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BIOLOGICAL PROCESSES FOR THE REDUCTION OF ALCOHOL IN WINES

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BIOLOGICAL PROCESSES FOR THE REDUCTION OF ALCOHOL IN WINES

by P.M. Smith

Key words: aeration, sugar and alcohol reduction, temperature, selected yeast strains, sensory suitability, combined aerobic/anaerobic fermentation, oxidation

A study was conducted into the effect of varying levels of aeration and temperature on the reduction of sugar and production of alcohol in Muller Thurgau grape juice by selected yeast strains. In addition, an assessment of the suitability of combining aeration with anaerobic fermentation to produce a reduced alcohol wine with acceptable colour and taste was undertaken.

Of seven yeast strains which had shown promising results at reducing sugar levels in either apple juice or orange juice, three; *Pichia stipitis, Candida tropicalis* and *Saccharomyces cerevisiae*, individually produced significant alcohol reductions when fermented at 30°C under controlled aeration.

By combining short-term controlled aeration, to reduce the sugar content of the grape juice, with anaerobic fermentation using an active dried wine yeast, wines with acceptable taste and 25 to 30% less alcohol were produced. These wines did, however, exhibit the deep golden colour indicative of oxidation.

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1.1 LOW OR REDUCED ALCOHOL BEVERAGES

1.1.1 INTRODUCTION

The concept of reducing alcohol intake while still enjoying the pleasures of wine is tempting given todays health conscious society. The mid-1980's saw an enormous boom in the health-food industry as more people strove to conform to recommendations to improve their health; while drink-driving campaigns also made the population reconsider their alcohol intake.

Crown Health Enterprises throughout New Zealand are involved with alcohol awareness education, and the attitude of these organisations is often manifested in such areas as liquor licensing. Although the Liquor Licensing Authority has held that Health Enterprise concerns over possible increases in drink driving and foetal abnormality because of such things as extended trading hours are not relevant considerations when contemplating the grant of a liquor license (An Application by Lorraine Elsie Harley, LLA Decision 1046/91), an Applicant for a liquor license may be required to complete a questionaire for the Crown Health Enterprise regarding "host responsibility". Responses to questions regarding the availability of food and non-alcoholic beverages and dealing with or serving people who are intoxicated often determine whether a Board considers opposing the grant of a liquor license. The availability of low alcohol products also appears to be encouraged by the CHE's.

Surveys in the United Kingdom in the late 1980's showed that drinking patterns were changing towards a decrease in alcohol consumption. This decrease was clear among both sexes and across most age groups. Not only was the amount of alcohol consumed changing, but also the type of alcoholic drink consumed. There has been a marked switch in recent years from the consumption of beers and spirits to wines. (Howley & Young 1992).

Partly to meet the demand from drivers, and partly to cater for the health or diet conscious (once alcohol is

removed, wine is low in calories), wine producers have begun to experiment with low and de-alcoholised wines and beverages.

1.1.2 WHAT ARE LOW OR REDUCED ALCOHOL BEVERAGES?

"Liquor" is defined in the Sale of Liquor Act 1989 as "any fermented, distilled, or spiritous liquor (including spirits, wine, ale, beer, porter, honeymead, stout, cider and perry) that is found on analysis to contain 1.15% or more alcohol by volume." (Section 2).

The Food Regulations (FR) 1984 are a little more helpful in dealing with the labelling of wine and wine products (R.225):-

-A wine or sparkling wine cannot be referred to as "reduced alcohol" unless it contains not more than 6.5% alcohol and not less than 1.15% alcohol (R.225 (5A))

-A wine, sparkling wine, wine based drink, or wine cooler cannot be referred to as "low alcohol" unless it contains not more than 1.15% alcohol (R.225 (5B))

-The words "reduced alcohol" cannot be used on the label of any wine product other than wine or sparkling wine, and "light" or "lite" cannot be used on the label of any wine or wine product in relation to a claim for alcohol content (R.225 (5C) and (5D)).

These standards are different in degree to those defined in the United Kingdom, which are:-

-de-alcoholized: not more than 0.5% alcohol by volume

-low-alcohol: any product which has an alcoholic strength of between 0.5 and 1.2% alcohol by volume

-reduced-alcohol: a product containing more than 1.2% but less than 5.5% alcohol by volume

(Howley & Young 1992)

However, whereas in New Zealand, "wine" is defined as "the product of the partial or complete alcoholic fermentation of grape juice: grape juice and other portions of grapes: or the reconstituted product of concentrated grape juice and drinking water" (FR 1984: R.219 (1)); in the United Kingdom, if fermentation is prematurely arrested, the drink cannot be described as wine where the alcohol content is less than 5.5% alcohol by volume. Such a beverage must be labelled a "partially fermented must" or "low alcohol drink" in the U.K. We in New Zealand can give it the more preferable descriptor "wine". (Rowe 1989), but would not be able to export it as such to the United Kingdom.

1.1.3 WHY THE INTEREST IN LOW ALCOHOL WINES?

In the United Kingdom when low alcohol wines first appeared on the market they were associated with healthy eating and sensible drinking, especially when associated with drink/driving. The "wines" were low in calories, low in fat, low in alcohol, low in cholesterol and high in fibre.

The real impetus for low alcohol products in the U.K received a boost from the Customs and Excise Department. In 1988 it decided that drinks containing less than 1.2% alcohol by volume would be duty free, and a reduced duty would be payable on drinks containing less than 5.5% alcohol by volume. Most low alcohol wines therefore became cheaper than their traditional counterparts.

A rapid growth in sales of low alcohol wines was predicted but did not eventuate. A polytechnic survey on consumer attitudes to low alcohol wines (LA) carried out in South London drew the following conclusions:

-the pattern of purchase of LA wines was different from wine. LA products were usually purchased by or for the person who had to drive at a party season such as Christmas, where they were seen as more socially acceptable than drinking soft drink.

-it was younger, less knowledgeable, consumers who mostly bought LA wines. Seasoned wine drinkers found very few LA products acceptable as wines. (Keough 1991).

In New Zealand a few foreign low alcohol wines are available in the liquor stores and supermarkets, however no local wine producers have yet been tempted to emulate the product. The Customs and Inland Revenue Departments are obviously not inundated with requests for information as to whether lower duties would be applicable for LA wines in New Zealand, and during personal communications were not able to give a definitive answer in this regard.

The real reason for the failure of LA products to take the wine market by storm may be that the early products did not taste good. Often they were made from inferior grapes that would otherwise go to distillation, and the method of their manufacture lead to major flavour loss. Unlike the brewing industry where low alcohol beers have been accepted for sometime, the wine industry has been slow to use new technologies to produce a low alcohol product of comparable taste and quality to the traditional product. (Rowe 1989).

1.1.4 TYPES OF TECHNOLOGY COMMERCIALLY AVAILABLE FOR REDUCING ALCOHOL IN WINES AND GRAPE MUSTS

Two of the earliest methods for alcohol reduction were distillation and partial fermentation, and these methods probably still produce the majority of low alcohol wines on the market. (Lord 1994).

1.1.4.1 Distillation

This process takes a full strength wine and heats it so that the alcohol boils off leaving behind the other components of the wine. The problem with this method is that much of the flavour of the wine is lost with the alcohol, in itself a major flavouring element, leaving little of the original product other than the sugar. (Lord 1994: Rowe 1989).

1.1.4.2 Partial Fermentation

Partially fermented grape musts are really the beginning of wines, with nothing added and nothing taken away. The aroma, fruit, flavour, acidity and the colour of the grape remain intact, and there is some body and structure to the wine. The sugar is not fully fermented out however, as the fermentation process is stopped at the desired alcohol content. In the U.K this is generally 5.5% so as to attract the lower duty. The wine has to be stabilized in some way to prevent further fermentation, and usually this is achieved by pasteurisation and the addition of sulphur dioxide. This method of production is used when the product is the low-alcohol version of a wine that is traditionally sweet. (Rowe 1989: Lynch 1988).

Newer techniques for alcohol removal include reverse osmosis, spinning cone column technology, various blends of grape and fruit juices, and enzymatic treatment of grape juice.

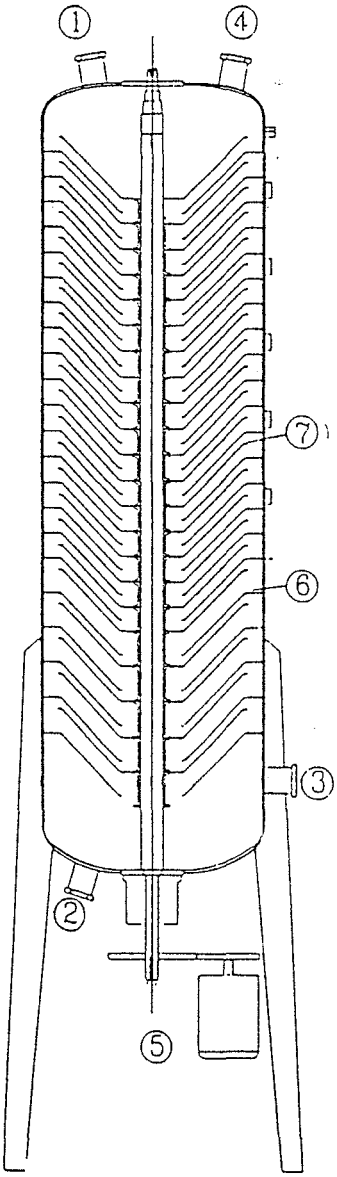
1.1.4.3 Reverse Osmosis

In this process the wine is filtered through a porous cylindrical membrane which permits the smaller molecules of water and alcohol to pass through, while retaining the other wine components in a concentrated form. The concentrate is then diluted with water or occasionally with the addition of grape juice. (Lynch 1988). Ariel Blanc and Ariel Cabernet Sauvignon, both available in New Zealand are made by this process.

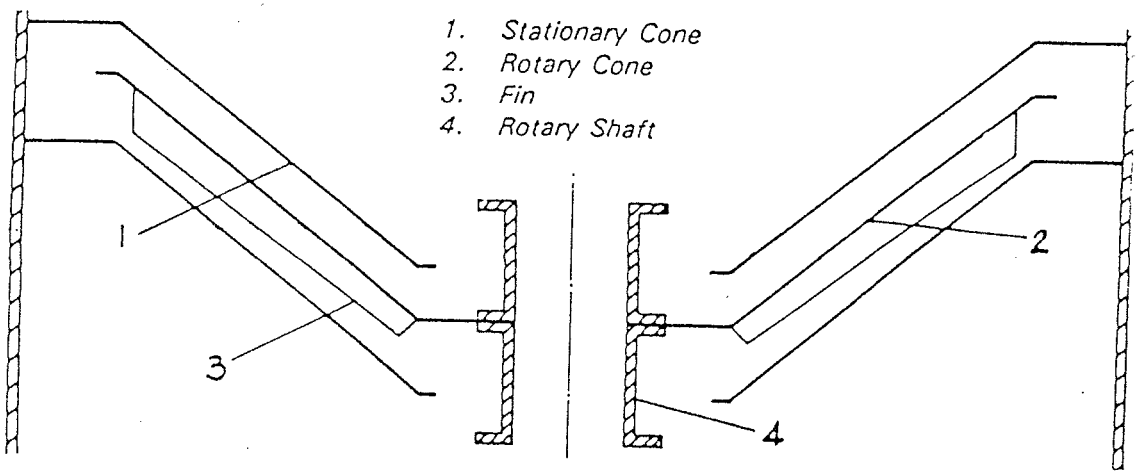
1.1.4.4 Spinning Cone Column Technology

The spinning cone column is a "multi-stage, counter current, gas-liquid contacting devise consisting of a stack of alternating spinning and stationary cones". Liquid, fed from the top of the column, is directed onto the base of a spinning cone from where centrifugal forces propel it radially and tangentially to the outer wall of the column. An underflow weir redirects the liquid down the nearest stationary cone and onto the succeeding spinning cone. In this way the liquid stream passes over every cone set in a very thin, highly turbulent film. A counter current gas flow, steam or inert gas, is directed up the column at which time the mass transfer of volatiles from the liquid to the

vapour stream occurs (Figure 1.1.4.4.a). In addition, on the underside of the spinning cones is a series of fan blades which greatly increase the vapour path length, increasing the mass transfer efficiency of the system, and converting the column to a multi-stage centrifugal fan (Figure 1.1.4.4.b). (Gray 1993).



Mechanical layout of Spinning Cone Column.



1. *Stationary Cone*
2. *Rotary Cone*
3. *Fin*
4. *Rotary Shaft*

Cross section of cone set.

1. *Product in*
2. *Product out*
3. *Gas in*
4. *Gas out*
5. *Rotating shaft*
6. *Fixed cones*
7. *Rotating cones*

Figures 1.1.4.4.a and 1.1.4.4.b

(Source: Gray 1993)

As applied to the wine industry the spinning cone column acts as follows:

> "Wine is pumped into the top of a column still and as it passes down the length of the still the wine forms a thin film on the cones. In a few seconds many of the alcohol elements in the wine evaporate. They are caught by nitrogen gas rising from the bottom of the column, collected and brought back to liquid form. This process takes place at room temperature. The remaining wine is then heated to 60°C, and the last of the alcohol is driven off.
>
> The winemaker is left with two batches of wine. The first has almost all its flavour elements intact and about one half its natural alcohol still in place. The second batch has no alcohol remaining. The two are blended together to give a reduced alcohol wine." (Lord 1994).

Penfolds, though its Seppelts subsiduary markets a reduced alcohol Chardonnay (6% alcohol) made by this method called Method Encore which is occasionally available in New Zealand. Orlando market a Chardonnay and a Cabernet Sauvignon in Australia with the alcohol levels reduced to about 6.2%.

1.1.4.5 Blends of grape and fruit juice

Wine coolers have been available in New Zealand for some time. These beverages which are manufactured in both sparkling and still styles employ various mixes of (mainly) apple juice and grape juice to produce a beverage with an alcohol content of around 4 - 7%. Really, these products employ partial fermentation combined with raw juice addition.

Of possible interest to New Zealand, given our surplus kiwi fruit production, research in Italy has found a novel way of disposing of surplus grape juice and undersized and damaged kiwi fruit, by mixing the two to produce a fermented beverage with a low alcohol content. (Anelli et al. 1986).

The kiwi fruit is harvested while still unripe, treated with pectinase and pressed to give a final yield of 65% juice.

A mixture of 70% white grape juice and 30% kiwi fruit juice is then inoculated with selected yeasts (*Saccharomyces cerevisiae*) and fermented at low temperatures (12°C). After 6 - 7 days when an alcohol content of 5% is reached, fermentation is stopped through pasteurisation, further grape juice is added (25%) and the product is bottled.

The final composition of the "kiwine" is as shown in Table 1.1.4.5. The beverage is a clear, pale yellow colour verging on green, with a slightly sour taste due to the high acid content. Although slightly sweet like a wine cooler, the kiwine balances the sugar with a higher acid composition so that the final product is closer in style to a wine than a soft drink.

Analytical data on "Kiwine".

pH	3,2
Alcohol (% v/v)	5,0
Specific weight	1,0481
Reducing sugars (g/l)	80,0
Total acidity (g/l as tart. acid)	11,8
Volatile acidity (g/l as acetic acid)	0,4
Total SO_2 (mg/l)	90,0
Free SO_2 (mg/l)	1,5
Bound SO_2 (mg/l)	88,5
Total nitrogen (g/l)	1,0
Dry matter without sugar (g/l)	22,3
Ash (g/l)	3,1
Ash alcalinity (meq/l)	38,08
Malic acid (g/l)	3,05
Lactid acid (g/l)	2,14
Citric acid (g/l)	1,60
Quinic acid (g/l)	1,32
Acetic acid (g/l)	0,51
Succinic acid (g/l)	0,22
Ascorbic acid (g/l)	0,06
Tartaric acid (g/l)	3,37
Glycerine (g/l)	4,90
2-3-Butyleneglycol (mg/l)	310.0

Table 1.1.4.5

(Source: Anelli et al. 1986)

1.1.4.6 Enzymatic treatment of grape juice

Villettaz (1986) has reported a method of producing low alcohol wines by means of a glucose oxidase/catalase treatment of grape juice. The enzyme system containing both glucose oxidase and catalase is obtained by a controlled fermentation of an *Aspergillus niger var.*

The basic concept is that the glucose fraction (50% of the sugar) contained in grape juice is converted to gluconic acid by an enzymatic treatment. Two types of enzyme are used in the concept:

1. a glucose oxidase which converts glucose to gluconic acid in the presence of oxygen

   $$2 \text{ glucose} + 2H_2O \rightarrow 2 \text{ gluconic acid} + 2H_2O_2$$

2. a catalase which converts the hydrogen peroxide formed during the first step into water and oxygen

   $$2H_2O_2 \rightarrow 2H_22O + O_2$$

   The oxygen formed is immediately reused by the glucose oxidase.

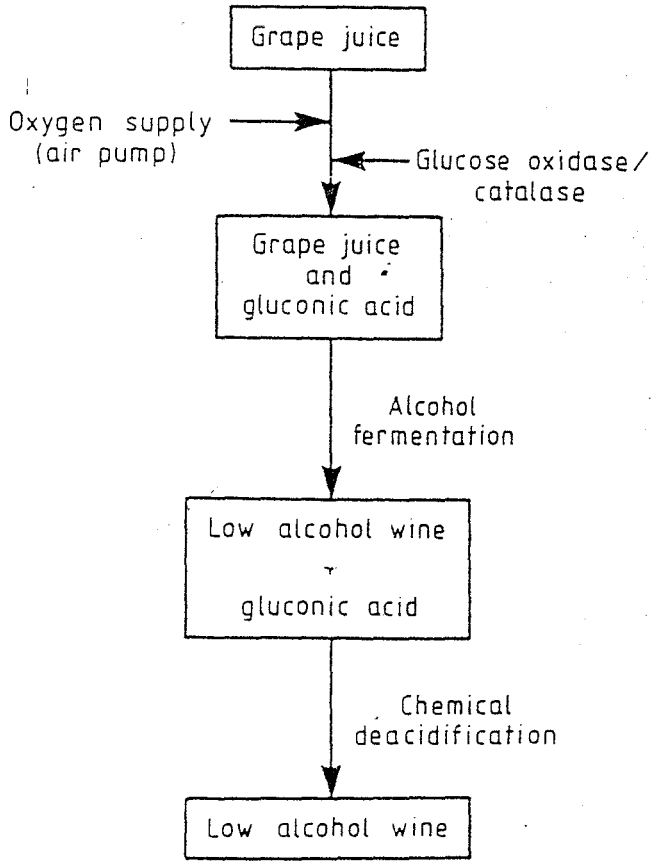
The overall enzymatic reaction can be written as follows:

$$\text{grape juice} \xrightarrow{\text{glucose-oxidase} \\ \text{catalase}} \text{acidified grape juice}$$

(glucose & fructose) (gluconic acid & fructose)

Since gluconic acid is not converted to alcohol by wine yeasts, the wine obtained after fermentation of the treated juice contains up to 50% less alcohol than a conventionally made wine. The excess of gluconic acid can be removed from the low alcohol wine by chemical deacidification.

The whole process is summarised in Figure 1.1.4.6



Low alcohol wine production by enzymatic treatment of grape juice

Figure 1.1.4.6

(Source: Villettaz 1986)

Research at Lincoln University to determine the optimum reaction conditions for the glucose oxidase/catalase (GOD/CAT) treatment, and to evaluate the effect of the technology on wine composition and quality has found that:

1. pH appears to be the primary limiting factor in both rate and extent of conversion of glucose to gluconic acid

2. given the need for constant aeration of the juice, once O_2 saturation is achieved, the extent of mixing becomes an important reaction-limiting factor

3. deacidification of grape juice using calcium carbonate prior to GOD/CAT addition significantly increases the rate and extent of glucose conversion

4. gluconic acid in the finished wine appears to have a positive effect on "mouth-feel" and "body" - attributes often critised as being absent from reduced alcohol products

5. colour stability in the finished wine may be superior to traditionally made wine due to polyphenol oxidation during juice aeration

6. GOD/CAT wines showed the same intensity of aroma as traditionally made wine and in taste tests they rated higher for acid, salt and citrus flavours, but lower for sweet taste

7. excessive gluconic acid production appears to be the major limitation of the glucose oxidase/catalase system. (Pickering et al. 1993 and 1994)

1.1.4.7 Biological Methods

None of the technologies available for alcohol reduction in wines are purely biological. If some of the sugar could be oxidised to CO_2, by yeasts for instance, then the remainder fermented to alcohol, a reduced alcohol product may be produced which retains the traditional flavour and aroma of a full strength wine.

1.2 RESEARCH INTO THE REDUCTION OF SUGARS IN FRUIT JUICES USING BIOLOGICAL METHODS

Given that the reduction in the natural sugar content of fruit juices and wines carries with it diet and health connotations, it was probably only a matter of time before research into a more "natural" method of obtaining such a sugar reduction was undertaken.

In the beverage sector, the manufacture of low calorie products had long been practised by the full or partial use of calorie-free sweeteners instead of sugar to produce alcohol free drinks, and by diluting with water to produce fruit nectars. The composition and structure of the final product was altered by these methods thus affecting the nutritional value. Dilution reduced the nutrient density, while a reduction of only the sugar, increased it. The aim therefore was to produce fruit juices with reduced caloric value while fully maintaining the content of their valuable ingredients. (Kolb et al. 1993).

Reasearch in Switzerland in the 1980's resulted in a patented process for the preparation of low-sugar or sugar-free fruit juices based on continuous or semi-continuous culture with a yeast. (Kappeli 1989).

Essentially, freshly pressed apple juice with a sugar content of about 10% had several vitamins added as complementary nutrients and was then sterile filtered. Three different processes were undertaken using one of each of three yeasts, namely *Saccharomyces cerevisiae*, *Candida tropicalis* and *Trichosporon cutaneum.*

In each case a 3 litre bioreactor with a mechanical foam cutter was filled with apple juice and inoculated with a prepared culture of one of these three yeasts. The temperature of the unit was adjusted to 30°C, the revolutions of the stirring column to 150rpm,and those of the foam cutter to 1000rpm. Ventilation of the unit was adjusted so that the partial pressure of oxygen was above 50% air saturation. The reactor was run until all sugar was consumed, or for the continual production of sugar-free apple juice, could be continually resupplied with sterile juice. These conditions resulted in the metabolism of sugar to CO_2 and water, rather than to ethanol.

The apple juice obtained using any of the yeasts was practically sugar-free (less than 0.1% sugar) and did not have an increased alcohol content. Alone it was said to be a pleasantly acidic tasting, refreshing drink. Alternatively calorie-free sweeteners could be added along with aroma substances and CO_2 to produce a sparkling beverage.

Following on from the early patent research using yeasts, German studies demonstrated that a selective decomposition of 50% of the sugar in fruit juices was possible with the aid of microorganisms without-

1. a reduction of the ingredients contributing towards value and taste, or

2. the necessity to add any "auxiliary substances" (neither special nutritive substances for the micro-organisms employed nor an anti-foam agent or similar substance).

(Kolb et al. 1993)

In a broad screening study of 120 yeast strains, several were chosen which showed a minimum of adverse effects on the sensory and functional qualities of a juice.

Orange juice which had been previously pasteurized was incubated with yeast cultures each with an original cell count of 10^3 - 10^6 cells/ml, and shaken at temperatures of 22 and 27°C at a frequency of 100 - 150rpm.

In addition to sensory suitability, sugar decomposition of a minimum of 50% within 5 days was selected as a minimum criterion for further study of the yeast strains (Figures 1.2.a and 1.2.b).

Yeast Stocks suitable for further Processing

1st Screening step				2nd Screening step		
Designation of yeast stock	sugar decomposition in approx. %	in days	Total acidity (calculated as CS) difference	sugar decomposition in approx. %	in days	Total acidity (calculated as CS) difference
Candida chilensis, diddensii, freyschussii, dendronema, melinii, sake, shehatae; silvicola, solani, tenuis	59 - 100	4- 7	67 - 77	43 - 80	3 - 4	+1/+2 (increasing)
Debaryomyces vanriji	100	5	decreasing	70	4	-3/-5 (decreasing)
Hansenula californica, henricii, polymorpha	53 -81	3 - 6	decreasing	74 - 90	4	±0 - -2 (decreasing)
Kloeckera africana	54	3	unchanging	-	-	-
Leucosporidium capsuligenum	76	4	decreasing	-	-	-
Metschnikowia pulcherrima	73	3	unchanging	-	-	-
Pachysolen tannophilus	68	5	unchanging	-	-	-

The stocks underlined were also processed in the 2nd sreening step.

1st Screening step				2nd Screening step		
Designation of yeast stock	sugar decomposition in approx. %	in days	Total acidity (calculated as CS) difference	sugar decomposition in approx. %	in days	Total acidity (calculated as CS) difference
Pichia farinosa, rhodanensis, stipitis, strassburgensis, vini, var. mel., wickerhamii	42 - 79	3	increase/ decreases	67 - 77	3 - 7	-2 - -5 (decreasing)
Saccharomyces aceti, capensis carlsbergensis, cerevisiae var. cerevisiae, ellipsoideus	68 - 100	1 - 3	increasing	100	1 - 2	+1/+2 (increasing)
Saccharomycodes ludwigii	100	2	increasing	95	2	±0/+2 (increasing)
Schizosaccharomyces japonicus, japonicus var. versatilis, malidevorans, octosporus, pombe	55 - 99	1 - 2	unchanging	100	2	±0 (unchanging)
Schwanniomyces occidentalis	97	2	unchanging	85 - 95	2	+0,5 (increasing)
Torulopis versatilis	91	1	unchanging	90	1	+1 (increasing)
Trichosporon pullulans	50	5	unchanging	-	-	-

The stocks underlined were also processed in the 2nd screening step.

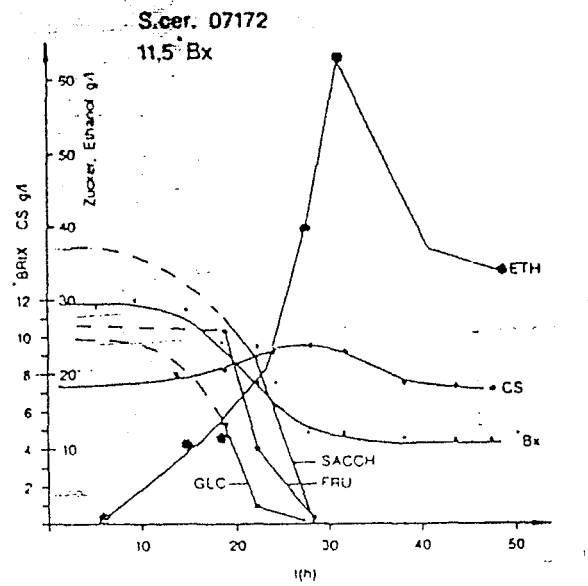
Figures 1.2.a and 1.2.b

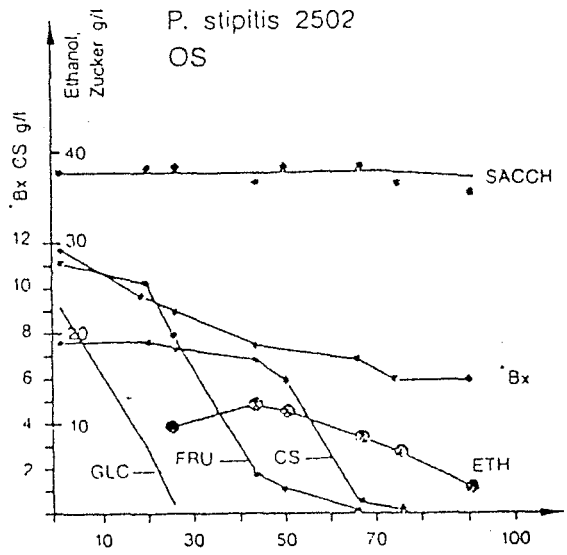
(Source: Kolb et al. 1993)

The aeration of the juices by shaking reduced the tendancy of some yeast strains to produce ethanol, however in this regard some strains were more successful than others. In the case of *Saccharomyces cerevisiae* the sugars glucose, fructose and saccharose were successively decomposed within about 30 hours without any change on acid concentration. However, an increase in the ethanol content of the juice was inevitable unless strong aeration was applied, leading to considerable losses in vitamin C. Sensory changes were also clearly evident in juice fermented by this yeast.

Pichia stipitis, on the other hand, decomposed glucose first and then, after a considerable delay, the fructose. Saccharose was not decomposed at all. A reduction in the acidity of the juice occurred only after the decomposition of glucose, therefore making control of the acid content dependent on the fermentation period. The ethanol content obtained under fermentation was minor and could be avoided by adjusting aeration during sugar decomposition.

Sensory evaluation of the juices also found *P. stipitis* to be superior to *S. cerevisiae*. Slightly bitter tones were always evident towards the end of the fermentation in tests with *S. cerevisiae*. Figure 1.2.c follows the course of fermentation of these two yeasts.





