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ASPECTS OF CELL DEATH AND AUTOLYSIS IN
SACCHAROMYCES CEREVISAE

A thesis presented
in partial fulfilment of the requirements
for the degree
of Master of Science
in Microbiology at
Massey University

Susan Christine Hornblow
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The kinetics of cell death and autolysis of twenty two haploid yeast strains were examined over a period of eight months in wine and synthetic media. Eight distinct patterns of cell death were observed using methylene blue staining and sample plating for viable cells. The rate of death was both yeast strain dependent and influenced by environmental factors such as temperature, nutrient supply and the presence or absence of ethanol.

The activity of extracellular killer yeast toxin concentrated by ultrafiltration was examined under various environmental conditions. Toxin activity was pH and temperature dependent. Concentrations of ethanol greater than 2% completely inhibited killer toxin activity. A difference of 12 hours was detected between a yeast cell becoming incapable of reproduction as the result of killer toxin action and this inability becoming discernible by methylene blue staining. A maximum kill of 97 - 99% was obtained independent of cell or toxin concentration. Toxin induced death was accompanied by the release of arginine and lysine. A bioassay was developed to quantify the amounts of arginine and lysine released.
"I drink it when I'm happy and when I'm sad.
Sometimes I drink it when I'm alone.
When I have company I consider it obligatory.
I trifle with it if I'm not hungry and drink it when I am.
Otherwise I never touch it - unless I'm thirsty."

- Madame Lilly Bollinger
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INTRODUCTION
2.10 AUTOLYSIS


1. Living cells, capable of fermenting sugar and undergoing rapid budding

2. Living yeast cells, in a retarded state, slow reproduction occurring

3. Living cells, incapable of reproducing

4. Dead cells, yielding to autolysis

Autolysis is the process of cellular degradation catalysed by intracellular enzymes after the death of the cell, and is utilized by man to produce the aroma and bouquet that typifies champagne. The key to the champenoise process is the prolonged ageing of bottle fermented champagne in contact with the yeast. Ageing is a slow process traditionally taking four years at low temperatures, during which time autolysis of the yeast cells occurs and cellular products are released into the wine (Margheri, Versini, Serra, Giannotti, Pellegrini and Mattarei, 1984).

During fermentation, amino acids are sequentially removed from the must (Rose and Keenan, 1981). Within the first six days of fermentation there is a drastic reduction in the free amino acid levels with the exception of proline (Suarez, Polo and Llaguno, 1979). The concentration of amino acids increases by 2 to 30% of the initial values in the must by the completion of the fermentation. The increase in free amino acids has been attributed to secretion by yeast and to cellular autolysis (Kluba, Mattick and Hackler, 1978). The most prominent increase is in proline concentration (Ough 1968).
A second explanation is that of passive excretion of amino acids into the wine by living cells (Morfax and Dupay, 1966).

Yeast autolysis, as determined by a renewed increase of amino acids in the wine, occurs after a latent period of 12 months (Surez et al, 1979). Joslyn (1955) recognised that changes in the flavour and stability of fermenting must can occur even before extensive autolysis of the yeast cells takes place. He found that excretion of amino acids, vitamins and other cellular constituents by viable yeasts in the early stages of autolysis have a noticeable effect on flavour. Substances produced during autolysis have been found to enhance the organoleptic quality of champagne. Changes in the composition of aroma substances was studied using gas chromatography, and the character of sparkling wine was attributed to volatile components of relatively high boiling points (Molnar, Oura and Suomalainen, 1981). It was estimated that the neutral fraction contributed significantly (54.6%) to the total aroma of flavour concentrate isolated from yeast autolysate (Hajslova, Velisek, Davidkk, and Kubelka, 1980).

Volatile compounds are released during the first year of ageing. Upon prolonged ageing the evolution of benzaldehyde is particularly prominent, reaching 4 mg/l after 16 years. The flavour of wine has been shown to be favourably altered by addition of benzaldehyde (Loyaux, Roger and Adda, 1980).

