the response of the yeast cells to the fluoride. A drop in cell numbers appeared to exaggerate this effect. This relationship between fluoride lethality and low pH has been described in many studies of the toxicity of fluoride against bacteria (Belli *et al.*, 1995., Kashket and Kashket, 1985., Kaufmann and Bartholmes, 1992., Marquis, 1977., Marquis, 1989., Marquis, 1995).

It was not possible to comprehensively show a relationship between cell density and fluoride lethality. A more quantitative approach is needed. Clearly there is no 'all or none' effect in fluoride inhibition.

Table 2.4.1, Growth response^a of Wine Yeast Strains on YM agar with varying levels of NaFl at pH 6.8, cells not diluted. Incubated at 30°C for 48 hours.

Na Fl (mg/L)	Fl ⁻ (mg/L)	RS1	RS2	RS10	UCD- 615	AWRI- 354	EC1118
0	0	+++	+++	+++	+	+++	+++
100	45	+++	+++	+++	+	++	+++
200	91	+++	+++	+++	+	++	+++
300	136	+++	+++	+++	+	++	+++
400	181	+++	+++	+++	+	++	+++
500	226	+++	+++	+++	+	++	+++
600	271	+++	+++	+++	+	++	+++
700	316	+++	+++	+++	+	+	+++

^a no growth,-; poor growth,+; fair growth.++; good growth,+++

Table 2.4.2, Growth response^a of Wine Yeast Strains on YM agar with varying levels of NaFl at pH 4.2, cells not diluted. Incubated at 30°C for 48 hours.

Na Fl (mg/L)	Fl ⁻ (mg/L)	RS1	RS2	RS10	UCD- 615	AWRI- 354	EC1118
0	0	+++	+++	+++	+++	+++	+++
100	45	+++	+++	+++	+++	++	+++
200	91	+++	+++	+++	+++	++	+++
300	136	+++	+++	+++	+++	+	+++
400	181	+++	+++	+++	+++	+	+++
500	226	+++	+++	+++	+++	-	+++
600	271	++	+++	+++	+++	-	+++
700	316	++	+++	+++	+++	-	+++

^a no growth,-; poor growth,+; fair growth.++; good growth,+++

Table 2.4.3, Growth response^a of Wine Yeast Strains on YM agar with varying levels of NaFl at pH 6.8, cells diluted 1 in 100. Incubated at 30°C for 72 hours.

Na Fl (mg/L)	Fl ⁻ (mg/L)	RS1		RS2		RS10		UCD- 615		AWRI- 354		EC1118	
0	0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
100	45	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
200	91	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
300	136	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
400	181	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
500	226	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
600	271	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
700	316	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++

^a no growth,-; poor growth,+; fair growth,++; good growth,+++

Table 2.4.4, Growth response^a of Wine Yeast Strains on YM agar with varying levels of NaFl at pH 4.2, cells diluted 1 in 100. Incubated at 30°C for 120 hours^b.

Na Fl (mg/L)	Fl ⁻ (mg/L)	RS1		RS2		RS10		UCD- 615		AWRI- 354		EC1118	
		48	120	48	120	48	120	48	120	48	120	48	120
		0	0	+++	+++	+++	+++	+++	+++	-	+++	+++	+++
100	45	++	++	++	++	++	++	-	+++	-	+	++	++
200	91	++	++	++	++	++	++	-	+++	-	++	++	++
300	136	++	++	++	++	++	++	-	+++	-	1.5	++	++
400	181	+	+	++	++	+	++	-	+	-	-	+	+++
500	226	-	0.5	++	+++	+	++	-	+	-	-	+	++
600	271	-	-	+	++	+	+	-	+	-	-	+	+
700	316	-	-	+	++	+	++	-	+	-	-	-	+

^a no growth,-; poor growth,+; fair growth,++; good growth,+++

^b initial incubation was only for 48 hours, then extended to 120 hours

note: numbers in table are an average of actual colony counts on the agar plates.

Table 2.4.5. Growth response of Wine Yeast Strains (diluted) to varying levels of NaFl at pH 3.3. Incubated at 30°C for 3 days.

	Fl- (mg/L)	0	45	91	136	181	226	271	316	360
RS1	1x10 ⁵ †	FG	-	-	-	-	-	-	-	-
	1x10 ⁴	FG	-	-	-	-	-	-	-	-
	1x10 ³	FG	-	-	-	-	-	-	-	-
RS2	1x10 ⁵	FG	G-	G-	G-	SC	SCS	SCS	-	-
	1x10 ⁴	FG	G-	G-	G=	SC	SCS	-	-	-
	1x10 ³	FG	G-	G-	SC	SC	-	-	-	-
RS10	1x10 ⁵	FG	G-	G-	G=	SCS	SCS	-	-	-
	1x10 ⁴	FG	G-	G=	SC	SCS	-	-	-	-
	1x10 ³	FG	SC	SC	SC	-	-	-	-	-
UCD-615	1x10 ⁵	FG	G-	G=	G=	G=	G=	G=	G=	G=
	1x10 ⁴	FG	G-	G=	G=	G=	G=	SC	SC	SC
	1x10 ³	FG*	SC	SC	SC	SCS	SCS	SCS	SCS	SCS
AWRI-354	1x10 ⁵	FG	-	-	-	-	-	-	-	-
	1x10 ⁴	FG	-	-	-	-	-	-	-	-
	1x10 ³	FG	-	-	-	-	-	-	-	-
SR50±	1x10 ⁵	FG	G-	G-	G-	G=	G=	G=	G=	G=
	1x10 ⁴	FG	G-	G-	G-	G=	G=	G=	SC	SC
	1x10 ³	FG*	SC	SC	SC	SC	SC	SCS	SCS	SCS

KEY: Full Growth,FG; Growth, but not as much as control,G-; Growth but not as much as G-, G=; Single colonies,SC;Single colonies under the stereoscope, SCS: Single colonies for control, *; Complete killing, -

± SR50 *Kloeckera apiculata* replaces EC1118., †, CFU/mL

Table 2.4.6. Growth response of Wine Yeast Strains (diluted) to varying levels of NaFl at pH 6.8. Incubated at 30°C for 3 days.

	Fl- (mg/L)	0	45	91	136	181	226	271	316	360
RS1	1x10 ⁵ †	FG	FG	FG	FG	FG	G-	G-	G-	G-
	1x10 ⁴	FG	FG	FG	FG	FG	G-	G-	G-	G-
	1x10 ³	FG	FG	FG	FG	FG	G-	G-	G-	G-
RS2	1x10 ⁵	FG	FG	FG	FG	FG	FG	FG	G-	G-
	1x10 ⁴	FG	FG	FG	FG	FG	FG	FG	G-	G-
	1x10 ³	FG	FG	FG	FG	FG	FG	FG	G-	G-
RS10	1x10 ⁵	FG	FG	FG	FG	FG	G-	G-	G-	G-
	1x10 ⁴	FG	FG	FG	FG	FG	G-	G-	G-	G-
	1x10 ³	FG	FG	FG	FG	FG	G-	G-	G-	G-
UCD-615#	1x10 ⁵	FG	G-	G-	G-	G-	G-	G-	G=	G=
	1x10 ⁴	FG	G-	G-	G-	G-	G-	G-	G=	G=
	1x10 ³	FG	G-	G-	G-	G-	G-	G-	G=	G=
AWRI-354	1x10 ⁵	FG	G=	G=	G=	G=	G=	G=	G=	SC
	1x10 ⁴	FG	G=	G=	G=	G=	G=	G=	G=	SC
	1x10 ³	FG	G=	G=	G=	G=	G=	G=	G=	-
SR50±	1x10 ⁵	FG	FG	FG	FG	FG	FG	FG	FG	FG
	1x10 ⁴	FG	FG	FG	FG	FG	FG	FG	FG	FG
	1x10 ³	FG*	FG	FG	FG	FG	FG	FG	FG	FG

KEY: Full Growth,FG; Growth, but not as much as control,G-; Growth lighter than previous result, G=; Single colonies,SC; Complete killing, -
 ±SR50 *Kloeckera apiculata* replaces EC1118; # Incubated at 30°C for 5 days., †. CFU/mL

TABLE 2.1 YEAST STRAINS USED IN THIS STUDY.

STRAIN NAME	TAXONOMY	COMMENTS
RS1 ^a	<i>Sacchromyces cerevisiae</i>	Montrachet strain; UCD#522
RS2 ^a	<i>Sacchromyces bayanus</i>	Pasteur Champagne strain; UCD #595
RS10 ^a	<i>Sacchromyces bayanus</i>	Premier Cuvée strain (formerly Prise de Mousse); Universal Foods isolate.
EC1118 ^b	<i>Sacchromyces bayanus</i>	Prise de Mousse strain; I.O.C. Champagne.
AWRI354 ^c	<i>Hansenula saturnus</i>	From Centraalbureau voor Schimmelcultures (C.B.S.), Deft, Holland.
UCD615 ^d	<i>Brettanomyces sp.</i>	
SR50 ^e	<i>Kloeckera apiculata</i>	

^a Red Star™ strains, obtained from Universal Foods Corporation, Milwaukee, Wisconsin.

^b Lalvin™ strains, obtained from Lallemant Australia, North Adelaide, South Australia.

^c From the Australian Wine Research Institute, Glen Osmond, South Australia.

^d From R.E. Kunkee, Dept. Enology & Viticulture, U.C. Davis, California.

^e From the Dept. of Microbiology & Genetics Culture Collection, Massey University, Palmerston North, New Zealand.

3. MINIMUM INHIBITORY CONCENTRATION EXPERIMENTS.

3.1 INTRODUCTION

Although earlier work gave a general idea of the effect of a broad range of fluoride concentrations at differing pH and cell densities, it did not elucidate the type of action fluoride has on the yeast. There are two proposed mechanisms a) fluoride inhibition, where upon the fluoride is affecting the metabolic activity of the yeast i.e slowing fermentation or b) fluoride toxicity i.e outright killing of the cells. The effect of fluoride on the yeast may also be a combination of both inhibition and toxicity depending upon the dose and conditions affecting the yeast cells. It should be noted that conditions of winemaking are themselves already inhibitory to many yeast strains.

Sodium Fluoride versus Cryolite as Fluoride Source

The range of fluoride concentrations from section two were very broad and the sensitivity at the lower end of concentration was double (45 mg/L fluoride) the recorded levels of fluoride found to cause stuck fermentations (15-20 mg/L fluoride) (Morenzoni, 1991, personal communication). In more recent experiments researchers have found that even lower levels of fluoride may cause slowed or stuck fermentations (Wahlstrom *et al*, 1994., Wahlstrom *et al*, 1996).

This next set of experiments were performed in YM broth media, which is closer to 'real world' wine conditions yet allows for optimal demonstration of fluoride effect. The levels of fluoride were reduced and two sources of fluoride were compared, firstly from NaFl and secondly from BDH Cryolite. As previously noted the fluoride ion of the compound Cryolite does not disassociate very well in water. The results are reported in this section.

Minimum Inhibitory Concentration

This set of experiments were performed to ascertain the minimum inhibitory concentration (MIC) of the fluoride on the selected yeast in YM broth. The MIC test is commonly used to test chemotherapeutic agents and provides important

information on inhibitory effect *in vitro*. To perform the MIC test, a standard inoculum of the test microbe was added to a series of dilutions (including controls) of the chemotherapeutic agent (in this case, fluoride). The inoculated dilutions were then incubated and observed for microbial growth. The lowest concentration of agent in which no growth occurs is the MIC (Van Denmark and Balzing, 1987).

The disassociation of fluoride from sodium fluoride compound was also examined. The Merck Index reports that freshly prepared aqueous solutions have an alkaline reaction caused by partial hydrolysis. The pH of freshly prepared saturated solutions is 7.4 (The Merck Index, 1989). The addition of NaF into YM media may cause the media pH to change. This section reports on this aspect.

3.2 MATERIALS AND METHODS

3.2.1 Yeast Culture Strains Used

Six yeast strains were used, including commercially available strains of *Saccharomyces cerevisiae* and *S. bayanus* and various "wild strains" which can be isolated from vineyards and wineries. These yeast strains used are described in Table 2 and are maintained in the Yeast Culture Collection of the Food and Fermentation Laboratory, Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand. These yeast were:-

- Red Star™ Montrachet *Saccharomyces cerevisiae* RS1
- Red Star™ Cuvee *Saccharomyces bayanus* RS2
- Red Star™ Prise de Mousse *Saccharomyces bayanus* RS10
- *Brettanomyces* species UCD-615
- *Hansenula saturnus* AWRI-354
- *Kloeckera apiculata* SR50

3.2.2 Culture Media

YM Broth/Agar

YM broth was prepared by dissolving 21 g of commercial YM broth (Difco) in 1 L of DI water. The pH was approximately 6.2 ± 0.2 and the media consisted of the

following (per litre): Yeast extract, 3g., malt extract, 3g., peptone, 5g, and dextrose, 10g. The pH was adjusted to requirement (pH 3.3 or 6.8) with 50% H₃PO₄ or 30% KOH. 10 mL volumes of YM broth was dispensed into 20mm x 150mm Kimax™ test tubes. The remaining YM broth was divided into 500mL aliquots in 1L Schott™ glass bottles. The media was autoclaved at 15lb or 15 minutes. Once the media had cooled, 0.553 g of NaFl was added to each bottle (500mg fluoride/ L of YM broth) or Cryolite powder 0.543 g of AlF₆Na₃ powder added. The media was kept at 30° C for two days to check for sterility.

The YM broth with fluoride was serially diluted as described in section 3.3.1

3.2.3 Preparation of Yeast Cultures

The yeast cultures were prepared as described in Section 2.2.3. Yeast used for the MIC experiment were subcultured into YM broth pH 3.3.

3.2.4 Reagents

Sodium Fluoride

BDH 'AnalaR' grade Sodium Fluoride was added to sterile YM broth to a final concentration of 500mg/L of fluoride (0.553g NaFl in 500mL sterile YM broth).

Cryolite

BDH Cryolite (Greenland) Powder was added to sterile YM broth for a final concentration of 500mg/L of flouride.

3.3. GROWTH STUDIES

3.3.1 Experimental Procedure

Sodium Fluoride verses Cryolite as Fluoride Source

YM broths supplemented with NaFl or Cryolite were serially diluted. 0.1mL of a 1x10⁶ CFU/mL yeast culture was added to duplicate tubes of sterile YM broth (pH

3.3). A positive control (no fluoride added) and negative control (media only) were included. The broth cultures were incubated at 30°C for 6 days.

Results were compared to the positive control and recorded as:- good growth "+++", fair growth "++", poor growth "+" or no growth "-".

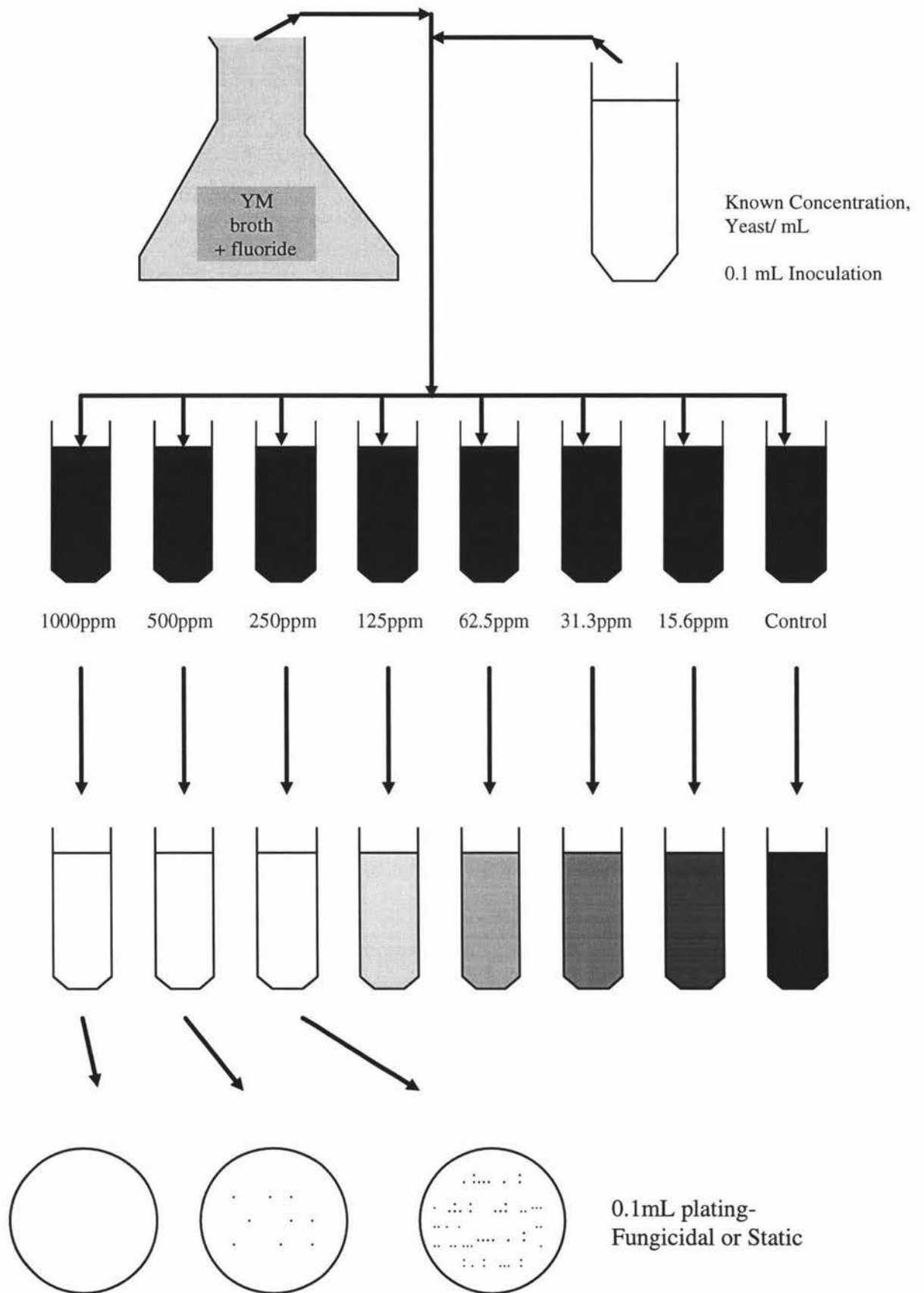
Minimum Inhibitory Concentration

Sterile supplemented YM broth (500mg/L) was serially diluted following the diagrammatic scheme in Figure 3.3, using the sterile 10mL YM broths. This was done aseptically and one set of dilutions was pH tested. The results of the pH testing are shown in Table 3.4.1.

0.1mL of 1×10^6 CFU/mL of each culture was added to duplicate tubes of sterile supplemented YM broth (pH 3.3). A positive control (no fluoride added) and negative control (media only at pH 3.3) were included. The broth cultures were incubated at 30°C for 6 days, and then the optical density was measured in duplicate. A Spectronic 20 was used at the wavelength of 600nm. The zero was set with 3mL of water and the blank set with 3mL of YM broth pH 3.3. Samples were vortexed to disperse cells. If the optical density was over 0.5, the sample was diluted with YM broth.

Samples that showed an absorption at 600nm of 0.05 and below (ie. no visible growth) were spread plated (0.1mL) in duplicate onto YM agar (pH 6.8) and grown up at 30°C for 3 days. All results were recorded.

Figure 3.3
Schematic Diagram of Serial Dilutions for MIC Experiment.



3.4. RESULTS AND DISCUSSION

Sodium Fluoride verses Cryolite as Fluoride Source

The results from Tables 3.4.2 and 3.4.3 show that fluoride from Cryolite has less of an effect than the fluoride from NaFl on the selected yeasts. Fluoride from NaFl clearly affects the yeast at a lower concentration. Complete killing for all yeast was noted between 15 to 62.5mg/L of fluoride (Table 3.4.2). In contrast the Cryolite showed complete killing only for RS1 and AWRI-354 (Table 3.4.3). Complete killing was shown for RS1 at 31.25mg/L fluoride (Table 3.4.2) compared to 500mg/L for complete killing with Cryolite (Table 3.4.3). AWRI-354 was the most sensitive yeast with a minimum inhibitory concentration of 15mg/L (NaFl as source) compared to 124mg/L (Cryolite as source). In both cases the most sensitive yeasts were RS1 and AWRI-354.

The results from this experiment only give an indication of the differences between fluoride sources as the measurement of growth were qualitative not quantitative.

TABLE 3.4.1 pH VALUES OF YM BROTH USED IN MIC TEST

Fl mg/L	500	250	125	62.5	31.25	15.63	7.8	Control
pH	4.95	4.56	4.13	3.84	3.67	3.57	3.54	3.49

Table 3.4.2. Growth response^a of selected yeast incubated at 30°C for six days in YM broth pH 3.3. Fluoride source NaFl.

Fluoride mg/L	Control -ve*	Control +ve #	15	31.25	62.5	125	250	500
RS1	-	+++	++	-	-	-	-	-
RS2	-	+++	++	+	-	-	-	-
RS10	-	+++	++	-	-	-	-	-
UCD 615	-	+++	++	++	-	-	-	-
AWRI 354	-	+++	-	-	-	-	-	-
SR50	-	+++	+++	++	-	-	-	-

^a no growth,-; poor growth, +; fair growth, ++; good growth, +++

Negative Control - YM broth 500mg/L no yeast added; * Positive Control YM broth no fluoride, yeast added.

Table 3.4.3. Growth response^a of selected yeast incubated at 30°C for six days in YM broth pH 3.3. Fluoride source Cryolite.

Fluoride mg/L	Control -ve#	Control +ve*	15	31.25	62.5	125	250	500
RS1	-	+++	+++	+++	++	++	+	-
RS2	-	+++	+++	++	++	++	++	+
RS10	-	+++	+++	+++	++	+	+	+
UCD 615	-	+++	++	++	++	++	++	++
AWRI 354	-	+++	+++	++	+	-	-	-
SR50	-	+++	+++	++	++	++	++	+

^a no growth, -; poor growth, +; fair growth, ++; good growth, +++

Negative Control - YM broth 500mg/L no yeast added; * Positive Control YM broth no fluoride, yeast added.

Minimum Inhibitory Concentration

The results were tabulated and are shown in Figures 3.4.1 to 3.4.6. There was no growth shown from any of the YM spread plates prepared from broths with an OD of less than 0.05 indicating that the effect of the fluoride upon the yeast in this experiment was fungicidal (Table 3.4.4). The pH values of each of the supplemented broths are shown in Table 3.4.1. This set of experiments were the first in liquid broth media at pH 3.3. These two factors allow closer comparisons with the true conditions of a grape fermentation.

