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

A Thesis in Partial Fulfillment of
the Requirements for the Degree of
Master of Science in Microbiology at
Massey University, New Zealand

BRUCE DICK

1984

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 $\pm$ 10,000 daltons (*Est 2*), 60,000 $\pm$ 12,000 daltons (*Est 3*), 73,000 $\pm$ 15,000 daltons (*Est 1*), and 113,000 $\pm$ 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 methanesulfonate 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.

ACKNOWLEDGEMENTS

I acknowledge first and foremost my supervisor, Dr. R.J. Thornton, who throughout my studies gave well-appreciated encouragement and advice, and who also taught me yeast hybridization techniques and corrected my proofs to an acceptable state. I would like to thank Dr. J. Shaw and Mr. J. Allen of the Plant Physiology Division, D.S.I.R., Palmerston North, for invaluable help in Gas Liquid Chromatography, particularly for production of standards, and making time available for me to do my sample analysis on their GLC Unit. I also acknowledge the help given by Mr. G. Ionis for discussions on Polyacrylamide Gel Electrophoresis, and help in the early stages of this work. I lastly specifically acknowledge Mrs. E.J. Baxter for her excellent typing and preparation of this thesis for submission.

I would like to thank all members of the Microbiology and Genetics Department, Massey University, Palmerston North, for their help throughout my work, and in no small way for making my studies possible. Lastly I would like to thank all the post-graduate students, past and present, particularly Miss D. Harvey, for their friendship and goodwill throughout, and for making my work more tolerable when it lagged.

CONTENTS

Page

SECTION THREE: RESULTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

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:

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

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

---

[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 p-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.

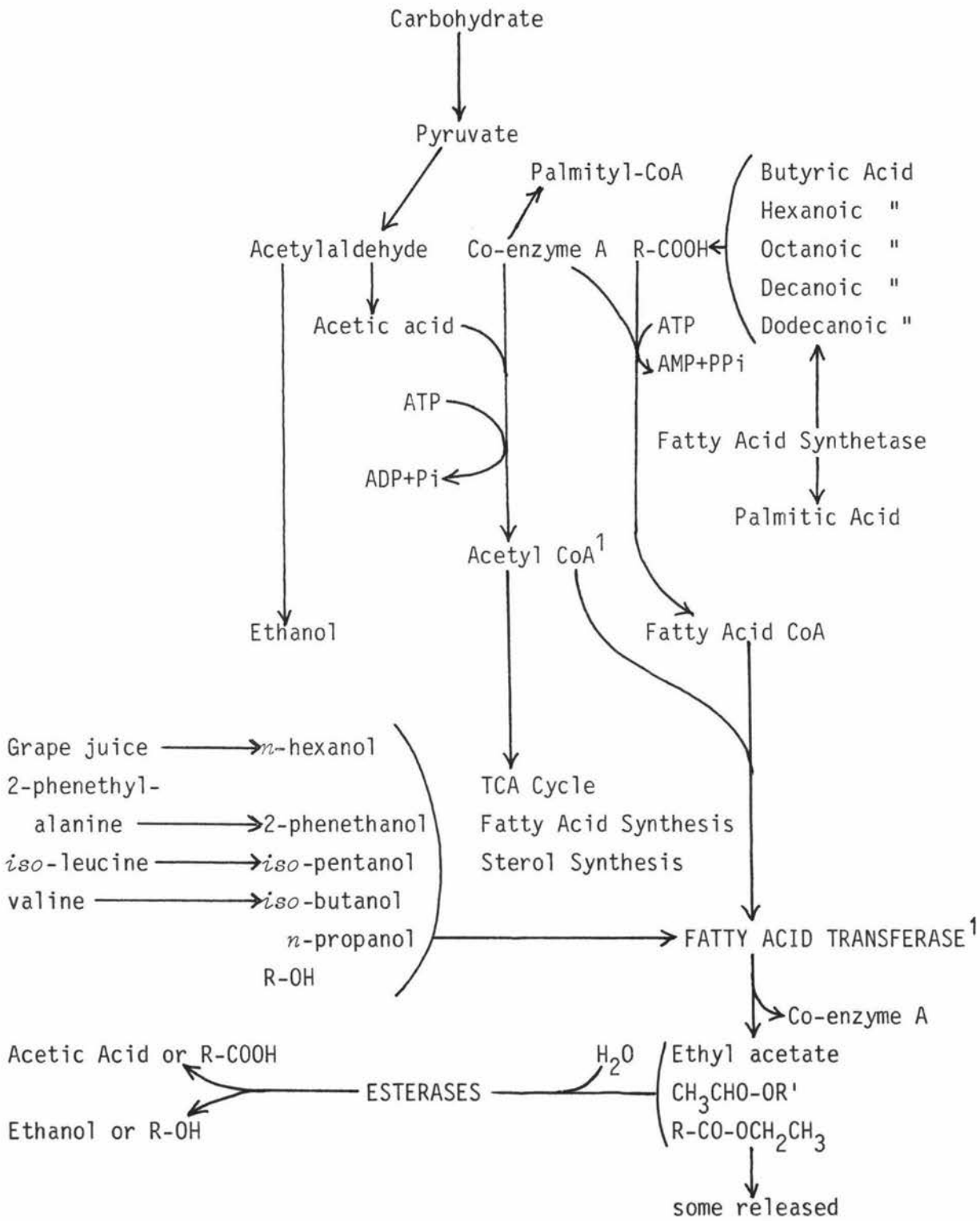
Aliphatic and aromatic esters are synthesised during growth by a membrane-bound enzyme, a fatty acid transferase, by alcoholysis of acyl CoA derivatives. Though untested, reversibility of the reaction may be possible, as seen with lipases. Like esterases, this enzyme probably has a wider range of substrates 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.