KEY

Sacch - saccharose
Glc - glucose
Fru - fructose
Eth - ethanol
°Bx - ° Brix
Cs - sucrose

Course of sugar and acid degradation and values for alcohol in vibrating flask fermentation

Figure 1.2.c

(Source: Kolb et al. 1993)

To obtain a better calorie reduction in these orange juices further work was carried out to find a mutant of *P. stipitis* which could also invert saccharose to glucose and fructose. If only fructose was left in the juice it was found to be sweeter tasting, while giving a calorie reduction of approximately 50% (Figure 1.2.d).

	original juice	juice sugar-reduced used		
		Pichia stipitis wild stock	Pichia stipitis wild stock + Invertase	Pichia stipitis mutant
Glucose (g/l)	22,7	0,0	0,0	7,1
Fructose (g/l)	24,6	24,1	37,5	34,7
Saccharose (g/l)	40,1	39,7	0,0	1,3
Citric acid (g/l)	8,8	6,2	8,4	6,5
Potassium (mg/l)	1960	1620	1690	1637
Calcium (mg/l)	84	85	83	79
Magnesium (mg/l)	104	96	96	90
Vitamin C (mg/l)	481	450	455	455
Ethanol (g/l)	0,0	0,0	0,0	0,0
Sugar reduction (%)	-	27,1	57,1	50,7
Caloric value (MJ/l kcal/l)	1,600 (376)	1,165 (274)	0,747 (175)	0,817 (192)
Nutrient density for Vitamin C (mg/MJ)	301	386	609	557

Analysis values of partially sugar-reduced orange juices in comparison to original juice

Figure 1.2.d

(Source: Kolb et al. 1993)

The process was tested in laboratory scale using standard technical fermenters (10 litre batches) for a series of fermentations of orange (from concentrate), apple (from concentrate) and grape juice. After separation of the biomass and reconstruction of the aroma, sugar reduced juices could be obtained with an almost unchanged sensory profile, with the exception of reduced sweetness and the benefit of no additives and low calories.

1.3 PROPOSED RESEARCH

Kolb et al. (1993) reported obtaining sugar reduced fruit juices with almost unchanged sensory profiles using yeasts under controlled aeration.

The purposes of this study were:-

1. to examine the effects of varying levels of aeration and temperature on the reduction of sugar and production of alcohol in grape juice by selected yeast strains, and

2. to assess the sensory suitability of combining short-term aeration with anaerobic fermentation to produce a reduced alcohol wine.

1.4 THE MICRO-ORGANISMS OF WINEMAKING

1.4.1 INTRODUCTION

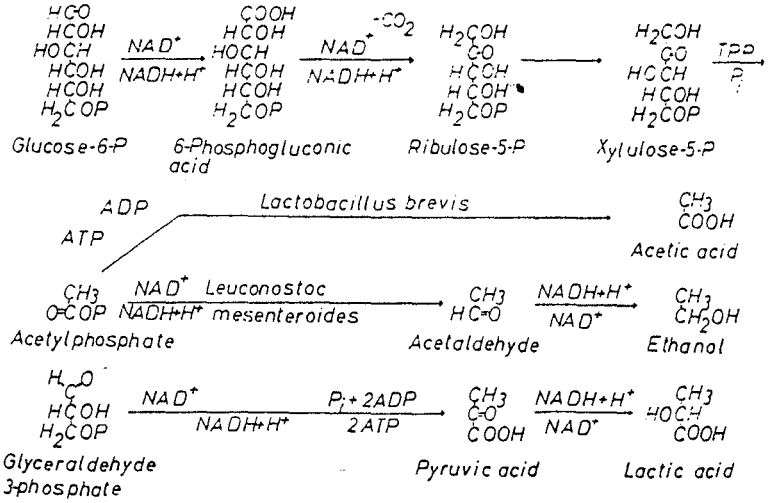
A great diversity of micro-organisms are able to use the grape sugars for growth. The most significant of these are shown in Table 1.4.1.

Microbial group	*Significance*
Yeasts	Alcoholic fermentation; spoilage; autolysis; deacidification
Killer yeasts	Alcoholic fermentation; spoilage control
Lactic acid bacteria	Malolactic fermentation; spoilage; autolysis
Acetic acid bacteria	Spoilage; stuck fermentations
Fungi	Botrytized wines; spoilage; corky taints
Bacillus, *Clostridium* spp.	Spoilage
Actinomyces, *Streptomyces* spp.	Earthy, corky taints
Bacteriophages	Disrupt malolactic fermentation

(Source: Fleet 1993)

1.4.1.1 Lactic Acid Bacteria

The end products of the metabolism of sugars by heterofermentative lactic acid bacteria are ethanol, acetic acid and lactic acid. (Figure 1.4.1.1).



(Source: Sponholz 1993)

Although some of these bacteria play a key role in the malolactic fermentation of wines, they are primarily known as spoilage agents due to their ability to significantly increase the acidity of wines. For this reason, they would not be considered suitable for use in sugar reduction per se.

1.4.1.2 Acetic Acid Bacteria

Acetic acid bacteria cause the vinegary spoilage of wine through the oxidation of ethanol to acetaldehyde and acetic acid. The metabolism of glucose by these bacteria produces gluconic acid. (Fleet 1993).

Excessive growth of these bacteria on grapes significantly alters the chemical composition of the juice, especially by increasing the concentrations of acetic acid, gluconic acid and dihydroxyacetone. These changes, as well as subsequent growth of the bacteria in the grape juice, can affect the growth of yeasts during alcoholic fermentation. Therefore, they are unsuitable for sugar reduction. (Drysdale & Fleet 1989).

1.4.1.3 Fungi

Fungi (other than yeasts) are normally associated with grape and wine spoilage. Some filamentous fungi are parasites capable of penetrating the tissue of the grape and developing at its expense. The most notable of these fungi is *Botrytis cinerea.* It draws the nutrients necessary for its growth from living cells of the berry, causing considerable modification in the chemical composition of the whole grape. Must from rotten grapes is characterised by very high sugar levels.

While the fungus is under the grape skin, it degrades little sugar due to lack of oxygen. Once it comes out of the grapes and has unlimited oxygen however, it degrades the grape sugars to gluconic acid which is not degraded by yeasts during alcoholic fermentation. This acid environment favours the growth of yeasts like *Kloeckera apiculata* and *Torulopsis stellata* over the traditional wine yeast *Saccharomyces cerevisiae.* These yeasts often produce greater quantities of volatile components which are considered detrimental to wine aroma and flavour. (Fleet 1993).

Musts from infected grapes also contain acetic acid bacteria. The formation of glycerol and acetic acid by the fungi, coupled with the higher sugar content of the juices make them unsuitable for reduced alcohol study. (Doneche 1993).

1.4.1.4 Bacillus and Clostridium spp.

Clostridia are obligate anaerobic, spore-forming bacteria that develop in wines with high pH (>pH 4.0). They ferment sugars, producing n-butyric acid, acetic acid, carbon dioxide and hydrogen. The n-butyric acid in particular gives wine an undesireable taint of ranciness. (Sponholz 1993). Clostridia are found only in heavily deacidified juices or juices with a low acid content, therefore, are likely to be more of a problem in a hot climate where grapes reach maximum ripeness and retain a low acid content.

Growth of Bacillus species is accompanied by significant increases in volatile and total acidity. They are considered primarily spoilage organisms because volatile acidity is associated with aroma and flavour faults in wine. (Fleet 1993).

1.4.1.5 Yeasts

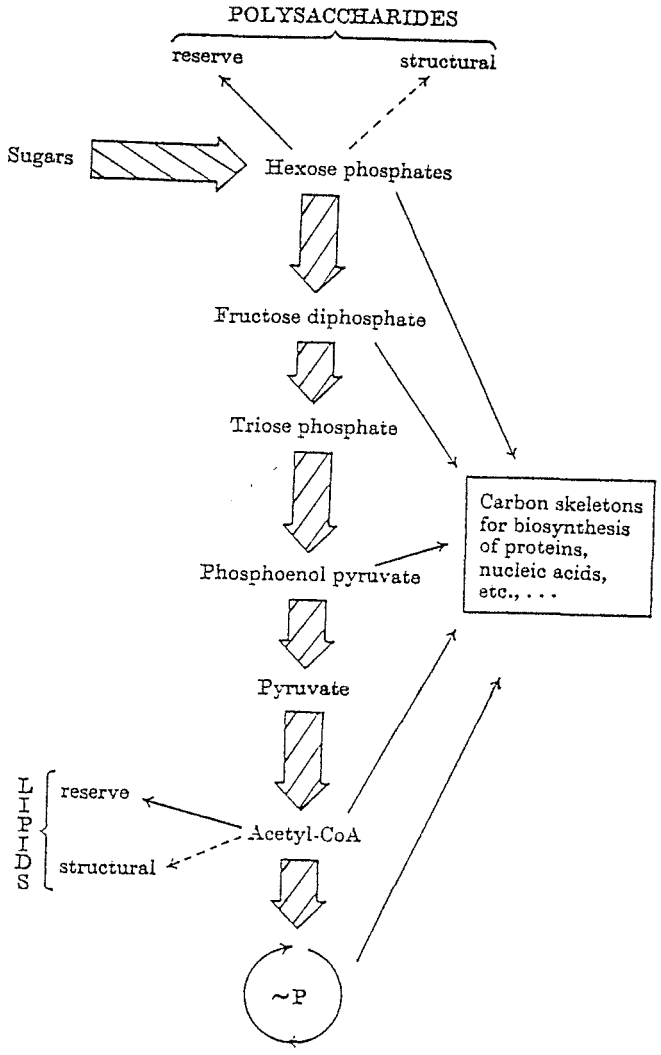
Yeasts metabolise the grape sugars into ethanol, carbon dioxide and other metabolic end-products that contribute to the chemical composition and sensory quality of wine. Their role in conducting alcoholic fermentation has never been challenged. However, the species of yeasts present, their mode of growth and the chemical changes they produce are influenced by winemaking procedures. No two wine fermentations will be identical.

1.4.2 SUGAR UTILISATION BY YEASTS AND THEIR REQUIREMENTS FOR OXYGEN

The ability to grow in the presence or absence of air is important when differentiating yeasts. Although all species grow on glucose aerobically, only about half of them can use glucose anaerobically to produce ethanol. (Fiechter et al. 1987).

1.4.2.1 Anaerobic Metabolism

Glucose and certain other sugars are widely utilized as a major source of energy by yeasts, and with few exceptions, when glucose is utilised as the source of energy - and eventually carbon - the glycolytic pathway is the backbone of its degradation system. As is illustrated by Figure 1.4.2.1, most of its steps are common to anaerobic fermentation and to aerobic utilisation through the citric acid cycle. (Sols et al. 1971).



. Schematic outline of metabolism in yeast of sugar as energy and carbon source.

Figure 1.4.2.1

(Source: Sols et al. 1971)

The oxidation processes which occur in the absence of air, where the role of molecular oxygen as terminal electron acceptor is accomplished by some other inorganic substance such as nitrate or sulphate is defined as an oxido-reduction process. In "true" anaerobic metabolism, the electron donor and the electron acceptor arise from the same molecule and so in order to be fermentable, a compound needs to be in an intermediate state of oxidation. Carbohydrates meet these requirements and are at the origin of most fermentations produced by microorganisms. (Sols et al. 1971).

1.4.2.2 Alcoholic Fermentation

While the pathway of fermentation of glucose as far as the pyruvate stage is identical with that used in respiration, what distinguishes fermentation from respiration is the ultimate fate of pyruvate. Anaerobically, pyruvate is decarboxylated to pyruvate decarboxylase to give acetaldehyde and subsequently the acetaldehyde is reduced to ethanol by alcohol dehydrogenase, completing the conversion of sugar to alcohol. (Bisson 1993).

Anaerobic processes are much less energy efficient than aerobic ones. That is why many micro-organisms when moved from anaerobic to aerobic conditions will drastically reduce their rate of sugar catabolism and switch to aerobic respiration, a regulatory phenomenon known as the Pasteur effect.

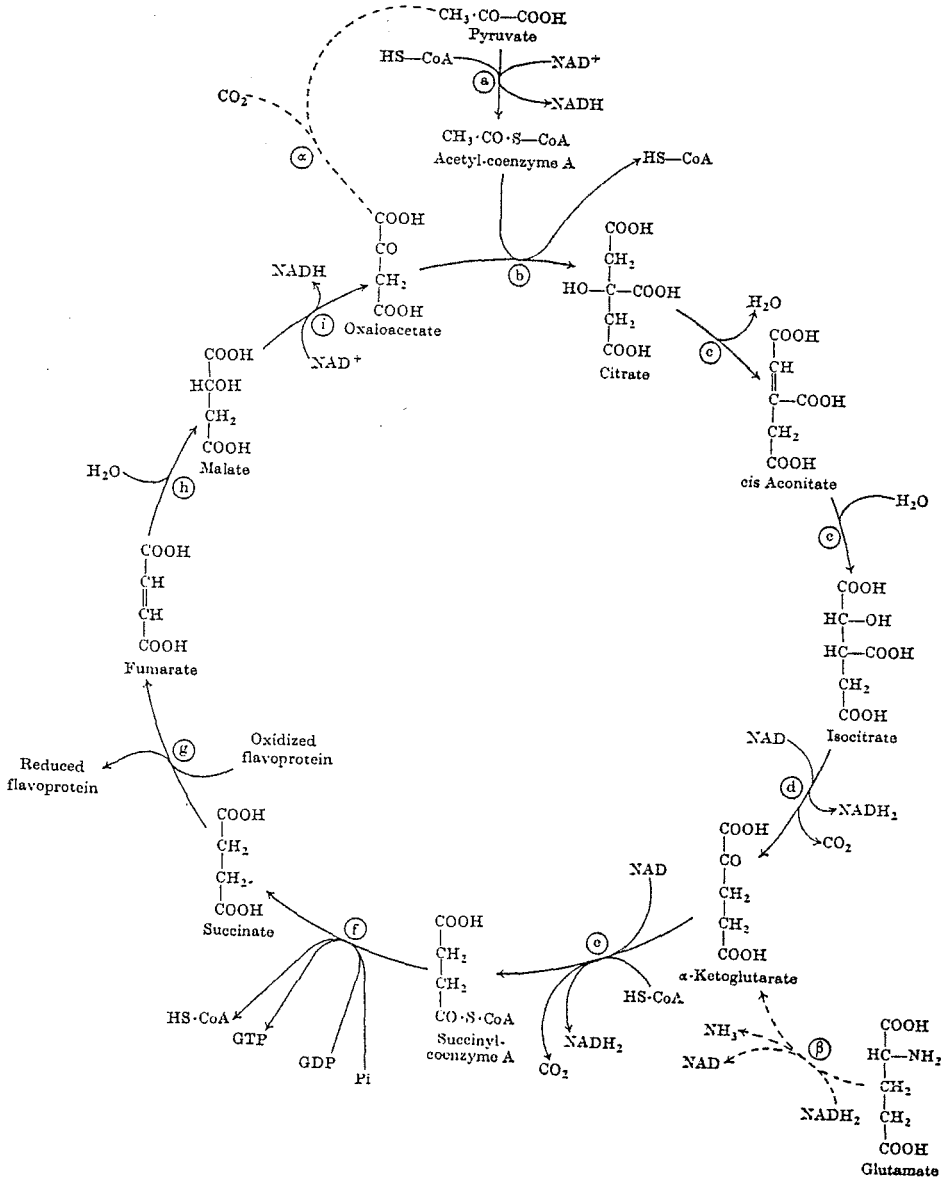
There are a number of yeasts however, including *Saccharomyces cerevisiae,* which produce an unexpected reaction in the presence of even moderate levels of glucose. Respiration is repressed and fermentation is induced whether or not O_2 is present. This is known as the "Crabtree Effect" by analogy to similar observations made by H.G. Crabtree in 1929 on glucose metabolism by cultured tumour cells. (Fiechter et al. 1981).

1.4.2.3 Aerobic Metabolism

The pathway for the oxidation of nutrients in aerobic conditions is the citric acid or Krebs cycle. Carbohydrate, amino acid and lipid derivatives are oxidised to carbon dioxide and water, and when coupled to the respiratory chain, act as the main energy producers of the yeast cell.

The cycle as shown in Figure 1.4.2.3 is that concerned with energy production, namely the generation of acetyl-CoA. The acetyl-CoA is then fed into the "Tricarboxylic Acid Cycle" (TCA) where it is metabolised to CO_2 and H_2O.

The energy released by this process is transferred to and stored in the form of high energy electrons in the molecules NADH and $FADH_2$. (Doelle 1981).



Citric acid cycle. The figure includes a pathway linking glycolysis and the citric acid cycle: (a) pyruvate dehydrogenase complex; (b) citrate synthase; (c) aconitase; (d) isocitrate dehydrogenase; (e) α-ketoglutarate dehydrogenase; (f) succinate thiokinase; (g) succinate dehydrogenase; (h) fumarase; (i) malate dehydrogenase; (α) pyruvate carboxylase; (β) glutamate dehydrogenase.

(Source: Sols et al. 1971)

Considering the destiny of the pyruvate arising from glucose, yeasts may be divided into two groups; obligate aerobes and facultative anaerobes.

1.4.2.4 Obligate aerobes

These yeasts are unable to utilize glucose in the absence of oxygen, therefore, their metabolism is exclusively respiratory, and pyruvate is funnelled into the citric acid cycle to be oxidised. This group includes all species of *Rhodotorula, Saccharomycopsis* and *Sporobolomyces.* Some species of *Torulopsis, Pichia* and *Hansensula* also belong to this group.

1.4.2.5 Facultative anaerobes

This group is able to utilize glucose under aerobic or anaerobic conditions. In anaerobic conditions they metabolise glucose to ethanol (the classical alcoholic fermentation), while during aerobic growth both fermentation and respiration may contribute to the catabolism of glucose. Depending on the magnitude of this contribution, two subgroups can be identified:-

a) Fermentative yeasts

During aerobic growth, respiration accounts for less than 10% of the glucose catabolism of these yeasts. The remainder of the carbon feed appears in three exudates, namely ethanol, glycerol and pyruvate. Yeasts of the genera *Saccharomyces* and *Schizosaccharomyces* are included in this subgroup.

b) Respiratory yeasts

The great majority of facultative anaerobes belong to this subgroup. During aerobic growth, less than 30% of the metabolised glucose is fermented. These types show relatively fast growth and high yields of biomass under unrestricted oxygen supply. Under such conditions, ethanol is not released. Typical examples are species in the genera *Candida* and *Pichia.*

(Gancedo & Serrano 1989).

Both obligate aerobes and facultative anaerobes are likely to be found in grape juice at some stage during its progression into wine.

1.4.3 WHAT ARE THE "WINE YEASTS" AND HOW DOES THE ENVIRONMENT AFFECT THEIR GROWTH?

The yeasts responsible for alcoholic fermentation originate from either of three sources:

(i) the surface of the grape;

(ii) surfaces of winery equipment; and

(iii) inoculum cultures.

(Fleet & Heard 1993)

Generally, they are facultative anaerobes.

Mature sound grapes, asceptically crushed, will give a must with a total yeast population in the range of between 10^3 - 10^6 colony forming units (cfu) per ml. (Parrish & Carroll 1985).

Spontaneous or natural fermentations of grape juice are characterised by a succession of indigenous species of yeast beginning with the genera *Kloeckera*, *Hansenula*, *Hanseniaspora*, *Candida* and *Pichia*. During the most active stages of the fermentation and toward the end, these yeasts are usually replaced by *Saccharomyces cerevisiae*, the strongest fermenter. (Reed & Nagodawithana 1991).

1.4.3.1 The principal phases of yeast growth

There are three principal phases in the yeast growth cycle of *Saccharomyces cerevisiae* during fermentation:

a) a limited phase of multiplication that lasts for 2 - 3 days and carries the cell population from initial values of 10^4 - 10^6 cfu/ml to 10^8 -10^9cfu/ml;

b) a stationary phase where the viable cell level remains approximately constant and which lasts about eight days; and

c) a decline phase where the viable cell population progressively reduces to a level around 10^5 cfu/ml. This last phase may last several weeks. (Ribereau-Gayon 1985).

The total growth of yeasts in a wine fermentation is limited and corresponds to about 4 or 5 generations.(Ribereau-Gayon 1985: Fleet & Heard 1993).

1.4.3.2 Environmental changes

The principal phases of yeast growth vary with each fermentation and are influenced by such factors as:

clarification of the grape juice

addition of sulphur dioxide / early elimination of oxygen

the temperature of fermentation

the composition of the grape juice

inoculation with selected yeasts,

and interactions with other microorganisms.

(Fleet & Heard 1993)

For the purposes of my study, the effects of temperature and oxygen on yeast growth are important.

1.4.3.3 Temperature

The temperature at which the alcoholic fermentation is conducted affects:

(i) the rate of yeast growth and, consequently, the duration of fermentation;

(ii) the extent to which different yeast species contribute to the fermentation; and

(iii) the biochemical reactions of the yeasts which ultimately determines the chemical composition and sensory quality of the wine. (Fleet & Heard 1993).

The rate of yeast growth and speed of alcoholic fermentation increase as the temperature increases, with maximum rates generally occurring at temperatures between 20 - 25°C. (Amerine et al. 1980: Benda 1982).

A series of studies by Ough and co-workers who examined the fermentation rates of grape juice over the range 10 - 33°C after inoculation with *Saccharomyces cerevisiae*, found the length of yeast growth to be inversely proportional to the temperature. An increase of 4.7 times in yeast growth rate between 10° and 21°C, therefore, produced an increase in fermentation rate of about two-fold. (Ough 1964: 1966a: 1966b).

The sensitivity of *Saccharomyces cerevisiae* to ethanol is enhanced at temperatures above 30°C, sometimes leading to premature cessation of fermentation at high temperatures. In contrast, the indigenous yeasts (*Kloeckera apiculata* and *Candida stellata* for instance) exhibit an increased tolerance to ethanol at the lower fermentation temperatures of 10 - 15°C, and may even exhibit faster growth rates than *S. cerevisiae*, allowing them to dominate fermentations under these conditions. (Van Uden 1989: Fleet & Heard 1993).

1.4.3.4 Oxygen Availability

Growth of wine yeasts in the complete absence of oxygen probably cannot take place.

Before fermentation commences, grape juice may be saturated with oxygen, although this concentration is not very great, only about 8 - 9mg/l. A small amount of this oxygen is needed by wine yeasts to induce the formation of mitochrondria, the citric-acid-cycle enzymes, the respiratory electron transport system, and the corresponding effect of fermentation, the Pasteur effect. (Traverso-Rueda & Kunkee 1982). The remainder may be required to assist in the synthesis of at least three cellular constituents of the yeast. These are the unsaturated fatty-acyl

residues in lipids, sterols and nicotinic acid. The actual role of these three constituents in yeast growth is uncertain, although some researchers have considered them to be growth stimulants. (Rose 1987).

The addition of sterols to a wine must increases the concentration of sterols in the cells and increases the viability of the population and the speed of fermentation. (Ribereau-Gayon 1985).

Traditional winemaking requires the exclusion of oxygen from the must at an early stage of the fermentation, and anaerobic yeast growth. Oxygen dissolved in wine is consumed with time, reaching zero in about 6 days at 30°C. (Singleton 1987). The reasons for this exclusion are that over-oxidation causes the formation of aldehyde, giving wines bitter flavours by causing complexes of the aldehyde and colouring matter, primarily in white wines. In the early stages of juice or must preparation, browning is primarily the result of enzymatic oxidation of naturally occurring phenolic substances. The enzyme catalyzing oxidative browning is polyphenol oxidase (PPO). (Amerine & Joslyn 1970).

A purely aerobic fermentation of grape juice therefore, would not produce a wine with desireable aroma or colour. The flavour of an oxidised wine is also likely to be bitter. A combination of aerobic growth and anaerobic fermentation however, may produce a wine with acceptable taste and a less pronounced colour.

1.4.4 BATCH AND CONTINUOUS CULTURE OF YEASTS

The method of culture of yeasts also impacts on growth. Most studies on metabolism of yeasts as well as any industrial utilisation of these microorganisms requires their cultivation in liquid media by either batch or continuous culture. (Fiechter et al. 1987).

In batch processes, a vessel is filled with all starting materials and subsequently inoculated with a micro-organism. Biochemical conversion takes place in the vessel over a time period that may range from a few hours to several days, during which the number of organisms in the reactor increases. Ultimately, the reactor is emptied and a new batch is started. Growth in batch culture, such as a wine fermentation, is a spontaneous process. Its development is

uncontrolled once started.

Continuous culture differs from batch culture in that a steady state is maintained in the reactor vessel over a theoretically unlimited period of time. In this process, the reactants are constantly supplied to the reactor and the products (and cells) are continuously removed at the same rate. Accordingly, the number of cells in the reactor remains constant since as many cells are removed from the vessel as are generated by growth. (Fiechter et al. 1987).

1.4.4.1 Batch growth

Batch culture represents a closed system (except for gas flow) in which cells multiply until some nutrient is exhausted or some metabolite accumulates to toxic concentrations. The developing population passes successively through a number of distinct phases - lag, accelerating, exponential, decelerating, stationary and decreasing - each of which is characterised by specific metabolic processes. (Pirt 1975).

The most characteristic feature of the lag phase seems to be a change in synthesis of rRNA and ribosomal proteins, which enables the cells to grow successively with a rate adapted to the new environmental conditions. No significant increase in biomass occurs during this phase.

The accelerating phase is a short intermediate period between the lag and exponential phases in which biomass increases from 0 to maximum levels.

Under optimal conditions, cells then enter the exponential growth phase during which they proliferate at a maximal rate. Essential for exponential growth are an excess of all essential nutrients, no hidden limitations like incomplete mixing or precipitation of medium components, the capability of all cells to proliferate, and the absence of inhibitors.

When a significant variable such as the concentration of one nutrient or a toxin no longer supports growth at the maximal rate, the culture enters the decelerating phase. When the substrate is exhausted no growth occurs and the

stationary phase is entered. The duration of this phase is difficult to predict as the cells respond to starvation conditions by degradation of redundant cell components. Cell lysis may supply enough nutrients to allow other cells to survive or even to divide, thus maintaining the overall size of the population for some time.
Usually the decreasing (death) phase of a cell population follows an exponential decay. (Fiechter et al. 1987).

The main disadvantages of batch culture are that the environmental conditions of such a system are not constant. There is a high concentration of glucose at the start of the fermentation.

Nutrient, substrate, product and biomass concentrations change continuously and even a strict control of the external parameters (temperature, pH value, pressure, aeration) does not give reproducible results.

1.4.4.2 Continuous culture

A continuous culture represents an open system (Figure 1.4.4.2) in which nutrients are constantly supplied and an equal fraction of the reaction mixture, including cells, is withdrawn. The rate of microbial growth is not fixed but varies with the availability of nutrients. Therefore, it is possible to fix the specific growth rate of cells at any value from zero to maximum. (Monod 1942).

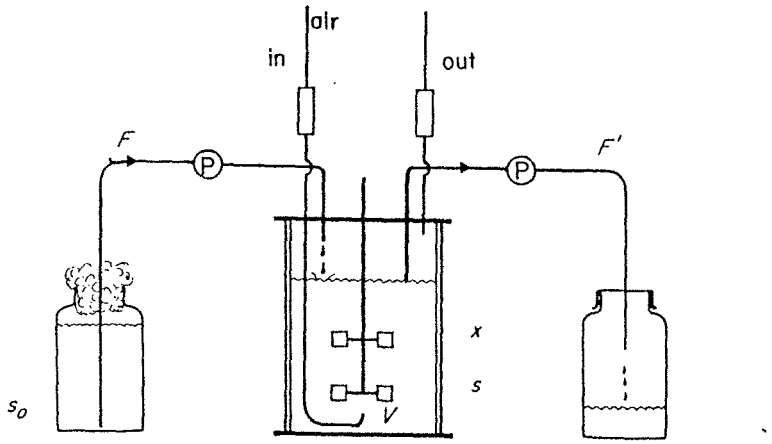


Figure 1.4.4.2

A diagram of a continuous culture. Fresh medium with a certain substrate concentration (S_0) is fed by a metering pump (P) to the bioreactor at a constant rate (F). The liquid volume (V) contains cells at the concentration x and residual substrate s, as well as the gas phase. Culture liquid is removed at a rate F', which equals the medium feed F under steady-state conditions. The important variable of the system is the dilution rate, which is defined as the medium flow per unit volume ($D = F/V$).

(Source: Fiechter et al. 1987)

One prerequisite for successful application of the continuous culture is the availability of a theoretical basis for growth. The mathematical formulation of growth allows the researcher not only to test, by comparison of calculated and measured parameters, whether the culture is actually behaving as expected, but also allows a prediction of the further development of the culture. Also, once the interdependence of the various parameters has been established, those variables which are not easily accessible may be calculated from on-line measured variables and used for process control. The most important parameters determining growth in continuous culture are:-

(i) biomass balance and dilution rate - the change in biomass over time is determined by the amount of biomass formed in the reactor and the amount of biomass removed from the reactor: or

increase in biomass = growth - output

(ii) substrate balance - the change in the concentration of a substrate in the reactor during an (infinitesimally) small time interval is determined by:

a) the concentration of the substrate in the medium,

b) the amount of substrate removed from the reactor, and

c) the amount of substrate consumed by the growing culture, or

net increase = input - output - substrate metabolized

(iii) critical dilution rate - theoretically, the maximum specific growth rate is observed when the concentration of the limiting substrate approaches zero

(iv) organism specific parameters - the growth of any strain under defined environmental conditions is determined by 3 organism-specific parameters namely,

a) maximal specific growth rate,

b) saturation constant, and

c) yield constant.

These parameters vary with different media as well as with different organisms and are an indication of how fast an organism grows on the medium and how effectively a substrate is assimilated.

(v) steady state - a continuous culture is a self-regulating system in which the equilibrium of growth rate and dilution rate becomes independent of time, thus permitting the recovery of cells in a defined physiological state at any time.

(vi) deviations from chemostat theory - any deviations from the theoretically expected growth pattern may be caused by physical, chemical or biological effects. Examples of physical effects include wall growth and incomplete mixing. A typical chemical effect is precipitation of medium components resulting in various limitations which may not be easily evaluated. Biological events leading to a deviation are often strain-specific and usually reflect metabolic peculiarities. (Fiechter et al. 1987).

