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STUDIES ON THE NON-SPECIFIC ESTERASES OF *SACCHAROMYCES CEREVISIAE*

A Thesis in Partial Fulfillment of
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ABSTRACT

Twenty wine-making and three laboratory strains of *Saccharomyces cerevisiae* were examined for non-specific esterases by Polyacrylamide Gel Electrophoresis. All wine-making strains contained the fast alleles of the *Est 1* and *Est 2* loci, confirming there is a selective advantage for the *Est 1f* and *Est 2f* genes in these strains. Only one wine-making strain carried the *Est 3* and *Est 4* genes, which was a much lower frequency than that published. The three laboratory strains all contained the *Est 1f* and *Est 2s* genes. A new non-specific esterase band, labelled *Est 5*, was identified by using a modified staining technique, which was apparently of low molecular weight as it travelled with the tracking dye front.

Fast and slow alleles of *Est 1* and *Est 2* were determined to be charge allozymes. *Est 2* proteins were considered to be polymeric, probably dimeric, and the *Est 1* proteins to undergo post-translational modification. Difficulty in resolving the *Est 4* band was overcome by adding Triton X-100 to cell suspensions before disruption, indicating this esterase protein may be particulate bound. Molecular weights were determined by Ferguson Plots to be 51,000 ± 10,000 daltons (*Est 2*), 60,000 ± 12,000 daltons (*Est 3*), 73,000 ± 15,000 daltons (*Est 1*), and 113,000 ± 23,000 daltons (*Est 4*).

No isolates of *S. cerevisiae* for comparison of allele frequencies could be made from mature locally-grown grapes, indicating that this species is rare in the New Zealand environment, which is in accordance with published studies.

No "inducible" non-specific esterases were found in strains examined at different stages in the life cycle, or by growth in different media. The level of esterase activity in cells increased throughout aerobic growth in liquid media, but was quickly lost during fermentation. Esterase activity during sporulation also decreased.

A non-specific esterase mutant was induced by ethyl methane-sulfonate and detected by the hydrolysis of α-naphthyl acetate incorporated into solid medium. This mutant lost expression of both *Est 1f* and *Est 2s* , as did subsequent mutants produced by hybridisation. Segregation of esterase-deficient to esterase-proficient spores after

hybridisation, showed that two unlinked loci were involved in esterase suppression, both genes being unlinked to *ade 1*, *Est 1* and mating type locus *MAT*. It is hypothesised these genes are a suppressor (*SUP*) and a mutated regulator (Reg^{Est-}).

Gas Liquid Chromatography was used to quantitatively determine volatile ester concentrations produced during fermentation. Selected wine-making strains and diploid strains produced by micromanipulation and having different non-specific esterase compositions were fermented to the limit of their ethanol tolerance in Reisling Sylvaner grape juice and Complete Defined Medium. Ethyl acetate, ethyl propanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate, 2-phenethyl acetate, *n*-hexyl acetate and *iso*-pentyl acetate were all quantitated. A maximum error of $\pm$30% was determined for differences in ester concentration between two fermentations using the same strain. Correction for differences in fermentation ability by different strains was attempted, and the resulting ester concentrations compared qualitatively. Results indicate that differences in volatile ester concentrations between strains are not due to the esterase composition. The non-specific esterases probably have little if any influence on wine bouquet as the majority of ester production is late in fermentation when esterase activity has ceased.

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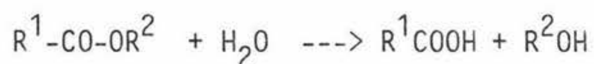
INTRODUCTION

1.1 Esterases

Esterases are ubiquitous enzymes in all organisms examined (eg. bacteria, fungi, protozoa, insects, molluscs, vertebrates) and are detected by the hydrolysis of esters:



$$R^1\text{-CO-OR}^2 + H_2O \dashrightarrow R^1COOH + R^2OH$$

Ethyl and acetate esters are split in all organisms by several esterases, which normally are not artifacts of one enzyme. Their biological functions are generally unknown. Esterases have been classified into groups depending on substrate utilisation and sensitivity to numerous compounds. Two groups of esterases with known cellular activity are readily distinguished. Lipases, which are able to hydrolyse or synthesise glycerol esters, break down triglycerides and diglycerides to release long chain fatty acids, and can be detected by hydrolysis of glycerol tributyrin. Cholinesterases, found in multicellular animals, hydrolyse esters of choline. Acetylcholinesterases readily hydrolyse acetylcholine, a neural transmitter, while pseudocholinesterases preferably hydrolyse butylcholine (Wheeler *et al.*, 1972). Those found in *Saccharomyces cerevisiae* hydrolyse esters of simple alcohols and fatty acids have been termed carboxylic acid hydrolases (Schermers *et al.*,., 1976), but are normally referred to as non-specific esterases. This seems a more appropriate name because of the wide range of substrates readily hydrolysed - including many "artificial" esters - and because the main cellular functions of these enzymes have not yet been resolved.

The earliest detection of esterase activity in yeasts was with *Hansenula anomola*, determinations of residual ethyl acetate being by a hydroxamic acid test (Smith and Martin, 1963). Most subsequent investigations have been carried out with *S. cerevisiae* , activity being detected colourimetrically by the hydrolysis of "artificial" substrates ρ-nitrophenyl esters and naphthyl esters. The naphthyl ester method is also used to detect esterase bands by Polyacrylamide Gel Electrophoresis. Sites of activity, physiological influences, substrate specificity, and other influences on enzyme activity have also been studied using naturally-produced fatty acid esters, detecting the free acids enzymatically or by Gas Liquid Chromatography.

1.2 Genetic Analysis of Esterases from *Saccharomyces cerevisiae*

Polyacrylamide Gel Electrophorisis (PAGE) was used to separate five non-specific esterase bands from laboratory strains (Berking and Hauschild-Rogat, 1970). Analysis demonstrated that the five bands represented four genes, corresponding to two loci segregating independently each with two alleles, and labelled as *Est 2f* (fast allelle), *Est 2s* (slow allele), *Est 1f*, and *Est 1s*, in the order of the fastest to slowest migrating enzyme bands. The fifth band was a hybrid enzyme occurring in strains heterozygous for *Est 2*. The *Est 2* locus has been located closely linked with the *ade 2* locus (0.31 centimorgans apart) on chromosome 15, but the location of *Est 1* has not been determined (Strobel and Wohrmann, 1972). The *Est 1* alleles have subsequently been shown to segregate as three bands which migrate as a distinct unit (Wohrmann and Lange, 1980).

Wine-making strains of *S. cerevisiae* isolated in Europe, were found to carry only the fast alleles (*Est 1f*, *Est 2f*), and about two thirds of these strains also carried two further genes; labelled *Est 3* and *Est 4*; which were expressed constitutively or inducibly together. These new genes segregated independently from each other and were not linked to either the *Est 1* or *Est 2* loci (Wohrmann and Lange, 1980). Twenty strains of *S. cerevisiae* [1] were examined by PAGE by Campbell *et al.* (1972). All had a common fast band, undoubtedly *Est 2* and probably *Est 2f*, but only two strains showed a second band, presumably an *Est 1* allele. One other strain (described as *Saccharomyces chevalieri*) had a second band probably representing the *Est 3* gene, and five strains (described as *Saccharomyces uvarum*) an unidentifiable fast-migrating band.

Alleles of the *Est 1* locus had no apparent effect on the rate of vegetative reproduction, whereas the *Est 2f* allele caused a definite selective advantage in a series of haploid strains produced by micromanipulation and possessing different *Est 1* and *Est 2* alleles. *Est 1s* homozygotes exhibited an increased meiotic rate but the *Est 2* locus had

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[1] Classification of Barnett *et al.* (1983).

no demonstrable influence in hybridised diploid strains with different *Est 1* and *Est 2* allele combinations (Strobel and Wohrmann, 1975).

Selective advantages occurred for certain alleles in competition experiments where two strains were grown together (20°C and 29°C), in two inbred haploid lines differing only in esterase marked gene blocks, which were partly or totally reversed at higher incubation (38°C). Selective advantage of each gene block, however, was applicable only for each inbred line (Strobel and Wohrmann, 1977).

1.3 Esterase Fractionation by Column Chromatography

Schermers *et al.* . (1976) found two esterase activity peaks (probably *Est 1* and *Est 3*) by gel filtration in a 55-75% saturated ammonium sulphate protein fraction from a brewery strain. Molecular weight determinations gave values of 130,000 (*Est 1*) and 67,000 daltons (*Est 3*). Five esterase activity peaks have been identified using bakers yeast cell-free protein samples, and these have been labelled as E_{1A} , E_{1B}, E_2, E_3 and E_4. Esterases E_{1A}, E_{1B} , E_2 and E_3 almost certainly represent respectively *Est 4*, *Est 1* , *Est 3* and *Est 2* of Wohrmann and Lange (1980). Molecular weight determinations were 230,000 (E_{1A}), 90,000 (E_{1B}), 45,000 (E_2), 22,000 (E_3) and 6,000 daltons (E_4), and temperature optima, pH optima (all about pH 8.0), % activity of each enzyme from the total esterase activity (Suomalainen, 1980), as well as esterase profiles from different ammonium sulphate fractions, inhibition by several inhibitors, and hydrolysis of different acyl length (C2-C12) esters have also been determined for each enzyme (Parkkinen, 1980).

In protein fractions, E_2 (*Est 3*) represented about 68% total esterase activity using ρ-nitrophenyl acetate as substrate. E_4 has been suggested to be a proteolytic fragment of one of the other enzymes because of its low molecular weight, high heat stability, and increased activity in fractions treated with zymolase. E_{1A} (*Est 4*) is thought to be particulate bound, as it is partly removed by high centrifugation, and shows loss of activity with re-chromatography (Parkkinen, 1980). The observed correlation between molecular weights suggests possible monomer-multimeric relationships between the esterases, but positive (Schermers *et al.*, 1976) or negative (Parkkinen, 1980) evidence for this hypothesis is insufficient for either conclusion.

1.4 Location, Substrate Specificity, and Enzyme Activity of Non-specific Esterases in *Saccharomyces cerevisiae*.

Two esterase fractions with totally different specificities have been identified from bakers yeast. Those hydrolysing 2-oxo-gluteric acid diethyl ester, but not ρ-nitrophenyl or naphthyl esters, have not been studied, but are known to be located inside the yeast cell (Parkkinen *et al.*, 1978). The second esterase fraction, termed here as the non-specific esterases, hydrolyse ρ-nitrophenyl and naphthyl esters instead of 2-oxo-gluteric acid diethyl ester.

The non-specific esterases occur in both cell wall digests and sphaeroplast lysates, but no activity is involved with the cell envelope of sphaeroplasts (Wheeler and Rose, 1973; Parkkinen *et al.*, 1978). Activity on both sides of the cell membrane involves the same five esterases from Column Chromatography. Protein fractions from either side of the envelope have similar activity profiles with ρ-nitrophenyl acetate (Suomalainen, 1981), though specificity towards several substrates differed between the intracellular and periplasmically located esterase fractions (Parkkinen and Suomalainen, 1982a). Depending on the method of evaluation and substrate used, 40-80% (reliable values 50-65%) of the total non-specific esterase activity is located outside the plasma membrane, and the activity is loosely bound to the cell wall or is located in the periplasmic space (Parkkinen *et al.*, 1978), but is not liberated into the medium. The suggestion that the bulk of the enzyme activity is located outside the cell membrane is supported by histochemical staining (Wheeler and Rose, 1973). Activity inside the cell is thought to be primarily located in vacuoles (Malile and Weimken, 1967).

Many esters have been found to be hydrolysed. Those tested include α-naphthyl acetate, β-naphthyl esters (fatty acids C2-C12), ρ-nitrophenyl esters (fatty acids C2-C12), ethyl esters (fatty acids C2-C12), various acetate esters, and also $_{14}C$ labelled sterol esters (Wheeler and Rose, 1973). Cell-free esterase fractions show differences in substrate specificity due to both alcohol moiety and fatty acid moiety carbon chain length (Parkkinen, 1980). Different activity profiles result when different carbon length acyl groups (C2-C12) are esterified to produce ethyl, ρ-nitrophenyl, or β-naphthyl esters (Parkkinen and Suomalainen, 1982a). Activity towards different esters

is also confused by the variation between use of whole cells and different protein extracts (Parkkinen *et al.*, 1978).

Very little hydrolysis of acetate esters, ethyl acetate, *iso*-pentyl acetate, and 2-phenethyl acetate, occurred with intact cells. Activity with ethyl esters increased markedly with fatty acyl group length to ethyl octanoate, which was hydrolysed twice as readily as ethyl decanoate and quadruple that of ethyl hexanoate. However, isolated esterase fractions overall showed greatest activity towards ethyl propanoate, ethyl pentanoate, and ethyl hexanoate (Parkkinen and Suomalainen, 1982a).

Ethyl octanoate hydrolysis occurred at over 50% maximum relative esterase activity in the pH range 3.0 to 9.0, greatest activity occurring between pH 7.0 and pH 9.0 with intact cells in buffered solutions (Parkkinen and Suomalainen, 1982a). With esterase protein fractions, little activity remained below about pH 6.0 (Parkkinen, 1980). At varied ethanol concentrations (5%, 10%, 15% v/v) hydrolysis of ethyl octanoate with an isolated E_2 (*Est 3*) fraction was slowed and the final residual ester concentration increased with increased alcohol concentration. Equilibria between esters and acids in ethanol solutions has also been shown, the equilibrium attained depending not only on the concentrations of esters, acids and alcohols, but also on the pH, the final ester concentration remaining higher at lower pH's. The molar ratio of ethyl octanoate/octanoic acid in esterase protein extract solutions is about the same as that found in fermentation solutions under the same conditions (Parkkinen and Suomalainen, 1982b).

A comparison of final ester concentration in worts fermented by different brewing strains and the specific activity of their esterases, showed that ester concentration and esterase activity were proportional to each other in some simple manner (Schermers *et al.*, 1976).

1.5 Esterases of Other Yeasts and Fungi

A survey of a large group of yeasts (94 strains) by Campbell *et al.* (1972) showed that yeast species could be grouped into accepted taxonomic groups according to esterase patterns, and it was suggested that strictly aerobic yeasts produced more bands (up to four) than facultative anaerobes (one or two bands).

Crude protein extracts from *Hansenula anomala* did not show ester synthesising ability (Tabachnick and Joslyn, 1952; Smith and Martin, 1963) but the cell-free extracts showed hydrolysis towards a wide range of aliphatic and aromatic esters over a wide pH range (Smith and Martin, 1963).

Protein extracts from a strain of *Brettanomyces bruxellosis* had similar esterase activity profiles at different pH's to *S . cerevisiae* , with maximum activity at pH 7.6 for hydrolysis of ethyl acetate, and little activity remained below pH 4.0. Levels of activity in whole cells were dependent on growth media, aeration rate and temperature. The esterases were not released into the culture media or into cell washings. Activity (per 300 ml medium) increased during growth and began declining before cessation of the log phase of growth. Specific esterase activity (per mg dry weight cells) began declining early in the growth phase, activity levels and periods of change varying with the growth media (Spaepen and Verachterl, 1982).

In *Candida lipolytica* , intracellular esterase activity increased with growth, and again little or no activity occurred in the media. Esterases were detected in *Aspergillus niger* only in late conidiation when intracellular lipids were decreasing (Lloyd *et al* ., 1971). Four esterases have been highly purified (Iwai *et al.*, 1983), which hydrolyse aliphatic esters of short chain fatty acids and acetate esters of phenols, but not methyl esters of aromatic carboxylic acids or acetate esters of aromatic alcohols (Okumura *et al.*, 1983).

Intensities of esterase bands on polyacrylamide gels varied with age of culture in a yeast-like fungus and new bands were also detected with arthrospore formation, suggesting important functions in different growth phases. Intensity, but not number of esterase bands, increased in *C. lipolytica* and *A. niger* from the early exponential to late exponential growth phase in liquid media (Lloyd *et al.*, 1971). *Candida utilis* bands varied between one and three, depending on concentrations and composition of the artificial liquid media used, but not due to the pH (Campbell *et al.*, 1972).

Esterase extracts from a yeast-like fungus, *C . lipolytica* and *A. niger* all hydrolysed glycerol tributyrin. Intracellular lipid decreased

when esterase activity increased and in *C. lypolytica* growth on media containing glycerol tributyrin showed induction of esterases. These observations suggested that esterases may be linked with lipid metabolism (Lloyd *et al.*, 1971).

1.6 Synthesis of Cellular Esters by *Saccharomyces cerevisiae*.

Early workers showed that levels of esters found in fermented media could not be accounted for by the spontaneous condensations of organic acids and alcohols, but were formed by the activity of yeast cells (Davies *et al.*, 1951; Peel, 1951). Crude protein extracts from *S. cerevisiae* and other yeasts showing esterase hydrolysing ability did not synthesise esters by simple condensation; esters were formed by alcoholysis of Coenzyme A organic acid derivatives in protein extracts when fatty acyl CoA's were added to the reaction mixture (Howard and Anderson, 1976; Yoshioka and Hashimoto, 1981). The enzyme involved, a fatty acid transferase, is located in the cell membrane, and the purified enzyme showed the greatest activity at pH 7.0 to 8.0, 30°C, and activity towards alcohols of C1 to C6 carbon length (Yoshioka and Hashimoto, 1981).

Purified E_2 (*Est 3*) from brewing yeasts when compared with crude protein extracts readily synthesised ethyl acetate (Schermers *et al*., 1976) and ethyl octanoate (Suomalainen, 1981) without addition of acyl CoA's, and to set up equilibria either through hydrolysis or synthesis of ethyl aliphatic esters with their acids and ethanol (Parkkinen and Suomalainen, 1982a).

The rate of synthesis of different esters in fermentation of sugar solutions varied significantly between strains or groups of strains (Nykanen and Nykanen, 1977; Nordstrom, 1964b). Significant differences in ethyl acetate formation have also been shown with ethanol concentration (Nordstrom, 1962a), pH, and acetic acid concentration (Nordstrom, 1961). In synthetic medium containing lower fatty acids, all tested strains gave the same distribution between corresponding ethyl esters. None of the strains were able to form ethyl esters from propionic acid, *iso*-butyric acid, or *iso*-pentanoic acid (Nordstrom, 1964b).

The distribution of esters formed during sugar fermentations between cells and medium varied in relation to the carbon length in ethyl esters. Acetate esters and ethyl esters up to and including ethyl hexanoate are almost completely released from intact cells, while ethyl dodecanoate is not released at all (Nordstrom, 1964c; Nykanen *et al* ., 1977). More esters are released at higher fermentation (Suomalainen, 1981), the proportion of ester retention also depending on the strain (Nykanen *et al*., 1977). Lower fatty acids, particularly the intermediate ones (C4 to C10) show high toxicity for yeast. It has been suggested than an important function of ester synthesis in growing cells is the removal of these troublesome fatty acids (Nordstrom, 1964c).

In early growth ethyl acetate was not formed as the fatty acid transferase activity decreases, and insufficient amounts of alcohols are available as substrate, though acetyl-CoA is present. Increase in ethyl acetate was due to decreased consumption of acetyl-CoA and availability of alcohols (Thurston *et al*., 1981a; Yoshioka and Hashimoto, 1984a). Competition for metabolism of acetyl CoA can greatly affect ethyl acetate production (Nordstrom, 1963a) as does competition for Co-enzyme A in production of various ethyl esters through their respective acyl CoA's (Nordstrom, 1962b, 1963b). Fatty acids of varying chain length compete in ester synthesis, either for Co-enzyme A, or for the fatty acid transferase (Nordstrom, 1964a, 1964c).

The rate of synthesis of esters increased markedly in fermentation when synthesis of lipids stopped (Thurston *et al*., 1981a; Thurston *et al*., 1982). The effect of lipids on synthesis of esters, fusel alcohols and medium chain fatty acids in worts, could be explained solely by the unsaturated fatty acid fraction (Taylor *et al* ., 1979). Saturated fatty acids only slightly inhibit the transferase activity, while unsaturated fatty acids, though stimulatory for growth (Taylor *et al*., 1979), strongly inhibit the enzyme and ester synthesis, suggesting a control by fatty acid composition in the cell membrane for transferases (Yoshioka and Hashimoto, 1983). Formation of flavour esters is linearly correlated to melting points of incorporated fatty acids, and in Sake yeast the relationship between *iso*-pentyl acetate formation and % linoleic acid in cellular fatty acids is inversely proportional (Ishikawa and Yoshizawa, 1979). Addition of linoleic acid caused a shift from medium to long chain fatty acid synthesis. Reduction in ester synthesis by linoleic acid is direct by inhibiting ester-

producing enzymes, or by allowing alternate use of acetyl CoA for synthesis of saturated fatty acids (Thurston *et al.*, 1981b; Thurston *et al.*, 1982). A deficiency of nitrogenous compounds also results in the inhibition of enzyme activity by causing increased accumulation of unsaturated fatty acids (Yoshioka and Hashimoto, 1984b).

1.7 Esters in White Wines

Esters are important aroma components of white wines, and have a major influence on wine quality. The main origin of esters, as is the case for the majority of the aroma components, is from the yeast fermentation, only small amounts originating from the grape must. Final ester levels in the complex "naturally" fermented beverage vary greatly due to the numerous conditions applied during fermentation. Major influences on ester concentrations have been shown to be due to fermentation temperatures (Daudt and Ough, 1973a), yeast strain, and grape variety, with initial SO_2 levels (Daudt and Ough, 1973b), grape maturity, sugar content, and juice clarity (Houtman *et al.*, 1980) also having signif-icant influences.

Numerous studies have been carried out over the last twenty years by Gas Liquid Chromatography (GLC), identifying compounds from the volatile fractions of grape juice, wines (eg. Chaudhary *et al.*, 1968), worts, beers, other fermented beverages and distillates. These are reviewed by Schreier (1979), who lists 52 esters that have been identified from grapes, the majority of which are present in only trace amounts. Almost 100 esters have been detected in white wines and from quantitative studies (eg. Suomalainen and Nykanen, 1966) 16 have been found to occur in greater than trace amounts. These are listed in Table 1.

Though most quantitated esters are generally in concentrations of 0.1 - 10 mg per litre, many have a definite influence on the bouquet and aroma of wines, as indicated by odour thresholds from an odour-stripped white wine with individual ester additions (de Wet, 1978). Ethyl hexanoate, ethyl octanoate, ethyl decanoate, *iso*-pentyl acetate, *n*-hexyl acetate and 2-phenethyl acetate are known to have enhancing effects on odour quality and intensity. Below 100 mg per litre ethyl acetate has no significant influence, and about this concentration there is a decrease in bouquet quality, but increase in intensity (Van der Merwe and van Wyk, 1981).

Table 1. Quantitatively Measurable Esters of White Wines, and their Odour Thresholds.

Esters	Recorded Concentrations [1] (mg/l)	Odour Thresholds [2] (mg/l)
Ethyl formate *	trace - 8	155.2
Ethyl acetate *	30 - 285	12.3
Ethyl propanoate *	trace - 1.2	1.84
Ethyl hexanoate *	0.1 - 2.0	0.08
Ethyl octanoate *	0.2 - 2.38	0.58
Ethyl decanoate *	trace - 0.71	0.51
Ethyl dodecanoate *	0.0 - 0.4	
2-Phenethyl acetate *	0.0 - 2.6	1.51 - 1.80
n-Propyl acetate *	0.0 - 0.26	4.74
iso-Propyl acetate *	trace - 0.35	
iso-Pentyl acetate *	0.4 - 9.52	0.16
n-Hexyl acetate *	0.0 - 2.0	0.67
Ethyl 2-hydroxy-propanoate	10 - 400	
iso-Pentyl 2-hydroxy-propanoate	trace - 0.6	
Diethyl succinate	0.8 - 20	
Methyl anthranilate	trace - 3	

[1] values from Daudt and Ough (1973a, 1973b); de Wet (1978), Houtman *et al*., (1979); Schreier (1979) and van Wyk (1981).

[2] values from de Wet (1978).

* in volatile fraction

1.8 Summary

All *Saccharomyces cerevisiae* strains contain two esterases produced from two unlinked loci, each locus is represented by either a slow or fast allele. Wine-making strains possess fast alleles of both loci (*Est 1f* and *Est 2f*), and commonly have two further genes from unlinked loci, which also occur in brewing and baking yeasts. In some wine strains these genes (*Est 3* and *Est 4*) appear to be induced, which could be explained by slight changes in growth conditions, or life cycles, as demonstrated with other yeasts and fungi. Extrapolation suggests that the non-specific esterases may have an important function during growth.

Isolated esterases show a wide and varied variety of esters hydrolysed, though only one, E_2 (*Est 3*), has been demonstrated to have synthesising ability. It is almost certain that esters tested for hydrolysis do not represent the full range of substrates utilised by these enzymes.

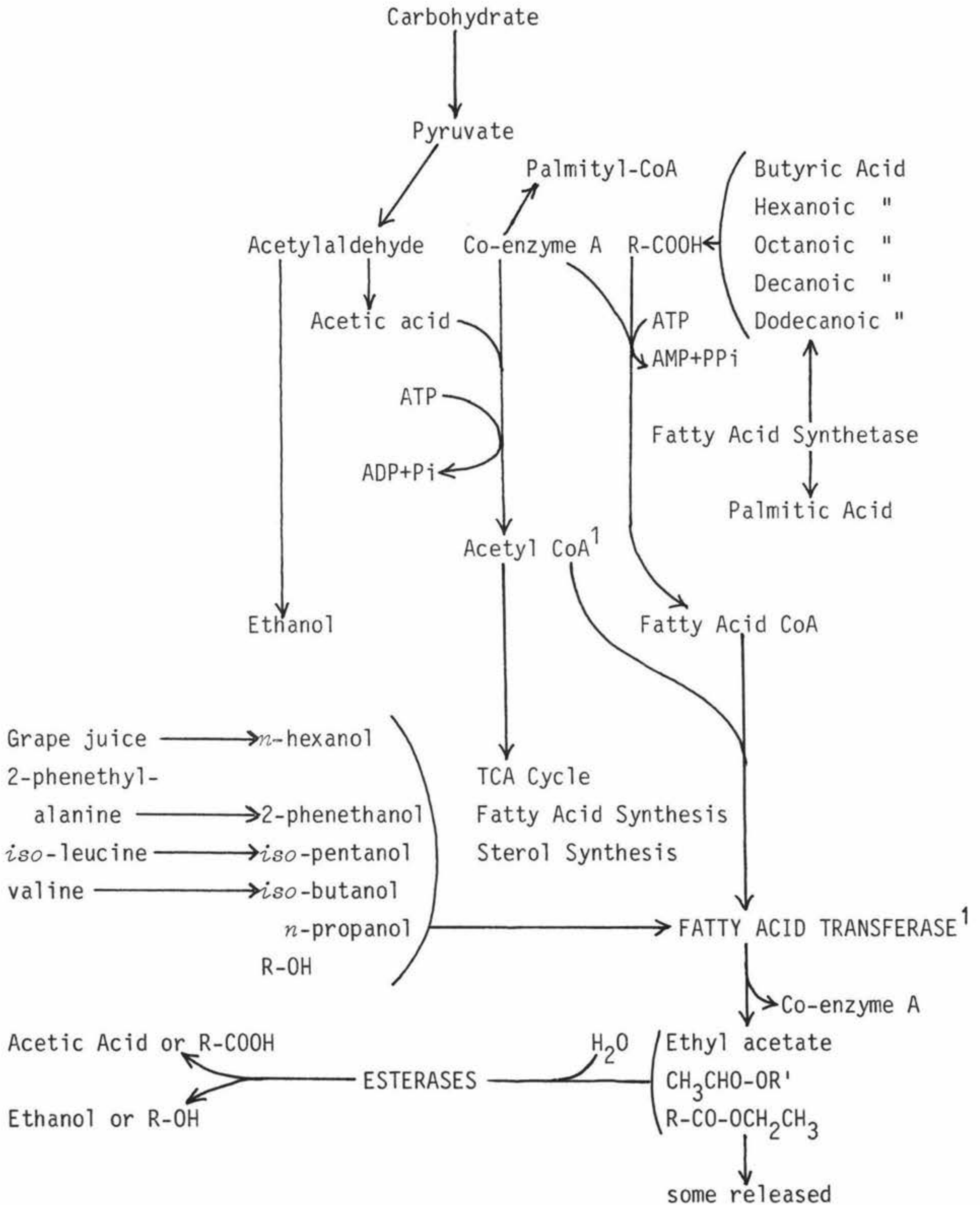
Aliphatic and aromatic esters are synthesised during growth by a membrane-bound enzyme, a fatty acid transferase, by alcoholysis of acyl CoA derivatives. Though untested, reversibility of the reaction may be possible, as seen with lipases. Like esterases, this enzyme probably has a wider range of substrates than tested, and though it is considered a means of removing fatty acid toxicity from the cell, its main biological function remains unresolved from present studies.

Grape must is a complex growth medium, and in white wines a major determining factor is the level of volatile compounds, particularly esters, which occurs in the final product. Final levels of esters, in equilibrium with their alcohol and fatty acid, have been shown to be influenced by numerous factors. Some of those demonstrated include:- yeast strain, grape variety, sugar levels, acidity, alcohol concentration, temperature, period of growth, levels of lipids, competition for Co-enzyme A and acetyl CoA, between fatty acyl CoA's, and competition for substrates by enzymes (fatty acid transferase and esterases). Availability of alcohols and fatty acids must also be an important influence. Acetic acid and alcohols are end products of fermentation, the fusel alcohols being produced by decarboxylation of the corresponding α-keto acids (Nordstrom, 1963c). Fatty acids are

produced by Fatty Acid Synthetase, and *n*-hexanol is released from grapes. Some of these influences on ester synthesis and hydrolysis in *S. cerevisiae* are depicted in Figure 1.

Many points are still open to investigation about esterase activity and ester production during fermentation. Three lines of investigation were chosen for this study. Firstly, studies on the distribution of different esterases in sub-populations of *S. cerevisiae*, particularly the *Est 1* and *Est 2* fast alleles and *Est 3* and *Est 4* in wine-making strains. Secondly, studies on the influences on esterase gene expression and esterase activity of anaerobic and aerobic fermentations over a period of time during fermentation. Thirdly, studies on the influences of different esterase genes on levels of esters produced during fermentations of grape juice and Complete Defined Medium, as yeast strains of specific esterase gene composition could be constructed using hybridisation or cross-breeding techniques.

Figure 1. Synthesis and Hydrolysis of Ethyl Acetate, Ethyl Esters and Acetate Esters by *Saccharomyces cerevisiae* During Sugar Fermentations.



1 Suppression by unsaturated fatty acids.

MATERIALS AND METHODS

2.1 General Media

General Growth Medium (GGM)

Yeast Extract	10 g	
Difco Bacto-peptone	10 g	Made up to 1 litre with
Glucose	20 g	distilled water.

50 ml samples were placed into 100 ml flasks, and autoclaved at 70 kPa (10 psi) for 10 minutes.

Yeast Morphology Agar (MYGP)

Difco Malt Extract	3 g	
Yeast Extract	3 g	
Difco Bacto-peptone	5 g	Made up to 1 litre with
Glucose	10 g	distilled water.
Agar	20 g	

Autoclaved at 105 kPa (15 psi) for 15 minutes.

Glucose Nutrient Agar (GNA) Presporulation Medium

Difco Nutrient Agar	2.3 g	
Yeast Extract	1 g	Made up to 100 ml with
Glucose	5 g	distilled water.
Agar	0.5 g	

Dispensed into clean bijou bottles, autoclaved at 70 kPa (10 psi) for 10 minutes, and slanted before cool.

Potassium Acetate (PA) Sporulation Medium

Potassium Acetate	1 g	Made up to 100 ml with
Yeast Extract	0.25 g	distilled water.
Agar	3 g	

Dispensed into clean bijou bottles, autoclaved at 70 kPa (10 psi) for 10 minutes, and slanted before cool.

Minimal Medium (MM)

Difco Yeast Nitrogen base (without amino acids)	7 g	Made up to 1 litre with
Glucose	20 g	distilled water.
Agar	30 g	

Autoclaved at 105 kPa (15 psi) for 15 minutes.

When used with adenine-requiring mutants, adenine was added at a level of 20 mg per litre.

2.2 Polyacrylamide Gel Electrophoresis (PAGE)

Esterases can be visualised after PAGE by appropriate staining using naphthyl esters as substrate, and coupling the naphthol produced by the enzymatic reaction to diazotized aromatic amines to yield intensely-coloured products. This was accomplished using α-naphthyl acetate as substrate stained with Fast Blue B Salt.

The experimental PAGE technique used a modified Ornstein (1964) discontinuous buffer in vertical slab gels on a Studier (1973) style PAGE apparatus.

2.2.1 Yeast strains examined for esterase genetic compositions

Twenty Southern Hemisphere wine-making strains of *Saccharomyces cerevisiae* were chosen for study from those held by the Microbiology and Genetics Department, Massey University. Four standards of known esterase composition but unknown origin were received from Wohrmann, K., and Lange, P. Four haploid laboratory strains were also examined. Strains, sources, and comments are listed in Table 2.

2.2.2 Protein extraction and storage for esterase genetic composition analysis

A procedure similar to that of Wohrmann and Lange (1980) was used for cell growth. Test strains were streaked onto MYGP plates for isolated colonies. Six large isolated colonies were selected. Four were inoculated into 50 ml of GGM, one into 50 ml of MYGP broth (MYGP agar minus agar), and one into 50 ml of CDM, all in 100 ml flasks. Inoculated flasks were incubated at 30°C on an orbital shaking platform for 24 to 48 hours.

Cultures were centrifuged at 2,000 G on a SS34 rotor in a Sorvall RC2-B centrifuge for 10 minutes. The cells were washed twice by resuspension in 40 ml distilled water, and centrifuged at 2,000 G for 10 minutes. The cells were finally resuspended in 5 ml of sample buffer. The cell suspensions were standardised to a standard turbidity using a Klett-Summerson Photoelectric Colorimeter with a Blue 42 filter. This was accomplished by using a 1/100 dilution, correcting to a turbidity of 30 units. The cell suspension was corrected to an equivalent turbidity of 3000 units.

Table 2. Yeast Strains[1] Examined for Esterase Genetic Composition

Wine-making Strains	Source	Comments
R[2]28	New Zealand	
R80	New Zealand	
R92	South Africa	
R93	South Africa	*Saccharomyces chevalier*[4]
R99	South Africa	
R107	South Africa	
R108	South Africa	
R109	South Africa	
R176	South Africa	
R177	South Africa	
R179	South Africa	
AWRI[3]62	Australia	
AWRI63	Australia	
AWRI65(R171)	Australia	Strongly flocculent
AWRI79	Australia	
AWRI89	Australia	
AWRI93	Australia	
AWRI105	Australia	
AWRI116(R11)	Australia	
R2	Australia	

Table 2 (continued)

Other strains	Source	Comments
Control Est 1s/1s 2s/2s	Wohrmann, K. & Lange, P.	Standard
Control Est 1f/1f 2f/2f	Wohrmann, K. & Lange, P.	Standard
Control Est 1s/1f 2s/2f	Wohrmann, K. & Lange, P.	Standard, heterothallic
Control Est 1f/1f 2f/2f 34	Wohrmann, K. & Lange, P.	Standard
R92/2/47	From a R92/X2928 cross	haploid **a** mating, *ade1*, *gal1*
X 2928	Laboratory strain	haploid, α mating, *ade1* *gal1*, *ura3*, *his2*, *leu1* *met14*
S-1α		haploid, standard mater type α
S-2**a**		haploid, standard mater type **a**

[1] All strains diploid unless stated.

[2] R = Ruakura, New Zealand.

[3] AWRI = Australian Wine Research Institute.

[4] Now included in *Saccharomyces cerevisiae* (Barnett *et al.*,1983).