Wine has an elevated pH, and increased concentrations of amino acids, ash phosphates, B vitamins, pantothenic acid, P.D. factor and ribose after nine months ageing on the lees compared to ageing without yeast contact (Margheri, Versini, Della Serra, Giannotti, Pellergrini and Mattarei, 1984). Other substances found to be
excreted during autolysis are: peptides, polypeptides, purine and pyrimidene bases, proteases, and other enzymes (Babayan, Latov, Belikov and Kalumyanz, 1984; Joslyn, 1955; Jund, Chevallier and Lacroute, 1977; Molnar, Oura and Suomalainen, 1980). All of the substances released during ageing and the composition of the base wine contribute to the bouquet of the finished champagne.

The release of amino acids into champagne and the changes of composition with time have been most extensively examined. The rate of autolysis and the concentrations of free amino acids in autolysates of different yeast strains is variable despite the similar amino acid compositions of cell protein in different yeast strains (Kluka, 1985; Martini, Martini and Miller, 1979) which explains anomalies between different reports of amino acid concentrations in aged champagne. All reports agree that the overall effect is an increase in total amino acid concentrations in wines aged on the lees. Small increases and small decreases in the amounts of individual amino acids have been observed during ageing (Margheri, Versini and Gianotti, 1984).

Phenolic substances can be formed by means of enzymatic hydroxylation and methylation of aromatic amino acids and by degradation of the resulting products (Hajslova et al, 1980). It has been suggested that the observed increase then decrease of alanine and arginine concentrations between the 12th and 24th months of ageing is due to transformation of amino acids by deamination, with the liberation of ammonical nitrogen; alternatively certain amino acids may act as precursors of aromatic compounds (Feuillat and Charpentier, 1982). Investigation of the influence of the length of contact time showed that the greatest enrichment of amino acids occurs between the 6th and 12th month of ageing. 12% more amino acids were
present after six months than in the base wine, and from 12 to 24 months the percentage of free amino acids rose from 24.5% to 25.6%. Major variations occur during the first three months of the secondary fermentation, with amino acid concentrations increasing after 15 months, and surpassing initial levels after 43 months ageing (Suarez et al, 1979).

The distribution of amino acids has been found to be dependent on the actual production method, particularly the extent of yeast contact time. Commercial charmat and champenoise style sparkling wines both have comparable organic acid and alcohol levels, and vary only in total and amino nitrogen composition (Colagrande) and Mazzoleni, 1977). Methionine and tryptophan were found in higher levels in champenoise style wines, while isoleucine, proline and ornithine have higher levels in charmat wines.

A linear relationship exists between the length of storage and the degree of autolysis, with the autolytic rate increasing by 6 to 7 per cent for a 10°C rise in temperature (Molnar et al, 1980). They suggested that elevated temperature may be unfavourable for the organoleptic quality of the wine; as cell components dissolved in the wine are not bound by secondary chemical reactions, the resulting sparkling wine may have a strong yeast flavour. Increasing the temperature caused acceleration of the rate of loss of enzyme activity (Molnar et al, 1980). After 20 days of storage at 10°C a considerable part of the total enzyme activity is lost, and after 80 to 100 days no demonstrable activity was detected that could influence further development of the wine. Protease activity was an exception, in that it increased in activity with storage. Heating of yeast autolyysate containing free amino acids and reducing sugar leads
to intensive browning (Davidek, Hajslova, Kubelka and Velisek, 1979), increase in alkylpyrazine concentration (a product of browning reactions), and a nutty caramel like character (Hajslova et al, 1980). Organoleptic studies suggested that champagne aged at 10°C had more favourable properties than wine stored at higher temperatures, which was attributed to the presence of volatile substances at the lower temperatures (Molnar et al, 1981).

Reports on the extent of cytolysis associated with autolysis vary. Joslyn (1955) reported loss of protoplasm occurred during autolysis, while the cell wall rigidity was retained. Margheri et al (1984A) reported no hydrolysis of yeast cells during champagne ageing. But Perez-Leblin, Reves, Martinez and Lahoz (1982) reported autolysis as being the degradation of the cytoplasm and lysis of cell walls. Molnar et al, (1980B) recorded loss of cells as a means of calculating the degree of autolysis.

That autolysis has a genetic basis is evidenced by the mapping of nuclear genes responsible for cell lysis (Stateya and Venkov, 1981). A ten fold difference between the slowest and the most rapidly autolytic strains under the same conditions supports genetic involvement (Kulka, 1953).

This report examines the rate of death of different strains of Saccharomyces cerevisiae. Autolysis is examined using cell death as an indicator of the rate of ageing in wine and synthetic media. No attempt has been made to determine the importance of cellular products to the organoleptic quality of champagne.