This experiment has demonstrated a clear effect of fluoride on all yeast cultures in the YM broth environment. The most resistant yeast was the wild yeast *Kloeckera apiculata* which has grown to nearly 40% of full growth at 15.6 mg/L of fluoride and had a MIC of 62.5 mg/L of fluoride. The most sensitive yeast was wild yeast AWRI-354 *Hansenula saturnus* with a MIC of 15.6 mg/L of fluoride. The most sensitive commercial yeasts were RS1 and RS2.

However, the dramatic effects of fluoride observed in YM broth do not reflect the complex conditions of normal grape fermentations. This experiment does give a clear indication of a tolerance threshold of each of the different yeast in YM broth conditions.

TABLE 3.4.4 MINIMUM INHIBITORY CONCENTRATION

YEAST	Fluoride ion (mg/L)
RS1	31.3
RS2	31.3
RS10	62.5
UCD615	31.3
AWRI354	15.6
SR50	62.5

It is likely that YM broth does not have the same buffering effect of grape juice. The must contains many more constituents that may provide some level of

protection for the yeast from the fluoride. Some of the fluoride may be complexed by other compounds in the must, the yeast may flocculate/bind to particle matter and this may also alter the effectiveness of the fluoride. On the other hand the very nature of particulate grape juice may aid in the concentration of fluoride.

For these reasons, the inhibitory effect of fluoride on yeast in grape juice should be examined.

Figure 3.4.1 RS1 Growth vs. Fluoride Concentration

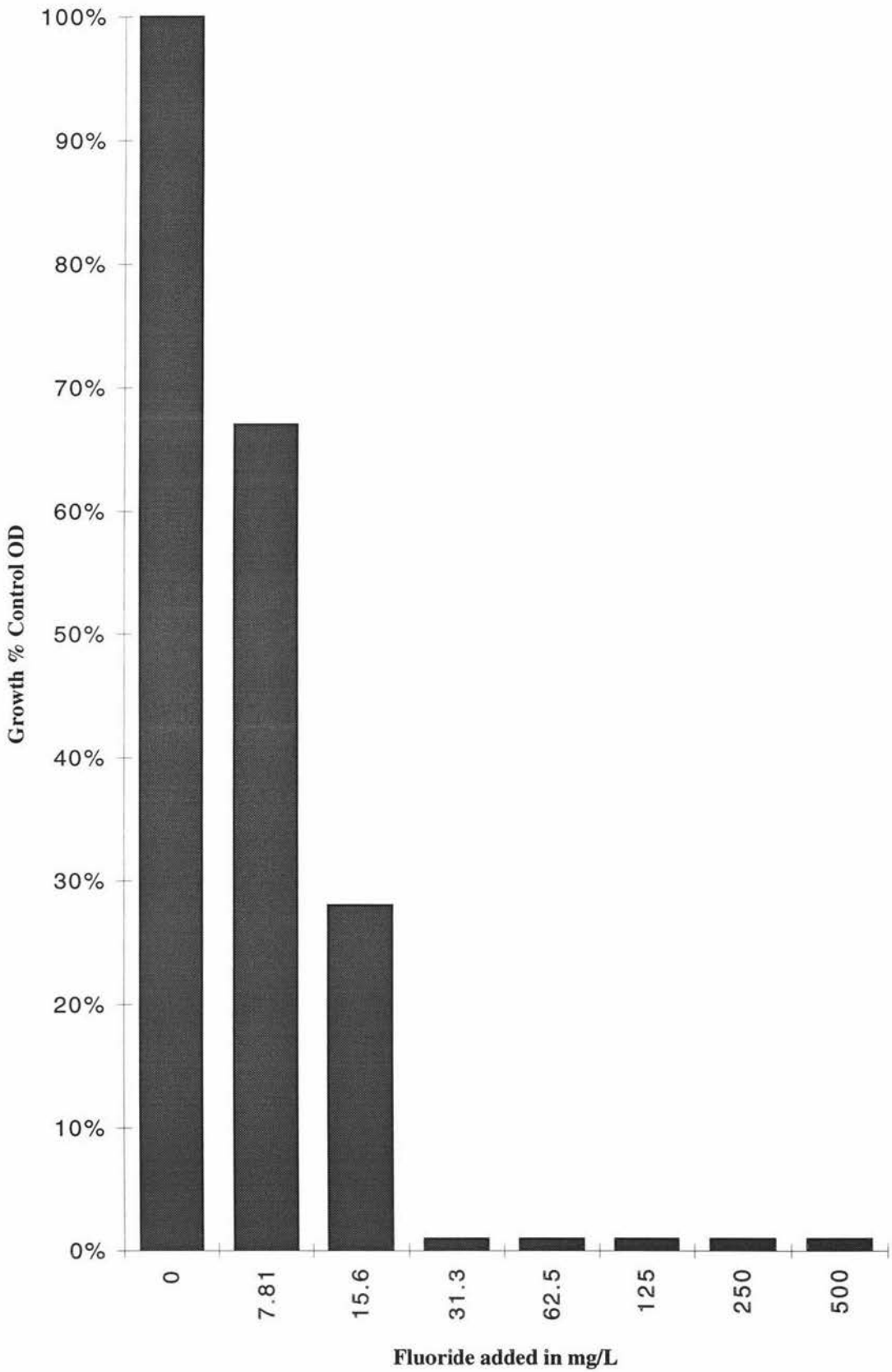


Figure 3.4.2 RS2 Growth vs. Fluoride Concentration

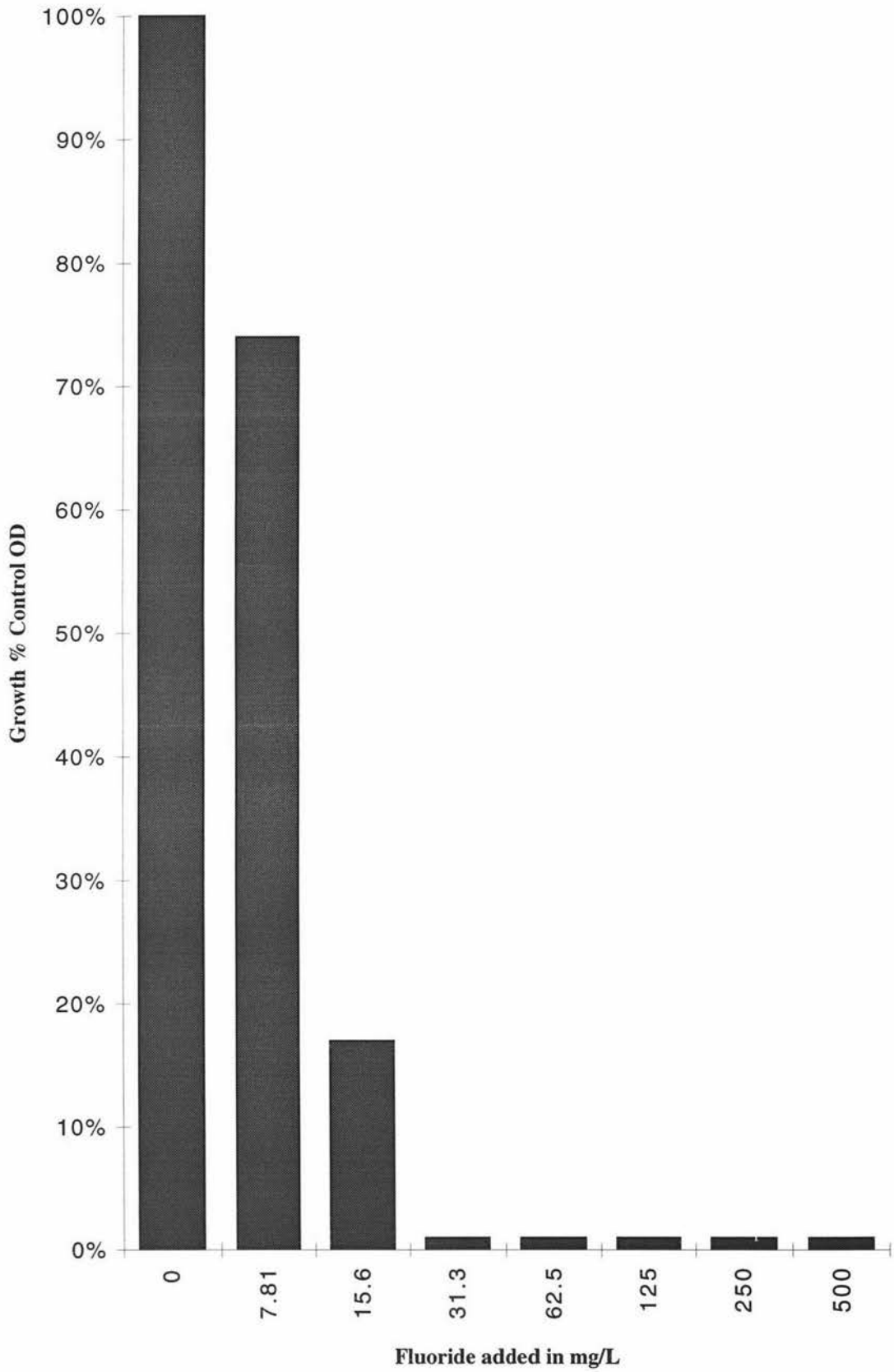


Figure 3.4.3 RS10 Growth vs. Fluoride Concentration

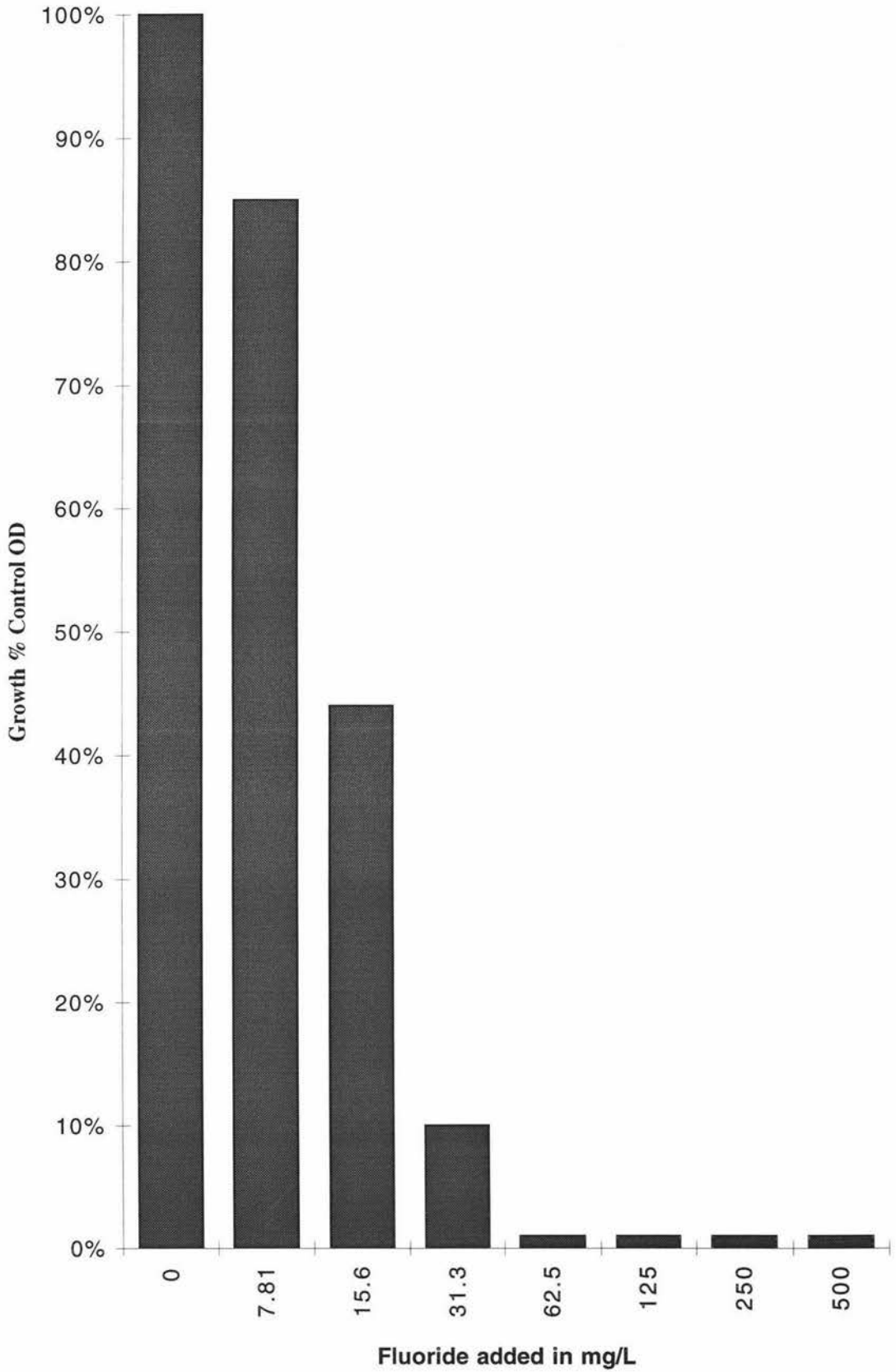


Figure 3.4.4 UCD-615 Growth vs. Fluoride Concentration

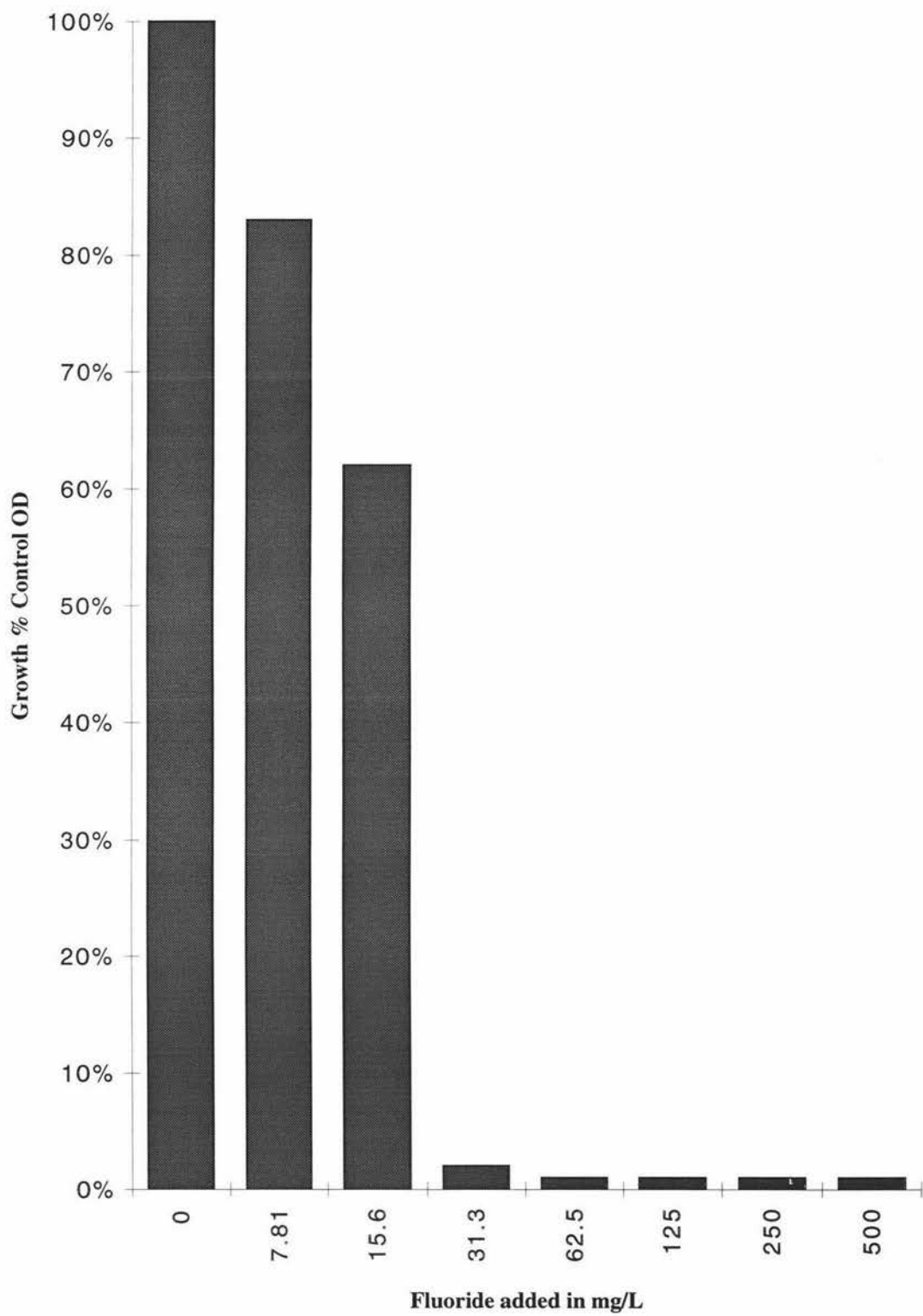


Figure 3.4.5 AWRI-354 Growth vs. Fluoride Concentration

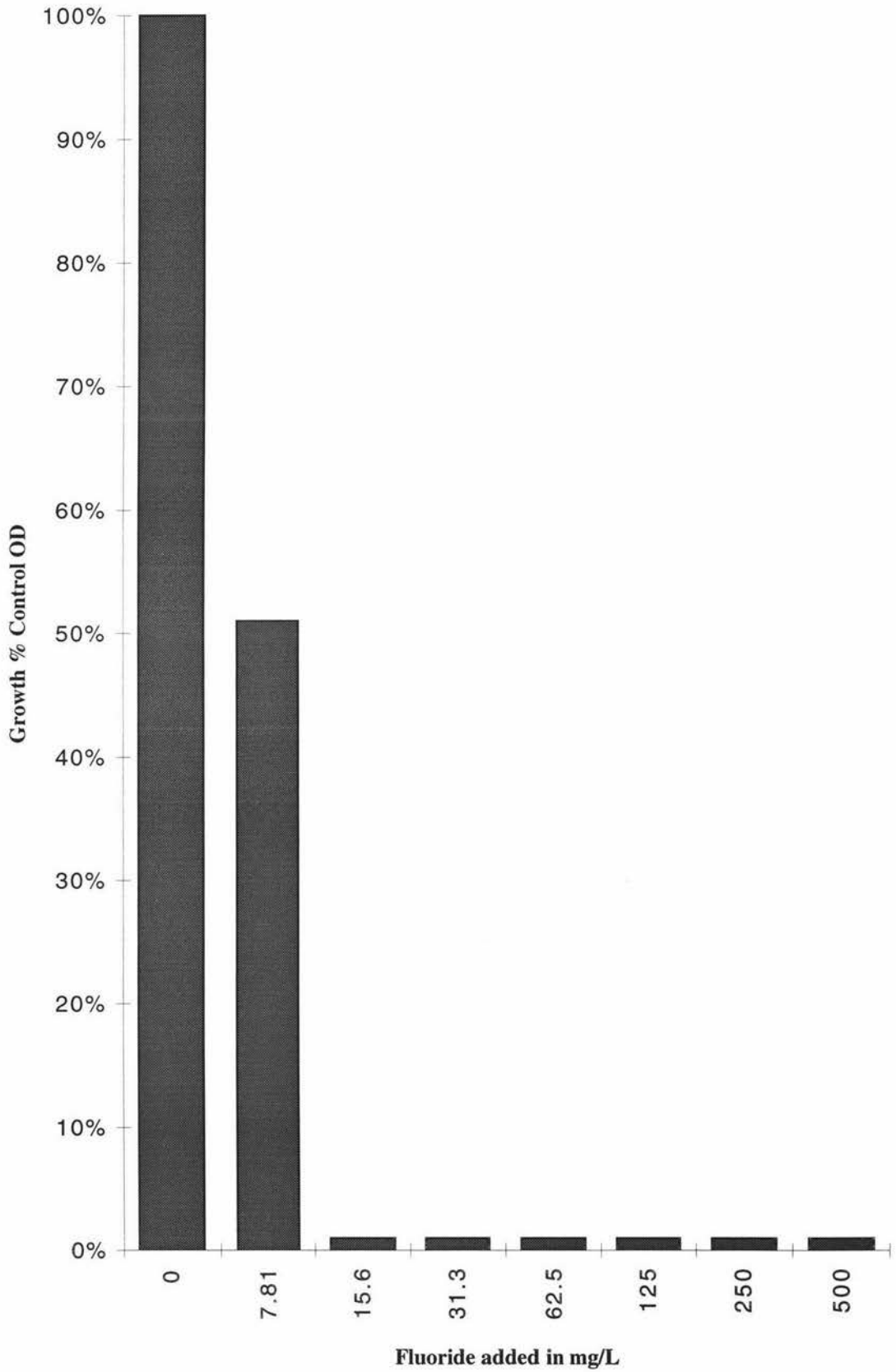
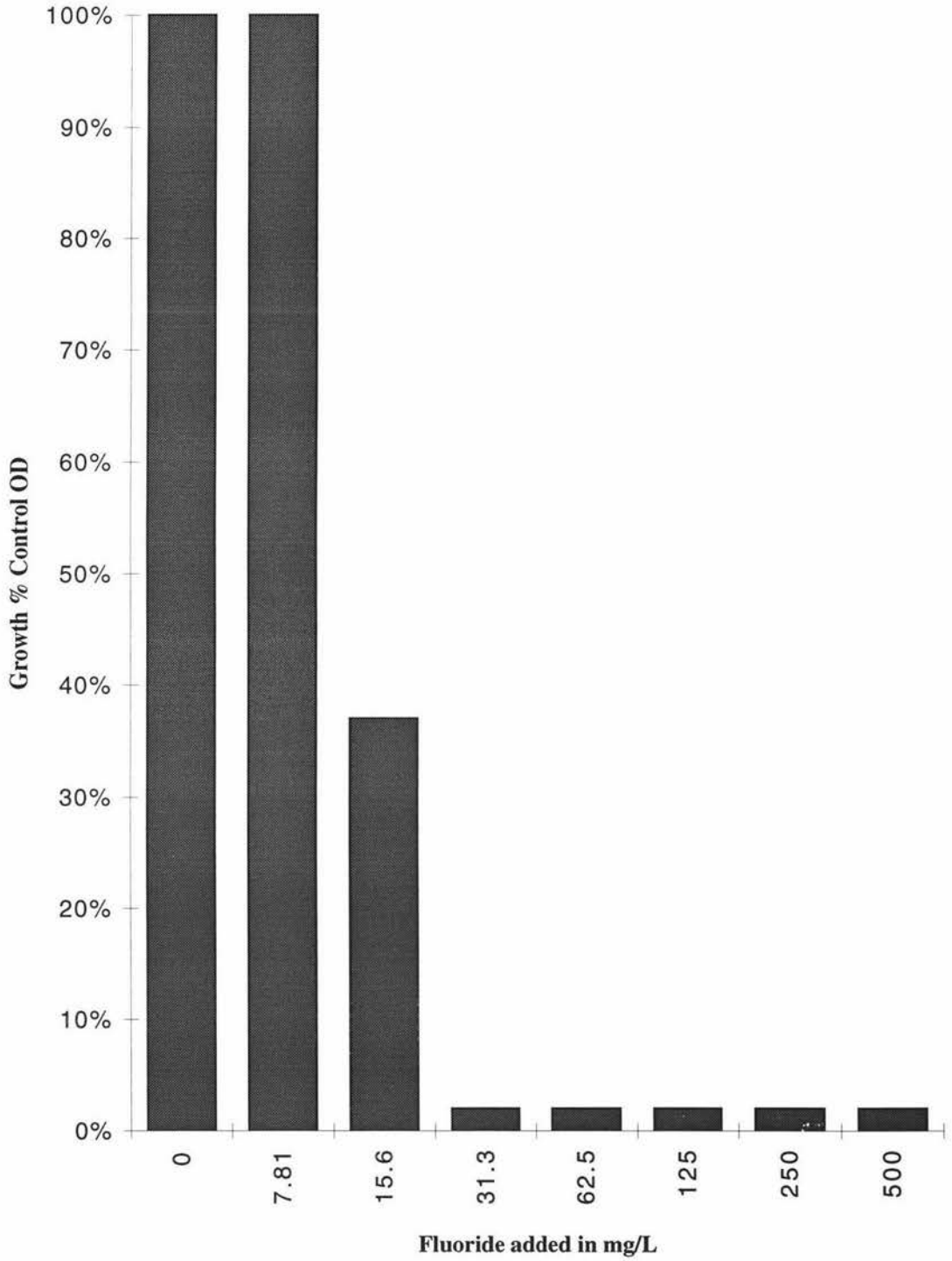


Figure 3.4.6 SR50 Growth vs. Fluoride Concentration



4. PRELIMINARY LABORATORY GRAPE JUICE FERMENTATION'S

4.1 INTRODUCTION

The first part of this study looked at the effect of fluoride on yeast in a range of conditions. It was found that at a lower pH and to some extent, at a lower cell density, fluoride had a more noticeable affect on the yeast. The lower pH was in the range of wine making conditions i.e. pH 3.0-4.0 (Rankine, 1989). It was not clear if the effect of fluoride was fungistatic/fungicidal or a combination of both. There was an indication that over time, in some cases, the effect was fungistatic.