Cells were broken by using an Aminco French Cell placed in an industrial press set to a working pressure of 155 to 180 x 10^3 kPa (10 to 12 tons per square inch). To avoid clumping and reduction of french cell efficiency, cell suspensions were thoroughly suspended by vortex mixing before pressing. Cellular debris was removed by centrifugation in a SM24 rotor at 10,000 G for 20 minutes. Aproximately 3 ml of supernatant was removed by drawing into a 5 ml disposable syringe through a hypodermic needle. The supernatant was placed in clean bijou bottles with tight-sealing lids, and stored at -70°C until required.

Throughout the protein extraction procedure, cells and supernatant were kept as cool as possible to minimise possible esterase enzyme degradation by proteases.

2.2.3 Protein extraction modifications for other PAGE experiments

To check on possible band variations over a long period of incubation, three strains were placed in flasks of GGM, and one flask of each was extracted at 1, 3, 5, 7, 9, 11, 13 and 15 days as described.

Difficulty in resolving the *Est 4* band of Wohrmann and Lange (1980) was encountered. Strains containing *Est 4* were adjusted to an equivalent turbidity of 6000 units, with 1% or 2% Triton X-100 (a non-ionic detergent) added before or after cellular disruption by the French Cell, to determine if increased *Est 4* could be produced.

To check on possible band variations during sporulation, strains were grown on GNA and PA slants as for sporulation in Section 2.3.1. After 3 days' growth on PA slants, cells were harvested, suspended in distilled water, washed as described, and suspended to an equivalent turbidity of 6000 units before protein extraction.

2.2.4 Preparation of stock solutions

Running Gel Acrylamide

Acrylamide	30 g
Methylene bis acrylamide	0.5 g
Distilled, deionised water to	100 ml

Stacking Gel Acrylamide

Acrylamide	30 g
Methylene bis acrylamide	1.6 g
Distilled, deionised water to	100 ml

To prepare the acrylamide solutions, the acrylamide was dissolved in about 70 ml distilled water and stirred till the solution had returned to room temperature. The methylene bis acrylamide was added, dissolved, and the solution made up to 100ml. The solution was filtered through a single layer of Whatman No. 1 filter paper, and stored at 4°C in the dark for up to three months.

Tris-Glycine Reservoir Buffer

Trizma base	6.07 g
Glycine	28.8 g

Made up to one litre with distilled, deionised water.

Lower Tris, or Running Gel Buffer pH 8.8 (1.5M Tris HCl)

Trizma base	18.17 g
12N HCl to	pH 8.8
Distilled, deionised water to	100 ml

Upper Tris, or Stacking Gel Buffer pH 6.8 (0.5M Tris HCl)

Trizma base	6.06 g
12N HCl to	pH 6.8
Distilled, deionised water to	100 ml

Sample Buffer pH 6.8

This is 25% strength stacking gel buffer pH 6.8, diluted with distilled deionised water.

Both Tris HCl gel buffers were prepared by adding the Trizma Base to 70 ml distilled deionised water, dissolved, adjusted to the correct pH with HCl, and diluted to 100 ml. After all buffers had been checked for correct pH, and adjusted if necessary, they were stored at $4^{o}C$ for up to one month.

Bromophenol Blue Tracking Dye

Bromophenol Blue	0.05 g	
Glycerol	40 ml	Stored at
Distilled water to	50 ml	room temperature.

2.2.5 Preparation of Polyacrylamide Gels

The slab polyacrylamide gels were produced between two glass plates held apart by 1.5 mm thick perspex spacers. One glass plate (back plate) had a section removed to allow contact between the gel and the upper reservoir buffer (see Figure 2). Before plate assembly each plate was washed in detergent (1-5% Extran) and thoroughly rinsed with distilled water. Spacers and track-forming combs were also cleaned with detergent, thoroughly rinsed, and dried. After several runs the glass plates could be immersed overnight in chromic acid (5 g sodium dichromate dissolved in 5 ml distilled water, and added slowly with continuous stirring to 100 ml of conc. H_2SO_4). This ensured a high level of plate cleanliness, but was not found to be necessary.

For assembly, one plate was placed on the bench, and a small continuous beading of petroleum jelly (produced from a disposable syringe) was placed along the two sides and the bottom a few millimetres from the plate edge. The perspex spacers were firmly pressed into place along these three edges, the bottom one being placed on first. Another beading of petroleum jelly was then added onto the perspex spacers. The second plate was lowered onto the spacers, and also pressed on firmly. The assembled plates were finally clamped onto a perspex stand by use of

3-inch "Bulldog" clips. Before the gel solutions were added, the plates were checked for complete sealing, especially where the perspex spacers contacted each other. Plates were handled by the edges throughout to avoid surface contamination.

After comparative tests with 5.0%, 7.5% and 10% running acrylamide gels, 7.5% running gel acrylamide was selected to carry out enzyme analysis. These were produced by adding the stock solutions at room temperature to a small conical flask in the order given in Table 3. All work with unpolymerised poly-acrylamide was with disposable gloves including handling of plates before eletrophoresis. After addition of ammonium persulphate and tetraethylmethylethyldiamine (TEMED), the solution was thoroughly shaken to produce even polymerisation. Immediately after preparation the as-yet unpolymerised solution was poured between the plates making sure no air bubbles were trapped. Those trapped could be removed by rocking the unpolymerised gel. When a running gel length of 105 to 110 mm was reached, the gel was placed in a vertical position and immediately overlaid with several mls of distilled water to allow polymerisation to occur under anaerobic conditions. The running gel was normally left at room temperature ovenight (approximately 18 hours) to allow complete polymerisation. Alternately, gels could be used after polymerisation for about one hour at room temperature.

The stacking gel solutions at room temperature were poured into a conical flask in the order given in Table 3, and thoroughly mixed as before. The water overlay was poured off the running gel and 2 to 3 ml of as-yet unpolymerised stacking gel added to wash. This washing enabled good adherence between the gels. The remaining unpolymerised stacking gel was poured between the plates until the top of the back plate was reached. Finally, the track-forming comb was pushed between the plates into the stacking gel approximately 15 to 18 mm. Polymerisation usually occurred in 30 minutes. The comb was removed, and the wells and plates rinsed before use.

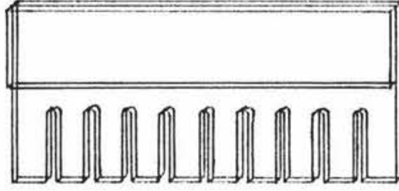
The polymerised gel, spacers and plates were unclamped from the stand, and the bottom spacer was removed to allow contact between the running gel and the bottom reservoir buffer. Two beads of petroleum jelly were added to the Studier-style PAGE apparatus (Figure 3), one half-way up the stand, the other in a ⊔ shape at the top. These ensured that buffer did not leak behind the plates thus causing a

Figure 2. Gel Forming Equipment at 1/2 Natural Size.

From front: back glass plate, perspex spacers and track-forming comb, front glass plate, gel-forming stand.

Glass plates are of heavy glass, 130 x 170 mm; the back plate with a 100 x 20 mm section removed. Perspex spacers are 12 mm wide, spacers and comb being made of 1.5 mm thick perspex. The comb has 7 mm wide tracks formed by cutting 3 mm grooves to a depth of 18 mm.

Plates and spacers are held together by petroleum jelly; these are held to the gel-forming stand by 3 inch "bulldog" clamps.



shorting of the electric current. The plate assembly was clamped to the electrophoresis apparatus by "bulldog" clips, the back plate in contact with the vertical stand. Sufficient reservoir buffer pH 6.3 was added to both upper and lower reservoirs to cover both ends of the gel. Air bubbles were removed fom the lower end by using a syringe with a bent hypodermic needle filled with reservoir buffer. A check was made to make sure the perspex spacers had not moved, enabling buffer to pass between spacers and gels.

Table 3. Preparation of Polyacrylamide Gels (made in the order given).

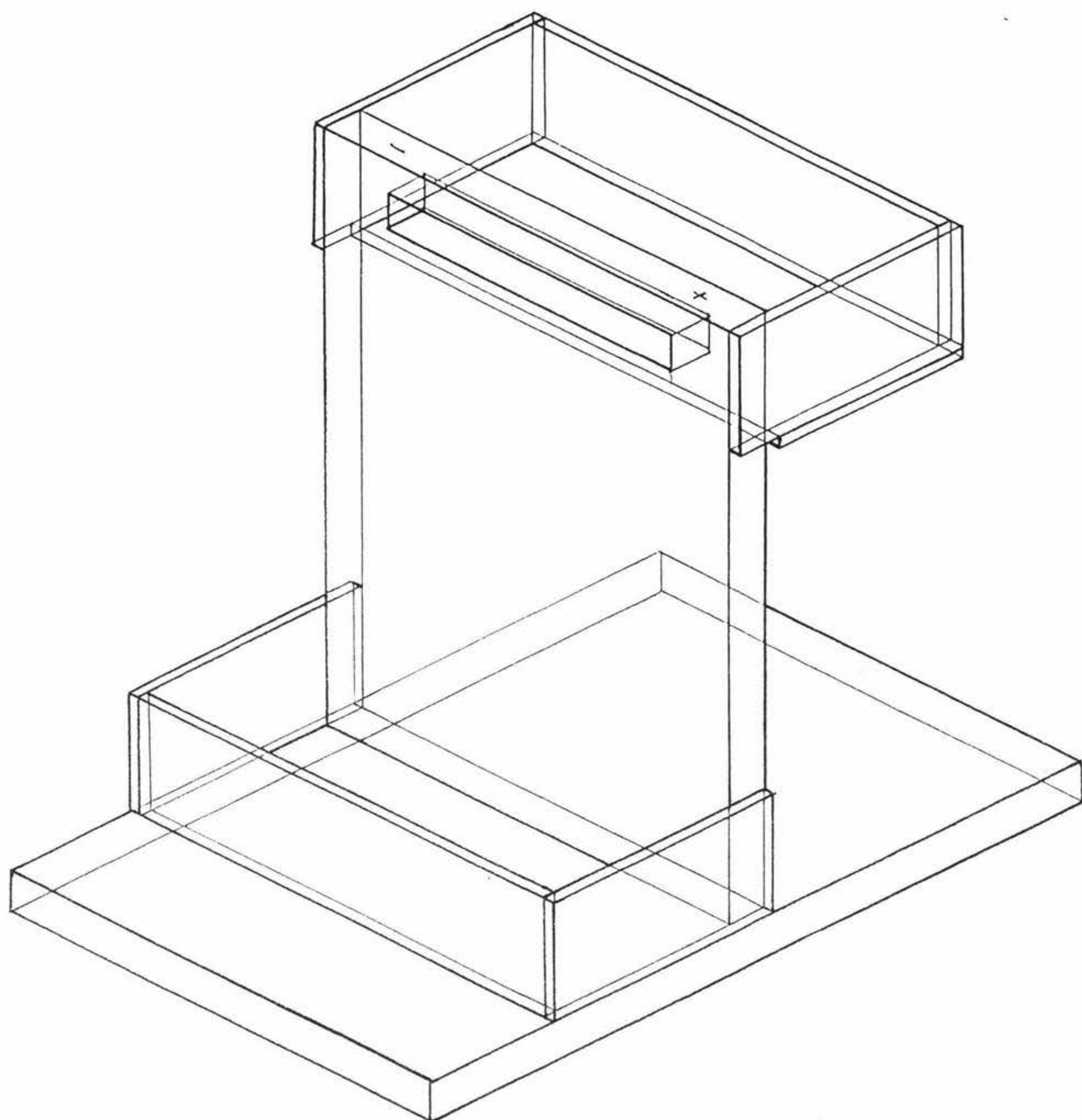
Solutions	Running Gel 5.0%	Running Gel 7.5%	Running Gel 10.0%	Stacking Gel
Running Gel buffer pH 8.8	5.0 ml	5.0 ml	5.0 ml	-
Stacking Gel buffer pH 6.8	-	-	-	2.5 ml
Running Gel Acrylamide	3.33 ml	5.0 ml	6.66 ml	-
Stacking Gel Acrylamide	-	-	-	1.2 ml
Distilled deionised water	11.66 ml	10.0 ml	8.33 ml	6.3 ml
Ammonium persulfate[1]	0.05 ml	0.05 ml	0.05 ml	0.03 ml
TEMED[2]	0.01 ml	0.01 ml	0.01 ml	0.01 ml
Approximate volumes	20.0 ml	20.0 ml	20.0 ml	10.0 ml

[1] A 10% (w/v) solution in distilled deionised water made fresh each day.

[2] Tetraethylmethylethyldiamine; stored at $4^{o}C$.

Figure 3. Studier Style PAGE Apparatus at 1/2 Natural Size.

The negative lead going to the bottom well, passes from the connecting terminal located in the vertical perspex slab, through a groove on the left-hand side, into the reservoir and across the entire width. The positive lead going to the top reservoir well, passes from the connecting terminal through the vertical perspex slab into the top reservoir and across the entire width. Both leads were of platinum wire. The electrophoresis apparatus was made entirely of perspex.



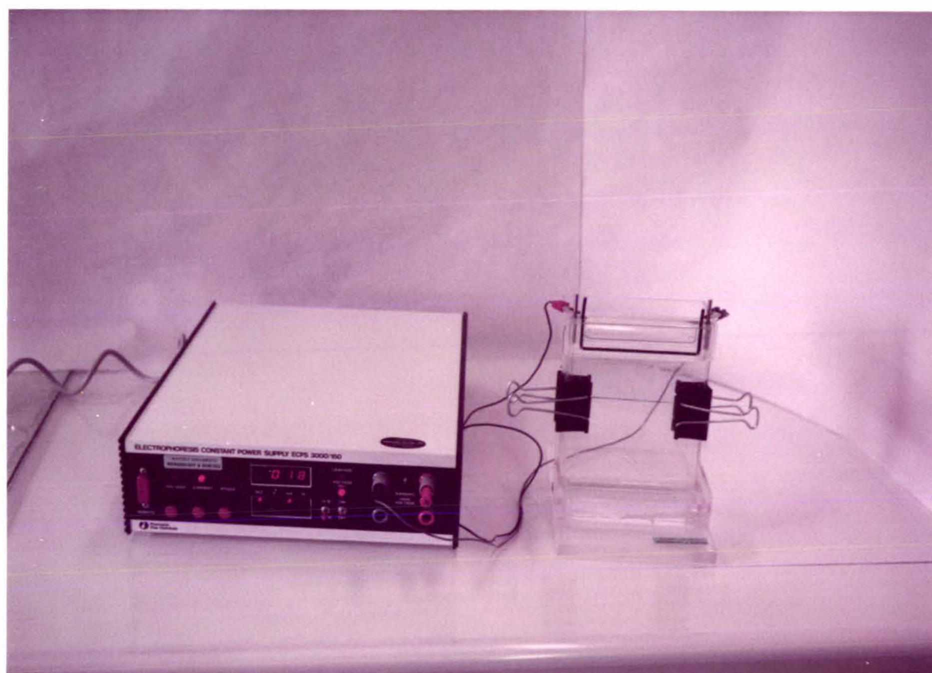
2.2.6 Running of Polyacrylamide Gels

Protein samples were thawed at room temperature just before required. 20 μl of bromophenol blue tracking dye was added to 180 μl of thawed protein sample, the tracking dye representing 10% of the total volume. When the protein and tracking dye had mixed, samples were added to each stacking gel well by use of an automatic adjustable pipette in a slow, even motion. The addition of glycerol in the tracking dye makes the sample heavier than the reservoir buffer, causing it to sink to the bottom of the wells. Loading was as close to the bottom of each well as possible to avoid excessive mixing of sample with reservoir buffer. The track-forming comb produced 10 wells. Those not loaded with sample were loaded with an equal volume of sample buffer with 10% tracking dye solution. This helped tracks to run parallel. Initially 10 μl, 20 μl, 30 μl, 40 μl, 50 μl and 60 μl samples were tested to determine the optimum loading. All subsequent experiments used 50 μl sample loading.

After sample addition, the electrophoresis apparatus was connected to a power pack (Pharmacia ECPS 3000/150); the top reservoir being negative, the bottom reservoir (direction of electrophoresis) being positive. The power pack was set at a constant current of 12 milliamps, and the samples were run until they reached the stacking gel/running gel interface. When this occurred, the current was increased to 18 milliamps and maintained at this level until the tracking dye was about 3 to 5 mm from the bottom of the running gel. The power pack was turned off, and the glass plate/gel sandwich removed from the electrophoresis apparatus. The glass plates were carefully separated and one plate and spacers removed. The bottom left-hand corner of the exposed gel was removed from the running gel to enable gel orientation. The stacking gel was separated from the running gel and removed. Finally the running gel was removed from the remaining glass plate into a plastic container for staining, by lifting one corner, inverting, and allowing the gel to peel off under its own weight.

Plate 1. Running a Polyacrylamide Gel.

The gel, enclosed in the perspex spacers and glass plates, has been attached to the electrophoresis apparatus by two 3 inch "bulldog" clips, after the bottom spacer had been removed. The tracking dye front has already passed out of the stacking gel, and can be seen moving through the running gel as a blue line.


ELECTROPHORESIS CONSTANT POWER SUPPLY ECPS 3000/150
018

2.2.7 Gel Staining for Non-specific Esterases

PAGE esterase staining methods are all very similar, the technique described here, derived from Lawrence *et al.* (1960), is the method of Campbell *et al.*, (1972) with minor modifications. A second method was later developed in an attempt to resolve a possible fifth esterase band, labelled *E4*. by Parkkinen (1979), distinguished by sephadex chromatography using ρ-nitrophenyl acetate (ρNPA) as substrate.

Standard PAGE Esterase Staining Method:

40 mg of α naphthyl acetate (αNA) were dissolved in 4 ml of 50% (volume/volume) acetone in distilled water. This was added to 96 ml of 0.1M tris-maleate buffer pH 6.4. The gel was added and allowed to hydrolyse the αNA for 20 minutes at room temperature. 100 mg of Fast Blue B Salt were dissolved in 20 ml of Tris-maleate buffer pH 6.4 and added after 20 minutes. Staining occurred for a further 40 minutes, then the staining solution was poured off and the gel stood in tap water to destain. The gel was placed in fresh water each day and examined after three days.

Modified PAGE Esterase Staining Method:

30 mg of ρNPA were added to 80 ml of 0.1M Tris-maleate buffer pH 6.4. The gel was added and allowed to hydrolyse the ρNPA for 20 minutes at room temperature. 40 mg of αNA were dissolved in 4 ml of 50% (volume/volume) acetone in water and added to 16 ml of Tris-maleate buffer pH 6.4. This was added to the reaction mix after 20 minutes for a further 20 minutes for hydrolysis of the αNA. 100 mg of Fast Blue B Salt dissolved in 20 ml of Tris-maleate buffer pH 6.4 were finally added after 40 minutes, and staining allowed to occur for a further 20 minutes. The staining solution was then poured off and the gel stood in tap water to destain. The gel was placed in fresh tap water each day and examined after three days.

In both methods, the 0.1M Tris-maleate buffer pH 6.4 was made just before required. The αNA and Fast Blue B Salt were stored at 4°C, and the αNPA at -20°C. 0.1M solutions of Trizma base and maleate were also stored at 4°C.

2.2.8 Molecular Weight Determinations by PAGE

Native protein PAGE cannot directly be used for molecular weight (MW) determinations, since the proteins migrate not only by protein size, but also by protein charge. Good estimations can, however, be achieved by constructing Ferguson Plots, where standard proteins are run with the unknowns at different total acrylamide concentrations (%T) against R_f values calculated from the tracking dye front, and slopes or Retardation Coefficients (K_R) calculated by least Mean Squares Regression. A standard curve of K_R against MW can then be produced for the standard proteins.

Protein standards were dissolved in PAGE sample buffer pH 6.8 at a concentration of 1.0 mg per ml, and Triton X-100 added to 1%. Protein standard solutions were stored at -16°C ready for use. Proteins used are listed in Table 4. Both standard proteins and yeast extracts; R93, R92 and a hybrid Est 1s/1s 2s/2s strain, were loaded at 50 μl on the same gel. The standards were grouped together, as were the yeast extracts. After PAGE, the gel was cut between the two groups of proteins, the yeast extracts being stained by the standard αNA method, and the protein standards by the Coomassie Brilliant Blue total protein stain.

Table 4. Protein Standards for MW Determinations of Esterases by PAGE.

Protein	Source	MW	
Myoglobin	bovine muscle	17,500	3 isoenzymes
Albumin	human serum	69,000	3 isoenzymes
Hexokinase	yeast	102,000	2 subunits (51,000)
Phosphoglucose Isomerase	bakers yeast	120,000	4 subunits (30,000)
Alcohol Dehydrogenase	yeast	140,000	4 subunits (35,000)

Gels of acrylamide concentrations; 5%, 5.6%, 7%, 7.5%, 9%, 11%, 13% and 15% were run to produce Ferguson Plots. Care was taken to use the same stock solutions, protein solutions, temperature, and other conditions, so that differences in R_f values were due only to the change in total acrylamide (%T).

Coomassie Brilliant Blue Protein Stain

*iso*propanol	250 ml
Glacial acetic acid	100 ml
Coomassie Brilliant Blue R	0.4 g

Made up to 1 litre with distilled water, stored at room temperature.

Gels were placed in 100 ml of the total protein stain and left overnight. The stain was poured off, the gel rinsed, and destained in several washes of 7% acetic acid to destain for several days, until no more stain left the gel.

2.2.9 Recording of Results

Primary recording was carried out by examining the gels visually after three days' destaining in water. The gel was placed on an illumination box with a opal-white screen, where appropriate R_f values calculated from the tracking dye front and stacking gel/running gel interface were recorded.

Permanent records were by gel photography or densitometer recordings. For photography, selected gels were wetted and placed on an illumination box. Care was taken to avoid trapping air bubbles. Photographs were taken with a standard 35 mm black and white film using normal exposure times. A straw-coloured filter was used to increase film sensitivity to the red stain.

For densitometer readings, each gel track was cut to approximately 10 mm widths, thoroughly wetted, and placed on clean ISCO glass slab gel carrier plates. An ISCO type 6 Optical Unit with a 580 nm filter conneted to an ISCO Model UA-5 monitor was used. The gel guides on the optical unit were fitted with 0.50 mm photometric slit inserts. The optical unit run at the fast running speed of 150 cm per hr without a blank in the reference cell, and the monitor set to a chart speed of 150 cm per hr with a range sensitivity of 0.5.

2.3 Wild Yeast Isolation

The isolation of wild *Saccharomyces cerevisiae* strains from locally-grown grapes was attempted to enable comparison by PAGE of wine-making and environmentally-selected *S. cerevisiae* strains. Three different isolation techniques were tried. The first relied on selection of yeast under highly acidic conditions (pH 3.5 - 3.8) excluding most bacterial growth, the second enriching micro-organisms that could grow fermentatively under acidic conditions (pH 4.5 - 4.8), and the last, a novel method using ethanol (2% volume/volume) with antibiotics for selectivity.

The yeasts that were isolated were then examined macroscopically and microscopically, and possible *S. cerevisiae* isolates were passed through a series of physiological and biochemical tests for identification.

2.3.1 Isolation, Purification and Morphological Examination

Isolation Using Acidic Conditions:

Two grapes were added aseptically to 50 ml of GGM in 100 ml flasks with the pH adjusted at 3.5 to 3.8 with 1.0M HCl. Flasks were incubated at 30°C for three days.

Isolation using *Saccharomyces* Enrichment Medium:

Glucose	8.0%	
KH_2PO_4	6.5%	Made up with distilled water.
Yeast Extract	0.5%	

The final pH was adjusted at 4.5 to 4.8 with 1.0M HCl before autoclaving. Medium was autoclaved in septum-stoppered glass bottles at 105 kPa (15 psi) for 10 minutes. Two grapes were added, the bottles topped up with spare sterile medium, and the glass stoppers replaced excluding air bubbles. Bottles were incubated at 30°C for five to seven days.

Selective Isolation Using Ethanol and Antibiotics:

To 50 ml GGM in 10 ml flasks were added 1.0 ml absolute ethanol (2% ethanol in GGM) and 500 units Penicillin and 1000 units Streptomycin

filter sterilised in 2.0 ml distilled water (10 units/ml Penicillin and 20 units/ml Streptomycin). Two grapes were added and flasks were incubated at 30°C for three days.

After incubation of the inoculated media, dilutions were plated onto MYGP plates, and incubated at 30°C for 48 hours. Colonial types that were unlikely to be *Saccharomyces cerevisiae* (pigmented, powdery and spreading) were ignored, and remaining discrete colonies examined microscopically. Possible *S. cerevisiae* isolates were chosen after examination (multilateral budding on a narrow base, moderate-sized cells, spherical/oval to elongate, no mycelium or well-developed pseudomycelium, and no odd-shaped ascospores or basidiospores), grown up for 48 hours at 30°C on MYGP plates, and placed in tests as shown in the scheme in Figure 4.

2.3.2 Identification of Isolates

Fermentation Tests:

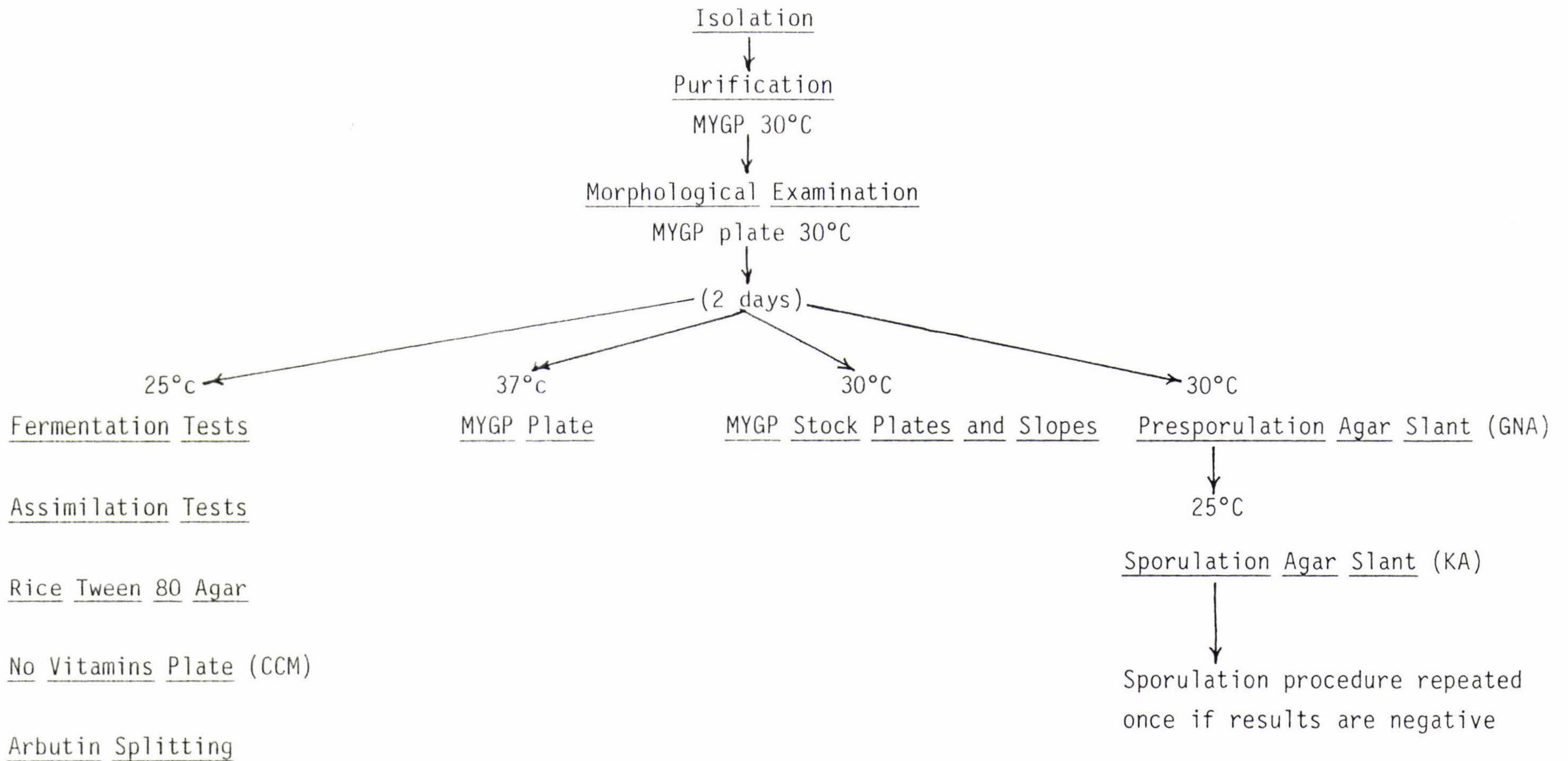
100 ml bromcresol purple indicator solution were added to 900 ml distilled water, and 9.0 ml amounts dispensed into test tubes with Durham tubes. 20% carbohydrate solutions in distilled water were filter sterilised, and 1.0 ml added to each autoclaved test tube. Inoculated tubes were incubated for up to three weeks at 25°C. Positive fermentation was taken as production of gas, acid production (purple to yellow colour change) taken as a positive utilisation. The following carbohydrates were tested:-

glucose	lactose
galactose	trehalose
maltose	raffinose
sucrose	cellobiose

Assimilation Tests:

3.35 g Difco Yeast Nitrogen Base (without amino acids) were added to 450 ml distilled water with bromcresol purple indicator and autoclaved. 50 ml filter-sterilised 20% carbon compound solutions were

Figure 4. Identification Scheme for Wild Yeast Isolates



added just before pouring. Cultures were spotted onto the surface and plates incubated at 25°C for up to two weeks.

For utilisation of nitrogen compounds, 5.85 g Difco Yeast Carbon Base were added to 45 ml distilled water with bromcresol purple indicator and autoclaved. 50 ml filter-sterilised 20% nitrogen compounds solutions were added just before pouring. Utilisation of carbon and nitrogen compounds was taken as positive with both growth and colour change. Compounds tested were:-

glucose (control)

maltose	melibiose
galactose	lactate
sucrose	D-xylose
lactose	ribose
trehalose	inositol
raffinose	inulin
cellobiose	mannitol
melezitose	salicin
ethanol	rhamnose
L-arabinose	erythritol

potassium nitrate	potassium nitrite
ethylamine HCl	

Growth with no Vitamins:

Complete Mineral Medium (CMM) was made by mixing together the mineral components of CDM, and adding 2.0% agar and bromcresol purple indicator before autoclaving. Plates were incubated for up to two weeks at 25°C. Positive results were taken as visible growth with acid production.

Arbutin Splitting:

0.5% arbutin was added to a 0.1% Yeast extract and 2% agar solution then autoclaved. Before pouring, one drop of 1.0M $FeCl_3$ was added to each plate. Inoculated plates were incubated for up to two weeks at $25^{o}C$. Positive results showed a dark brown halo in the agar around colonies. (Figure 4 - Identification Scheme for Wild Yeast Isolation.)

Growth at $37^{o}C$:

Cultures were inoculated onto MYGP plates and incubated at $37^{o}C$ for one week.

Sporulation:

Cultures were streaked onto GNA slants and incubated at $30^{o}C$ for 40 to 44 hours. Cultures were then streaked onto PA sporulation slants and incubated at $25^{o}C$ for up to three weeks. Slants were initially examined after five days, then on every alternate day. If no sporulation was visible after two weeks, the sporulation procedure was repeated once. Cells were examined unstained in distilled water, and by ascus staining with Malachite Green at two weeks.

Morphological Conversions:

Cultures were streaked into the agar of plates of Taschdjians Rice Tween 80 Agar, and sterile coverslips placed over part of the streak. Plates were incubated at $25^{o}C$ and examined at one and two weeks. Areas, both exposed and covered, were examined for pseudohyphae, hyphae, sexual structures, and chlamydospore production.

2.3.2 Identification of Isolates

After completion of all the tests, isolates were placed into genera by use of the key of Lodder (1970). Speciation was accomplished by using both keys of Lodder (1970) and lists of biochemical tests of Barnett and Pankhurst (1974). A final check on current classification was made by referring to Barnett *et al.* (1983).

2.4 Induction of Esterase Deficient Mutants

Many means of inducing mutants in eukaryotic organisms have been found to be effective on yeasts in general, and *Saccharomyces* strains in particular. Two of these, ultraviolet irradiation (UV) and ethyl methanesulfonate[1] (EMS) were chosen in an attempt to induce esterase mutants. The method of Loprieno and Clarke (1965) was used for UV induction, and that of Lindegren *et al.* (1965) for EMS induction. Possible mutants were identified by growth on agar plates with incorporation of αNA, and visually estimating the level of hydrolysis after staining.

2.4.1 Induction by Ultraviolet Irradiation

Yeast strains X2928 and R92/2/47 were incubated for 48 hours on MYGP plates. One loopful of cells (5×10^6 to 2×10^7 cells) was thoroughly suspended in 5 ml 0.1M citric acid and 0.2M $Na_2HPO_4.H_2O$ buffer pH 7.0 and placed in a sterile glass petri dish. Irradiation was carried out in a UV box 35 cm from a Philips Fluorescent UV tube (574 P/40 OA, TUV 15W) at about 27°C. Irradiation was begun by removing the petri dish lid to expose the cells to the UV rays, and replaced to stop irradiation. At appropriate times 0.5 ml samples were removed and added to 4.5 ml sterile distilled water. Appropriate dilutions were plated out onto MYGP plates. All sampling and platings out were done in a darkened room with only red light illumination. This was to avoid photo-reactivation repair of damaged DNA. Plates were sealed in a cardboard box, incubated at 25°C, and examined at four days.

For production of UV kill curves, samples were removed from UV irradiation at zero time, 20, 40, 60, 80, 100, 120 and 180 seconds, and plated onto MYGP and MM + 40 mg per l adenine. Percent auxotrophic mutants of percent surviving Colony Forming Units (CFU) was calculated as the difference of colonies between MYGP and MM + 40 mg per l adenine plates. Each dilution was plated four times for calculation of a mean value.

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[1] Methanesulfonic acid ethyl ester.

2.4.2 Induction by Ethyl methyl sulfonate

Yeast strains X2928 and R92/2/47 were incubated on MYGP plates for 48 hours. One loopful of cells (5 x 10^6 to 2 x 10^7 cells) was suspended in 9.2 ml of sterile 0.2M phosphate buffer pH 7.0 and 0.5 ml of filter-sterilised 40% glucose in distilled water was added. 0.3 ml of Sigma EMS was added, and the mixture shaken before incubation at 30°C in a shaking water bath. At appropriate times 0.2 ml aliquots were removed, and added to 9.8 ml of autoclaved 6% sodium thiosulphate for 10 minutes, to stop further EMS activity. After inactivation, cells were further diluted in sterile distilled water, and plated onto MYGP. Plates were incubated at 25°C and examined at four days.

For production of an EMS kill curve for strain R92/2/47, samples were removed at zero time, 20, 40, 60, 80, 100 and 120 minutes. Each dilution was plated onto four plates, and from two runs a mean calculated.

2.4.3 Isolation of Induced Esterase Mutants

Esterase Mutant Isolation Method:

Selected colonies from the MYGP plates were stabbed into two plates in identical patterns; one MYGP, the other MYGP with added αNA in a glass petri dish. To find the best concentration of αNA, trials were carried out using 1 μg αNA per ml, 10 μg αNA per ml and 100 μg α NA per ml. Plates were incubated at 30°C for 48 to 72 hours.

1 mg per ml of Fast Blue RR Salt was added to 50/50 (volume/volume) chloroform in methanol (made fresh daily), and 5 to 7 ml were poured onto the MYGP plus αNA plates. Glass petri dishes were used as plastic plates were dissolved by chloroform. Plates were examined after one hour. Around the colonies a deep red halo was observed; the diameter ofeach halo was taken as an indication of esterase activity when compared to colony size on the MYGP master plates. Colonies with small halos but good colonial growth were seleted as possible esterase mutants and streaked onto fresh MYGP plates, ready for growth and protein extraction, to enable confirmation by PAGE of true esterase mutants.

