GLYCEROL PRODUCTION BY VARIOUS STRAINS OF SACCHAROMYCES CEREVISIAE

A Thesis presented in Partial Fulfilment
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ABSTRACT

The influence of yeast strain, fermentation procedure and media on cell growth and the production of glycerol and ethanol was studied. Two fermentation procedures were compared

(a) fermentation at a constant temperature of 15°C and

(b) fermentation at higher temperatures (15-20°C)

maintaining a constant rate of sugar utilization. Three wine-making yeasts and three high glycerol producing hybrid yeasts were fermented on two types of grape juice and a synthetic [control] media.

The effect of the fermentation procedure on glycerol, ethanol production and cell growth was variable and appeared to depend on the yeast strain. Comparison of the yeast strains showed glycerol production to vary considerably depending on the yeast this effect was also dependent on the media. The yeast strain is important for maximum fermentation efficiency in a specific grape juice.

Selective hybridisation of pure culture wine yeasts was employed to develop yeast strains capable of maximum glycerol yield, without jeopardising ethanol production in Muller Thurgau and in Chenin Blanc grape juices.

Improved yields were achieved, but those yeasts selected for fermentation in one type of grape juice did not give outstanding yields when fermented in the other type of grape juice. This suggests that for wine-making it is possible to tailor yeasts for fermentation in specific grape juices.

The addition of sulphur dioxide [0-300 ppm] and its influence on glycerol and ethanol production was studied using a wine-making yeast and a high glycerol producing hybrid. The effect was strain dependent and as expected, the addition of sulphur dioxide to the wine-making yeast showed enhanced glycerol production and depressed ethanol production. However, the converse was apparent with the high glycerol producing hybrid.

The addition of glycerol to the media prior to fermentation at levels of 0 to 20 g/l was tested in an attempt to simulate the

conditions of grapes attacked by the fungus Botrytis cinerea [noble rot]. No inhibition or stimulation of glycerol or ethanol production was apparent by either the wine-making yeast or the high glycerol producing hybrid yeast tested.

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AIMS OF THIS INVESTIGATION

The research work undertaken as part of this project involved the investigation into factors affecting glycerol production during alcoholic fermentation by Saccharomyces cerevisiae.

Glycerol is a major fermentation end product believed to contribute to the 'body' of a wine and may be associated with prolonging the stability of a wine kept in storage. Glycerol production has been shown to depend quantitatively on the yeast strain (Radler & Schutz 1981; Rankine & Bridson 1971); grape variety (Eschenbruch & Fisher, 1983; Rankine & Bridson 1971); temperature of fermentation (Ough et al 1972; Hickinbotham & Ryan 1948; Wootton et al 1983; Rankine & Bridson 1971) and of sulphur dioxide (Ough et al 1972; Rankine & Bridson 1971).

Areas investigated included:

(1a) **Comparative fermentation trials**

In different grape juices and in a synthetic completely defined medium at 15°C using three wine yeasts (MD26, AWI60, AWI80) and three hybrid yeasts (XGL74, XGL78, XGL81 - developed for their abilities to produce high levels of glycerol). Daily sampling of the fermenting juice and media provided information on sugar utilization as well as the production of glycerol and ethanol. Viable counts taken daily during the first half of the fermentation process allowed the comparison of cell growth with glycerol production.

(1b) As in (a) but, controlling the fermentation rate by altering the temperature in order to maintain a constant rate of sugar utilization (approximately 1-1½ Brix/day).

(2) **Sulphite addition**

Determination of the effect that the additional sulphite to the must has on glycerol production at the level of sulphur dioxide used in wine making. Comparative trials utilizing a synthetic medium and a Chenin Blanc grape juice using a wine yeast and a high glycerol producing hybrid yeast.

(3) **The effect of glycerol prior to fermentation**

To study the effect of glycerol, already present in the must prior to fermentation, on the production of glycerol producing

hybrid yeast (this experiment was an attempt to simulate the condition of grapes attached by the fungus Botrytis cinerea, thus already containing glycerol).

(4) **The effects of hybrid yeasts**

Running a hybridization programme, whereby, for three successive generations four lines of yeast strain were developed from two high glycerol producing yeasts. The strains were selected for each of the following categories:

(i) high glycerol production in Muller Thurgau grape juice

(ii) high glycerol and high ethanol production in Muller Thurgau grape juice

(iii) high glycerol production in Chenin Blanc grape juice

(iv) high glycerol and ethanol production in Chenin Blanc grape juice.

The aim was to achieve the maximum yield of glycerol possible, and the maximum yield of glycerol with no loss in ethanol production. This was carried out in two different grape juices.

INTRODUCTION

1. GLYCEROL IN WINES

Glycerol is a trihydroxyalcohol, which in its pure form, is a colourless, odourless, sweet-tasting viscous liquid. Since Pasteur's time it has been recognized as a major byproduct of alcoholic fermentation and is considered to be an important factor in the 'smoothness' and 'body' of wine, possibly increasing the stability of bottled wines. Thus a high level of glycerol is usually considered desirable. (Amerine & Joslyn, 1970).

There are two ways by which glycerol can be formed in wines:

- by the fungus Botrytis cinerea under cool and dry conditions forming 'noble rot'. Such botrytised graps contain glycerol, and wines may be produced from these containing glycerol levels of up to 20 g/l
- by yeasts during fermentation

However, the amount of glycerol produced during the fermentation process appears to depend on many factors, such as yeast strain, grape variety, fermentation conditions and temperature. Most dry red wines contain more glycerol than dry white wines (this may be due in part to the warmer temperatures required initially to extract the colour from red grapes, and to the higher sugar content of the red graps). Flor sherries contain much less glycerol than dry wines (as do bacterially contaminated wines), because the flor forming bacteria utilize glycerol. The same occurs in wines which have undergone a malo-lactic fermentation (Rankine et al 1971).

In experiments where different strains of S. cerevisiae are fermented under controlled conditions of pH, grape juice composition, temperature and sulphur dioxide level, it was shown that glycerol production is affected by the yeast strain used. It was hypothesised by Nordstrom (1968), that glycerol production is a reductive process, counteracting the oxidative processes of cell growth, which suggests that the majority of glycerol is produced during the initial stage of fermentation, when yeast growth is at a maximum. Although there has been a general acceptance of Nordstrom's hypothesis, further work by Radler & Schutz (1981) has led to another hypothesis whereby glycerol

production is in competition with ethanol production for the reduced co-enzyme NADH (Nicotinamide adenine dinucleotide).

Fermentation process conditions have also been implicated in the production of glycerol. It is believed that changes in the fermentation temperature, sulphur dioxide level and pH of the grape juice can influence the final level of glycerol produced.

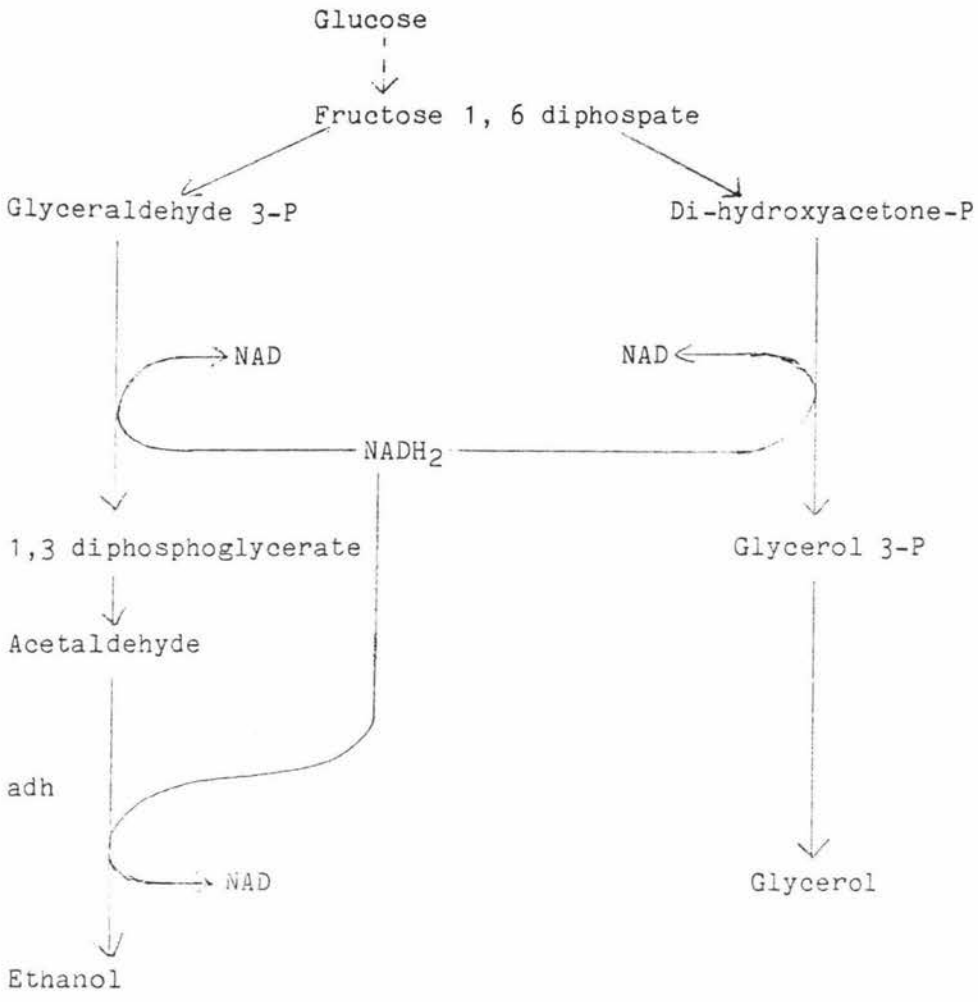
2. GLYCEROL PRODUCTION - ENZYMATIC PATHWAYS

Ralder & Schutz (1981) proposed the following hypothesis for glycerol production based on competition between alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase for NADH.