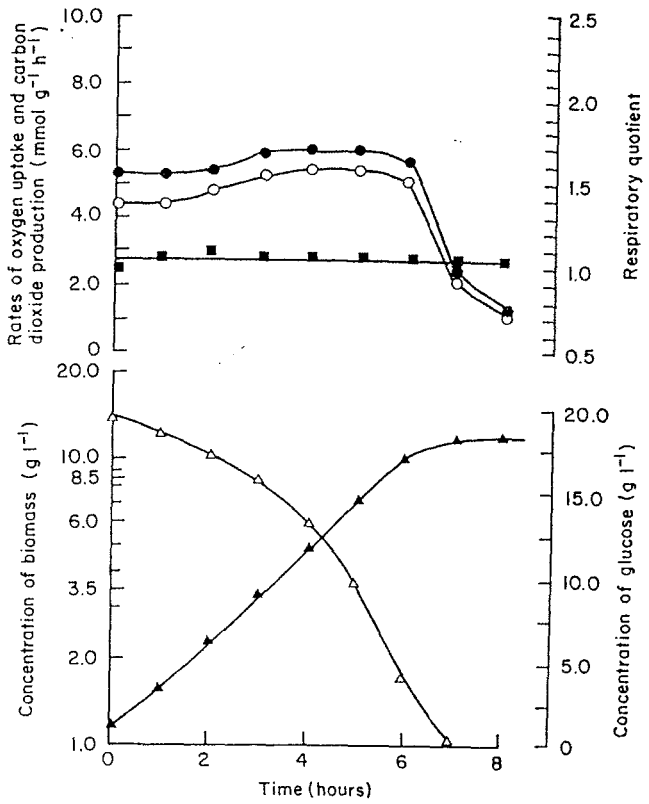
1.4.4.3 Cultivation of Yeasts

Three of the yeast strains likely to be used in my study have been grown in batch and continuous culture by Fietcher et al.(1987). These were *Saccharomyces cerevisiae, Trichosporon cutaneum* and *Candida tropicalis*. Only *T. cutaneum* did not follow the general characteristics about to be described, probably because the strain used for my research was capable of ethanol production under conditions of oxygen limitation.

A. BATCH CULTURE

In batch culture, as in traditional wine fermentation, where the carbon source (glucose) was present in excess concentration, the three yeasts used by Fietcher et al. (1987) did not show a uniform growth pattern. Differences were due not only to different specific growth rates, but originated also from differences in the metabolic pathways used for glucose utilisation and their regulation. (Fietcher et al. 1981).

1. Trichosporon cutaneum exhibited a single-phase growth curve (Figure 1.4.4.3.a), in which the rate of oxygen uptake equals the rate of carbon dioxide production. No product is generally formed that could serve as substrate for a second growth phase. As a consequence, a high yield of biomass on sugar is characteristic of *T. cutaneum.* (Fiechter et al. 1987).

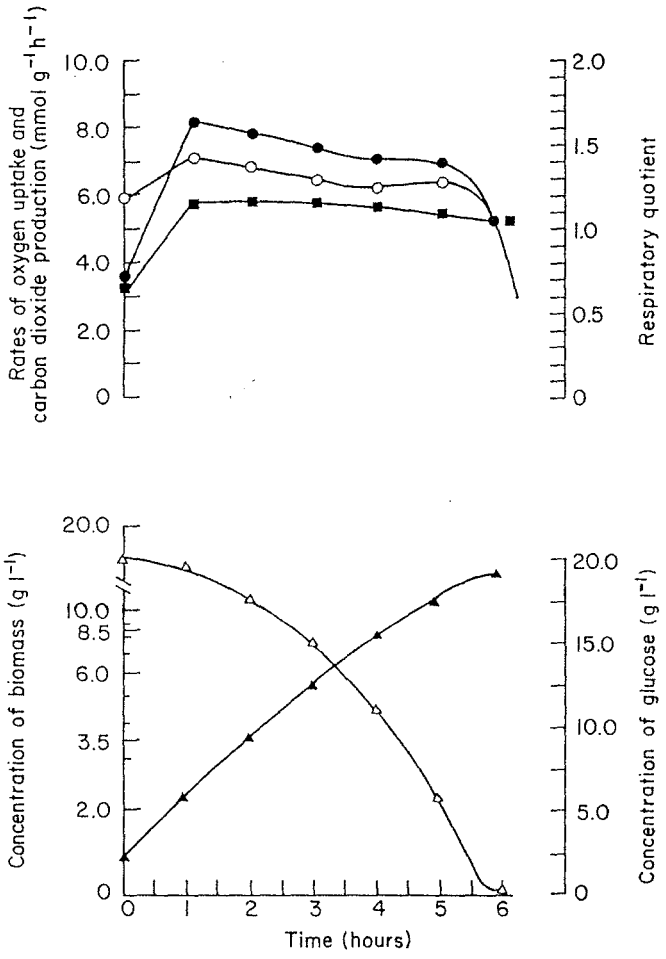


Batch growth kinetics of *Trichosporon cutaneum* in medium containing 20 g glucose l^{-1}. Biomass (▲) was formed at the expense of substrate utilization (Δ). Rate of carbon dioxide production (●) was close to the rate of oxygen uptake (○), yielding a respiratory quotient (■) slightly above unity. The maximum growth rate was 0.40 h^{-1}. The pH value of the culture was maintained at 5.0 and the aeration rate was 0.67 volume air (volume liquid)$^{-1}$ min^{-1}.

Figure 1.4.4.3.a

(Source: Fiechter et al. 1987)

2. *Candida tropicalis* generally resembles *T. cutaneum* in its growth kinetics. A single phase growth curve is observed in well-aerated reactors, and there is no product formed which can be further metabolised (Figure 1.4.4.3.b).

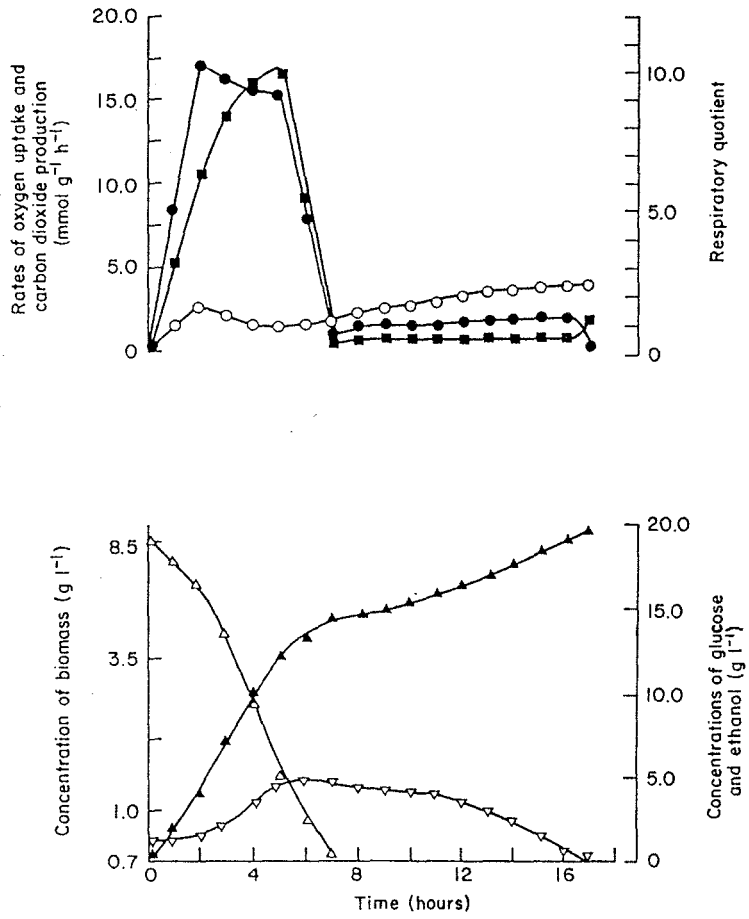


Batch growth kinetics of *Candida tropicalis* in medium containing 20 g glucose l^{-1}. The same single-phase growth is observed with this yeast as with *Trichosporon cutaneum* . The maximum growth rate is also in the same range (0.40 h^{-1}). Cultural conditions are identical to those described ▲ indicates biomass, △ substrate concentration, O oxygen uptake rate, ● carbon dioxide production rate and ■ respiratory quotient.

Figure 1.4.4.3.b

(Source: Fiechter et al. 1987)

3. Saccharomyces cerevisiae differs from the other two strains in that it exhibits double-phase (or diauxic) growth kinetics batch culture (Figure 1.4.4.3.c). This results from the ability of this yeast to metabolize glucose glycolytically during the first growth phase, producing ethanol which is subsequently metabolized oxidatively.



Batch growth kinetics of *Saccharomyces cerevisiae* in medium containing 20 g glucose l^{-1}. The double-phase growth is clearly indicated. In the first growth phase, ethanol (▽) is formed, and is used in the second growth phase as substrate. Owing to ethanol production, the rate of carbon dioxide production (●) exceeds the rate of oxygen uptake, yielding a respiratory quotient (■) above unity. In the second growth phase, the rate of oxygen uptake is higher than that of carbon dioxide production because of the stoicheiometric equation for ethanol oxidation. The maximum growth rate for the first growth phase was 0.35 h^{-1} and for the second growth phase was 0.10 h^{-1}. Overall culture conditions were the same as for *Trichosporon cutaneum* and *Candida tropicalis* Δ indicates glucose concentrations.

Figure 1.4.4.3.c

(Source: Fiechter et al. 1987)

4. Ethanol production and oxygen limitation in batch culture

In glucose-utilizing cultures of *T. cutaneum* growth proceeds only so long as sufficient oxygen is available. This yeast is generally unable to generate energy for growth except by respiration. In contrast, according to Fiechter et al. 1987), insufficiently aerated shake-flask cultures of glucose-utilizing *C. tropicalis* do form ethanol. The ethanol formation has been shown to be due to oxygen limitation rather than the presence of excess glucose. It is only ethanol formation during the first growth phase in batch cultures of glucose-utilizing *S. cerevisiae* which can be directly attributed to the presence of glucose, regardless of the availability of oxygen.

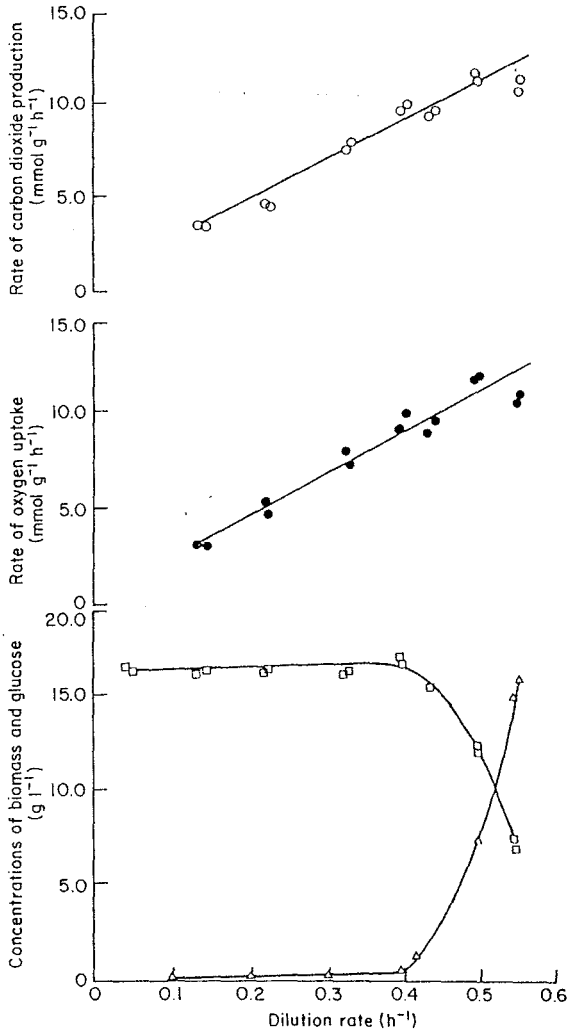
B. CONTINUOUS CULTURE

Continuous processes have been developed for red winemaking, and are successfully used for the production of sparkling wine in Russia, but are not extensively used.

Yeast growth in continuous culture, as reported by Fiechter et al. (1987), again produced contrasts between the respiratory yeasts and *Saccharomyces cerevisiae.*

1. Respiratory yeasts: *Trichosporon cutaneum* and *Candida tropicalis*

A plot of the biomass formation by *T. cutaneum* versus dilution rate (Figure 1.4.4.3.d) shows that steady states were obtained up to a dilution rate of $0.55h^{-1}$, and indicate a critical dilution rate of 0.55 to $0.60h^{-1}$. This is higher than the maximal growth rate of $0.40h^{-1}$ observed in batch culture. Glucose accumulated in the culture liquid at dilution rates greater than 0.40^{-1} and the residual glucose that was not metabolised caused a steady-state decrease in biomass concentration. The rates of oxygen uptake and carbon dioxide production increased linearly with increasing dilution rates with maximum uptake rates observed at approximately 11.0 $mmolg^{-1}h^{-1}$. These rates were also considerably higher than those observed in batch culture (5.5 $mmolg^{-1}h^{-1}$).



Continuous cultivation of *Trichosporon cutaneum* in medium containing 30 g glucose l^{-1}. The critical dilution rate is approximately 0.60 h^{-1}. Glucose (△) accumulates in the reactor at dilution rates higher than 0.40 h^{-1}. Rates of oxygen uptake (●) and carbon dioxide production (○) are equal over the entire range of dilution rates tested.

Figure 1.4.4.3.d

(Source: Fiechter et al. 1987)

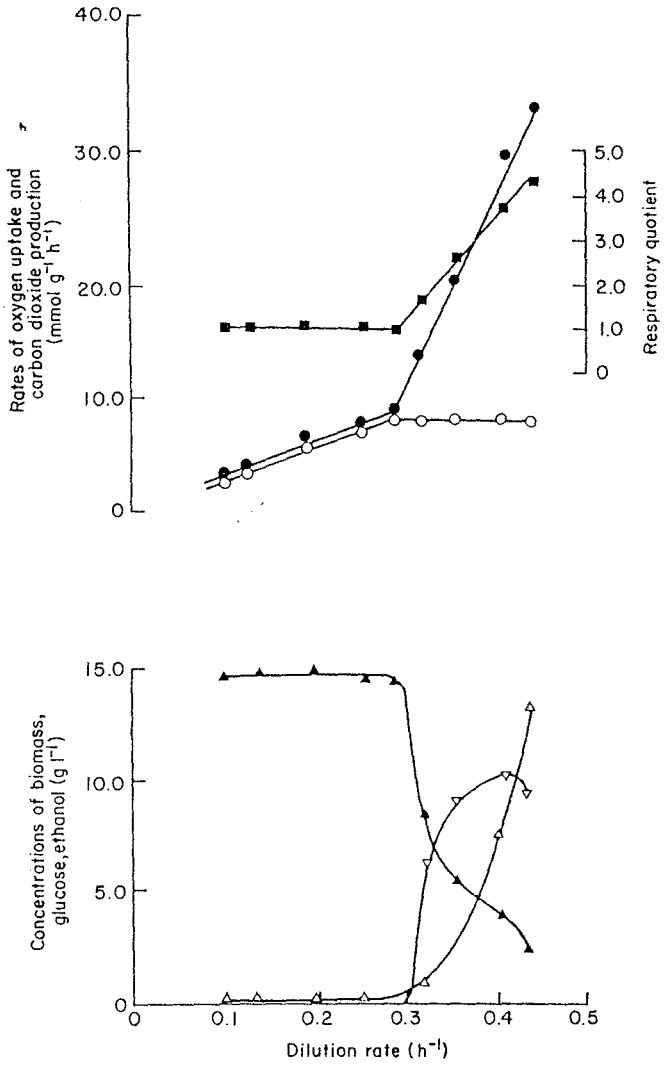
The ultimate decline in biomass occurred when oxygen became limiting. As *T. cutaneum* can utilize one mol of glucose only if 6 moles of oxygen are available, with increasing oxygen limitation, increasing amounts of residual glucose were found in the culture filtrate. (Kappeli & Fiechter 1982).

C. tropicalis produced similar results when not subjected to oxygen limitation. In this yeast glucose uptake rate and respiration are co-ordinated so that aerobic ethanol does not occur unless respiration is artifically decreased by oxygen limitation. (Petrik et al. 1983).

2. *Saccharomyces cerevisiae*

When this yeast was cultivated in continuous culture two metabolically different responses could be distinguished when the overall culture parameters were recorded as a function of dilution rate (Figure 1.4.4.3.e). At low dilution rates, glucose breakdown occurred oxidatively and only biomass and carbon dioxide were formed. This type of metabolism resembled *T. cutaneum* and *C. tropicalis*. The yield of biomass was high and the rate of CO_2 production equalled the rate of oxygen uptake.

When the dilution rate was successively increased, a particular rate (D_r) was reached above which the rate of oxygen uptake no longer increased linearly with increasing dilution rates, but levelled off. Above D_r *S. cerevisiae* began to metabolize glucose reductively and produce ethanol. The energy-rich ethanol formed caused a drop in the yield of biomass to about one-quarter the level found at low dilution rates. This yield was comparable to that found in the first phase of batch culture. (Fiechter et al. 1987).



Continuous cultivation of *Saccharomyces cerevisiae* in medium ; containing 30 g glucose l^{-1}. The plot is characterized by two regions. Up to a dilution rate of 0.30 h^{-1}, the substrate is degraded oxidatively; at dilution rates above 0.30 h^{-1}, ethanol is produced by cells. The latter state is characterized by a rate of carbon dioxide production that exceeds the rate of oxygen uptake and a respiratory quotient above unity. ▲ indicates concentration of biomass, △ of glucose, ▽ of ethanol, O rate of oxygen uptake, ● rate of carbon dioxide production and ■ respiratory quotient.

Figure 1.4.4.3.e

(Source: Fiechter et al. 1987)

Whereas batch processes are still favoured in the brewing and winemaking industries especially for the assistance yeasts can give to the development of flavours and aromas over time, continuous culture offers a greater versatility for other applications. Continuous culture is used for metabolic studies requiring great amounts of cells, mass production of chemicals by yeasts, and for biomass production. Although the products of fermentation can be modified by limiting the amount of glucose or oxygen available to the yeast for growth by batch culture, continuous culture may be better at achieving these ends, and have a role in the production of reduced alcohol wines and beverages.

2.0 MATERIALS AND METHODS

The overall equation for alcoholic fermentation is:

$$C_6 H_{12} O_6 \rightarrow 2C_2 H_5 OH + 2CO_2$$

180 92 88

(Gay-Lussac 1815)

By this calculation, 180 parts by weight of sugar is thereby converted in fermentation into 92 parts by weight of alcohol and 88 parts of carbon dioxide. By dividing the weight percent of alcohol by its specific gravity (0.7893), we can obtain the percent of alcohol by volume. We would expect under normal wine fermentation conditions therefore, that 182g/l sugar would convert to about 11.65% alcohol. A reduced alcohol wine will be anything under this figure. (Rankine 1991).

The raw juice for experimentation is Muller Thurgau from Marlborough with a °Brix level of 17 and pH of 3.2. This Brix level equates to approximately 182g/l sugar. (Vine 1981).

2.1 THE YEASTS

The yeast strains chosen for this research were among those which had shown promising results at reducing sugar levels in either apple juice (Kapeli 1989) or orange juice (Kolb et al. 1993), namely:

Saccharomyces cerevisiae

Schizosaccharomyces pombe

Saccharomycodes ludwigii

Candida tropicalis

Pichia stipitis

Trichosporon cutaneum

Candida shehatae

S. cerevisiae, *S. pombe*, *S. ludwigii* and *T. cutaneum* were sourced from Lincoln University, and were of unkown origin.

C. tropicalis was obtained from the New Zealand Reference Culture Collection in Porirua and has its origins in the American Type Culture Collection (ATCC 13803).

P. stipitis and *C. shehatae* were obtained from the Centraalbureau voor Schimmelcultures (Yeast Division) in the Netherlands and have the reference numbers CBS 5774 and CBS 5813 respectively.

A description of each of these yeasts as it appears in Kreger-van Rij (1984) is attached as Appendix I.

2.2 CULTURE TECHNIQUES AND SAMPLE PREPARATION

2.2.1 CULTURE TECHNIQUES

Yeast cultures, which had been stored on agar slopes were streaked out onto Malt Extract Agar (MEA) of the following composition:-

Malt extract, powdered	20g
Peptone	1.0g
Glucose	20g
Agar	20g
Water, distilled	1 litre

The cultures were incubated at 25°C for 48 hours.

Colonies from the MEA were then grown in 10ml nutrient broth of the following composition:-

Peptone 190 (pancreatic digest of gelatin)	3.5g
Beef extract	3.5g
Yeast extract	1.0g
Distilled water	1000ml.

Incubation was again at 25°C for 48 hours.

To accustom the yeast cultures to grow in grape juice, a further nutrient bulking media was prepared consisting of:-

Sucrose	182g
oxoid mycological peptone	10g
Yeast extract	5g
Distilled water	1000ml.

The pH of this further nutrient media was adjusted to 3.2 by the addition of 4N H_2SO_4, so that the pH and sugar level of the media equalled those of the grape juice used for this series of experiments.

The 10ml nutrient broth (and cultures) was transferred into 80mls. of the nutrient bulking media and incubated at 25°C (*T. cutaneum* at 30°C) until the required cell counts were obtained.

Bacterial contamination of yeast cultures grown with aeration was seen as a problem. Throughout the series of experiments, therefore, samples of juice were taken every 48 hours, streaked onto nutrient agar and incubated at 25°C for two weeks to check for growth of undesirable organisms. The nutrient agar had the following composition:-

Peptone 190 (pancreatic digest of gelatin)	5.0g
Beef extract	5.0g
Yeast extract	1.0g
Agar	12.0g
Distilled water	1000ml.

Preparation of the active dried *Saccharomyces cerevisiae* yeast culture used for the combined aerobic/anaerobic fermentation was undertaken by rehydrating the granules in 10ml warm water (35 - 40°C) for 30 minutes, then addition to 80 ml of juice and incubation at 30°C for 24 hours prior to inoculation.

2.2.2 SAMPLE PREPARATION

During experimentation samples were drawn at least daily under aseptic conditions, and frozen immediately for later examination.

After thawing and microscopic examination, the samples were centrifuged for 5 minutes at 4640*g* to facilitate yeast biomass extraction prior to sugar and/or alcohol determination.

2.3 CHEMICAL METHODS OF SUGAR AND ALCOHOL ANALYSIS IN MUSTS AND WINE

2.3.1 DETERMINATION OF REDUCING SUGAR BY REBELLEIN METHOD

The advantages of the Rebellein Method over other methods for the determination of reducing sugar are:-

1. that the end point of titration (cream-white) is easily detected, and
2. the titration does not have to be carried out on a boiling solution.

The method is based on reacting the sugars in the wine sample (after dilution if necessary) with excess alkaline cupric (Cu++) tartrate. The concentration of the Cu++ remaining after reaction is then determined by titration. From this the amount of Cu++ that has reacted with the sugar can be determined and hence the sugar concentration. The Cu++ concentration in the Rebellein Method is determined by reducing it with iodine ($2Cu^{++} + 2I^{-} \rightarrow 2Cu^{+} + I_2$) and estimating the iodine with standard thiosulphate solution ($I_2 + 2S_2O_3 \rightarrow 2I^{-}+S_4O_6^{=}$).

Wines that have completely fermented will not have a reading of zerogL^{-1} reducing sugar since there are non-fermentable reducing sugars present in grape juice that when carried through to the wine will be analysed by this chemical method. Hence wines that have been fermented dry may give values between zero and 2gL^{-1} reducing sugar depending on the actual concentration of the non-fermentable reducing sugars present. (Iland 1988).

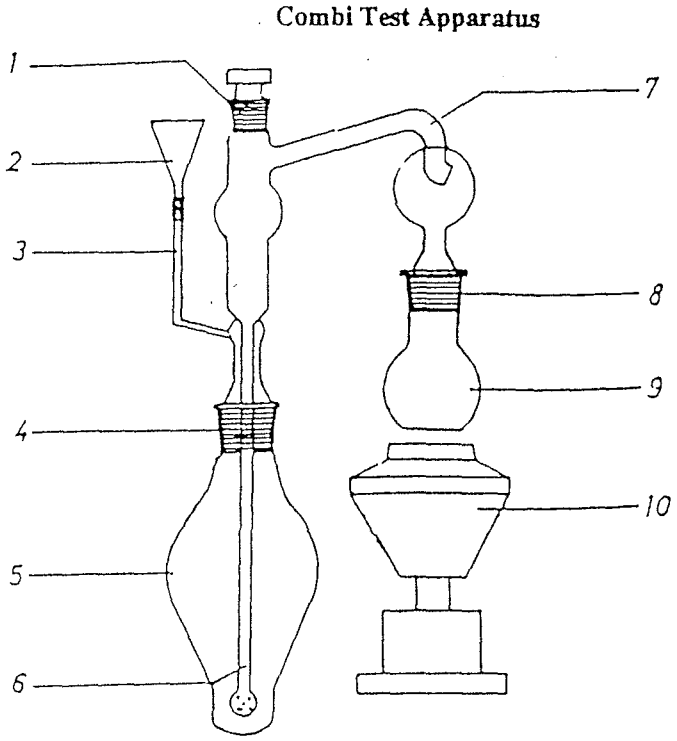
The analysis method and reagents required for reducing sugar by the Rebellein Method are attached as Appendix II.

This method was used during experimentation to follow the reduction of sugars prior to completion of fermentation.

2.3.2 DETERMINATION OF ALCOHOL AND SUGAR WITH THE COMBITEST PROCESS (COMBI METHOD)

The combitest process was used to determine final sugar and alcohol levels in all experimental wines.

The distillation of alcohol and the simultaneous decomposition of sugar are carried out by this method using a single integrated analysis with the same apparatus (Figure 2.3.2).



1. Stopper
2. Funnel
3. Steam escape tube
4. Ground connection
5. Receiving flask
6. Steam entry tube
7. Distillation bridge with foam trap
8. Ground connection
9. Boiling flask
10. Electric reflector burner (or gas burner)

Figure 2.3.2

(Source: Combitest, Fritz Merkel, notes accompanying Apparatus)

2.3.2.1 Sugar determination

In the Combi Test, a diluted wine sample is boiled with an excess of this oxidising agent. The Cu^{++} left unreacted is then acidified and reacted with excess iodide, I^-. The iodine, I_3^- generated can then be titrated with standardised thiosulphate, $S_2O_3^{2-}$. While the reactions between Cu^{++} and I^- and between I_3^- and $S_2O_3^{2-}$ are stoichiometric, the initial reaction between sugar and Cu^{++} is not. Consequently, a standardised procedure using reagents of a specified composition together with a critically timed boiling period is required. Given the non-stoichiometric nature of the sugar oxidation the titre volume of the $S_2O_3^{2-}$ must be used in conjunction with a patented table to obtain the sugar content of the original sample (Table 2.3.2.1). (Van Dam 1979).

REDUCING SUGAR BY COMBI TEST - using 10ml of 1:10 diluted sample
(Blank Titre - Sample titre) x 3.76 = g/l

Volume of 0.1M Thiosulphate (difference) ml

	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0	0.00	0.38	0.75	1.13	1.50	1.88	2.26	2.63	3.01	3.38
1	3.76	4.14	4.51	4.89	5.26	5.64	6.02	6.39	6.77	7.14
2	7.52	7.90	8.27	8.65	9.02	9.40	9.78	10.15	10.53	10.90
3	11.28	11.66	12.03	12.41	12.78	13.16	13.54	13.91	14.29	14.66
4	15.04	15.42	15.79	16.17	16.54	16.92	17.30	17.67	18.05	18.42
5	18.80	19.18	19.55	19.93	20.30	20.68	21.06	21.43	21.81	22.18
6	22.56	22.94	23.31	23.69	24.06	24.44	24.82	25.19	25.57	25.94
7	26.32	26.70	27.07	27.45	27.82	28.20	28.58	28.95	29.33	29.70
8	30.08	30.46	30.83	31.21	31.58	31.96	32.34	32.71	33.09	33.46
9	33.84	34.22	34.59	34.97	35.34	35.72	36.10	36.47	36.85	37.22
10	37.60	37.98	38.35	38.73	39.10	39.48	39.86	40.23	40.61	40.98
11	41.36	41.74	42.11	42.49	42.86	43.24	43.62	43.99	44.37	44.74
12	45.12	45.50	45.87	46.25	46.62	47.00	47.38	47.75	48.13	48.50
13	48.88	49.26	49.63	50.01	50.38	50.76	51.14	51.51	51.89	52.26
14	52.64	53.02	53.39	53.77	54.14	54.52	54.90	55.27	55.65	56.02
15	56.40	56.78	57.15	57.53	57.90	58.28	58.66	59.03	59.41	59.78
16	60.16	60.54	60.91	61.29	61.66	62.04	62.42	62.79	63.17	63.54
17	63.92	64.30	64.67	65.05	65.42	65.80	66.18	66.55	66.93	67.30
18	67.68	68.06	68.43	68.81	69.18	69.56	69.94	70.31	70.69	71.06
19	71.44	71.82	72.19	72.57	72.94	73.32	73.70	74.07	74.45	74.82
20	75.20	75.58	75.95	76.33	76.70	77.08	77.46	77.83	78.21	78.58
21	78.96	79.34	79.71	80.09	80.46	80.84	81.22	81.59	81.97	82.34
22	82.72	83.10	83.47	83.85	84.22	84.60	84.98	85.35	85.73	86.10
23	86.48	86.86	87.23	87.61	87.98	88.36	88.74	89.11	89.49	89.86
24	90.24	90.62	90.99	91.37	91.74	92.12	92.50	92.87	93.25	93.62

(Source: Van Dam 1979)

2.3.2.2 Alcohol determination

All reactions involved in the alcohol determination are stoichiometric and proceed in accordance with the following balanced equations:

$$3CH_3CH_2OH + 2Cr_2O_7^{2-} + 16H^+ \rightarrow 3CH_3COOH + 4Cr^{3+} + 11_2O$$

$$Cr_2O_7^{2-} + 14H^+ + 9I^- \rightarrow 2Cr^{3+} + 7H_2O + 3I_3^-$$

$$2S_2O_3^{2-} + I_3^- \rightarrow S_4O_6^{2-} + 3I^-$$

The boiling procedure from the sugar determination also serves to quantitatively distill the alcohol in the sample into a receiving flask where it is oxidised to acetic acid by acidified dichromate, $Cr_2O_7^{2-}$. Iodide, I^- is then added to the unreacted $Cr_2O_7^{2-}$ to again yield I_3^- which can be titrated with $S_2O_3^{2-}$ until the mixture reaches a pale blue colour. The titre volume is then used to look up a table to obtain the % alcohol by volume of the sample (Table 2.3.2.2).