Modified Mutant Isolation Method:

After a suitable mutant had been isolated by the previous method, the following technique was developed and gave similar results when the mutant, X2928 and R92/2/47 were compared.

Cells were plated at a suitable dilution onto MYGP plates containing 10 g αNA per ml. Plates were incubated at 30^oC for 48 to 72 hours, when colonies were clearly visible. A 0.4% soft agar overlay containing 1 mg Fast Blue B Salt per ml was added, and plates incubated again at 30^oC for a further 24 hours. Deep red halos were visible on examination. Halo size was again taken as an indication of esterase activity when compared to colony size.

2.5 Yeast Hybridisation

Conventional techniques of yeast hybridisation were employed to breed yeast strains of defined esterase allele composition (Mortimer and Hawthorne, 1969; Thornton, 1981).

Haploid cultures of different *Est 1* and *Est 2* alleles and mating type compositions were produced from the heterothallic control Est 1f/1s 2f/2s. Diploid cells were produced by mating various haploid strains from the haploid library and with a mutant esterase strain from R92/2/47. Esterase compositions of haploid strains were determined by PAGE.

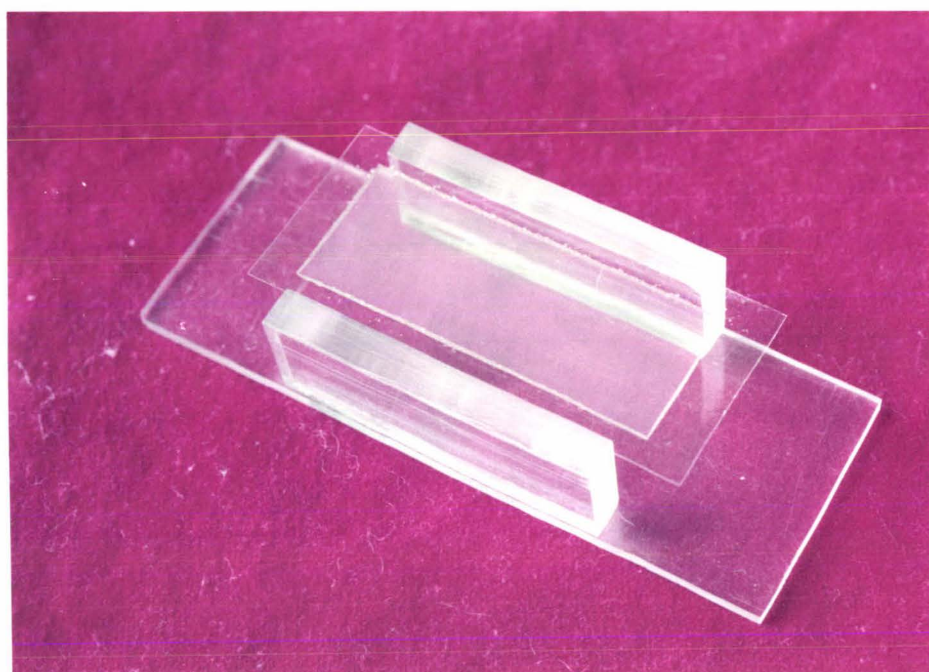
A Prior manual yeast micromanipulator and a Reichert-Jung Micro Star 110 fixed stage microscope placed on an antivibration table were used for micromanipulation. The micromanipulator was placed so that it could be used with the left hand, and was fitted with a blunt-ended glass needle. Dissection was done on an inverted agar slab placed in a perspex dissecting chamber (Plate 2).

2.5.1 Preparation of Cells

Sporulation was induced in diploid yeast strains by incubation on GNA presporulation agar slants at 30°C for 40 to 44 hours with the lids loosened. A large inoculum was streaked onto PA sporulation slants and incubated at 25°C. Slants were checked at five days for sporulation. The culture was recycled through the sporulation induction procedure if sporulation had not occurred. A small amount of sporulating culture was removed at the sixth to ninth day from the slant by a sterile toothpick, and placed into 0.2 ml of 20% (volume/volume in sterile distilled water) Sigma ρ-glucuronidase (snail juice enzyme). The cells were thoroughly mixed by vortexing and incubated at 30°C for 20 to 30 minutes. The ρ-glucuronidase causes partial digestion of the ascus wall, allowing easier separation of the ascospores from the ascus (Johnston and Mortimer, 1959). The mixture was lightly vortexed and used immediately. Spore suspensions were stored for several days at 4°C for re-use if necessary.

Plate 2. Dissection Chamber with Inverted Agar Slab.

Note the trimming of the slab to fit in the dissection chamber.



Haploid cells were incubated on MYGP at 30^{0}C for 24 to 48 hours. Haploid mutants, because of their slower growth, were incubated for 48 to 72 hours. A small amount of culture from an isolated colony was placed in 0.2 ml sterile distilled water. Cells were thoroughly vortexed before use to avoid clumping. Suspensions were used immediately, or stored for only a few hours, as cells soon began to bud.

Mating types were determined by incubating haploid yeast strains and standard mater strains S-1α and S-2**a** on MYGP plates at 30^{0}C for 24 hours. Small amounts of culture were mixed separately with both S-1α and S-2**a**. Resulting growth was checked at 24 hours for the presence of zygote formation (zygote formation with S-1α shows mating type **a**, with S-2a then mating type α).

Esterase composition was determined by PAGE as described in Section 2.2.2. Cells were suspended in sample buffer pH 6.8 at an equivalent absorbance of 30 at 400 nm, and disrupted by the French pressure Cell with 1% Triton X-100 detergent.

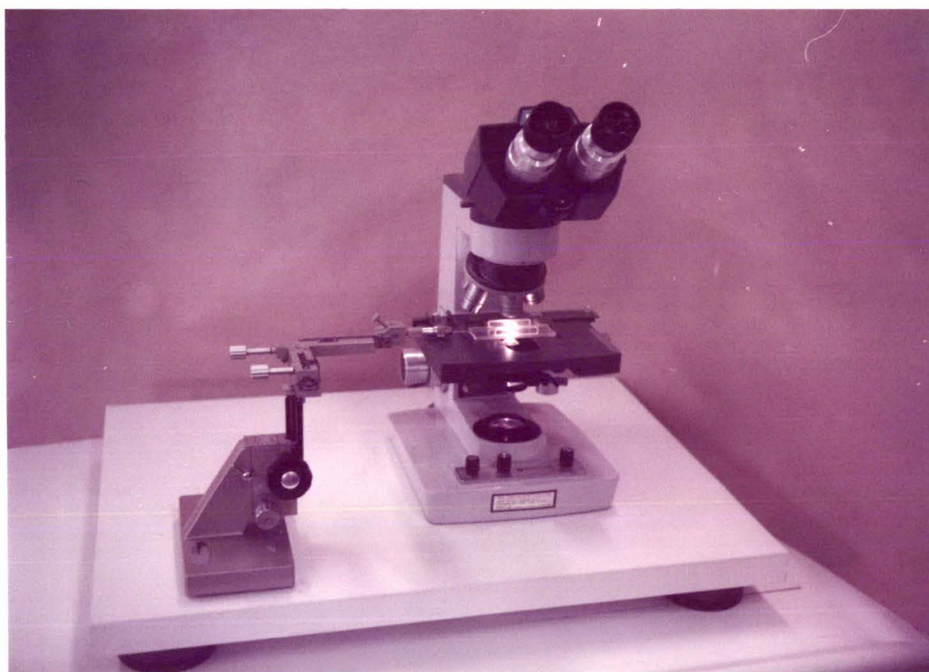
2.5.2 The Micromanipulator Procedure

MYGP containing 3.0% agar was used for production of agar slabs. The medium was autoclaved as 10 ml samples in universal bottles. When required, a universal bottle was placed in boiling water and the medium redissolved. Approximately 0.7 to 0.9 ml of medium was aseptically pipetted onto ethanol-sterilised and flamed 22 x 50 mm glass coverslips, which had been placed on a sterilised bench surface and covered with a sterilised glass petri dish lid. 3 to 5 mm of agar was removed from all edges with another sterile coverslip prior to streaking. This produced a flat slab for micromanipulation, and enabled the slab to sit flat in the dissecting chamber. Slabs were used within two hours before they dehydrated.

For use, along the long edges was streaked a culture or cultures, and the slabs placed inverted in the dissecting chamber. The dissection needle was centred in the microscopic field prior to micromanipulation. Needles were produced from 3 mm soda glass rods, two being heated together in a bunsen flame, melted together, and quickly drawn apart at a slight angle. Micromanipulation was carried out at x300 magnification. The needle was moved only vertically when centred by the Prior

Plate 3. Micromanipulation of Yeast Cells.

The manual micromanipulator is set up on the left-hand side, the fixed-stage microscope on the right, both sitting on an antivibration table. The needle is in place and centred, with the perspex dissecting chamber with inverted agar slab ready for micromanipulation.



manipulator, and movement of the agar slab and cells was by the microscope stage slide controls. Following micromanipulation, the coverslips were removed from the dissecting chamber and the agar slab removed with a sterilised spatula. Slabs were placed onto MYGP plates for incubation at 30^{0}C with the cells uppermost.

2.5.3 Micromanipulation of Yeast Cells

For production of the haploid strain library, control Est 1f/1s 2f/2s, mutant X *Est 1s 2s*, and mutant X *Est 1s 2f* strains were streaked along one edge of the agar slab. An ascus was picked up and placed at a recorded point on the agar slab. The ascus was broken by gently tapping with the dissecting needle, and spores were placed separately across the slab at 2 to 3 mm intervals. Different asci were dissected at 2 to 3 mm along the slab, care being taken to record the positioning of each cell. Normally five or six asci were dissected on the agar slab.

In the production of the diploid strains, the two mating types were placed on alternate long edges of the agar slab. Positioning was carefully recorded, and one cell of each mating type deposited at each reference. Cells were moved until they rested in contact. Excessive movement and pressure on the cells was avoided as cell viability was reduced.

Ascus formation and lack of zygote formation with standard mater strains and ascus formation confirmed that mating had taken place.

2.6 Esterase Activity Determinations After Growth in Liquid Media

Esterase activity was determined by the use of artificial esters and registering their hydrolysis by spectrophotometry. α-Naphthyl acetate and β-nitrophenyl acetate were used as substrates, and initially modified methods of α-NA hydrolysis after Wheeler *et al.* . (1972) and ρ-NPA hydrolysis after Parkkinen (1980) were tested. Both were found to give satisfactory results, but the ρNPA was used as it was simpler and more reproducible.

Esterase activity was measured as μmoles ρ-nitrophenol (ρNP) produced per 0.5 ml samples per 30 minutes. These values were converted to and recorded as ρNP Units, defined here as:

One ρNP Unit equals one n mole ρNP produced per minute per 1.0 ml sample.

2.6.1 Aerobic Growth of Control Est 1f 2f 3 4 in GGM

Control Est 1f 2f 3 4 was grown on MYGP plates at $30^{o}C$ for 24 hours. Cells were suspended in sterile GGM, and diluted to an equivalent absorbance of 0.8 at 400 nm. 1.0 ml of suspension was added to ten 100 ml flasks containing exactly 50 ml of GGM, and incubated at $30^{o}C$ on an orbital bed. Two flasks were removed on day 1, and one flask on days 2, 3, 4, 5, 7, 9, 11 and 15 ready for protein extraction as described in Section 2.2.2. Extracts were stored at $-70^{o}C$ until determinations could be made.

2.6.2 Fermentation by R92 and R2 in CDM and Grape Juice

Wine-making strains R92 and R2 were grown in 50 ml samples of CDM and Reisling Sylvaner grape juice {∿20% sugar and 75 mg/l SO_2) for three days at $20^{o}C$. Cell suspensions were diluted to an equivalent absorbance of 8.0 at 400 nm, and 25 ml were added to 800 ml of the respective media. Flasks were fitted with gas traps and placed at $20^{o}C$ to ferment. 30 ml samples were removed at 2 and 4 days, and 20 ml samples at 6, 9, 12, 15 and 18 days. Cell density was calculated before protein extraction, and the samples from the four media were adjusted to an equivalent absorbance of 8.0 at 400 nm. Protein samples were prepared as in Section 2.2.2, with the alteration that washed cells were placed in 0.1M phosphate buffer pH 6.8 instead of sample buffers. Protein and esterase determinations were made immediately after extraction.

2.6.3 Cell Density Determinations

Cell densities were determined from absorbances at 400 nm and from Klett units using a blue filter. Sterile four-day cell suspensions of R93, R92 and R2 were made and dilutions were plated onto four MYGP plates each. Absorbances and Klett readings of the undiluted

suspensions and serial dilutions from each strain were measured in duplicate.

A 1/100 dilution using a 0.05 ml aliquot was made of the washed cells suspended in the appropriate buffer for cell density determinations in growth experiments. Readings were made in a Spectrometer 20 at 400 nm, the volume of remaining suspension measured, and the suspension corrected to the required equivalent absorbance. Cells per ml of medium were estimated by adding 0.05 ml to the measured volume, finding the cell density of the cell suspension from the standard curve, and correcting the volume to the sample volume removed.

2.6.4 Protein Determinations by the Biuret Method

Protein samples were diluted 1/10 with distilled water. 1.0 ml samples were added to 4.0 ml Biuret Reagent, thoroughly mixed, and left for 30 minutes at room temperature to allow colour development before being read at 550 nm in a Beckman DB Spectrophotometer.

A standard curve was produced by dissolving Bovine Serum Albumin in 0.1M phosphate buffer pH 6.8 to make an 80 mg protein per ml solution. This was diluted with distilled water to make a range of concentrations between 8.0 and 80 mg protein per ml. 1.0 ml samples were then added to 4.0 ml Biuret Reagent, mixed, and left for 30 minutes at room temperature before reading at 550 nm in a Spectronic 20. Four samples of each concentration were read, and the mean and range plotted to produce the standard curve.

2.6.5 Esterase Determinations by a ρ-Nitrophenyl Acetate Method

A stock solution of ρNPA was made by adding 90.6 mg of ρNPA to 50 ml absolute ethanol (0.1M ρNPA). The stock solution was stored for up to one week at $4^{o}C$ or four weeks at $-20^{o}C$. For determinations, 1.0 ml stock ρNPA was added to 4.0 ml distilled deionised water just before use. The following protocol was set up:

Distilled deionised water	3.0 ml
0.1M phosphate buffer pH 6.8	1.0 ml
ρNPA (20 mM)	0.5 ml
Protein sample (in buffer)	0.5 ml
Total	5.0 ml

The distilled deionised water, phosphate buffer, and ρNPA solution were mixed together and allowed to reach 25°C, before the protein sample, also at 25°C, was added. The final amount of ρNPA in the reaction mixtures was 10 μmoles. The reaction mixtures were incubated at 25°C in a waterbath, and readings at 400 nm were taken on a DB Spectrophotometer at zero time, 5, 10, 15, 20, 25 and 30 minutes. A reading at zero time was essential as a measurable absorbance was due to the protein sample at this wavelength. Before each series of five minute readings, the spectrophotometer was zeroed with the blank and samples were thoroughly mixed. μMoles ρ-nitrophenol (ρNP) produced per 0.5 ml sample per 30 minutes were determined by observing the increase in absorbance over 30 minutes from a plot of the readings and reading off a standard curve of ρNP against absorbance.

A standard curve of ρNP was made by adding 55.64 mg of ρNP to 50 ml absolute ethanol (0.08M ρNP). This solution was used immediately. 1.0 ml stock solution was added to 9.0 ml of distilled deionised water just before use, giving a solution containing 8 μmoles ρNP per ml. Samples were diluted with distilled deionised water to give values of 0.5 to 8.0 μmole ρNP per ml. The following protocol was set up:

Distilled deionised water	3.0 ml
0.1M phosphate buffer pH 6.8	1.5 ml
ρ-Nitrophenol	0.5 ml

Mixtures were thoroughly mixed, left for three to five minutes, and read in a Spectronic 20 at 400 nm. The procedure was repeated twice to produce the standard curve.

2.7 Gas Liquid Chromatography for Volatile Esters

2.7.1 Sample Preparation

Diploid yeast strains were grown anaerobically in two media; Reisling Sylvaner grape juice (20% sugar, 75 mg per l SO_2) and Complete Defined Medium (CDM). Wherever possible 1.8 litres of medium were fermented, otherwise sample lots were reduced to 900 ml. The following strains were grown in both media:

R93 (*Est 1f/1f 2f/2f 3 4*) hybrid Est -/- -/- [1]
R92 (*Est 1f/1f 2f/2f*)
R2 (*Est 1f/1f 2f/2f*)

The four strains listed below were grown only in grape juice:

hybrid Est 1f/1f 2s/2s
hybrid Est 1s/1s 2f/2f
hybrid Est 1f/(1f) 2s/(2s) [2]
hybrid Est 1s/(1f) 2f/(2s) [2]

The following strain was grown only in CDM:

hybrid Est 1s/1s 2s/2s

For growth of hybrid Est -/- -/- , adenine at 40 mg per litre was add to both media.

Yeasts were grown up aerobically on an orbital shaker at 30°C for 48 hours on the corresponding media. To each flask of fermentation media, a 3% to 5% volume of yeast suspension was added and flasks were fitted with gas traps.

Fermentations were carried out at 15°C for five to six weeks. Samples were then chilled at 4°C to arrest cell activity after fermentation, and stored at 4°C until samples could be utilised.

900 ml samples were centrifuged at 2500 G for 15 minutes on a Sorval RC-3 centrifuge in a HG-4L rotor, and repeated once, to remove all cells. Samples were stored stoppered at 4°C until ester extraction.

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[1] diploid mutant. [2] Bracketed alleles from an esterase mutant.

Ethanol levels were determined by the Alcohol Dehydrogenase Enzymatic Method of Bernt and Butmann (1970), as reproduced in Appendix Three.

2.7.2 Ester Extraction

For analysis, the method of Killian and Ough (1979) was followed, using the simultaneous steam distillation/extraction apparatus of Likens and Nickerson (1964). Redistilled *n*-pentane was used as the extraction solvent, and was stored at 4^{0}C over anhydrous sodium sulphate until required. A high level of purity was obtained by redistilling AnalaR *n*-pentane through a long column (80 cm) packed with glass Fenske Helices at 42^{0}C.

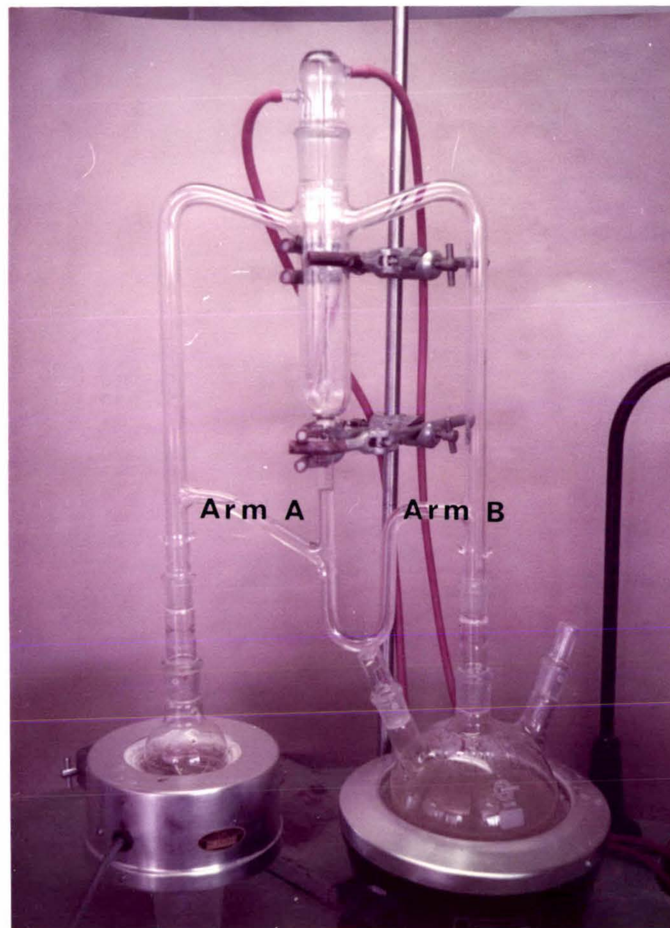
60 ml of redistilled *n*-pentane was added to the pentane reservoir (100 ml flask) of the Likens and Nickerson apparatus. The reservoir was connected by an adaptor to return arm A (solvent arm). Exactly 800 ml of sample was added to the sample reservoir (one litre flask) and adjusted to 12% ethanol (volume/volume) with AnalaR absolute ethanol. The sample reservoir was connected, also via an adaptor, to return arm B (sample arm), and 1.0 ml redistilled *n*-pentane containing exactly 1.2 mg each of two internal standards: ethyl nonanoate and *n*-pentyl acetate, was added. The condenser was turned to a fast flow-rate and heating mantles placed under the two reservoirs to produce a strong boil of each reservoir. Pentane was allowed to condense first to fill up the return arms. Once sample condensation began in return arm B, a one-hour distillation/extraction was carried out. After one hour, the mantles were turned off and the reservoirs allowed to cool before the condenser was turned off.

The redistilled *n*-pentane remaining in the Likens and Nickerson apparatus was collected by unclamping and tilting the whole apparatus (minus the sample reservoir), so that the solvent flowed out of the solvent return arm (with a small amount of sample).

After extraction of the esters into the solvent, the redistilled *n*-pentane was transferred to a 100 ml separatory funnel containing 20 ml of 1.0M aqueous sodium chloride. The funnel was shaken for 30 seconds, and the two phases allowed to separate. The water layer was drawn off, and the *n*-pentane poured into a 250 ml glass-stoppered flask. The water phase, containing any ethanol, was replaced into the separatory

Plate 4. Simultaneous Steam Distillation/Extraction Apparatus of Likens and Nickerson.

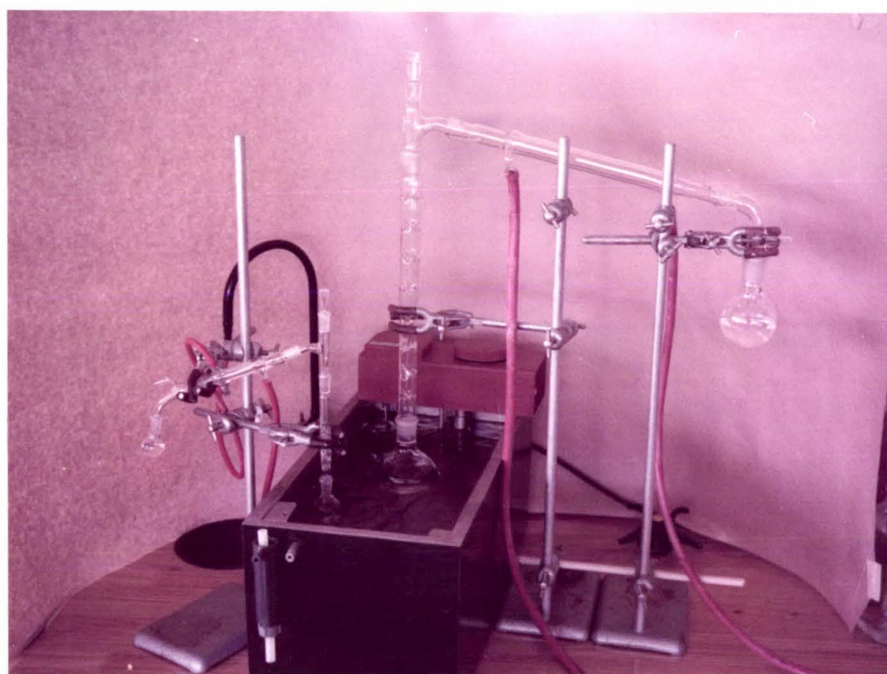
The apparatus is in use, with both thermal jackets turned on and condensation on the condenser. Pentane is returned to the small solvent reservoir through arm A, with sample returning through arm B to the large sample flask.



Arm A
Arm B

Plate 5. Distillation of *n*-Pentane Leaving Esters as Residues.

Both distillation apparatus fitted with Vigreux columns are in use, the *n*-pentane is being distilled off at $47^{o}C$ leaving behind the higher boiling esters.



funnel and any remaining esters were re-extracted twice with further 20 ml portions of redistilled *n*-pentane. These *n*-pentane washes were combined with the original *n*-pentane sample.

Extracts were dried overnight over 7 g anhydrous sodium sulphate at $4^{o}C$. The extracts were filtered through one layer of Whatman No. 1 filter paper and the sodium sulphate washed with several mls fresh redistilled *n*-pentane.

Extracts were distilled through a distillation apparatus (Quickfit glassware) using a 40 cm Vigreux column at $47^{o}C$ over an adjustable waterbath until the volume was reduced to 8 to 10 ml. This residue was transferred to a Quickfit microdistillation apparatus, with the 10 cm column (C2/00) converted to a Vigreux column. The volume was reduced to 0.9 to 1.1 ml, also at $47^{o}C$. The final residue was transferred to a septum-stoppered vial, and stored at $4^{o}C$ until analysed by gas liquid chromatography (GLC).

All glassware was thoroughly rinsed after each run and air dried. After several days' use glassware was placed in detergent (Extran) and hot water to soak overnight. Glassware was thoroughly rinsed in tap water and rinsed in distilled water. Finally each piece of equipment was rinsed with acetone and air dried before use.

2.7.3 Gas Chromatographic Analysis

The volatiles (esters) were separated and examined on a Hewlett Packard model 5840 A Gas Chromatograph, fitted with a 50 m fused silica capillary column containing a Carbowax 20M liquid phase. The injection port heater block was set at $220^{o}C$, the flame ionisation detector heater block at $250^{o}C$. The oven was temperature programmed at $5^{o}C$ per minute between $50^{o}C$ (starting temperature) and $200^{o}C$. Under these conditions, suitable GLC traces were obtained with a nitrogen gas flow of approximately 2 cm^3 per minute, a 1.0 1 sample loading, chart speed of 1 cm per minute, and a chart attenuation of 2^8 (standards 2^{10}).

Ester peaks were identified by comparing retention times (R_T) of aroma sample peaks with prepared ethyl esters and acetate esters mixtures on the same day. Standard and sample peaks were considered identical when R_T values varied by ± 0.03 minutes or less. Peaks were

electronically integrated and amounts were calculated in mg per litre, but results were not corrected for molar response facors. Ester peaks with retention times below and including *n*-hexyl acetate were quantitated against *n*-pentyl acetate (IS 1), and esters with retention times greater than *n*-hexyl acetate were quantitated against ethyl nonanoate (IS 2).

Where 1.8 litres of medium had been fermented, two samples were extracted and compared. Duplicate controls of CDM and Reisling Sylvaner grape juice were used. GLC under the conditions described was also used to check the purity of the internal standards and redistilled *n*-pentane

2.7.4 Production of Ester Standards

Ethyl Esters Synthesis:

The seven acids in Table 2.4 were dissolved in 50 ml of absolute ethanol, and refluxed in the presence of 3 to 5 drops of concentrated sulphuric acid for three hours. Collection of the esters was done by the following steps in a 200 ml separatory funnel:

1) Approximately 50 ml distilled water was added, and shaken for 30 seconds;

2) 50 ml ether was added to the funnel, shaken for 30 seconds, and the non-solvent phase drawn off;

3) The ether containing the esters was washed with approximately 20 ml 1.0M sodium hydroxide, shaken for 30 seconds, and the water phase drawn off;

4) The ethyl ester standards were finally washed with equal volumes of distilled water three times.

The standards in ether were dried overnight over 5 g anhydrous sodium sulphate and stored in a stoppered bottle until required.

Table 5. Acids used in Ethyl Esters Synthesis.

Acids	Volume	Acids	Volume
Acetic acid	1.4 ml	Octanoic acid	1.0 ml
Propionic acid	1.5 ml	Decanoic acid	1.0 ml
Butanoic acid	1.5 ml	Dodecanoic acid	1.0 ml
Hexanoic acid	1.0 ml		

Acetate esters synthesis:

Acetate esters were formed by adding the eight alcohols listed in Table 6 to 20 ml of 1:1 (v/v) pyridine:acetic anhydride mixture, and left for 48 hours at room temperature to react. Collection of the esters was the same as for ethyl ester standards, with the addition of washing with approximately 20 ml 1.0M hydrochloric acid after washing with 2 ml 1.0M sodium hydroxide.

Table 6. Alcohols used in Acetate Esters Synthesis.

Alcohol	Volume	Alcohol	Volume
Ethanol	0.8 ml	*iso*-pentanol	1.4 ml
iso-propanol	1.0 ml	*n*-pentanol	1.4 ml
n-propanol	1.0 ml	*n*-hexanol	1.6 ml
iso-butanol	1.2 ml	2-phenethanol	1.0 ml

2.7.5 Production of internal standards

n-Pentyl acetate was prepared as described above for acetate ester synthesis. The internal standard (IS 1) was purified by distillation at 147°C, and checked for purity by GLC.

Ethyl nonanoate was prepared as described for ethyl ester synthesis. The internal standard (IS 2) was partly purified by distillation at 225°C, and was further purified by distillation on spinning band column. Purity was checked after both distillation steps by GLC.

RESULTS

3.1 Polyacrylamide Gel Electrophoresis

3.1.1 Experimental Systems

Protein samples were prepared where possible by following Wohrmann and Lange (1980), as were gels (Ornstein, 1964; Davis, 1964). Minor differences in buffers and gel preparations (Hames, 1981) were incorporated for quicker and simpler preparation. Instead of the disc electrophoresis used by Wohrmann and Lange (1980) and multi-phase buffers of Berking and Hauschild-Kogat (1970), the equipment used for this study was for slab gel electrophoresis. A modified Reid and Bieleski (1968) vertical apparatus (or Studier-type) was used throughout. Slab gels have several advantages over disc gels, in particular a large number of samples can be run under identical conditions enabling samples to be directly compared.

Ammonium persulphate was used as the polymerising catalyst in the stacking gel instead of riboflavin. Excess persulphate ions can reduce the activity of some sensitive enzymes (Hames, 1981) but the results suggested that this did not occur.

The nomenclature of Wohrmann and Lange (1980) was used for esterase enzymes. The fastest migrating protein was *Est 2*, followed by *Est 3* , *Est 1* (which segregates as three distinct bands), and lastly *Est 3* (see Plate 8). The fast alelles, *Est 1f* and *Est 2f*, migrated closer to the tracking dye front than the slow alleles *Est 1s* and *Est 2s*.

Three gel concentrations were tested for the best resolution: 5.0%, 7.5% and 10.0%, and all gave good esterase band separation. A 7.5% gel concentration was chosen for all subsequent work as it correlated more closely with published work. Some R_f values obtained under the conditions described are listed in Table 7. These relative mobility values are only approximate, as variations of $\pm$0.04 between gels were encountered, even though conditions were standardised as far as possible. Such differences are due to slight variations in gel concentration and environmental conditions between runs, but caused no difficulty in evaluating results as a standard was run in each gel.

Table 7. R_f Values of Esterase Bands on Polyacrylamide Gels.

	Gel Concentration		
	5.0%	7.5%	10.0%
Est 1s	0.67	0.45	0.32
Est 1f	0.73	0.50	0.35
Est 2s	0.85	0.60	0.45
Est 2f	0.95	0.70	0.50

A gel loading of 50 μl was selected. The greater the sample loading the heavier the staining, enabling easier evaluation of results. 50 μl loading was the most practicable volume since a 60 μl loading tended to overflow from the tracks before electrophoresis. Large sample loading caused some streaming (see Plates 6 and 7), but this did not affect the determination of esterase bands.

During initial esterase staining with α-naphthy acetate (αNA), it was found that the background gel stained less intensely when the Fast Blue B Salt was added separately at a different time from the substrate, αNA. This enabled bands to be more readily visualised and photographed, and this modification was made part of the standard staining method. All bands stained an intense deep red, the *Est 2* and *Est 3* bands tending to be a dark crimson, while the *Est 1* and *Est 4* bands a deeper brownish-red.

Staining was quite variable, some gels stained very strongly, while others so faintly that the bands were almost indistinguishable from the background gel. No satisfactory explanation was found, though such variability is common with specific stains and is probably due in part to slight changes in stains and the environment between runs. Decreased staining was not due to a lack of esterase penetration into the gels as little or no activity was observed in the wells, or at the gel interface.

Est 2 and *Est 3*, the two fastest migrating loci, consistently stained clearly with the *Est 3* band staining particularly heavily. Densitometer readings were not routinely used to quantitate band activities because of band streaming and equipment inaccuracy, but when they were used, recordings clearly showed that *Est 3* (Figure 5) represented a large proportion of the total esterase activity in strains containing this enzyme.

All *Saccharomyces cerevisiae* strains tested had both *Est 1* and *Est 2* genes present. All strains were also homozygous (carrying only one allele) for each locus.

A new band appeared half-way between *Est 2f* and *Est 2s* bands in the heterothallic control Est 1f/1s 2f/2s. This was a hybrid protein of the two alleles, showing that *Est 2* locus proteins are polymeric, probably dimers. *Est 1* locus proteins resolved as three bands each, the fastest-travelling band (isoenzyme) often being fainter than the other two. The mobility of the faster *Est 1s* and slower *Est 1f* isoenzymes were indistinguishable. This was considered coincidental, as Wohrmann and Lange (1980) were able to clearly distinguish each band in strains heterozygous for *Est 1*.

3.1.2 Esterase Composition of Selected Strains

The esterase compositions of the 20 wine-making strains, 4 laboratory strains and 4 controls are listed in Table 8. All *S. cerevisiae* wine-making strains except R93 (previously classified as *S. chevalieri*) were *Est 1f/1f 2f/2f*, R93 being *Est 1f/1f 2f/2f 3 4* [1]. The four laboratory strains were *Est 1f 2s*, and the controls as labelled. It is evident that the fast alleles *Est 1f* and *Est 2f*, are both selected for in wine-making strains. Slow alleles are known to be relatively common in laboratory strains.

In the laboratory strains studied here, only the slow allele *Est 2s* was present, but a statistical analysis of its importance could not be determined because of the small sample size.

---

[1] Zygosity of *Est 3* and *Est 4* unknown

Table 8. Esterase Composition of Selected Yeast Strains

Wine-making Strains	Esterase Composition
R2	*Est 1f/1f 2f/2f*
R92	" " "
R93	*Est 1f/1f 2f/2f 3 4*[1]
R99	*Est 1f/1f 2f/2f*
R107	" " "
R108	" " "
R109	" " "
R176	" " "
R177	" " "
R179	" " "
AWRI62	" " "
AWRI63	" " "
AWRI65 (R171)	" " "
AWRI79	" " "
AWRI89	" " "
AWRI93	" " "
AWRI105	" " "
AWRI116 (R11)	" " "
R28	" " "
R80	*Est 1f/1f 2f/2f*

Other Strains	Esterase Composition
Control Est 1s 2s	*Est 1s/1s 2s/2s*
Control Est 1f 2f	*Est 1f/1f 2f/2f*
Control Est 1f/1s 2f/2s	*Est 1f/1s 2f/2s*
Control Est 1f 2f 3 4	*Est 1f/1f 2f/2f 3 4*[1]
R92/2/47	*Est 1f 2s*
x2928	" " "
S-1α	" " "
S-2**a**	" " "

[1] Zygosity of *Est 3* and *Est 4* unknown

Plate 6. Esterase Patterns of Selected Yeast Strains on Polyacrylamide Gels.

From left to right:

X2928	(*Est 1f 2s*)
Control	Est 1f 2f
Control	Est 1f/1s 2f/2s
Control	Est 1f 2f 3 4
R28	(*Est 1f/1f 2f/2f*)
R80	(*Est 1f/1f 2f/2f*)

Plate 7. Esterase Patterns of Selected Yeast Strains on Polyacrylamide Gels.