2.20 EFFECT OF ETHANOL ON SACCHAROYMCES CEREVISIAE

Saccharomyces cerevisiae strains are widely used in the fermention-
tation of carbohydrates to produce ethanol because of their resistance to ethanol. All strains do not have equal ethanol tolerance, indicating that ethanol tolerance is partly genetic (Gray, 1941). Strains are able to grow in 8% to 12% (v/v) ethanol, survive up to 15% (v/v), and ferment up to 12% (v/v) with some sake strains capable of fermenting 20% (v/v) ethanol (Rose, 1980; Rose, 1983).

Ethanol causes an increase in cytoplasmic membrane fluidity and a decrease in membrane order as the result of a complex combination of physical effects both directly on the membrane and on the membrane environment (Mitchaelis and Mitchaelis, 1982; Janoff and Miller, 1982). Ethanol being a short-chain alcohol is expected to be dominated by its polar function (Ingram and Buttke, 1984), causing an increase in the intracellular pH and a decrease in the strength of hydrophobic interactions disrupting membrane packaging (Rottenberg, Waring and Rubin, 1981). It has been demonstrated that ethanol inhibits sugar uptake in S. cerevisiae by changing the lipid environment of the plasma membrane (Leao and van Uden, 1982). Ethanol increases the membrane fluidity and increases permeability to ions and to small metabolites (Janoff and Miller, 1982). These effects are increased with increasing temperature (Kleinons, Lee, Bord, Haak, and Woods, 1979).

The membrane lipids are also the site of resistance to ethanol. S. cerevisiae strains adapt to ethanol by increasing the membrane mono-saturated fatty acid composition (Bevan, Charpentier, and Rose, 1982). The presence of specific sterols and fatty acid residues enhances resistance to ethanol (Thomas, Hossack and Rose, 1978). The presence of unsaturated fatty acids in media acts as an essential growth factor for brewing yeasts when insufficient oxygen is
available for fatty acid biosynthesis (Thompson and Ralph, 1967).

The presence of ethanol may also affect the relationship between the yeast and the physical environment. It has been proposed that the observed decrease of yeast resistance to ethanol as the temperature increases is due to modification of sites located on the cell membrane that determine maximum growth temperature, making the cells more temperature sensitive (von Uden and de Cruz Durote, 1981, cited by Ingram, 1984).

2.30 KILLER YEAST IN SACCHAROMYCES CEREVISIAE

Makower and Bevan (1963) first reported the phenomena of Saccharomyces cerevisiae strains which produce an extracellular toxin that is lethal to sensitive cells. The killer character is expressed as one of three distinct phenotypes; killer, neutral or sensitive. Killer cells (K⁺, R⁺) produce toxin which kills sensitive cells, (K⁻ R⁻). Neutral strains (K⁻ R⁺) are immune to toxin, and do not produce toxin. To kill, toxins must be structurally distinct from those toxins produced by sensitive strains, since the specific immunity system of the killed strain is active against its own toxin, but not against other toxins (Rodgers and Bevan, 1978). Thirteen classes of killer yeast have been identified using killer and resistant phenotypes (Young and Yagi, 1978). Three killer groups are found in the genus Saccharomyces cerevisiae; K₁, K₂ and K₃. The K₁ toxin kills K₀ sensitive cells and K₂ and K₃ strains (Bevan and Makower, 1963). K₂ strains first discovered in Russian wines are active against K₀ and K₁ strains (Naumov and Naumov, 1973). Toxins of the K₃ group kill K₀, K₁ and K₂ strains (Young and Yagi, 1978). The
different classes of toxins produced can be distinguished on the basis of pH optima for activity, temperature stability, and relative susceptibility to protease action (Young and Yagiw, 1978). Killer mutants have been isolated in which the toxin is defective, and sensitive strains may mutate to resistance (Al-Aidroos and Bussey, cited by Rodgers and Bevan, 1978). Killing activity has also been identified between yeast strains of different genera (Bussey and Skipper, 1975; Bussey and Skipper, 1976; Rodgers and Bevan, 1978; Kandel and Stern, 1979).