The analysis method and the reagents required for sugar and alcohol determination by the Combi Method are attached as Appendix III. (Combitest, Fritz Merkel: Van Dam 1979).

In undertaking analysis by either the Rebellein or Combi method throughout this series of experiments, at least dual testing of samples was undertaken and in most cases three tests of each sample were made, and the average result used.

TABLE 2.3.2.2

Alcohol % by Volume using 25 ml Dichromate solution

Vol.	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
5	15.11	15.09	15.08	15.06	15.05	15.03	15.02	15.00	14.99	14.97
6	14.96	14.95	14.93	14.92	14.90	14.89	14.87	14.86	14.84	14.83
7	14.81	14.80	14.78	14.77	14.76	14.74	14.73	14.71	14.70	14.68
8	14.67	14.65	14.64	14.62	14.61	14.60	14.58	14.57	14.55	14.54
9	14.52	14.51	14.49	14.48	14.46	14.45	14.44	14.42	14.41	14.39
10	14.38	14.36	14.35	14.33	14.32	14.30	14.29	14.28	14.26	14.25
11	14.23	14.22	14.20	14.19	14.17	14.16	14.14	14.13	14.11	14.10
12	14.09	14.07	14.06	14.04	14.03	14.01	14.00	13.98	13.97	13.95
13	13.94	13.93	13.91	13.90	13.88	13.87	13.85	13.84	13.82	13.81
14	13.79	13.78	13.77	13.75	13.74	13.72	13.71	13.69	13.68	13.66
15	13.65	13.63	13.62	13.61	13.59	13.58	13.56	13.55	13.53	13.52
16	13.50	13.49	13.47	13.46	13.44	13.43	13.42	13.40	13.39	13.37
17	13.36	13.34	13.33	13.31	13.30	13.28	13.27	13.26	13.24	13.23
18	13.21	13.20	13.18	13.17	13.15	13.14	13.12	13.11	13.10	13.08
19	13.07	13.05	13.04	13.02	13.01	12.99	12.98	12.96	12.95	12.93
20	12.92	12.91	12.89	12.88	12.86	12.85	12.83	12.82	12.80	12.79
21	12.77	12.76	12.75	12.73	12.72	12.70	12.69	12.67	12.66	12.64
22	12.63	12.61	12.60	12.59	12.57	12.56	12.54	12.53	12.51	12.50
23	12.48	12.47	12.45	12.44	12.43	12.41	12.40	12.38	12.37	12.35
24	12.34	12.32	12.31	12.29	12.28	12.26	12.25	12.24	12.22	12.21
25	12.19	12.18	12.16	12.15	12.13	12.12	12.10	12.09	12.08	12.06
26	12.05	12.03	12.02	12.00	11.99	11.97	11.96	11.94	11.93	11.92
27	11.90	11.89	11.87	11.86	11.84	11.83	11.81	11.80	11.78	11.77
28	11.76	11.74	11.73	11.71	11.70	11.68	11.67	11.65	11.64	11.62
29	11.61	11.59	11.58	11.57	11.55	11.54	11.52	11.51	11.49	11.48
30	11.46	11.45	11.43	11.42	11.41	11.39	11.38	11.36	11.35	11.33
31	11.32	11.30	11.29	11.27	11.26	11.25	11.23	11.22	11.20	11.19
32	11.17	11.16	11.14	11.13	11.11	11.10	11.08	11.07	11.06	11.04
33	11.03	11.01	11.00	10.98	10.97	10.95	10.94	10.92	10.91	10.90
34	10.88	10.87	10.85	10.84	10.82	10.81	10.79	10.78	10.76	10.75
35	10.74	10.72	10.71	10.69	10.68	10.66	10.65	10.63	10.62	10.60
36	10.59	10.58	10.56	10.55	10.53	10.52	10.50	10.49	10.47	10.46

	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
37	10.44	10.43	10.41	10.40	10.39	10.37	10.36	10.34	10.33	10.31
38	10.30	10.28	10.27	10.25	10.24	10.23	10.21	10.20	10.18	10.17
39	10.15	10.14	10.12	10.11	10.09	10.08	10.07	10.05	10.04	10.02
40	10.01	9.99	9.98	9.96	9.95	9.93	9.92	9.90	9.89	9.88
41	9.86	9.85	9.83	9.82	9.80	9.79	9.77	9.76	9.74	9.73
42	9.72	9.70	9.69	9.67	9.66	9.64	9.63	9.61	9.60	9.58
43	9.57	9.56	9.54	9.53	9.51	9.50	9.48	9.47	9.45	9.44
44	9.42	9.41	9.40	9.38	9.37	9.35	9.34	9.32	9.31	9.29
45	9.28	9.26	9.25	9.23	9.22	9.21	9.19	9.18	9.16	9.15
46	9.13	9.12	9.10	9.09	9.07	9.06	9.05	9.03	9.02	9.00
47	8.99	8.97	8.96	8.94	8.93	8.91	8.90	8.89	8.87	8.86
48	8.84	8.83	8.81	8.80	8.78	8.77	8.75	8.74	8.73	8.71
49	8.70	8.68	8.67	8.65	8.64	8.62	8.61	8.59	8.58	8.56
50	8.55	8.54	8.52	8.51	8.49	8.48	8.46	8.45	8.43	8.42
51	8.40	8.39	8.38	8.36	8.35	8.33	8.32	8.30	8.29	8.27
52	8.26	8.24	8.23	8.22	8.20	8.19	8.17	8.16	8.14	8.13
53	8.11	8.10	8.08	8.07	8.05	8.04	8.03	8.01	8.00	7.98
54	7.97	7.95	7.94	7.92	7.91	7.89	7.88	7.87	7.85	7.84
55	7.82	7.81	7.79	7.78	7.76	7.75	7.73	7.72	7.71	7.69
56	7.68	7.66	7.65	7.63	7.62	7.60	7.59	7.57	7.56	7.55
57	7.53	7.52	7.50	7.49	7.47	7.46	7.44	7.43	7.41	7.40
58	7.38	7.37	7.36	7.34	7.33	7.31	7.30	7.28	7.27	7.25
59	7.24	7.22	7.21	7.20	7.18	7.17	7.15	7.14	7.12	7.11
60	7.09	7.08	7.06	7.05	7.04	7.02	7.01	6.99	6.98	6.96
61	6.95	6.93	6.92	6.90	6.89	6.88	6.86	6.85	6.83	6.82
62	6.80	6.79	6.77	6.76	6.74	6.73	6.71	6.70	6.69	6.67
63	6.66	6.64	6.63	6.61	6.60	6.58	6.57	6.55	6.54	6.53
64	6.51	6.50	6.48	6.47	6.45	6.44	6.42	6.41	6.39	6.38
65	6.37	6.35	6.34	6.32	6.31	6.29	6.28	6.26	6.25	6.23
66	6.22	6.20	6.19	6.18	6.16	6.15	6.13	6.12	6.10	6.09
67	6.07	6.06	6.04	6.03	6.02	6.00	5.99	5.97	5.96	5.94
68	5.93	5.91	5.90	5.88	5.87	5.86	5.84	5.83	5.81	5.80
69	5.78	5.77	5.75	5.74	5.72	5.71	5.70	5.68	5.67	5.65
70	5.64	5.62	5.61	5.59	5.58	5.56	5.55	5.53	5.52	5.51
71	5.49	5.48	5.46	5.45	5.43	5.42	5.40	5.39	5.37	5.36
72	5.35	5.33	5.32	5.30	5.29	5.27	5.26	5.24	5.23	5.21
73	5.20	5.19	5.17	5.16	5.14	5.13	5.11	5.10	5.08	5.07
74	5.05	5.04	5.03	5.01	5.00	4.98	4.97	4.95	4.94	4.92
75	4.91	4.89	4.88	4.86	4.85	4.84	4.82	4.81	4.79	4.78
76	4.76	4.75	4.73	4.72	4.70	4.69	4.68	4.66	4.65	4.63

77	4.62	4.60	4.59	4.57	4.56	4.54	4.53	4.52	4.50	4.49
78	4.47	4.46	4.44	4.43	4.41	4.40	4.38	4.37	4.35	4.34
79	4.33	4.31	4.30	4.28	4.27	4.25	4.24	4.22	4.21	4.19
80	4.18	4.17	4.15	4.14	4.12	4.11	4.09	4.08	4.06	4.05
81	4.03	4.02	4.01	3.99	3.98	3.96	3.95	3.93	3.92	3.90
82	3.89	3.87	3.86	3.85	3.83	3.82	3.80	3.79	3.77	3.76
83	3.74	3.73	3.71	3.70	3.68	3.67	3.66	3.64	3.63	3.61
84	3.60	3.58	3.57	3.55	3.54	3.52	3.51	3.50	3.48	3.47
85	3.45	3.44	3.42	3.41	3.39	3.38	3.36	3.35	3.34	3.32
86	3.31	3.29	3.28	3.26	3.25	3.23	3.22	3.20	3.19	3.17
87	3.16	3.15	3.13	3.12	3.10	3.09	3.07	3.06	3.04	3.03
88	3.01	3.00	2.99	2.97	2.96	2.94	2.93	2.91	2.90	2.88
89	2.87	2.85	2.84	2.83	2.81	2.80	2.78	2.77	2.75	2.74
90	2.72	2.71	2.69	2.68	2.67	2.65	2.64	2.62	2.61	2.59
91	2.58	2.56	2.55	2.53	2.52	2.50	2.49	2.48	2.46	2.45
92	2.43	2.42	2.40	2.39	2.37	2.36	2.34	2.33	2.32	2.30
93	2.29	2.27	2.26	2.24	2.23	2.21	2.20	2.18	2.17	2.16
94	2.14	2.13	2.11	2.10	2.08	2.07	2.05	2.04	2.02	2.01
95	2.00	1.98	1.97	1.95	1.94	1.92	1.91	1.89	1.88	1.86
96	1.85	1.83	1.82	1.81	1.79	1.78	1.76	1.75	1.73	1.72
97	1.70	1.69	1.67	1.66	1.65	1.63	1.62	1.60	1.59	1.57
98	1.56	1.54	1.53	1.51	1.50	1.49	1.47	1.46	1.44	1.43
99	1.41	1.40	1.38	1.37	1.35	1.34	1.32	1.31	1.30	1.28

3.0 EXPERIMENTAL PROCEDURE

3.1 INTRODUCTION

Having fermented all the yeast cultures to dryness (less than 2% residual sugar) in the nutrient bulking media to ensure their viability, the following experimental fermentations were carried out.

3.2 FERMENTATION AT 25°C

Once cell numbers of all yeast cultures had reached a minimum of 3.0×10^6 in the nutrient bulking media, 90mls. was added to 1 litre of grape juice and shaken at 120rpm on a Gallenkamp Orbital Shaker #3746 at 25°C. Samples were taken at least daily and frozen for later analysis. Sugar levels were monitored throughout the fermentation process and dryness was reached in all cases within 14 days.

Final alcohol levels were recorded to ascertain the effectiveness of aeration at this temperature and speed of shaking.

3.3 CONTINUOUS STIRRED FERMENTATION OF *SACCHAROMYCES CEREVISIAE* AT 25°C

Saccharomyces cerevisiae with cell numbers of 3.2×10^6 in 90mls. of nutrient bulking media was added to an initial volume of 3 litres of grape juice in a Biotec LP100 fermenter (constantly stirring).

The fermenter was stirred at 300rpm and juice run through at approximately $0.60h^{-1}$. Exact precision in timing of the juice run through was not possible as some variation between the time setting and actual dosage was detected.

Samples were taken from juice exiting the system.

Sugar and alcohol levels were recorded throughout the process to ascertain the effect of aeration of this yeast through continuous rather than batch culture. Yeast cell counts were also taken from most samples to indicate the growth phases of this yeast during fermentation.

Fermentation was continued for 10 days.

3.4 FERMENTATION AT 30°C

Using all yeast cultures and with cell numbers between 4.0 x 10^6 and 9.6 x 10^7 in bulking media,, further 1 litre fermentations were carried out in 3 litre flasks on the orbital shaker. This time shaking was at 150rpm at a temperature of 30°C. A further change to the fermentation described in 3.2 was that cotton wool bungs were used in the flasks instead of a cover of aluminium foil.

Sugar and alcohol levels were monitored throughout the fermentation, and cell counts taken from all samples.

Fermentation was ended after 9 days.

Fermentation at 30°C was repeated in duplicate with *P. stipitis, C. tropicalis* and *S. cerevisiae* to test reproducibility. During the further 9 day fermentation, pH and TA were also monitored.

3.5 COMBINED AEROBIC/ANAEROBIC FERMENTATIONS USING *SACCHAROMYCES, PICHIA/SACCHAROMYCES* AND *CANDIDA/SACCHAROMYCES*

Starting with *Pichia stipitis, Candida tropicalis* and *Saccharomyces cerevisiae* cultures with cell numbers of between 4.0 x 10^7 and 6.0 x 10^7 in bulking media; 1 litre volumes of juice were combined with 90ml. bulking media + yeast in 3 litre flasks. They were stirred at a speed of 150rpm at 30°C for 18 hours.

The juices were then centrifuged for 15 minutes at 6370*g* to facilitate yeast biomass extraction.

The juices were added to 1 litre flasks and inoculated with the *Saccharomyces cerevisiae* active dried wine yeast marketed as "Fermivin" SF by Gist-brocades (France). The recommended dosage rate of 0.765g/1 litre supplied by the manufacturer was followed.

Fermentation was allowed to proceed anaerobically until the fermentation appeared to be complete according to Rebellein analysis.

Sugar, alcohol, pH and TA were monitored at 18 hours, prior to the start of the anaerobic fermentation, two weeks into the fermentation, and at the completion of fermentation.

Basic sensory analysis of the aroma and flavour of the final product was undertaken by sniff and taste testing. The opinions of a number of experienced wine tasters were pooled to give the results.

For comparison purposes, a purely anaerobic fermentation of 1 litre of grape juice was carried out using the active dried wine yeast at the recommended dosage rate of 0.765g/l. Sugar, alcohol, pH and TA were monitored at 18 hours and two weeks into the fermentation, and at the completion of the fermentation.

Sensory analysis of this anaerobic fermentation assisted in assessing the final product of the mixed aerobic/anaerobic fermentations.

4.0 RESULTS

4.1 FERMENTATION AT 25°C

While all of the yeast cultures showed some reduction from the expected normal alcohol percentage for a wine fermentation (11.65%), only *S. cerevisiae, P. stipitis* and *C. tropicalis* showed any promising trend. (Table 4.1.1).

Sensory analysis carried out on the final products of this fermentation showed all to have highly volatile, earthy aromas and the meaty, caramel, bitter tastes indicative of oxidation.

4.1.1 - ALCOHOL DETERMINATION

YEAST	TITRE (av.)	ALCOHOL % (v/v) (av.)
S. cerevisiae	61.80	6.83
C. shehatae	55.50	7.75
T. cutuneum	56.5	7.56
S. pombe	52.10	8.24
C. tropicalis	62.90	6.67
S. ludwigii	53.60	8.03
P. stipitis	63.40	6.60

4.2 CONTINUOUS STIRRED FERMENTATION OF *S.CEREVISIAE* AT 25°C

A steady state of constant cell mass, sugar concentration and alcohol concentration was reached for only a short period of time between samples 7 and 11 during this fermentation. The higher than desirable dilution rate ($0.6h^{-1}$ as opposed to $0.3h^{-1}$ recommended by Fiechter et al. 1987), led to the production of 4 - 5% (v/v) alcohol. (Table 4.2.1). A further reduction in alcohol percentage for *S. cerevisiae* from that obtained in batch culture was however obtained. Given an ability to restrict the dilution rate more, a better result would hopefully have been obtained.

4.2.1 - Sugar, alcohol and cell count determinations

SAMPLE	DATE	HOUR	YEAST COUNT	TOTAL SUGAR	TOTAL ALCOHOL
1	7/10	0	$3.20x10^6$	182g/l	0% (v/v)
2	8/10	23.5	ND	110	2.46
3	9/10	47.5	ND	71.82	4.08
4	10/10	69.0	ND	65.62	5.33
5	10/10	73.0	$1.04x10^8$	66.56	5.46
6	11/10	92.0	$2.08x10^8$	67.49	5.62
7	11/10	98.0	$2.12x10^8$	57.34	5.38
8	12/10	120.0	$8.0 x10^8$	42.59	5.06
9	13/10	140.0	$8.8 x10^8$	27.83	4.74
10	13/10	147.0	$8.0 x10^8$	36.47	4.47
11	14/10	164.0	$3.2 x10^8$	43.62	4.19
12	14/10	169.0	$1.48x10^8$	39.48	4.16
13	15/10	194.5	$1.68x10^8$	64.30	4.87
14	17/10	236.0	$1.48x10^8$	65.80	6.07

ND - no determination

4.3 FERMENTATION AT 30°C

Table 4.3 Final sugar and alcohol determinations from all yeast cultures were as follows:

YEAST	SUGAR	ALCOHOL
	g/l (av.)	**% (v/v)(av.)**
S. cerevisiae	0	7.85
C. shehatae	0	8.94
T. cutuneum	0	8.96
S. pombe	0.75	7.88
P. stipitis	52.26	1.28
C. tropicalis	3.01	5.23
S. ludwigii	1.88	7.79

A summary of the full results from *P. stipitis, C. tropicalis* and *S. cerevisiae* cultures appear in Tables 4.3.1.1, 4.3.1.2 qnd 4.3.1.3. Graphs of sugar reduction and alcohol production during fermentation appear in Appendix IV. Given the promising results from these three cultures, in terms of alcohol reduction, fermentation at 30°C was repeated with them, in duplicate, to test reproducibility.

A summary of the results of this further 30°C fermentation are given in Tables 4.3.3.1, 4.3.3.2 and 4.3.3.3.

Graphs of sugar reduction and alcohol production, and pH and TA appear in Appendix V.

4.3.1.1 - *PICHIA STIPITIS*

SAMPLE	HOUR	SUGAR g/l	ALCOHOL %(v/v)	CELL COUNT
	0	182.0	0	
1	19	150.4	<0.128	4.0×10^7
2	25	155.88	<0.128	2.4×10^7
3	49	127.08	0.521	2.56×10^8
4	73	124.84	0.727	4.8×10^8
5	91	120.32	0.674	6.8×10^8
6	97	108.28	0.575	6.4×10^8
7	118	105.28	0.473	6.4×10^8
8	143	112.80	0.637	5.6×10^8
9	163	106.04	0.596	6.0×10^8
10	169	96.26	0.929	7.2×10^8
11	187	72.57	0.819	1.2×10^9
12	193	53.40	1.28	4.0×10^8

4.3.1.2 - *SACCHAROMYCES CEREVISIAE*

SAMPLE	HOUR	SUGAR g/l	ALCOHOL % (v/v)	CELL COUNT
	0	182	0	
1	19	109.04	2.80	3.6×10^7
2	25	72.57	3.64	5.2×10^7
3	49	34.22	5.32	9.2×10^7
4	73	10.90	6.04	8.4×10^7
5	91	6.39	7.36	1.04×10^8
6	97	6.02	7.24	1.04×10^8
7	118	6.39	6.86	1.68×10^8
8	143	6.02	7.60	2.76×10^8
9	163	5.64	7.47	3.4×10^8
10	169	6.02	7.46	2.56×10^8
11	187	5.26	7.75	2.16×10^8
12	193	0	7.85	2.2×10^8

4.3.1.3 - *CANDIDA TROPICALIS*

SAMPLE	HOUR	SUGAR g/l	ALCOHOL % (v/v)	CELL COUNT
	0	182	0	
1	19	139.88	1.055	4.4×10^7
2	25	108.28	1.092	6.0×10^7
3	49	94.76	1.79	6.4×10^7
4	73	79.72	2.64	1.32×10^8
5	91	58.66	3.01	1.48×10^8
6	97	47.38	3.17	1.40×10^8
7	118	21.06	4.15	2.04×10^8
9	163	6.02	4.30	1.80×10^8
10	169	5.26	4.28	1.52×10^8
11	187	4.89	4.70	1.72×10^8
12	193	3.01	5.23	1.68×10^8

4.3.3.1 - *PICHIA STIPITIS* (A)

HOUR	pH	TA	SUGAR g/l	ALC. % (v/v)	CELL COUNT
0	3.22	6.889	182	0	8.0×10^7
19	3.329	4.015	142.88	0.364	5.2×10^7
24.5	3.271	4.089	109.80	0.492	6.8×10^7
48.5	3.128	4.441	107.54	1.095	1.8×10^8
72.5	3.217	3.854	116.56	0.868	2.24×10^8
96.5	3.245	3.259	97.76	0.880	ND
118	3.217	3.475	94.0	0.871	1.4×10^8
139	3.230	3.367	89.48	0.977	ND
144.5	3.265	2.614	78.20	0.919	2.6×10^8
168.5	3.297	3.820	69.94	1.023	ND
187	3.295	3.690	75.95	0.805	1.52×10^8

ND - no determination

4.3.3.1 - *PICHIA STIPITIS* (B)

HOUR	pH	TA	SUGAR g/l	ALC. % (v/v)	CELL COUNT
0	3.22	6.889	182	0	8.0×10^7
19	3.182	4.740	142.28	0.449	8.8×10^7
24.5	3.234	4.503	127.08	0.564	1.04×10^8
48.5	3.162	4.108	129.34	0.639	2.16×10^8
72.5	3.181	3.845	115.06	0.537	2.48×10^8
96.5	3.208	3.489	110.54	0.561	ND
118.5	3.216	3.520	117.32	0.508	1.92×10^8
139	3.188	3.811	120.32	0.580	ND
144.5	3.199	3.408	109.04	0.626	2.32×10^8
168.5	3.216	3.520	74.44	0.701	ND
187	3.232	3.690	75.95	0.800	1.88×10^8

ND - no determination

4.3.3.2 - *CANDIDA TROPICALIS* (A)

HOUR	pH	TA	SUGAR g/l	ALC. % (v/v)	CELL COUNT
0	3.22	6.889	182	0	6.8×10^7
19	3.331	3.474	90.68	1.071	1.24×10^8
24.5	3.329	3.526	74.44	1.103	7.9×10^7
48.5	3.241	3.658	71.44	1.425	8.8×10^7
72.5	3.233	3.885	38.36	1.461	8.4×10^7
96.5	3.206	3.480	39.48	1.464	ND
118.5	3.237	3.949	38.36	1.490	1.28×10^8
139	3.308	3.996	45.12	1.508	ND
144.5	3.207	3.666	36.84	1.511	1.44×10^8
168.5	3.246	3.660	10.53	1.63	ND
187	3.238	3.796	13.91	1.86	1.52×10^8

ND - no determination

4.3.3.2 - *CANDIDA TROPICALIS* (B)

HOUR	pH	TA	SUGAR g/l	ALC. % (v/v)	CELL COUNT
0	3.22	6.889	182	0	6.8×10^7
19	3.341	4.045	103.78	1.159	8.4×10^7
24.5	3.281	4.186	97.76	1.285	8.8×10^7
48.5	3.206	4.093	78.96	1.483	1.04×10^8
72.5	3.116	4.597	60.92	1.462	8.4×10^7
96.5	3.135	4.339	52.64	1.444	ND
118.5	3.154	4.842	40.60	1.509	1.36×10^8
139	3.155	4.422	40.60	1.518	ND
144.5	3.141	4.344	29.32	1.520	1.6×10^8
168.5	3.184	4.210	9.02	1.31	ND
187	3.223	4.266	9.78	1.28	1.68×10^8

ND - no determination

4.3.3.3 - *SACCHAROMYCES CEREVISIAE* (A)

HOUR	pH	TA	SUGAR g/l	ALC. % (v/v)	CELL COUNT
0	3.22	6.889	182	0	5.6×10^7
19	2.946	5.974	68.44	1.387	1.08×10^8
24.5	3.189	4.723	51.89	3.52	1.04×10^8
48.5	3.049	5.860	9.02	4.57	9.6×10^7
72.5	3.080	7.152	5.64	3.96	7.6×10^7
96.5	3.090	8.173	6.02	3.76	ND
118.5	3.097	8.913	5.64	3.83	1.0×10^8
139	3.109	8.868	6.39	3.93	ND
144.5	3.142	8.755	5.64	4.06	1.24×10^8
168.5	3.108	10.270	3.76	4.19	ND
187	3.115	10.011	2.26	4.19	8.4×10^7

ND - no determination

4.3.3.3 - *SACCHAROMYCES CEREVISIAE* (B)

HOUR	pH	TA	SUGAR g/l	ALC. % (v/v)	CELL COUNT
0	3.22	6.889	182	0	5.6×10^7
19	3.132	3.865	63.16	1.435	5.2×10^7
24.5	3.380	3.849	33.46	2.87	6.4×10^7
48.5	3.057	5.647	9.02	4.59	7.2×10^7
72.5	3.068	6.861	4.89	4.52	5.2×10^7
96.5	3.087	8.152	4.89	5.17	ND
118.5	3.111	9.625	4.14	5.37	9.2×10^7
139	3.071	10.123	4.51	5.21	ND
144.5	3.095	9.964	4.89	5.51	1.0×10^8
168.5	3.131	8.914	1.88	5.56	ND
187	3.131	8.673	2.26	5.59	8.4×10^7

ND - no determination

The higher temperature of fermentation and/or better aeration of the yeast cultures during the first 30°C fermentation resulted in lower alcohol production for *Pichia stipitis* and *Candida tropicalis* but not for *Saccharomyces cerevisiae*. Regardless of the temperature and amount of aeration however, *P. stipitis* was the slowest fermenter, failing to complete the conversion of sugar during any of the aerobic fermentations.

Yeast cell count numbers indicate that the cultures took little time to adjust to the grape juice environment, having first been propagated in an artificial medium with the same sugar and pH. This propagation amounted to the first phase of yeast growth referred to in 1.4.3.1. The stationary and beginning of the decline phases can be seen in the cell counts. (Tables 4.3.1.1, 4.3.1.2, and 4.3.1.3).

Lower alcohol concentrations were obtained for all three yeast cultures from the second 30°C fermentation than for the first, underlining the variable nature of the batch culture. For each yeast culture, second A and B fermentations were almost identical, indicating that the external environment (such as temperature fluctuations) of the fermentation is important in obtaining reproducible results.

Oxidative reactions and microbiological growth are linked to pH. Juice from healthy white grapes with a pH of 3.0 to 3.3 will be resistant to oxidation, encourage the growth of desirable micro-organisms, inhibit microbial spoilage and accentuate the colour, fruitiness and balance of the wine generally. (Rankine 1993). The pH range for all second 30°C samples was between 3.049 and 3.431 with the end pH for each fermentation within the range 3.115 to 3.238, agreeing with plate analysis that no bacterial contamination had occurred. (Appendix V: 4.3.5.1, 4.3.5.2 and 4.3.5.3).

Usual values for total acidity in wine are 4 to 8g/l. (Rankine 1993). With an initial acidity of 6.889g/l in all cases, *P. stipitis* fell to 3.690 in both fermentations and *C. tropicalis* to 3.796 (A) and 4.266 (B). *S. cerevisiae* showed an increase in TA to 10.011 (A) and 8.673 (B). As no bacterial contamination had occurred this increase may have been due to the production of acetic acid during fermentation.

Kolb et al. (1987) found a reduction in the acidity of orange juice fermented using *P. stipitis* which occurred only

after the decomposition of the glucose. The grape juice results do not agree with this proposition. The major reduction in acidity during *P. stipitis* fermentation occurred within the first 18 hours when sugar levels were high and therefore glucose was still present. (Table 4.3.3.1 and Appendix V, 4.3.4.1).

The results from *P.stipitis* and *C. tropicalis* indicate that no or little acid is produced by these primarily aerobic yeasts.

Some yeasts however, do produce acetic acid during fermentation, and the quantity of acetic acid increases as the temperature increases. (Castelli 1941). Acetic acid formation by strains of *S. cerevisiae* is affected by sugar concentration and pH, with some strains being able to produce in excess of 1g/l. (Monk & Cowley 1984). The origin of the *S. cerevisiae* used in this series of experiments was unknown, and could have been one capable of acetic acid formation. *S. cerevisiae* as a species would appear not to produce appreciable amounts of any other acid during alcoholic fermentation. (Fleet 1993). This acid formation occurs early during fermentation and its production stops as soon as sugar is fermented. (Radler 1993).

The fermentation results indicate that acetic acid may have been produced by *S. cerevisiae*, and that when fermentation was terminated, production had stopped causing the TA readings to decline. (Table 4.3.3.3 and Appendix V, 4.3.5.3).

Under anaerobic fermentation the changes to TA (lower for *P. stipitis* and *C. tropicalis* and higher for *S. cerevisiae)* would likely be reflected in the sourness of the finished wine. In these aerobic experiments however, the end products of all 30°C fermentations were dark gold in colour and bitter to taste.