R93	(*Est 1f/1f 2f/2f 3 4*)
R99	(*Est 1f/1f 2f/2f*)
R107	(*Est 1f/1f 2f/2f*)
R92	(*Est 1f/1f 2f/2f*)
AWRI65 (R171)	(*Est 1f/1f 2f/2f*)
AWRI63	(*Est 1f/1f 2f/2f*)
AWRI62	(*Est 1f/1f 2f/2f*)
Control	Est 1s 2s

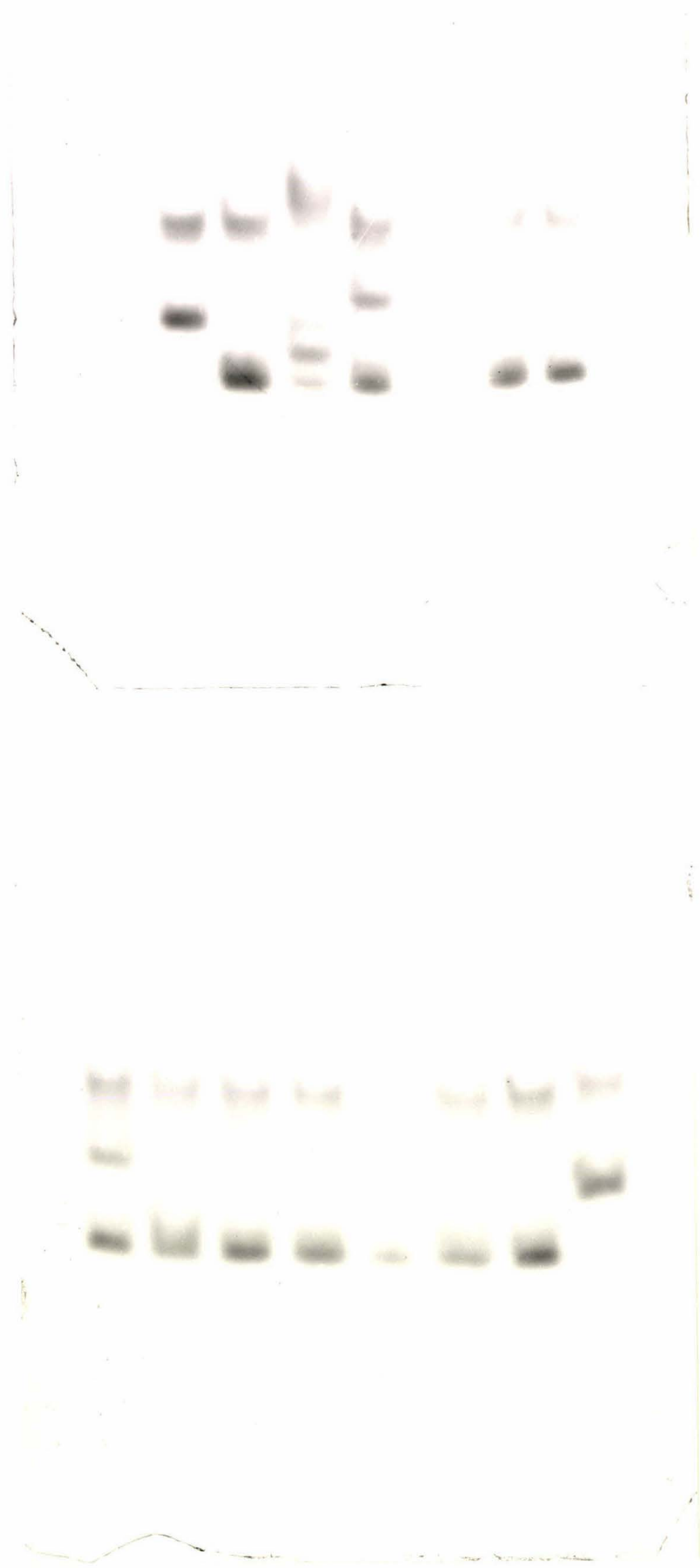

Est 1s
Est 1f
Est 3
Est 2s
hybrid
Est 2f

Table 9. Frequency of Esterase Alleles and Genes in Wine-making *S. cerevisiae* Strains.

	Total Strains	*Est* *1f/1f 2f/2f*	*Est* *1f/1f 2f/2f* *3 4*	"inducible" *Est 3 Est 4*
Wohrmann & Lange (1980)	40	37.5%	45%	17.5%
This study	20	95.0%	5%	-

The abundance of the *Est 3* and *Est 4* genes differed markedly from those published by Worhmann and Lange (1980). These are summarised in Table 9. A statistically significant difference between the two populations occurs, no "inducible" *Est 3 Est 4* strains (those that did not produce *Est 3* and *Est 4* proteins in all extracts tested) were recorded, and the total strains containing the two genes (*Est 3*, *Est 4*) recorded by Wohrmann and Lange (1980) differed markedly from this study.

Campbell *et al.* (1972) found that esterase band patterns of *Candida utilis* could change in aerobic growth due to slight changes in medium. For this reason, each strain was grown on three different media and extracted. No inducible enzymes were stimulated by such changes, and esterase bands detected were always constituitive during growth.

3.1.3 Results of Other PAGE Experiments

Strains R28, R92 and control Est 1f 2f 3 4 were checked for the possibility of band intensity and numbers changing with age of culture. In all three strains, no new bands appeared and bands present did not disappear. The intensity of all bands increased in protein extracts up to nine days' aerobic growth. Activity of bands appeared to be synchronous with each other; no band became more intense in comparison to any other.

Difficulty in resolving the *Est 4* band was not corrected by the addition of Triton X-100 non-ionic detergent to solubilize protein extracts after disruption by the French Cell. *Est 4* band activity increased, however, when 1% or 2% Triton X-100 was added before cell disintegration (Plate 8).

Plate 8. Effects of Addition of Triton X-100 on Esterase

Extraction before French Cell Pressuring.

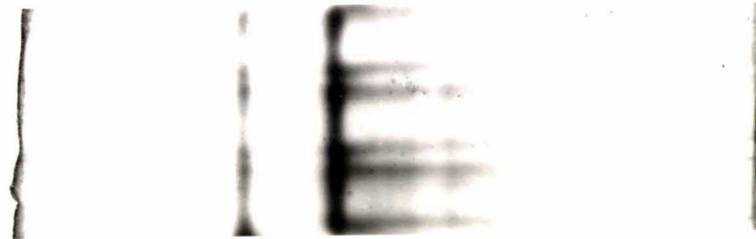
Track 1 - Strain R93 without Triton X-100.

Track 2 - Strain R93 with 1% Triton X-100.

Track 3 - Strain R93 with 2% Triton X-100.

Figure 5. Densitometer Recordings of Esterase Bands.

Strain R93 with 1% Triton X-100, as photographed above.



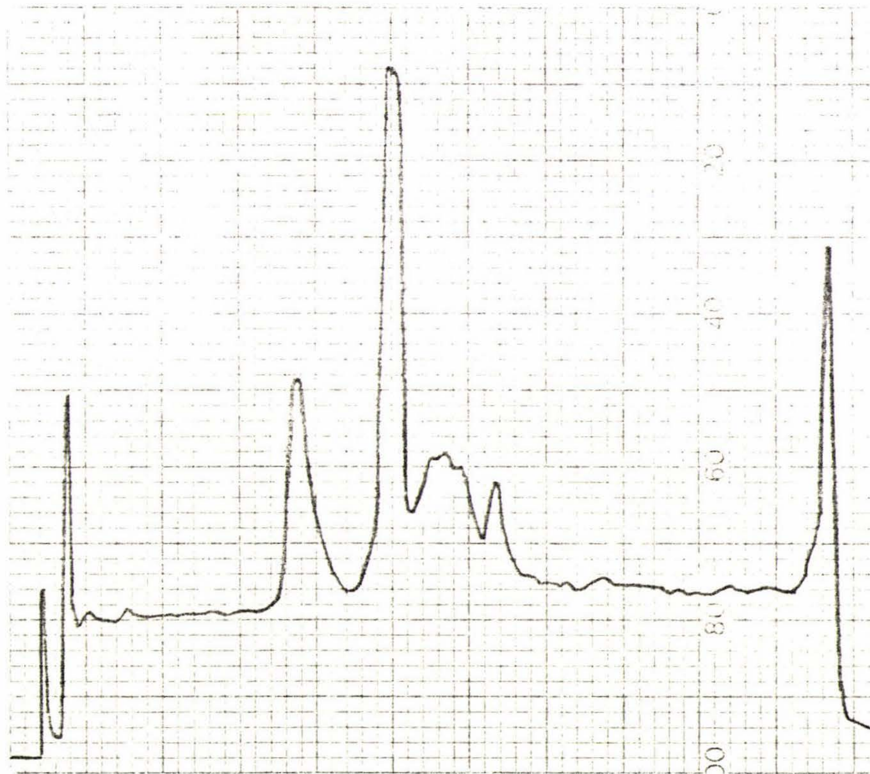
Track 1

Track 2

Track 3

Est 3 *Est 4*

Est 2f *Est 1f*



Extracts from three-day sporulating cells of strains R2 and R93 showed greatly decreased activity of esterase bands by PAGE and no new bands were observed. The αNA utilising esterases thus appeared not to be significant in reproductive development. Unsporulated and sporulated cells were not separated before protein extraction.

The modified PAGE staining method was developed to check for a possible fifth enzyme not readily utilising αNA as substrate (Parkkinen, 1980). A new band, labelled here as *Est 5*, was resolved travelling just behind the tracking dye front. It was present in all of the limited number of strains tested (R28, R92, R99, R176, AWRI65 and control Est 1f 2f 3 4). The band was extremely sharp, but stained only very weakly red, and in some gels it could not be resolved. It was not possible to photograph the band satisfactorily or record it with a densitometer.

3.1.4 Molecular Weight Determinations

Proteins in native polyacrylamide gels migrate through the gel by the relationship:

$$\log_{10} R_f = \log_{10} Y_0 - K_R T$$

where:

- R_f = relative mobility
- Y_0 = relative free mobility which would be obtained in a gel of zero concentration
- K_R = retardation coefficient
- T = total concentration of polyacrylamide.

From Ferguson plots, where $\log_{10} R_f$ is plotted against total polyacrylamide concentration (%T), the retardation coefficient (K_R) becomes the slope of the line from plotted points at different %T. K_R values are proportional to the effective molecular surface area, which with globular proteins (partial specific volumes of 0.73 to 0.74 ml per g) is indirectly proportional to the molecular weight (MW).

For Ferguson plots, R_f values from at least seven gel concentrations were determined for both standards and esterases, and lines produced by Least Mean Squares Regression to calculate precise values of K_R

(Andrews, 1981). Ferguson plots were produced for esterases *Est 1f*[1], *Est 1s*[1], *Est 2f, Est 2s, Est 3* and *Est 4*, but not for *Est 5*. Calculated K_R values are listed in Table 10. These values are only relevant for the conditions described, and accurate for the buffers and stock solutions used.

Table 10. Retardation Coefficients (K_R) of Protein Standards and Yeast Esterases

Esterases	K_R	Protein Standards	K_R	
Est 1f	0.0624	Myoglobin	0.036[1]	0.035
Est 1s	0.0634	Hexokinase	0.0505[2]	0.076
Est 3	0.0564	Alcohol Dehydrogenase	0.093[2]	0.061
Est 2f	0.0528	Human Albumin	0.0685	
Est 2s	0.0525	Phosphoglucose Isomerase	0.080	
Est 4	0.0795			

[1] isozymes [2] different polymers

Ferguson plots of the αNA utilising esterases are shown in Figure 6. They demonstrate that proteins from the four loci separate primarily because of different molecular weights, *Est 4* being the largest (greatest K_R). Alloenzymes of *Est 1* and *Est 2* have nearly identical retardation coefficients, migrating separately because of different charge (y axis intercept). The alleles are charge allozymes, as opposed to molecular weight allozymes or charge/molecular weight allozymes.

The three bands of *Est 1f* and *Est 1s* were found to travel as a constantly definable unit in different gel concentrations, showing that bands differed in charge, not MW. The *Est 1* proteins must therefore undergo post-translational modification of one or more amino acid

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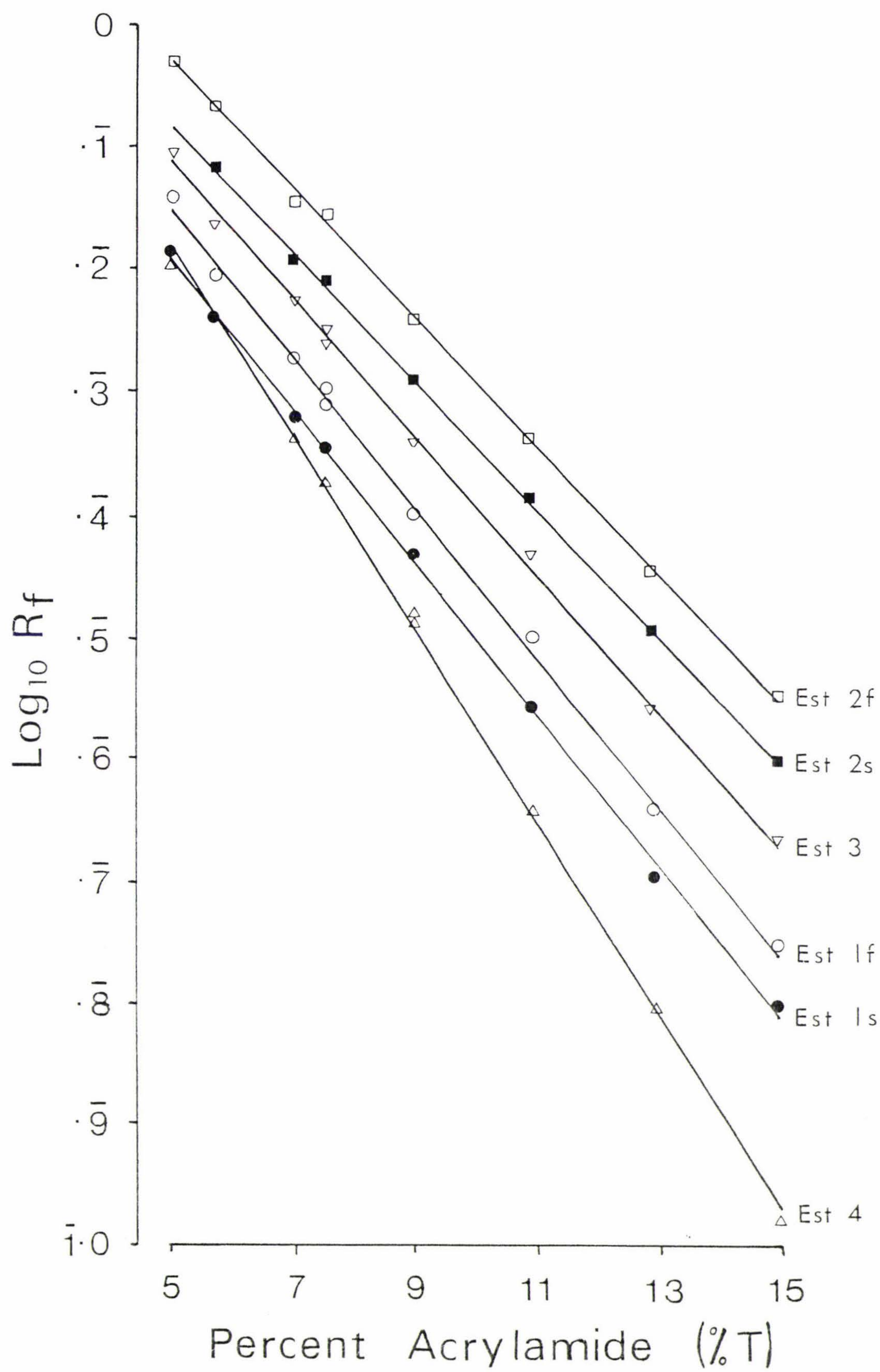
[1] Middle band

Figure 6. Ferguson Plots of Yeast Non-specific Esterases


0
·1̄
·2̄
·3̄
·4̄
·5̄
·6̄
·7̄
·8̄
·9̄
1̄·0
Log10 Rf
5
7
9
11
13
15
Percent Acrylamide (%T)
Est 2f
Est 2s
Est 3
Est 1f
Est 1s
Est 4

residues (hydroxylation, covalent attachment of glycoside moieties, or phosphorylation), rather than major structural modifications such as cleavage.

Retardation coefficients of the standard proteins were plotted against their MW, are shown in Figure 7. A standard curve was calculated by Least Mean Squares Regression from the eight points. Molecular weight determinations for the esterases were determined from this standard curve, and are listed in Table 11. A mean value of K_R for *Est 1* and *Est 2* was used from the two alleles. Errors involved are large because of the indirect relationship of MW to K_R, and therefore an average error of 20% was accepted (Bryan, 1977) for each value.

Table 11. Molecular Weight Determinations of Yeast Esterases

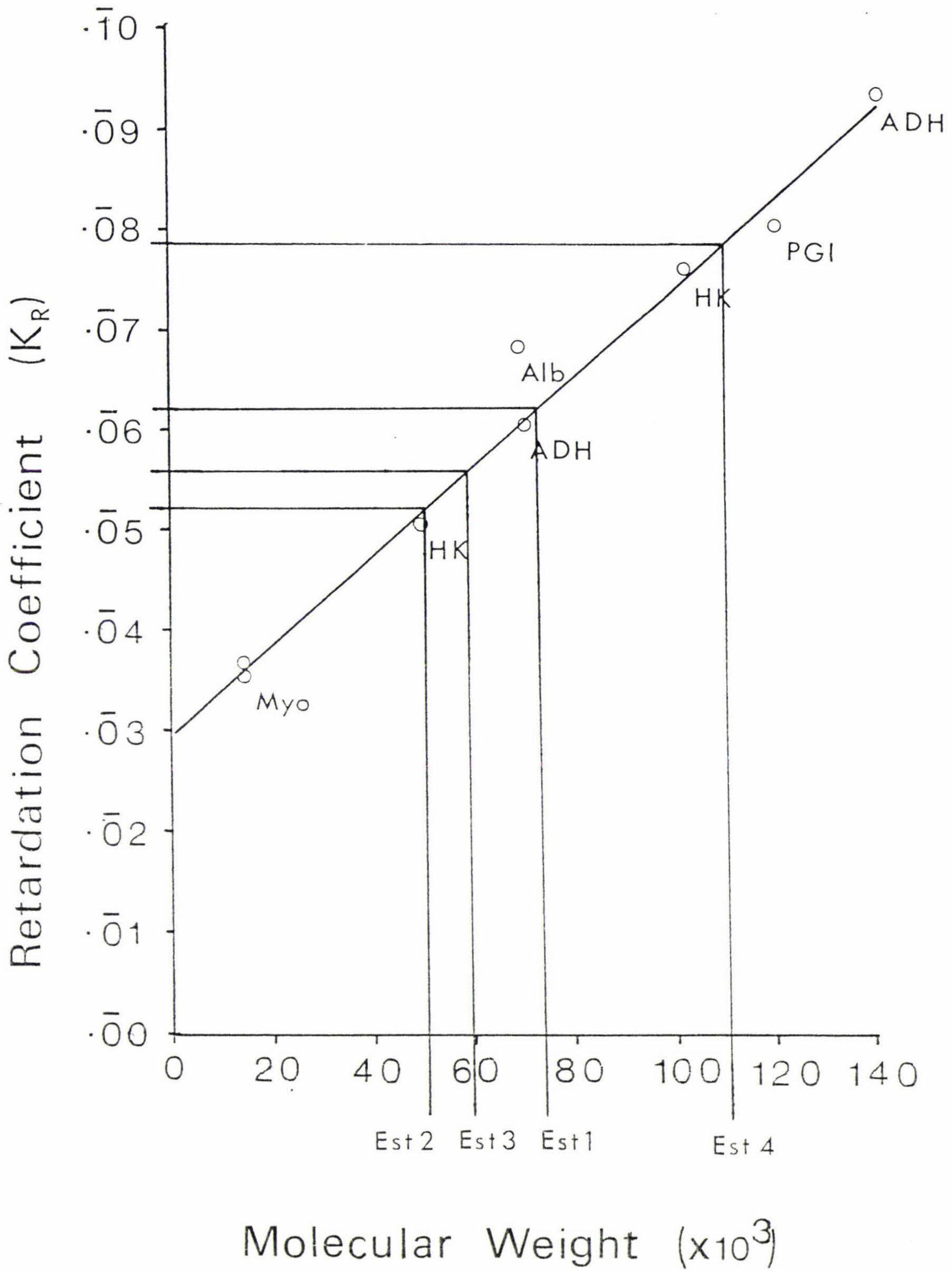
Esterase	Molecular Weight
Est 2 [1]	51,000 ± 10,000 daltons
Est 3	60,000 ± 12,000 daltons
Est 1 [1]	73,000 ± 15,000 daltons
Est 4	113,000 ± 23,000 daltons

[1] Both alleles

Figure 7. Calculation of Molecular Weights of Yeast Esterases.

From Retardation Coefficients (K_R).

Myo - myoglobin; HK - hexokinase;
ADH - alcohol dehydrogenase;
Alb - albumin; PGI - phosphoglucose isomerase.



·1̄0
·0̄9
·0̄8
·0̄7
·0̄6
·0̄5
·0̄4
·0̄3
·0̄2
·0̄1
·0̄0
Retardation Coefficient (K_R)
ADH
PGI
HK
Alb
ADH
HK
Myo
0 20 40 60 80 100 120 140
Est 2 Est 3 Est 1 Est 4
Molecular Weight ($x10^3$)

3.2 Wild Yeast Isolation

Yeasts were isolated on all three selective media tested, but none of the several hundred isolates screened and identified was found to be *Saccharomyces cerevisiae*. Species that were identified are listed in Table 12.

Table 12. Yeast Species Isolated from Locally-grown Mature Grapes.

High Acidity (pH 3.5-3.8) Isolation	*Saccharomyces* Enrichment Medium	Ethanol and Antibiotics Selective Isolation
Kloeckera apiculata	*Candida sake*	*Pichia membranaefaciens*
Candida sake		*Candida solani*
Candida famata [1]		*Candida famata*[1]
		Candida krusei [2]

[1] Asexual stage of *Debaromyces hansenii* (previously *Torulopsis candida)*

[2] Asexual stage of *Issatchenkia orientalis*.

Saccharomyces spp. are known to be difficult to isolate from a mixture of yeasts of other genera (Beech and Davenport, 1971), but this difficulty was expected to have been overcome by the use of several selective media. Other technical reasons for lack of *S. cerevisiae* isolation were expected to have been minimised by the use of multiple samples from several locations, large numbers of platings, screening of numerous samples, and use of a large number of tests for identification. Representative colonies of all morphological yeast types were also identified to the species level, to check that *S. cerevisiae* was not being selected against during the morphological screening stage.

The six species that were isolated are all yeasts that are commonly associated with grapes and fermentation.

3.3 Induction of an Esterase Deficient Mutant

3.3.1 Mutagen Systems

Kill curves for ultraviolet irradiation (UV) and ethyl methane-sulfonate (EMS) were produced as percent surviving colony forming units (CFU) against time. Plate counts were carried out at four days to allow sufficient time for petite mutants to grow, colonies being too small to count at two days. Petite colonies which were white in colour (red parents) did not appear until after 36 hours, and represented almost 10% of the total surviving cells at levels of 1% or less surviving CFU. The percent auxotrophic mutants was recorded as the difference in counts between platings onto MYGP and MM + 40 mg adenine per 1 (1.0 to 2.5 x 10^2 CFU per plate), rather than the difference on the two media from replica plating (Mortimer and Hawthorne, 1969). Mutation treatments were carried out under conditions which produce the greatest percentage of auxotrophic mutations.

Kill curves for UV mutagenesis and percent induced mutants of surviving CFU of R92/2/47 are shown in Figure 8. Strains R92/2/47 and X2928 showed similar lethality to UV treatment. The light source (Philips fluorescent UV tube) could not be calibrated in ergs per mm^2 per second as a measure of dose rate. Maximum auxotrophic mutation appeared to occur at about 10% surviving CFU, so 70 seconds was used for esterase mutation induction.

The kill curve for EMS mutagenesis of R92/2/47 differed little to that of Lindegren *et al.* (1965). Percent induced mutants of percent CFU could not be determined because of variability between counts. Mutation inductions were carried out at 60 minutes on the basis that greatest proportion of auxotrophic mutants occurs at approximately 10% surviving CFU.

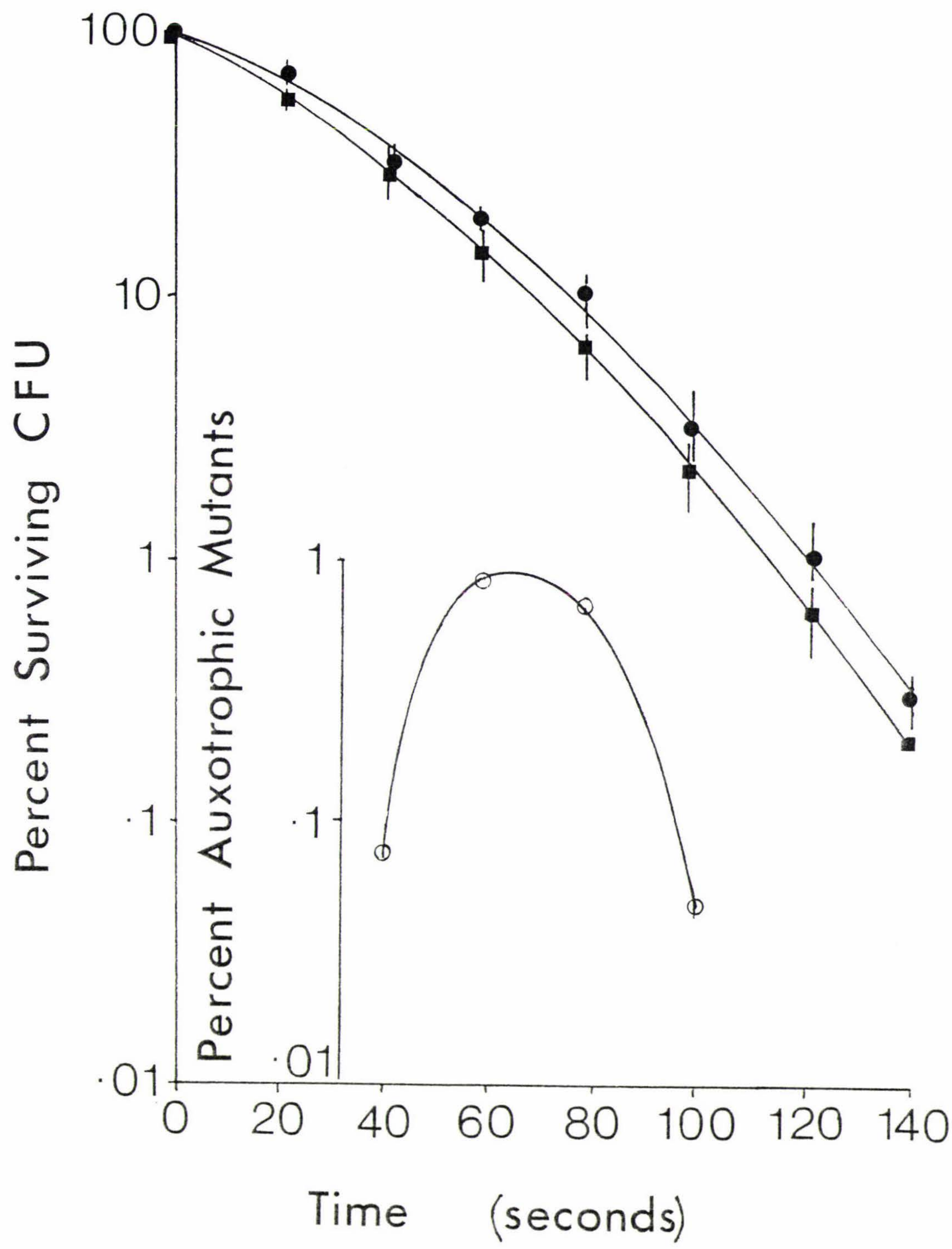
3.3.2 Isolation of an Esterase Mutant

Screening trials determined that a concentration of 10 µg αNA per ml in MYGP was the most suitable concentration for isolating esterase mutants. 100 µg αNA per ml stained the surrounding agar too deeply with Fast Blue RR Salt, while 1 µg αNA per ml occasionally stained too lightly to make comparisons of esterase activity between colonies (Plate 9).

Figure 8. Ultraviolet Irradiation Kill Curves and Auxotrophic Mutant Induction Curve at 27°C.

Bars represent range of counts

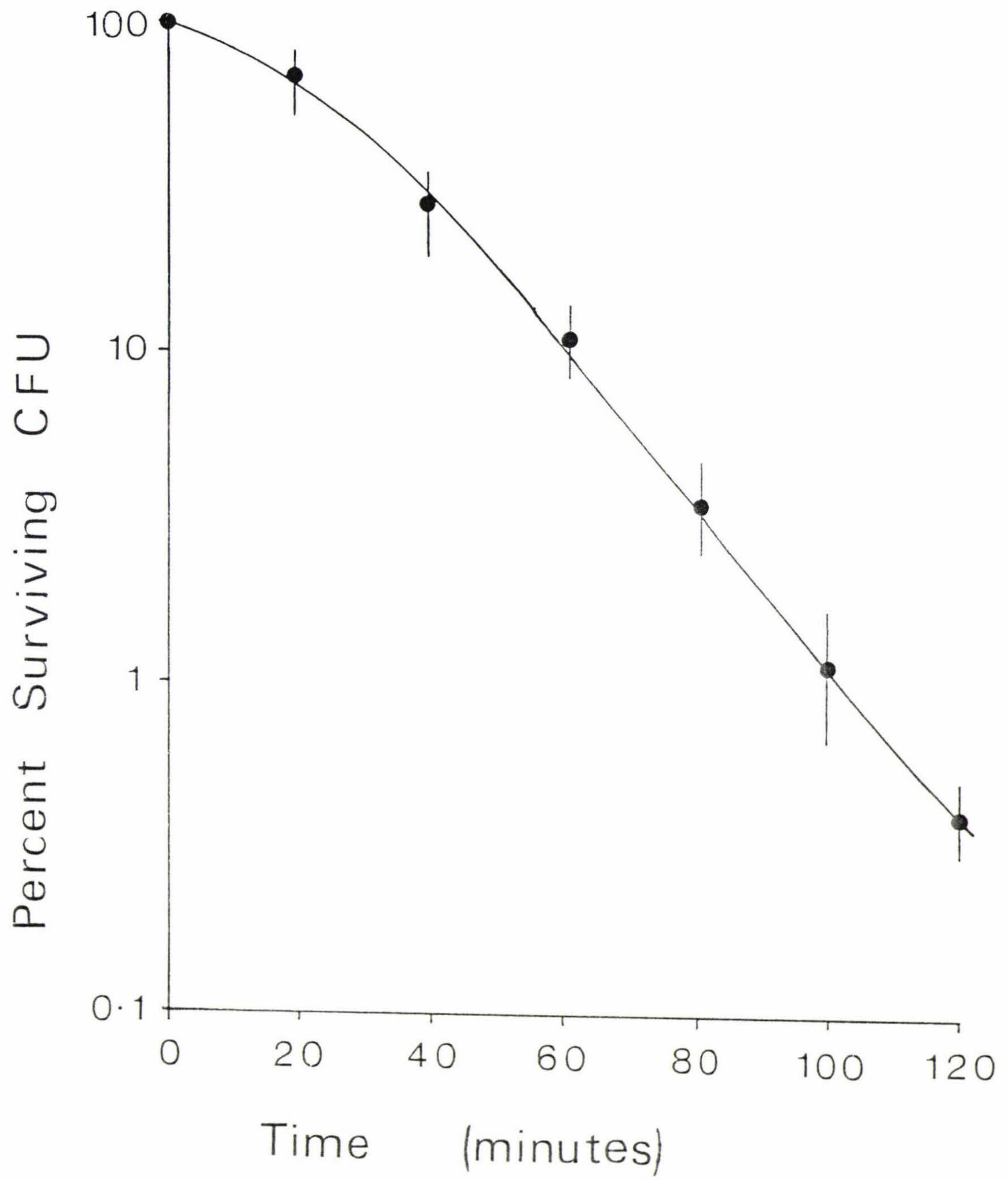
●-● R92/2/47; ■-■ x2928; O-O R92/2/47 percent auxotrophic mutants.



100
10
1
·1
·01
Percent Surviving CFU
Percent Auxotrophic Mutants
1
·1
·01
0
20
40
60
80
100
120
140
Time (seconds)

Figure 9. Ethyl Methane Sulfonate Kill Curve of R92/2/47 at 30^{0}C.

Bars represent range of counts.


100
10
1
0·1
Percent Surviving CFU
0
20
40
60
80
100
120
Time (minutes)

After mutagenesis, 400 isolated isolates of different colonial sizes and colour (including petite mutants) were pricked out and screened. Forty isolates that appeared to have decreased esterase activity were examined for esterase composition by PAGE. An EMS induced esterase mutant (labelled EMS-2) showed a marked decrease in esterase activity when compared with the parental type, R92/2/47 (Plate 9).

EMS-2 formed white colonies which were smaller than the parent R92/2/47 (Plate 10). Growth was poor in liquid and on solid media. Some spontaneous revertants occurred with the first replating onto MYGP, and were easily identified by the expression of the *ade 1* mutation (red pigment production). An isolated revertant was found to have regained its full αNA esterase composition (*Est 1f Est 2s*) when examined by PAGE.

On microscopic examination, cells of R92/2/47 tended to be oval, slightly variable in size, with mean measurements of 4.8 x 4.2 µm. EMS-2, however, was more uniform in size, spherical, with mean measurements of 4.2 x 4.0 µm.

Plate 9. Isolation of Induced Esterase Mutants.

Upper photograph: MYGP control plate showing size and colour of colonies.

Lower photograph: Test plates containing 1 μg αNA per ml MYGP, 10 μg αNA per ml MYGP and 100 μg αNA per ml MYGP.