Then in conditions that were more conducive to showing the effect of fluoride, such as using simple liquid media that is commonly used to propagate yeast, results were obtained that gave an indication of the MIC of fluoride against yeasts.

The second part of this study concentrated on the effect of fluoride in more realistic conditions so that the laboratory results may have more legitimate proclivity in 'real world' situations.

These experiments were performed in concentrated grape-juice with the juice chemistry and conditions as close to a realistic fermentation as possible. The intention was to assess the effect of fluoride at 30 and 300mg/L on the normal fermentation ability of a yeast in grape juice. This was done to test the experimental procedure and to give an indication of the sensitivity of yeast to the fluoride during fermentation. The results are reported. Then the experiments were repeated and the range of concentrations of fluoride added to the grape juice was extended. The results are reported in this section. The comparisons will be monitored by CFU/mL.

Finally two commercial yeast were examined during the first twelve hours of their fermentation to investigate the initial response of the yeast to different levels of fluoride. The yeast chosen to examine the effect of 30mg/L of fluoride was RS1 as it appears to be the most sensitive commercial yeast. The other yeast chosen was RS2, as it showed an initial growth slightly better than the control in earlier experiments. The results are reported.

4.2 MATERIAL AND METHODS

4.2.1 Yeast Strains Used

The yeast strains used were as described in Section 3.2.1. These yeast were:-

- Red Star Montrachet *Saccharomyces cerevisiae* RS1
- Red Star Pasteur Champagne *Saccharomyces bayanus* RS2
- Red Star Prise de Mousse *Saccharomyces bayanus* RS10
- *Brettanomyces* species UCD-615
- *Hansenula saturnus* AWRI-354
- *Kloeckera apiculata* SR50

4.2.2 Culture Media

YM Broth/Agar

YM broth was prepared by dissolving 21 g of commercial YM broth (Difco) in 1L of DI water. The pH was approximately 6.2 ± 0.2 and the media consisted of the following (per litre): Yeast extract, 3g., malt extract, 3g., peptone, 5g, and dextrose, 10g. The pH was adjusted to requirement (pH 3.3 or 6.8) with 50% H_3PO_4 or 30% KOH. All agar plates used were YM agar at pH 6.2 as described in section 2.2.2.

Grape Juice

Concentrated grape juice from Brewmaker™ Basic White (Light Dry) wine kit was diluted with milli-Q water until the soluble solids level was 18° Brix. This measurement was determined by a temperature correcting Brix Refractometer (Atago). The pH was adjusted to 3.4 with 50% H_3PO_4 , using an Orion Research, model 501 ionalyser calibrated with buffer solutions of 4.0 and 7.0 (Mallinkroft). The grape juice was sterilised at 121°C, 15lbs for 15 minutes in 300mL, 285 and 270mL aliquots in a bench top pressure cooker. Care was taken to sterilise the grape juice in a small bench top pressure cooker as larger steam autoclaves can damage the juice concentrate (The Oxoid Manual, 1990). Heat treatment of complex media such as grape juice can result in nutrient destruction, either by

direct thermal degradation or by reactions between the medium components (Oxoid Manual, 1990). According to Cannon, (1994), the total SO₂ of the juice was 15mg/L. It was noted that the grape juice produced a precipitate after pH alteration to 3.4 and heat treatment.

4.2.3 Reagents

Sodium Fluoride

BDH Sodium Fluoride 'AnalaR'.

Stock solution of NaFl was made by adding 0.663 g of NaFl to 1 litre of sterile water for a concentration of 300mg/L fluoride.

For fluoride concentrations:- a) 300mg/L fluoride, 0.199 grams of NaFl powder added to the 300mL sterile grape juice, b) 30mg/L fluoride - added 30mL of stock solution to 270mL of sterile grape juice, c) 15mg/L fluoride - added 15mL of stock solution to 285mL of sterile grape juice, d) 3mg/L fluoride added 3 mL of stock solution to 300mLs of sterile grape juice.

The NaFl powder was not sterilised before adding to the grape juice aliquots. The prepared grape juice with fluoride added was incubated at 30°C for 2 days, 0.1mL samples were spread-plated (as described in section 4.3.1.) onto YM agar plates to check for sterility. All samples tested were sterile.

Peptone Water 0.1%

Peptone water for dilutions were prepared as described in section 2.2.6

4.2.4 Maintenance and Preparation of Yeast for Starter Cultures

All yeast used were maintained and prepared as described in section 2.2.5. The yeast used as starter cultures were subcultured from YM plates into 50mL of YM broth, pH 3.3 and incubated for 48 hours at 30°C. The incubated yeast culture was sampled, diluted in 0.1% peptone water and counted on haemocytometer for total colony forming units per mL (CFU/mL).

4.3 GROWTH STUDIES

4.3.1 Experimental Procedure

A 1% inoculation of the starter yeast culture was added to the appropriate prepared bottle of grape juice (300mL). The bottles were gently mixed, allowed to stand for a few minutes and are mixed again. 5mL was aseptically removed from each bottle into a sterile test tube. From this 5 mL sample, a portion was serially diluted and spread-plated in duplicate as appropriate.

The CFU/mL of each inoculated bottle were enumerated every 24 hours for 6 consecutive days (ie day 0 through to day 5) or as appropriate. An appropriate serial dilution of the sample to be tested was spread in 0.1mL aliquots on the surface of sterile YM agar plates with a sterile glass spreader to isolate colonies. All testing was done in duplicate. The YM plates were grown at 30° C until the colonies on the control plates had grown sufficiently to be counted. All plates were then counted where appropriate and recorded. This method of measuring CFU/mL showed the viable cell count.

Initial fermentations were performed to gauge the effect of fluoride then the experiments were repeated to confirm results.

4.3.2 Twelve Hour Survival Curve

Experimental procedure followed section 4.3.1 but the fermentations were sampled every hour for 12 hours and only 0.5mL samples were taken.

4.4 RESULTS AND DISCUSSION

During this study the ability of the yeasts to grow at pH 3.3 has appeared to improve over time as the cultures have been consistently grown up on YM at this low pH (results not shown). Typically the yeast cultures grow to a population of approximately 1×10^7 CFU/mL if grown at 30°C for 2 days. A 1% inoculation therefore allows for approximately 1×10^5 CFU/mL in each fermentation. Usually

in a normal fermentation the initial numbers are closer to 1×10^6 CFU/ml. This difference was not relevant for laboratory fermentations as the comparison was between the growth rate of the trial yeast relative to the growth rate of the control yeast.

Figures 4.4.1 to 4.4.6 summarise the effect of fluoride on the growth on selected yeasts during the first set of fermentations. The fluoride concentrations were 30 and 300mg/L.

RS1 Figure 4.4.1

The control culture of RS1 *Saccharomyces cerevisiae* shows a typical growth curve for a fermentative yeast peaking at day 4. At 30mg/L there appears to be a gradual fungistatic effect with growth restricted to less than 1×10^1 CFU/mL from day 2 to 4 where upon the log CFU/mL quickly increases on day 5 to log CFU/mL 5.52. This effect may be a fungicidal/fungistatic reaction to the fluoride i.e initial population has been killed off and the few survivors that can tolerate the fluoride grow after a few days. At 300mg/L there appears to be a fungicidal effect with no growth throughout the six day fermentation period. Initial contact at this level of fluoride has brought about an instant decline of yeast cell numbers such that on Day 0 the viability of the cells is reduced by approximately 98 per cent. The initial population for 300mg/L is considerably less than that of the control and this may have occurred by instant contact killing due to the high level of fluoride.

RS2 Figure 4.4.2

This figure shows RS2 *S.bayanus* with a typical growth curve over the 6 days. At 30mg/L of fluoride there appears to be a drop in population from log CFU/mL 4.73 to 2.69 but by day 4 it is equivalent to the control, appearing to have reached its optimum growth level. At 300mg/L the yeast appears more resistant to the fluoride than RS1 as it appears it takes 2 days for killing to occur. Again the initial population on day 0 appears to have been reduced by contact killing.

RS10 Figure 4.4.3

RS10 *Sacchromyces bayanus* at 30mg/L fluoride shows a definite drop in cell numbers until the end of the first 24 hours. The yeast cell numbers then increase dramatically. This may have been due to either a developing tolerance to fluoride, or the slow recovery of the remaining viable fluoride resistant cells. Fluoride at 300mg/L was fungicidal within the first 24 hours to RS10 as it was to RS1.

UCD-615 Figure 4.4.4

This yeast control culture showed an initial slight decline in cell numbers. 30mg/L of fluoride reduced the viable count of this yeast in the first 24 hours, but the cell numbers subsequently increased, although not to the magnitude of the control. At 300mg/L it appears that the initial contact had killed all the cells. Duplicate testing would be required to confirm this.

AWRI-354 Figure 4.4.5

The AWRI-354 control shows a gradual increase in cell numbers over the 6 day time span, typical of the *Brettanomyces* species. (it is not a strong fermentative yeast). The growth curve of AWRI-354 at 30mg/L shows a decline that gradually slows into a lag phase. On day 3 the CFU/mL starts to slowly increase but sharply drops to the baseline on day 4. At 300mg/L fluoride there is an immediate drop in cell numbers in the first 24 hours of incubation.

SR50 Figure 4.4.6

The control of SR50 showed a slight lag in growth over the first 24 hours. During fermentation of grape juice supplemented with 30 mg/L fluoride the concentration of viable yeast dropped to baseline levels at 24 hours, before increasing rapidly to over 1×10^7 CFU/mL at 48 hours. This die off must be repeated to confirm the results. The same drop in CFU/mL was observed at 300mg/L with no recovery of the culture for the remainder of the 6 days incubation.

Fluoride had a marked effect on all yeast strains at both 30 and 300mg/L. At 300mg/L approximately 90% of yeast were killed on initial contact with the

exception of SR50. All yeast except SR50 and RS2 were reduced to 10 CFU/mL (the limit of sensitivity of the procedure) within 24 hours.

All Yeast 30mg/L Figure 4.4.7

The effect of fluoride on some yeast was still significant at 30mg/L. Yeasts RS2, RS10, UCD-615 and SR50 all show an initial decline phase followed by a phase of growth that (which) is similar to their normal growth curves. Yeasts RS1 and AWRI-354 were the most sensitive yeast, both having a distinctive lag phase followed by a phase of growth. AWRI-354 appeared, from this data, to succumb to the effect of fluoride after day 4, whereas RS1 increased at a growth rate similar to its control.

These experiments were repeated to incorporate fluoride concentrations of 3mg/L and 15mg/L (Figures 4.4.8 to 4.4.13).

RS1 Figure 4.4.8

This yeast shows little appreciable differences in growth rate between the control and 3mg/L. Growth rates at 30 and 300mg/L are equivalent to those in Fig 4.4.1. At 15mg/L of fluoride there is a reduction in viable cell numbers followed by a lag phase during which the cells typically repair and restore followed by a gradual log phase that peaks on day 5. The peak is similar to the control's on the last day but the growth rate is not recovered to the level of the control. The results indicate that this is the most susceptible commercial yeast to the addition of fluoride.

RS2 Figure 4.4.9

At 3mg/L fluoride the population growth curve was similar to the control although the growth rate over the first day was a little higher. The effect on the yeast at 15mg/L was minor with the growth rate a fraction less than the control over the first day. Fluoride at 30mg/L delayed the onset of cell growth by reducing the viable cell count but by day 4 the cell numbers had increased to a range comparable with that of the control. These results indicate that this commercial yeast is most resistant to fluoride in grape juice.

RS10 Figure 4.4.10

The results obtained at 30 and 300mg/L were similar to those in figure 4.4.3. At 15mg/L the initial growth rate was very slow, but it built up to a rate comparable to the control by day 3. The population growth curve for the control and 3mg/L were almost identical.

UCD-615 Figure 4.4.11

The samples treated with 3 and 15mg/L fluoride showed higher population growth curves, respectively, than the control. The control initially dropped slightly in numbers then slowly increased to a peak at day 4. At 30mg/L fluoride the cells numbers dropped by over 99% in the first day but recovered to levels obtained at 3 or 15mg/L of fluoride. There was complete killing at 300mg/L and initial contact dropped cell numbers by 2 log cycles.

AWRI-354 Figure 4.4.12

The control showed a slow growth rate peaking on day 5. Cell numbers in the 3mg/L sample peak above control cell counts at day 3. At 15 and 30mg/L the effect of the fluoride was dramatic and on day 5 the fluoride concentration of 30mg/L had reduced the numbers of cells to baseline. The effect of 300mg/L was fungicidal and once again day 0 contact had reduced the viable cells by approximately 2 log cycles.

SR50 Figure 4.4.13

The growth curve of SR50 at 3mg/L fluoride was approximately half a log cycle higher than the control after day 1. In the first 24 hours of incubation in 15 and 30mg/L samples there was a decline in cell numbers but after this time period the growth curves recover to a level comparable with the control. The higher the fluoride concentration the steeper the death rate. For SR50 the fungicidal effect was noted after 2 days, at a fluoride concentration of 300mg/L.

All Yeast 15mg/L Figure 4.4.14

The effect of fluoride on yeast varied greatly dependant on the yeast strain. This is illustrated in Figure 4.4.14 where a variety of trends have emerged upon fluoride challenge of 15mg/L. In some cultures this amount of fluoride caused an initial die-off that was overcome by time (RS1 and SR50), some showed a growth rate slightly lower than the control (RS2, RS10, UCD-615) and one was compromised such that it did not recover, at least over the time span shown (AWRI-354).

The preparation of growth curves requires a number of manipulations (sampling procedure, serial dilutions, spread-plating of dilutions, counting and recording of many hundreds of plates) to arrive at the final result. For this reason it is difficult to measure the repeatability and accuracy of these trials. Rather, these results indicate trends of the effect of the fluoride on the yeast. More precise measurements could be done with absorbance vs time or other enzymatic experiments but as these experiments were unprecedented at the time we chose to follow growth rate with CFU/mL. The results give an excellent indication of trends of effect in the different selected yeast at differing levels of fluoride challenge.

12 Hour Fermentations

The next set of results looked specifically at two commercial yeast, that is: RS1, for the apparent sensitivity to fluoride, and RS2, for its robustness against fluoride.

Figure 4.4.15 is a graph of the first 12 hours of fermentation of RS1 30mg/L. This graph shows that the effect of fluoride on the yeast was apparent in the first hour of contact and the cell numbers steadily declined. At hour 10 there was a small increase in cell numbers followed by a drop to the sensitivity limit then an increase to 10 colony forming units per mL by hour 12.

The effect of 3mg/L fluoride on RS2 was investigated (Figure 4.4.16) over the first 12 hours. Over the first 6 hours the growth rate was increasing slowly with slightly variable results for both fermentations. There appeared to be no detrimental effect on the yeast at 3mg/L, in fact the yeast's growth was slightly better than the control from hour 6 to 12.

Results suggest substantial variation among the six selected yeasts with respect to fluoride sensitivity during fermentation. In fact, the more tolerant of the yeast showed a better than control growth rate at low levels of fluoride. The sensitive yeasts showed a delayed multiplication in the grape juice, with a drop in population density in the first instance followed by a recovery to cell numbers similar to the control. The severity in loss of initial population densities, and the time delay to recover, appears to be related to the initial dosage of fluoride in the grape juice.

Higher concentrations of fluoride in cultures of the selected yeasts consistently produced an apparent fungicidal effect. Samples exposed to 300mg/l were plated onto a non-selective media (YM) at pH 6.2 and no colonies grew, indicating a true fungicidal effect. These trends of fluoride effect on the yeasts have been consistent throughout the experiments.

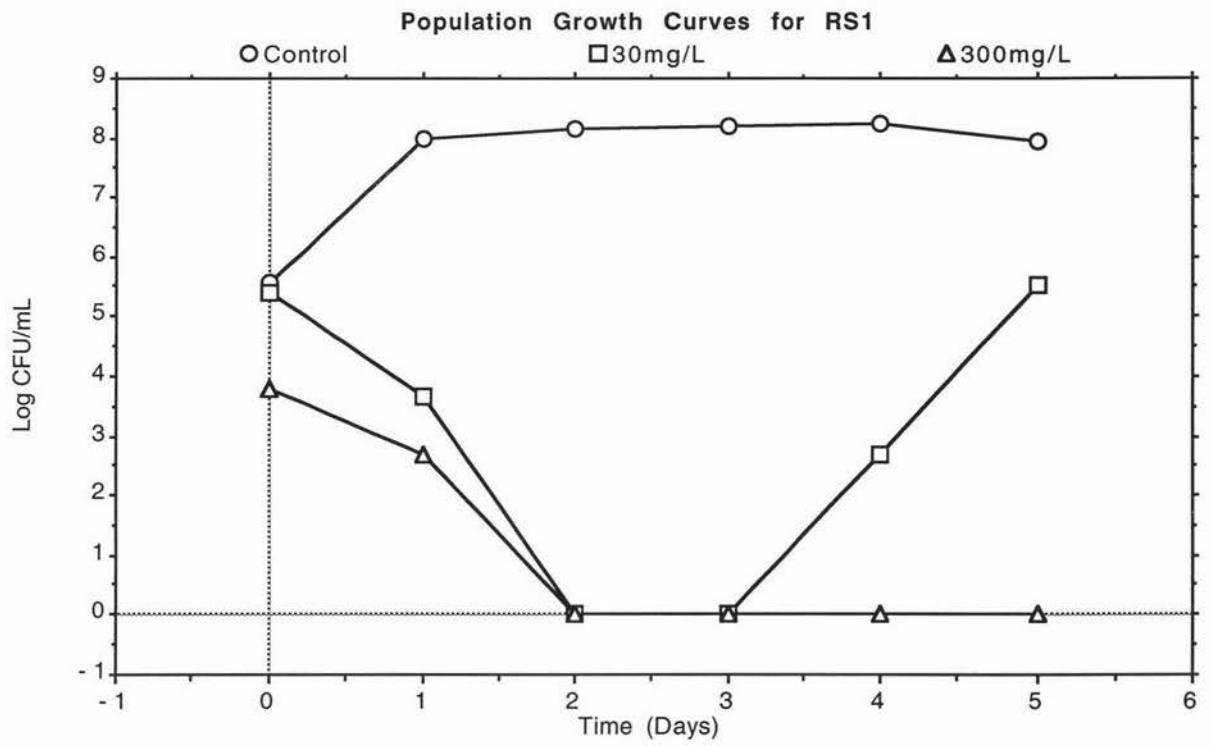


Figure 4.4.1 Growth of yeast RS1 versus varying fluoride concentrations (mg/L).

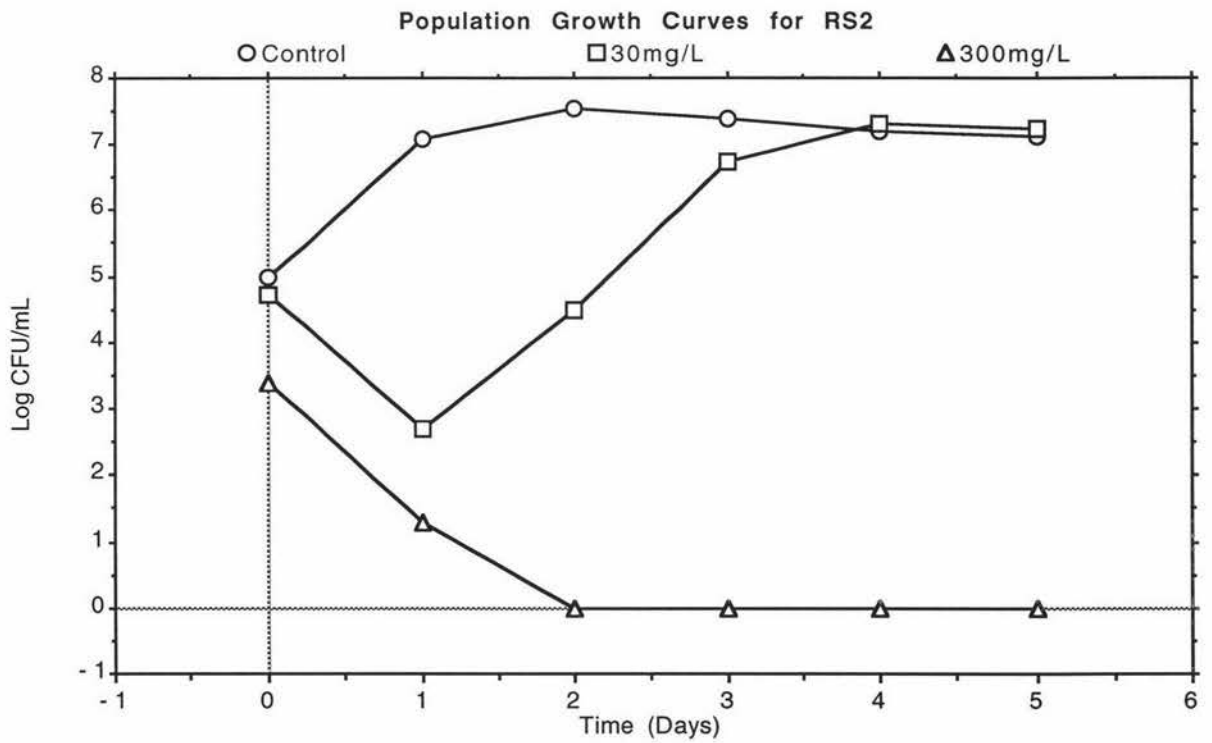


Figure 4.4.2 Growth of yeast RS2 versus varying fluoride concentrations (mg/L).