4.4 COMBINED AEROBIC/ANAEROBIC FERMENTATION USING *SACCHAROMYCES, PICHIA/SACCHAROMYCES* AND *CANDIDA/SACCHAROMYCES*

Juice analysis at the start of this series of experiments gave identical figures to those for the second 30°C fermentations, namely:-

Sugar	182g/l
Alcohol	0% (v/v)
pH	3.22
TA	6.889.

Changes to these components at day 1 after 18 hours of aerobic fermentation and before the start of the anaerobic fermentation, and at days 14, 21 and 28 of the anaerobic fermentation are given in Tables 4.4.1, 4.4.2, 4.4.3 and 4.4.4. The results at the same points in time for the purely anaerobic fermentation using *S. cerevisiae* active dried wine yeast are also shown in the tables.

4.4.1 - Day 1, individual sugar, alcohol, pH and TA determinations

YEAST	TIME (Days)	pH	TA (g/l)	SUGAR (g/l)	ALCOHOL (%v/v)
P. stipitis (1)	1	3.157	7.866	144.38	0.370
P. stipitis (2)	1	3.141	7.189	143.64	0.373
C. tropicalis (1)	1	2.998	8.523	131.60	0.414
C. tropicalis (2)	1	3.022	8.416	134.60	0.390
S. cerevisiae	1	2.980	8.205	136.12	2.88
*S. cerevisiae**	1	3.293	7.761	133.86	0.725

*Control - totally anaerobic

4.4.2 - Day 14, individual sugar, alcohol, pH and TA determinations

YEAST	TIME (Days)	pH	TA	SUGAR (g/l)	ALCOHOL (%v/v)
P. stipitis (1)	14	3.054	6.168	30.08	8.35
P. stipitis (2)	14	3.045	7.009	43.24	6.95
C. tropicalis (1)	14	2.967	7.192	78.21	4.38
C. tropicalis (2)	14	2.979	7.170	76.33	4.78
S. cerevisiae	14	2.931	7.092	74.07	6.28
*S. cerevisiae**	14	3.086	6.672	14.29	9.11

*Control totally anaerobic

4.4.3 - Day 21, individual sugar, alcohol, pH and TA determinations

YEAST	TIME (Days)	pH	TA	SUGAR (g/l)	ALCOHOL (%v/v)
P. stipitis (1)	21	2.976	7.951	1.88	8.23
P. stipitis (2)	21	2.966	7.306	3.76	8.11
C. tropicalis (1)	21	2.931	8.056	61.29	5.55
C. tropicalis (2)	21	2.957	9.243	64.30	6.07
S. cerevisiae	21	2.905	7.729	37.98	6.63
*S. cerevisiae**	21	3.184	7.638	0.0	10.85

*Control totally anaerobic

4.4.4 - Day 28, remaining sugar, alcohol, pH and TA determinations

YEAST	TIME (Days)	pH	TA	SUGAR (g/l)	ALCOHOL (%v/v)
P. stipitis (1)	28	-	-	-	-
P. stipitis (2)	28	-	-	-	-
C. tropicalis (1)	28	2.976	7.152	2.63	7.71
C. tropicalis (2)	28	2.905	8.914	1.88	7.76
S. cerevisiae	28	3.095	6.861	1.13	7.85
*S. cerevisiae**	28	-	-	-	-

*Control totally anaerobic

4.4.1 - Day 1

Over the complete series of aerobic fermentations at 30°C, *P. stipitis* showed the most consistent, although slowest, short-term sugar reduction. At 18 to 19 hours, the range was 150.40g/l to 142.28g/l sugar remaining. The ranges for *S. cerevisiae* and *C. tropicalis* respectively were 136.12g/l to 63.16g/l, and 139.88g/l to 90.68g/l. This slow sugar reduction by *P. stipitis* is probably due to its inability to decompose all of the grape sugars. It is able to ferment glucose predominantly. (Kolb et al. 1993).

Alcohol accumulation after 18 hours of aerobic fermentation was consistent with earlier 30°C fermentations for *P. stipitis* and *S. cerevisiae*. The lower accumulation results for *C. tropicalis* are probably indicative of better aeration of the flasks.

A reduction in pH and increase in TA was recorded for all aerobic fermentations after 18 hours.

At the start of the anaerobic fermentation therefore, juice sugar levels had been reduced by 25 to 35%, with *P. stipitis* and *C. tropicalis* showing alcohol accumulation of less than 1%.

It is interesting to note that after 18 hours of anaerobic fermentation the *S. cerevisiae* active dried yeast had greater sugar reduction and lower alcohol accumulation than the aerobic fermentation of *S. cerevisiae* obtained from plate culture.

4.4.2 - Day 14

Considerable variation in the speed of sugar reduction can be seen between the fermentations. The control (totally anaerobic fermentation) was almost complete at 14.29g/l, while those begun with *C. tropicalis* and *S. cerevisiae* still retained 74.07 to 78.21g/l sugar. (Table 4.4.2).

4.4.3 - Day 21

The fermentations begun with *P. stipitis* were complete, yielding 8.23% and 8.11% alcohol (v/v) respectively. The control fermentation produced a final alcohol reading of 10.85% (v/v). Final acidity and pH readings for these three fermentations were within normal wine ranges, and no microbial contamination was observed.

The fermentations begun with *C. tropicalis* did not produce the expected sugar reduction. Contamination of the anaerobic fermentation with *C. tropicalis* yeast occurred despite centrifugation of the juice between aerobic and anaerobic stages. This contamination was observed during combi analysis of the Day 21 samples; the oxidising agent having turned brick red/brown during boiling instead of remaining blue/green as typical when analysing a wine sample fermented by *S. cerevisiae*. Cell analysis confirmed almost equal numbers of *C. tropicalis* and *S. cerevisiae* present in the juice at this stage.

S. cerevisiae continued a steady sugar reduction, although at a slower rate than the control fermentation. (Table 4.4.3).

4.4.4 - Day 28

All fermentations were complete. Those begun with *C. tropicalis* producing final alcohol readings of 7.71% (v/v) and 7.76% (v/v) respectively; and *S. cerevisiae*, 7.85% (v/v). Final pH readings for these three fermentations were acceptable, however *C. tropicalis* (2) produced a higher than expected acidity reading. (Appendix VIII, 4.4.4).

Cell analysis of the *C. tropicalis* fermentations showed almost total domination of the end product by *S. cerevisiae*.

The early aerobic exposure of all yeasts, coupled with anaerobic fermentation, reduced alcohol levels by 25 to 30% when compared to the control.

4.4.5 - Sensory Analysis

Sensory evaluation of the wines produced from the combined aerobic/anaerobic fermentation was carried out.

To the eye there was an immediate difference between the control and partially aerated wines. The control being a light straw colour, while all of the other fermentations were the deep gold indicative of oxidation. All wines appeared clear, however, a small amount of crystalline sedimentation was observed in *P. stipitis* and *C. tropicalis* wines after three weeks in the bottle.

All wines, with the exception of *S. cerevisiae*, had an acceptable bouquet. The control and *P. stipitis* wines displayed tropical aromas, while *C. tropicalis* had an apple - like aroma. None of these aromas were intense.

S. cerevisiae smelled like wet socks, indicating high sulphur levels, which was surprising considering no sulphur dioxide had been added at any time during fermentation. The aroma of this wine was objectionable.

The four basic primary taste sensations are sweet, acid, salty and bitter. The control wine showed a combination of sweet and acid; *P. stipitis* (1) and (2) exhibited acid and slightly bitter tastes; *C. tropicalis* (1) and (2) were sweet, and *S. cerevisiae* was bitter.

The tropical and apple aromas of the control, *P. stipitis* and *C. tropicalis* carried through to the taste of these wines. *S. cerevisiae* exhibited a rotten fruit taste.

The general opinion of six experienced wine tasters was that these wines tasted more like "wine" than partially fermented or dealcoholised products.

CONCLUSIONS

Controlled Aeration

Under controlled aeration, yeast strains use sugars at different speeds. Some, like *P. stipitis*, are slow fermenters. Others, like *C. tropicalis* and *S. cerevisiae*, show considerable variation in the speed of decomposition of grape sugars.

While similar amounts of sugar may be decomposed, the amount of alcohol produced during the first 18 to 19 hours of aerobic fermentation is yeast strain dependent. The primarily aerobic yeasts, *P. stipitis* and *C. tropicalis*, generally produce less than 1% alcohol, whereas *S. cerevisiae*, a facultative anaerobe, can produce up to 3%.

The amount of alcohol produced during aerobic fermentation by the yeasts is also temperature and/or oxygen dependent. *P. stipitis* and *C. tropicalis* produce less alcohol when well aerated at 30°C than when less well aerated at 25°C. Alcohol production by *S. cerevisiae* is unaffected by this temperature variation or by the amount of aeration.

The external environment of the fermentation is important in obtaining reproducible results. More similarity in alcohol production figures were obtained from two batches of simultaneously fermented wines than from another earlier batch fermented at the same shaker speed and temperature.

Controlled aeration did not adversely affect the grape juice pH, indicating that juice from healthy grapes remains resistant to bacterial contamination even in the presence of oxygen. The titratable acidity values however, indicated that aeration is likely to affect the acid taste of wine. The primarily aerobic yeasts, *P. stipitis* and *C. tropicalis*, produced wines with reduced acidity, while *S. cerevisiae* produced an acid, probably acetic acid, during the fermentation to end with higher acid readings.

Combined Fermentation

The percentage sugar reduction during aeration ultimately determined the final alcohol reduction. Sugar reduction of 25 to 35% during aeration was reflected in a 25 to 30% alcohol reduction after fermentation, regardless of the speed of sugar utilisation.

The wines produced by the combined aerobic/anaerobic fermentation of *P. stipitis* and *C. tropicalis* had an acceptable taste and aroma, and the final acidity of the wines was improved over those produced purely by aeration. None of the wines had an acceptable colour, however, and since all of these fermentations were undertaken without sulphur dioxide addition, there is no way of assessing whether the degree of oxidative browning that occurred could have been reduced by SO_2 dosing at the start of anaerobic fermentation.

Despite contamination of the fermentations begun with *C. tropicalis* with this yeast during the anaerobic fermentation, the wines produced were not adversely affected.

Reduced alcohol wines can therefore, be produced by combining short-term controlled aeration by selected yeast strains with anaerobic fermentation by wine yeasts.

If the colour problem could be overcome, a wine with a more acceptable taste to the "serious" drinker than a partially fermented or dealcoholised wine, yet with less alcohol than one fermented traditionally, could be produced.

The wines produced by the combined aeration/anaerobic fermentation were not "reduced alcohol" wines as defined by law (less than 6.5% alcohol). However, if a proceedure could be developed to ensure the maximum sugar reduction by yeasts like *C. tropicalis* and *S. cerevisiae* during short-term aeration, thus maximising the alcohol reduction, the further 1% reduction necessary to meet the legal criteria could be achieved using this method.

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APPENDIX I

2.1 Description of yeast species

Genus 22. *Saccharomyces* Meyen ex Reess

by D. Yarrow

Vegetative reproduction by multilateral budding. Cells globose, ellipsoidal or cylindrical. Pseudohyphae may be formed but not septate hyphae.

The vegetative phase is predominantly diploid (or of higher ploidy), conjugation occurring on or soon after germination of the ascospores, diploid ascospores may be formed.

Ascospores globose to short ellipsoidal, with a smooth wall, usually one to four, occasionally more, per ascus. Asci persistent.

Vigorous fermentation.

Starch-like compounds are not produced.

No growth with nitrate as sole source of nitrogen.

Standard description of *Saccharomyces cerevisiae*

Growth in malt extract: After 3 days at 25°C the cells may be globose or subglobose (5.0–10.0) × (5.0–12.0) µm, ellipsoidal to cylindrical (3.0–9.5) × (4.5–21.0) µm, elongated cells of more than 30 µm in length may be present, single, in pairs, short chains or clusters (Figs. 96, 97). A sediment and occasionally a ring are present. After one month at 20°C a sediment is present, sometimes also a ring and a film.

Growth on malt agar: After one month at 20°C the streak culture is butyrous,

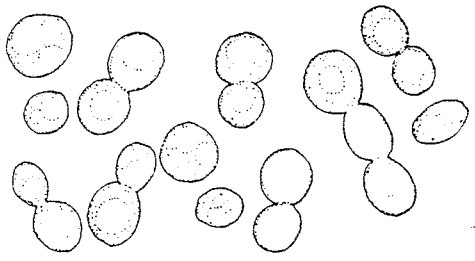


Fig. 96 – *Sacch. cerevisiae*, CBS 1171
After 3 days in malt extract
(From 'The Yeasts', ed. I, 1952)

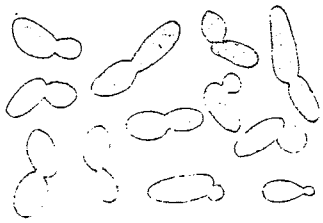


Fig. 97 – *Sacch. cerevisiae*, CBS 380
After 3 days in malt extract
(From 'The Yeasts', ed. I, 1952)

cream to slightly brownish, slightly raised and smooth with light striations or raised and folded, often sectored, glossy or dull.

Slide cultures on corn meal- and morphology agar: Pseudohyphae may be formed, they may be rudimentary or well branched.

Formation of ascospores: Vegetative cells are transformed direct into asci containing one to four, occasionally more, globose to short ellipsoidal ascospores (Fig. 98).

Acetate agar was the best medium for inducing sporulation.

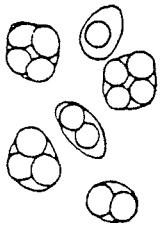


Fig. 98 – *Sacch. cerevisiae*
After 1 week on Gorodkowa agar
(From 'The Yeasts', ed. I, 1952)

Fermentation:

Glucose	+	Maltose	v
Galactose	v	Lactose	–
Sucrose	v		

Assimilation of carbon compounds:

Galactose	v	Raffinose	v	Erythritol	–
Sucrose	v	Soluble starch	v	Ribitol	–
Maltose	v	D-Xylose	–	D-Mannitol	v
Cellobiose	–	L-Arabinose	–	Succinic acid	– or + s
Trehalose	v	D-Ribose	–	Citric acid	–
Lactose	–	L-Rhamnose	–	Inositol	–

Assimilation of nitrate: –

Assimilation of ethylamine·HCl: –

Assimilation of cadaverine·2 HCl: –

Growth in vitamin-free medium: v

Growth on 50% (w/w) glucose-yeast extract agar: v

Growth at 37°C: v

Genus 21. *Pichia* Hansen

by C.P. Kurtzman

a. DIAGNOSIS OF THE GENUS

Asexual reproduction is by multilateral budding on a narrow base. Some species may also form arthrospores. Cells are spheroidal, ellipsoidal, or elongate and occasionally may be tapered, but they are not ogival in shape. Pseudohyphae or, to a limited extent, true hyphae may be present.

Asci produce one to four (infrequently more) ascospores that may be hat-shaped, hemispheroidal, Saturn-shaped, or spheroidal with a smooth surface. Generally asci are dehiscent, but occasionally they are persistent. Asci are unconjugated, or if conjugated, they may show conjugation between bud and parent or between independent cells. Hyphal or pseudohyphal cells may serve as asci, but they do not become swollen or spindle-like. Asci are not borne on ascophores. Species are homothallic or heterothallic.

Sugars may or may not be fermented.

Nitrate is not assimilated.

Standard description of *Pichia stipitis*

Growth on 5% malt extract agar: After 3 days at 25°C, the cells are spheroidal to ovoidal (2.2–4.5) × (2.5–6.0) μm, and occur singly or in pairs. Growth is cream-colored, dull-glistening, smooth to faintly wrinkled and butyrous.

Growth on the surface of assimilation media: Rings and incomplete pellicles may form.

Dalmau plate culture on morphology agar: After 7 days at 25°C, growth under the coverglass shows abundant and well branched pseudohyphae with occasional blastospores. True hyphae are not formed. Aerobic growth is light tan, smooth to finely wrinkled, dull-glistening, butyrous, and with an entire to lobed margin.

Formation of ascospores: Preceding ascus formation there may be conjugation between a parent cell and a bud or between independent cells or the ascus may be unconjugated but with a protuberance. Asci produce two hat-shaped spores and these are released soon after formation.

Spores were observed on 5% malt extract agar.

Fermentation:

Glucose	+s	Lactose	–
Galactose	+s	Raffinose	–
Sucrose	–	Trehalose	v
Maltose	+s		

Assimilation of carbon compounds:

Galactose	+	Raffinose	–	Erythritol	+
Sucrose	+	Soluble starch	+	Ribitol	+
Maltose	+	D-Xylose	+	D-Mannitol	+
Cellobiose	+	L-Arabinose	+	Succinic acid	+
Trehalose	+	D-Ribose	+	Citric acid	+
Lactose	+	L-Rhamnose	+	Inositol	–

Additional carbon compounds tested: L-Sorbose – or +s, melibiose –, melezitose +, inulin –, D-arabinose +s, D-glucosamine · HCl +, glucitol +, α-methyl-D-glucoside +, salicin +, potassium D-gluconate v, DL-lactic acid +

Assimilation of nitrate: –

Growth in vitamin-free medium: – or +w

Growth in 10% sodium chloride plus 5% glucose in yeast nitrogen base: –

Growth at 37°C: +

Genus 25. *Schizosaccharomyces* Lindner

by D. Yarrow

a. DIAGNOSIS OF THE GENUS (SLOOFF, 1970b)

Cells globose to cylindrical, reproducing by fission: true septate hyphae may develop and break up into arthrospores.

Asci are produced usually by somatic conjugation of vegetative cells. Ascospores may be liberated at an early stage. Spores in the asci are globose or short ellipsoidal, seldom cylindrical. They develop by swelling; during maturation the shape may change to reniform (*Schizosaccharomyces japonicus* var. *versatilis*). The ascospores may infrequently appear to be slightly roughened when observed under the light microscope.

Fermentative ability present.

Nitrate not assimilated.

Positive starch test in Wickerham's liquid medium.

Standard description of *Schizosaccharomyces pombe*

Growth in malt extract: After 3 days at 25°C the cells are globose, ellipsoidal or cylindrical (3.0–5.0) × (5.0–15.0–24.0) µm, single, in pairs or small groups (Fig. 117). A sediment is present. After one month at 20°C a sediment is present and sometimes a thin ring.

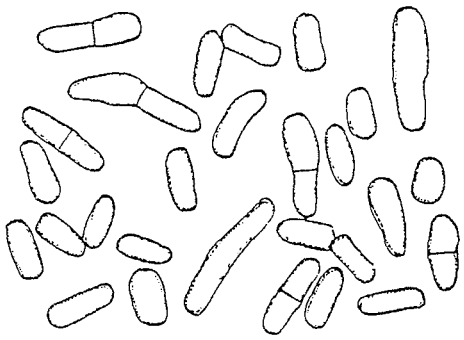


Fig. 117 – *Schiz. pombe*
After 3 days in malt extract
(From 'The Yeasts', ed. I, 1952)

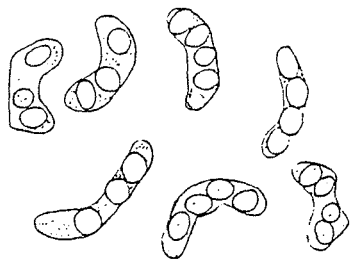


Fig. 118 – *Schiz. pombe*
On malt agar
(From 'The Yeasts', ed. I, 1952)

Growth on malt agar: After one month at 20°C the streak is brownish, dull or glistening, slightly raised, striated, the margin is entire or sinuous.

Slide cultures on potato- and corn meal agar: A few short chains of cells may be observed. Asci may be present.

Formation of ascospores: Conjugation of vegetative cells precedes the formation of evanescent asci containing two to four globose to ellipsoidal ascospores (Fig. 118). The released ascospores may cohere in small groups. Haploid heterothallic strains may be encountered.

Sporulation was observed on malt-, potato- and corn meal agar.

Fermentation:

Glucose	+	Lactose	
Galactose	–	Melibiose	–
Sucrose	+	Raffinose	+ 1/3
Maltose	+		

Assimilation of carbon compounds:

Galactose	–	Raffinose	+	Erythritol	–
Sucrose	+	Soluble starch	–	Ribitol	–
Maltose	+	D-Xylose	–	D-Mannitol	–
Cellobiose	–	L-Arabinose	–	Succinic acid	–
Trehalose	–	D-Ribose	–	Citric acid	–
Lactose	–	L-Rhamnose	–	Inositol	–

Assimilation of nitrate: –

Growth in vitamin-free medium: –

Growth on 50% (w/w) glucose-yeast extract agar: +

Growth at 37°C: +

G + C: 42 mol%, one strain (Rost & Venner, 1964)

Genus 4. *Candida* Berkhout

by Sally A. Meyer, D.G. Ahearn and D. Yarrow

a. DIAGNOSIS OF THE GENUS

Cells vary in shape: globose, ovoid, cylindrical to elongate, rarely apiculate, ogival, triangular or flask-shaped. Reproduction usually by multilateral budding. Pseudomycelium absent, rudimentary or well developed; true mycelium present in some species. Chlamydospores may be present; ascospores, teliospores, ballistospores and arthrospores absent.

Visible pigmentation due to carotenoid pigments absent.

Extracellular polysaccharides may be formed and may give a green to purple color reaction with iodine.

Inositol assimilation variable. Inositol positive strains form pseudomycelium.

Fermentation absent or present.

Standard description of *Candida shehatae*

Growth in glucose-yeast extract-peptone water: After 3 days at 25°C the cells are ovoid, (2–4.5) × (3.5–6) μm; cylindrical and pseudohyphal cells may also occur (Fig. 331) (van Uden & Buckley, 1970).

Growth on glucose-yeast extract-peptone agar: After one month at 25°C the streak culture is cream-colored, glistening, soft and smooth or slightly reticulated (van Uden & Buckley, 1970).

Dalmau plate culture on corn meal agar: The pseudomycelium consists of ramified chains of curved pseudohyphae bearing blastospores in varying amounts in more or less verticillated positions (Fig. 332) (van Uden & Buckley, 1970).

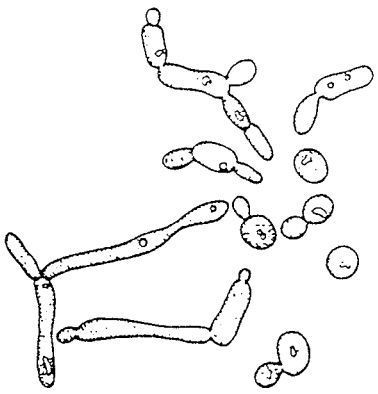


Fig. 331 – *C. shehatae*
In glucose-yeast extract-peptone water
(From 'The Yeasts' ed. II, 1970)

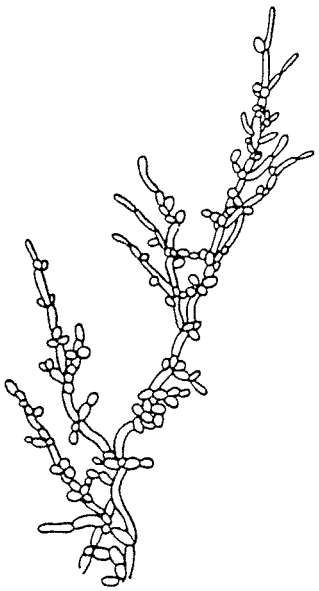


Fig. 332 – *C. shehatae*
Dalmau plate culture on corn meal agar
(From 'The Yeast' ed. II, 1970)

Fermentation:

Glucose	+	Maltose	+
Galactose	+	Lactose	–
Sucrose	–	Trehalose	+

Assimilation of carbon compounds:

Galactose	+	Melezitose	+	Ribitol	+
L-Sorbose	+	Soluble starch	+	Galactitol	–
Sucrose	+	D-Xylose	±	D-Mannitol	+
Maltose	+	L-Arabinose	v	D-Glucitol	+
Cellobiose	+	D-Arabinose	v	Salicin	+
Trehalose	+	D-Ribose	+	DL-Lactic acid	+
Lactose	+	L-Rhamnose	v	Succinic acid	+
Melibiose	–	Glycerol	±	Citric acid	+
Raffinose	–	Erythritol	v	Inositol	–

Splitting of arbutin: +
Assimilation of nitrate: –
Growth in vitamin-free medium: v
Growth at 37°C: +
Color reaction with DBB: –
Coenzyme Q system: Q 9 (Yamada & Kondo, 1972)
G + C: 41.2 mol%, one strain (Nakase & Komagata 1971f)

Standard description of *Candida tropicalis*

Growth in glucose-yeast extract-peptone water: After 3 days at 25°C the cells are globose, short-ovoid to long ovoid, (4.3–7.2) × (5.8–10.8) µm (Fig. 348). A sediment, a ring and islets are present.

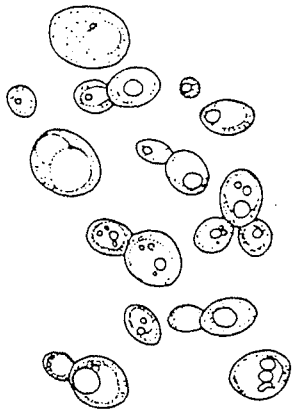


Fig. 348 – *C. tropicalis*
In glucose-yeast extract-peptone water
(From 'The Yeasts', ed. II, 1970)

Growth on glucose-yeast extract-peptone agar: After one month at 25°C the streak culture is cream-colored, off-white to grey, dull, soft, smooth and creamy or wrinkled and tough. A mycelial border is evident.

Dalmau plate culture on corn meal agar: Pseudomycelium is abundantly formed and consists of long, branched pseudohyphae bearing blastospores singly, in short chains and clusters (Fig. 349). True mycelium also may occur.

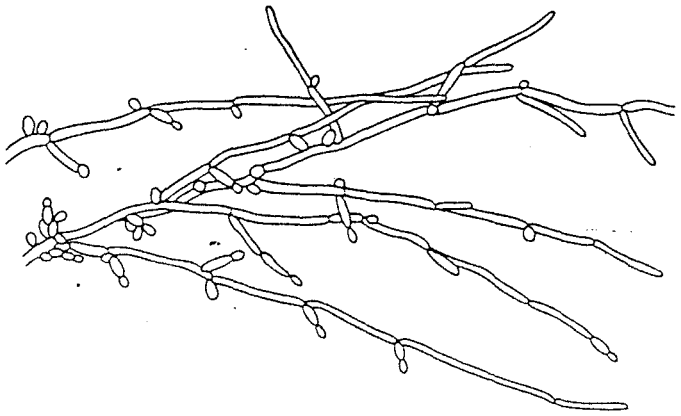


Fig. 349 - *C. tropicalis*
Dalmau plate culture on corn meal agar (From 'The Yeasts', ed. II, 1970)

Fermentation:

Glucose	+	Maltose	+		
Galactose	+	Lactose	–		
Sucrose	v	Trehalose	+ or s		

Assimilation of carbon compounds:

Galactose	+	Melezitose	v	Ribitol	v
L-Sorbose	v	Soluble starch	+	Galactitol	–
Sucrose	v	D-Xylose	+	D-Mannitol	+
Maltose	+	L-Arabinose	+ w or –	D-Glucitol	+
Cellobiose	v	D-Arabinose	–	Salicin	v
Trehalose	+	D-Ribose	v	DL-Lactic acid	v
Lactose	–	L-Rhamnose	–	Succinic acid	+
Melibiose	–	Glycerol	v	Citric acid	v
Raffinose	–	Erythritol	–	Inositol	–

Additional carbon compound tested: Inulin –
Splitting of arbutin: v
Assimilation of nitrate: –
Growth in vitamin-free medium: –
Growth at 37°C: +
Color reaction with DBB: –
Coenzyme Q system: Q 9 (Yamada & Kondo, 1972)
G + C: 35.9–36.1 mol%, 3 strains (Meyer et al., 1975)

Genus 16. *Trichosporon* Behrend

by N.J.W. Kreger-van Rij

a. DIAGNOSIS OF THE GENUS (DO CARMO-SOUSA, 1970c)

Budding cells of various shapes. Pseudomycelium well-developed or reduced. Septate mycelium and arthrospores always present, usually abundant. Ring and pellicle often formed on liquid media.

Ascospores, basidiospores and teliospores not produced.

Asexual endospores may be formed.

Fermentation present or absent.

Standard description of *Trichosporon cutaneum* (do Carmo-Sousa, 1970c)

Growth in glucose-yeast extract-peptone water: After 3 days at 25°C true mycelium and arthrospores are always present (Fig. 391). Width and length of the arthrospores are variable. Budding cells may be abundant or scarce, or even absent in strains which have been maintained in culture collections for a long time. The yeast cells are spherical to oval, (3.5–7) × (3.5–14) µm. Budding may be on a narrow or a broad base; it is stimulated by growing the cultures on a shaker. A pellicle is often present; it is cream colored or white, smooth, wrinkled, undulating, rugose or folded, thin or thick, often creeping, sometimes tending to sink to the bottom. In the absence of a pellicle, a ring is sometimes formed; it may be incomplete or thick. The sediment is butyrous or flocculent.

Growth on glucose-yeast extract-peptone agar: After one month at 25°C the streak culture is white to yellowish, seldom with a brownish tinge, smooth, wrinkled, rugose or folded, velvety, powdery, hairy or coremial; dull, moist or shiny; of a pasty to tough texture, with an entire or irregular radially folded border, fringed with mycelium. In 3–4-week-old cultures chlamydospores may be observed (van der

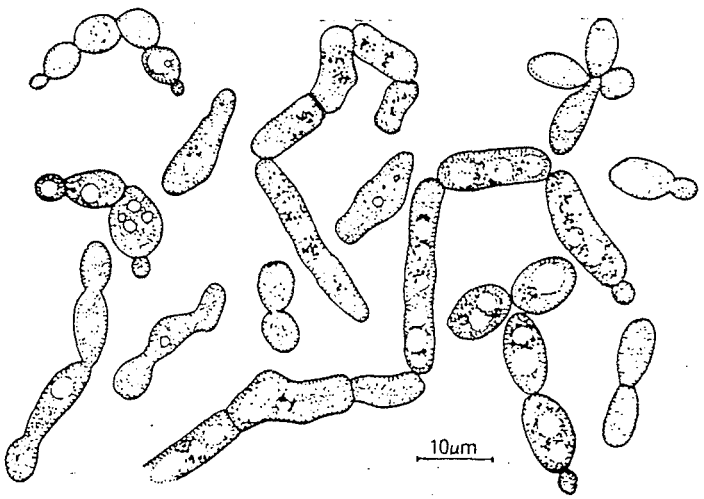


Fig. 391 – *Tr. cutaneum*
After 3 days in glucose-yeast extract-peptone water (From 'The Yeast' ed. II, 1970)

Walt, 1970i). The strain of *Trichosporon dulcitum* (CBS 508.67) is exceptional in forming chlamydospores after 3 days in potato water.