Glycerol is formed from sugar during glycolysis to ethanol and carbon dixoide (refer below). Fructose 1,6 diphosphate is formed from hexose sugar and is split by aldolase to form a mixture of 95% dihydroxyacetone phosphate (DHAP) and 5% glyceraldehyde-3-phosphate (GA3P). Although GA3P is present in smaller quantities it is preferentially oxidized and phosphorylated by glyceraldeyde-3-phosphate dehydrogenase (GA3Pdh) in the presence of NAD to 1,3 diphosphoglyceric acid (1,3 DPGA) with the release of NADH. The 1,3DPGA is then dephosphorylated to 3-phosphoglyceric acid (with the release of ATP) and is eventually converted through pyruvic acid to acetaldehyde.

In the presence of the above formed NADH, acetaldehyde is reduced by alcohol dehydrogenase (adh) to ethanol (with the release of NAD).

Glycerol is derived from DHAP which is enzymatically reduced using NADH to glycerol 3-phosphate (G3P) and is then converted by a phosphatase to glycerol:



Experimental evidence supports this theory, although in many cases factors such as the regulation of the enzyme by ions or metabolites present in the media need be considered. Correlations have been observed between high G-3Pdh activity and high glycerol production, as well as the converse. However, further research on enzyme activities and their regulation is necessary before firm conclusions can be reached.

3. INFLUENCE OF GRAPE VARIETY AND YEAST STRAIN ON GLYCEROL PROCUCTION

The typical yeast strain has been implicated in playing a major role in the production of glycerol. Under identical conditions of fermentation (i.e. similar inoculum size, pH, grape variety, sulphur dioxide level and temperature) different yeast strains have been distinguished as high or low glycerol producers. In addition, the grape variety and its stage of maturity influences the level of glycerol production (Hickinbotham & Ryan, 1948; Rankine & Bridson 1971; Ough et al 1972).

While there appears to be a correlation between high sugar levels in the must, and high levels of glycerol produced (Radler & Schutz 1981) the relationship appears complex. Some yeast strains appear to vary in terms of their sugar to glycerol conversion efficiency when fermented in different grape juices. The variation in efficiency of conversion even occurs when the different grape varieties are of the same sugar level (Rankine & Bridson 1971). A correlation between stage of maturity and level of glycerol production is supported by the results of Rankine & Bridson (1971). This may be the result of higher sugar and nutrient levels associated with fully mature fruit.

As previously stated the yeast strain has an important influence on glycerol production. Radler & Schutz (1982) observed that a high level of acetaldehyde produced early in the fermentation process is associated with a high yield of glycerol. They hypothesised from this observation that high acetaldehyde levels result in more NADH being made available for glycerol production, whereas low glycerol producing yeasts were associated with a lower initial level of acetaldehyde, and hence, less NAD.

Despite the variations observed when comparing the levels of glycerol produced by different yeast strains in different grape juices, the amount produced under wine-making conditions fluctuated very little (i.e. 1/10 to 1/15 of the amount of ethanol formed), regardless of the grape juice's sugar content.

Why yeasts produce glycerol at all is uncertain, Nordstrom (1968) has proposed that while yeast growth is an oxidative process during anaerobic fermentation, a reductive process (i.e. glycerol production) is necessary to maintain the redox balance within the cell.

4. FERMENTATION CONDITIONS AND GLYCEROL PRODUCTION

It is widely accepted that glycerol production is influenced by the fermentation conditions. Research workers have studied the effect of varying such conditions as the fermentation temperature, sulphur dioxide level, pH, grape sugar level, yeast type, quantity of inoculum and the quantities of growth factors in the medium. (Rankine & Bridson, 1971; Ough et al, 1972; Gentilini & Cappelleri, 1950).

The effect of the fermentation temperature on glycerol production is controversial. Claims have been made that higher fermentation temperatures reduce glycerol production, however, most research indicates glycerol production increases with temperature.

Most winemakers favour lower fermentation temperatures (i.e. 15°C) for the production of white wines, thus reducing the loss of volatile components and minimising the activity of contaminating micro-organisms. Red wines are fermented at higher temperatures for better colour extraction from the grapes and improved flavour. Glycerol yields also tend to be higher.

An hypothesis has been put forward by Rankine & Bridson (1971), suggesting higher glycerol yields are associated with higher fermentation temperatures due to increased phosphatase activity whigh dephosphorylates α-glycerophosphate to glycerol. This has not been substantiated experimentally.

Investigation into glycerol production is complicated by the fact that glycerol formed in laboratory scale fermentations tends to be less than that produced in commercial scale opertions (Hickinbotham & Ryan, 1948).

The effect of pH on glycerol production has been studied, in relation to the addition of sulphur dioxide. Neutral and alkaline pH levels cause high levels of glycerol production. Rankine & Bridson (1971) suggested that the effect of pH on glycerol formation is a result of dismutation of ethanol, acetaldehyde and acetic acid rendering acetaldehyde less available at high pH levels. Addition of 100 ppm sulphur dioxide at pH levels of 3.3-3.8 typically winemaking conditions, has also been associated with increased glycerol production.

The influence of grape variety on glycerol production has been considered in terms of variation in the levels of micronutrients in the different grape juices (of comparable sugar levels). Experimental evidence suggested a correlation between glycerol production and the presence or absence of certain micronutrients (Rankine & Bridson, 1971, Radler & Schutz, 1981).

Although pH, sulphur dioxide level, oxygen availability and fermentation temperature influence glycerol formation within the

'normal' conditions of fermentation these factors are not important. More significant changes can be included in winemaking procedures by altering the yeast genotype and selecting yeast strains with high glycerol-3-phosphate dehydrogenase activity.

5. SULPHUR DIOXIDE AND ITS INFLUENCE ON GLYCEROL PRODUCTION

Sulphur dioxide is important in winemaking due to its ability to impart:

- antimicrobial activity against spoilage micro-organisms
- antioxidant properties
- binding of free acetaldehyde to give a fresher flavour in white wines
- assistance in colour extraction from red grapes

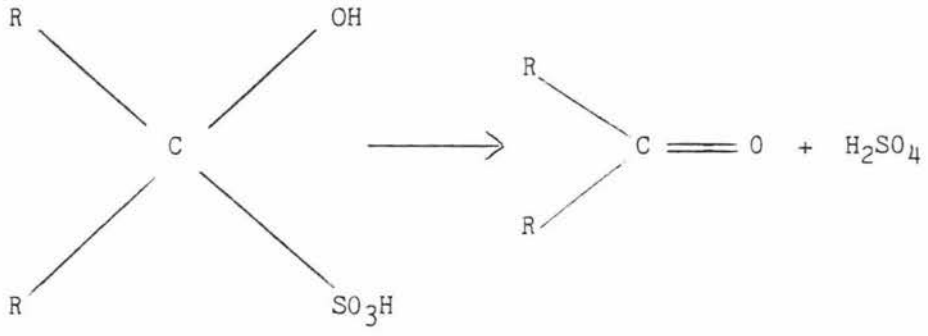
These functions require the presence of free sulphur dioxide as much of the sulphur dioxide added is bound to substances such as acetaldehyde, pyruvate and sugars present in grape juice. Since there are legal limitations* on the total sulphur dioxide content (i.e. free + bound SO_2) of wines, the nature of the sulphur dioxide binding substances and the levels present are of practical importance.