0 R93

0 Revertant

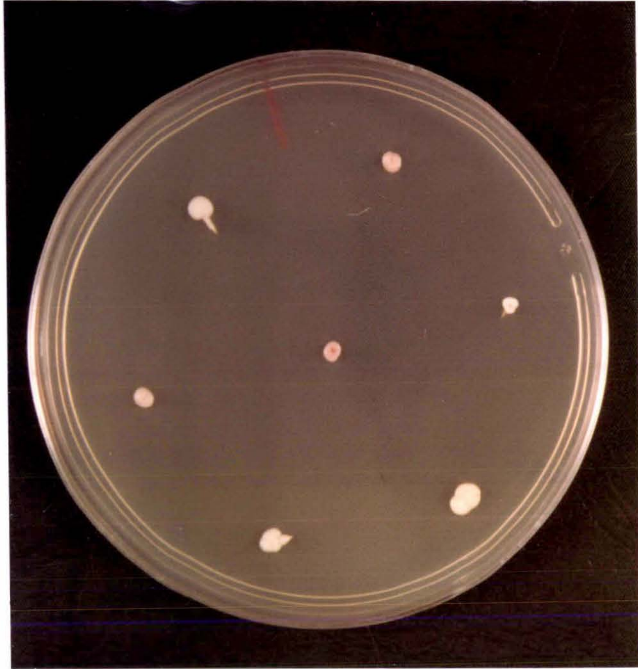
0 R92/2/47

0 x2928

EMS Esterase
0 Mutant

0 R2

0 R92



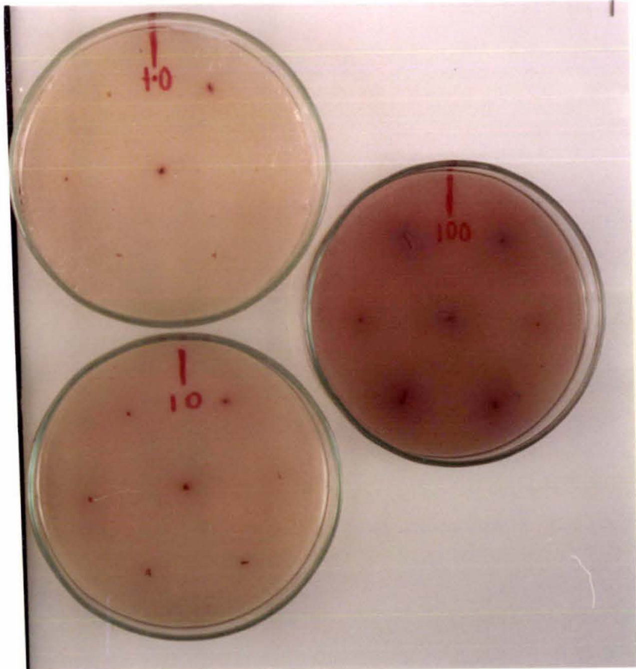
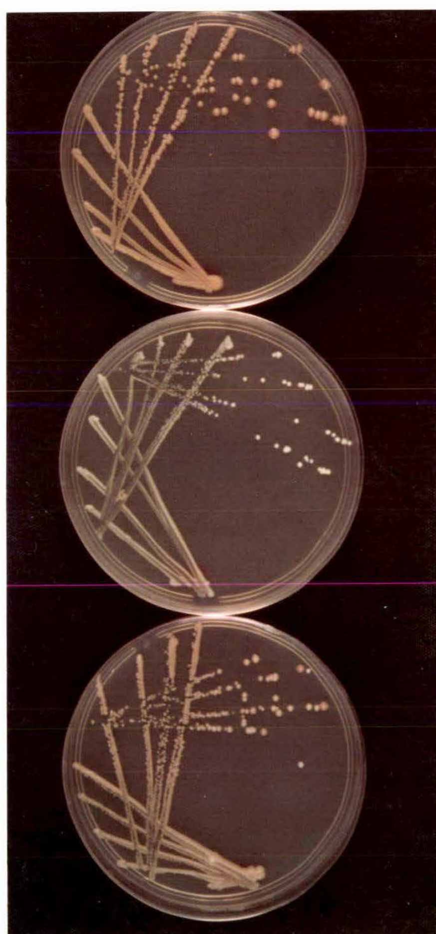

1·0
100
10

Plate 10. Comparison of Colonial Growth of R92/2/47, EMS-2, and a Spontaneous Revertant.

Upper: R92/2/47; middle: EMS-2; lower: spontaneous revertant.

Note the small size and white colour of mutant EMS-2.



3.4 Yeast Hybridisation

Heterothallic control Est 1f/1s 2f/2s was used to produce diploid strains with different esterase compositions for fermentation and gas liquid chromatography studies. On dissection, alleles and genes segregated independently (Plate 12) as expected (Strobel and Wohrmann, 1972).

The ethyl methanesulfonate induced esterase mutant (EMS-2) had unexpectedly lost both esterase bands (*Est 1f 2s*) when examined by PAGE (Plate 11). This suggested that the mutation was in a regulatory gene that controlled both esterase loci synchronously. An initial cross EMS-2 x *Est 1s 2s* α (derived from control Est 1f/1s 2f/2s) was produced and sporulated. All the resulting mutants showed the loss of both band activities (*Est 1* and *Est 2*) as observed with EMS-2. Further, mutant to non-mutant segregation did not occur at a 1:1 ratio, but rather a 1:3 ratio, indicating that gene expression in mutant strains was due to two genes. From the small number of asci dissected, the mutant did not appear to be linked to the following loci; *Est 1* , mating type *MAT*, or *ade 1*.

For further analysis two crosses of EMS-2 x *Est 1s 2f* α (derived from control Est 1f/1s 2f/2s) were sporulated and spores dissected out. On analysis by PAGE, no spores were found to be esterase mutants. This result suggested that there may have been the introduction of new genetic material with the *Est 1s 2f* α strain, which then influenced one or both genes involved in the lack of expression of esterases in mutant strains. A likely system for this gene control is discussed in Section 4.2.

During micromanipulation, EMS-2 and derived mutants were found difficult to manipulate as they would not "stick" to the dissection needle. After manipulation, mutants were found to have low viabilities of about 45% to 70% (normal cells over 85%), decrease in viability probably in part being due to cell damage during attempts to manipulate them.

For fermentations, diploid mutant strains were produced by crossing EMS-2 with a produced mutant (α mating type). Three diploid strains were produced which did not mate with either standard mater, S-1α, and S-2**a**, but would not sporulate for confirmation. The strains retained the loss of non-specific esterase activity when examined by PAGE (Plate 11).

Plate 11. Colour Photograph of a αNA Stained Polyacrylamide Gel.

Tracks loaded from left to right:

R92/2/47, EMS-2 (esterase mutant), hybrid Est -/- -/-, R92/2/47.

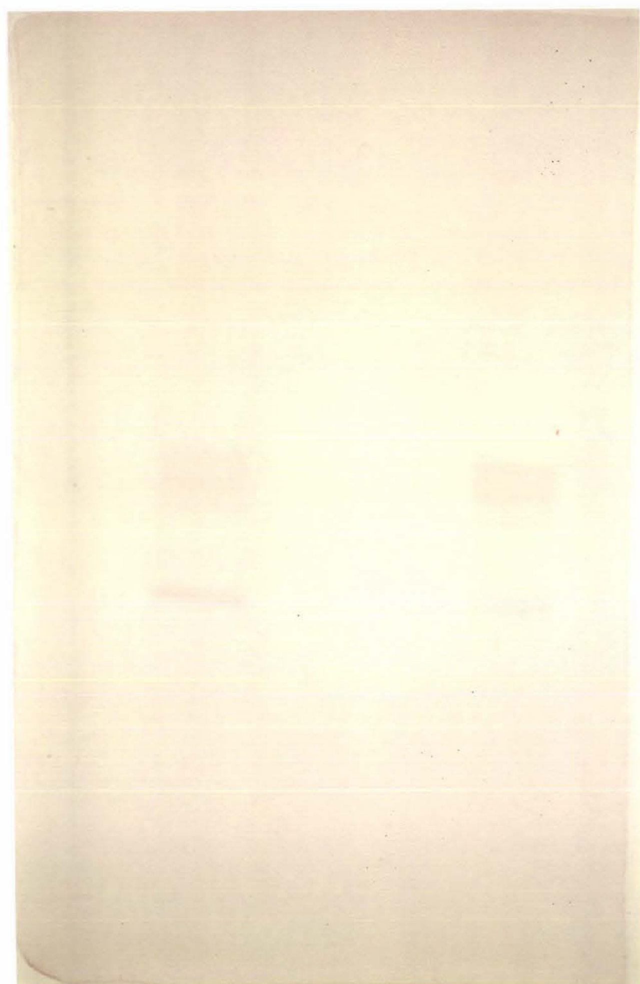


Plate 12. Esterase Composition of Some Ascospores Dissected from Three Asci of Control Est 1f/1s 2f/2s.

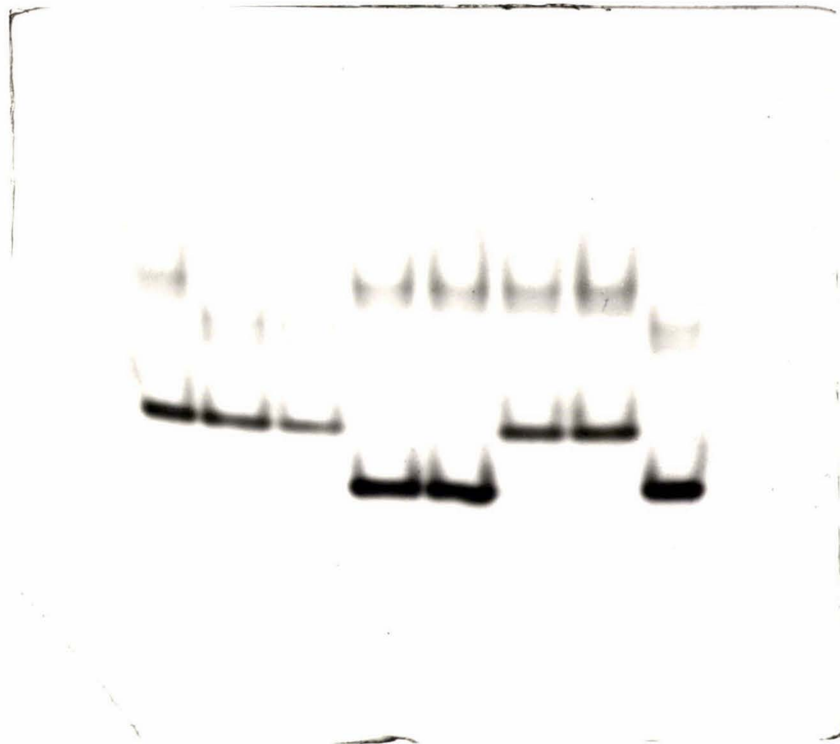
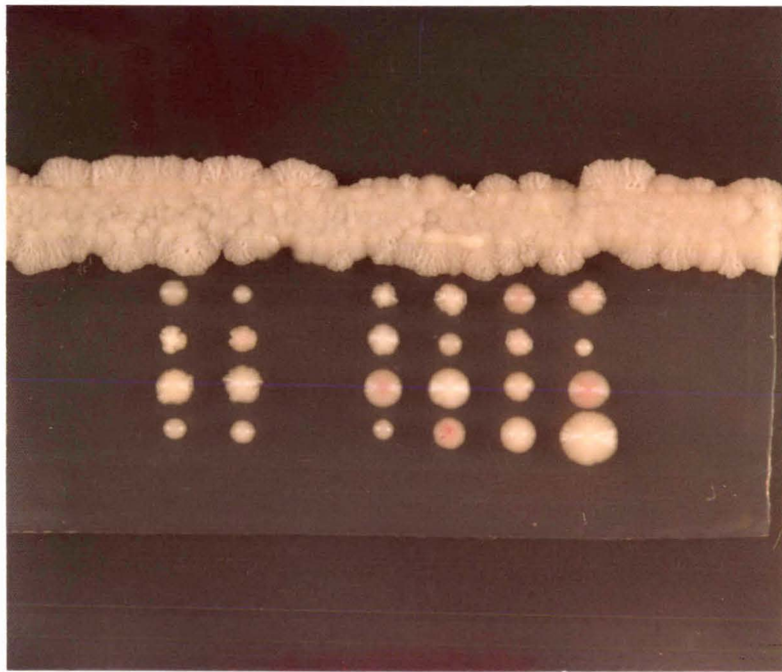


Plate 13. Dissection of Asci to give Haploid Strains.

Here, six asci have been dissected and grown up from a cross made between EMS-2 (esterase mutant) and *Est 1s 2s* α (derived from control Est 1f/1s 2f 2s). EMS-2 carried the *ade 1* marker, resulting in pink colouration of some colonies (analysis Appendix One, Table B).


Spore
d
c
b
a
Asci 1 2 3 4 5 6

3.5 Esterase Activity after Growth in Liquid Media

In this series of studies, the ester hydrolysing ability of yeast protein extracts were studied over a period of time. Two experiments were carried out; the first to follow esterase levels in GGM for cell growth esterase pattern identification, and the second anaerobically, to understand what might be occurring during must fermentation.

A standard cell density was established by taking the means of strains R2, R92 and R93 at absorbance 0.150 (400 nm) from duplicate runs (R2 = 1.96 x 10^6 cells, R92 1.87 x 10^6 cells, R93 = 1.77 x 10^6 cells) and determining an overall mean. The error was accepted as that between the means. At absorbance 0.150 (Klett 30 units) cell density was 1.87 $\pm$ 5% x 10^8 cells per ml. The Biuret method, despite relataive lack of sensitivity and accuracy because of reactions with other cellular components such as amino acids, was used for protein determination since it offered rapid and reproducible estimates in the presence of relatively high concentrations of protein. The dye binding method of Bradford (1976) was tried, but was not used because of low reproducibility and high sensitivity requiring 1/100 dilutions.

ρ-nitrophenol (ρNP) determinations were highly reproducible. Standard curves of cell density, protein and ρNP are produced in Appendix Two.

3.5.1 Aerobic Growth of Control Est 1f 2f 3 4 in General Growth Medium at 30°C

Cell density was recorded over 15 days. Cells per ml medium increased during the first 11-12 days, after which a gradual decline was observed. Protein per unit cells reached a maximum level at approximately 9 days, as did levels of ρNP units per unit cells (Figure 10). The decline in protein levels probably coincided with the depletion of nutrient.

A steady state of approximately 3 ρNP units per mg protein was established after 5 days and persisted until at least the 11th day.

Figure 10. Growth Measurements of Control Est 1f 2f 3 4 under Aerobic conditions in General Growth Medium.

●-● cells/ml medium

■-■ protein/9.3 x 10^7 cells

▲-▲ ρNP units/9.3 x 10^7 cells.

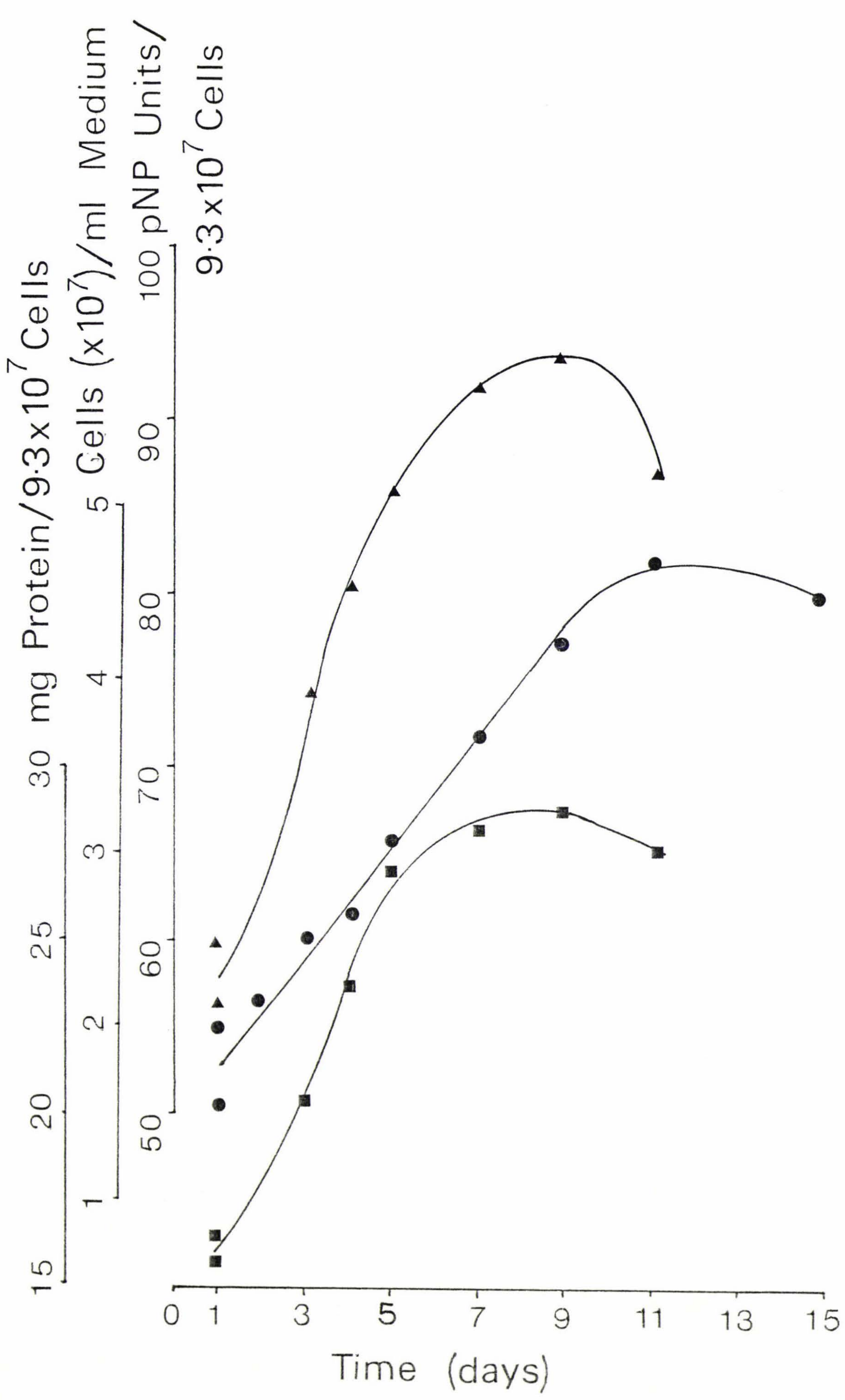
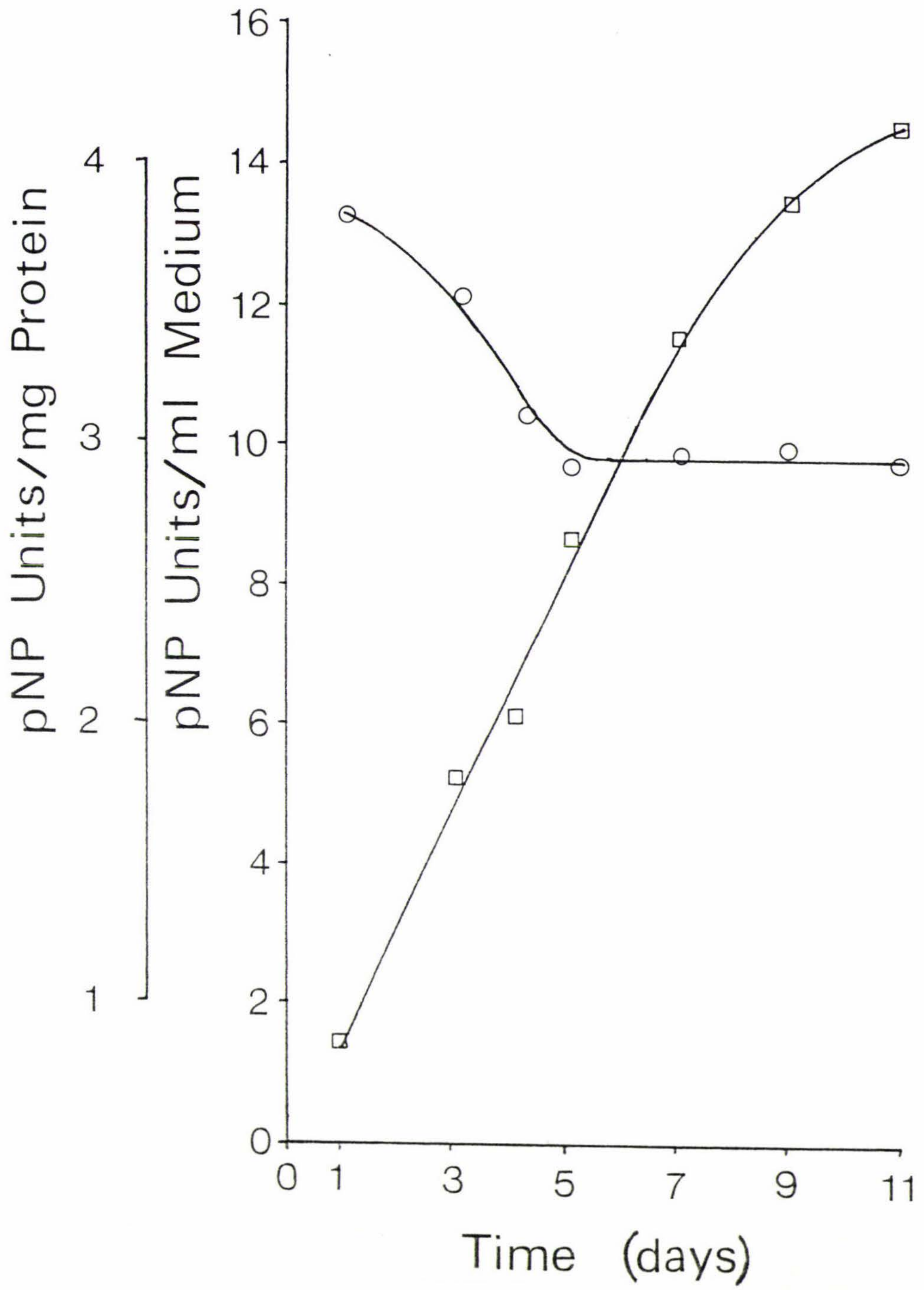

15 20 25 30 mg Protein/9·3x10^7 Cells
1 2 3 4 5 Cells (x10^7)/ml Medium
50 60 70 80 90 100 pNP Units/
9·3x10^7 Cells
0 1 3 5 7 9 11 13 15
Time (days)

Figure 11. Esterase Activity of Control Est 1f 2f 3 4 During Aerobic Growth in General Growth Medium.

0-0 ρNP units/mg protein;

□-□ ρNP units/ml medium.


pNP Units/mg Protein
pNP Units/ml Medium
Time (days)
16
14
12
10
8
6
4
2
0
4
3
2
1
0
1
3
5
7
9
11

The initial 5-day period probably represented the period required for the yeast cells to adapt to the new environment after growth on solid media. This steady state suggests an intimate association between esterase activity during growth.

ρNP units per ml medium increased linearly throughout growth to about 8 days. Esterase activity per ml medium continued to increase, but at a decreasing rate, at least to 11 days (Figure 11).

3.5.2 Fermentation by R92 and R2 in Complete Defined Medium and Grape Juice at $20^{\circ}C$

Cell densities increased to about 9 days during fermentation in CDM, and slightly later in Reisling Sylvaner grape juice. The initial growth during the first 2 to 4 days was due in part to the presence of oxygen in the medium and the head space which promoted aerobic growth. The population densities in grape juice reached about twice that observed in CDM (Figure 12).

The mg protein per unit cells also increased for the first 2 to 4 day period and then declined to a stationary level during nutrient depletion. In CDM, protein per unit cells continued to drop to a very low maintenance level during starvation (Figure 13). During growth, protein levels were at least 1.5 times higher in CDM fermentation, due to the increased requirement for anabolic enzyme activity for growth from only glucose and minerals.

Levels of ρNP units per unit cells in R92 grape, R2 grape, and R92 CDM, declined throughout the recording period, and little or no activity was detected at 15 days. Strain R2 in CDM, however, had increasing esterase activity up to 6 days' growth, before activity declined at a rate similar to that of other trials (Figure 14).

With R92 grape, R2 grape, and R92 CDM, similar trends of ρNP units per mg protein were observed as with control Est 1f 2f 3 4 during aerobic growth. An initial adaptive period of about 4 days occurred before cellular control of esterase activity to the new environment was reached. A steady state occurred between 5 and 9 days, the level of ρNP units per mg protein depending on the strain and medium used. Higher levels of activity occurred in grape juice, probably due to the greater

cell growth which took place in this medium. R92 CDM showed increased activity throughout the growth phase, then decreased rapidly after 9 days during the stationary or starvation phase of growth. No steady state occurred for R2 in CDM, and steady states for the other samples were higher than control Est 1f 2f 3 4 during aerobic growth (Figure 15).

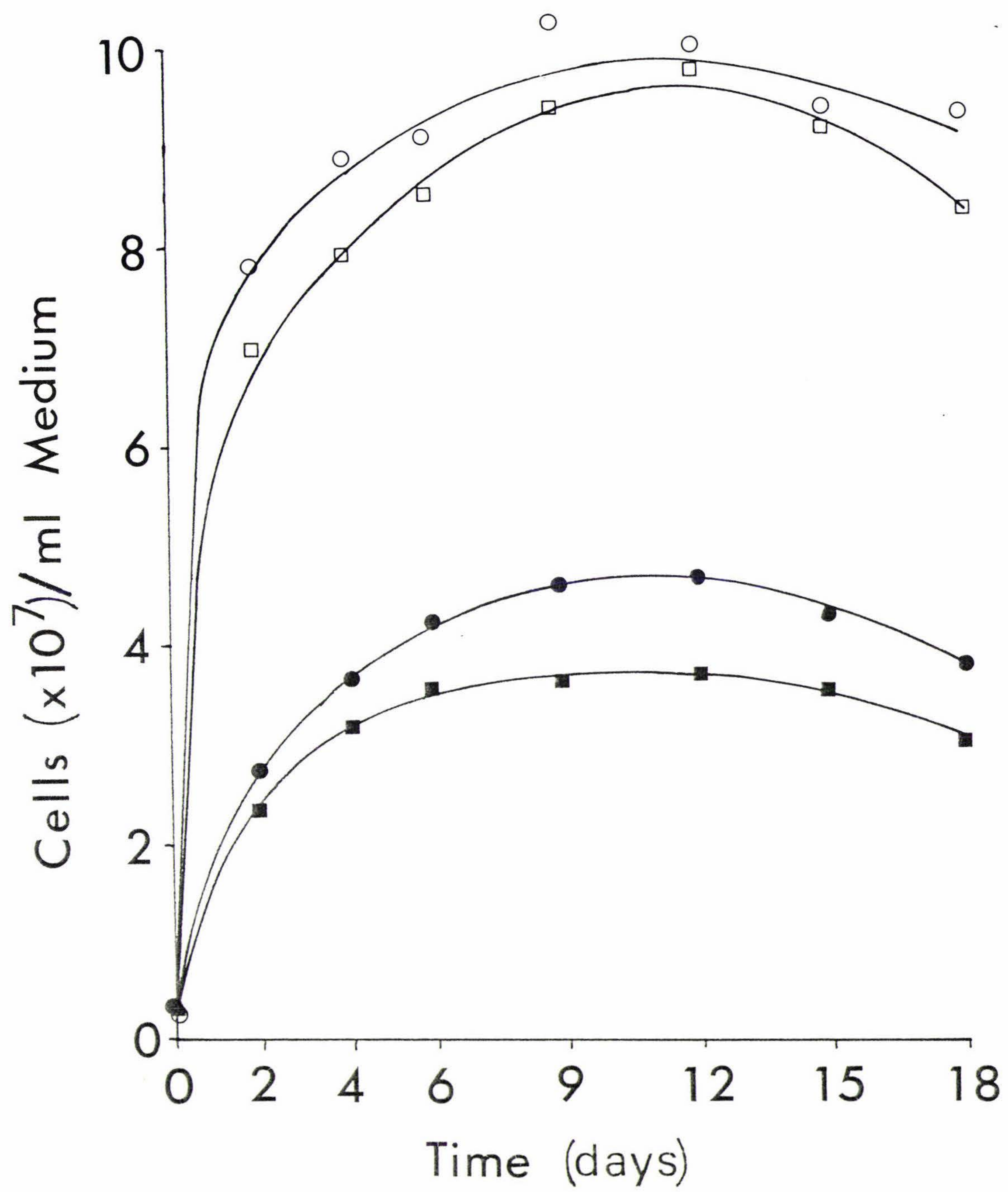
Figure 12. Cell Densities of Wine-making Yeast Strains R2 and R92 During Fermentation.

O-O R2 grape juice:

□-□ R92 grape juice;

●-● R2 CDM;

■-■ R92 CDM.



Cells (x10^7)/ml Medium
0
2
4
6
8
10
0
2
4
6
9
12
15
18
Time (days)

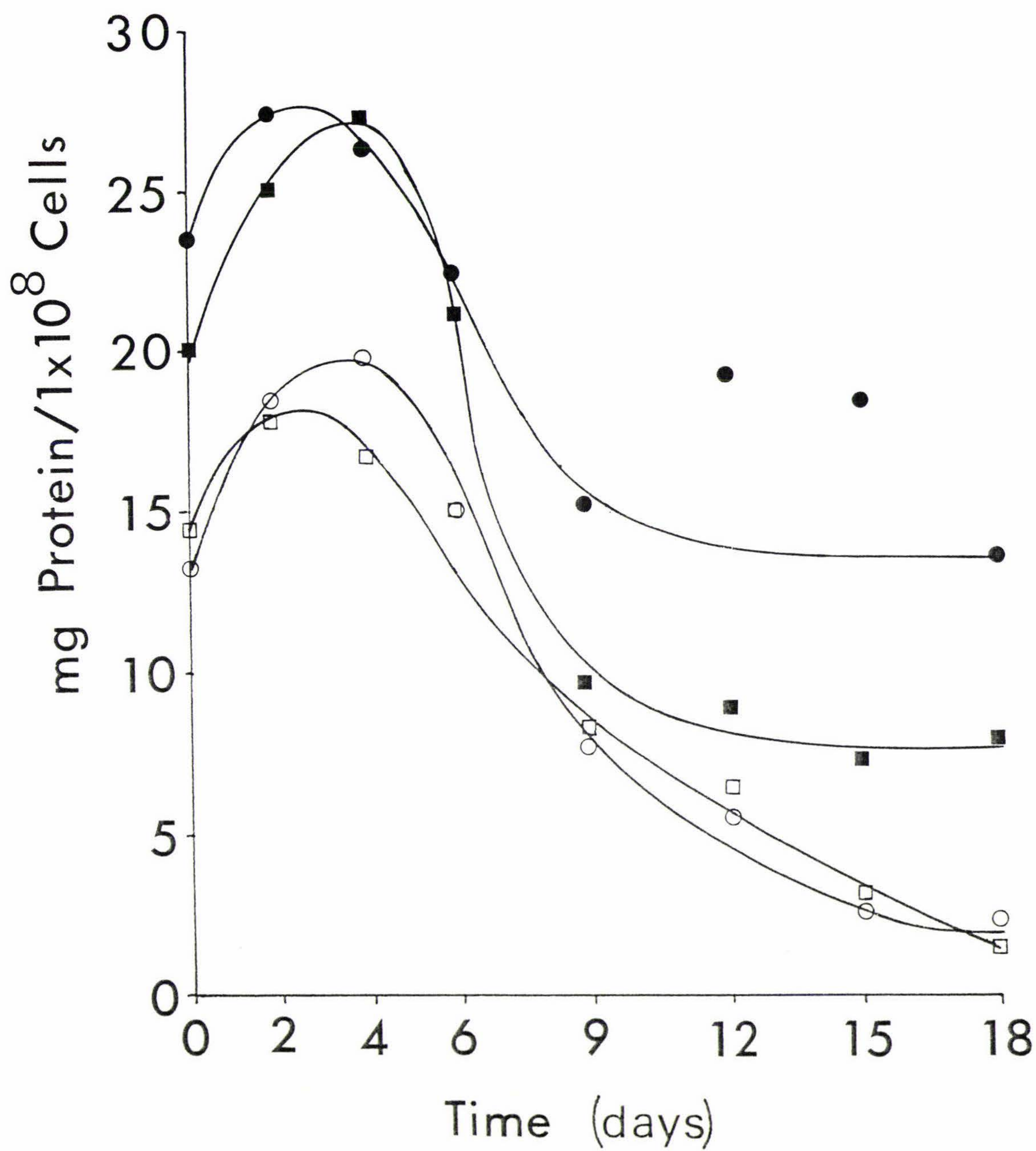
Figure 13. Cellular Protein Levels in Wine-making Yeast Strains R2 and R92 During Fermentation.

○-○ R2 grape juice;

□-□ R92 grape juice;

●-● R2 CDM;

■-■ R92 CDM.



30
25
20
15
10
5
0
mg Protein/1x10^8 Cells
0 2 4 6 9 12 15 18
Time (days)

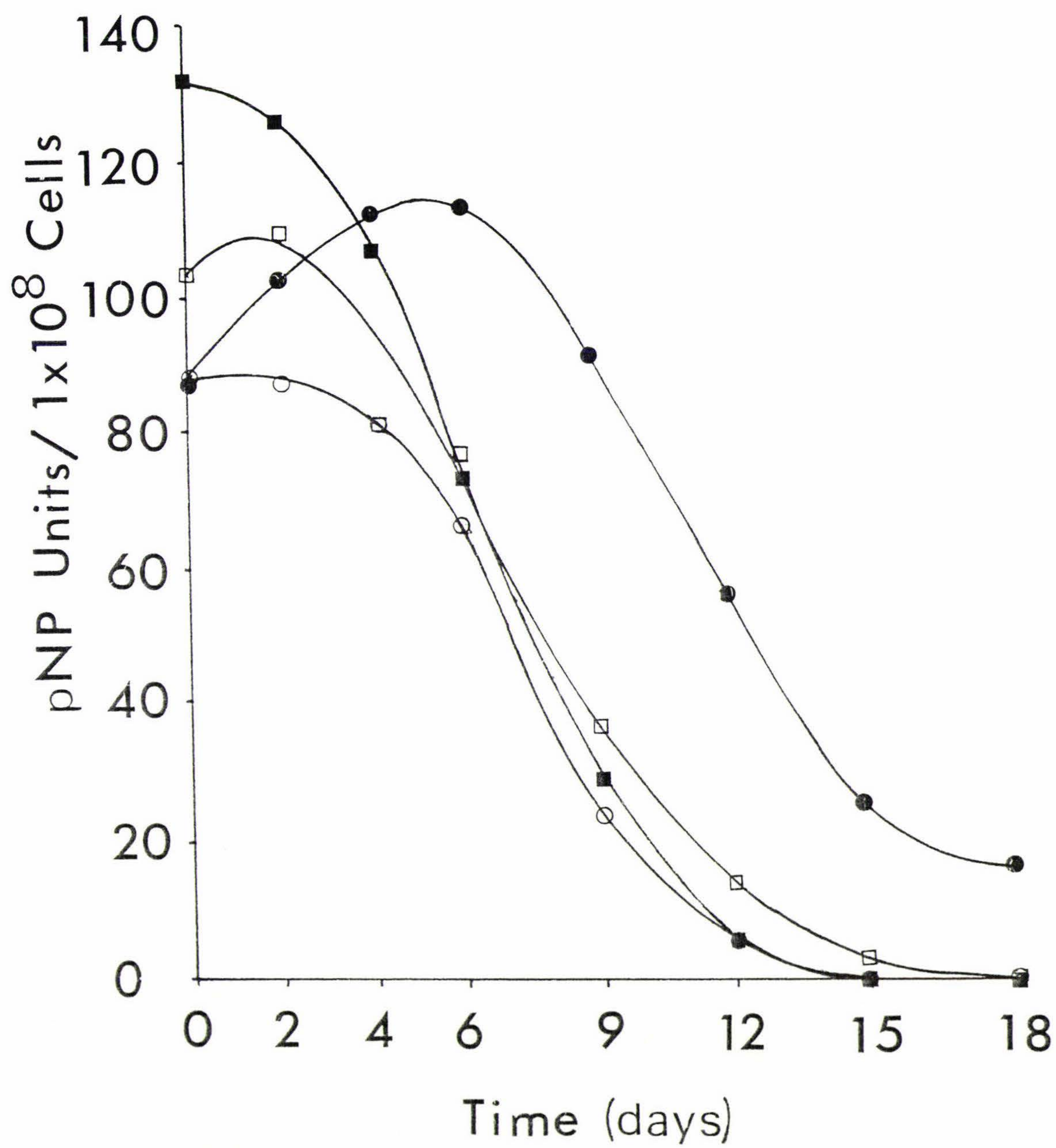
Figure 14. Esterase Activity per Unit Cells of Wine-making Yeast Strains R2 and R92 During Fermentations.

O-O R2 grape juice;

□-□ R92 grape juice;

●-● R2 CDM;

■-■ R92 CDM.



pNP Units/ 1x10^8 Cells
140
120
100
80
60
40
20
0
0
2
4
6
9
12
15
18
Time (days)

Figure 15. Esterase Activity per mg Protein of Wine-making Yeast Strains R2 and R92 During Fermentations.

O-O R2 grape juice;

□-□ R92 grape juice;

●-● R2 CDM;

■-■ R92 CDM.