Slide cultures on potato- and corn meal agar: True mycelium is abundant; arthrospores of variable size may be abundant or scarce (Figs. 392 and 393). Pseudomycelium often occurs with blastospores in chains or clusters. Blastospores may also be formed on the true hyphae or from the arthrospores. In some isolates, the mycelium and arthrospores assume a bizarre shape with very few blastospores. Giant cells may be present; they are conspicuous in the strain of *Tr. loubieri* (CBS 252.61). In the hyphal septa dolipores with a primitive cap have been observed (Kreger-van Rij & Veenhuis, 1971b).

Formation of asexual endospores: Asexual endospores may be formed (Fig. 394). Spherical, ellipsoidal or pear-shaped cells may contain one to six or more endospores of various sizes, spherical or ellipsoidal in shape. These endospores were formed in very recently isolated cultures on glucose-yeast extract-peptone agar after 3 months at 25°C. The capacity of forming endospores by protoplasmic cleavage was apparently lost after a few transfers. Endospores formed by internal budding may occur. The endospores were observed in slide cultures after 3 weeks at 25°C and on slants after one week at 24°C.

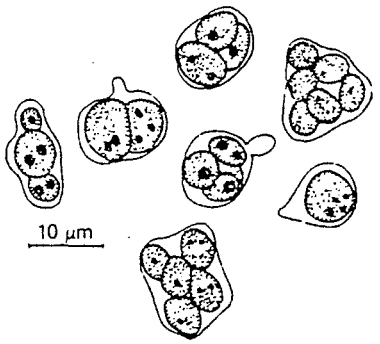


Fig. 394 – *Tr. cutaneum*
Endospores after 3 months on glucose-yeast extract-peptone agar
(From 'The Yeasts' ed. II, 1970)

Fermentation: –

Assimilation of carbon compounds:

Galactose	+ (–)	Raffinose	v	Erythritol	v
Sucrose	+ (–)	Soluble starch	+ (–)	Ribitol	v
Maltose	+ (–)	D-Xylose	+	D-Mannitol	v
Cellobiose	+ (–)	L-Arabinose	+ (–)	Succinic acid	v
Trehalose	v	D-Ribose	+ (–)	Citric acid	v
Lactose	+	L-Rhamnose	v	Inositol	+

Splitting of arbutin: +

Assimilation of nitrate: –

Assimilation of nitrite: v

Growth in vitamin-free medium: –

Growth on 50% (w/w) glucose-yeast extract agar: +w or –

Growth at 37°C: v

Urease: +

Color reaction with DBB: +

G + C: 60 mol%, CBS 5580; 57 mol%, *Tr. loubieri*, CBS 252.61; 57.5 mol%, *Geotrichum vanriji*, CBS 439.64; 60 mol%, *Tr. amycelicum*, CBS 186.38 (all by Guého, 1979)

Genus 23. *Saccharomycodes* Hansen

by M.W. Miller and H.J. Phaff

a. DIAGNOSIS OF THE GENUS

Large, diploid, lemon-shaped (apiculate) or elongate cells, which reproduce by bipolar budding on a very broad base (bud-fission). Pseudomycelium, if present, is poorly developed.

The spores are spheroidal with a smooth wall; a narrow ledge may be visible. Asci usually contain four spores, occurring in two pairs; upon germination members of each pair ordinarily conjugate. In some strains spores may germinate without conjugation.

In liquid media a sediment and a ring are formed.

Sugars are fermented; nitrate is not assimilated.

Standard description of *Saccharomycodes ludwigii*

Growth in malt extract : After 3 days at 25°C the cells are lemon-shaped with blunt tips, sausage-shaped, curved, broad-oval, or elongated with a swelling in the

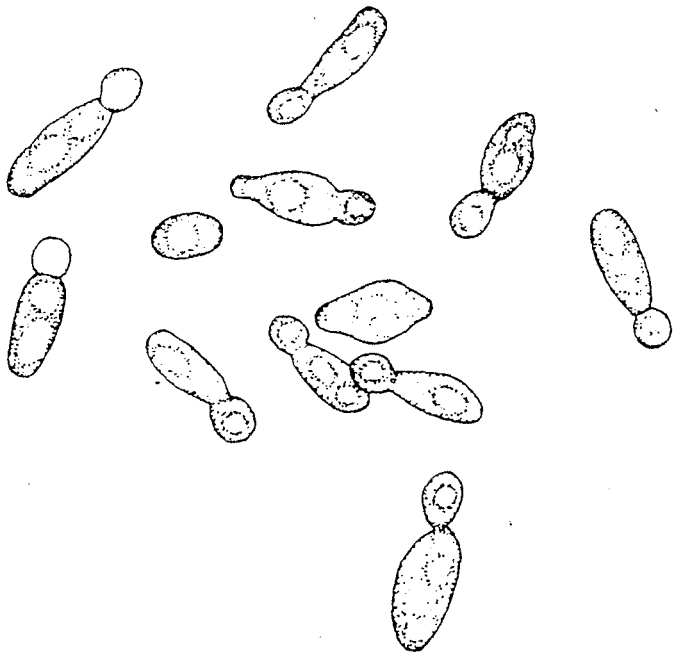


Fig. 100 – *S'codes ludwigii*
After 3 days in malt extract (From 'The Yeasts', ed. II, 1970)

middle, (4–7) × (8–23) μm, and occasionally longer, single or in pairs and sometimes in groups of three. Reproduction occurs by bipolar budding on a broad base, followed by fission (bud-fission). Cells in the process of budding resemble bowling pins (Fig. 100). A sediment and a slight ring are present. After approximately one month a sediment and a ring in evidence.

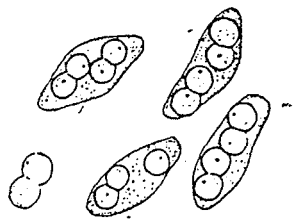


Fig. 101 – *S'codes ludwigii*
On malt agar (From 'The Yeasts', ed. I, 1952)

Growth on malt agar: After 3 weeks the streak culture is cream colored to tan, nearly smooth, semi-glossy, low convex with an irregular border.

Slide culture on potato agar: Aerobically a few short chains of cells. Under the coverslip the pseudomycelium is somewhat better developed consisting of branched chains of elongated cells resembling the 'Mycocandida' type.

Formation of ascospores: The spores are spheroidal, smooth with a narrow subequatorial ledge which is visible in sections in the electron microscope (Kreger-van Rij, 1969b); usually there are four spores per ascus in two groups of two (Fig. 101). Ascus walls do not rupture or lyse when the spores are mature. For germination of spores see section a., Diagnosis of the genus.

Sporulation is good on Gorodkowa agar.

Fermentation:

Glucose	+	Lactose	–
Galactose	–	Melibiose	–
Sucrose	+	Raffinose	+
Maltose	–		

Assimilation of carbon compounds:

Galactose	–	Raffinose	+	Erythritol	–
Sucrose	+	Soluble starch	–	Ribitol	–
Maltose	–	D-Xylose	–	D-Mannitol	–
Cellobiose	+	L-Arabinose	–	Succinic acid	±
Trehalose	–	D-Ribose	–	Citric acid	–
Lactose	–	L-Rhamnose	–	Inositol	–

Additional carbon compounds tested: Melibiose –, glucono-δ-lactone –, calcium 2-ketogluconate –, potassium 5-ketogluconate –

Assimilation of nitrate: –

Growth in vitamin-free medium: –

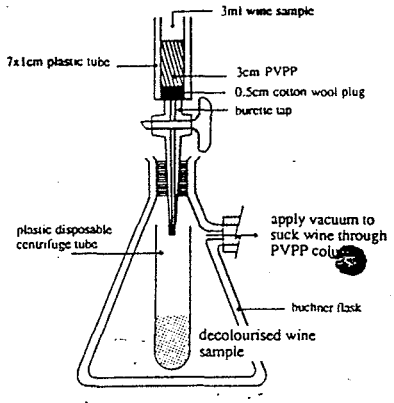
Growth on 50% (w/w) glucose-yeast extract agar: –

Growth at 37°C: v

G + C: 38.3 mol%, one strain (Nakase & Komagata, 1971g)

APPENDIX II

2.3.1 Analysis method - Rebellein



Sample Preparation

Red wines need to be decolourised as per method given for the Lane and Eynon or by using the system shown opposite which is appropriate for decolourising small volumes of wine.

Procedure (Blank Analysis)

1. Pipette 10.0 mL of Z_1 and add 5 mL Z_2 into a 125 mL conical flask.
2. ◆Add three boiling chips.
3. Pipette 2.0 mL of distilled water into the flask.
4. Heat the flask and allow the solution to boil for 30 seconds, allow the solution and flask to cool.
5. When the solution has reached room temperature add 10 mL of Z_3, Z_4, Z_5 in that order.
6. Titrate the mixture in the conical flask with Z_6, shaking the flask throughout the titration, until the solution turns cream (the end point).
7. Record the titre value (blank titre). This should be in the range 29-31 mL and will vary slightly for each particular set of reagents prepared.

Procedure (Sample Analysis)

Repeat above procedure (steps 1-7) but instead of 2.0 mL distilled water use 2.0 mL of treated wine sample. Record the titre value (sample titre).

Calculation (Rebelein Method)

Reducing Sugar (gL^{-1}) = Dilution Factor x (Blank Titre — Sample Titre)

Errors may occur due to...

Failure to decolourise red wines.

Incorrect judgement of the end point - take as the first obvious change to cream.

Wines containing sugar content greater than 20 gL^{-1} require dilution so that an accurate volume of Z_6 may be delivered on titration. If the sugar content is 30 gL^{-1} or greater then there is no excess Z_1 to titrate with Z_6, hence no sensible measurement can be achieved, on an undiluted sample.

CHEMICAL	CHEMICAL FORMULA	REAGENT PREPARATION	REAGENT ADDITION
Rebelein			
Z_1		Accurate	Accurate
Z_2		"	Approximate
Z_3		-	-
Z_4		-	-
Z_5		-	-
Z_6		-	Accurate
Wine		Decolourised for reds	-

Reagents for Reducing Sugar by Rebelein Method

Z_1 Solution (Prepare Accurately)

Measure approximately 600 mL distilled water into a large beaker. Add carefully 1.0mL of concentrated sulphuric acid (H_2SO_4) to the 600 mL of distilled water and mix thoroughly. Weigh 41.92 grams of copper (cupric) sulphate ($CuSO_4.5H_2O$). Dissolve the copper sulphate in some of the sulphuric acid solution, and quantitatively transfer (using the sulphuric acid solution for transfer) to a one litre volumetric flask. Add the remaining sulphuric acid solution to the volumetric flask. Rinse the beaker containing the sulphuric acid with distilled water and transfer this to the volumetric flask. Make to the mark with distilled water. Mix thoroughly. Store in sealed container.

Z_2 Solution (Prepare Accurately)

Weigh 250 grams of sodium potassium tartrate. Dissolve this in about 600 mL distilled water. Weigh 80 grams sodium hydroxide (NaOH) and CAREFULLY add this to the sodium potassium tartrate solution. CARE — heat will be generated on dissolving. It may be necessary to stand the beaker in a cold water bath. Quantitatively transfer the cooled mixture to a one litre volumetric flask. Make to the mark with distilled water. Mix

thoroughly. Store in a sealed plastic container. If storing in glass use a plastic not a glass top.

Z_3 Solution (Prepare Accurately)

Measure approximately 600 mL distilled water into a large
* beaker. Add to the distilled water, with mixing 100 mL of 1 M NaOH (CARE) (see preparation notes in sulphur dioxide reagent preparation section). Mix thoroughly. Weigh 300.0 grams of potassium iodide (KI). Dissolve the potassium iodide in a portion of the 600 mL sodium hydroxide solution.

Transfer the KI solution quantitatively (with the sodium hydroxide solution) to a one litre volumetric flask, and add any remaining sodium hydroxide solution to the volumetric flask. Add distilled water and make to the mark. Mix. Store in sealed plastic container

Z_4 Solution (Accuracy not Essential)

* Measure (measuring cylinder) 175 mL of concentrated (H_2SO_4) [98% w/w]). Add carefully and slowly, with stirring, the H_2SO_4 to approximately 1825 mL of cold distilled water in a large beaker. Mix thoroughly. It may be necessary to stand the beaker in an ice bath during the mixing operation. Store in a sealed glass container.

Z_5 Solution (Accuracy not Essential)

Weigh 20.0 grams of potassium iodide (KI) and 10 grams starch in separate beakers. Measure (measuring cylinder) 10 mL of 1 M
* NaOH (CARE) (see preparation notes in sulphur dioxide reagent preparation section) in approximately 500 mL distilled water. Use this NaOH solution to dissolve the KI and starch and quantitatively transfer each solution to the same volumetric flask.Add any remaining NaOH solution to the volumetric flask. Make to volume with distilled water. Mix thoroughly. Store in sealed plastic container. If storing in glass use a plastic not glass top.

Z_6 Solution (Prepare Accurately)

Weigh 13.777 grams of sodium thiosulphate ($Na_2S_2O_3.5H_2O$). Dissolve in distilled water and quantitatively transfer to a one litre volumetric flask. Add 50 ml (measuring cylinder) of 1 M
* NaOH (CARE) (see preparation notes in sulphur dioxide reagent preparation section) to the volumetric flask. Add distilled water to make to the mark. Mix thoroughly. Store in a sealed plastic container.

APPENDIX III

2.3.2.2 - Preparation of reagents for combi test and standardised analysis method

Appendix

Preparation of Reagents

1. **Alkaline copper EDTA solution.**
   (a) Dissolve 25.3 g AR copper sulphate pentahydrate, $CuSO_4.5H_2O$, in 100 mL of distilled water.
   (b) Dissolve 37.2 g disodium ethylene diamine tetraacetic acid (EDTA) in 250 mL of distilled water.
   (c) Boil 300 mL of distilled water in a 2 L beaker. Add slowly, 105.9 g anhydrous sodium carbonate, Na_2CO_3.
   (d) Add with constant stirring the EDTA solution and then the copper sulphate solution to the sodium carbonate solution.
   (e) Leave to cool.
   (f) Dilute to 1 litre with distilled water.

2. **Potassium iodide solution (30% to make 1 litre).**
   (a) Dissolve 300 g potassium iodide, KI, in approximately 800 mL of distilled water.
   (b) Dilute to 1 litre in a measuring cylinder with distilled water.
   (c) Store in a dark bottle.

3. **Sodium starch glycollate solution (1% to make 100 mL).**
   (a) Dissolve 1 g of sodium starch glycollate in approximately 80 mL of distilled water. The solution may need heating to dissolve the compound.
   (b) Cool, and dilute to 100 mL in a measuring cylinder with distilled water.

4. **Acidified potassium dichromate solution (0.07257 *M*)**
   (a) Dissolve 21.35 g of AR potassium dichromate, $K_2Cr_2O_7$, in about 350 mL of distilled water.
   (b) Add CAREFULLY 585 mL of concentrated nitric acid, HNO_3, SLOWLY with constant stirring. Nitric acid is very corrosive and potassium dichromate is a strong oxidising agent, so the mixture should be treated with care. Clean up spills immediately and if a spilt on hands wash immediately with large amounts of water.
   (c) Dilute to 1 litre with distilled water.

5. **Standardised sodium thiosulphate (0.1 *M*)**
   Vials may be purchased (e.g. from BDH) and these should be made up in a volumetric flask according to the directions supplied.

Report

1. Record the titre values for each of the sugar and alcohol determinations.
2. Use Table 1 to deduce the sugar content of the wine sample and the unknown standard.
3. Use the balanced chemical equations given in the introduction and the data below to calculate the alcohol content (% alcohol v/v) of the samples analysed. (Show your calculations).

Data

Density of ethanol at various temperatures

10°C	0.79788 g/mL
15°C	0.79367 g/mL
20°C	0.78945 g/mL

Procedure

Part 1. Sample Preparation

(a) Pipette 10 mL of wine into a 100 mL volumetric flask.
(b) Dilute to volume with distilled water and mix well.

Part 2. Standardised Analysis Method

(a) Using a pipette filler, pipette 20 mL of alkaline copper EDTA reagent into the boiling flask (see Figure 1.)
(b) Pipette 10 mL of the diluted sample (wine or unknown standard) into the boiling flask.
(c) Add 3 to 4 antibumping granules and connect the boiling flask to the apparatus.
(d) Using a pipette filler, pipette 25 mL of acidified dichromate reagent into the receiving flask.
(e) Connect the receiving flask to the apparatus, insert the stopper, and apply heat to the boiling flask.
(f) Begin timing as soon as the contents of the flask start to boil.
(g) Boil for exactly 5 minutes, then remove the heat source and remove the stopper quickly, so that the contents of the receiving flask do not suck back into the boiling flask (explosion hazard !).
(h) Lower the receiving flask until it is clear of the liquid and rinse the steam entry tube with distilled water.
(i) Place both flasks in a cold water bath and allow to cool.

Part 3. Sugar Titration

(a) To the cooled boiling flask mixture, slowly add using a burette 30 mL of 5*N* sulphuric acid. The mixture gives off carbon dioxide gas so care should be taken that none is lost through excessive effervescence.
(b) To the boiling flask add 10 mL of potassium iodide solution and 2 mL of sodium starch glycollate solution. The solution turns brown upon the addition of the sodium starch glycollate.
(c) Titrate dropwise with 0.1*M* (0.1*N*) sodium thiosulphate solution until the mixture turns white. Record the titre value.

NOTE The sugar calculation requires the use of a blank titration. This is obtained using 10 mL of distilled water in place of the diluted wine sample in Part 2 above, and then proceeding through Parts 2 and 3 as before.

To obtain the percentage sugar in the wine, subtract the titre value obtained for the sample from the blank titre. Use this difference value when consulting Table 1 to obtain grams of sugar per litre of wine.

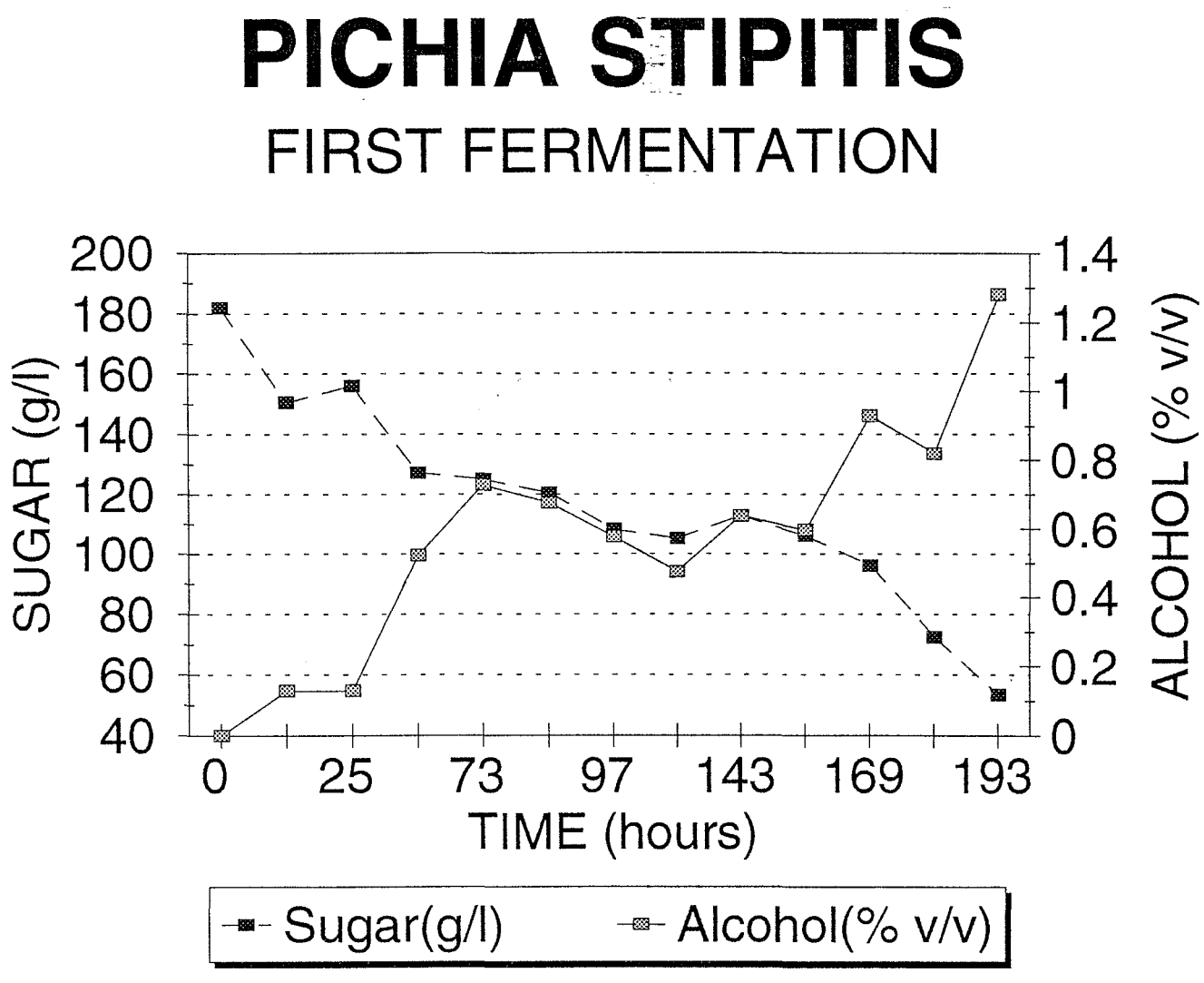
Part 4. Alcohol Titration

(a) Dilute the contents of the receiving flask to volume (140 mL) with distilled water.
(b) Add 10 mL of potassium iodide solution and 2 mL of sodium starch glycollate to the receiving flask. The solution turns a olive-brown colour.
(c) Titrate dropwise with 0.1*M* (0.1*N*) sodium thiosulphate solution until the mixture reaches a pale blue colour. Record the titre value.