2.3.1 Genetics of the Killer System

Two species of cytoplastically inherited dsRNA molecules are seen in killer yeast strains, both of which are necessary for the killer phenotype (Sommers and Bevan, 1969; Berry and Bevan, 1972; Mitchell, Bevan and Herring, 1973). Both dsRNA molecules are separately encapsulated as virus-like particles (Herring and Bevan, 1974; Kane, Pletras and Bruenn, 1979). The larger L-molecule (or $P_1$) consists of two equal strands of RNA, with a constant molecular weight of $2.5 \times 10^6$ to $3.4 \times 10^6$, and is found in cells of all killer phenotypes (Wickner, 1980). $L$ dsRNA codes for the major capsid protein, and is present if the $m$ or $s$ plasmids are present (Hopper, Bostian, Rowe and Tipper, 1977). The helper $L$ dsRNA plasmid has no known dependence on other plasmids and confers no known phenotype on the host cell apart from the effect on $m$ dsRNA plasmid maintenance (Tipper and Bostian, 1984). The smaller $m$ plasmid ($P_1$) codes for the killer toxin polypeptide (Bostian, Hopper, Rogers and Tipper, 1980), and contains the immunity component (Sommers, 1973). The $m$ plasmid
is found in all killer toxin producing strains, it has never been found in the absence of the L plasmid. Sensitive strains contain the L plasmid only. $K_1$, $K_2$ and $K_3$ killers carry physically distinct species of mdsRNA with molecular weights of $1.1 \times 10^6$ to $1.7 \times 10^6$; $1.0 \times 10^6$; and $0.87 \times 10^6$ respectively (Bevan, Herring and Mitchell, 1973; Vodkin, Katterman and Fink, 1974; Wickner and Leibowitz, 1976; Young and Yagui, 1978). Study of $m_1$ deletion mutants has shown that $m_1$ dsRNA acts as a template coding for a polyadenylate tail needed for protoxin polypeptide translation. Genes have been identified that are responsible for maintenance and replication of the dsRNA sequence (Thiele, Hanning and Leibowitz, 1984). A third type of dsRNA(s) is present in suppressive strains (Vodkin et al, 1974). Killer dsRNA plasmids are maintained at a relatively constant copy number. Transmission occurs by cytoplasmic mixing during budding, mating and other forms of cell fusion. The role of dsRNA in killer systems has been reviewed by Tipper and Bostian (1984).

Chromosomal genes code for proteins involved in the maintenance and regulation of the plasmids, and the expression of the mdsRNA coded proteins (Table 1). The properties of mutations affecting the killer phenotype have been reviewed (Wickner, 1980; Bruenn, 1980). 29 chromosomal genes are involved in the maintenance of the mdsRNA plasmid (mak genes). They are scattered on 15 of the 17 yeast chromosomes. Mutations in any of these mak genes results in loss of killer ability. In each case the mdsRNA is lost, and the L plasmid retained. Each mak gene is thought to have a host specific function independent of the presence or absence of mdsRNA.

Four chromosomal genes (ski 1 to ski 4) regulate killer plasmid replication, a recessive mutation in any one of these genes results
Table 1. Chromosomal Genes Affecting the Killer Character of *Saccharomyces cerevisiae*
I Expression.

kex 1, kex 2; \( K_1^{-}R_1^{+} \) \([KIL-K_1]\)

kex 2 gene also needed for mating by strains

rex 1 \( K_1^{-}R^{-} \) \([KIL-K_1]\)

regulation of expression

II Killer Plasmid Maintenance

mak 1, mak 3 - mak 28; \( K_1^{-}R_1^{-} \) \([KIL-0]\)

mak 1 and mak 16 are temperature sensitive for growth

mak 3 and mak 10 mutants lose genetic cytoplasmic determinants \([NEX]\) \([HOK]\) and \([EXL]\)

pet 18 needed for growth and mitochondrial maintenance

spe 2 needed for spermidine and spremine biosynthesis, sporulation and optimum growth

mkt 1 needed for \([KIL-K_2]\) maintenance if \([NEX]\) is present

III Regulation

ski 1 -ski 4; \( K_1^{+}R_1^{+} \) \([KIL-K_1]\)

certain ski mak double mutants are \( K_1^{+} \) or \( K_1^{++} \); ski mutants can maintain \([KIL-sd_1]\)

KRB 1 \( K_1^{+}R_1^{+} \) \([KIL-K_1]\)

dominant: bypasses need for mak 7 or pet 18

Plasmid genes are in brackets in capital letters

Chromosomal genes in small letters.
in an increase in production of killer toxin indirectly by increasing the plasmid copy number, conferring the super-killer phenotype (Toh-e, Guerry and Wickner, 1978; Siddiqui and Bussey, 1981). The ski5 gene is a recessive mutant that appears to lead to toxin over secretion through a defect in the cell surface (Bussey, Steinmetz and Saville, 1983).