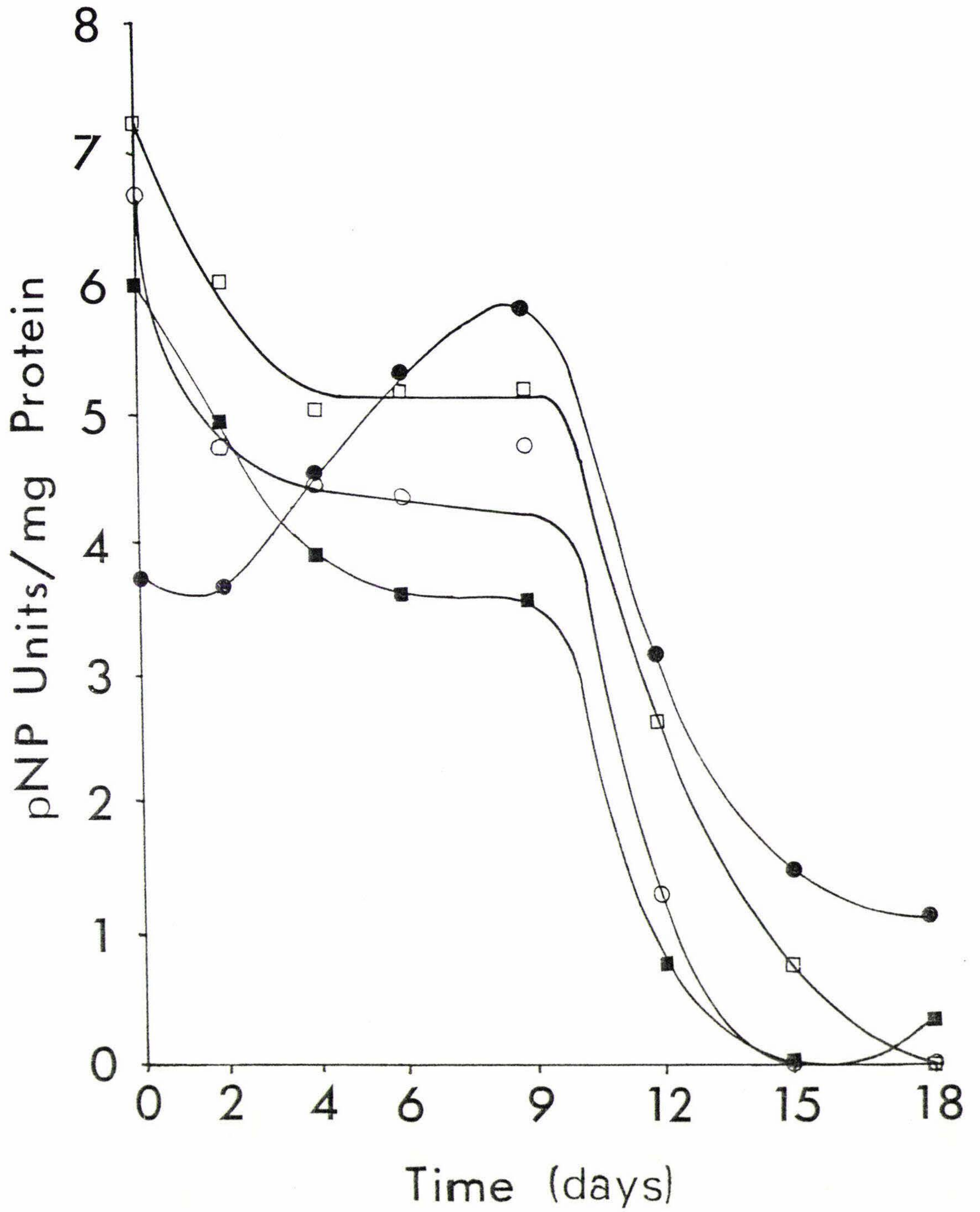

8
7
6
5
4
3
2
1
0
pNP Units/mg Protein
0
2
4
6
9
12
15
18
Time (days)

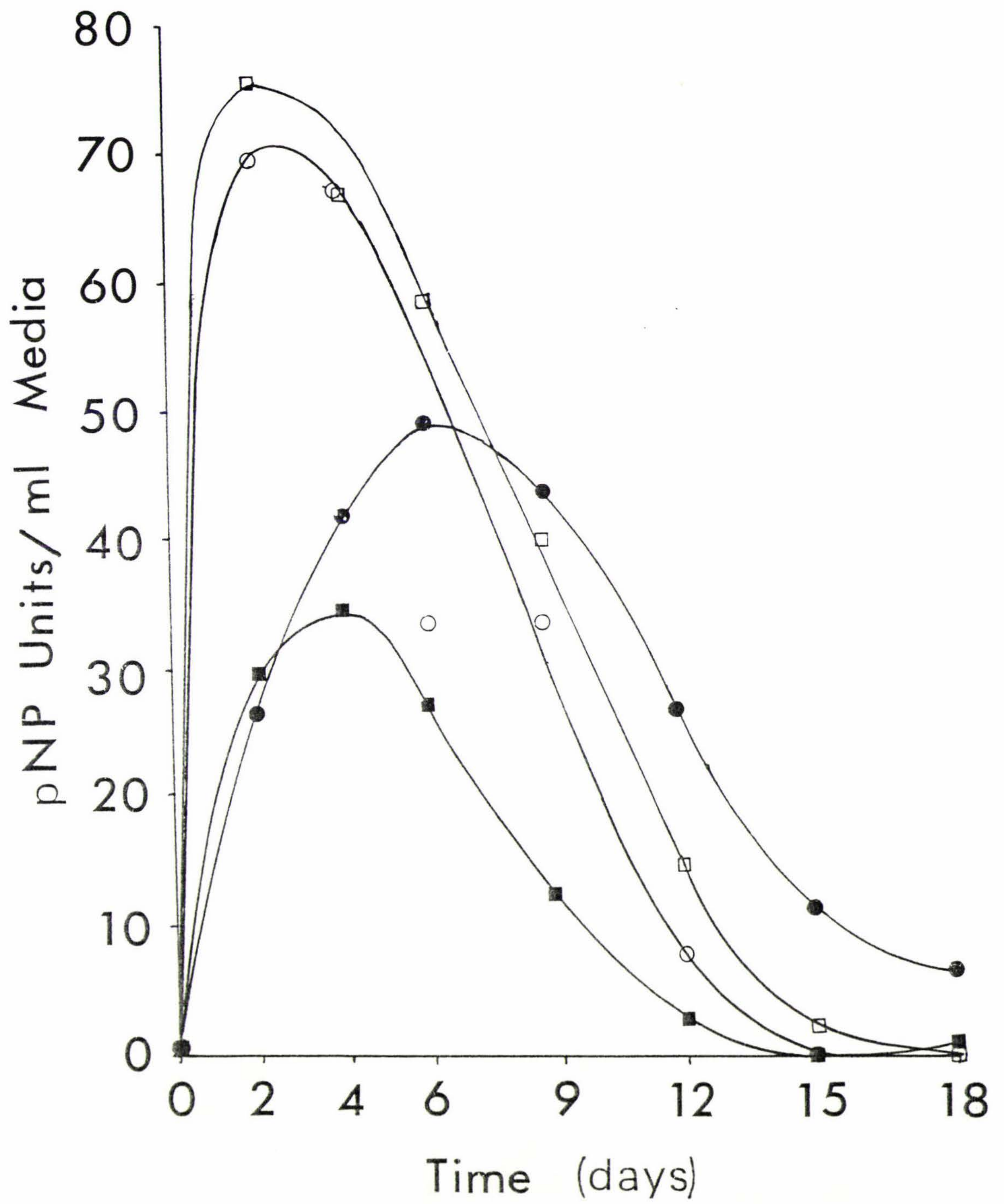
Figure 16. Esterase Activity per ml Medium of Wine-making Strains R2 and R92 During Fermentations.

O-O R2 grape juice;

□-□ R92 grape juice;

●-● R2 CDM;

■-■ R92 CDM.



pNP Units/ml Media
80
70
60
50
40
30
20
10
0
0
2
4
6
9
12
15
18
Time (days)

3.6 Gas Liquid Chromatography (GLC) for Volatile Esters

Fermentation of 900 ml or 1.8 litres (divided into two aliquots for sampling) media with yeast strains R93, R92, R2, hybrid Est -/- -/-, hybrid Est 1f/1f 2s/2s, hybrid Est 1s/1s 2f/2f, hybrid Est 1f/(1f) 2s/(2s), and hybrid Est 1s/(1f) 2f/(2s) in Reisling Sylvaner grape juice (20% fermentable sugars), and strain R93, R92, R2, hybrid Est -/- -/-, and hybrid Est 1s/1s 2s/2s in Complete Defined Medium (10% glucose) was carried out and samples prepared as described.

For rapid quantitative measurement of esters produced in fermentations the procedure of Killian and Ough (1979) was selected. This involved using redistilled *n*-pentane as the extraction solvent, and though this solvent was found extremely difficult to handle (boiling point 37°C), it was necessary because of its good selectivity for esters, and exclusion of ethanol when in high concentrations (Williams and Tucknott, 1973). Care was taken with redistilling, as the impurities in AnalaR *n*-pentane were detected up to mg amounts after distillation of 100 ml to 1 ml. For this study, a Carbowax 20M liquid phase was used instead of a SP2100 liquid phase, and the oven programme changed. Good resolution and differentiation of the ester peaks occurred, as can be seen from selected gas chromatograms; Figures 17 to 22.

The two internal standards (IS) were added in a 1 ml aliquot of redistilled *n*-pentane from a reservoir to produce as little error as possible. An initial analysis showed that direct application by syringe involved error of about $\pm$ 8% when internal standards were compared to each other. The extractibility of standards was not determined.

Concentrations of esters determined form the appropriate internal standard, were not corrected for molar response factors, as only comparative, and not absolute, values were required.

A modified Kutscher and Steudel type liquid-liquid extractor was available, and tested. This was not used because of the long time required for satisfactory extraction (two to four hours), and an inadequate length in the solvent collection tube to force the *n*-pentane through the sintered glass base.

3.6.1 Experimental Systems

In analysis of samples, no *iso*-propyl acetate was detected. Ethyl propanoate, *iso*-butyl acetate and *n*-propyl acetate, were not detected, or detected at only low levels. In samples that contained measurable amounts of these three esters it was found that some contamination of the redistilled *n*-pentane had occurred, but at only low levels and only with those esters under study. Because of only trace detections and contamination, these esters were not included in further analysis.

From three runs of an R2 grape juice sample (Table 13), loading and gas chromatograph errors were found to be about $\pm$ 4%. With comparisons of extracts from the same fermentation (Tables 13 and 14), maximum errors increased to about $\pm$ 20%.

Ester concentrations were not able to be compared directly between strains because of the different levels of growth and fermentation. A correction factor was thus employed. Plots of $\log_{10}$ ethyl acetate (mg per l) against % ethanol (v/v) for both Reisling Sylvaner grape juice and CDM (adding 1.0 mg per l ethyl acetate to each value to include the CDM control) were produced, and a linear correlation observed. Since % ethanol was determined with some accuracy, and ethyl acetate at least in part being dependent on ethanol levels, a Least Squares Regression Line was calculated for each medium (slope = 0.10 CDM, slope = 0.07 grape juice). R92 was chosen for use as a standard for comparisons (grape juice = 11.9% ethanol, CDM = 5.7% ethanol). Correction values were calculated by finding the theoretical ethyl acetate concentration at the actual % ethanol levels, and dividing this by the corrected ethyl acetate concentrations of R92 (grape juice = 18.2 mg per l, CDM = 3.7 mg per l). This was done from an ethyl acetate (mg per l) versus % ethanol (v/v) plot with media standard concentrations subtracted (Appendix One, Table F). R92 grape juice, R92 CDM, and R93 CDM ester concentrations were not corrected.

From two separate fermentations of R2 in grape juice, a total maximum error of about $\pm$ 30% was calculated, and this was used as the error limits of each adjusted value (Table 15).

Figures 17 - 19. Representative GLC Traces of Volatile Compounds from Complete Defined Medium Fermentations.

Figure 17: CDM Control.

Figure 18: R92.

Figure 19: Hybrid Est -/- -/-.

Figure 17

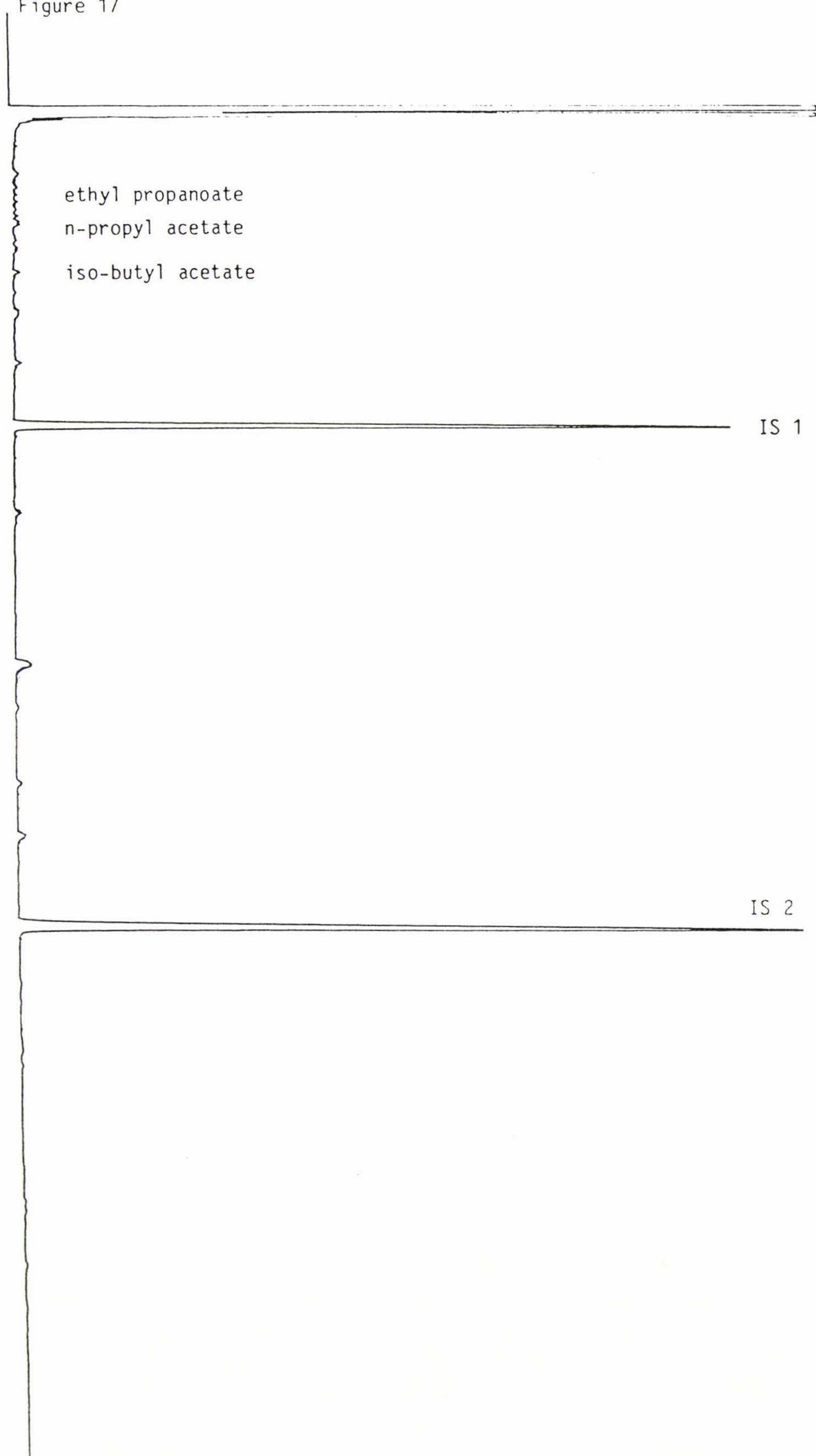


Figure 18

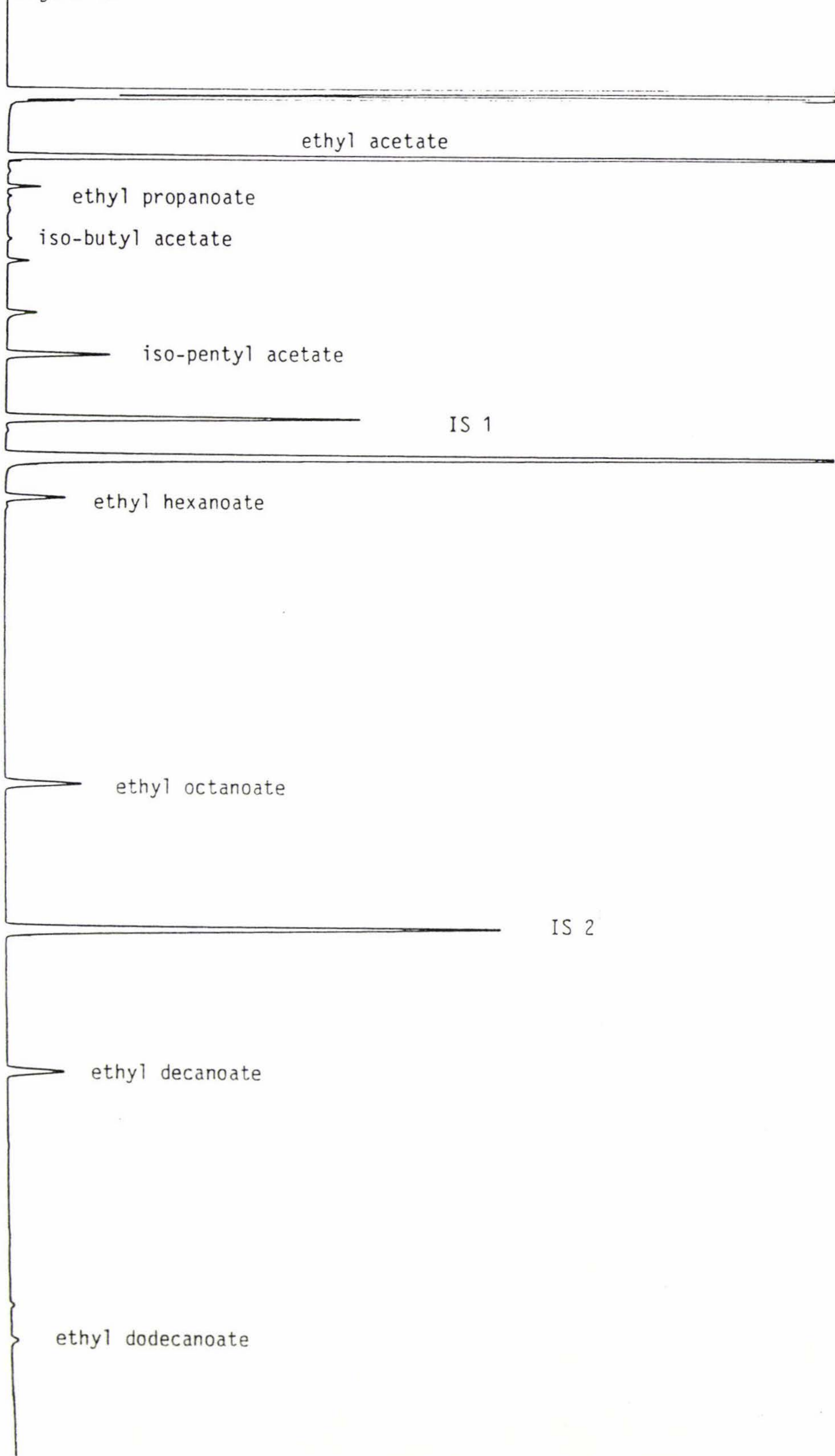
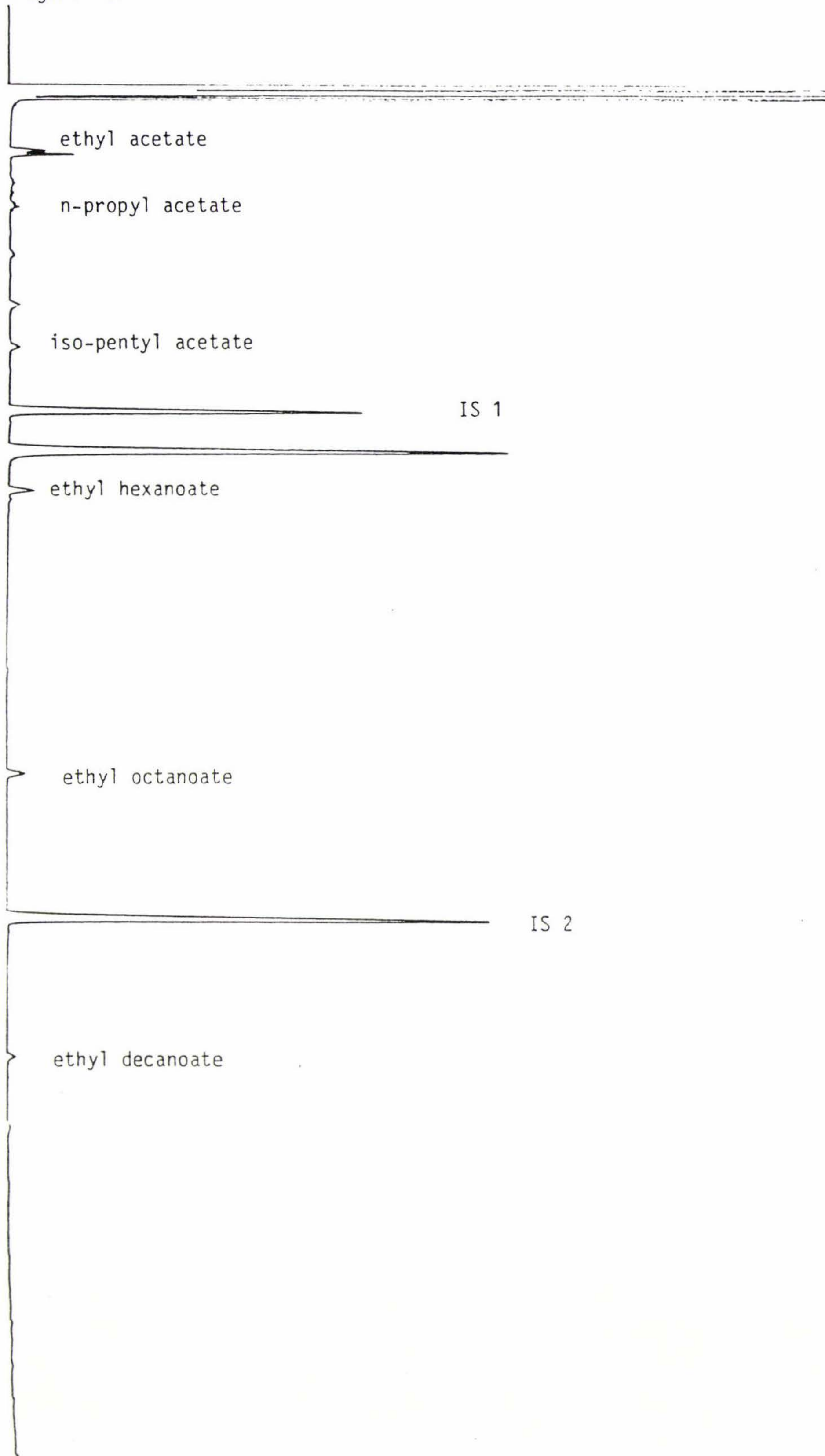


Figure 19.



Figures 20 - 22. Representative GLC Traces of Volatile Compounds from Reisling Sylvaner Grape Juice Fermentations.

Figure 20: Grape Juice Control.

Figure 21: R92.

Figure 22: Hybrid Est -/- -/-.

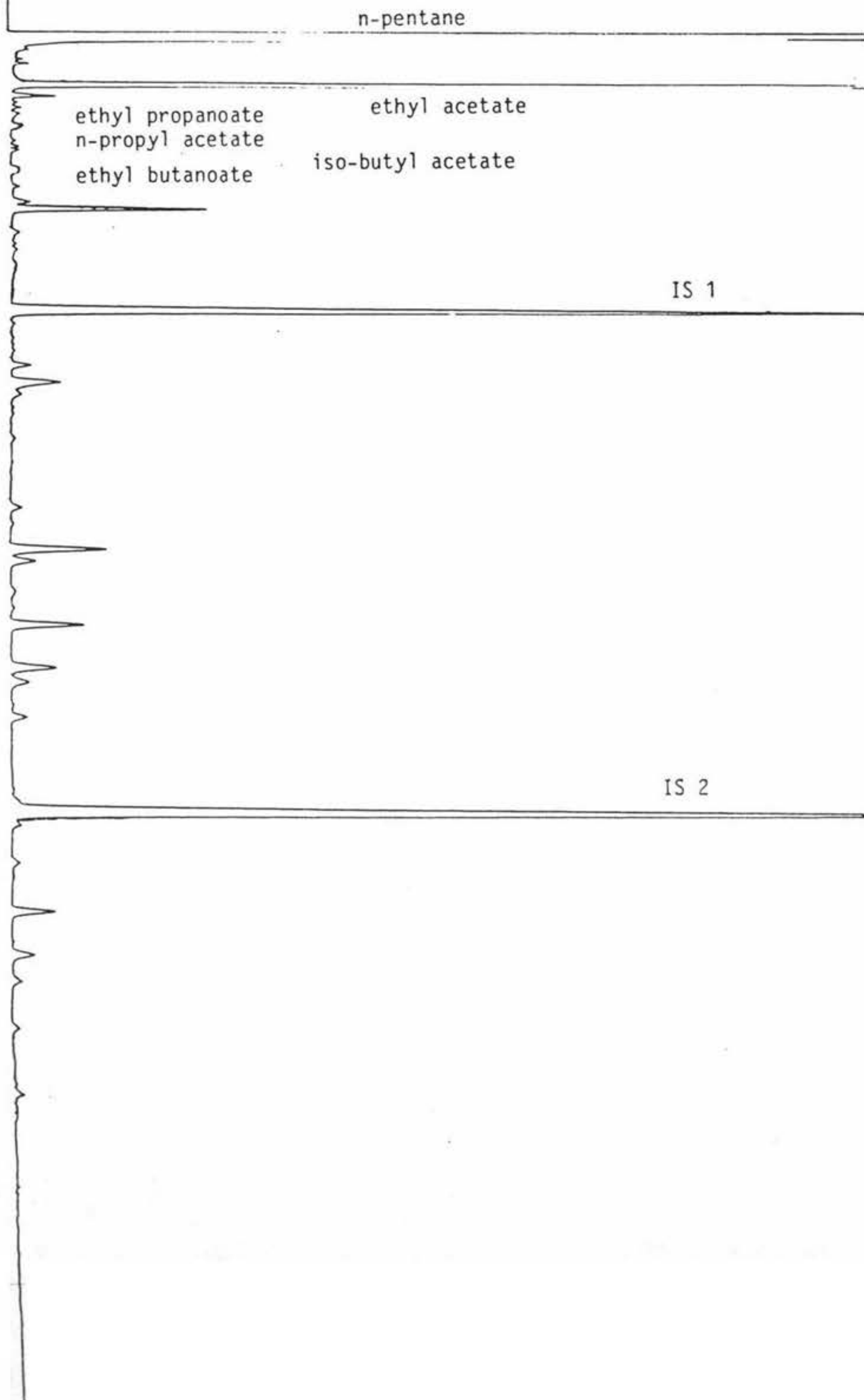


Figure 20
n-pentane
ethyl acetate
ethyl propanoate
n-propyl acetate
iso-butyl acetate
ethyl butanoate
IS 1
IS 2

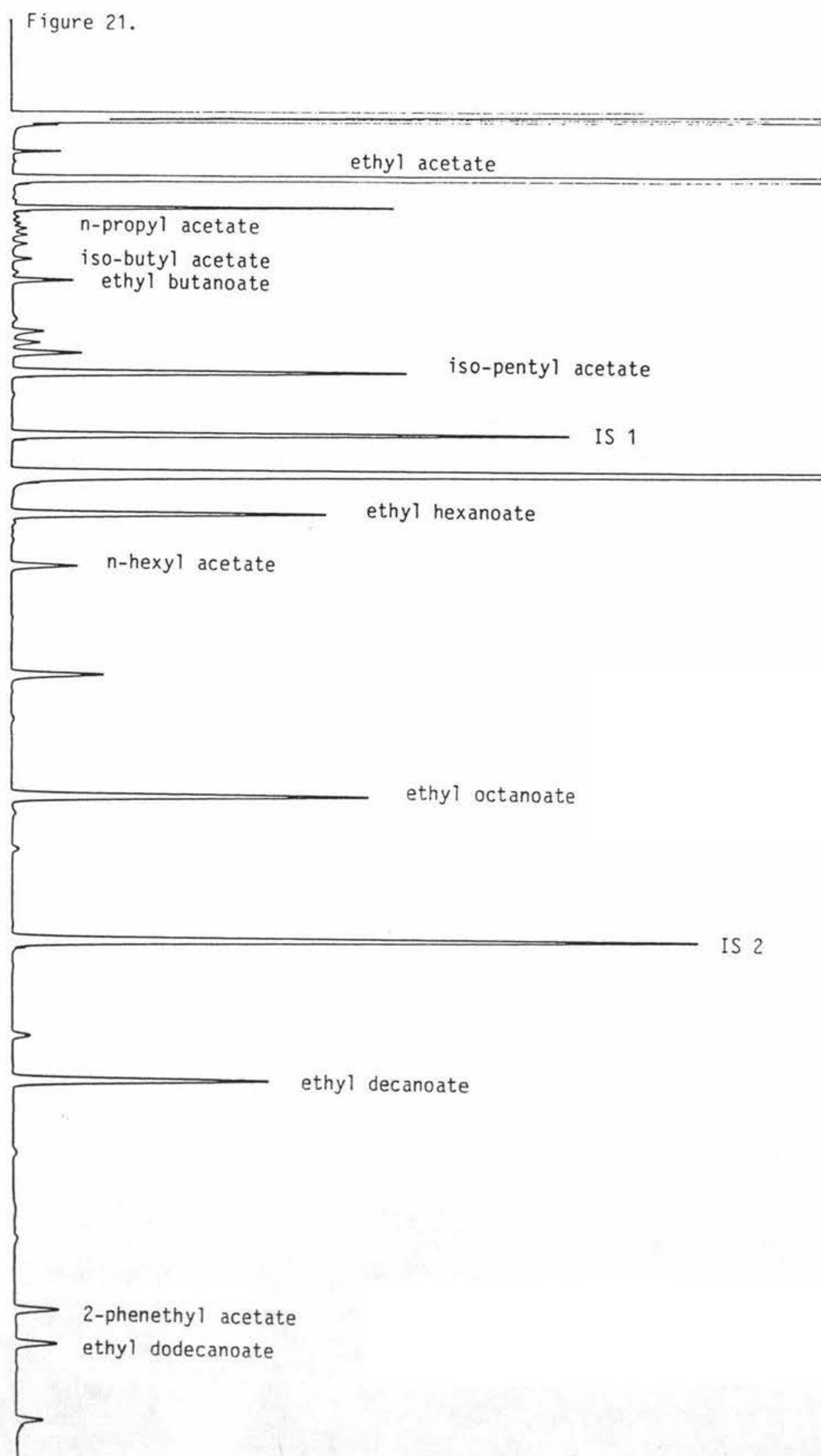


Figure 21.
ethyl acetate
n-propyl acetate
iso-butyl acetate
ethyl butanoate
iso-pentyl acetate
IS 1
ethyl hexanoate
n-hexyl acetate
ethyl octanoate
IS 2
ethyl decanoate
2-phenethyl acetate
ethyl dodecanoate

Figure 22.

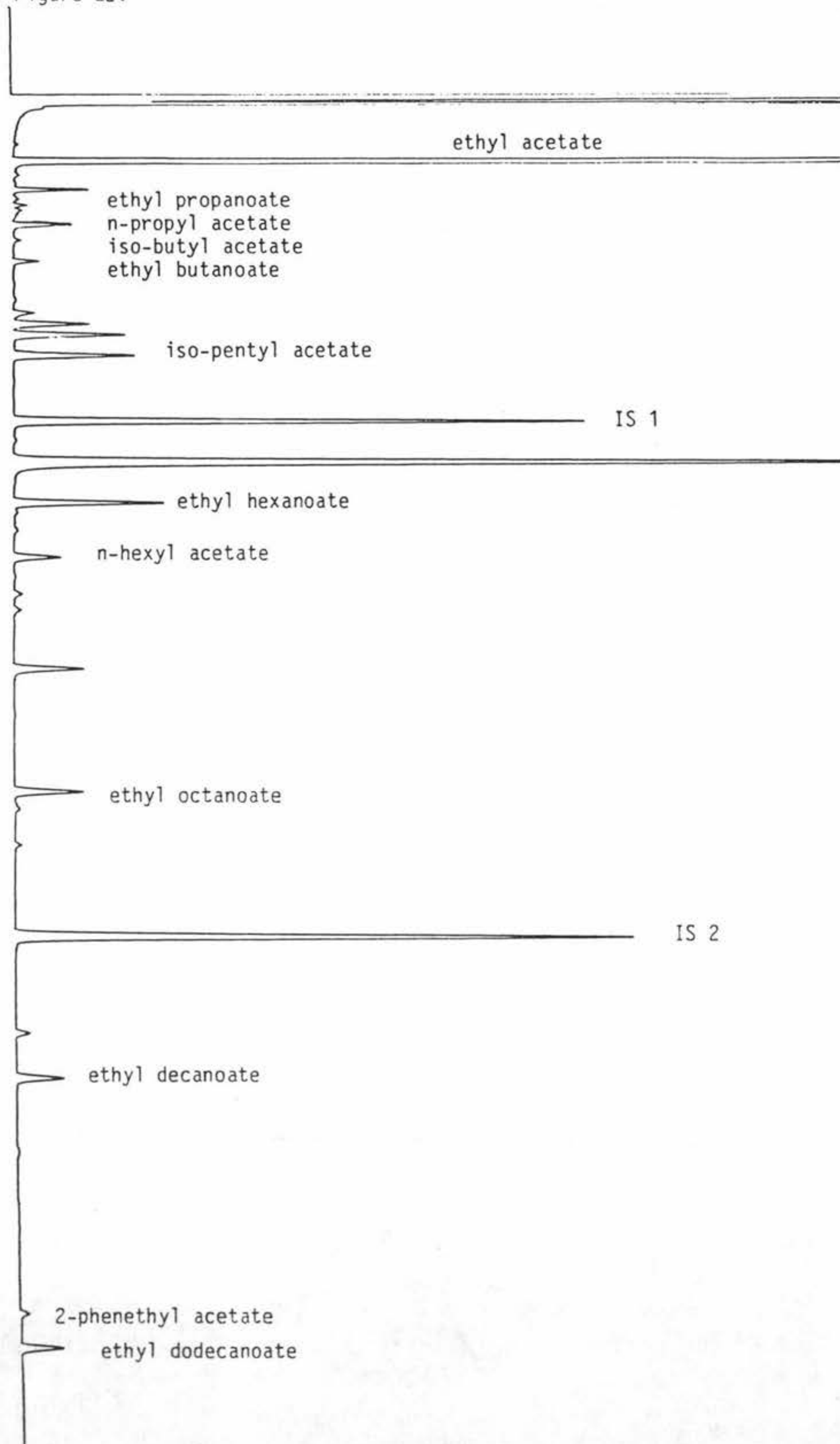


Table 13. Concentrations of Identified Esters by Gas Chromatography, from Grape Juice and Grape Juice Fermentations, with Ethanol Determination.