* Legal limit of sulphur dioxide in wines (NZ Food Reg, 1984) -

(i) 200 ppm in wines with a residual sugar level (calculated as sucrose) of not more than 5 g/l

(ii) 300 ppm in wines with a residual sugar level of more than 5 g/l but not more than 30 g/l.

Work by Burroughs & Sparks (1972, 1981) suggested that the sulphur dioxide binding substances identified in white wines contains either one or two carbonyl groups which can react reversibly with sulphur dioxide to form carbonyl bisulphite compounds (hydroxysulphonic acids) e.g. acetaldehyde bisulphite. This reaction reaches an equilibrium depending on the pH, temperature and concentration of the components concerned:



Grape juice contaminated with wild yeast or bacteria may contain high levels of sulphur dioxide binding substances (especially acetaldehyde) which will consume any free sulphur dioxide present and leave insufficient free to protect the wine. Thus it is desirable to keep the wine as bacteria-free as possible, keeping sulphur dioxide binding substances, particularly acetaldehyde, to a minimum.

The various forms of sulphur dioxide vary in their toxicity to micro-organisms (Rankine & Pocock 1971):

H_2SO_3 - very toxic

HSO_3^- - toxic

SO_3^- - non-toxic

bound SO_2 - almost non-toxic

The form of sulphur dioxide in the wine is strongly influenced by the pH of the wine - at low pH H_2SO_3 is prevalent, while at high pH the non-toxic SO_3^{2-} form is favoured.

Prior to fermentation, the sulphited must is reduced so that the sulphur dioxide content is less than 50 ppm (higher levels can be

detected organoleptically in the finished product), to prevent problems being encountered in initiating fermentation. Again, prior to bottling, the sulphur dioxide level is checked to ensure sufficient (both free and bound) is present to preserve the wine - while maintaining levels within the legal limit and ensuring that it can not be detected organoleptically.

The addition of bisulphite to high pH fermentations held at high pH's have been employed commercially to produce glycerol. By binding acetaldehyde, the NADH is made available for glycerol-3-phosphate dehydrogenase to convert dihydroxyacetone phosphate to glycerol-3-phosphate which is then dephosphorylated to glycerol (refer p. 6-7). The quantity of sulphite used is about one hundred times that used in wine making, and the pH is alkaline. Glycerol production in winemaking conditions appears little influenced by the relatively low sulphite addition to the grape juice. (Ough et al 1972).

6. GLYCEROL PRODUCTION IN BOTYRYTISED GRAPES

It is widely accepted that glycerol production is influenced by grape variety and stage of maturity, and has found to be associated with the levels of sugar and micronutrients present in the grapes (Rankine & Bridson, 1971).

Glycerol production may also be influenced by the amount of glycerol present in the grapes prior to fermentation. High levels of glycerol (up to 20 g/l) have been recorded, prior to fermentation in grapes attacked by the fungus Botrytis cinerea. These initial glycerol levels may enhance or repress production of glycerol by the winemaking yeasts used in the fermentation process.

7. SELECTIVE HYBRIDIZATION TO IMPROVE GLYCEROL PRODUCTION

Selective hybridization has been used to develop wine yeast strains of improved fermentation qualities (Thornton 1982), and to minimise the undesirable characteristics such as H_2S formation (Eschenbruch et al 1982). The efficiency of grape sugar to ethanol conversion has been improved using this technique (Thornton 1982) as has the efficiency of conversion to glycerol (Eustace & Thornton, in press).

Hybridization uses the haploid stage of the yeast life cycle, and allows haploid cells derived fromd ifferent strains of S. cerevisiae to be mated thus giving rise to a new diploid yeast strain with properties derived from both parent strains. Multigenic properties such as the conversion of sugar to ethanol or glycerol can then be improved.

Sporulation can be induced in diploid yeast cells. This meiotic process gives rise to the formation of four haploid ascospores within an envelope, the complete structure being called an ascus. Two of the ascospores are of a mating type and two of α mating type. The ascus is dissected and the ascospores isolated by micromanipulation (Thornton, 1982). On nutrient media the ascospores germinate to form haploid clones. mating type is determined by zygote formation when each spore clone is mixed with a and α mating tester strain. Only strains of opposite mating type, i.e. a and α can form zygotes.

Genetic variation may be readily detected in haploid strains since only one copy of each chromosome and thus, each gene, is present.

Fermentation trials using haploid strain segregants permit the selection of those strains with the most desirable properties (i.e. greatest production of glycerol, or greatest production of glycerol with high ethanol production also).

Selected haploid strains can be crossed with haploids selected from other parent strains. The diploid strains formed have properties derived from both parent strains. This provides a broad gene pool of the hybrid yeast enabling undesirable properties to be eliminated out, and in addition allows for the development of yeasts capable of glycerol yields greater than either parent strain.

Radler & Schutz (1981) suggested that glycerol is produced at the expense of ethanol. It is considered desirable in this hypothesis to select for strains which product high levels of glycerol, with no loss in efficiency of ethanol production, as well as for strains which are selected only for their abilities to produce high levels of glycerol.

MATERIALS AND METHODS

1. GENERAL MEDIA AND YEAST STRAINS

1.1 Media components

1.1.1 Complete defined medium (CDM)

Major mineral solution

Glucose	100 g
NH_4Cl	1 g
$CaCl_2.2H_2O$	0.7 g
KCl	0.6 g
Na_2SO_4	0.3 g
$MgCl_2.6H_2O$	0.4 g
$Na_2HPO_4.7H_2O$	1.8 g
Citric acid	1.8 g

Made up to 1 litre with distilled water

Minor mineral stock solution

H_3BO_3	50 mg
$MnCl_2.4H_2O$	46.8 mg
$FeCl_3.6H_2O$	20 mg
$Na_2MoO_4.2H_2O$	20 mg
ZnCl	19 mg
KI	10 mg
$CuCl_2.2H_2O$	2.7 mg

Made up to 100 ml with distilled water

Vitamin stock solution

Inositol	200 mg
Thiamine HCl	40 mg
Pyridoxine HCl	40 mg
Biotin	0.2 mg
Calcium pantothenate	20 mg
Folic acid	4 mg
Vitamin B12 (cyanobalamin)	10 mg

Made up to 100 mls with distilled water, filter sterilized and stored at 4°C.

1 ml Minor mineral stock solution was added to 1 litre of Major mineral solution, the pH is adjusted to 4.2 with 10M HCl. The

medium was autoclaved at 105 kPa (15 psi) for 5 minutes. To the sterile Complete mineral media 1 ml vitamin stock solution was added.

1.1.2 Yeast morphology agar (MYGP)

Difco malt extract	3 g
Yeast extract	3 g
Difco Bacto-Peptone	5 g
Glucose	10 g
Agar	20 g

Made up to 1 litre with distilled water. Autoclaved at 105 kPa (15 psi) for 15 mins.

1.1.3 Glucose nutrient agar (GNA) - presporulation media

Difco nutrient agar	2.3 g
Yeast extract	1 g
Glucose	5 g
Agar	0.5 g

Made up to 100 ml with distilled water. Dispensed into clean bijou bottles autoclaved at 70 kPa (10 psi) for 10 mins and slanted before cooled.

1.1.4 Potassium acetate (PA) - sporulation media

Potassium acetate	1 g
Yeast extract	0.25 g
Agar	3 g

Made up to 100 ml with distilled water. Dispensed into clean bijou bottles, autoclaved at 70 kPa (10 psi) for 10 mins and slanted before cooled.

1.2 Yeast strains and maintenance

The yeast strains used in this study are listed in Table 1. They were grown up on MYGP agar at 30°C for 48 hours and maintained in the haploid or diploid vegetative state on MYGP agar at 4°C. The yeasts were sub-cultured at intervals of 2-4 months.

TABLE 1

YEAST INDEX (all are strains of Saccharomyces cerevisiae)

Strain	Source	Glycerol Prodn.
MD26	A commercial wine yeast from Montana Wines (NZ) Limited	Low
AWI60)	Wine yeasts from the Australian	Low
AWI80)	Wine Research Institute	Low
XGL74)	Hybrid yeasts developed in this	High
XGL78)	Laboratory for their ability to	High
XGL81)	produce high levels of glycerol	High
XGA)	Yeasts developed in the first generation	High
XGB)	of the hybridisation programme in this	High
XGC)	project.	High
XGD)	Yeasts developed in the second	High
XGE)	generation of the hybridisation	High
XGF)	programme in this project	High
XGG)		High
XGH)	Yeasts developed in the third	High
XGI)	generation of the hybridisation	High
XGJ)	programme in this project	High
XGK)		High
Sl-α)	Haploid yeast strains developed in this	-
)	Laboratory to determine the mating types	
Sl-a)	of unknown haploid strains	-

2 ANALYTICAL TECHNIQUES

2.1 Sugar analysis by hand-held Refractometer

Sugar levels were measured using an Atago hand-held sugar refractometer capable of measuring sugar levels from 0 to 32° Brix with an accuracy of ± 0.2° Brix.