Two chromosomal genes KEX1 and KEX2 in addition to mdsRNA are needed for production and excretion of toxin (Wickner, 1974A). Mutants defective in these genes are K-R+ and maintain the m plasmid.

Three chromosomal genes kre1, kre2 and kre3 (killer resistance) are necessary for killing of a sensitive strain (Al-Aidroos and Bussey, 1978). Mutants defective in kre1 or kre2 have decreased binding of the toxin to sensitive cells.

A third class of nuclear mutations affecting the killer character are the rex mutants (regulation of expression). These 'suicide' strains produce toxin but are sensitive to their own toxin. These K-R- strains can grow normally above pH 4.8 (Wickner, 1974A).

Mutations of killer plasmid genes may effect plasmid maintenance or production of toxin. The recognised mutant phenotypes are summarised in Table 2 (Wickner, 1981).

2.32 Curing of a Killer Strain

Killer strains may be cured of the ability to produce toxin by growth at 37 to 40°C (Wickner, 1974B), by 5-fluorouracil treatment (Mitchel et al, 1973), or by growth in the presence of 13.3μg per plate of cycloheximide, slightly less than that needed to prevent growth (Fink and Styles, 1972). The resulting clones were mixed or
Table 2. Killer Plasmind Genotypes
[KIL-K₁] and [KIL-K₂]  wild type K₁ and K₂ killer plasmids
[KIL-O]  no killer plasmid
[KIL-n₁]  plasmid conferring resistance to K₁ toxin but not toxin production
[KIL-ts]  Killing is temperature sensitive
[KIL-i]  Confers toxin production but not resistance

[KIL-s]  Defective-interfering plasmid (suppressive) prevents replication of [KIL-K]
(Sommers, 1973)
(Vodkin et al, 1974)

[KIL-d]  defective maintenance and expression in haploid strains; normal in a/ diploid strains
(Wickner, 1976)

[KIL-b]  bypasses need for some mak genes; also confers super-killer phenotype
(Toh-e and Wickner, 1980)

[KIL-sk]  confers superkiller phenotype

[KIL-sd]  ski⁻ dependent plasmid, can be maintained only in ski⁻ strains
(Toh-e and Wickner, 1979)

[KIL-kd]  deletion mutant of a non-essential region of [KIL-k₁]
fully cured, and bred true over repeated trials. Cycloheximide acts by inhibiting protein synthesis on cytoplasmic ribosomes (Hartwell, Hutchinson, Holland and McLaughlin, 1970), which it is assumed blocks plasmid replication while enabling chromosomal and mitochondrial replication, resulting in the killer plasmid being diluted out over successive generations (Fink and Styles, 1972). All type $K_1$ and $K_3$ killers are readily cured by cycloheximide treatment, all type $K_2$ killers are cured by incubation at elevated temperature. Curing results in the loss of the $m$ plasmid as shown by polyacrylamide gel electrophoresis (Young and Yagia, 1978).

2.3.3 Purification, Stabilisation and Characterization of Killer Toxin

The extracellular nature of killer toxin was first indicated by the killing of 39% of a sensitive cell culture after a four hour incubation in cell-free filtrates of buffered media in which killer cells had been grown (Bevan and Makower, 1963). A 40% purification of killer toxin from growth media was achieved by fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ followed by dialysis, gel filtration and ultrafiltration (Woods and Bevan, 1968). The purified toxin was characterized as an unstable, protease-sensitive macromolecule, stable within the narrow pH range of 4.6 to 4.8. Addition of gelatine slowed down the loss of activity. Addition of 20% glycerol to killer toxin concentrate enables retention of activity indefinitely at 5°C and pH 4 to 5 (Ouchi, Kawase, Nakano and Akiyama, 1978).