Strain	Sample	2-phenethyl acetate*	*n*-hexyl acetate	*iso*-pentyl acetate	*iso*-butyl acetate	*n*-propyl acetate	ethyl acetate	ethyl propanoate	ethyl butanoate	ethyl hexanoate	ethyl octanoate	ethyl decanoate		% ethanol (v/v)
GRAPE	sample 1	-	-	-	-	+	2.36	0.01	0.01	-	-	-	-	0.0
GRAPE	sample 2	-	-	-	+	0.01	2.10	+	0.01	-	-	-	-	0.0
R92		0.09	0.20	1.08	0.03	0.03	20.40	+	0.14	0.92	0.76	0.57	0.09	11.9
R2	sample 1	0.05	0.19	0.39	+	+	19.73	-	0.07	0.52	0.50	0.38	0.11	10.6
R2	sample 2 run 1	0.05	0.15	0.66	+	-	32.29	-	0.20	1.06	0.93	0.54	0.13	14.1
R2	sample 2 run 2	0.06	0.15	0.64	+	-	30.79	-	0.19	1.03	0.92	0.54	0.13	14.1
R2	sample 2 run 3	0.06	0.16	0.70	+	-	30.36	-	0.21	1.10	0.93	0.54	0.14	14.1
R93	extract 1	0.07	0.06	0.42	0.03	0.02	17.04	0.03	0.06	0.29	0.15	0.27	0.13	11.0
R93	extract 2	0.01	0.08	0.61	0.02	0.02	15.74	0.01	0.06	0.42	0.14	0.17	0.03	11.0
hybrid Est 1f/1f 2s/2s		0.01	0.13	0.28	-	-	7.70	-	0.03	0.23	0.09	0.04	+	4.9
hybrid Est 1s/1s 2f/2f		0.06	0.09	0.14	-	+	5.62	-	0.03	0.19	0.16	0.04	0.01	5.8 5.6
hybrid Est 1s/1s 2s/2s		-	-	+	-	-	4.36	-	-	-	-	-	-	2.4
hybrid Est -/- -/-		0.01	0.14	0.34	+	+	12.83	+	0.05	0.45	0.16	0.11	0.10	6.4
hybrid Est 1f/(1f) 2s/(2s)		0.01	0.03	0.16	-	+	12.60	-	0.18	0.79	0.49	0.20	0.10	10.2
hybrid Est 1s/(1f) 2f/(2s)		0.02	0.07	0.30	-	0.02	15.99	+	0.25	0.89	0.61	0.22	0.08	10.3
GRAPE	mean	-	-	-	-	0.01	2.23	0.01	0.01	-	-	-	-	0.0
R2	sample 2 mean	0.06	0.13	0.67	+	-	28.92	-	0.22	1.06	0.93	0.54	0.13	14.1
R93	extracts mean	0.04	0.07	0.52	0.03	0.02	14.16	0.02	0.05	0.36	0.15	0.34	0.08	11.0

* all measurements as mg per litre

+ trace - not detected

Table 14. Concentrations of Identified Esters by Gas Chromatography. From CDM and CDM Fermentations with Ethanol Determinations.

Strain	Sample	2-phenethyl acetate*	*n*-hexyl acetate	*iso*-pentyl acetate	*iso*-butyl acetate	*n*-propyl acetate	ethyl acetate	ethyl propanoate	ethyl butanoate	ethyl nexanoate	ethyl octanoate	ethyl decanoate	ethyl dodecanoate	% ethanol (v/v)
CDM		-	-	-	0.01	0.01	-	+	-	-	-	-	-	0.0
R92	extract 1	-	+	0.29	+	+	5.07	-	0.08	0.30	0.23	0.13	+	5.7
R92	extract 2	-	+	0.44	+	-	4.24	-	0.07	0.26	0.22	0.16	0.03	5.7
R2	extract 1	-	0.02	0.22	-	-	5.96	-	0.09	0.34	0.26	0.13	-	5.6
R2	extract 2	-	0.02	0.25	-	-	3.66	-	0.09	0.41	0.29	0.15	-	5.6
R93		0.03	-	0.72	+	-	2.40	+	0.12	0.45	0.45	0.53	0.03	5.7
hybrid Est 1f/1f 2f/2f		-	-	-	-	-	0.89'	-	-	-	-	-	-	1.6
hybrid Est 1f/1f 2s/2s		-	-	+	-	-	0.86	-	+	+	0.02	-	-	2.1
hybrid Est 1s/1s 2s/2s		0.04	-	0.63	0.04	0.03	2.01	0.02	0.11	0.35	0.18	0.21	0.03	3.2
hybrid Est -/- -/-		-	-	0.03	-	-	0.15	-	-	0.10	0.04	+	-	4.8
R92	extracts mean	-	+	0.38	+	+	4.66	-	0.08	0.28	0.23	0.15	0.02	5.7
R2	extracts mean	-	0.01	0.24	-	-	3.74	-	0.09	0.38	0.28	0.14	-	5.6

* All measurements as mg per litre

\+ trace - not detected.

Quantitative comparisons could not be made because of the large error and lack of statistical samples. Qualitative analysis was carried out for all strains against R92, and against strains of similar genetic parentage (and ethanol tolerance). Samples with overlapping error ranges were considered as showing no difference; others with decreased or increased concentrations, and the remainder with greatly decreased or increased levels where values were x2 or more. Comparisons are summarised in Table 16.

3.6.2 Comparisons of the Ester Productions from Different Diploid Yeast Strains in Grape Juice

In Reisling Sylvaner grape juice the two wine-making strains R92 and R2 showed similar ester productions in fermentation, R2 only differed in having a decreased *iso*-pentyl acetate level. R93 in comparison, though still a wine-making strain, had decreased levels of many esters (*n*-hexyl acetate, ethyl butanoate, ethyl hexanoate, ethyl octanoate), in particular ethyl octanoate, when compared to R92.

Hybrid strains when compared to each other generally had similar ester profiles. Hybrid Est 1s/1s 2f/2f had greatly increased levels of 2-phenethyl acetate, and hybrid Est -/- -/- greatly increased levels of ethyl dodecanoate, both when compared to hybrid Est 1f/1f 2s/2s. The two hybrids of identical non-specific esterase composition; hybrid Est 1f/1f 2s/2s and hybrid Est 1f/(1f) 2s/(2s), showed the most differences in ester concentrations of any grape juice comparisons, only the ethyl acetate and ethyl hexanoate concentrations being similar.

Hybrid strains compared to R92 generally showed more changes in ester concentrations than when compared to each other. All hybrids had similar levels to R92 of ethyl hexanoate and all decreased concentrations of ethyl decanoate. Four strains had similar levels of ethyl acetate (hybrid Est 1s/1s 2f/2f decreased concentration), and all strains different levels of 2-phenethyl acetate, all reduced concentrations except hybrid Est 1s/1s 2f/2f. Interestingly, hybrid Est -/- -/- had the least differences in ester concentrations of the five hybrid strains, when compared to R92.

Table 15. Adjusted Ester Concentrations with ± 30% Errors.[1]

Grape Juice	2-phen-ethyl acetate	*n*-hexyl acetate	*iso*-pentyl acetate	ethyl acetate	ethyl butanoate	ethyl hexanoate	ethyl octanoate	ethyl decanoate	ethyl dodecanoate
R92	0.06 - 0.12	0.14 - 0.26	0.76 - 1.40	14.3 - 26.4	0.10 - 0.18	0.64 - 1.20	0.53 - 0.99	0.40 - 0.74	0.06 - 0.12
R2	0.04 - 0.08	0.14 - 0.22	0.36 - 0.66	15.5 - 28.9	0.09 - 0.15	0.52 - 0.96	0.47 - 0.87	0.32 - 0.58	0.09 - 0.15
R93	0.03 - 0.07	0.06 - 0.10	0.44 - 0.82	12.0 - 22.2	0.04 - 0.08	0.31 - 0.57	0.13 - 0.23	0.29 - 0.53	0.07 - 0.13
hybrid Est 1f/1f 2s/2s	0.02 - 0.04	0.30 - 0.56	0.65 - 1.21	12.7 - 23.5	0.05 - 0.09	0.53 - 0.99	0.21 - 0.39	0.09 - 0.17	+
hybrid Est 1s/1s 2f/2f	0.12 - 0.22	0.18 - 0.34	0.28 - 0.52	6.9 - 11.7	0.04 - 0.08	0.38 - 0.72	0.32 - 0.60	0.08 - 0.16	0.02 - 0.04
hybrid Est -/- -/-	0.01 - 0.03	0.24 - 0.46	0.59 - 1.09	18.3 - 34.1	0.07 - 0.13	0.78 - 1.44	0.28 - 0.52	0.19 - 0.35	0.17 - 0.33
hybrid Est 1f/(1f) 2s/(2s)	0.01	0.03 - 0.05	0.15 - 0.27	9.5 - 17.7	0.15 - 0.29	0.72 - 1.43	0.45 - 0.83	0.18 - 0.34	0.09 - 0.17
hybrid Est 1s/(1f) 2f/(2s)	0.02 - 0.04	0.06 - 0.12	0.28 - 0.50	12.5 - 23.1	0.22 - 0.40	0.80 - 1.50	0.55 - 1.03	0.20 - 0.36	0.07 - 0.13
CDM									
R92	-	+	0.27 - 0.49	3.3 - 6.1	0.06 - 0.10	0.20 - 0.36	0.16 - 0.30	0.10 - 0.20	0.01 - 0.03
R2	-	0.01	0.17 - 0.33	2.7 - 5.1	0.06 - 0.12	0.27 - 0.51	0.20 - 0.38	0.09 - 0.19	-
R93	0.02 - 0.04	-	0.50 - 0.94	1.7 - 3.3	0.08 - 0.16	0.31 - 0.59	0.31 - 0.59	0.37 - 0.69	0.02 - 0.04
hybrid Est -/- -/-	-	-	0.03 - 0.05	0.14 - 0.26	-	0.10 - 0.18	0.04 - 0.08	0.02 - 0.04	-
hybrid Est 1s/1s 2s/2s	0.07 - 0.13	-	1.1 - 2.1	3.5 - 6.5	0.20 - 0.36	0.62 - 1.14	0.31 - 0.59	0.37 - 0.69	0.06 - 0.10

[1] All measurements as mg per litre.

Table 16. Qualitative Comparisons of Esters from Different Yeast Fermentations.

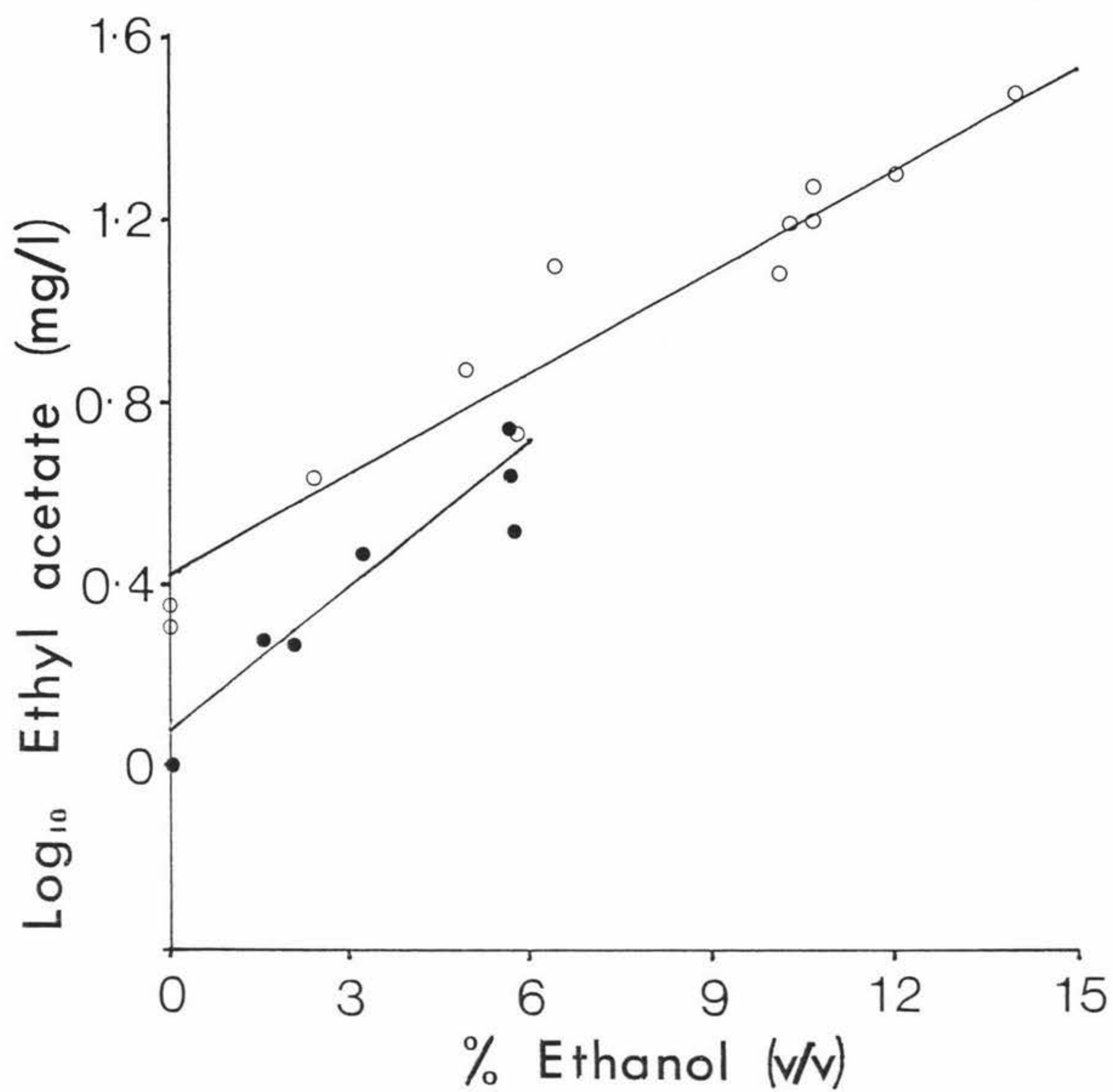
Grape Juice Comparisons	2-phen-ethyl acetate	*n*-hexyl acetate	*iso*-pentyl acetate	ethyl acetate	ethyl butanoate	ethyl hexanoate	ethyl octanoate	ethyl decanoate	ethyl dodecanoate
R92 *(Est 1f/1f 2f/2f)*	0.06-0.12[1]	0.14-0.26	0.76-1.40	14.3-26.4	0.10-0.18	0.64-1.20	0.53-0.99	0.40-0.74	0.06-0.12
R2 *(Est 1f/1f 2f/2f)* to R92	-	-	↓	-	-	-	-	-	-
R93 *(Est 1f/1f 2f/2f 3 4)* to R92	-	↓	-	-	↓	↓	↓↓	-	-
hybrid Est -/- -/- to hybrid Est 1f/1f 2s/2s	-	-	-	-	-	-	-	↑	↑↑
hybrid Est 1s/1s 2f/2f to hybrid Est 1f/1f 2s/2s	↑↑	-	↓	↓	-	-	-	-	↑
hybrid Est 1s/(1f) 2f/(2s) to hybrid Est 1f/(1f) 2s/(2s)	↑	↑	↑	-	-	-	-	-	-
hybrid Est 1f/(1f) 2s/(2s) to hybrid Est 1f/1f 2s/2s	↓	↓↓	↓↓	-	↑	-	↑	↑	↑↑
hybrid Est -/- -/- to R92	↓	-	-	-	-	-	↓	↓	↑
hybrid Est 1f/1f 2s/2s to R92	↓	↑	-	-	↓	-	↓	↓	↓
hybrid Est 1f/(1f) 2s/(2s) to R92	↓	↓↓	↓↓	-	-	-	-	↓	-
hybrid Est 1s/1s 2f/2f to R92	↑	-	↓	↓	↓	-	-	↓↓	↓
hybrid Est 1s/(1f) 2f/(2s) to R92	↓	↓	↓	-	↑	-	-	↓	-
CDM Comparisons									
R92 *(Est 1f/1f 2f/2f)*			0.27-0.49	3.3-6.1	0.06-0.10	0.20-0.36	0.16-0.30	0.10-0.20	0.01-0.03
R2 *(Est 1f/1f 2f/2f)* to R92			-	-	-	-	-	-	↑
R93 *(Est 1f/1f 2f/2f 3 4)* to R92			↑	↓	-	-	↑	↑	↑
hybrid Est -/- -/- to hybrid Est 1s/1s 2s/2s			↓↓	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓
hybrid Est -/- -/- to R92			↓↓	↓↓	↓↓	↓	↓↓	↓↓	-
hybrid Est 1s/1s 2s/2s to R92			↑↑	-	↑	↑	↑	↑	↑

- no difference ↓ decreased ↓↓ greatly decreased ↑ increased ↑↑ greatly increased [1] measurements mg per litre

Figure 23. Correlations of $\log_{10}$ Ethyl Acetate (mg per l) Against Percent Ethanol (v/v) from Grape Juice and Complete Defined Medium Fermentations.

O-O Reisling Sylvaner grape juice.

●-● CDM



1·6
1·2
0·8
0·4
0
Log₁₀ Ethyl acetate (mg/l)
0
3
6
9
12
15
% Ethanol (v/v)

3.6.3 Comparisons of the Ester Productions from Different Diploid Yeast Strains in Complete Defined Medium

Wine-making strains R2 and R92 in CDM had similar ester profiles, R2 differing from R92 only in having a greater concentration of ethyl dodecanoate. The third wine strain studied, R93, had similar concentrations of only ethyl butanoate and ethyl hexanoate, with increased concentrations of the other esters except ethyl acetate.

The double mutant hybrid Est -/- -/- showed reduced concentrations of most esters (most of them markedly) when compared to R92. Reduced levels of all esters becomes apparent when hybrid Est -/- -/- was compared to hybrid Est 1s/1s 2s/2s. Hybrid Est 1s/1s 2s/2s compared to R92, had increased concentrations of most esters (particularly *iso* - pentyl acetate), only ethyl acetate being at a similar level.

3.6.4 Comparisons of the Ester Productions from Different Diploid Yeasts Between Grape Juice and Complete Defined Medium

The four strains R92, R2, R93 and hybrid Est -/- -/- were fermented in both media. Comparisons against R92 showed differing responses of the other three strains due to the medium fermented. R2 showed few changes, with differences only in *iso*-pentyl acetate and ethyl dodecanoate concentrations. R93, in contrast, showed extensive changes in response to media. None of the esters showed the same changes in concentration.

Hybrid Est -/- -/- in grape juice had the most similar ester concentration profile to R92 of the hybrid strains. In CDM, however, reduced concentrations of nearly all the esters was observed.

DISCUSSION AND CONCLUSION

4.1 Non-specific Esterases by Polyacrylamide Gel Electrophoresis

The esterase allele composition of only a few *Saccharomyces cerevisiae* strains other than wine-making types have been published (Strobel and Wohrmann, 1972), but it seems Wohrmann and Lange (1980) had unpublished results from PAGE examinations of numerous other strains which suggested that there is selection for fast alleles in wine-making strains. If this is true, then this hypothesis is upheld by this study, since *Est 1f* and *Est 2f* alleles were ubiquitous in the 20 wine-making *S. cerevisiae* strains investigated. The three laboratory strains examined all had the *Est 2s* allele in contrast to the wine-making strains and, though this is too small a sample for a valid statistical analysis, it suggested that there could be allele variations between different sub-populations of *S. cerevisiae* brought about by different selective pressures.

Further investigations into the distribution of non-specific esterase alleles of *Est 1* and *Est 2* were prevented by the inability to isolate wild *S. cerevisiae* from yeast blooms on mature grape skins. This probably reflects a genuine absence, or at least the relatively rare occurrence, of this species on grapes. This conclusion is supported by the lack of isolates from large samples of New Zealand soils (di Menna, 1955; di Menna, 1965), and the rare isolation from fruits and berries worldwide (Phaff *et al.*, 1978). *S. cerevisiae* has been found in New Zealand vineyard soils and ripe grapes over summer, but not on vines or green grapes (Parle and di Menna, 1965). This summer occurrence in vineyards is believed to be due to reinoculation from the winery and addition of pumice to the soil.

Isolation of *S. cerevisiae* was hindered to a certain extent by the large number of tests required to identify yeast species. In the future this may be simplified by use of the system of Barnett *et al.* (1983), which indicates that only eleven tests (Appendix Three, Table G) are required to identify this species. Screening by nitrate growth and glucose fermentation would initially reduce the number of possible isolates.

Selection for the fast alleles *Est 1f* and *Est 2f* in wine-making strains may be intrinsic by the yeast, or extrinsic by the vintner. Though certain allele combinations in the hybrid inbreed strains seem to give a competitive advantage (Strobel and Wohrmann, 1977), this was only true for each inbreed line at certain temperatures. Such intrinsic selection does not appear to be the reason for fast allele selection in wine-making strains, since more significant selective influences, such as SO_2 and alcohol tolerance, and killer factors, appear to be operative. The *Est 1f* allele is known to give a selective advantage for vegetative reproduction (Strobel and Wohrmann, 1975), possibly enabling faster growth than *Est 1s* strains during grape must fermentation. Though wine strains can be induced to sporulate, and resulting spores have a favourable viability (Thornton and Eschenbruch, 1976), sexual reproduction is unimportant in the life cycle of these strains, which may give a selection against *Est 1s/1s* homozygotes, which have an increased rate of meiosis (Strobel and Wohrmann, 1975). Other intrinsic selective advantages, as yet not determined, may be responsible for the occurrence of the fast alleles. However, extrinsic selection is also possible, fast alleles giving a more desirable final product or fermentation pattern for the vintner.

All 60 wine-making *S. cerevisiae* strains now examined by PAGE have the alleles *Est 1f* and *Est 2f*. A major difference between this study and that of Wohrmann and Lange (1980) occurs when the frequency of the *Est 3* and *Est 4* genes are compared (Table 9). The reason for this difference was not determined, but three explanations can be postulated. Firstly, since the two samples were from geographically-isolated regions (Northern and Southern hemispheres), this may represent differences due to isolation. Secondly, Southern Hemisphere wine regions are much younger than their northern counterparts, and few strains with *Est 3* and *Est 4* in the southern regions may reflect lack of accumulation with time of these enzymes under wine-making conditions. Lastly, these enzymes may be extrinsically selected for or against, depending on the quality features required in different wines from different wine-making regions.

The view that *Est 4* is particulate bound (Parkkinen, 1980) is upheld here. Disruption of cells by the french cell was insufficient to cause release of *Est 4*, unlike the observations which employed a vibration homogenizer (Berking and Hauschild-Rogat, 1970). *Est 4* bands did not become visible when Triton X-100 was added to solubilize the protein

after cell disruption. However, addition of Triton X-100 before cell disruption permitted the release of the enzyme and the *Est 4* band could be detected. Esterases are not located in the cell membrane, so it would seem likely that this esterase is being released extracellularly from the cell wall in the extracellular fraction, as opposed to being located in the periplasmic space.

The detection of only one esterase band in most *S. cerevisiae* strains examined by Campbell *et al.* (1972) was probably due to low protein loadings onto the gels, resulting in the lack of detection of the poorer staining *Est 1* bands. One strain (described as *S. chevalieri*) had a seond band, *Est 3* , which is also heavily staining, but again no bands. The second fast band seen in *S. uvarum* strains cannot be equated with other non-specific esterase bands of *S. cerevisiae* . However, the classification of Barnett *et al.* (1983) is considered highly controversial, and many types now listed as *S. cerevisiae* may be reclassified as distinct species in the future.

Loci *Est 1* and *Est 2* are multiallelic, as is found with many esterase loci from higher animals. The alleles are charge allozymes as would be expected, rather than molecular weight or charge/molecular weight allozymes. Differentiation into different types of allozymes appear not to have been done for multiallelic esterase loci in other animals. Other studies have shown that esterases can be either monomeric (*Est 1* , *Est 3* and *Est 4*) or dimeric enzymes (eg. Zouros *et al.*, 1982) like *Est 2*, or undergo post-translational modification (Cochrane *et al.*, 1979) of amino acid residues (phosphoyrlation, acetylation, or covalent attachment of glycoside) as seen with *Est 1*.

A new band, labelled *Est 5*, was found to be present in all the strains tested. It travelled with, or just behind, the tracking dye front, suggesting that it was of very low molecular weight. Inability to satisfactorily resolve this band meant that studies of it could not be carried out, and it could not be determined if it was a breakdown product as suggested by Parkkinen (1980).

Esterase activity peaks from column chromatography using ρNPA as substrate almost certainly equate to PAGE bands identified by αNA hydrolysis. Both determination procedures have identified five esterases. The smallest, *Est 5* (E_4), does not readily hydrolyse αNA, while the

remaining four, *Est 1* (E_{1B}), *Est 2* (E_3), *Est 3* (E_2) and *Est 4* (E_{1A}), all hydrolyse both ρNPA and αNA. In column chromatography E_2 represents the majority of esterase activity as does *Est 3* in strains carrying this gene (Figure 5). The largest esterase, *Est 4* (E_{1A}), appears to be particulate bound in both cases.

Molecular weight determinations by Ferguson Plots and Sephadex column chromatography fractionation do not coincide. Determinations are compared in Table 17. Differences between the column chromatography estimations are ± 20%, and though this is relatively great considering the same technique was used, these differences could be possibly due to changes in volumes collected, sample preparation, and standard curve plotting using different protein standards. A greater variability can be expected when different techniques are used, which would account for some of the differences when column chromatography determinations are compared to those of PAGE. With PAGE, MW determinations with native (enzymatically active) proteins are only valid if the standard proteins used to generate calibration curves have the same shape with the same degree of hydration and partial specific volume (Hames, 1981). MW determinations are indirectly measured since proteins migrate through gels by their radius and charge. These problems may also account for some of the differences observed.

Table 17. Molecular Weight Determinations of Non-specific Esterases

Esterase	Schermers *et al.* (1976)[1]	Parkkinen (1980)[1]	PAGE
Est 5 (E_4)	-	6,000	-
Est 2 (E_3)	-	22,000	51,000 ± 10,000
Est 3 (E_2)	67,000	45,000	60,000 ± 12,000
Est 1 (E_{1B})	130,000	90,000	73,000 ± 15,000
Est 4 (E_{1A})	-	225,000	113,000 ± 23,000

[1] Sephadex column chromatography.

The possibility of monomeric/polymeric relationships between esterases *Est 1* and *Est 2* seems unlikely, though esterase gene duplication has been considered for other organisms, such as in *Drosophila* (Zouros *et al.*, 1982). The hypothesis of monomeric/polymeric relationships has been suggested primarily because of the observed multiplicative nature of Sephadex column chromatography MW determinations. The two constitutive esterases, *Est 1* and *Est 2*, do not show such a MW relationship by this study. For enzymatic activity, these enzymes are processed differently, *Est 2* becomes dimeric, and *Est 1* undergoing post-translational modification. Further, fast and slow alleles of each locus segregate independently from fast and slow alleles of the other loci. Esterases *Est 1* and *Est 2* are regulated together. This is probably a DNA conservative measure to control enzymes of similar physiological function, rather than from a duplication of genes and control sites. In *Est 3* and *Est 4*, MW determinations by PAGE do suggest a possible polymeric relationship, though this is less obvious from the values of Parkkinen (1980). Though both esterases occur together in wine-making strains, Wohrmann and Lange (1980) have found the loci are unlinked and segregate independently. Differences in substrate specificities and the effect of different inhibitors (Parkkinen, 1980) also suggests that none of the esterases are interrelated.

4.2 Esterase Expression and Control

In all haploid and diploid strains of *S. cerevisiae* evaluated for non-specific esterases by PAGE, the constitutive nature of the *Est 1* and *Est 2* genes supports the conclusion that these enzymes are physiologically important during aerobic cell growth. In aerobically-grown cells esterases were present throughout growth. In sporulation the esterases appear not to be involved. A small amount of activity was recorded from sporulated cultures, but this seems attributable to the unsporulated cells which were not separated from the ascospores, and which are able to grow on Sporulation Medium, though very poorly.

No new bands or loss of bands was noted by PAGE over a 15 day growth period in GGM. The intensity of bands increased over this period, and is in agreement with aerobic growth esterase determinations (Section 3.5.1). The intensities of bands in relationship to each other appeared to remain constant when densitometer tracings were compared. This

suggests that the non-specific esterases are regulated together rather than individually.

The possible inducible nature of esterases as suggested by Lloyd *et al.* (1971) with *Candida lipolytica* , was tested for by use of three media, CDM, MYGP broth, and GGM. In no instance was any additional esterase induced, or ones already present repressed. No "inducible" *Est 3 Est 4* wine-making strains were encountered in this study, so their possible inducible nature could not be studied. Since identical growth conditions were used for all protein extractions, no satisfactory explanations can be given for the results of Wohrmann and Lange (1980). Possibly small changes in pH, temperature, pre-incubation time, incubation time, or medium concentration may be enough to trigger expression of *Est 3* and *Est 4* genes in these "inducible" strains. In *Candida utilis* (Campbell *et al.*, 1972) induction of esterases was due primarily to the use of different peptones as nitrogen source.

Aerobic growth studies show that esterase activity per unit cell in a complex medium increases throughout growth, and began declining only with cessation of cellular activity rather than population growth. Similar increases of esterase activity during aerobic growth have been recorded for *Candida lipolytica, Aspergillus niger*, and a yeast-like fungus, with levels decreasing at the end of growth (Lloyd *et al* ., 1971).

Non-specific esterase activity per ml medium also increased during growth with an approximately tenfold increase in overall activity before cessation of cell growth. Esterase activity per mg protein during growth was in constant proportion with the total protein after an initial period required for the cells to adapt to the new growth conditions. The steady state of esterase to total protein suggests that esterase activity may be related with other cellular metabolism and control, possibly by competition for energy, cofactors, substrate, or through charge of nitrogen to carbon ratios.

With anaerobic fermentation in two media (CDM and grape juice), yeast populations increased to about 12 days, and protein per unit cells continually decreased after an initial three days during oxygen depletion. Esterase activity per unit cells of R92 in CDM and grape juice, and R2 in grape juice, also declined after the third day (aerobic

growth) with little activity remaining by 12 days though cell populations increased to about 15 days. Activity per unit cells was different for the two strains, showing that activity levels are not only dependent on the growth phase but also the yeast strain. Activity per mg protein showed activity levels partly depend on the strain as well as the medium used. Esterase activity per ml medium also reaches a peak at about three days during adaptive growth, with levels thereafter decreasing throughout fermentation and disappearing around 15 days. Esterase activity per mg protein follows the pattern of aerobic growth. There is an initial period during adaptation to the new medium, followed by a steady state, until the beginning of starvation around nine days' fermentation. Activity then rapidly declined during the log phase and had virtually disappeared after three more days.

Strain R2 in CDM did not follow the trends observed with R92 in CDM, or R2 and R92 in grape juice. Activity per unit cells and per ml media continued to increase to around six days before declining. The difference becomes evident when esterase activity per mg protein is followed. Activity increased throughout cellular growth and declined at the beginning of starvation, around nine days. In CDM, R2 thus appears to have a continuing increased demand for esterases, showing the growth medium probably has some influence on the expression of esterase production.

Such a control could be through the lipid and fatty acid or nitrogen content of the medium. Both have been shown to influence ester synthesis by a fatty acid transferase (Yoshioka and Hashimoto, 1983; Yoshioka and Hashimoto, 1984b). As already noted, the type of nitrogen source in the form of different peptones caused expression of different esterases in *C. utilis* , suggesting that nitrogen content of the medium may be the controlling factor in esterase production in *S. cerevisiae*.

With aerobic growth in GGM, activity per unit cell and per unit medium increases throughout cellular growth, and in fermentation of grape juice and CDM (apart from an initial period of oxygen utilisation) falls off with no activity remaining at the end of population growth. These differences cannot be explained by the use of different media, as GGM and grape juice both have glucose as the primary carbon and energy source, with non-limiting amounts of nitrogen, vitamins and other growth factors.

Differences do not reflect changes of pH and alcohol levels on the esterase levels. GGM has an initial pH of about 6.0, which in intact cells is within the range for optimum esterase activity (Parkkinen and Suomalainen, 1982a). With anaerobic fermentation large amounts of alcohol and acids are produced. Grape musts have low pH's (Reisling Sylvaner pH 2.95), while CDM is only slightly acidic (pH 6.5). Since esterase activity readily occurred in both media, it would seem that pH is not a controlling influence on esterase production. Differences in levels of activity indicate differences in requirements for different strains and in differing media.

Aerobic growth of *Brettanomyces bruxellosis* shows a different pattern of esterase activity (Spaepen and Verachtert, 1982) from *S. cerevisiae*. Activity per unit medium follows population growth, with activity decreasing well before that of the population, as with aerobic growth in *S. cerevisiae*. However, activity per unit cells followed the pattern of anaerobic fermentation, esterase activity decreasing well before the cessation of population growth and heads rapidly towards no activity. This indicates that different yeast have different control systems for esterase production, or non-specific esterases have different functions in different species.

In the non-specific esterase mutant EMS-2 (derived from R92/2/47) and other mutants produced by micromanipulation, both *Est 1* and *Est 2* were not expressed, indicating the mutation was in a regulatory system, and both loci were regulated in conjunction. Tetrad analysis of a cross between EMS-2 x *Est 1s 2s* α suggested that two genes were involved, as 2:2, 1:3 and 1:4 segregation was observed for esterase deficiency to esterase proficiency. These were from unlinked loci, both of which were unlinked to *ade 1* (chromosome I), mating type loci *MAT* (chromosome III), and *Est 1*. This cross did not determine if either gene was linked to the *Est 2* (chromosome XV) locus.

Lack of non-specific esterase activity in EMS-2 and derived mutants appears to be through a suppressor gene (*SUP*) and a mutated regulator (Reg^{Est-}). Numerous suppressors have been induced in *S. cerevisiae*, which are involved in the translational process and enable translation of mRNA containing genetic errors. The most common suppressors are tRNA's with changed nucleic acid sequences (not always in the anticodon), which are able to add amino acids to nonsense codons UAA, UAG

and UGA. Over 30 loci have been determined for these suppressors alone (Sherman, 1982). R92/2/47 (and EMS-2) must carry a *SUP* gene, while the other strains used in hybridisation are of the wild type (sup^{+}) lacking the suppressor. The induced mutation therefore was in the esterase regulator gene (Reg^{Est}), enabling the suppressor to translate its mRNA to produce active repression. The resulting 1:3 segregation in the EMS-2 x *Est 1s 2s* α cross can be seen in Table 18.

Table 18. Segregation of Esterase Suppressor and Regulator Loci in an EMS-2 x *Est 1s 2s*α Cross.