The sugar refractometer measures the critical angle of refraction of the sugar solution under test. A small volume sample was placed on the prism surface of the refractometer and on looking through the eyepiece of the refractometer, a boundary line which indicates the refractive index of the solution formed. The refractive index of the solution is dependent on the sugar concentration, which was measured by use of a scale (° Brix) in the refractometer. Sugar readings were corrected to 20°C.

High levels of alcohol present in the sample (i.e. more than 5% (w/v) were responsible for some inaccuracy in sugar levels measured by this technique.

2.2 High performance liquid chromatography (HPLC)

The simultaneous analysis of fermentation products by HPLC provided a faster and more accurate determination than could be achieved by enzyme analysis for individual components.

2.2.1 HPLC analysing equipment

A Shimadzu High Performance Liquid Chromatography System was used, consisting of an LC4A systems controller, a refractive index Detector (Model RID-2AS), an automatic sampler and a Chromatopac (Model C-R3A. The column employed was a Bio-Rad model 87H analytical column used in conjunction with a Brownlee Guard column.

2.2.2 Operating conditions

Glucose, fructose, ethanol and glycerol were passed through the column with a mobile phase of degassed (0.004 M) sulphuric acid (diluted in distilled, deionised water). The sample injection volume was 5 µl, which was passed through the column at a flowrate of 0.8 ml/minute, and a temperature of 65°C. The refractive index detector was set at a range of 4. The chromatopac chart speed was set on 0.5

cm/minute, with a peak attenuation of 1. The sample run time was 20 minutes.

2.2.3 Procedure: Preparation of standards for calibration

For days 1 to 9 of the fermentation, a high standard solution containing 3.5% glucose, 3.5% fructose, 0.25% glycerol, and 3.0% ethanol (all w/v) was prepared. From this a low standard was also made by using a 1:1 dilution of the high standard in deionised, distilled water. For days 10 to 25 of the fermentation, a high standard solution was prepared containing 0.2% glucose, 0.2% fructose, 0.5% glycerol and 4% ethanol (all w/v). The low standard was prepared as above by diluting the high standard 1:1 with deionised, distilled water.

2.2.4 Sample preparation

All samples were diluted 1:2 with distilled, deionised water. Both samples and standards alike were filtered prior to analysis.

2.2.5 Sample analysis

After calibrating the HPLC, the calibration data was stored in the memory of the Chromatopac. Prior to analysis each day, 5 µl of low standard, followed by 5 µl of high standard were injected and their chromatograms recorded. If the areas recorded per component under each peak, and subsequent factors calculated (to convert area into concentration) were within acceptable limits of those figures obtained during the calibration, filtered juice samples of 5 ul volume were injected into the column by the autosampler.

Concentration (unknown) = peak area (unknown) x factor.

Sample components were analysed in the order of glucose, fructose, glycerol and ethanol.

Figure 1 shows an example of a chromatogram with the high standard used for days 1 to 9 of the fermentation, and of a sample of partially fermented Chenin Blanc grape juice.

FIGURE 1 : Chromatograms of

(a) Standard Sample for Days 1 to 9 of the Fermentation Process

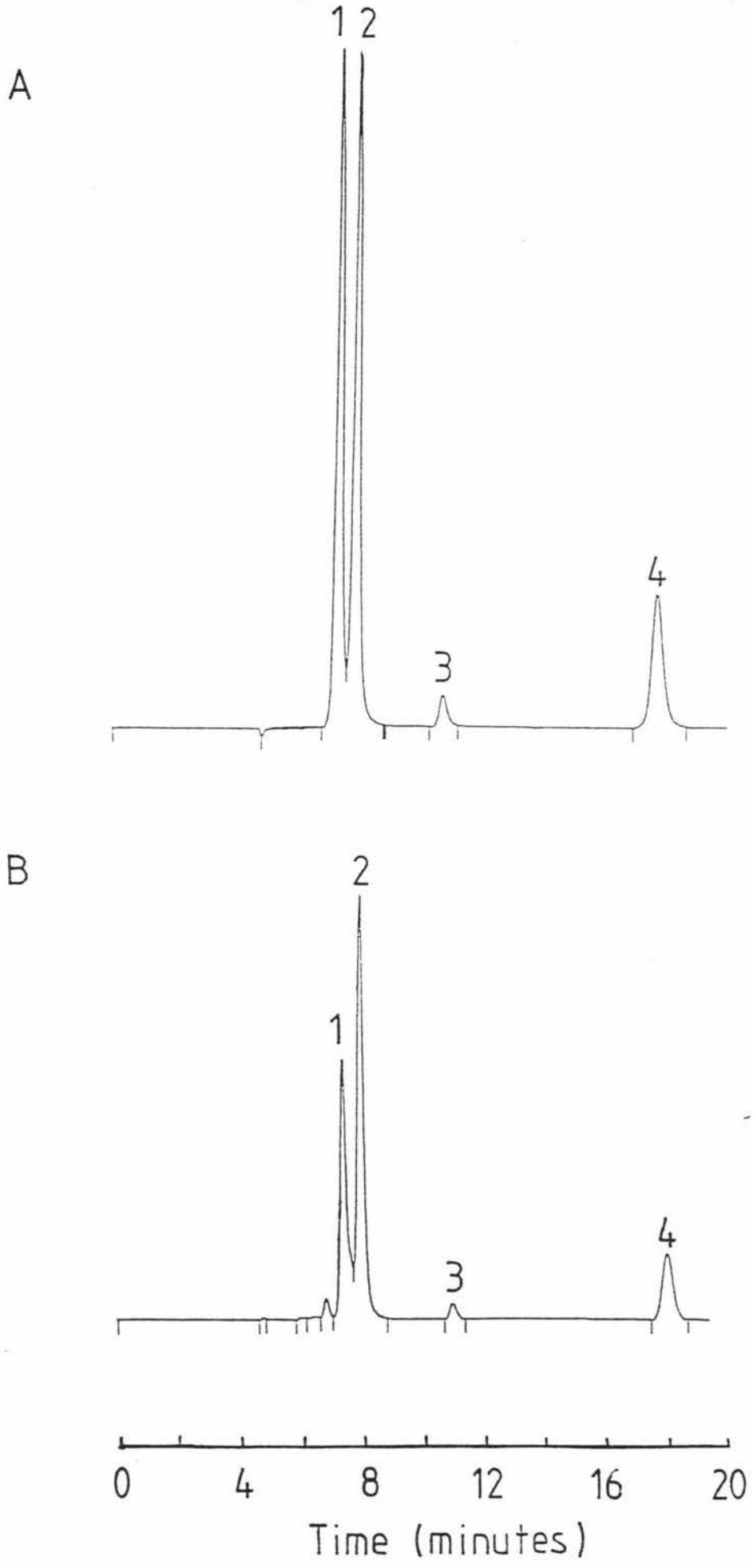
(b) Partially Fermented Sample of Chenin Blanc Grape Juice

Identified Peaks : 1 - Glucose

2 - Fructose

3 - Glycerol

4 - Ethanol


A
1
2
3
4
B
1
2
3
4
0
4
8
12
16
20
Time (minutes)

2.3 Sulphur dioxide analysis

2.3.1 Reagent preparation

Indicator

- Methyl Red 0.1 g
  Made up to 100 mls with 95% ethanol and filtered through Whatman's No.1 filter paper.
  Red colour = acid, yellow colour = alkali

- Hydrogen peroxide solution (prepare daily)

10 volume 3% H_2O_2	10 ml
Distilled water	80 ml
Methyl Red Indicator	0.5 ml

  0.01N NaOH added dropwise until solution just turns yellow. Then make up to 100 mls with distilled water.

- Phosphoric Acid solution

90% orthophosphoric acid	280 ml
distilled water	720 ml

- 0.01 N NaOH
  prepare daily from a 0.1N stock solution

2.3.2 Procedure

The technique used was developed by Rankine and Popcock in 1972. A 10 ml aliquot of the yellow 30% hydrogen peroxide solution was pipetted into a 50 ml round bottom flask, and connected to the bubbling tube of the apparatus (refer Plate 1).

A 20 ml aliquot of sample of the must or wine was pipetted into another 50 ml round bottom flask, followed by 10 ml of the 25% phosphoric acid solution. This was connected to the apparatus below the condenser as illustrated (refer Plate 1).

The water jet pump was turned on to allow air to be drawn through the apparatus at a rate of approximately 1 litre per minute. Aspiration at this flowrate was continued for 10 minutes.