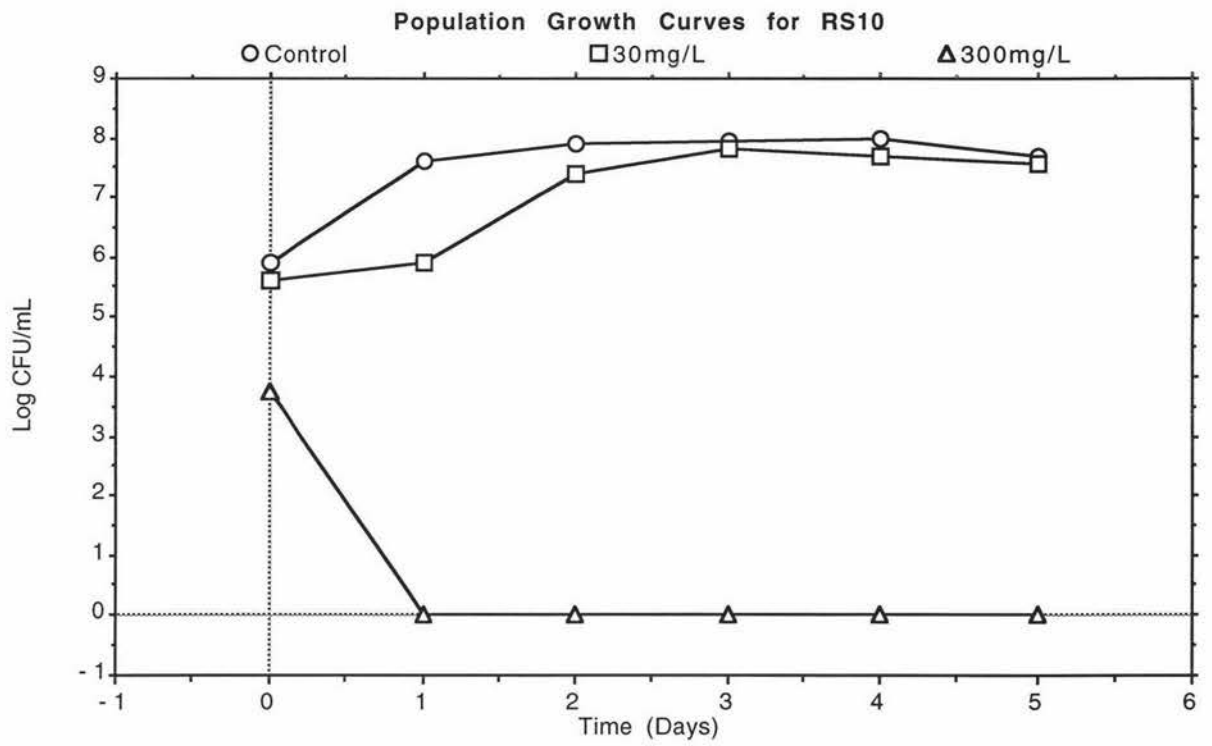


Figure 4.4.3 Growth of yeast RS10 versus varying fluoride concentrations (mg/L).

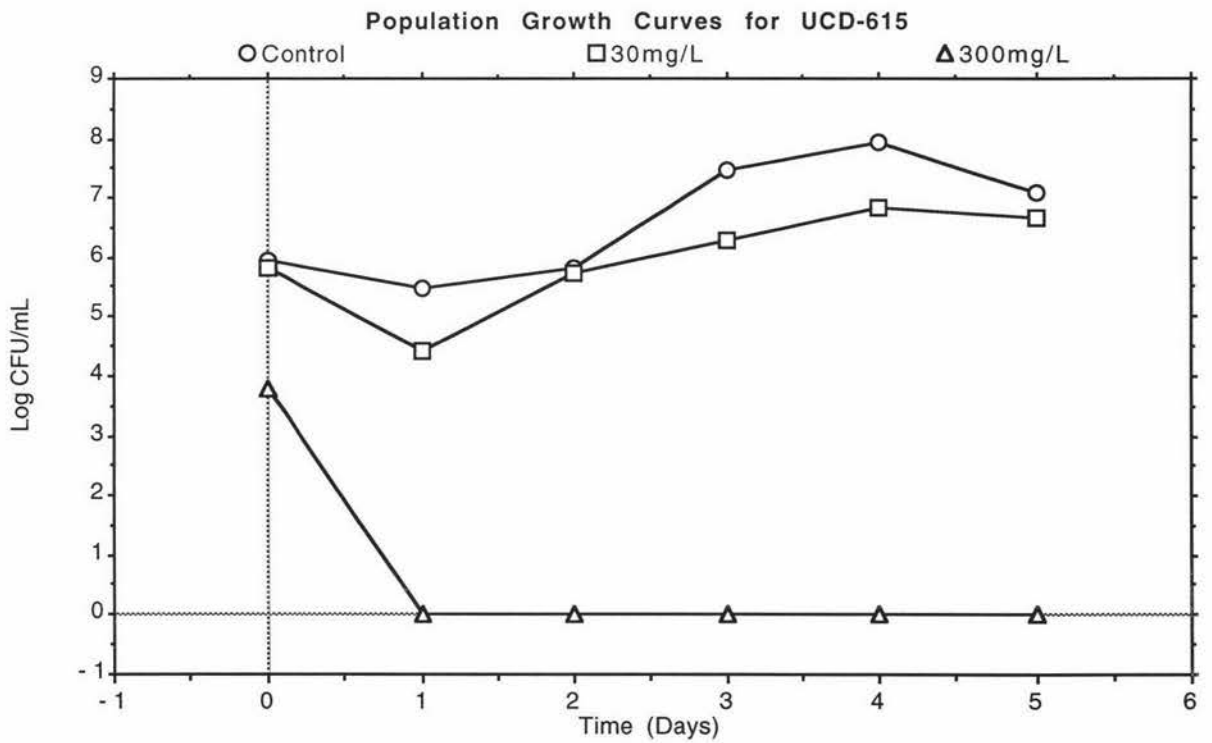


Figure 4.4.4 Growth of yeast UCD-615 versus varying fluoride concentrations (mg/L).

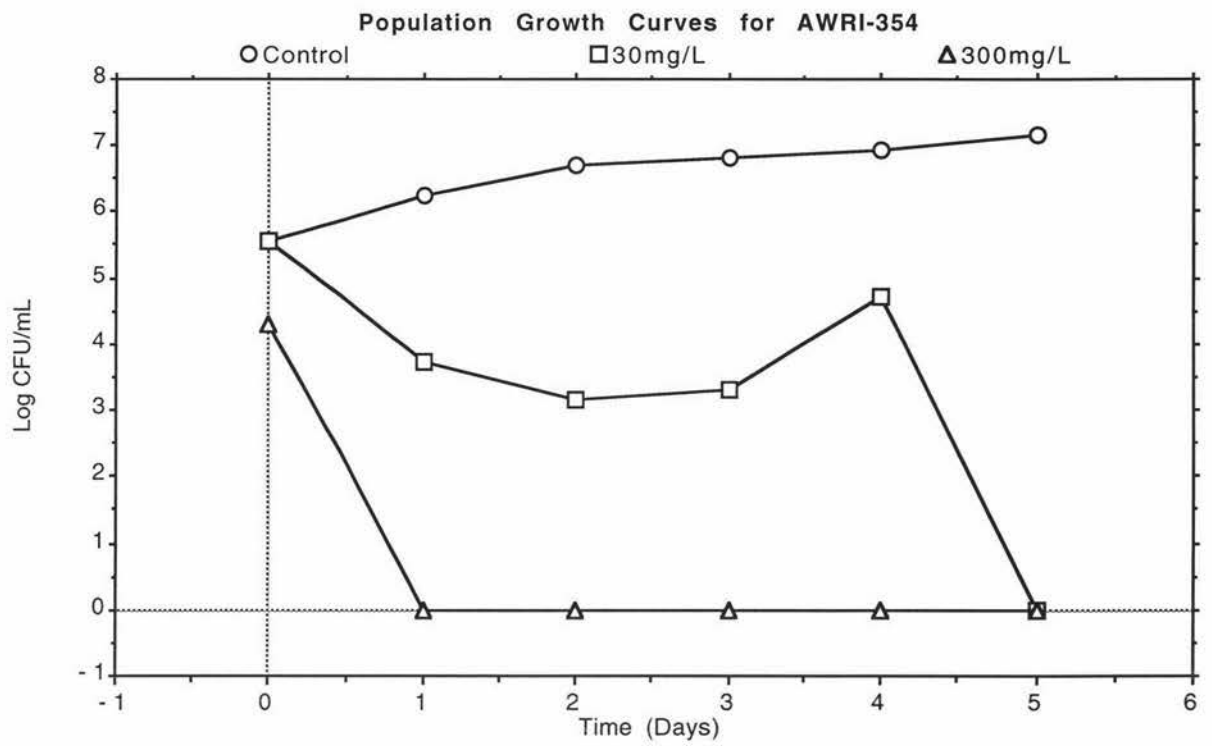


Figure 4.4.5 Growth of yeast AWRI-354 verses varying fluoride concentrations (mg/L).

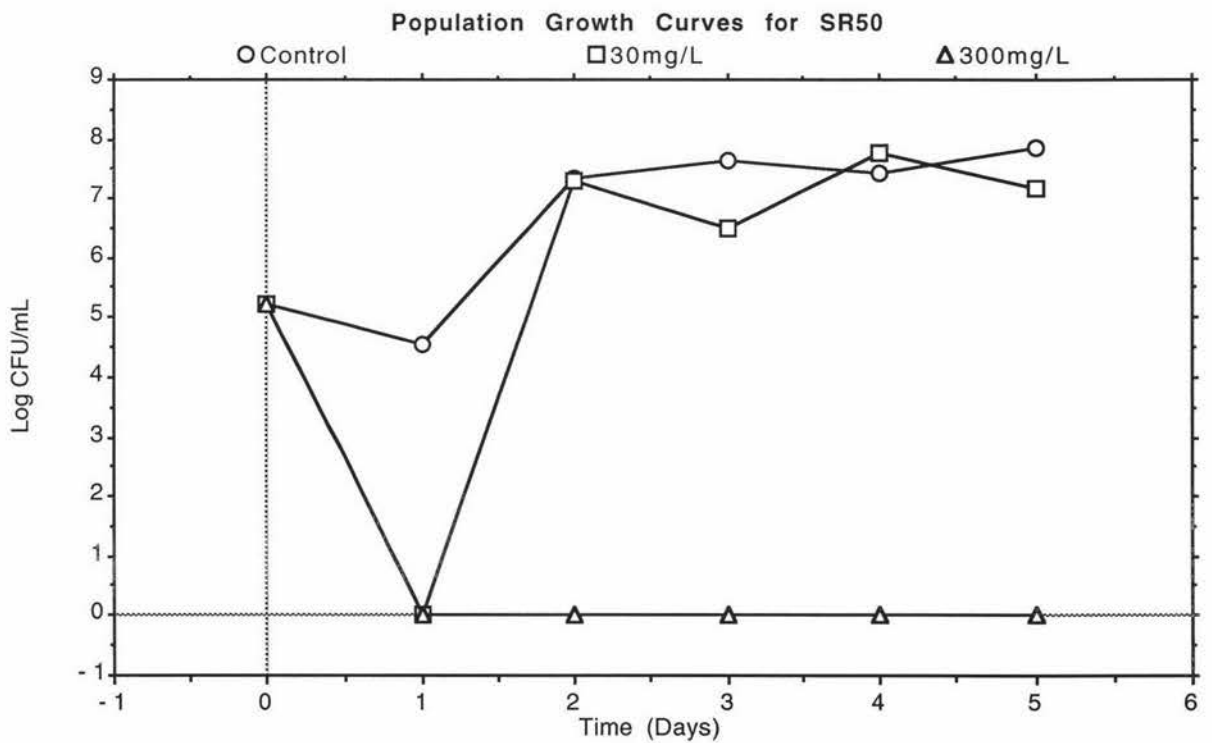


Figure 4.4.6 Growth of yeast SR50 verses varying fluoride concentrations (mg/L).

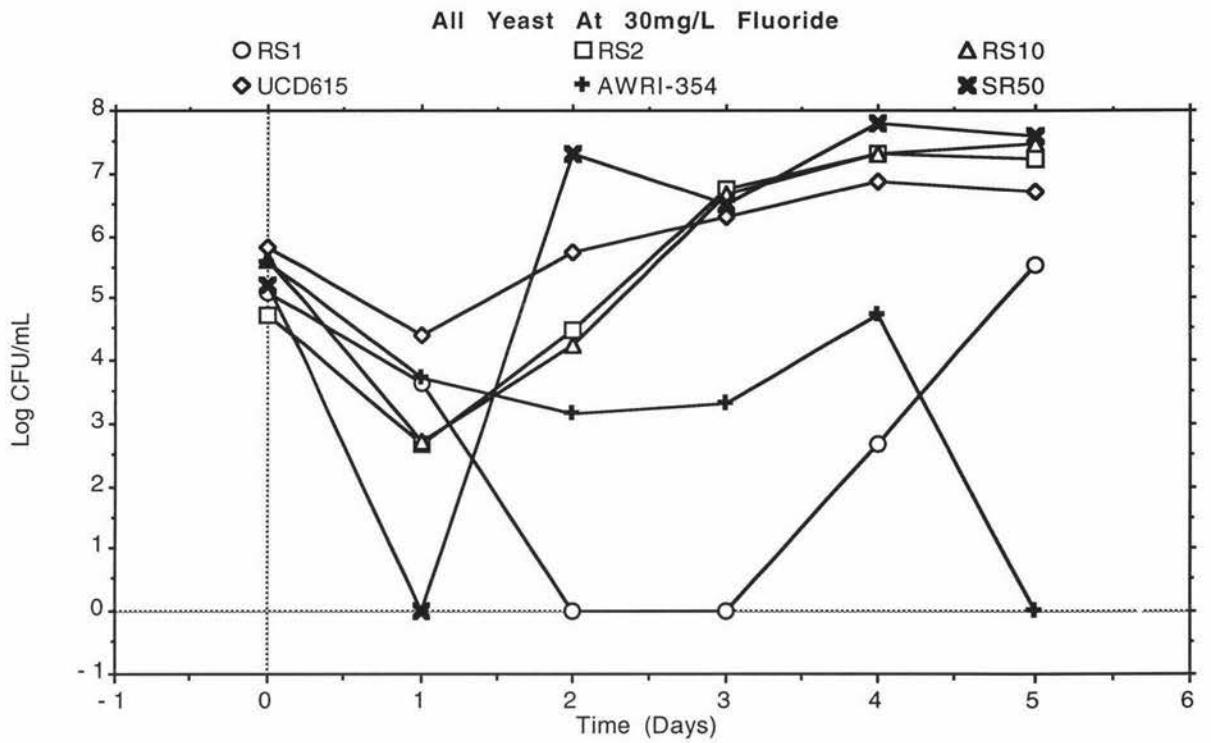


Figure 4.4.7 Growth of all yeast at 30mg/L fluoride in grape juice pH 3.4

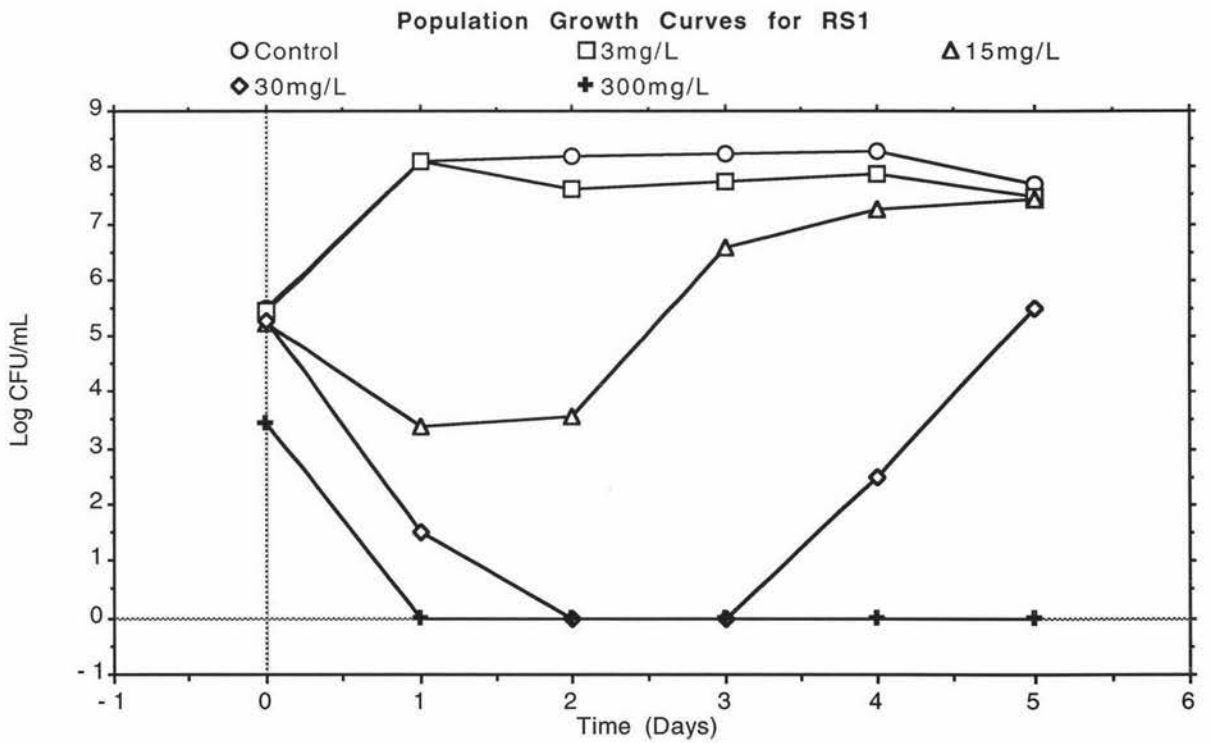


Figure 4.4.8 Growth of yeast RS1 versus varying fluoride concentrations (mg/L).

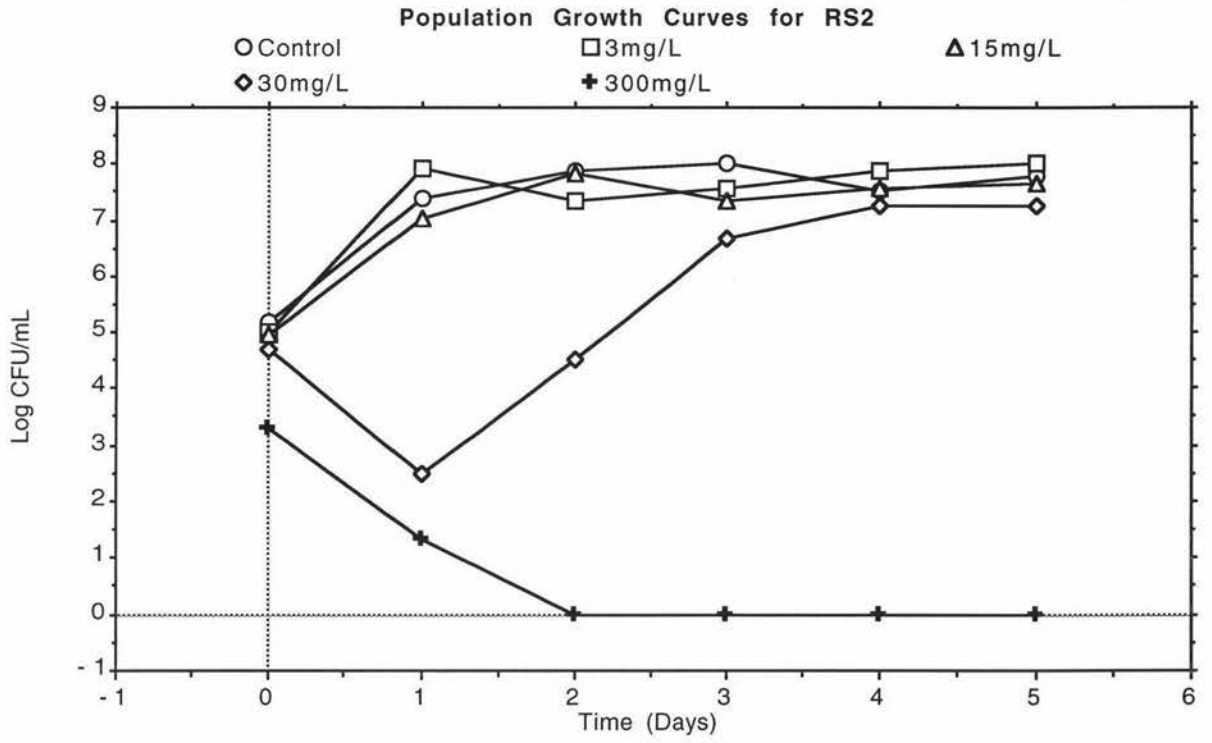


Figure 4.4.9 Growth of yeast RS2 versus varying fluoride concentrations (mg/L).