The possibility that killer toxin may be associated with a yeast virus was eliminated, and the toxin identified as being a protein with a protein to polysaccharide ratio of 3:1 (Bussey, 1972).
Further characterization was achieved by the successful purification of active toxin in high yields by concentration in polyethylene glycol followed by chromatography through glycerol-controlled-pore glass (Palfree and Bussey, 1979). The amino acid composition suggested that the toxin was a monomer with the active unit having a molecular weight of 11470, and activity within the pH range of 4.2 to 4.6. The presence of only two hexose residues per polypeptide supported the theory that the killer toxin was a protein. A more recent purification method avoided the use of harsh treatment and enabled concentration of the toxin from S. cerevisiae strain 28 that was unaltered by the purification procedure (Pfeiffer and Radler, 1982). The killer strain was grown in a modified synthetic-B media at 25°C for three days (Heerde and Radler, 1978). A 2,000 fold concentration was achieved by repeated ultrafiltration, followed by purification by ion-exchange chromatography. The purified toxin contained 111 amino acid residues, comparable to the concentration in the toxin purified by Palfree and Bussey, 1979). The amino acid composition suggested a molecular weight of 14045 for the active unit, and an isoelectric point of 4.5 to 4.8. The total toxin had a molecular weight of 16,000, and a protein to carbohydrate ratio of 9:1, indicating that the toxin is a glycoprotein. They suggested that the use of 4M urea in previous purification methods had caused the removal of the carbohydrate fraction without affecting toxin activity. A considerable fraction of partially purified toxin is inactive, only 10 to 30% is active (Hutchings and Bussey, 1983). 10% of the total extracellular protein is killer toxin (Wickner, 1981).
2.3A Toxin Secretion

The cytoplasm of many toxin producing strains do not appear to contain detectable amounts of toxin, this may be due to extraction methods used. The concentration of intracellular toxin extracted from *S. cerevisiae* strain 28 is a thousand times more concentrated than extracellular toxin (Pfeiffer and Radler, 1982). Extracellular and intracellular toxins are identical suggesting that killer toxin is produced internally and liberated unmodified into the environment. Toxin is thought to be produced as a protoxin that is processed by proteolytic cleavage (Wickner, 1981). The use of temperature sensitive (sec) mutants has enabled the defining of the secretory pathway of toxin in *S. cerevisiae* that is similar to secretion pathways found in mammals (Norvick, Ferro, Schekman, 1981). At restrictive temperatures, toxin secretion in sec mutants is blocked, giving evidence that glycosylation, extension of the toxin does occur, prior to the protoxin entering the lumen of the endoplasmic reticulum where cleavage occurs. It has been suggested that glucosylation involves the addition of a non-N-glycosidic-linked polysaccharide that is necessary for efficient toxin secretion (Bussey, Saville, Greene, Tipper and Bostian, 1983).

Two chromosomal genes *kex*₁ and *kex*₂ are essential for toxin secretion. Kex mutants retain resistance but the glycosylated protoxin is not properly processed or secreted (Wickner and Leibowitz, 1976; Bussey et al, 1983). Secretion of other proteins with the exception of the α-factor pheromone, are unaffected by kex mutations (Leibowitz and Wickner, 1976).
2.3.5 Mechanism of Toxin Action and Effect on Sensitive Cells

Three successive stages are recognised for killer toxin action on sensitive cells leading to cell death. The process takes 3 to 4 hours (Middlebeek, van de Laar, Hermans, Stumm and Vogels, 1980).

I Binding of the toxin to primary cell wall binding sites, requires kre 1 and kre 2 gene products

II Transmission of the toxin to reactive sites in the plasma membrane

III Functional change

Figure 1 shows a two stage model of toxin action (Bussey, 1981).

Toxin binds to β-(1-6) D glucan receptor sites on the cell wall by an energy dependent reaction (Hutching and Bussey, 1983). Binding is inhibited in the absence of metabolic energy and at low temperatures (Middlebeek, Laar et al, 1980). Examination of toxin binding to S. cerevisiae strain S14A showed that each cell has $1.1 \times 10^7$ cell wall receptor sites, but binding of only $2.8 \times 10^4$ toxin molecules is needed to kill a cell (Bussey, Saville, Hutchings and Palfree, 1979).