If free sulphur dioxide was present the hydrogen peroxide solution should turn red. The flask was removed from the bubbler which was

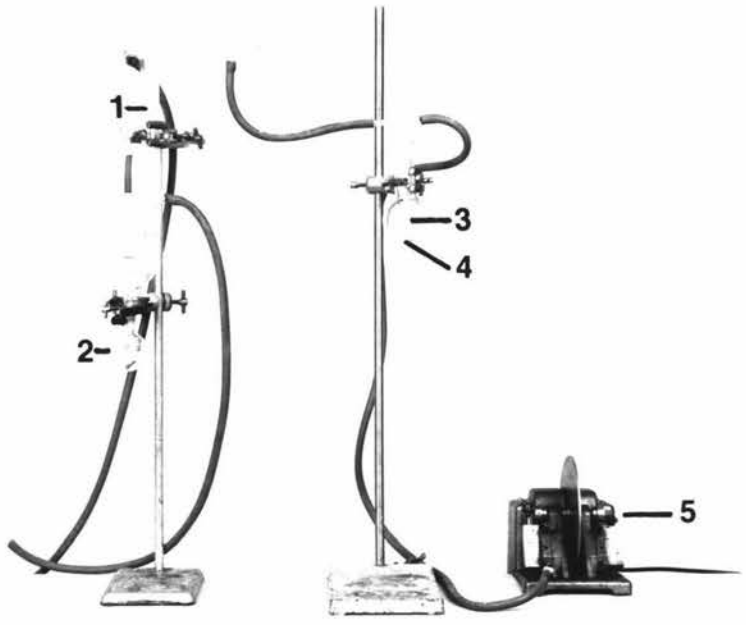


PLATE 1 : Sulphur Dioxide Analysis Apparatus

Key :-

1 - condenser
2 - flask containing sample
3 - bubbling tube
4 - hydrogen peroxide solution
5 - pump

rinsed into the flask with a little distilled water. The solution was titrated with the 0.01N sodium hydroxide, swirling it continuously to ensure the sodium hydroxide was well mixed, until the indicator just returns to its initial yellow colour. Bound sulphur dioxide (i.e. total sulphur dioxide - free sulphur dioxide) was determined on the same sample by using a fresh hydrogen peroxide indicator solution, and aspirating for 10 minutes at a flowrate of 1 litre per minute, while boiling the sample using a small flame. The solution was titrated as above.

2.3.3 Calculation to determine levels of SO_2 present in samples

1 ml 0.01 N sodium hydroxide = 0.32 mg sulphur dioxide, thus for the 20 ml wine sample the titration figure in mls is multiplied by 16 giving the sulphur dioxide level in mg/1 (ppm).

The total sulphur dioxide could be determined by adding the results for the free and bound sulphur dioxide levels, or in one operation by aspirating for 10 minutes a fresh 20 ml wine sample while boiling to release the bound and free sulphur dioxide.

2.4 Viable counting technique

Reagents:

0.1% Methylene Blue Stain solution

Methylene Blue (0.01 g) was dissolved in 10 ml distilled, deionised water, then add and dissolve Sodium citrate dihydrate (2g). Filter the solution and dilute the filtrate to 100 mls with distilled, deionised water.

Procedure:

2 ml of yeast suspension was diluted with an approximate volume of stain to give a total of 75-100 yeast per 1 mm^2 x 0.1 mm volume. To prepare the cells for counting, the yeast suspension was diluted as required, the mixture vortexed then left to stand for several minutes allowing the dead cells to take up the stain.

Cells were vortexed again to avoid clumping, and examined microscopically in the counting chamber.

When counting, mother cells with attached buds were counted as one cell only. Clumps of cells, even if only one cell is alive, were regarded as a single live cell, unless all the cells are dead.

Calculation:

$$\% \text{ of viable cells} = \frac{\text{no. of unstained cells}}{\text{total no. of cells}} \times 100$$

2.5 Statistical analysis for comparative fermentation trials

Fermentation trials were carried out in duplicate, using 3 winemaking (parent) yeast strains and 3 high-glycerol producing hybrid yeast strains. Statistical analysis to compare the production of glycerol and ethanol by these two yeast groups under a range of fermentation conditions, employed the 'Student's' t-test (Bailey 1981) for comparing the means of two small samples. This test was applied with $n_1 + n_2 - 2$ degrees of freedom and a rejection of 0.001 using the equation:

$$t = \frac{x_1 - x_2}{s\sqrt{\frac{1}{n_1} \times \frac{1}{n_2}}}$$

$$s = \sqrt{\frac{(n_1 - 1)\, s_1^2 + (n_2 - 1)\, s_2^2}{n_1 + n_2 - 2}}$$

x_1 and x_2 the mean values of glycerol (or ethanol) production from each group of yeasts. S_1 and S_2 the standard deviations of the production of each group. Differences in ethanol or glycerol production were considered significant at $P \leq 0.001$ if the calculated t-value was greater than the P value from the 'Student's' t-distribution.

3 EXPERIMENTAL TECHNIQUES

3.1 Fermentation procedure

Laboratory-scale fermentations were carried out maintaining similar conditions of pH, sulphur dioxide level and temperature to those employed for commercial wine-making.

The grape juices used were Vitis vinifera var. Chenin Blanc (CB), and Muller Thurgau (MT). In three of the experiments a synthetic medium referred to as Complete Defined Medium (CDM) was used as a control. Sugar levels of the media were recorded using a sugar refractometer or High Performance Liquid Chromatograph as indicated. Fermentations were carried out both aerobically and anaerobically as indicated in conical flasks.

3.1.1 Reducing the sulphur dioxide level of grape juice

The levels of sulphur dioxide (ppm) in the grape juices were measured as total (and free) using the technique described in Method 2.3. Hydrogen peroxide was added (0.075 mls of 30% H_2O_2 reduced the sulphur dioxide level control in grape juice by 40 ppm/l/day) to reduce the sulphur dioxide to 0-50 ppm, unless stated otherwise. The amount of total sulphur dioxide was again checked to ensure it was at the correct level.

3.1.2 Preparation of the media

pH and sugar levels of the media were recorded but not altered.

The grape juice and sterile complete defined medium was poured aseptically into sterile conical flasks (100 ml of medium into 150 ml flasks or 200 ml of medium into 250 ml flasks) and 8 mls was pipetted aseptically into sterile test tubes to build up the inoculum (1 test tube is used to inoculate 1 flask).

3.1.3 Yeast strains

Six strains of Saccharomyces cerevisiae have been used in these fermentation experiments

MD26 (1)	AWI60 (2)
AWIBO (3)	XGL74 (4)
XGL78 (5)	XGL81 (6)

The latter three yeasts are exceptionally high glycerol producing hybrids, which also produce relatively high levels of ethanol.

A large loopful of the yeast strain to be used was inoculated into 50 mls of MYGP broth and incubated at 30° or an orbital shaking platform for 48 hours.

The inocula were thoroughly mixed in the broth and 2 ml aseptically pipetted into a sterile test tube containing 8 ml of the fermentation medium. The inoculated test tubes were then incubated at 30°C for 24 hours.

3.1.4 Initiation of fermentation

The prepared test tubes were vortexed and the contents added to the media in the conical flasks. The inoculated flasks were placed in incubators at the required temperatures and left to stand until fermentation was complete. Fermentation to dryness was tested for by using 'Diastix' diabetic sugar indicator papers. (Miles Laboraties Inc, USA).

3.1.5 Sampling procedure

Some of the experiments required regular sampling of the media throughout the fermentation. Samples of 2 to 3 mls were taken aseptically using sterile pasteur pipettes for storage in bijou bottles (at -16°C so as to minimize loss of volatile components) prior to analysis. Samples were then prepared and analysed by HPLC as described in Method 2.2.

3.1.6 Constant temperature fermentation

This experiment was designed to study the production of glycerol, as a function of time, during the course of anaerobic fermentation at 15°C. All six of the yeast strains described in 3.1.3 were inoculated, in duplicate, into each of the Muller Thurgau and Chenin Blanc grape juices and into the Complete Defined Medium as a control.

The anaerobic fermentations were carried out using sterile glass fermentation traps containing sterile distilled water, which were inserted using rubber bungs into all 36 250 ml conical flasks.

Samples were taken from each flask at the same time each sampling day, until the fermentations were complete (after 25 days).

Samples were prepared for and analysed by HPLC and levels of sugar, glycerol and ethanol were recorded.