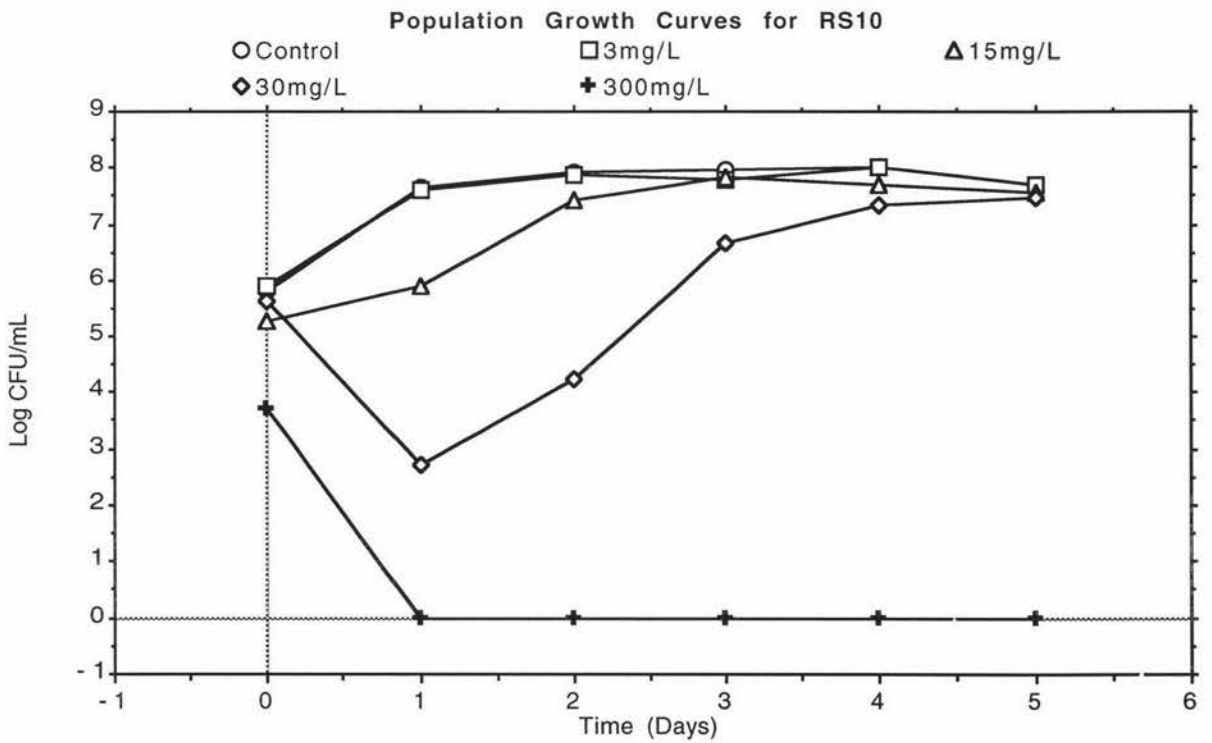


Figure 4.4.10 Growth of yeast RS10 versus varying fluoride concentrations (mg/L).

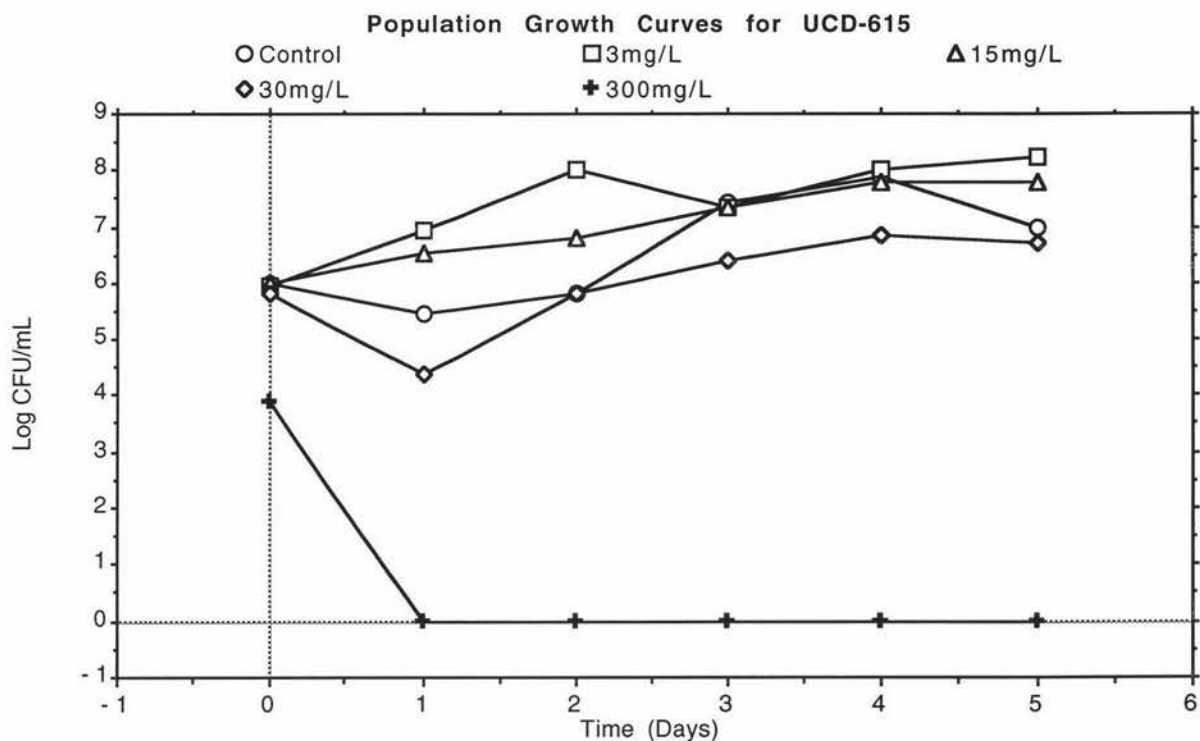


Figure 4.4.11 Growth of yeast UCD-615 verses varying fluoride concentrations (mg/L).

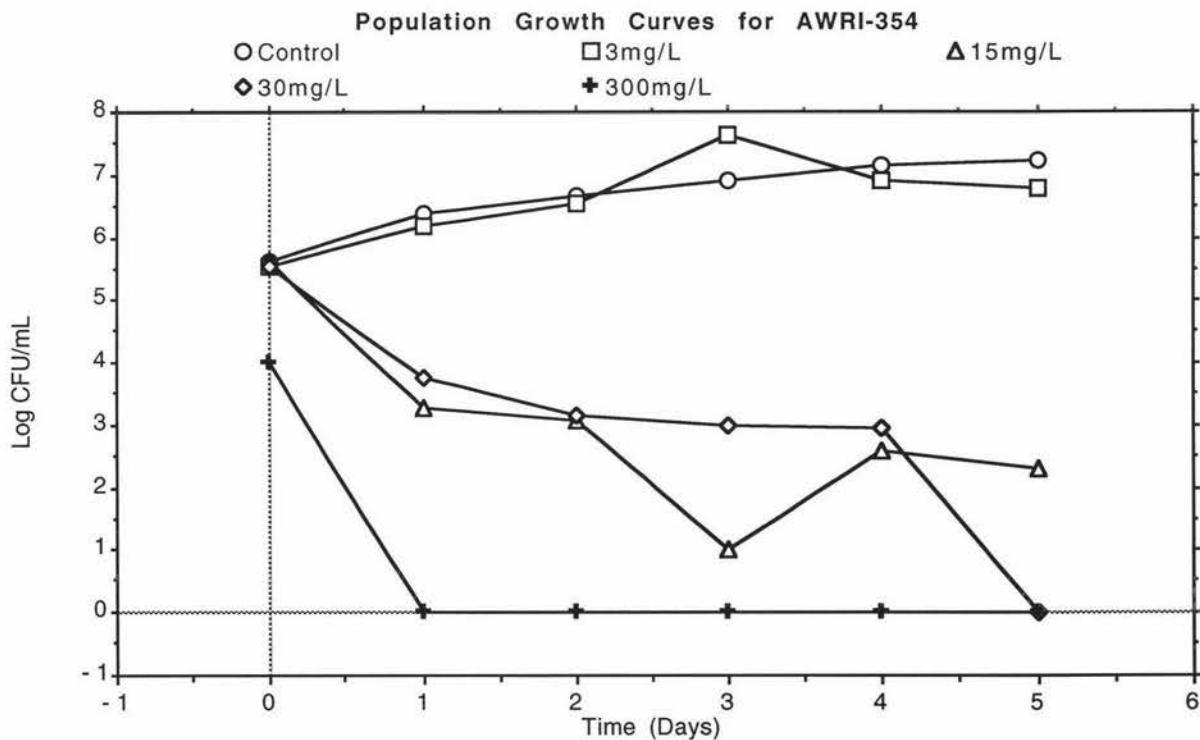


Figure 4.4.12 Growth of yeast AWRI-354 verses varying fluoride concentrations (mg/L).

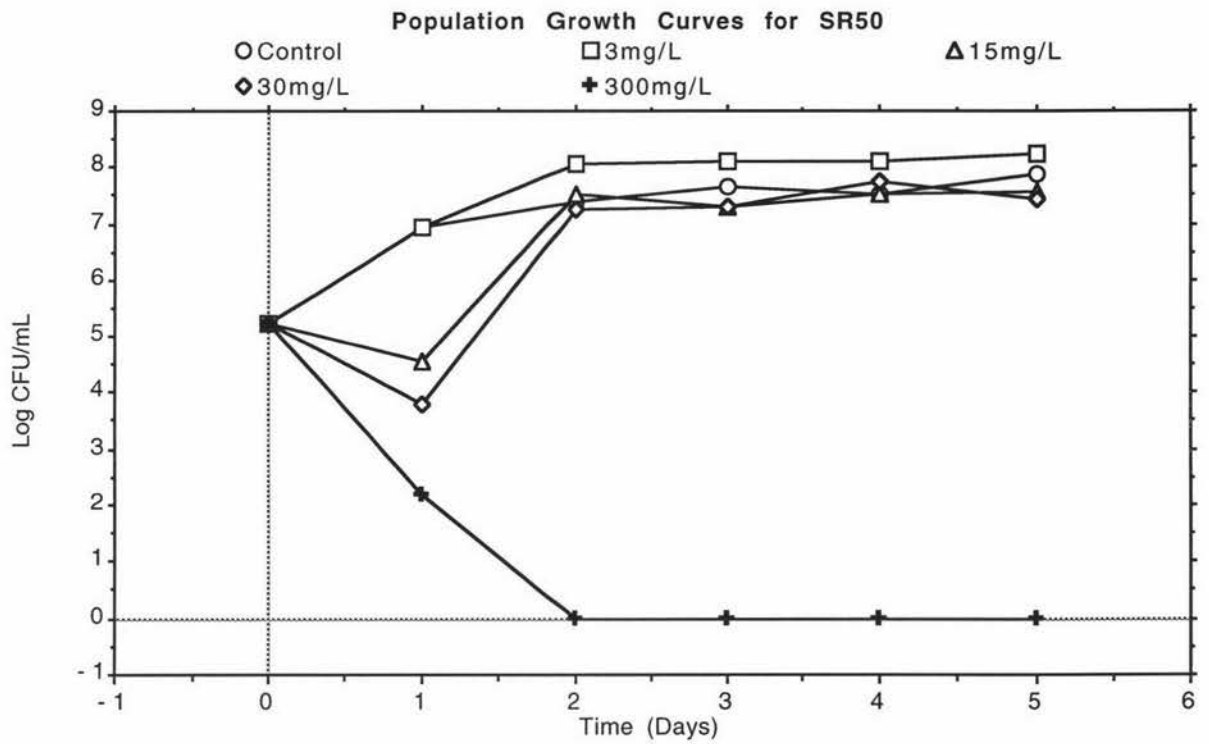


Figure 4.4.13 Growth of yeast SR50 versus varying fluoride concentrations (mg/L).

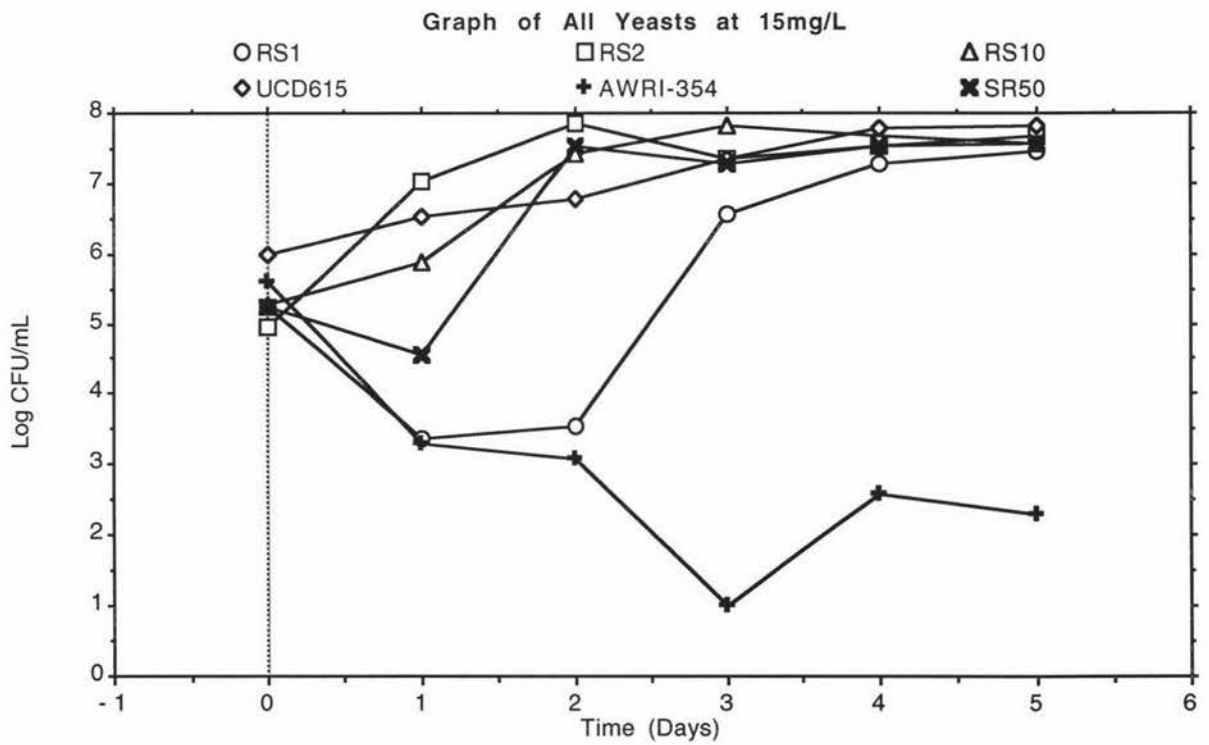


Figure 4.4.14 Growth of all yeast at 15mg/L fluoride in grape juice pH 3.4

5. REAL GRAPE JUICE FERMENTATIONS

5.1 INTRODUCTION

This section concentrates on the effect of fluoride on two commercial yeast one of which appears to be more sensitive and the other more tolerant. The grape juice used for this section of experiments was Chardonnay juice from The Misson Winery. This juice was left over from another Masterate project conducted in the same research laboratory and was kindly donated. The opportunity to use "real" grape juice allowed a closer comparison to real world conditions.

The first part of the experiment compared the growth of yeast in pasteurised Chardonnay grape juice supplemented with fluoride with data from the previous section to determine if there was any substantial differences between the two grape juice sources. The observations were recorded. Then comparisons were made between sterilised (section 4), pasteurised and non-pasteurised Chardonnay grape juices to gauge the effect of heat treatment on the constituents of the grape juice. This is because the heat treatment of the grape juice may reduce its nutritive value (Oxoid, 1990). The pasteurisation of the grape juice was performed to ensure that the only yeast involved with the fermentation was the selected yeast. However, as noted earlier, pasteurisation may affect the grape juice and alter the effect of fluoride on the yeast selected.

These experiments were performed on RS1 *S.cerevisiae* and the results were recorded.

The second part of this chapter focuses on the effect of a lower range of fluoride concentrations on the two selected yeasts. In 1989, the European wine advisory body, the Organisation International de Vin (OIV), had reduced the existing limit of fluoride in the wines imported to their countries from 3 to 1mg/L. The level of 3mg/L was reinstated in 1991 after further review. This experiment, therefore, firstly ascertained if low concentrations of fluoride (1,3,5 mg/L) affected the growth rate of the selected yeasts when compared to the non-supplemented control. Results, and the literature, tend to suggest that the action on yeasts may be fungicidal at higher concentrations. It also seems that as the concentration of fluoride drops, the action on yeasts becomes more fungistatic, interfering with the

yeasts ability to multiply. This effect is overcome by time, resulting in the yeasts population successfully propagating at a growth rate slightly less than to the control.

A set of experiments was also designed to examine the ability of the fluoride challenged yeast to ferment at a rate similar to the control, once they have started growing. Fluoride is known to act as an inhibitor against yeast enolase, thereby disrupting glycolysis. This was investigated by monitoring a fermentation in the presence of fluoride with measurements of growth rate (CFU/mL) and soluble solids (°Brix) and measuring any fluoride effects.

5.2 MATERIAL AND METHODS

5.2.1 Yeast Strain Used

The yeast strains used were as described in Section 3.2.1. The yeasts used for this section were:-

- Red Star Montrachet *Saccharomyces cerevisiae* RS1
- Red Star Pasteur Champagne *Saccharomyces bayanus* RS2

5.2.2 Culture Media

YM broth was prepared by dissolving 21 g of commercial YM broth (Difco) in 1 L of DI water. The pH was approximately 6.2 ± 0.2 and the media consisted of the following (per litre): Yeast extract, 3g., malt extract, 3g., peptone, 5g, and dextrose, 10g. The pH was adjusted to requirement (pH 3.3 or 6.8) with 50% H_3PO_4 or 30% KOH.

5.2.3 Grape Juice

The grape juice used in this section was Chardonnay juice (supplied by Mission Vineyard, vintage 1995). It was diluted with milli-Q water to a soluble solids concentration of 18° Brix. This measurement was determined by a temperature corrected Brix Refractometer (Atago). The pH was adjusted to 3.4 with 50% H_3PO_4 , using an Orion Research, model 501 ionalyser calibrated with buffer solutions of 4.0 and 7.0 (Mallinkroft). The grape juice was sterilised at 121°C (15lbs for 15 minutes), or pasteurised (10lbs for 10 minutes) as required, in

300mL, 285 and 270mL aliquots (in 500mL Schott bottles) in a bench top pressure cooker . Untreated juice was measured out with sterile measuring cylinders and decanted into sterile 500mL Schott bottles. The total SO₂ level of the juice was approximately 80mg/L after Ryan, 1996. Stock solution of NaFl was made by adding 0.663 g of NaFl to 1 litre of sterile water for a concentration of 300mg/L fluoride. Fluoride stock solution was added to the bottles of grape juice as appropriate. The prepared grape juice, supplemented with fluoride, was incubated at 30°C for 2 days. 0.1mL samples were spread-plated (as described in section 4.3.1.) onto YM agar plates to check for sterility. All samples tested were sterile. As earlier, it was noted that the grape juice produced a precipitate after pH alteration to 3.4 and heat treatment. Prepared grape juice was re-refrigerated at 4°C.

5.2.4 Maintenance and Preparation of Yeast for Starter Cultures

All yeast used were maintained and prepared as described in section 2.2.5. The yeast used as starter cultures for this section were subcultured from YM plates into 50mL of YM broth, pH 3.3. and incubated for 48 hours at 30°C. The yeast culture was sampled, diluted in 0.1% peptone water and counted on a haemocytometer to obtain total colony forming units per mL (CFU/mL).

5.3 GROWTH STUDIES

5.3.1 Experimental Procedure

Fluoride stock solution was added to the prepared grape juice (300mL), as appropriate, in duplicate. A 1% inoculation of the starter yeast culture was then added. The bottles were gently mixed and allowed to stand for a few minutes, were mixed again and then sampled, ie 5mL was aseptically removed into a sterile test tube. From this 5 mL sample, a portion was serially diluted and spread-plated in duplicate as appropriate. The soluble solids measurement was recorded at this time, if necessary. The bottles were then incubated at 30°C. All samples from this time on were taken at 30° C. As the fermentation progressed, bottles were more carefully mixed to ensure the yeast cells were evenly distributed throughout the medium without the liquid overflowing out of the bottle.

The cell count (CFU/mL) and soluble solids (°Brix, as required) were sampled every 24 hours for the required time (typically 6 consecutive days). An

appropriate dilution of the sample to be tested was spread in 0.1mL aliquots on the surface of sterile YM agar plates with a sterile glass spreader to isolate colonies. All testing was done in duplicate. The YM plates were grown at 30° C until the colonies on the control plates had grown sufficiently to be counted. All plates were then counted where appropriate and recorded. Colony morphology was checked as the plates were counted. Erroneous colonies were noted down and examined under the microscope.

5.4 RESULTS AND DISCUSSION

5.4.1 Comparison of Grape Juice Sources

The comparison of grape juice sources (Figure 5.4.1) showed that yeast growth patterns are similar in both the Brewmakers™ Whitewine grape juice (WGJ) and the Chardonnay grape juice (CGJ). The population growth curves of the two controls were similar but the CGJ fermentation had a 3-4 fold lower cell count than the WGJ over the fermentation period. The difference between the two controls may be related to the initial starter inoculations numbers (CGJ = 5.50 log CFU/mL and WGJ = 5.26 log CFU/mL). However the initial growth rate during the first 24 hours was higher in CGJ than WGJ (growth rate WGJ = 0.45, CGJ = 0.38).

At 15mg/L WGJ fermentation showed a lag phase from day 1 to 2 after the initial decline of cells in the first 24 hours. The population then grew from day 1, to a population peak of 7.45 log CFU/mL by day 6. The WGJ control's peak was approximately 2 fold larger than WGJ 15mg/L. The CGJ cell numbers dropped over the first 24 hours and then grew up to a fermentation high of 7.63 log CFU/mL, slightly lower than its corresponding control. It appears that the yeast in the CGJ had a sharper death curve yet recovered more rapidly, to a higher population than the WGJ culture.

The fermentations in both juices at 30mg/L fluoride had almost exactly the same population death curve, both reaching the lower sensitivity margin by day 2 but the CGJ had a 3 day lag period compared to the 24 hours lag of the WGJ fermentation, before entering log phase. Figure 5.4.3 showed similar variation of recovery time and indeed the time for the effect of fluoride to reduce the cell numbers to below the sensitivity range also varied from 1 to 3 days (Figures 4.4.1, 4.4.8 and 5.4.1). The growth rate for the two grape juice sources in the

first 24 hours, after the lag period, were similar, but one cannot be assured of the continuing growth rate for CGJ as the samplings only went to day 6.

5.4.2 Chardonnay Grape Juice Fermentations

Figure 5.4.2 shows population growth curves of yeast RS1 at fluoride concentrations of 15 and 30mg/L and two new fluoride concentrations of 5 and 10mg/L. At 10, 15 and 30mg/L there appeared to be a relationship between the concentration of fluoride challenge and the death curve. At 5mg/L the initial growth rate was slightly less than the control. The results at 30mg/L are equivalent to those in Figure 5.4.1.

5.4.3 Possible Reasons For Differences Between Grape Juices

Sulphur Dioxide (SO₂)

There was a considerable difference in the total SO₂ level in the two grape juice sources. The Brewmaker Whitewine™ grape juice only contained approximately 15mg/L SO₂ (Cannon, 1994) compared to the Chardonnay juice 80mg/L SO₂ (Ryan, 1996). Although as a wine yeast RS1 is selected for its tolerance of high SO₂ levels (Fugelsang, 1997) the combined effect of fluoride and SO₂ may have proved more restrictive to the yeast grown in the Chardonnay juice.