Toxin binds to sensitive and resistant cells. Sensitive mutants lacking cell wall receptor sites cannot bind toxin, so are resistant to killing. When spheroplast cultures of these mutants are treated with toxin, killing occurs, suggesting that binding of the toxin to the cell wall is necessary to allow killer passage through the cell wall to active sites on the cell membrane. Only 1% of the toxin bound to the cell wall is transferred to the membrane and is active in the killing (Bussey, Sherman and Sommers, 1973). Binding of toxin to the plasma-membrane results in changes of membrane permeability to small molecules allowing release of potassium ions, ATP and other
Figure 1. Two Stage Model of Toxin Action

T indicates toxin molecule; R, the glucan membrane receptor.
STAGE I

outside

inside

plasma membrane

STAGE II

outside

inside
small molecules allowing release of potassium ions, ATP and other small molecules. Coinciding with leakage is acidification of the cell interior and inhibition of active transport of amino acids (Middlebeek, Laar et al, 1980; Bussey and Skipper, 1975; Bussey and Sherman, 1973). Intracellular changes observed are the co-ordinate inhibition of protein and nucleic acid synthesis and inhibition of D-glucose incorporation into macromolecules (Bussey and Sherman, 1973). Increased turbidity of whole and spheroplast cultures is observed due to a reduction of cell volume coinciding with loss of intracellular ATP (Bussey, 1974). No cell lysis is observed even after several days (Bussey, 1972).

Toxin binds immediately to the cell wall with a lag period of 50 to 90 minutes before physiological changes occur (Skipper and Bussey, 1977; Middlebeek, Stumm and Vogels, 1980). Further evidence suggests that binding of toxin immediately disrupts the electrochemical proton gradient across the plasma membrane causing inhibition of leucine transport and blocking proton excretion (de la Pena, Barros, Gascón and Ramos, 1980). It is proposed that the observed effects are due to the irreversible formation of ion-permeable channels in the phospholipid bilayer membrane, similar to the mode of action of bacterial colicins of the E1 functional class. A single toxin molecule may be responsible for channel formation. The formation of a channel changes the existing membrane potential, disrupting the internal negative potential required for amino acid transport (Kagen, 1983).

Sensitive cells enter a transient state after toxin binding, at this stage killing may be enhanced by the addition of ADP (Kotani, Shinmyo and Enatsu, 1977). Alternatively cells may be rescued by
appropriate treatments such as incubation in yeast extract-peptone media supplemented with $\text{Ca}^{2+}$ (Kotani et al., 1977); by removal or inactivation of the toxin at elevated pH (Bussey, 1972); or by adjustment of the media to physiological conditions (pH 6.5, 50mM KCl) (Middlebeek, Crutzen and Vogels, 1980). Rescue of cells by physiological conditions implies that the loss of $\text{K}^+$ and $\text{H}^+$ and subsequent decrease of the internal pH, is responsible for the lethal effect of killer toxin (Kagen, 1983). Rescue of intoxicated cells may be accomplished only before functional damage has occurred (Middlebeek, Laar, 1980).

2.3.6 Toxin Immunity

The immunity component is presumably a protein coded for by the m plasmid, but is distinguished as a separate activity from toxin production. The rex$_1$ gene is also implicated in immunity, but its role is not understood. The mechanism of immunity is unknown. It is thought that immunity probably occurs at stage II of the killing process, and that the immunity protein may prevent membrane damage by inhibiting toxin recognition of the membrane receptor (Bussey, 1980).

2.3.7 Commercial Application of Killer Wine Yeasts

In the fermentation of wine a specific strain is added to the must to assure the desired quality of the product. Contamination by wild yeast in wine fermentation can be a serious problem resulting in off flavours and even killing of sensitive wine yeasts by wild killer strains. Although many commercially available wine yeasts are
killers, little attempt has been made to seek practical wine applications for killers, presumably due to the low pH of grape juice at which the killer effect is depressed. Inhibition of growth of contaminant killer sensitive cells in grape juice has been successfully achieved by cytoduction of super-killer RNA plasmids into a wine yeast (Seki, Choi and Ryu, 1985). Crossing of killer and wine strains has enabled the production of killer hybrids for use as starter cultures. Using such hybrids, sensitive *S. cerevisiae* strains can be killed during fermentation (Shodo, 1984).

This study examines the effect of temperature, pH and ethanol on the killing of $K_0$ cells by concentrated $K_1$ toxin to examine the potential application of killer toxin to induce natural ageing of charmat style champagnes.