3.1.7 Constant sugar utilization fermentation

This experiment was carried out as a comparison with the constant temperature fermentation, to determine whether or not glycerol production is influenced by temperature. The experimental procedure was identical to that used for the Constant Temperature Fermentation (Method 3.1.1), except that the temperature of incubation, for the 36 flasks, was monitored on a daily basis in an attempt to keep the rate of sugar utilization at approximately 1.5 Brix per day. Approximate sugar levels were checked using a sugar refractometer (Method 2.1). The temperature profile used for the fermentation was as follows:

Day 0 Fermentation commenced at 25°C
Day 1 Temperature lowered to 15°C
Day 6 Temperature raised to 16°C
Day 7 Temperature raised to 19°C
Day 9 Temperature raised to 25°C

The experiment was used to determine if relationship between glycerol production and sugar utilization existed, and can also indicate whether the final level of glycerol and/or alcohol produced is influenced by the fermentation procedure.

The samples were prepared over a period of 20 days and analysed by HPLC (as in Method 2.2) for sugar, glycerol and ethanol.

3.2 Effect of sulphur dioxide on glycerol production

To determine the effect of sulphur dioxide on an efficient glycerol-producing strain of yeast (XGL81), the yeast was inoculated into two series of 250 ml flasks, containing either, sterile Chenin Blanc grape juice (filtered through 0.45 u filter paper) or complete defined medium. The media were set at sulphur dioxide levels of 0 ppm, 50 ppm, 100 ppm, 150 ppm and 200 ppm in triplicate. Levels of both free (i.e. active sulphur dioxide) and bound sulphur dioxide were recorded in each case.

The Chenin Blanc was reduced to 0 ppm sulphur dioxide by the addition of hydrogen peroxide, then quantities of the Chenin Blanc and the complete defined medium were set aside and raised to the appropriate sulphur dioxide levels by the addition of sodium metabisulphite (the action of 200 mg/l sodium metabisulphite raised the sulphur dioxide level of the media by 100 ppm). The 24 flasks were then prepared for anaerobic fermentation by placing sterile glass fermentation traps containing sterile distilled water on the inoculated flasks.

Prior to inoculation, the addition of 0.01% by weight sterile Kieselguhr was added to each flask. The addition of particulate matter is believed to aid in the circulation of nutrients for yeast metabolism.

The fermentations were carried out under identical conditions at 15°C. This flasks with 100 ppm sulphur dioxide, or less, took 4 weeks to ferment to dryness. Those containing more took one to two weeks longer.

Samples were taken at the start of the fermentation and after the fermentation was complete. Prior to fermentation, pH and sugar levels were recorded. Samples were analyzed for sugar, ethanol and glycerol by high performance liquid chromatography.

3.3 Effect of glycerol (present in the media prior to fermentation) on glycerol production

Glycerol is not normally present in grape juice prior to fermentation unless it has been contaminated by bacteria or fungi, e.g. botrytised grapes. This experiment examined the effect of glycerol present in the medium prior to fermentation, at certain levels i.e 0 g/l, 5 g/l, 10 g/l, 15 g/l and 20 g/l, on the production of glycerol and ethanol during fermentation.

After reducing the sulphur dioxide level to 0-50 ppm, appropriate quantities of 99% Sigma grade glycerol were weighed into 500 ml volumetric flasks and made up to the mark with Chenin Blanc or complete defined medium as appropriate. Samples of 100 ml from each of these five concentrations were poured, in duplicate, into 150 ml conical flasks in preparation for aerobic fermentation.

The inocula were prepared using two yeast strains; MD26 (a relatively low glycerol producing yeast) and XLG81 (a very high glycerol producing yeast). These two yeasts were inoculated, in duplicate, into the ten different media and the fermentations carried out aerobically at 15°C, using cotton wool bungs to prevent contamination.

3.4 Yeast hybridisation

Yeast hybridisation by micromanipulation was employed to modify the properties of Saccharomyces cerevisiae to produce diploid strains capable of producing large amounts of glycerol and ethanol.

This technique takes advantage of the heterothallic yeast life cycle of the strains used here to select for high levels of glycerol. Haploid strains from different parent diploid strains are mated to form a diploid strain with properties different from that of either parent strain. However, homothallic wine yeasts, i.e. those with the ability to switch their mating type from a to α and vice versa following spore germination, create a problem as haploid cells are necessary for hybridisation. This is overcome by direct spore to cell matings, since the mating type switch does not occur until the third or fourth generation of growth following spore germination (Thornton, 1981).

A Prior manual yeast micromanipulator and a Reichert Jung Micro Star 110 fixed stage microscope placed on an antivibration table were used for micromanipulation (Plate 4). The micromanipulator was fitted with a blunt-ended glass needle (Plate 4) and positioned so it could be controlled by the left hand. Dissection and manipulation of the yeast cells was carried out on an inverted agar slab, trimmed to be supported, on a perspex dissecting chamber.

3.4.1 Preparation of the yeast cells for micromanipulation

Diploid yeast cells can be induced to sporulate so that haploid spores are available for micromanipulation. Saccharomyces cervisiae were induced to sporulate by incubation for 48 hours on GNA presporulation agar slants in bijou bottles at 30°C (with lids loosened to allow an adequate oxygen supply). A large inoculum of these cells at the logarithmic stage of growth were streaked onto PA sporulation slants and incubated at 25°C. Sporulation generally occurred after 5 days, but if none appeared after two weeks, the procedure was repeated.

The sporulating culture was prepared for micromanipulation (i.e. the ascus wall surrounding the spores is weakened by mild enzymatic digestion to release the four haploid spores).

Saccharomyces cerevisiae asci were digested by taking a small amount of sporulating culture from the potassium acetate slant using a sterile toothpick and placing them into 0.2 ml of 20% (v/v) sigma glucuronidase (from the snail Helix pomatia).

The cell suspension was mixed in a vortex and incubated at 30°C for 30 minutes. This left the ascus wall of most spore tetrads sufficiently weakened to allow the spores within to be released by micromanipulation. The mixture was vortexed again and used immediately.

Haploid cells were taken from fresh heterothallic cultures on MYGP agar using a sterile toothpick and put into 0.2 ml sterile distilled water and vortexed to prevent cell clumping. These cultures were used prior to the commencement of budding, to cross with other heterothallic haploid cells to produce yeasts capable of high glycerol production.

3.4.1.1 Yeast cell mating

Mating will only occur if the haploid cells are of opposite mating types. Where only heterothallic yeasts are concerned, mating type determinations are employed to ensure that the cells concerned are of the opposite mating type. This was achieved by incubating the strain in question with haploid tester strains of known mating types (i.e. S-1 α or an S-2 a) on MYGP at 30°C for 18 to 24 hours, and examining microscopicaly for zygote formation (mating type 'a' haploids form zygotes when crossed with S-1- α and vice versa). Plate 2 shows the typical dumbell shape of the zygote.

The resultant cross was fermented in 100 ml of grape juice in sterile 150 ml conical flasks using cotton wool bungs to prevent contamination. Analyses for glycerol and ethanol were carried out by high performance liquid chromatography and desired crosses were selected.

3.4.2 The micromanipulation procedure

Preparing the Agar Slab - a slab of 3% MYGP agar was prepared and trimmed aseptically. A loopful of the prepared cells was streaked along

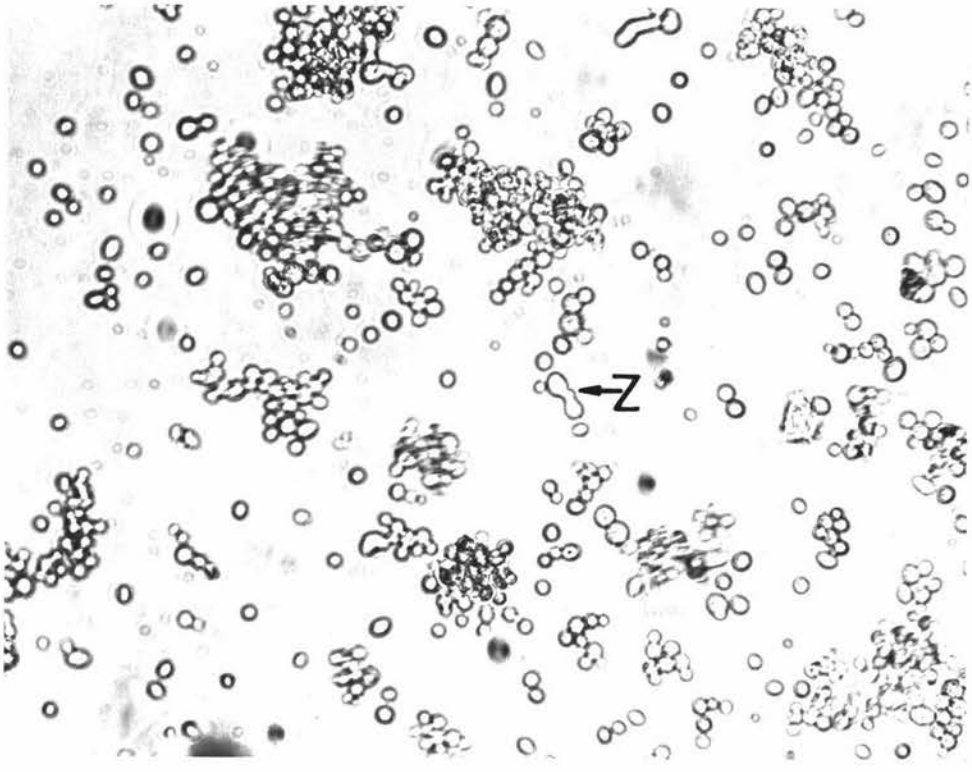


PLATE 2: Dumbell-Shaped Zygotes Formed During the Mating of Haploid Yeast Cells

one long edge of the agar slab. The slab was inverted and placed on the dissection chamber as shown in Plate 3A. (The dissecting needle had previously been centred in the microscopic field). Plate 4 shows the micromanipulation apparatus set up for use.