Ethanol

As it appears that the influence of the fluoride on the yeast is occurring early in the fermentation and the ethanol tolerance of RS1 is high, ethanol would probably not effect yeast growth in this situation.

Malic Acid and Titrable Acidity

There was also considerable variation in the levels of malic acid between the two grape juices i.e WGJ (0.62g/L) lower than CGJ (6.4g/L). The Chardonnay juice also had a titratable acidity of 10.88g/L but there were no statistics available on the WGJ.

Heat Treatment of Grape Juice

The WGJ was sterilised (121°C for 15 minutes) and the CGJ was pasteurised (110°C for 10 minutes). As mentioned in 4.2.2 the effect of high temperature / long time treatments on the constituents of the grape juice may be detrimental. Overheating is also a common cause for pH drift and precipitation (Oxoid Manual, 1990). The CGJ may have a lower total final population because of a combination of malic acid and higher levels of SO₂ but the growth rate of yeast in GCGJ was higher than WGJ because it was pasteurised, rather than sterilised.

Grape Juice Constituents

The WGJ was a product imported from England and is a processed concentrate. The CGJ was from a vineyard in New Zealand and had a makeup typical of grape juice produced in a cool climate region, that is high pH and low acidity. The CGJ was 'fresher' and less processed therefore more nutritious for the yeast.

5.4.4 Chardonnay Grape Juice Duplicate Fermentation.

When RS1 was challenged by fluoride at 30mg/L, it was dramatically compromised, as shown in Figures 4.4.1, 4.4.8 (WGJ) and 5.4.1, 5.4.2. It is apparent that there were some differences in effect due to the grape juice source and that in CGJ the lag phase was extended. Because of this extension and brief sampling period (6 days) the extent of growth after 6 days is not known. Figure 5.4.3 shows the effect of 30mg/L fluoride on two duplicate fermentations up to day 12. RS1 typically showed a drop off in cell numbers to below 1×10^1 CFU/mL within the first two days of fermentation, then a lag phase of one or two days. The extended fermentation time allows one to see that once the yeast started growing after the lag phase, it multiplied quickly. In fact the final cell counts for the duplicate fermentations were well over 1×10^8 CFU/mL (Figure 5.4.3). The repeatability of these two fermentations were not precise but the trends were repeated.

Figure 5.4.4 illustrates the growth curve of RS1 with fluoride challenge of 15mg/L showing excellent repeatability between the two separate fermentations. These fermentations reached an average peak count of Log CFU/mL 8.5 on day 7 showing that although the yeast had initially been compromised, it was able to recover very successfully over an extended time period.

5.4.5 Effect of Pasteurisation verses Non-Pasteurisation

Figures 5.4.5 to 5.4.8 compare the effect on RS1 culture growth, of pasteurised grape juice (P) with non-pasteurised grape juice (NP) focusing on a lower range of fluoride concentrations. The results show similar patterns in both the pasteurised and non-pasteurised trials, although some results do differ. In Figure 5.4.6 and Figure 5.4.7 RS1 in the non-pasteurised juice drops dramatically in numbers, unlike its equivalent in pasteurised juice. As the grape juice has been treated with SO₂, contamination from other microorganisms is unlikely. The colony morphology of the yeast plated from the non-pasteurised fermentation did not show any erroneous colonies, although colonies of other *Saccharomyces* species may have been difficult to differentiate.

5.4.6 Low Fluoride Chardonnay Grape Juice Fermentations

Figure 5.4.9 shows the population growth curves for RS1 with fluoride challenges of 1, 3, and 5 mg/L. Yeast growth curves were similar at all the different fluoride levels. Fluoride at 5mg/L showed slightly better growth than at other fluoride levels after day 1, dropping just below the control on the final day. There were no significant differences in the population numbers for RS1.

Figure 5.4.10 shows RS2 exposed to the same fluoride concentrations. Again, the culture growth rate is not greatly affected by such low fluoride concentrations. However at 5mg/L the growth curve showed a lower population from day 3 to the end of the fermentation.

It is difficult to measure the significance of growth rate variations observed at low fluoride concentrations. The sensitivity and accuracy of the method of testing as, discussed in section 4.4, indicates trends and to show differences in results at a more sensitive level more precise measurements need to be done.

Since it seems, from this study, that fluoride at low concentrations does not affect the population growth significantly, and yet fluoride concentrations as low as 3-5mg/L have been reported to induce stuck fermentations (Wahlstrom et al, 1996., Kunkee, 1991., Andris, 1990), the mode of action of the fluoride may be acting at a level not represented by these results. It is possible that the fermentation ability of the yeast is affected at the lower concentrations where it is not clear if the reduction in cell numbers is significant. Even when it is clear that the initial

population is reduced (10 and 15mg/L fluoride) sometimes severely (30mg/L), the recovering cells may grow well, but with reduced fermentation ability.

The next set of graphs combine the population growth curves of RS1 at a range of fluoride concentrations (Figure 5.4.11) with the level of sugar reduction (Figure 5.4.12) as the fermentation progresses. These results show that the yeast that fermented the best was the control. As the concentration of fluoride was increased the ability of the yeast to reduce sugars decreased. From these results a fermentation with only 1mg/L fluoride followed a fermentation rate extremely similar to that of the control. At fluoride 3, 5 and 10mg/L the fermentation rate was compromised by 5.5, 6.2 and 5.1% respectively. Fluoride at 15mg/L reduced the fermentation ability by over 27% despite the fact the population numbers were over 7 (log CFU/mL) from day 3 to day 6. At these population numbers we could expect fermentation to be carrying on as normal but evidence was to the contrary. The fermentation rate of the yeast RS1 at fluoride challenge 15mg/L was lower and less vigorous. Table 5.4.1 shows the differences as compared to the initial sugar level at day 1.

Table 5.4.1 Reducing Sugar Value (%RS) for RS1

Fl-	Control	1mg/L	3mg/L	5mg/L	10mg/L	15mg/L
Day 0	100	100	100	100	100	100
Day 1	88.64	88.50	89.62	90.57	95.6	98.5
Day 2	62.70	65.84	75.27	72.92	80.60	96.66
Day 3	55.13	56.12	65.59	62.80	67.03	93.33
Day 4	45.40	46.41	54.84	54.14	53.84	80
Day 5	41.40	46.41	47.31	48.02	52.74	72.77
Day 6	37.13	37.07	42.63	43.31	42.14	64.44

Previously our study had indicated that RS2 had more tolerance for fluoride (section 2.4., 4.4) which is clearly reflected in Figure 5.4.13. The corresponding measurements of RS2 fermentation is shown in Figure 5.4.14. The control sample had the best fermentation ability and was distinctly better than any of the other trials with fluoride. Unlike RS1 where fluoride at 1mg/L had no affect on fermentation rate, RS2 at 1mg/L was compromised and the fermentation rate difference was as high as 6.4% (Table 5.4.2).

Table 5.4.2 Reducing Sugar Value (%RS) for RS2

Fl-	Control	1mg/L	3mg/L	5mg/L	10mg/L	15mg/L
Day 0	100	100	100	100	100	100
Day 1	95.30	93.90	94.26	93.90	97.76	95.69
Day 2	74.19	77.71	78.20	80.57	85.88	83.87
Day 3	57.82	65.83	67.48	71.23	78.08	78.49
Day 4	45.11	51.10	51.79	55.74	65.81	63.97
Day 5	39.98	46.41	46.75	49.64	59.11	59.51
Day 6	36.38	38.85	40.01	43.17	52.81	51.61

Table 5.4.3 compares the final reducing sugar values (%RS) of RS1 and RS2. At 15mg/L RS1 fermentation ability is more effected than RS2, at the same fluoride concentration. Population cell densities and corresponding ability to ferment are not necessarily correlated.

Table 5.4.3 Comparison of Final Fermentation Day of RS1 and RS2 (Reducing Sugar Value %RS)**Fluoride added**

Day 6	control	1mg/L	3mg/L	5mg/L	10mg/L	15mg/L
-------	---------	-------	-------	-------	--------	--------

RS1

Day 6	37.13	37.07	42.63	43.31	42.14	64.44
-------	-------	-------	-------	-------	-------	-------

RS2

Day 6	36.38	38.85	40.01	43.17	52.81	51.61
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The results from section 5 have shown the effect of heat treatment and discussed the different grape sources. Yeast growth patterns are similar in both Brewmakers™ Whitewine grape juice and the Chardonnay grape juice.

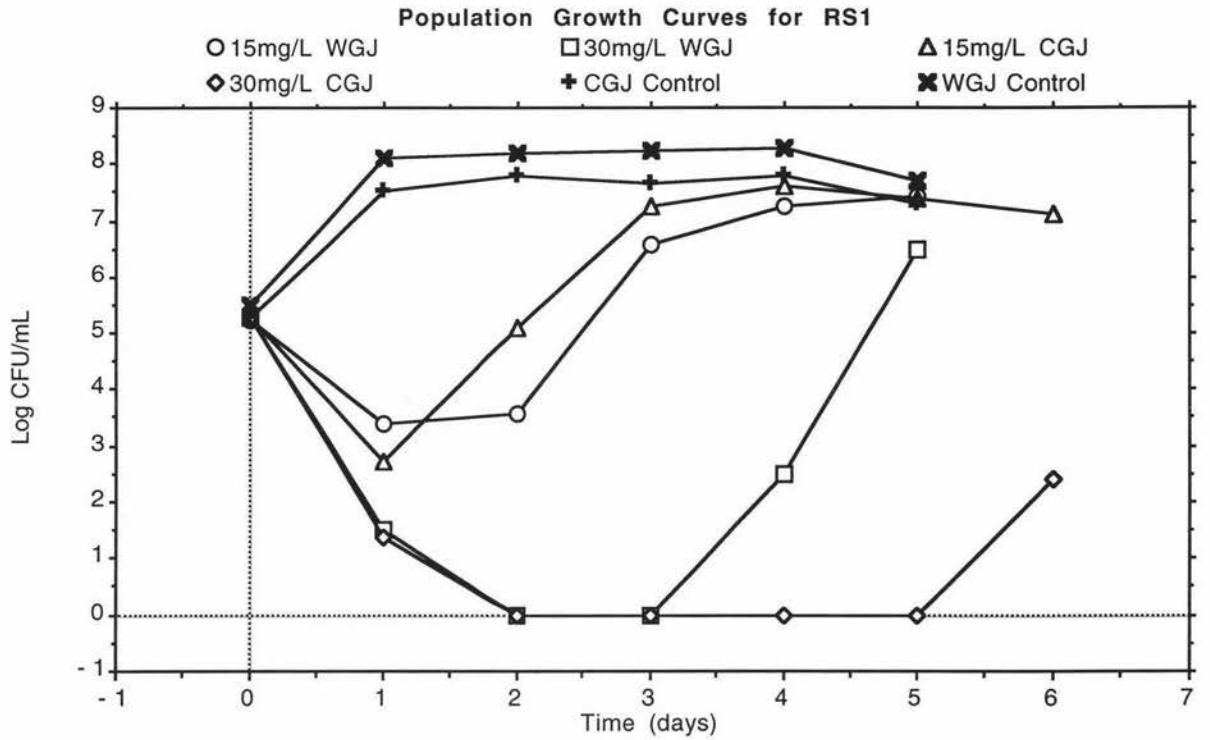


Figure 5.4.1 Comparison of Grape Juice Sources

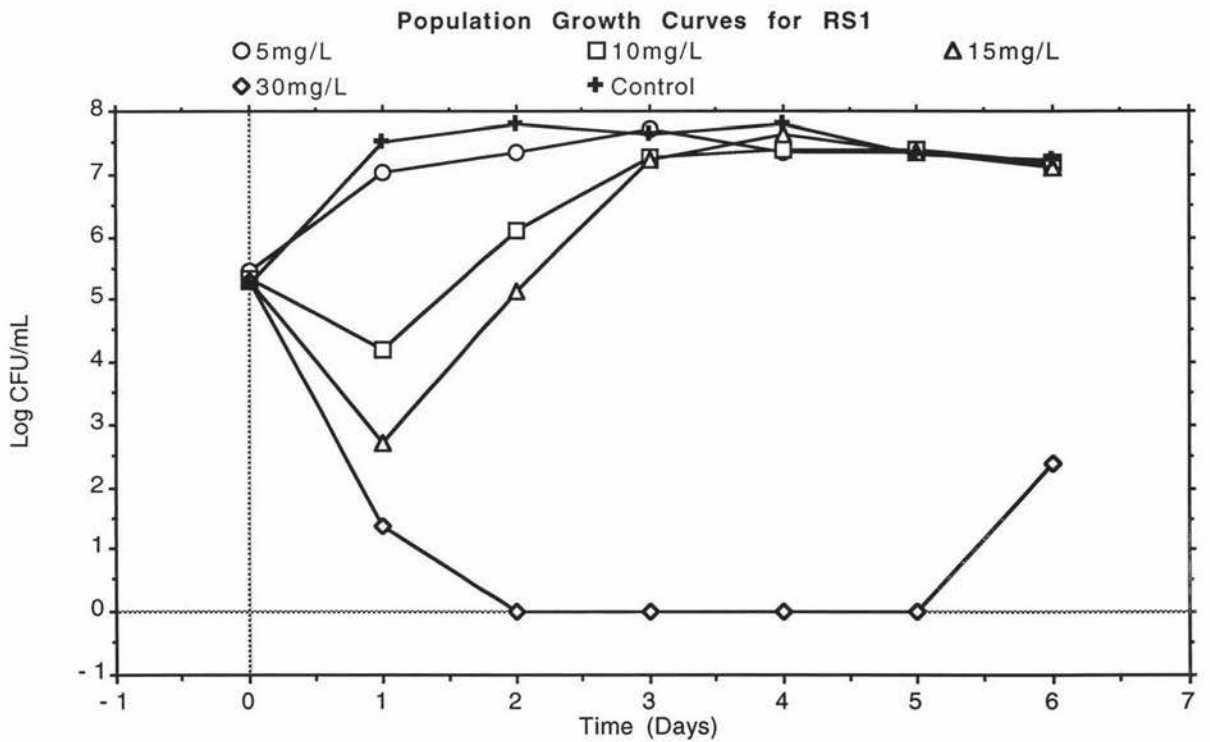


Figure 5.4.2 RS1 in Chardonnay grape juice with varying fluoride concentrations

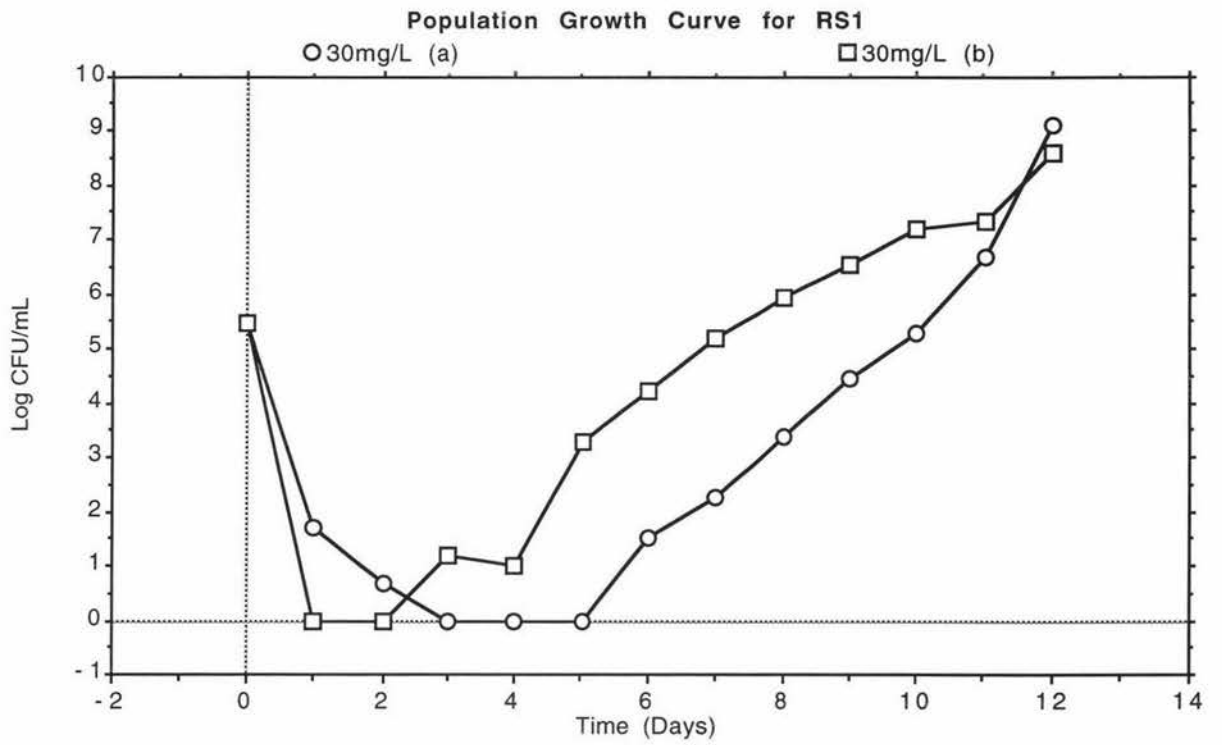


Figure 5.4.3 Growth of RS1 duplicates (a) and (b) at 30mg/L fluoride (pH 3.4)

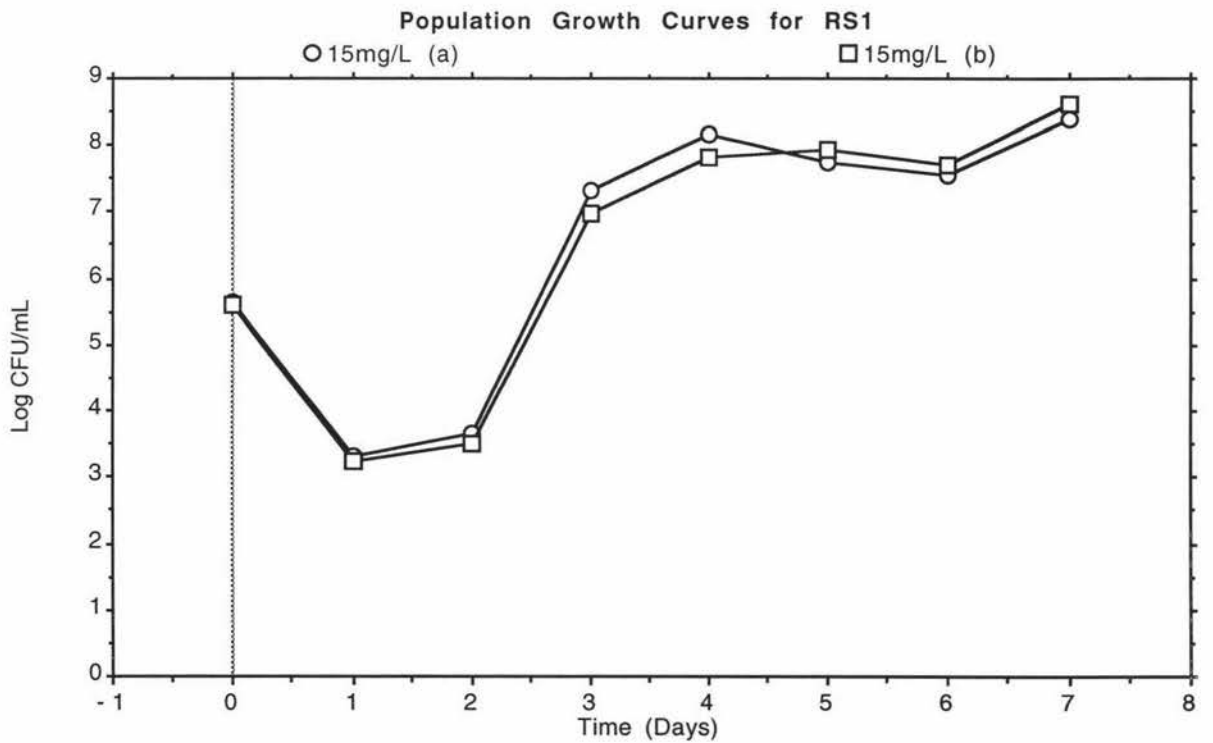


Figure 5.4.4 Growth of RS1 duplicates (a) and (b) at 15mg/L fluoride (pH 3.4)

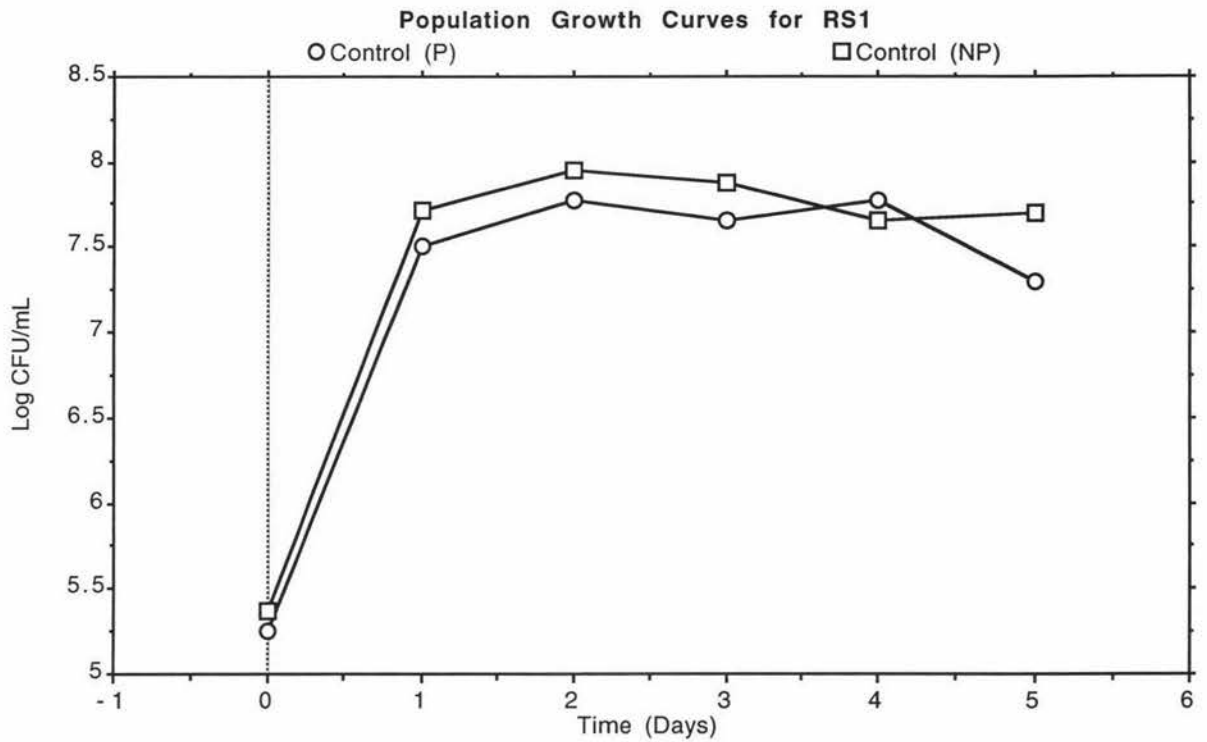


Figure 5.4.5 Comparison of Pasteurised (P) Verses Non Pasteurised (NP) Chardonnay Grape Juice