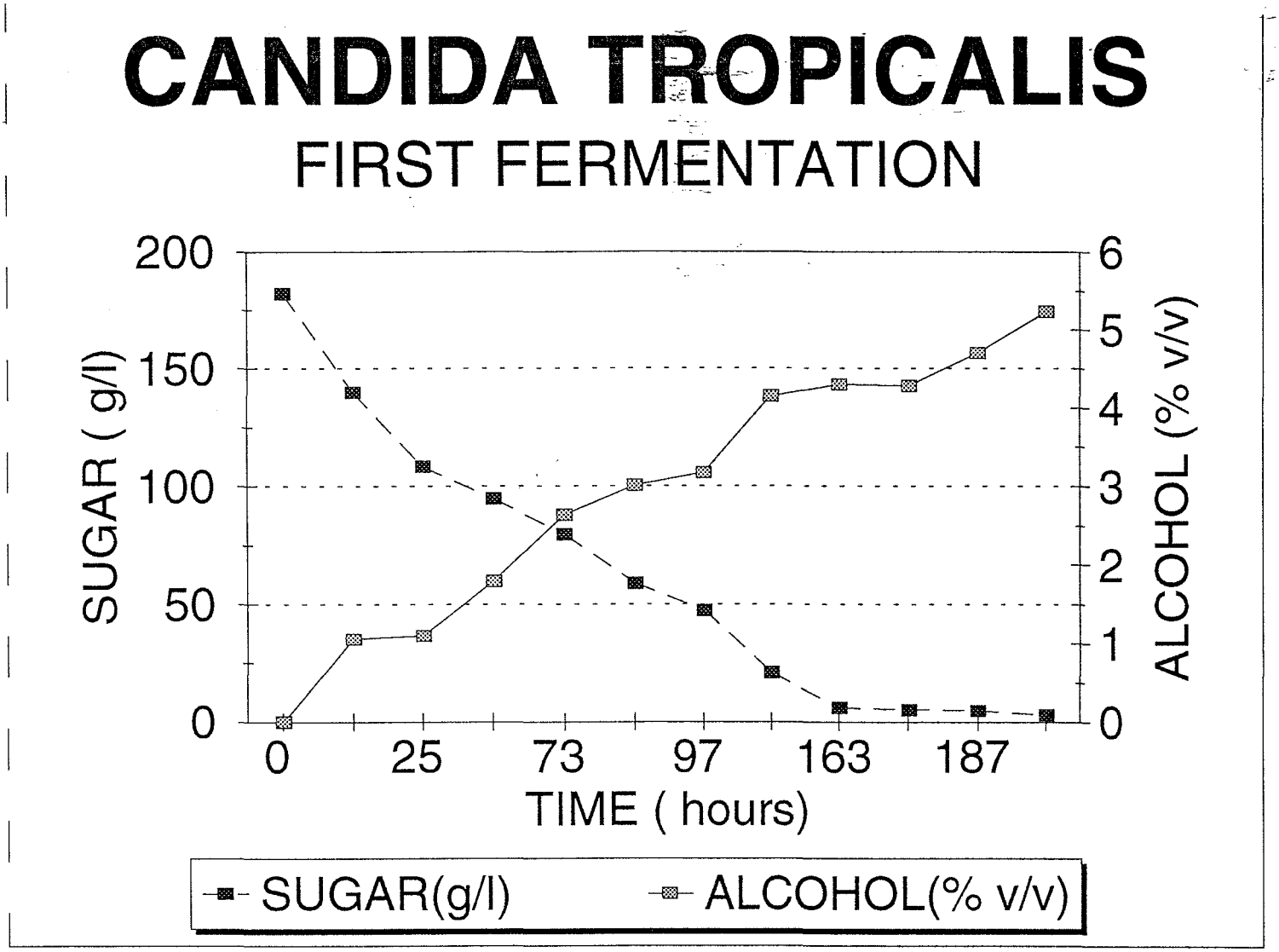
APPENDIX IV

4.3.2 - Sugar and alcohol during fermentation

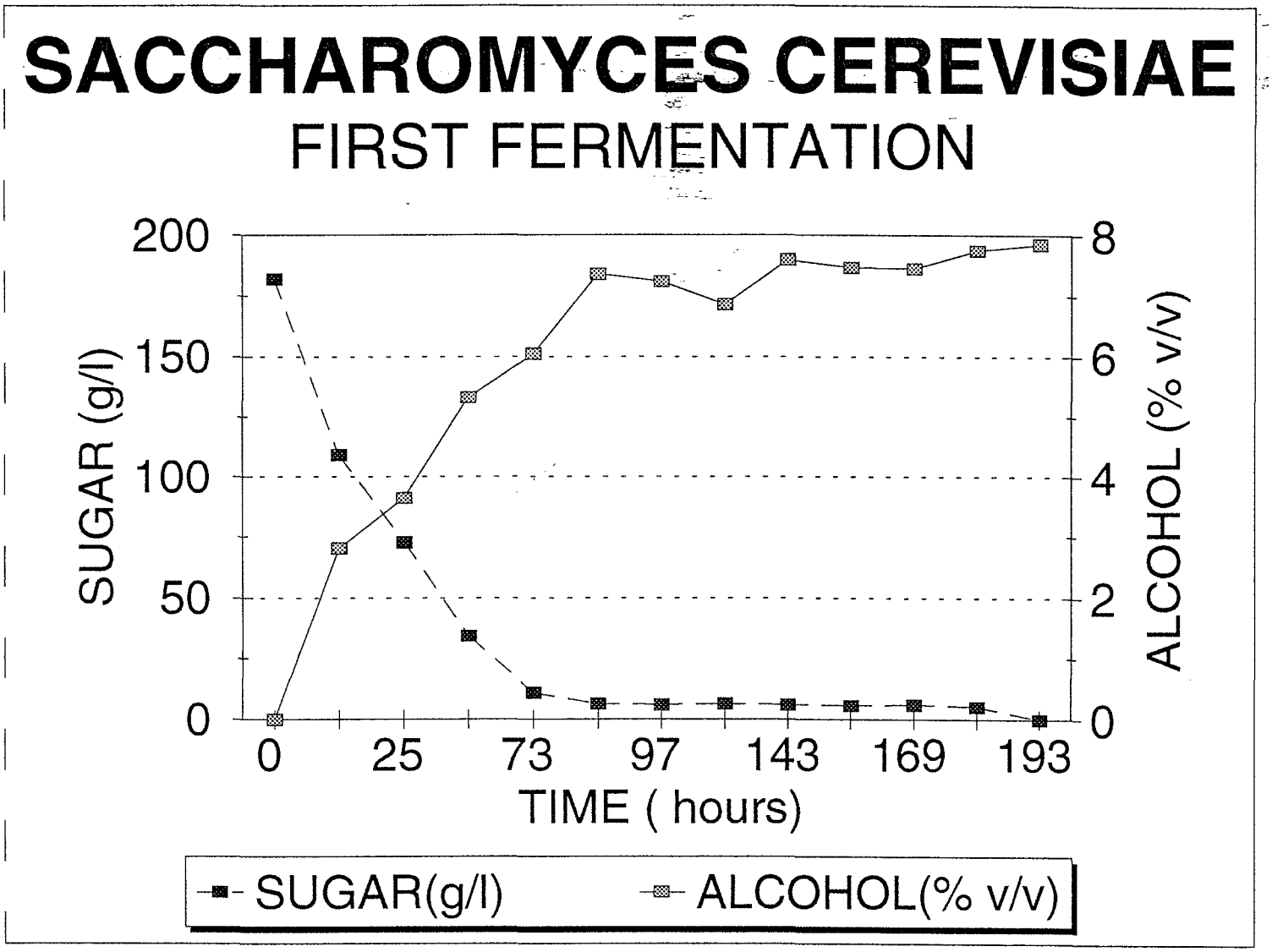
4.3.2.1 - *PICHIA STIPITIS*



4.3.2.2 - *CANDIDA TROPICALIS*

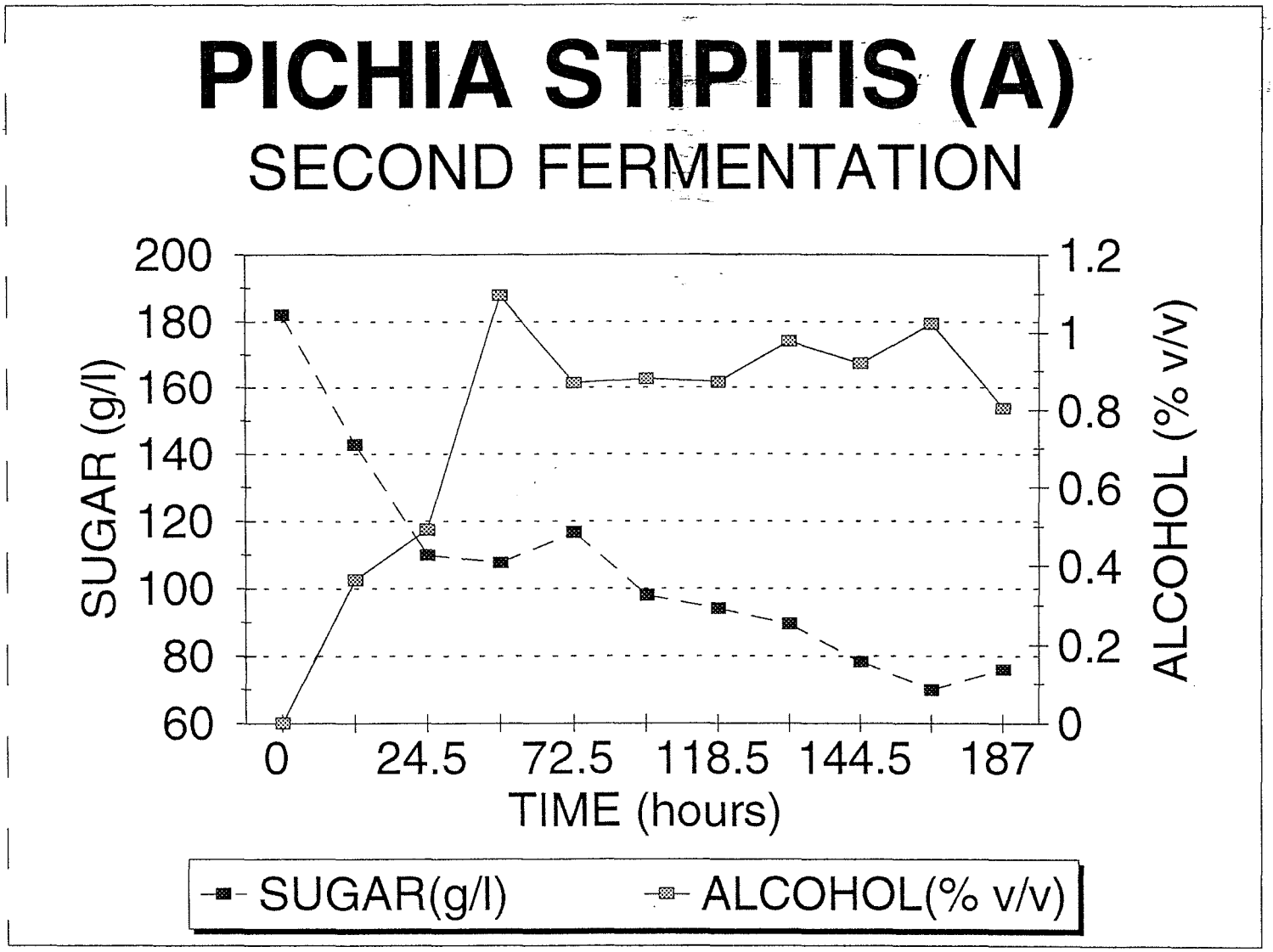


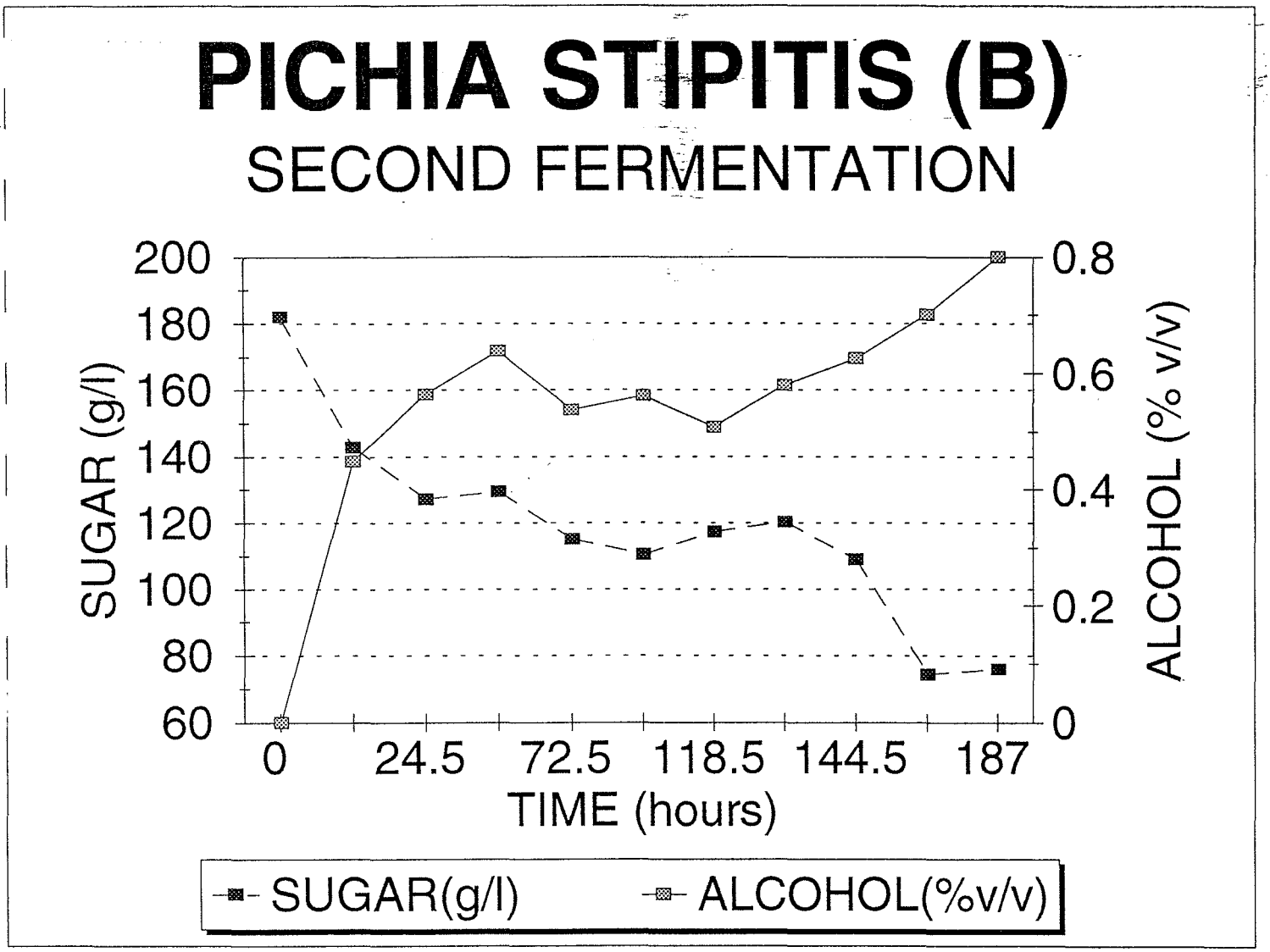
4.3.2.3 - *SACCHAROMYCES CEREVISIAE*



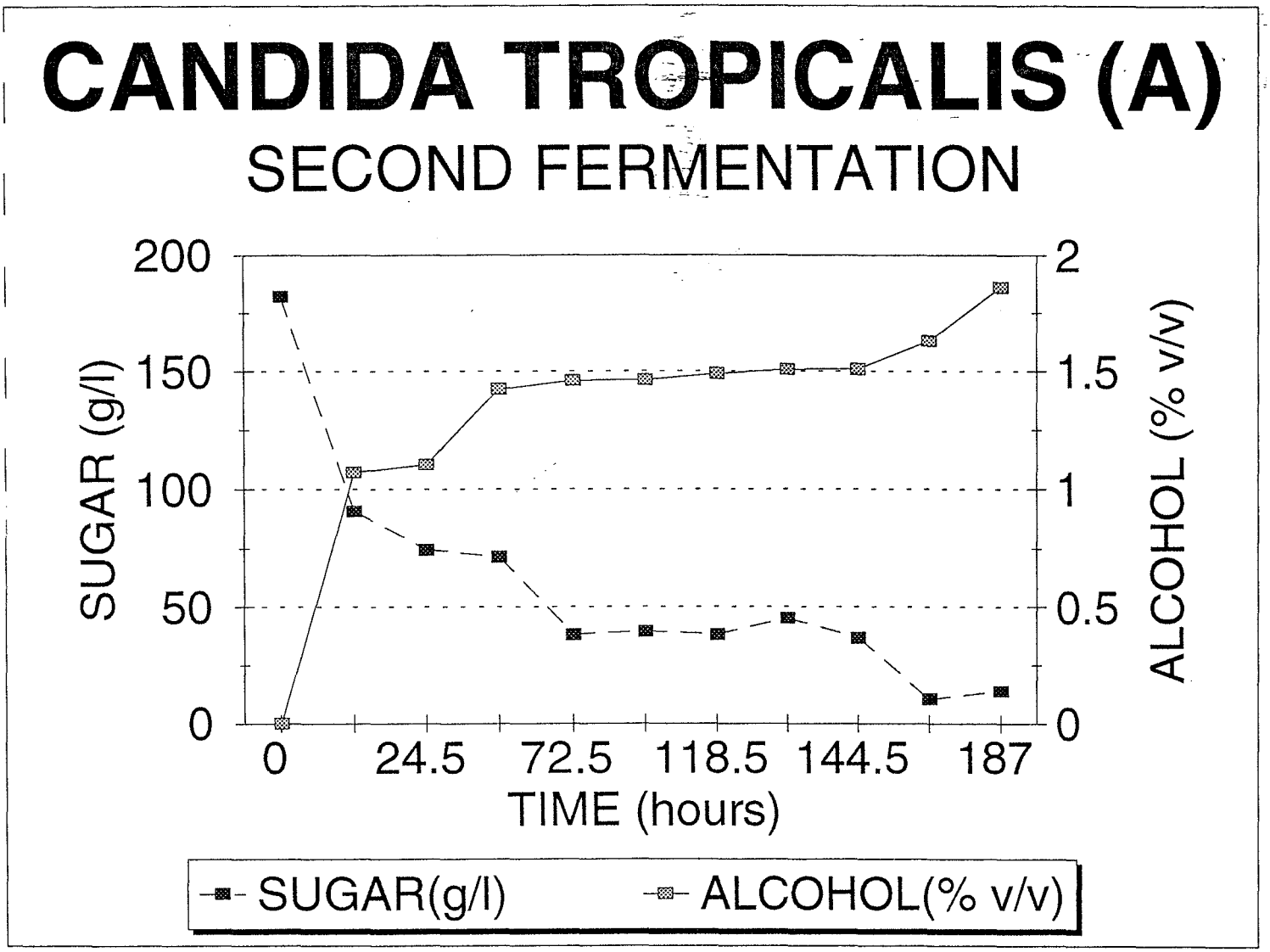
APPENDIX V

4.3.4 - Sugar and alcohol during fermentation



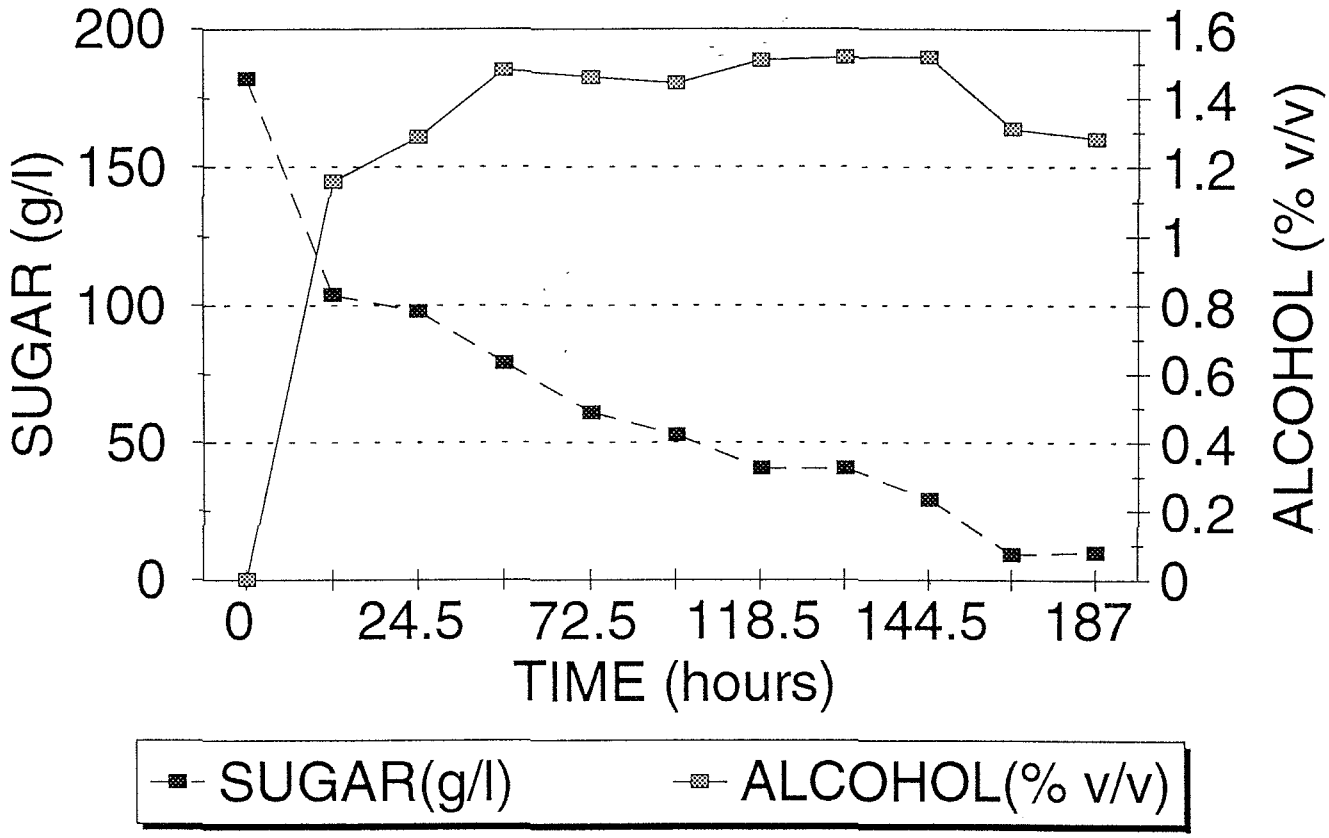

PICHIA STIPITIS (B)
SECOND FERMENTATION
SUGAR (g/l)
200
180
160
140
120
100
80
60
ALCOHOL (% v/v)
0.8
0.6
0.4
0.2
0
0
24.5
72.5
118.5
144.5
187
TIME (hours)
SUGAR(g/l)
ALCOHOL(%v/v)

4.3.4.2 - *CANDIDA TROPICALIS* sugar and alcohol during fermentation

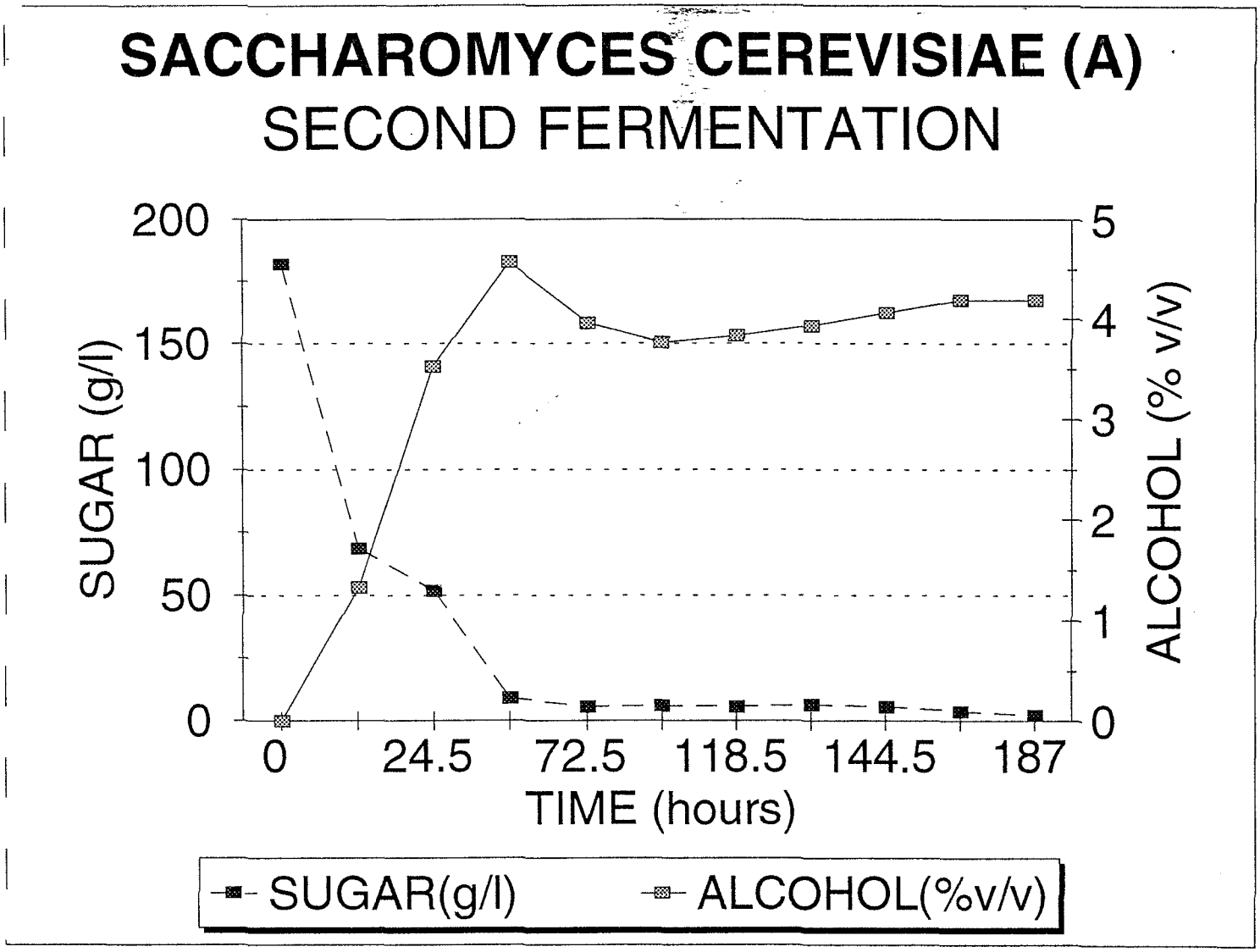


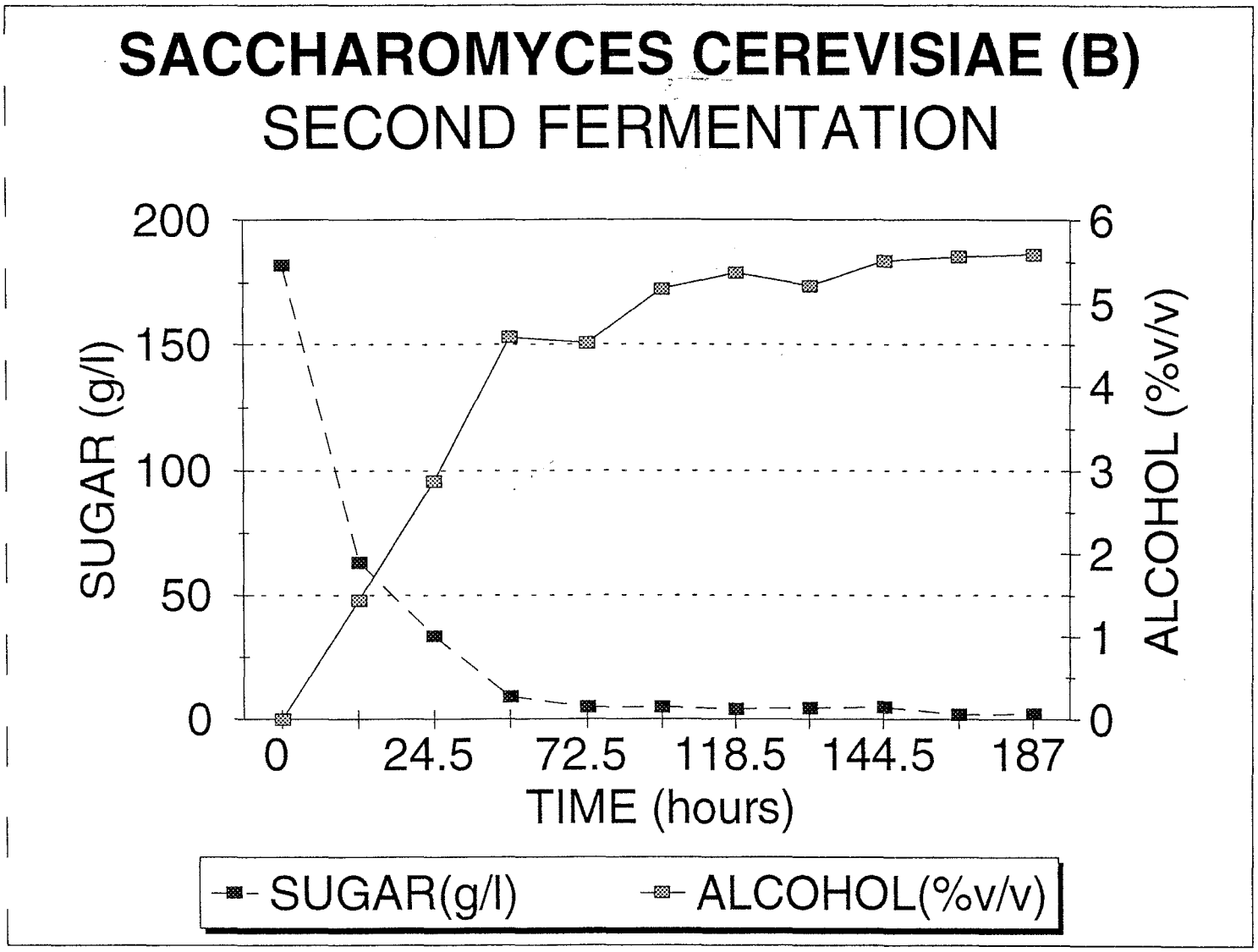
CANDIDA TROPICALIS (B)

SECOND FERMENTATION



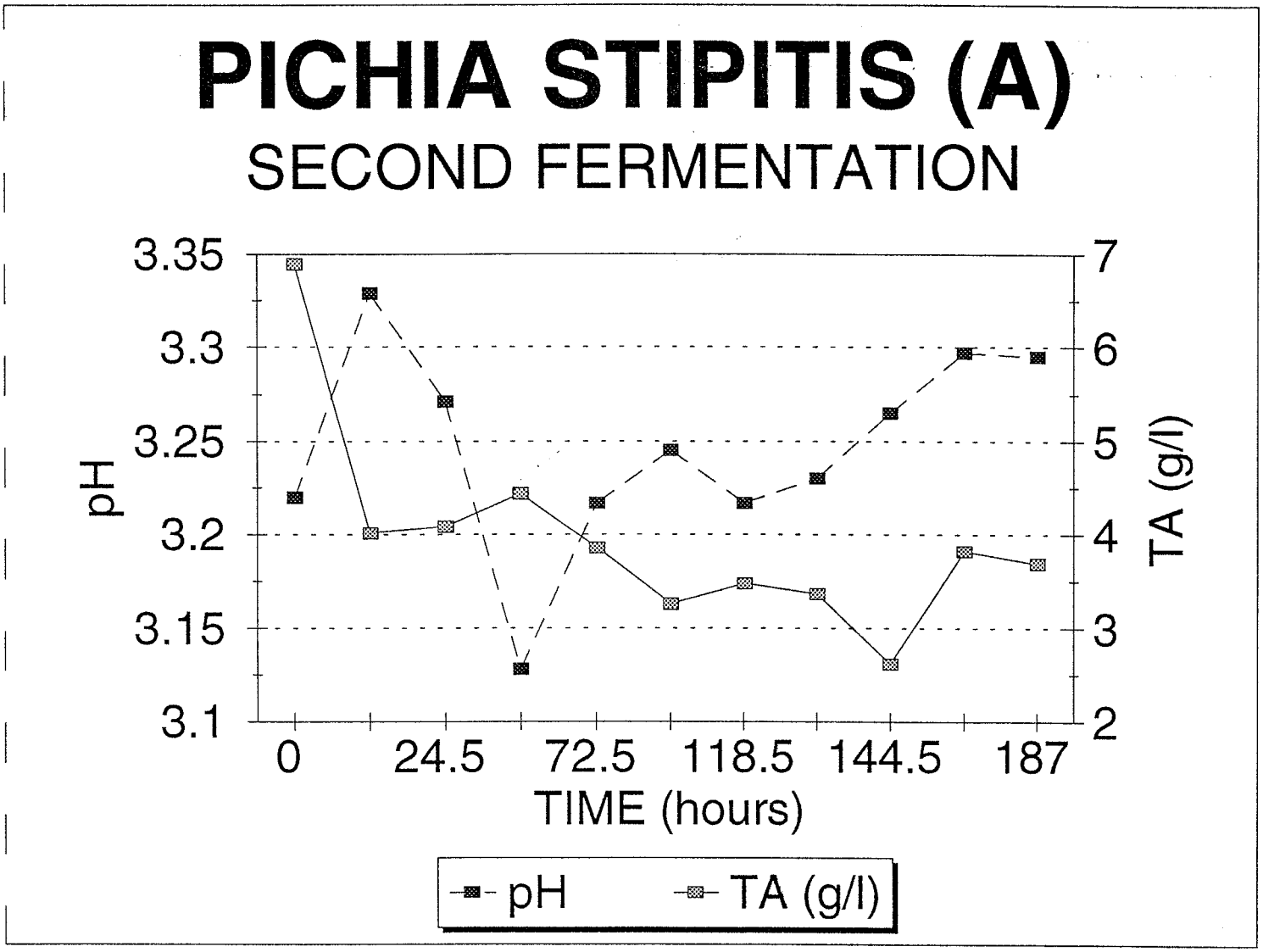
4.3.4.3 - *SACCHAROMYCES CEREVISIAE* sugar and alcohol during fermentation

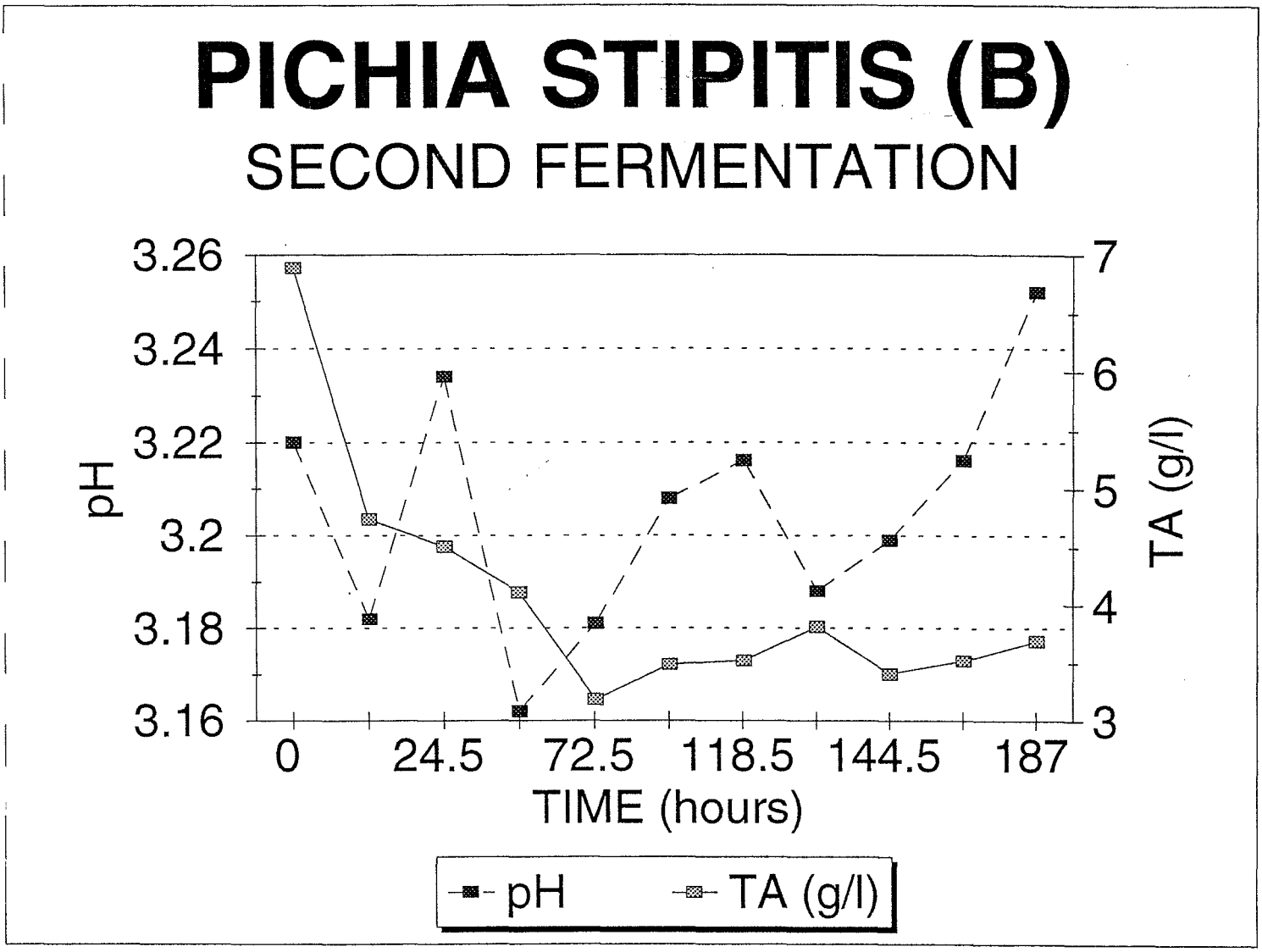



SACCHAROMYCES CEREVISIAE (B)
SECOND FERMENTATION
SUGAR (g/l)
200
150
100
50
0
ALCOHOL (%v/v)
6
5
4
3
2
1
0
0
24.5
72.5
118.5
144.5
187
TIME (hours)
SUGAR(g/l)
ALCOHOL(%v/v)