Micromanipulation was commenced at 300X magnification.

Needles were produced from 3 mm diameter soda glass rods, whereby 2 are heated together under a small flame, melted together and quickly drawn apart at a slight angle. The fine glass protruberance produced was then broken off to a length of approx 3 to 9 mm thus forming a fine needle slightly wider than the yeast cells (Plate 5).

Following micromanipulation, the coverslips were removed from the dissection chamber and the agar slabs removed from the coverslip (using a sterile spatula) then placed, cells uppermost, on to MYGP agar and incubated at 30°C for 48 hours (Plate 3B).

3.4.3 Selection for high glycerol producing yeasts by hybridisation

To produce haploid yeast cells and select for high glycerol producing strains of yeasts (i.e. XGL74 and XGL81), the sporulated strain was streaked down one long edge of the agar slab. An ascus was picked with a needle and placed at a noted location on the agar slab. The ascus wall was broken by gently tapping the dissecting needle and single spores were placed at 2 mm intervals across th slab, each time recording the location of the deposited spore. Asci were dissected at 3 mm intervals along the slab, again recording the position where each cell was placed. Normally 6 to 10 asci were dissected per slide. See Plate 3B.

In the production of diploid strains, the 2 mating types were streaked down alternate long sides of the agar slab. Single cells from one strain were placed at recorded locations on the agar slab, the single cells from the other strain were deposited at these locations and positioned gently so the two types of cell were in contact (rough treatment of the cells reduces viability).

Mating was detected by the ability of clone of cells selected for hybridisation to sporulate thus indicating the diploid life cycle.

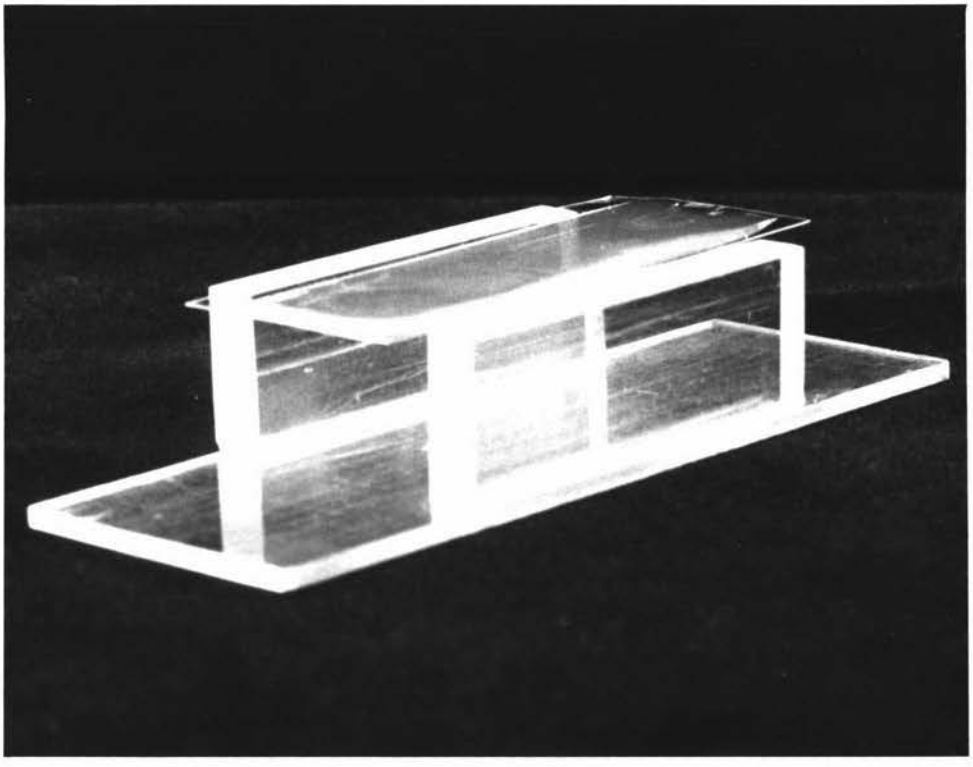


PLATE 3A: Dissection Chamber with Agar Slab in Position

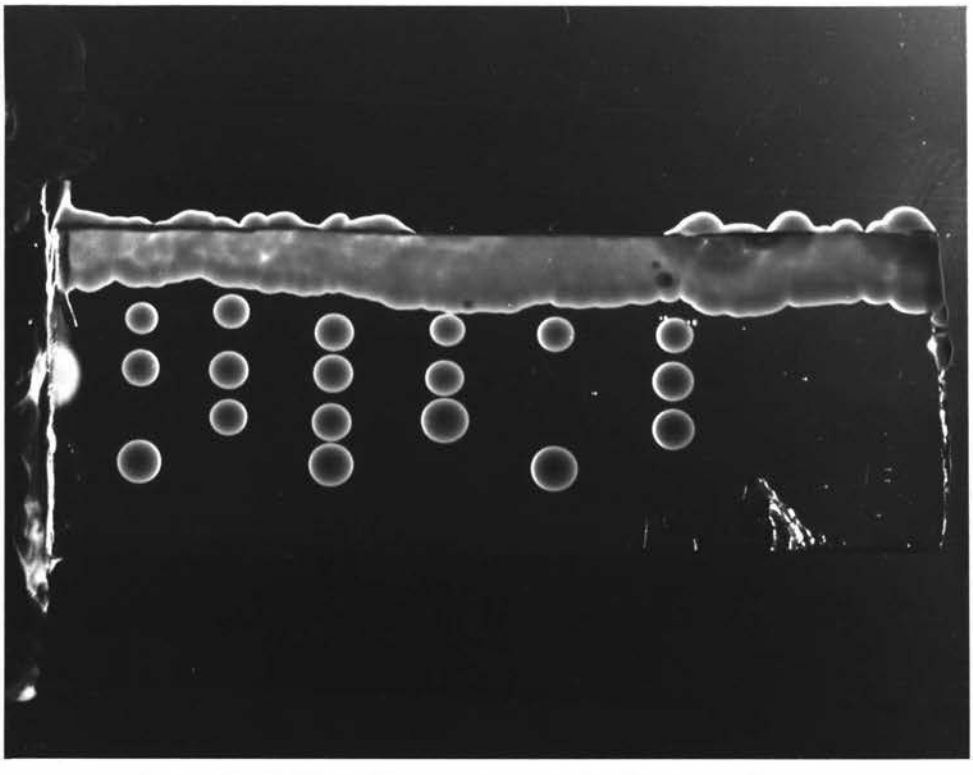


PLATE 3B: Agar Dissection Slab after Incubation, Showing the Growth of Spore Tetrads