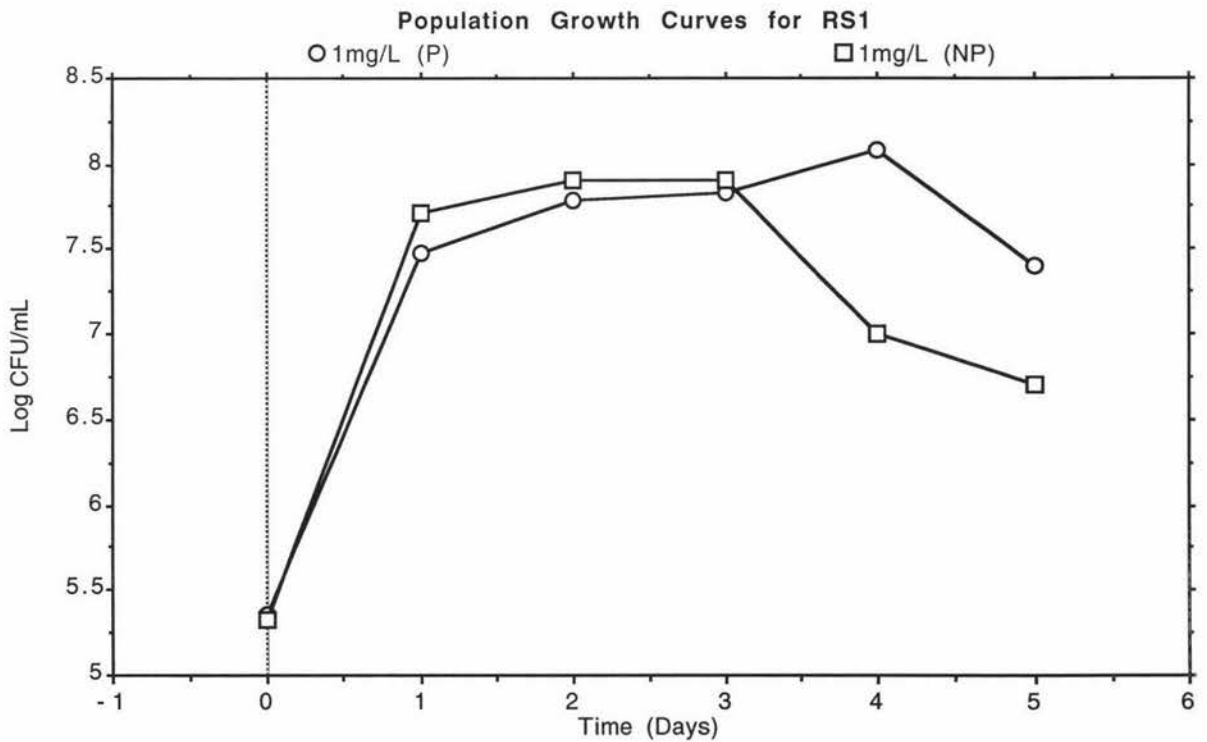


Figure 5.4.6 Comparison of Pasteurised (P) Verses Non Pasteurised (NP) Chardonnay Grape Juice

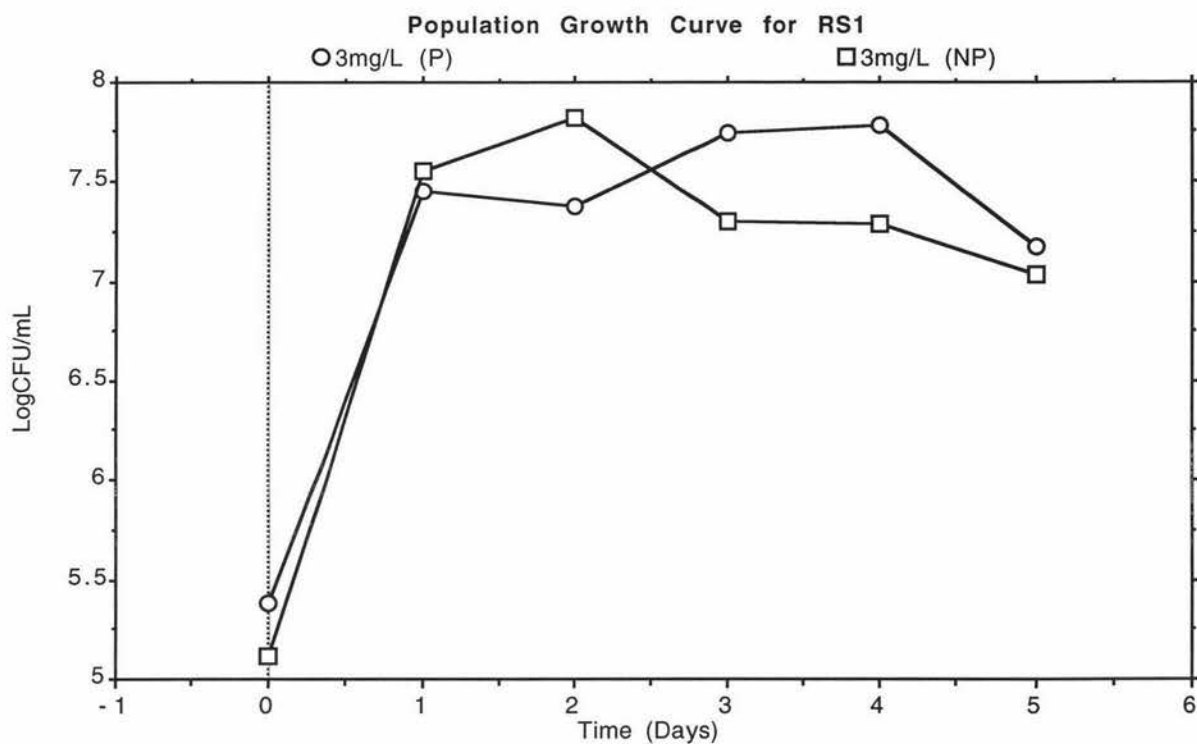


Figure 5.4.7 Comparison of Pasteurised (P) Verses Non Pasteurised (NP) Chardonnay Grape Juice

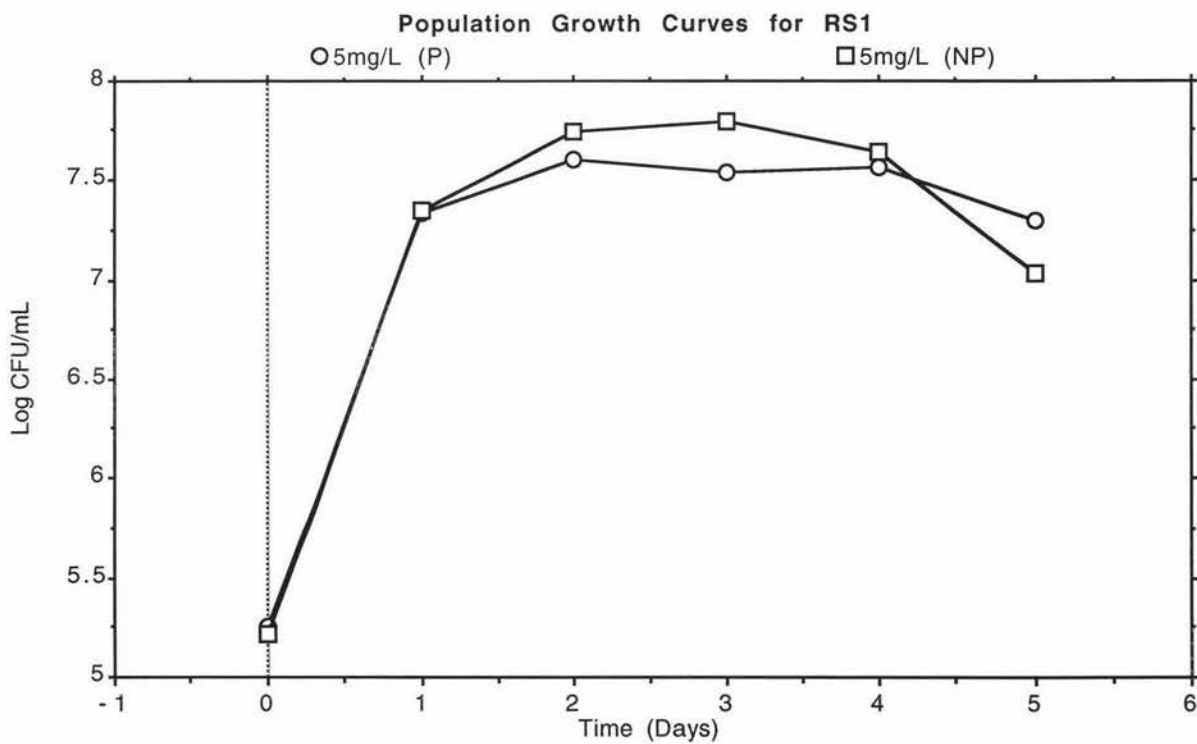


Figure 5.4.8 Comparison of Pasteurised (P) Verses Non Pasteurised (NP) Chardonnay Grape Juice

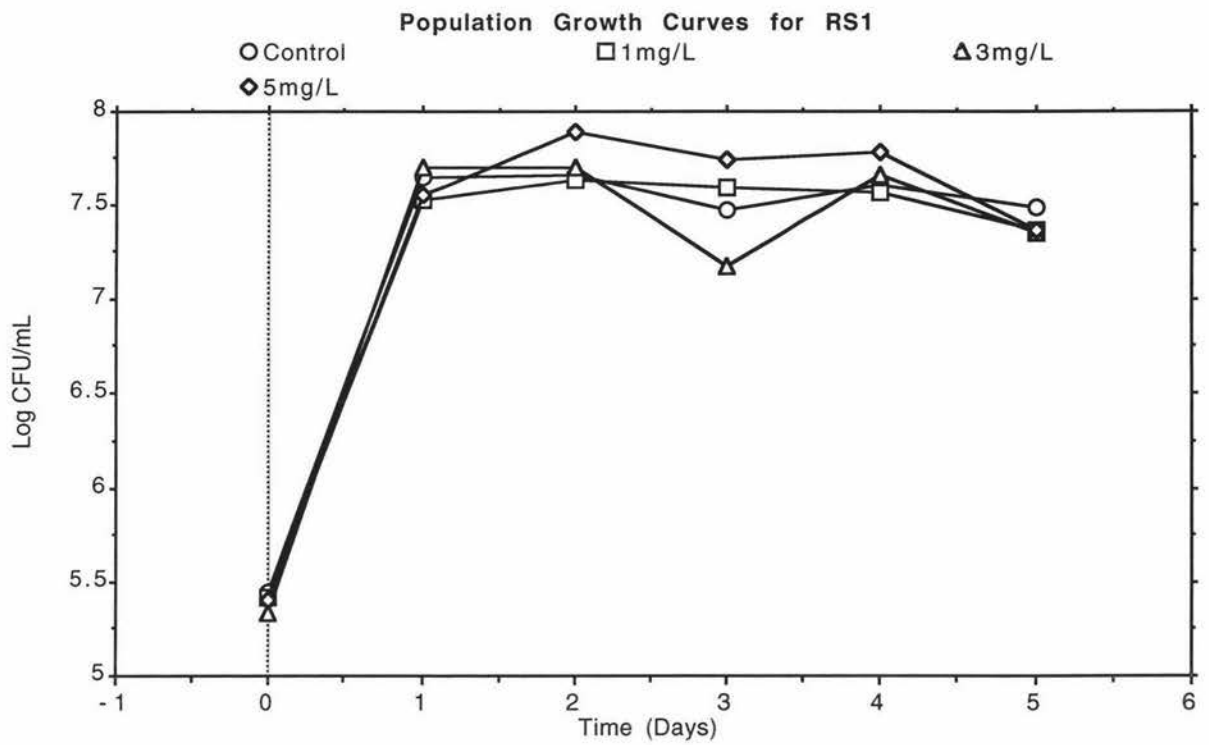


Figure 5.4.9 Growth of RS1 versus varying fluoride concentrations in Chardonnay Grape Juice

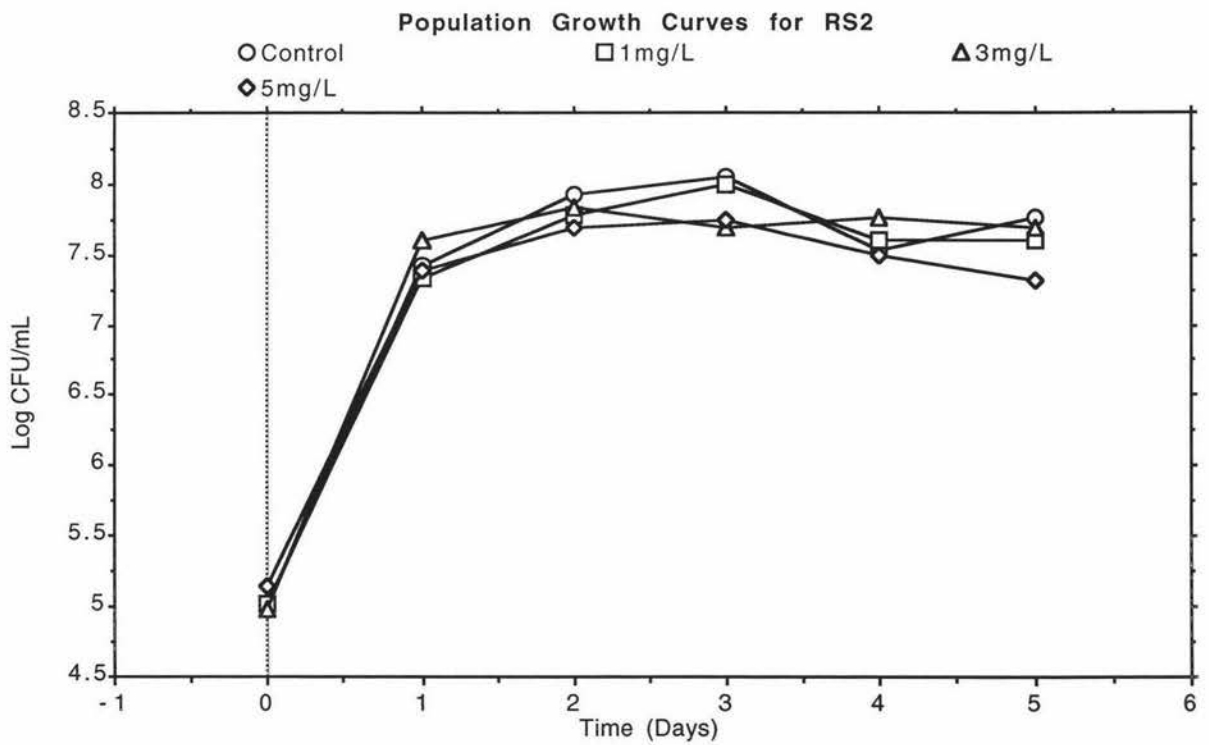


Figure 5.4.10 Growth of RS2 versus varying fluoride concentrations in Chardonnay Grape Juice

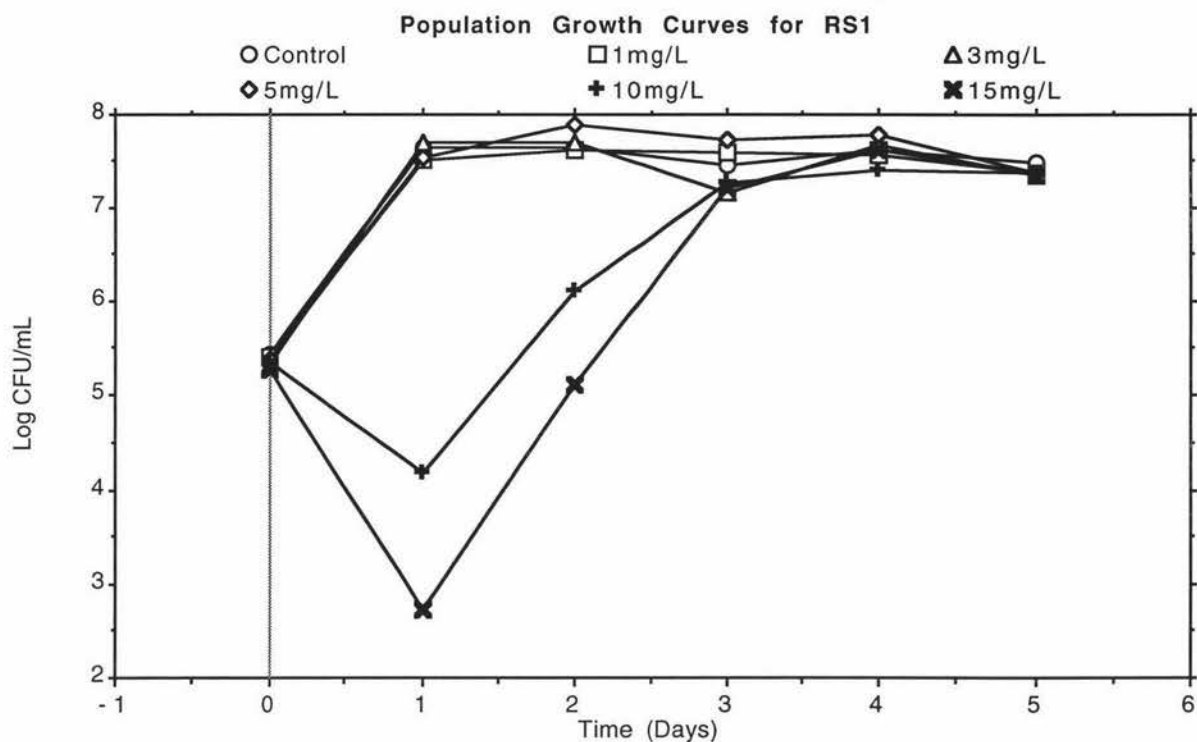


Figure 5.4.11 Growth of yeast RS1 versus varying fluoride concentrations (CGJ)

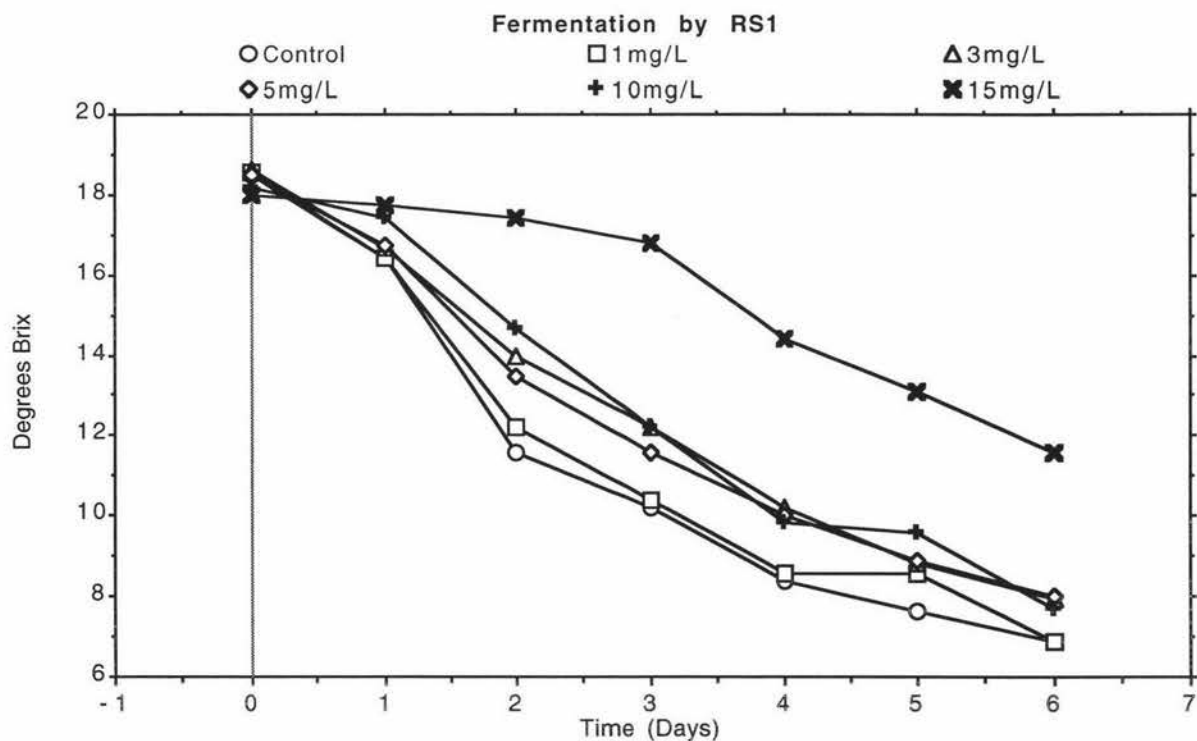


Figure 5.4.12 Fermentation of Chardonnay grape juice by RS1 at varying concentrations of fluoride

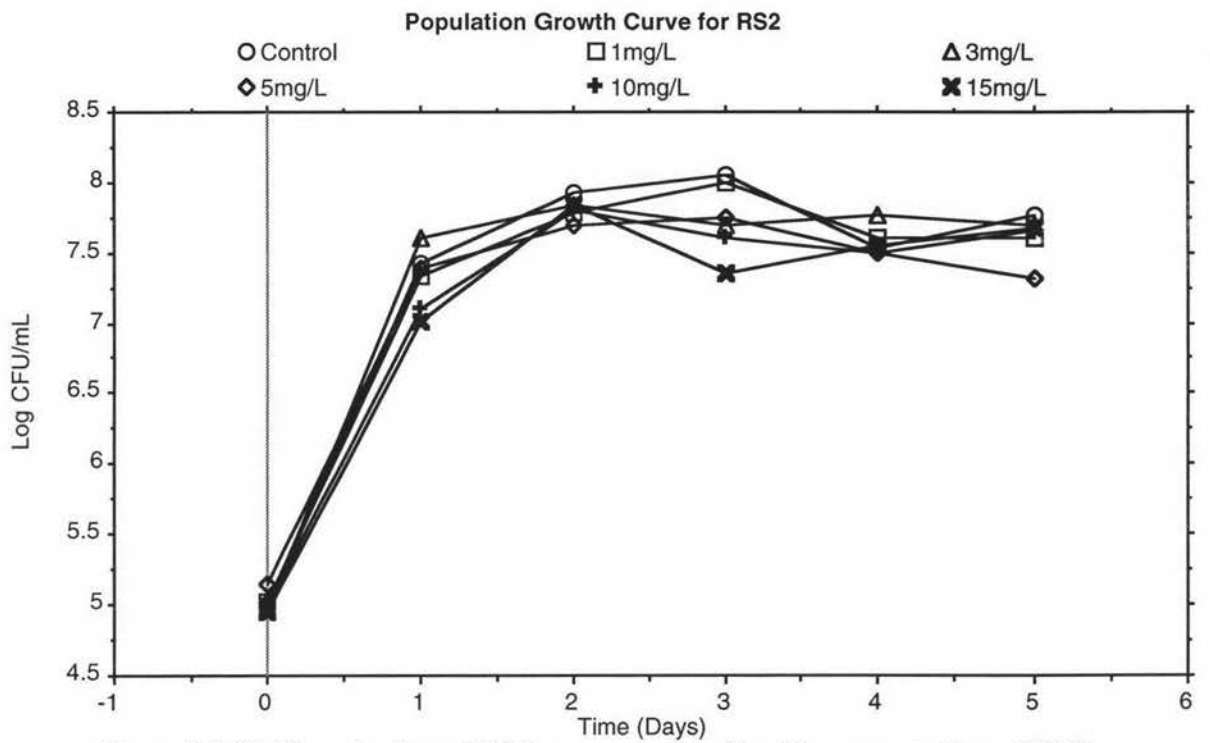


Figure 5.4.13 Growth of yeast RS2 verses varying fluoride concentrations (CGJ)