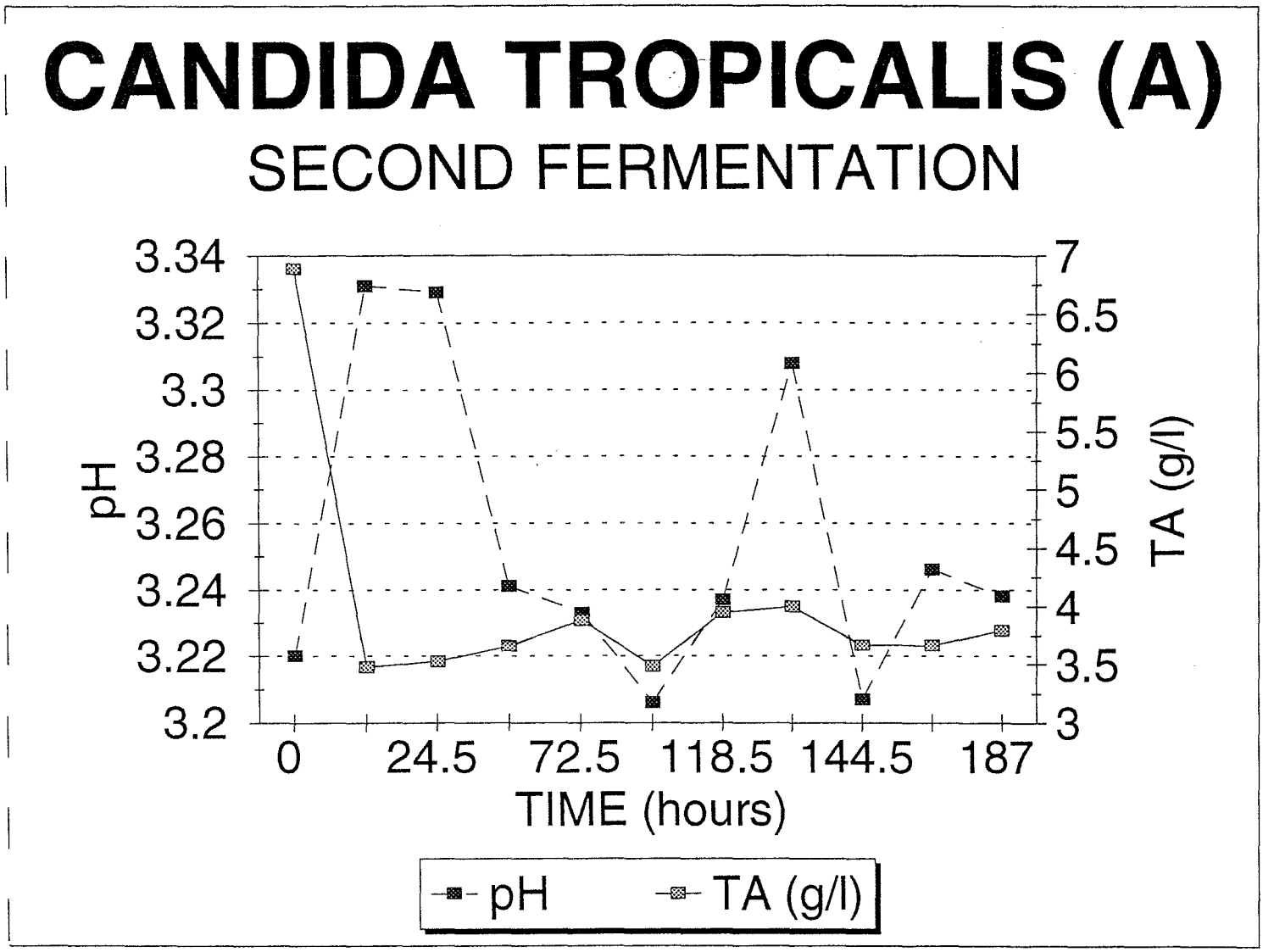
4.3.5 - pH and titratable acidity during fermentation

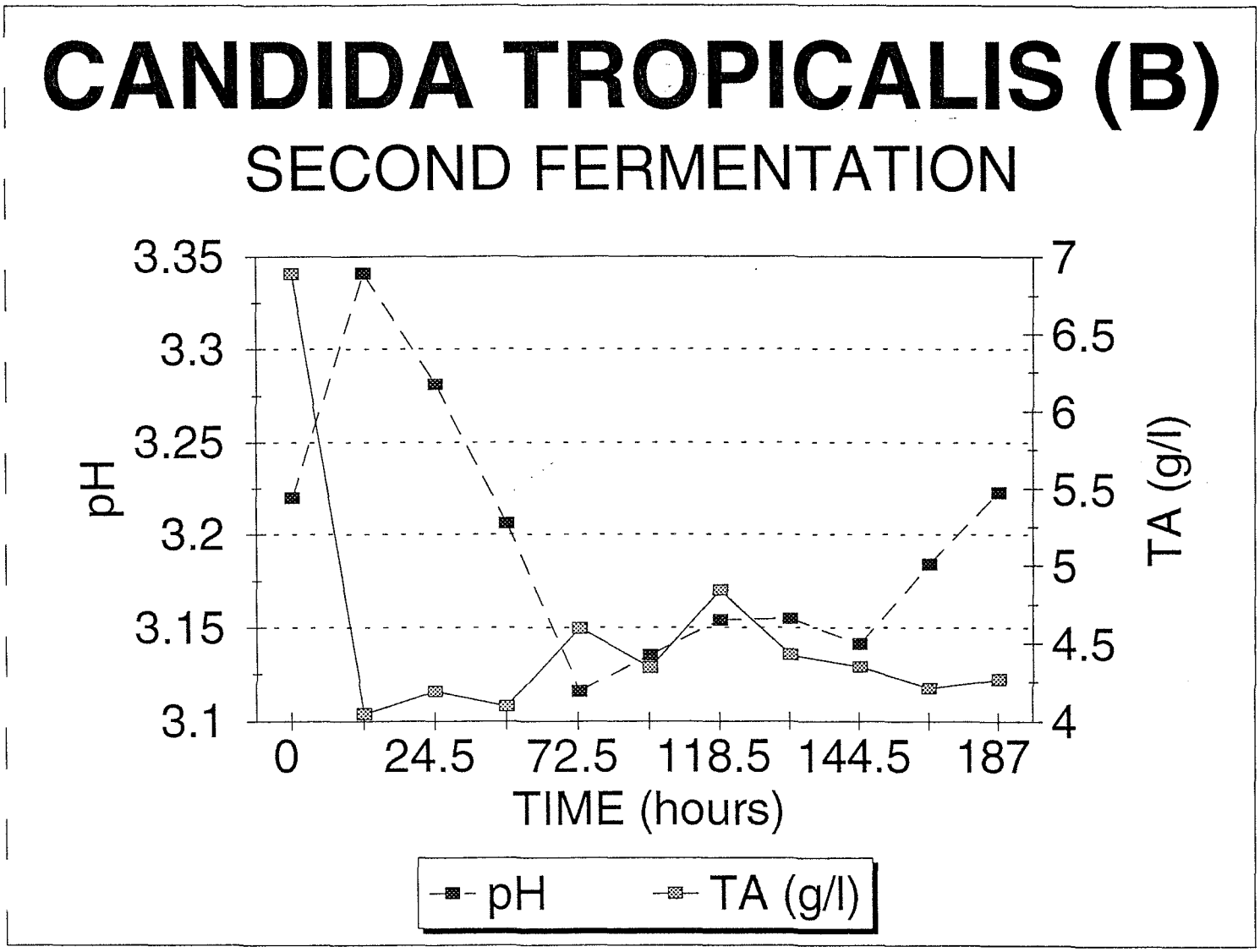
4.3.5.1 - *PICHIA STIPITIS*



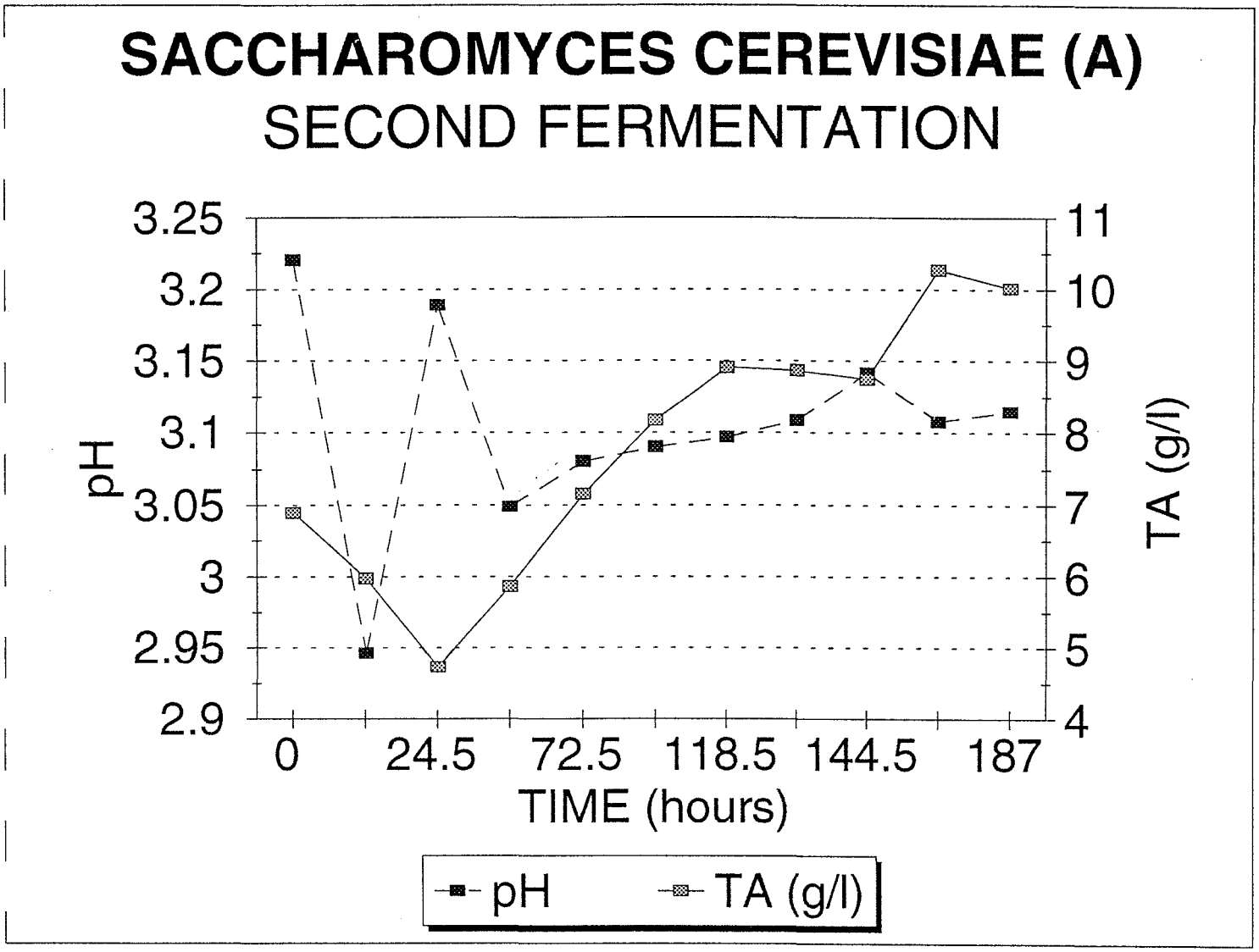

PICHIA STIPITIS (B)
SECOND FERMENTATION
pH
3.26
3.24
3.22
3.2
3.18
3.16
TA (g/l)
7
6
5
4
3
0
24.5
72.5
118.5
144.5
187
TIME (hours)
pH
TA (g/l)

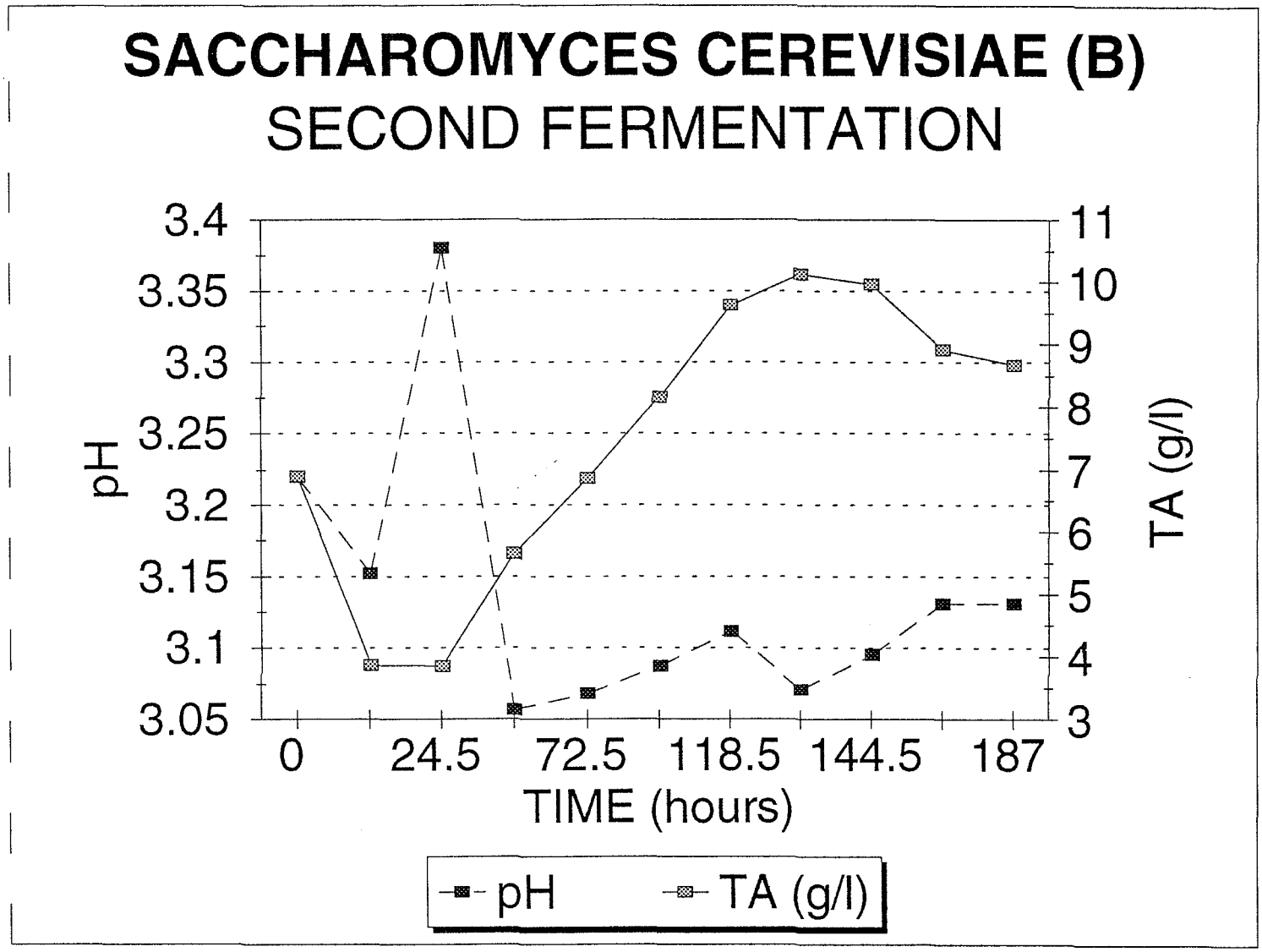
4.3.5.2 - *CANDIDA TROPICALIS* pH and TA during fermentation




CANDIDA TROPICALIS (B)
SECOND FERMENTATION
3.35
3.3
3.25
3.2
3.15
3.1
pH
7
6.5
6
5.5
5
4.5
4
TA (g/l)
0
24.5
72.5
118.5
144.5
187
TIME (hours)
pH
TA (g/l)

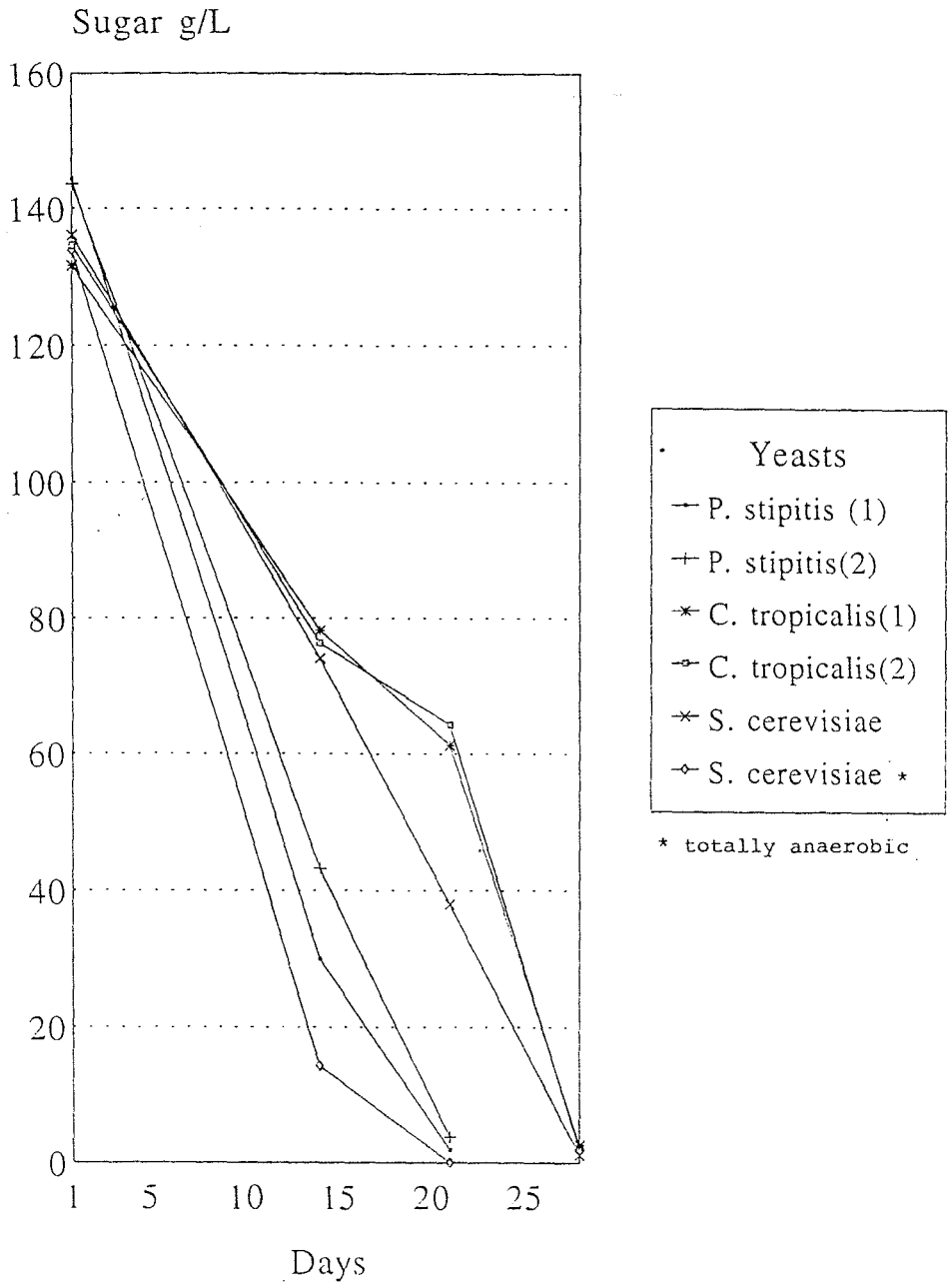
4.3.5.3 - *SACCHAROMYCES CEREVISIAE* pH and TA during fermentation



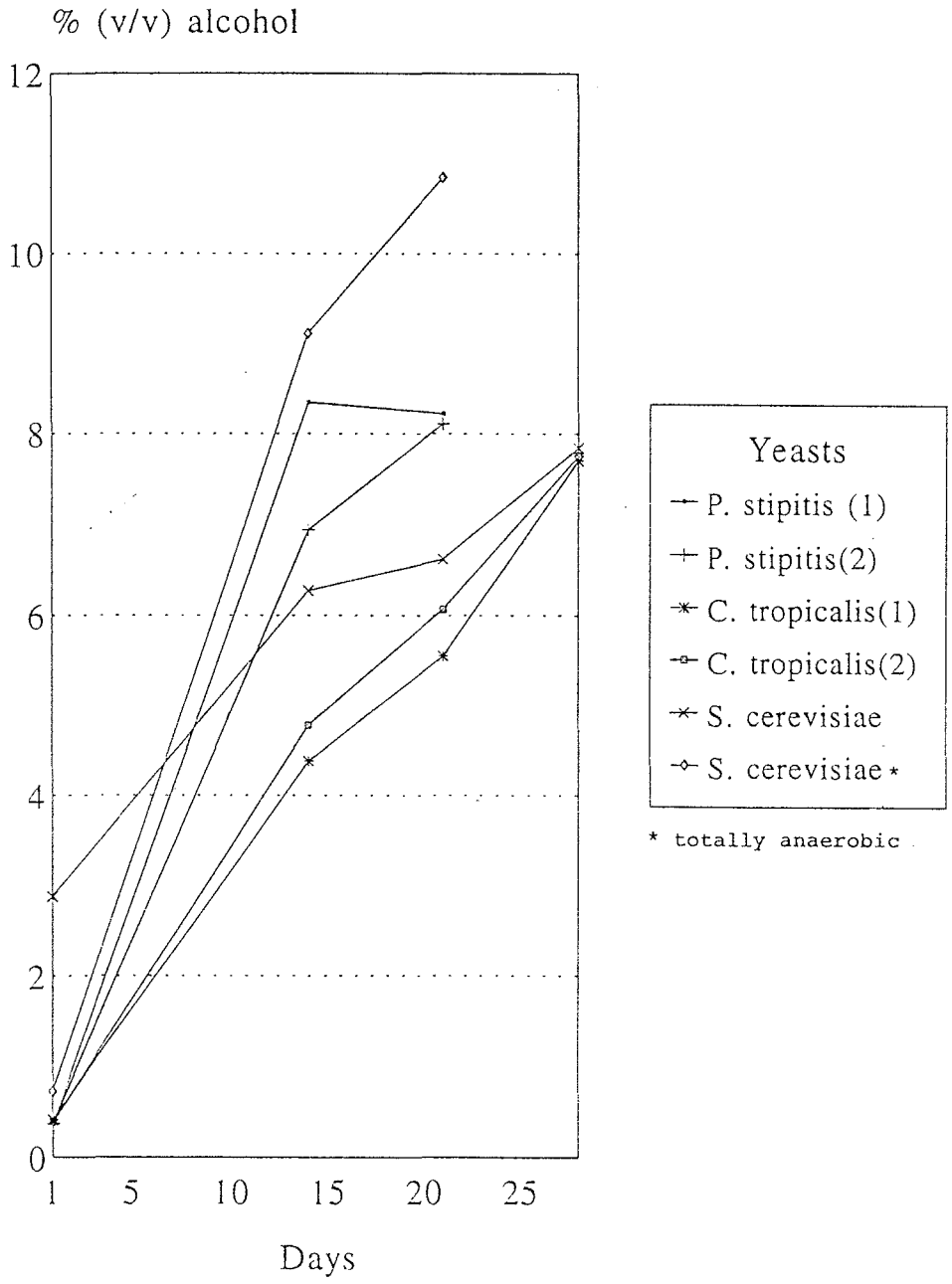

SACCHAROMYCES CEREVISIAE (B)
SECOND FERMENTATION
pH
TA (g/l)
3.4
3.35
3.3
3.25
3.2
3.15
3.1
3.05
11
10
9
8
7
6
5
4
3
0
24.5
72.5
118.5
144.5
187
TIME (hours)
pH
TA (g/l)

APPENDIX VI

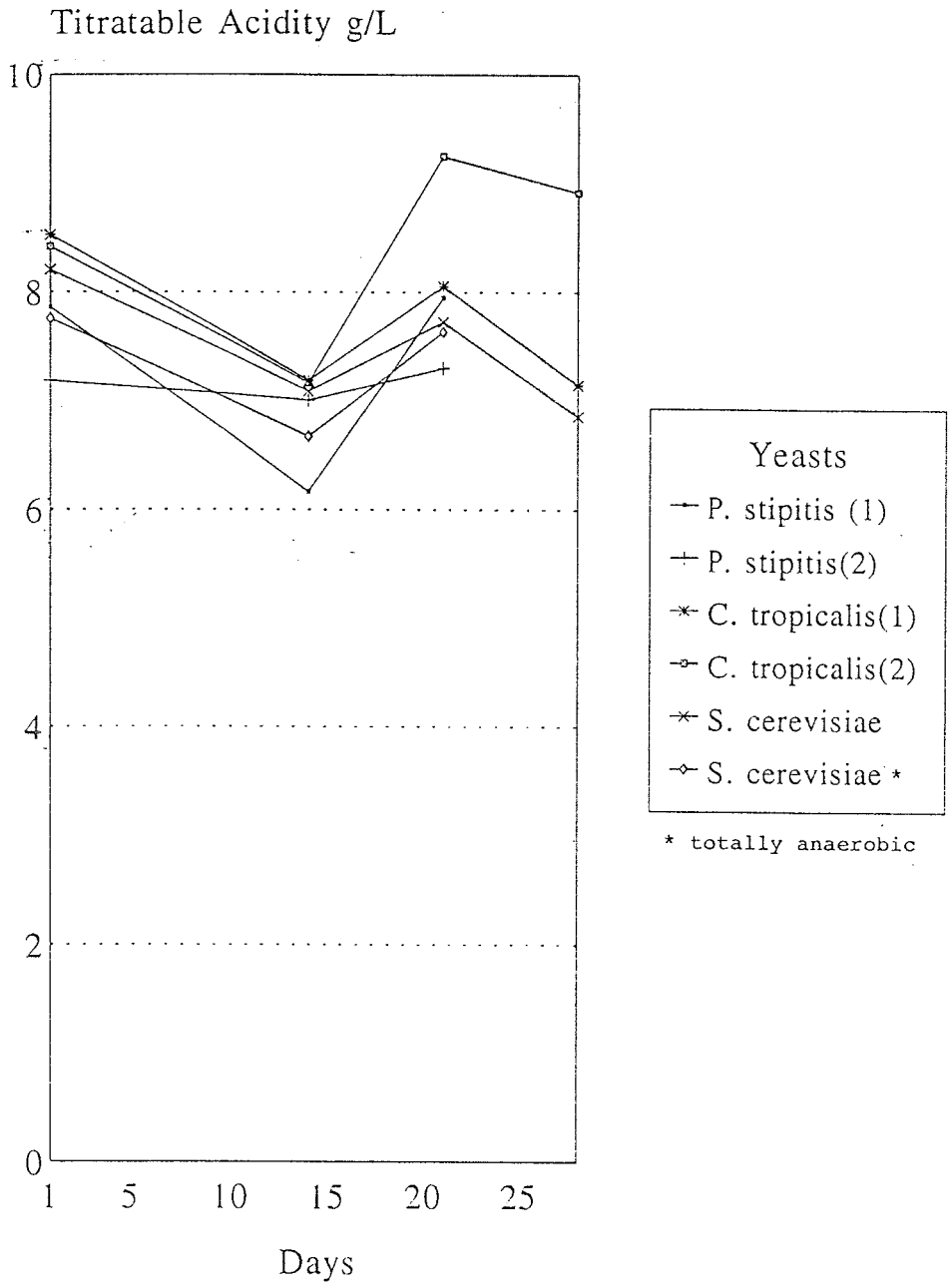
4.5.1 - Sugar during fermentation



4.5.2 - Alcohol during fermentation



4.5.3 - Titratable acidity during fermentation



4.5.4 - pH during fermentation