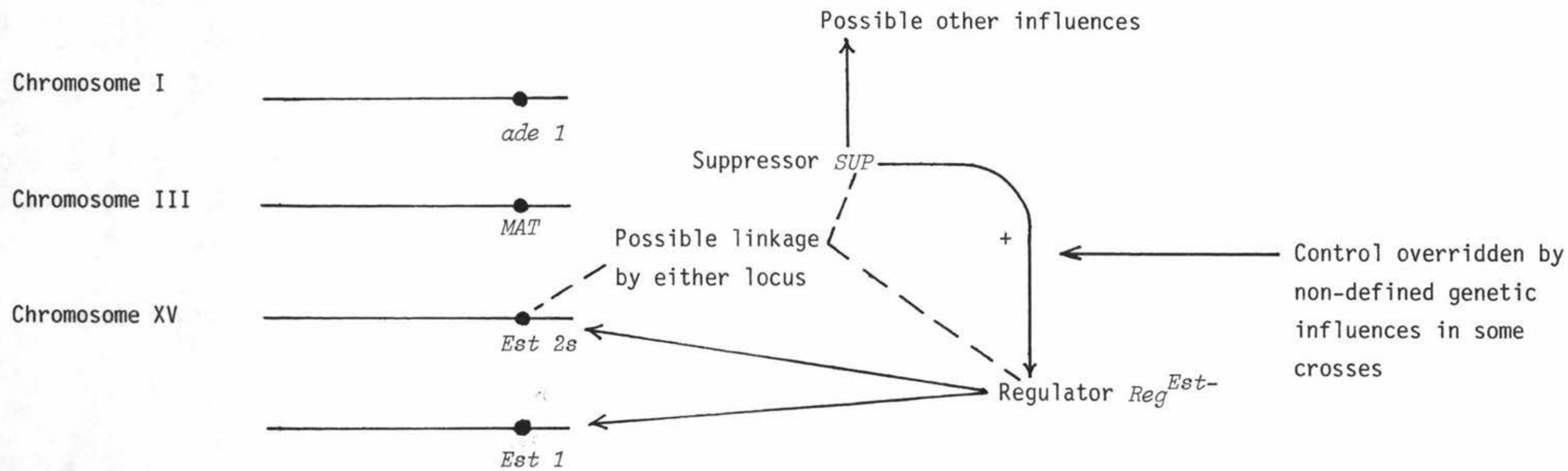
	Genetic composition	Esterase expression
Parental types		
Est 1s 2s α	sup^{+} Reg^{Est+}	proficient
EMS-2	*SUP* Reg^{Est-}	deficient
Segregates of crosses		
2:2	*SUP* Reg^{Est-}	deficient
	SUP Reg^{Est-}	deficient
	sup^{+} Reg^{Est+}	proficient
	sup^{+} Reg^{Est+}	proficient
1:3	*SUP* Reg^{Est+}	proficient
	SUP Reg^{Est-}	deficient
	sup^{+} Reg^{Est+}	proficient
	sup^{+} Reg^{Est-}	proficient
0:4	*SUP* Reg^{Est+}	proficient
	SUP Reg^{Est+}	proficient
	sup^{+} Reg^{Est-}	proficient
	sup^{+} Reg^{Est-}	proficient

In two EMS-2 x *Est 1s 2f* α crosses no esterase deficient strains were found. In these crosses, the *Est 1s 2f* α strain must have contained a suppressor modifier reducing or eliminating the expression of *SUP* (an antisuppressor) thus stopping translation of Reg^{Est-} mRNA. Numerous Mendelian segregating antisuppressors (eg. *asu* , *mod*, *sin*) have been recognised located at different loci, and at least one (*SIN 2*) pre-exists in some laboratory strains (Hawthorne and Leupold, 1974). Differences in the expression of suppressors are often observed in the progeny from different crosses, which indicates laboratory strains commonly contain different, undefined genetic backgrounds capable of influencing suppressors (Sherman, 1982). In addition, the ρ^- mitochondrial mutation has been suggested to modify the expression of certain nonsense suppressors (Ono *et al.*, 1979). Since no esterase deficient spores were observed from six asci from these crosses, it seems that antisuppressors from discrete chromosomal loci, which would have resulted in a 1:7 esterase deficient to esterase proficient segregation, were not involved. Possible supressor modification could therefore have been through undefined genetic background influences or mitochondrial mutations such as ρ^-. The suggested gene control for esterase mutants is presented in Figure 24.

Suppressors have also been shown to reduce sporulation, and in a hybridised diploid *SUP3/SUP3* strain asci formation was almost eliminated (Rothstein *et al.*, 1977). This observation could account for the lack of sporulation observed with hybridised diploid esterase deficient strains (hybrid Est -/- -/-).

During aerobic growth, cellular levels of free sterols (predominantly ergosterol) remain almost constant. Surplus sterols are converted to sterol esters by esterifying to long chain fatty acids, mostly palmitic (hexadecanoic) and stearic (octadecanoic) acids, and hydrolysed to release the sterols when required (Taketani *et al.*, 1978). Steryl esters are also formed during catabolite derepression (Quain and Haslam, 1979), and during the beginning of the stationary phase of the cell growth cycle (Bailey and Parks, 1975). Steryl esters are not incorporated into cell membranes, and appear to be for storage of sterols when there is excess production over the requirement for membrane assembly. The steryl esters are rapidly converted back to sterols when

Figure 24. Control of Non-specific Esterases in an EMS Induced Mutant and Derived Mutants.



Wild type - sup^{+}, $Reg^{Est^{+}}$

sterol synthesis is prevented, as in anaerobiosis (Quain and Haslan, 1979), when aerobically-grown cells are transferred to anaerobic growth, or during starvation (Taketani *et al.*, 1978).

It is suggested here that the main biological role of the non-specific esterases could be in sterol ester metabolism. Esterases containing protein fractions have been shown to hydrolyse steryl esters (Wheeler and Rose, 1973). The steryl esters are not membrane incorporated, as are the sterols, nor are the esterases, ensuring availability of substrate. In aerobically-grown cells increasing levels of esterase activity would keep sterols in continuous circulation, free sterol levels being maintained by some control system possibly residing in the esterases. At the end of aerobic growth and start of starvation, loss of esterase activity would allow accumulation of the esterified sterols. In anaerobic growth where steryl esters are not formed, esterase activity disappears as the stored sterols (esterified) are used up for growth.

Involvement of non-specific esterases in steryl ester metabolism could account for the fragile nature, slow growth and morphological changes in the esterase deficient mutant EMS-2. In these cells sterols would be esterified for storage away from the cell membrane, but would not be available to the cell for the co-ordination and correct functioning of the cell membrane because they lack the correct esterases to hydrolyse them.

4.3 Gas Liquid Chromatography Analysis of Esters Produced During Fermentations

The fundamental problems encountered in Gas Liquid Chromatography studies was the high error level, estimated at about ± 30% between different fermentations by the same strain. Of this, ± 4% was due to loading and instrumentation error, which is about the same as determined by Daubt and Ough (1973). The main error, about ± 16%, was the difference between extracts from the same fermentation, and appears to have been due to differences in each solvent extraction and distillation. Sample concentration by different distillation devices, especially microdistillation apparatus, are known to have a major influence on the loss of volitiles, mainly by efficiency of washing of the exposed vessel walls by the solvent (Bemelmans, 1981). Some error

is probably also involved in extraction by the steam distillation-extractor.

Liquid-liquid extractors have been used to selectively extract aroma components from alcoholic distillates (eg. Suomalainen and Nykanen, 1966). The method employed in this investigation, selective extraction with *n*-pentane in a Likens and Nickerson simultaneous steam distillation extractor followed by concentration by distillation, is now considered the best method for studying volatiles in low concentrations from alcoholic beverages (Bemelmans, 1981). Although this method has been found to have high efficiency in extracting esters (Killian and Ough, 1979), no studies have been done to determine the level of error. Since care was taken to extract esters under identical conditions, the $\pm$ 20% error must be considered normal. The remaining error in the $\pm$ 30% total was due to differences in fermentations by the same strain, and errors involved in correcting for different amounts of fermentation. All fermentations were with the same Reisling Sylvaner grape juice, but from different barrel fractions. Later fermentations utilised grape juice from the bottom of the barrel which, due to settling, had increased particulate matter and sugar (approximately 2% extra sugar), allowing increased fermentation, as can be seen by the differences in R2 fermentations (Table 13).

It must be remembered that levels of esters recorded here as mg per litre are not absolute, but are relative to the amount of internal standard (IS). Small MW esters such as ethyl acetate are at much higher concentrations than recorded because of different molar responses, as seen from Table 1.

Apart from the large errors, several other major problems were encountered. First was the problem of different ethanol tolerances by each strain. This caused different levels of fermentation, and hence ester formation. This problem could have been overcome by backcrossing four times with a selected strain to reduce the amount of genetic difference to a low level, and checking for esterase content. Alternately, all strains could have been stopped at the same level of ethanol production, but because of the low ethanol tolerance of the hybrid strains, this would have been unsatisfactory as it would result in only low levels of esters.

To enable comparison of ester production by different strains, scaling was used, where % ethanol (v/v) was plotted against $\log_{10}$ ethyl acetate, and each strain corrected to % ethanol of R92. The relationship of ethyl acetate to ethanol was linear, enabling good approximations to be determined. Similar plots using ethanol against other esters were constructed, but appeared not to show the same relationship. Wherever possible only strains of similar fermentation ability were compared to reduce error due to scaling.

Ethyl dodecanoate is not released, and ethyl decanoate and ethyl octanoate only partially released from living cells (Suomalainen, 1981), but these esters are found in significant levels in wines. Release of ethyl dodecanoate (and to a lesser extent ethyl decanoate and ethyl ocatanoate) occurs in late fermentation with cell death and breakdown. Some differences in concentration of these ethyl esters may have been due to the length of time fermented medium was stored before centrifugation, which varied up to eight days.

Stuck fermentations were a major problem, with many strains not listed in Section 2.7.1 being fermented. Since fermentation stopped at an early period when only some ethyl acetate had been synthesised, these strains were not further analysed. Results from CDM were not particularly useful because the low levels of fermentation resulted in only low concentrations of volatile esters which made quantification difficult.

The most important observation from comparisons (Table 16) is that strains with similar or identical non-specific esterase composition can differ markedly from each other for ester production. This is most clearly seen from the comparison of hybrid Est 1f/(1f) 2s/(2s) to hybrid Est 1f/1f 2s/2s in grape juice, which had more differences in ester concentrations than any other pair of strains compared. These differences show that levels of different esters produced by each strain in fermentation vary greatly, even in strains with identical esterase composition, and that ester concentrations depend largely on the availability of acids and alcohols produced by metabolic activity, rather than enzyme activity. These substrates are in general major metabolic end-products from anaerobic fermentation, acetic acid and ethanol from glycolysis, other acids from Fatty Acid Synthetase, and alcohols from

decarboxylation and transamination of amino acids (*n*-hexanol being contributed by the grape must).

It appears that non-specific esterases have little influence on the final concentrations of esters in wines. In anaeroabic fermentation with *S. cerevisiae* levels of esterases decline throughout and little activity remains during late fermentation when the majority of esters are formed. This is upheld by fermentation of hybrid Est -/- -/- in grape juice. With no esterase expression this strain produced comparable ester levels to other hybrid strains. In CDM, however, virtually no esters were produced. In CDM this strain may have been under physiological stress and, though alcohol was produced in large amounts, the fatty acids may have been rapidly utilised thus inhibiting ester synthesis.

In brewing, wort is aerated and the yeast cells probably have a high level of esterase activity. In brewing, esterase activity probably has an important influence on the final ester concentrations, and would account for the simple correlation between esterase activity and ester levels in fermented worts by different brewing strains (Schermers *et al.*, 1976).

Any selective advantages in wine strains for *Est 3* and *Est 4* genes does not seem to be related to improvement of quality of the aroma component of wines. Strain R93 (*Est 1f/1f 2f/2f 3 4*) when compared to the other two wine-making strains analysed, R2 and R92, produced inferior levels of esters. Further studies on the possible influences of *Est 3* and *Est 4* genes in fermentation was not carried out, and R93 could not be manipulated because of poor sporulation and low spore viability, in contrast to published results (Thornton and Eschenbruch, 1976).

R2, a commonly-used wine yeast strain in Australia, is considered a high ester producer. No differences in ester concentrations between R2 and R92 (widely used in New Zealand) was seen in these observations. Such a discrepancy could be accounted for by the differences in composition between New Zealand and Australian grapes. Many Australian grapes contain low levels of nitrogenous compounds in contrast to those of New Zealand, and these compounds are known to affect the fatty acid transferase activity (Yoshioka and Hashimoto, 1984b).

4.4 Conclusion

Four non-specific esterase loci are recognised in *Saccharomyces cerevisiae*, *Est 1*, *Est 2*, *Est 3* and *Est 4*. *Est 1* and *Est 2* have two alleles each, labelled as fast and slow alleles depending on their migration in PAGE. *Est 1* and *Est 2* genes are found in all strains, and are continuously expressed during aerobic growth, but not during anaerobic fermentation or sporulation. *Est 3* and *Est 4* genes have been found in brewing and bread-making yeast, a few wine-making strains, but not in laboratory strains, and appear to occur together. A fifth esterase identified by Sephadex column chromatography (E_4) and in this study by PAGE (labelled *Est 5*) is of very low molecular weight and may represent a breakdown product. Their biological functions are as yet undetermined, and though their ability to hydrolyse ethyl esters is well recorded, their main role may be in steryl ester metabolism.

Wine-making strains all contain the *Est 1f* and *Est 2f* genes, suggesting there is a strong selective advantage for the fast alleles, but as yet this selection is unknown. Influence of the esterases on the bouquet of wine by hydrolysis of esters is probably minimal, since esterase activity is rapidly lost well before completion of ester synthesis. Any selective advantage for the *Est 3* and *Est 4* also remains unknown, but probably does not exist in wine-making yeast as only 43% of strains examined carry these genes.

Several important wine characteristics have been changed by hybridisation using micromanipulation and significant improvements can be achieved for sulphur dioxide and ethanol tolerance, flocculation, and decreased foaming (Thornton, 1983). It was hoped that manipulation of the non-specific esterases would enable the aroma components of wines to be modified and improved, but these results suggest that such manipulation of the esterases has little, if any, influence. Increases in different esters may, however, be achieved by selectively crossing high ester-producing strains and isolating high ester-producing hybrids. Such increases would be due to increasing the levels of alcohols and fatty acids produced as metabolic end products. The widely-used wine-making strains, R2 and R92, had similar ester concentration profiles. This may indicate that certain combinations of ester concentrations may have an advantageous effect on the bouquet quality of white wines.

APPENDIX ONE

Table A. Relative Mobility (R_f) Values of Esterases and Protein Standards at Different Gel Concentrations (%T).

Protein	Gel Concentration							
	5.0%	5.6%	7.0%	7.5%	9.0%	11.0%	13.0%	15.0%
Est 4	0.65	-	0.46	0.44	0.34/ 0.33	0.23	0.16	0.10
Est 1s[1]	0.67	0.58	0.48	0.46	0.38	0.28	0.20	0.16
Est 1f[1]	0.74	0.63	0.54	0.51/ 0.50	0.40	0.32	0.22	0.18
Est 3	0.78	0.70	0.61	0.58/ 0.56	0.46	0.37	0.28	0.21
Est 2s	-	0.78	0.65	0.63	0.51	0.42	0.32	0.25
Est 2f	0.95	0.87	0.72	0.73	0.58	0.46	0.35	0.29
ADH	0.71	-	0.47	0.44	0.31/ 0.30	0.20	0.13	0.09
ADH	0.91	-	0.68	0.67	0.54	0.41	0.29	0.22
PGI	0.60	-	0.41	0.37	0.29	0.20	0.13	0.10
Hexokinase	0.36	-	9.25	0.23	0.18	0.13	0.09	-
Hexokinase	0.62	-	0.46	0.45	0.38	0.29	0.24	0.19
Human albumin	0.58	-	0.42	0.41	0.31	0.23	0.17	0.12
Myoglobin	0.68	-	0.58	0.56	0.49	0.42	0.35	0.30
Myoglobin	0.52	-	0.44	0.43	0.38	0.33	0.27	0.23

[1] Middle band

APPENDIX ONE

Table B. Mating Type, Esterase Composition, and *ade 1* Marker Analysis of Six Dissected Asci from an EMS-2 x *Est 1s 2s* α Cross

		Mating	Esterases		Marker
ascus 1 spore	a	α	-	-	(*ade 1*)[1]
	b	a	*1s*	*2s*	-
	c	a	*1f*	*2s*	-
	d	α	*1f*	*2s*	*ade 1*
ascus 2 spore	a	α	-	-	-
	b	a	*1f*	*2s*	-
	c	a	*1f*	*2s*	*ade 1*
	d	α	-	-	*ade 1*
ascus 3 spore	a	a	-	-	(*ade 1*)[1]
	b	a	*1f*	*2s*	*ade 1*
	c	α	*1f*	*2s*	-
	d	α	*1s*	*2s*	-
ascus 4 spore	a	α	*1s*	*2s*	*ade 1*
	b	a	*1f*	*2s*	-
	c	α	*1s*	*2s*	*ade 1*
	d	a	*1f*	*2s*	-
ascus 5 spore	a	a	-	-	-
	b	α	*1f*	*2s*	-
	c	a	*1s*	*2s*	*ade 1*
	d	α	*1s*	*2s*	*ade 1*
ascus 6 spore	a	a	*1f*	*2s*	-
	b	α	*1s*	*2s*	*ade 1*
	c	a	-	-	-
	d	α	*1s*	*2s*	*ade 1*

[1] Masked markers

APPENDIX ONE

Table C. Measured Parameters of control Est 1f 2f 3 4 during Aerobic Growth in GGM at 30°C.

Day	Cells/ml medium ($\times 10^7$)	mg protein 9.3 x 10^7 cells	nmρNP/30 minutes/0.5 ml sample	ρNP units[1]/ 9.3 x 10^7 cells	ρNP units/ mg protein	ρNP units/ ml medium
1	1.6	15.7	0.90	60	3.66[2]	4.3[2]
1	2.0	16.4	0.86	57		
2	2.2	-	-	-	-	-
3	2.5	20.6	1.11	74	3.53	7.5
4	2.6	23.6	1.19	79	3.26	7.5
5	3.1	27.0	1.27	85	3.20	9.8
7	3.7	28.6	1.37	91	3.23	12.0
9	4.1	29.0	1.41	94	3.25	13.9
11	4.7	27.8	1.33	87	3.23	15.1
15	4.5	-	-	-	-	-

[1] One ρNP unit = one nmole ρNP produced per minute per ml sample.

[2] Mean of the two values.

APPENDIX ONE

Table D. Measured Parameters of R2 and R92 during Fermentative Growth in CDM at $20^{o}C$.

R2 CDM	Day 0	2	4	6	9	12	15	18
Cells/ml medium (x 10^7)	0.3	2.8	3.7	4.3	4.6	4.7	4.3	3.8
mg protein/1 x 10^8 cells	23.8	27.2	25.9	21.7	15.6	19.0	18.8	13.4
μm ρNP/30 minutes/0.5 ml sample	1.36	1.52	1.23	1.72	1.37	0.84	0.38	0.24
ρNP units/1 x 10^8 cells	91	101	113	115	91	56	25	16
ρNP units/mg protein	3.8	3.7	4.4	5.3	5.8	2.9	1.3	1.2
ρNP units/ml medium	0.1	28	42	49	44	26	11	6
R92 CDM								
Cells/ml medium (x 10^7)	0.3	2.4	3.2	3.5	3.6	3.7	3.5	3.0
mg protein/1 x 10^8 cells	20.2	25.7	27.6	20.7	9.3	10.6	7.0	8.8
μm ρNP/30 minutes/0.5 ml sample	1.79	1.90	1.23	1.16	0.51	0.12	0	0.04
ρNP units/1 x 10^8 cells	119	127	109	77	34	8	0	3
ρNP units/mg protein	5.9	4.9	3.9	3.7	3.6	0.8	0	0.3
ρNP units/ml medium	0.2	30	35	27	12	3	0	1

APPENDIX ONE

Table E. Measured Parameters of R2 and R92 Fermentative Growth in Grape Juice at 20°C.

R2 grape	Day 0	2	4	6	9	12	15	18
Cells/ml medium (x 10^{7})	0.3	7.9	8.9	9.1	10.6	10.0	9.4	9.5
mg protein/1 x 10^{8} cells	13.4	18.7	19.5	14.4	7.5	6.6	2.6	2.8
μm ρNP/30 minutes/0.5 ml sample	1.35	1.32	0.95	1.05	0.46	0.12	0	0
ρNP units/1 x 10^{8} cells	90	88	85	70	31	8	0	0
ρNP units/mg protein	6.7	4.7	4.4	4.9	4.2	1.4	0	0
ρNP units/ml medium	0.2	70	67	33	33	9	0	0

R92 grape	Day 0	2	4	6	9	12	15	18
Cells/ml medium (x 10^{7})	0.3	7.1	7.9	9.1	9.5	9.8	9.2	8.4
mg protein/1 x 10^{8}	14.4	18.0	16.9	14.4	8.1	5.2	2.8	1.6
μm ρNP/30 minutes/0.5 ml sample	1.56	1.60	1.28	1.17	0.63	0.19	0.03	0
ρNP units/1 x 10^{8} cells	104	107	85	78	42	13	2	0
ρNP units/mg protein	7.2	5.9	5.0	5.2	5.2	2.5	0.7	0
ρNP units/ml medium	0.2	76	67	58	40	13	2	0

APPENDIX ONE

Table F. Ester Concentrations Adjusted to Specified Ethanol Levels from Yeast Fermentations.

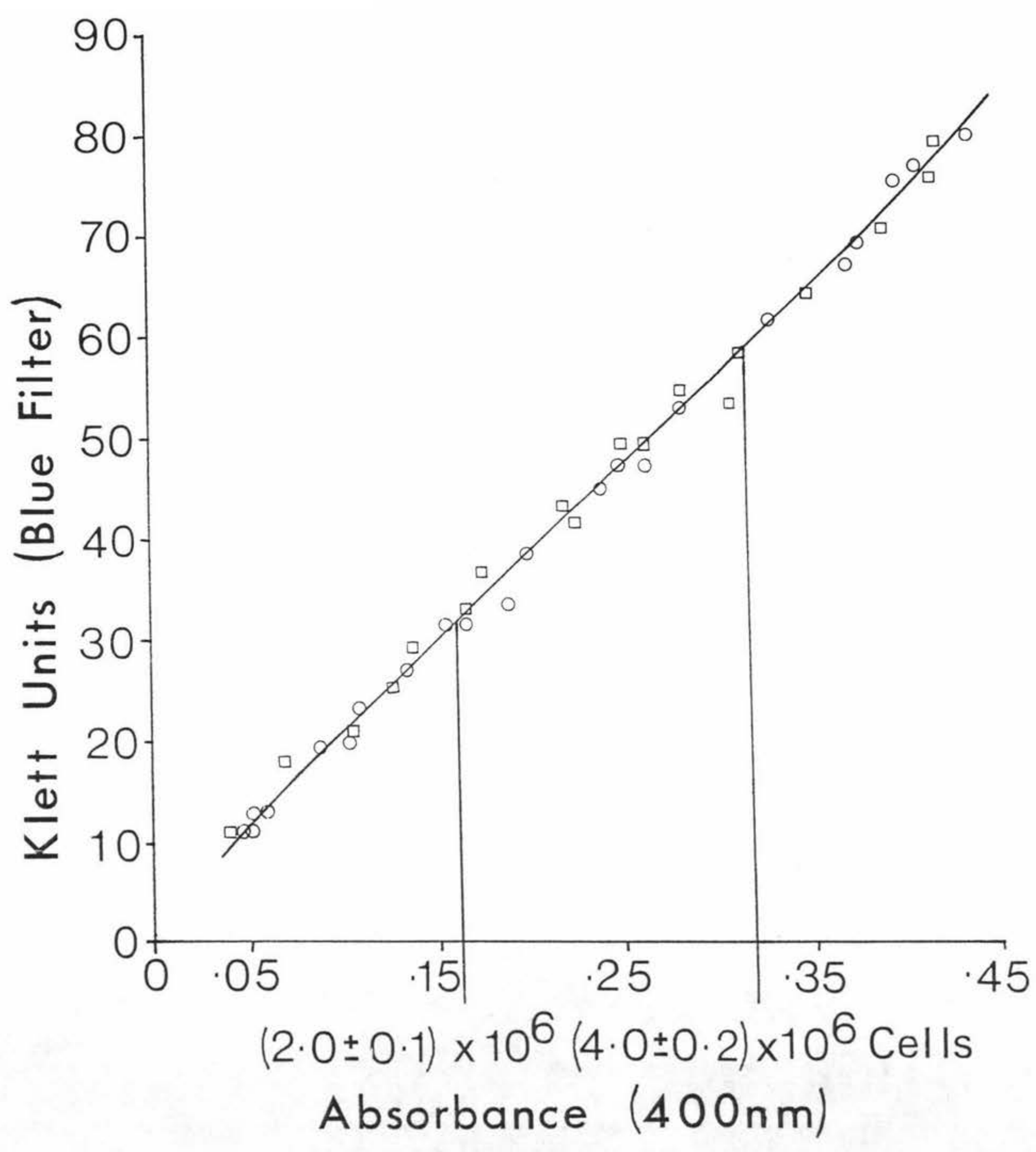
In Reisling Sylvaner Grape Juice									
R92	0.09	0.20	1.08	20.4	0.14	0.92	0.76	0.57	0.09
R2 sample 1	0.06	0.22	0.48	21.4	0.08	0.63	0.61	0.46	0.13
R2 sample 2	0.05	0.12	0.53	22.9	0.15	0.73	0.73	0.43	0.10
R93[1]	0.05	0.08	0.63	17.1	0.06	0.18	0.18	0.41	0.10
hybrid EST 1f/1f 2s/2s	0.03	0.43	0.93	18.1	0.07	0.76	0.30	0.13	+
hybrid EST 1s/1s 2f/2f	0.17	0.26	0.40	9.8	0.06	0.55	0.46	0.12	0.03
hybrid EST -/- -/-	0.02	0.35	0.84	26.2	0.10	1.11	0.40	0.27	0.25
hybrid EST 1f/(1f) 2s/(2s)	0.01	0.04	0.21	13.6	0.22	1.03	0.64	0.26	0.13
hybrid EST 1s/(1f) 2f/(2s)	0.03	0.09	0.39	17.8	0.31	1.15	0.79	0.28	0.10
R2 mean	0.06	0.17	0.51	22.1	0.12	0.68	0.67	0.45	0.12
In CDM									
R92[1]	-	+	0.38	4.7	0.08	0.28	0.23	0.15	0.02
R2[1]	-	0.01	0.25	3.9	0.09	0.39	0.29	0.14	-
R93	0.03	-	0.72	2.4	0.12	0.45	0.45	0.53	0.03
hybrid EST 1s/1s 2s/2s	0.10	-	1.58	5.0	0.28	0.88	0.45	0.53	0.08
hybrid EST -/- -/-	-	-	0.04	0.20	-	0.14	0.06	0.03	-

All measurements as mg per litre. [1] Means of two extracts.

APPENDIX TWO.

Figure A. Cell Density of Yeast Suspensions Measured against Absorbance (400 nm) and Klett Units (Blue Filter).

□ haploid *ade 1* strains; 0 diploid strains.

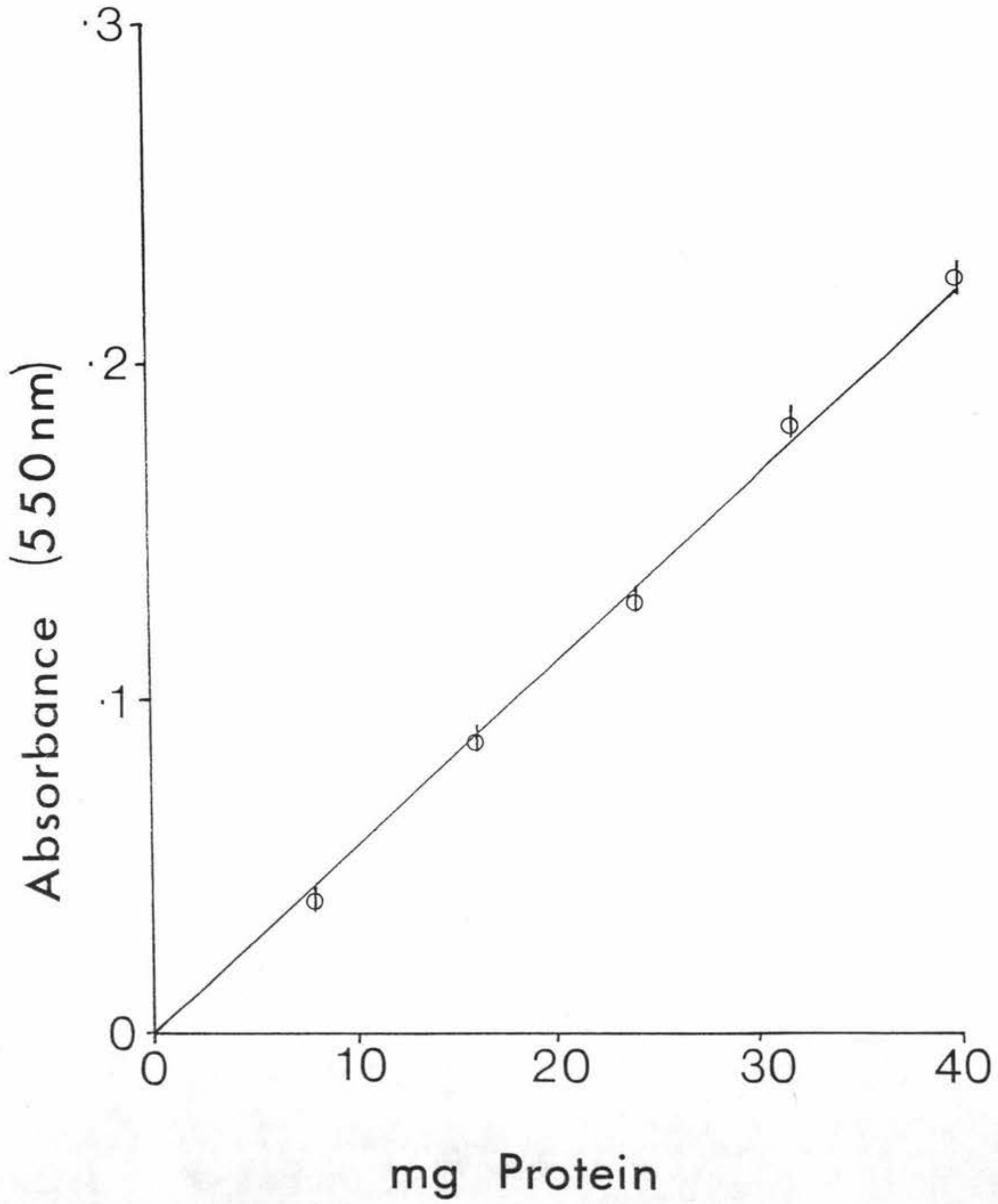


90
80
70
60
50
40
30
20
10
0
Klett Units (Blue Filter)
0
·05
·15
·25
·35
·45
(2·0±0·1) x10^6 (4·0±0·2)x10^6 Cells
Absorbance (400nm)

APPENDIX TWO.

Figure B. Biuret Protein Standard Curve.

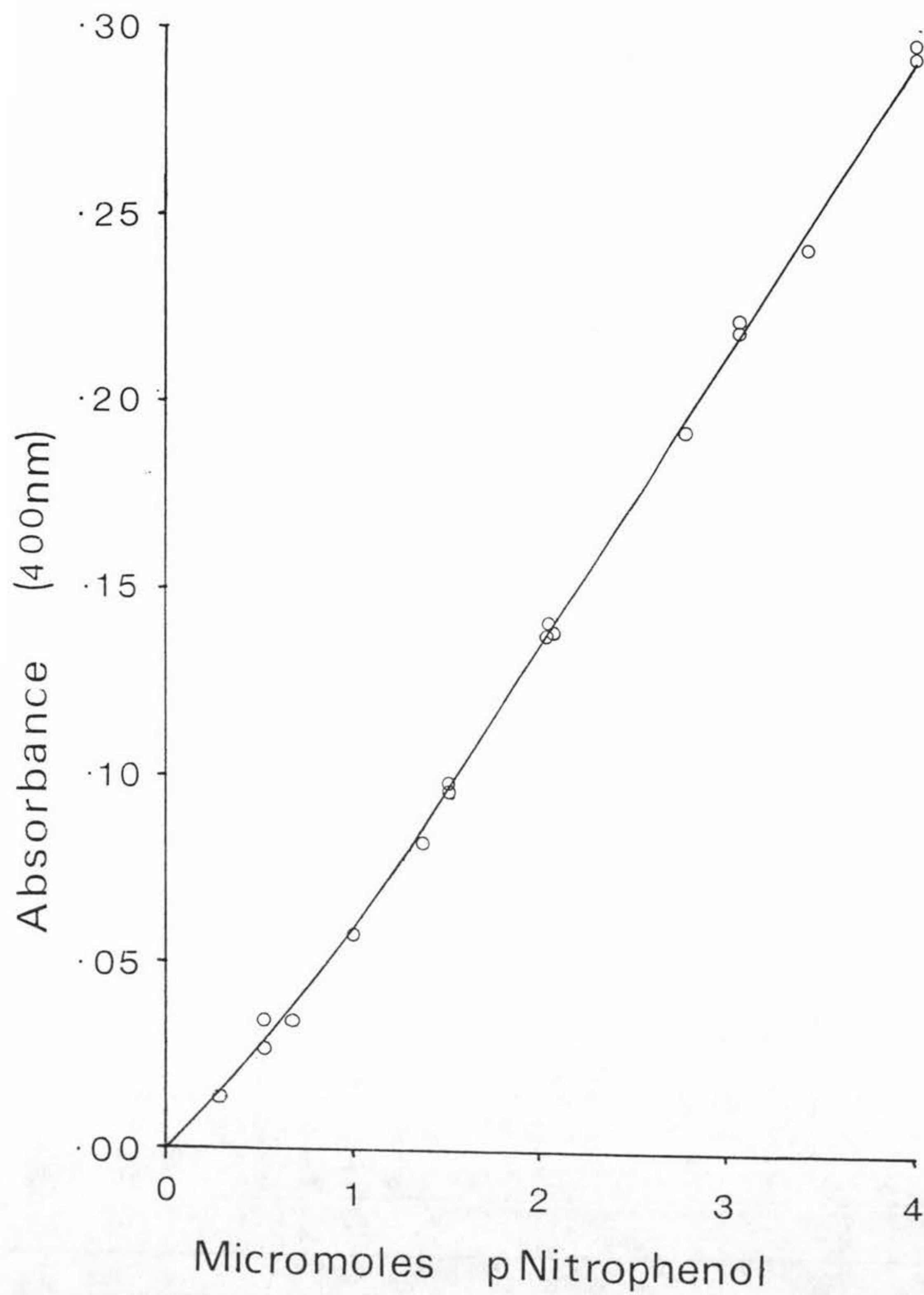
Bars represent range of readings.



Absorbance (550nm)
·3
·2
·1
0
0
10
20
30
40
mg Protein

APPENDIX TWO.

Figure C. ρ-Nitrophenol (ρNP) Standard Curve.



·30
·25
·20
·15
·10
·05
·00
Absorbance (400nm)
0
1
2
3
4
Micromoles pNitrophenol

APPENDIX THREE.

Enzymatic Ethanol Determinations.

Tris Buffer pH 9.7

Trizma Base	72.68 g	Diluted to one litre
Lysine HCl	73.08 g	with deionised water.

Adjust pH to 9.7 with 4M NaOH.

NAD* Cocktail (for 20 samples)

Tris Buffer pH 9.7	35 ml	Prepared fresh each
deionised water	35 ml	day and stored on ice
NAD*	30 mg	until required.

Alcohol Dehydrogenase (ADH)

8 mg ADH was added to 0.5 ml deionised water and stored on ice until required.

Wine samples were diluted 1/500 with deionised water. 3 ml cocktail were added per cuvette, then 50 ul of sample, and mixed. This was repeated for all samples, standards and blanks. A water blank was used to zero a Spectropotometer SP1800 at 340 nm. 20 ul ADH were finally added to each cuvette, allowed to stand two minutes, then read. Blanks contained cocktail and ADH, but no sample.

Ethanol concentration was determined by:

$$\text{mM ethanol} = \frac{\text{Final reading} - (\text{initial reading} + \text{blank})}{6.22} \times \frac{\text{Total Volume in cuvette}}{\text{Sample Volume}}$$

$$\text{mM ethanol} = [\text{Final reading} - (\text{initial reading} + \text{blank})] \times 9.87$$

$$\%\ \text{ethanol (V/V)} = \text{mM ethanol} \times 2.88$$

Table G. Tests Required for Identification of *Saccharomyces cerevisiae* from Yeasts Associated with Vineyards and Wine-making.[1]

Test	Growth
Nitrate Growth	-
Glucose fermentation	+
2-Keto-D-gluconate growth	-
Growth at 30°C	+
L-Lysine growth	-
Urea Hydrolysis	-
Growth without niacin	+
Cycloheximide (0.01%) resistance	-
Cadaverine growth	-
Erythritol growth	-
Citrate growth	-

[1] From Key 17, Barnett *et al.* (1983)

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