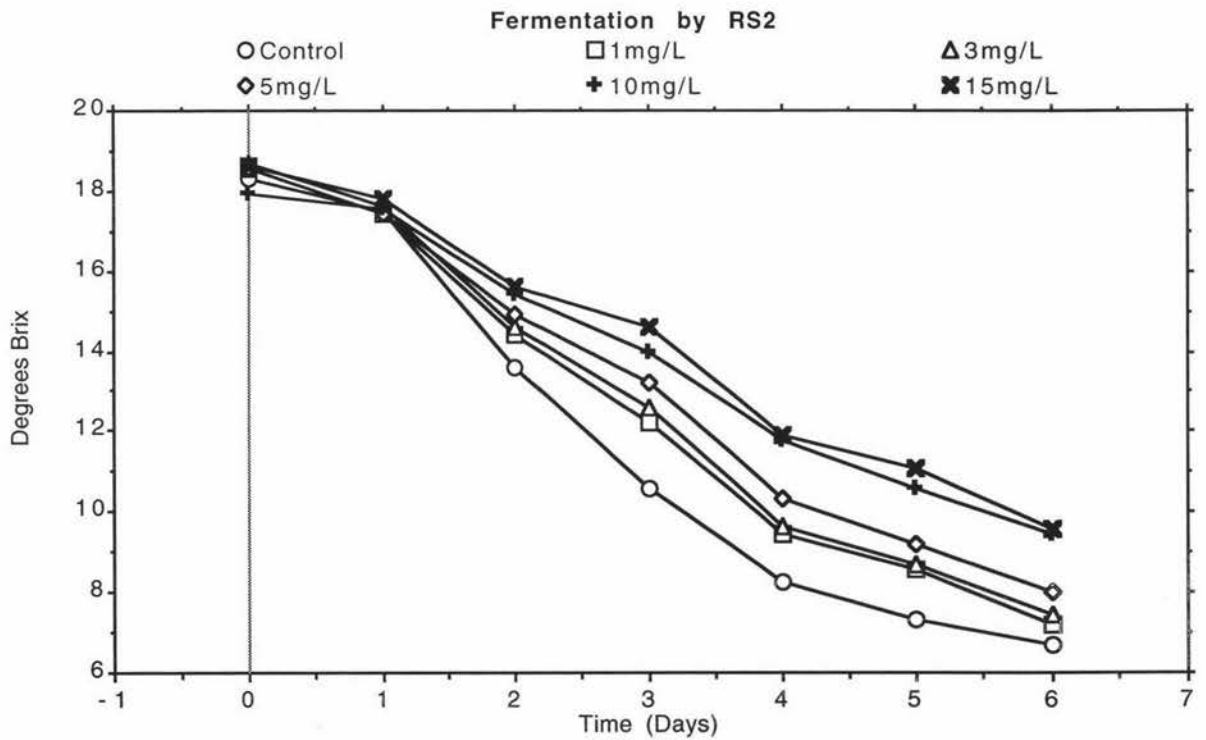


Figure 5.4.14 Fermentation of Chardonnay grape juice by RS2 at varying concentrations of fluoride

6. GENERAL DISCUSSION AND CONCLUSIONS

6.1 INTRODUCTION

The efficient conversion of grape juice sugars, glucose and fructose, to ethanol is critical in winemaking. The amount of sugar remaining in a wine after fermentation is determined by the style of wine required by the wine maker. Residual sugar is a characteristic that is desirable in sweet or dessert wines but undesirable in most table or dry wine, yet an overall balance of final sugar, ethanol and acid is vital to the finished product.

There are many variables that can interrupt and sometimes completely halt this vital conversion. One of the lesser known factors is the effect of fluoride on grape juice fermentations.

This study investigated the effect of fluoride on yeasts involved with winemaking. Initial experiments correlated with the findings of other authors, ie. that these fluoride effects are regulated by pH. The effect of fluoride on yeast growth and fermentation was also strain dependent.

Sections 4 and 5 investigated the effect of differing fluoride concentrations in small scale grape juice fermentations. This basic information could help winemakers select a yeast for fermentation and provide information from which more detailed studies may eventuate. During these studies the influence of grape juice source and heat treatment on fluoride inhibition were examined. This way the results from these experiments are more useful for practical application.

This final section discusses in depth the findings of this research firstly examining the initial experiments in sections 1 and 2. The availability of fluoride from Cryolite is discussed as this is key to the potential for fluoride 'pollution' of grape juice. Secondly, sections 4 and 5 compare our results to previously described effects of fluoride on yeast and bacteria from the literature. As the mechanism of fluoride affect on oral flora in dentistry is well understood and comprehensively reported, similarities of fluoride action on bacteria and yeast will be discussed.

Conclusions will be made and as this field of research is relatively unstudied. Brief suggestions for further research are noted.

6.2 SELECTION OF EXPERIMENTAL PARAMETERS

Through initial laboratory experiments using YM agar and YM broth we found that fluoride lethality was influenced by culture density and pH. The growth or no growth break point differs for each yeast and Table 6.2 summarises the minimum inhibitory concentration of fluoride for sections 2 and 3. This table shows that fluoride in liquid YM broth at pH 3.3 is more inhibitory to the yeast cells than fluoride in YM agar pH 3.3 and 4.3, respectively.

Table 6.2 MIC Values for Selected Yeast in mg/L fluoride.

YEAST	YM AGAR (pH 4.2)	YM AGAR (pH 3.3)	YM BROTH (pH 3.3)
RS1	271	45	31.3
RS2	>316	316	31.3
RS10	>316	271	62.5
UCD-615	>316	>360	31.3
AWRI-354	181	45	15.6
SR50	-	>360	62.5

There also appeared to be a time factor associated with fluoride toxicity. That is, over time the inhibitory effect of fluoride was reduced. This factor was initially identified in the preliminary agar experiments, as shown in Table 2.4.4, and was dependent on both the yeast strain and the dosage of fluoride. All of the yeasts showed improved growth over a 48-120 hour period up to the no growth break point. The commercial showed the least recovery with time (RS1, RS2, RS10, EC1118) and the wild yeast (UCD-615 and AWRI-354) showed the greatest improvement in growth.

The most sensitive yeasts to fluoride were RS1 and AWRI-354. The fluoride sensitivity of RS1 has been previously demonstrated (Wahlstrom et al, 1996., Morenzoni, 1991, personal communication). The most resistant yeast overall, as

shown in the initial experiments, was SR50. RS2 and RS10 were the most resistant commercial yeast strains.

The initial experiments were performed in simple laboratory media and the results only gave an indication of the effects fluoride challenge on yeast growth, not on the fermentation ability of the yeast.

As there are many new and different dynamics associated with grape juice fermentations, results of laboratory media experiments may not be directly applicable to small scale fermentations.

6.2.1 Availability of Fluoride From Cryolite

The different experiments carried out constitute only one approach to investigating the influence of Cryolite on wine yeasts. There is debate on the physiochemical availability of fluoride in real world conditions. Tables 3.4.2 and 3.4.3 showed that in YM liquid media Cryolite was considerably less inhibitory than sodium fluoride to the selected yeasts. It seems likely that a significant proportion of fluoride is bound in the Cryolite form and therefore not available to react with the yeast. Only at very high levels of fluoride challenge was there an inhibitory effect on yeast growth, and this effect yet again varied with each yeast. These results indicate that it is the effect of the fluoride ion itself that causes inhibition and not the Cryolite compound. Lunnardi *et al.* (1988) showed that fluoride inhibition of the F-ATPases of mitochondria and of *Escherichia coli* was due mainly to the AlF_4^- complex, which can mimic the phosphate group of ATP, to form an abortive form of ADP at the active, hydrolytic sites of the enzyme. At present Marquis (1995) reports that it is not clear if this kind of inhibition occurs within intact cells, mainly because aluminium is generally excluded from cells.

The transfer of fluoride from vine to vat has been demonstrated at vineyards that use Cryolite to control unwanted pests (Morenzoni, 1991, personal communication) showing that the fluoride ion is released from Cryolite. Numerous factors should also be taken into consideration when determining the level of fluoride residue in musts from Cryolite.

There is a correlation between application timing, with respect to harvesting, method and dosage to the fluoride content of the wine juice (Archer and Gauer, 1979., Ostrom and Petrucci, 1994). Ostrom and Petrucci, (1994) recorded fluoride levels in

untreated wines from 0.07 to 1.55mg/L. Regardless of harvest method or surfactant used, grapes treated with 8.98 kga.i./ha at bloom (Kryocide™) had significantly higher wine fluoride levels (0.34 to 3.02mg/L) than untreated wines. Additional applications of Kryocide™ at veraison, regardless of the amount applied at bloom, caused significantly higher levels of fluoride. An application of 8.98 kg a.i./ha at bloom plus 6.73 kg a.i./ha at veraison produced fluoride levels in Zinfandel of 9.75mg/L.

Environmental conditions including rain, wind and heat can affect this insecticide. After initial deposit on the plant, partial elimination of the product by runoff, leaching, or evaporation can result. The alteration to the must by racking and/or clarification may eliminate a proportion of the insecticide. The addition to the must of bentonite, glass beads or yeast hulls may allow the absorption of fluoride.

The physiochemical state of fluoride from Cryolite may be compared to the state of fluoride in plaque. Initially researchers thought that most of the fluoride was bound in plaque. Research by Ophaug *et al.*(1987), indicate that even if the fluoride is bound, it is readily released by mild acidification. Marquis (1995) noted that because of the need for specially formulated solutions with high ionic strength when using the fluoride electrode for assays, fluoride concentrations in plaque cannot be assessed directly but only in plaque removed and treated in vitro. The acidic conditions of wine making could easily allow for the release of the fluoride component from Cryolite, and as mentioned above, the physical and chemical conditions may also break the Cryolite down, releasing fluoride ions into the must.

6.3 COMBINED GRAPE JUICE FERMENTATIONS

6.3.1 Fluoride Action On Yeast

The inhibition demonstrated in sections 4 and 5 may be attributed to a sensitisation of the yeast cells to fluoride on a number of different levels, dependant on yeast type and the concentration of fluoride. Parallels may be drawn, to some extent, from the effect of fluoride on bacteria.

6.3.2 Enzyme Effects

Fluoride is known to effect a number of bacterial enzymes including glycolytic enolase and most of the detailed work on enolase inhibition by fluoride has been carried out with eukaryotic enzymes from yeast (Marquis, 1995). The initial studies of Warburg and Christian (1942) showed that fluoride in association with Mg^{2+} and phosphate is a potent, competitive inhibitor of this enzyme. Inhibitory concentrations of fluoride depend very much on concentrations of Mg^{2+} and phosphate (Marquis, 1995).

A more complex picture of fluoride inhibition has been found by Kaufmann and Bartholmes (1992), with both competitive and noncompetitive components. They found that phosphate greatly enhanced fluoride inhibition and inhibition was noncompetitive at fluoride concentrations below 10mM but competitive above that level.

It is a common practise for wineries to add diammonium phosphate as a supplement to for grape juices lacking in nitrogenous compounds (Kunkee, 1991). It is possible that the combination of fluoride and added phosphate increases the inhibitive effect of the fluoride ion. The limited research has not investigated such a linkage.

Curran *et al* (1994) reported that during the course of fluoride inhibition, enolase is inactivated. The inactivation could be completely reversed by phosphoenolpyruvate (PEP) and to a lesser degree 2-phosphoglycerate (2-PG), with full recovery of activity. Curran *et al*, (1994) also found that fluoride can act to irreversibly inhibit enolase of yeast, but this was only apparent in activity verse time plots, which showed a reduction in activity to zero after about 20 minutes, even though substrate was still at a high concentration in the reaction mix. The research concluded that fluoride inactivation of enolase did not occur in intact cells.

Fluoride is remarkable in terms of the wide variety of enzymes it affects. Fluoride acts to inhibit enzymes such as F-ATPases, sulphatases, catalase, phosphatases, phosphoglucomutases and ureases. Many of these enzymes are effected due to fluoride's ability to acidify the cytoplasm (Marquis, 1995).

Fluoride is a well-known inhibitor of eukaryotic pyrophosphatases (Marquis, 1995). The roll of pyrophosphatases in recycling phosphate from pyrophosphate yielding reactions, such as amino acid activation, is known, and at least for *Escherichia coli*,

the enzyme is essential for growth (Chen *et al*, 1990). This type of action may be reflected in the experiment results of sections 4 and 5 whereupon cell growth is retarded.

6.3.3 Membrane Effects and Fluoride Accumulation

The effect of low pH on fluoride inhibition is well documented for bacterial cells (Belli *et al*, 1995., Kashket and Kashket, 1985., Kaufmann and Bartholmes, 1992., Marquis, 1977., Marquis, 1989., Marquis, 1995.) and this pH dependancy is also demonstrated in yeast cells in the initial experiments in section 2. Fluoride is a weak acid with a pK_a value of approximately 3.2. Therefore when the pH of the YM media was lowered to 4.2 and 3.3, a significant proportion of the fluoride was in the form of HF. This form moves readily across cell membranes and membrane permeability is up to 10^7 times greater than F^- (Gutknecht and Walter, 1981).

Cryolite is one of the most popular pesticides because it has a long residual effect. Fluoride is concentrated in dental plaque and the oral bacteria can accumulate the fluoride from the environment (Marquis, 1995). The major basis for fluoride accumulation is the weak-acid nature of fluoride and the transmembrane chemical gradient (ΔpH) across cell membranes. If the external pH is lower than the cytoplasmic pH value, fluoride becomes protonated outside of the cell, moves across the membrane in the form of HF, and then dissociates in the cytoplasm. This dissociation releases F^- which can act as a metabolic inhibitor and the cytoplasm becomes acidified by the H^+ (Marquis, 1995). Therefore fluoride acts to dissipate the ΔpH (Kashket and Kashket, 1985) and to reduce the acid tolerance of the bacteria (Eisenberg *et al*, 1980., Marquis, 1995).

Part of the concentration of fluoride in dental plaque appears to be due to fluoride binding to various cell structures, mainly proteins (Marquis, 1995). In this context, it should be noted that grape must contains proteins and other nitrogenous material such as nucleic acids and polypeptides. Murphey *et al*. (1989) found 70 to 120mg/L of protein in juice of Gewürztraminer and Riesling with increasing pH in the 2.9 to 3.6 range. The majority of the proteins that can cause haze in white wine come from the grapes and at least some survive fermentation. Therefore clarification and racking of grape juice for white winemaking may help in the elimination of protein-associated fluoride residues from the juice. In contrast the pulping process of red winemaking may allow for higher levels of fluoride in the fermentation due to more adsorption

sites in the pulp and cap. It is not clear in the available literature if there is a tendency for slow or stuck fermentation to be more commonly associated with white winemaking than red. As we experimented solely with white grape juices the distinction cannot be made.

6.4 RESULTS OF THIS RESEARCH

The effect of fluoride on viable yeast cells counts was examined by the plating of treated cultures onto non-selective solid media. During this study yeast cell numbers in the grape juice supplemented with fluoride were reduced, dependant on the yeast strain and the concentration of the fluoride in solution. Trends of fluoride inhibition and/or toxicity were repeated for all of the yeasts examined.

It is obvious that a fungicidal effect is exerted on the yeast at a certain concentration of the fluoride ion (Table 6.2). In reality it would be highly unlikely that fluoride concentrations in grape juice or wine would reach levels of up to 300mg/L however levels as high as 15-20mg/L have been noted in wines, with associated objectionable smells and tastes associated (Morenzoni, 1991, personal communication).

In wines fermented in the presence of fluoride, most SO₂ is bound and very little is free suggesting that there is passage for the SO₂ molecule through the cell membrane (Morenzoni, 1991, personal communication). As previously mentioned fluoride binds to various cell structures, including cell membranes, such that localised areas of weakness are generated, and cell membrane permeability is altered. Cell mortality, reduced fermentation ability, and the 'off' organoleptic nature of the wines produced could all be related to cell membrane damage. Fluoride has been found to enhance turnover of cell wall peptidoglycans and in organisms with active autolytic systems this can induce lysis (Marquis, 1995).

At fluoride concentrations of 5 to 15mg/L, the more sensitive commercial yeast RS1 showed die off and then revival. At the same time reduced fermentation ability was recorded suggesting that the effect of fluoride has a double action. Firstly the fluoride causes a mortality of cells, the severity of which is dependant on the concentration of fluoride, media environment (pH, additives, particulate matter), cell numbers and yeast strain. Secondly, the fluoride compromises the effective conversion of sugars to

ethanol by affecting glycolytic enzymes, (research suggests primarily enolase). The fermentation rate is thereby altered by two factors; reduced cell density and inhibition of glycolysis.

Fluoride toxicity can be overcome as was demonstrated by RS1 when challenged by 30mg/L fluoride (Figures 5.4.1, 5.4.2. and 5.4.3). After a lag period the yeast cells appeared to repair and revitalise, adapting to the supplementation of fluoride. This may be because the fluoride over time is either bound to constituents within the media or is internalised in the yeast cells and therefore is no longer exerting an effect. These two theories are unlikely as research shows that fluoride has a long residual effect and is readily released into the media by low pH (Belli *et al*, 1995., Kashket and Kashket, 1985., Kaufmann and Bartholmes, 1992., Marquis, 1977., Marquis, 1989., Marquis, 1995)). In addition, the majority of yeast cells were lysed during this study, and one would expect fluoride to be released from the cytoplasm of these cells. In fact, the fluoride to yeast ratio was very high, in favour of the fluoride (fluoride 10mg/L approximately 1×10^{23} fluoride ions compared to yeast at 1×10^6 CFU/mL).

RS1 in grape juice supplemented with fluoride (30mg/L) can consistently regenerate from a very low population (less than 1×10^1 CFU/mL) that was dormant for 1 to 3 days. The mechanism by which this occurs is debatable, it may be related to the time needed to repair and revitalise the remaining cells, which by mutation, survived the fluoride challenge. A survival mechanism, whereby the glycolysis pathway is altered, may have come into effect.

Figures 5.4.12 and 5.4.14 examined the affect of fluoride on fermentation and compared those results with the corresponding population growth curves at differing fluoride challenges. The fermentations showed that fluoride added to grape juice can affect the fermentation ability of yeast cell populations. There is a point between 5 and 10mg/L fluoride for RS1 where the fluoride challenge causes the cell population to go into lag phase. At 10mg/L fluoride reduces cell numbers in the first 24 hours but then the population recovers.

RS2 showed a significant reduction in fermentation when challenged with 1 mg/L. It is interesting to note that this challenge has no effect on RS2 growth rate.

One might expect that over a longer fermentation period the samples supplemented with fluoride would eventually ferment to completion (Figures 5.4.12 and 5.4.14).

The experiments from section 5 did not show this as they only monitored the reducing sugars to day 6. However as extended fermentations are costly any batch of grape juice not fermenting to the desired level of sugar in the expected time is undesirable.

6.5 CONCLUSIONS

To conclude, the literature shows that fluoride can have two general types of effect on glycolysis. It can act as an enzyme poison, most probably inhibiting enolase, and then secondarily to inhibit the phosphotransferase system, which requires phosphoenolpyruvate. It seems that the arrest of glycolysis is circumvented by the yeast in these experiments albeit not to the yeasts normal fermentative capacity. This has been described in bacteria by Marquis, (1995).

From the experimental work conducted on the six yeast common to winemaking the following conclusions are drawn:

- The effect of fluoride on the yeast is increased with lower pH and lower cell densities.
- The effect of fluoride on yeast growth and fermentation is strain dependant.
- The effect of fluoride can be, in some cases, overcome by time. For example, RS1 yeast cells (30mg/L fluoride), after a lag period, appear to repair and revitalise, adapting to the supplementation of fluoride.
- RS1 was the most sensitive commercial yeast, RS2 was the most resistant commercial yeast.
- AWRI-354 was the most sensitive yeast overall.
- SR50 was the most resistant yeast overall.
- The wine yeasts used in these experiments had a 'break point' (MIC), where the level of fluoride effect went from being inhibitory to toxic. The MIC was dependent on the yeast.

6.6 FURTHER RESEARCH

This preliminary research has provided some good insights into the effect of fluoride on yeasts involved with winemaking. There are many areas that warrant further investigation simply because data on the effect of fluoride on yeasts is not comprehensive. With that in mind suggestions that logically follow on from this research are documented below.

- Further investigation of the fluoride concentration at which wine yeasts growth is affected. A wider range of wine yeasts could be used and a more specific range of fluoride concentrations.
- Follow fermentations using cell numbers (total and viable) and cell mass vs. time. Using various concentrations of fluoride would allow one to construct a plot of generation time vs fluoride concentration. If generation time is not proportional to increase in fluoride concentration, then possibly some type of saturation kinetics might be involved.
- There may be interest in producing a fluoride resistant yeast. In dental research evidence suggests that development of resistance to fluoride in the mouth flora does not occur (Marquis, 1995). This may be related to the natural variations in pH in plaque, usually with multiple cycles per day of acidification and alkalization. Fluoride has been used widely since World War II, without significant fluoride resistance occurring (Marquis, 1995).
- Factors associated with the process of winemaking should be investigated. For example, the incidence of stuck fermentations in both red and white winemaking.
- Determination of whether the effect of fluoride in red winemaking is a) increased due to fluoride accumulation in the pulp and cap., or b) reduced due to limited fluoride availability subsequent to adherence to particulate matter.
- Additives should be carefully monitored in winemaking to see if there is a correlation with increased fluoride inhibition.

- Investigate the glycolysis pathway of yeast challenged with differing levels of fluoride.

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