PLATE 4 : Micromanipulation Apparatus

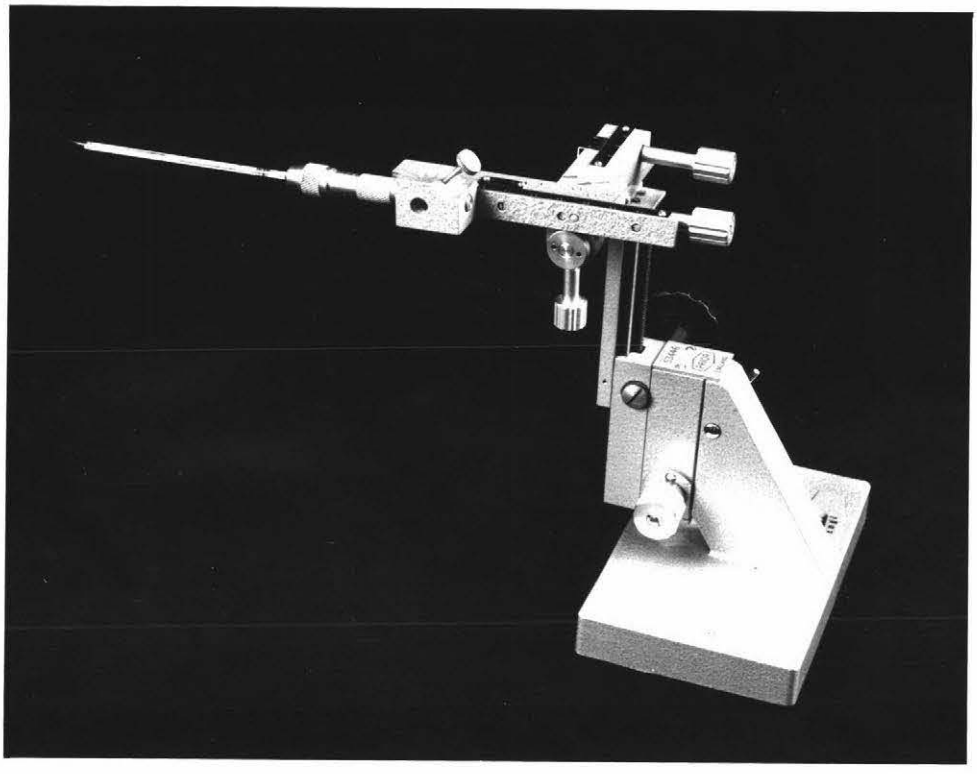


PLATE 5 : Micromanipulator and Dissection Needle

EXPERIMENTAL RESULTS

1. GLYCEROL PRODUCTION DURING FERMENTATION TO DRYNESS BY STRAINS OF SACCHAROMYCES CEREVISIAE

1.1 General outline

The effects of different parameters on the production of glycerol during fermentation to dryness by six strains of Saccharomyces cerevisiae were examined.

(a) Strains MD26, AWI60, AWI80 - referred to as parent strains and are moderage glycerol producers;

(b) Strains XGL74, XGL78, XGL81 - hybrid yeasts capable of producing large quantities of glycerol during alcoholic fermentation.

The following trials were conducted:

(1) Fermentation in three types of media (a synthetic completely defined medium and two grape juices - Muller Thergau and Chenin Blanc) at a constant temperature of 15°C;

(2) Fermentation in the three media described in (1) at a constant rate of sugar utilization;

(3) Fermentation by two yeasts (AW160 and XGL81) of completely defined medium and Chenin Blanc grape juice containing levels of sulphur dioxide ranging from 0 ppm - 300 ppm.

(4) Fermentation by two yeasts (MD26 and XGL81) of completely defined medium and Chenin Blanc grape juice containing initial levels of glycerol ranging from 0 - 20 g/l.

1.2 Fermentation trials at a constant temperature of 15°C

Sugar utilization, glycerol production, ethanol production and viable cell number were observed during the fermentation of three growth media by six yeast strains. The work was performed at a constant temperature and the procedures utilized have been previously described in Methods Section 2.2, 2.4 and 3.1.

Representative results for two of the yeast strains in the three media are presented in Figure 2 and their mean values for glycerol and ethanol production are detailed in Tables 2-7.

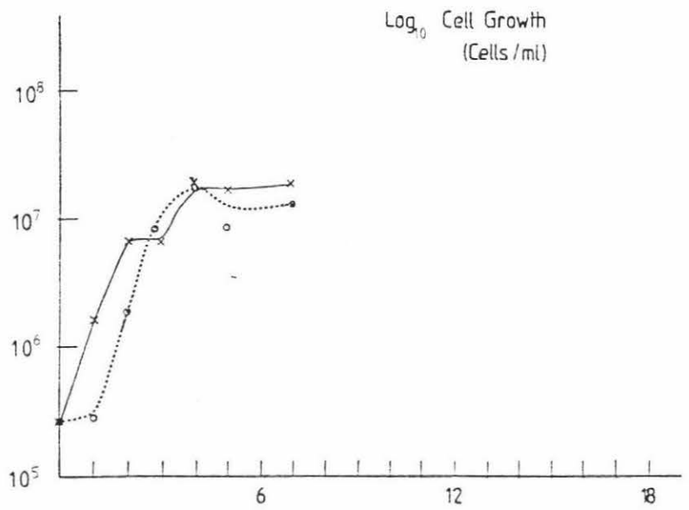
FIGURE 2 : Fermentation at Constant Temperature (15°C)

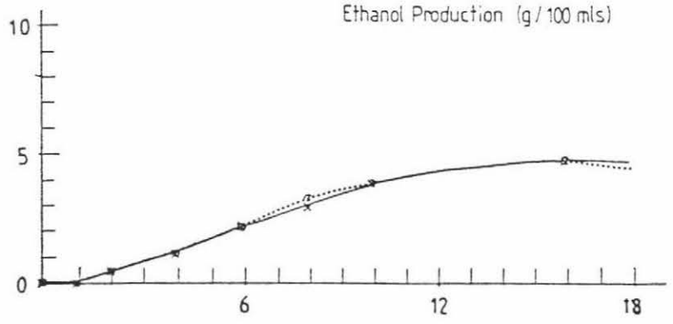
Key:-

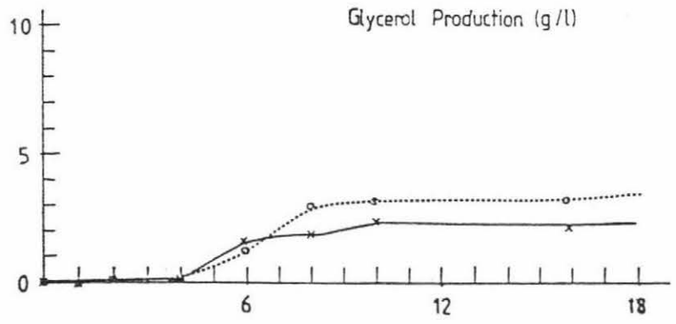
Yeast AWI 60 —×—

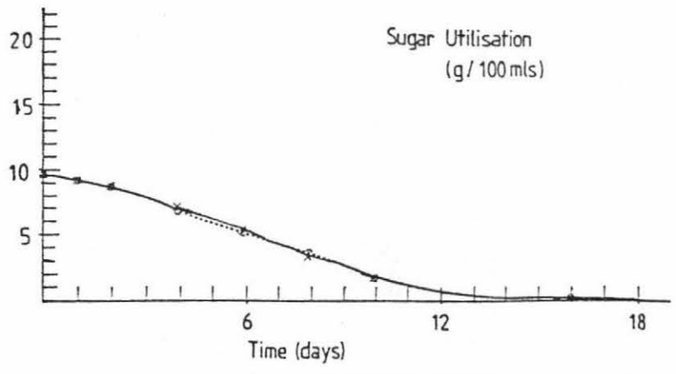
XGL 81 ··o·····

Complete Defined Media

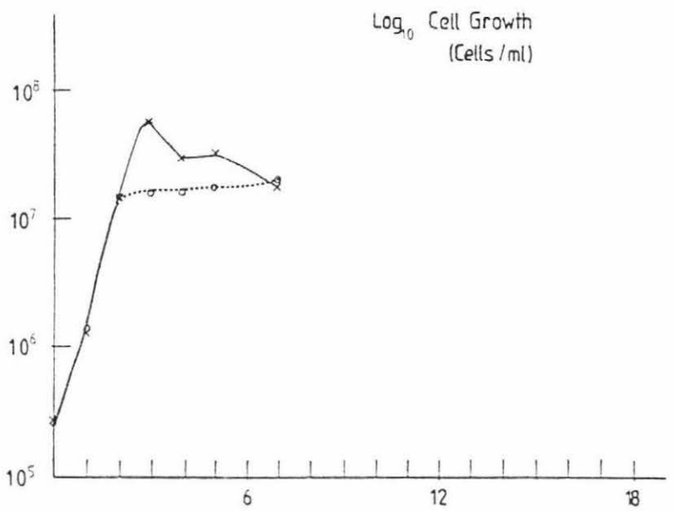


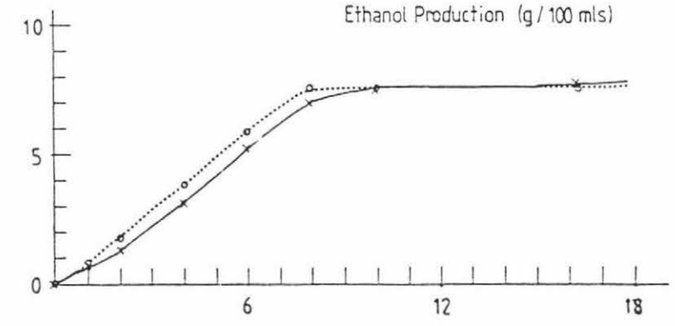


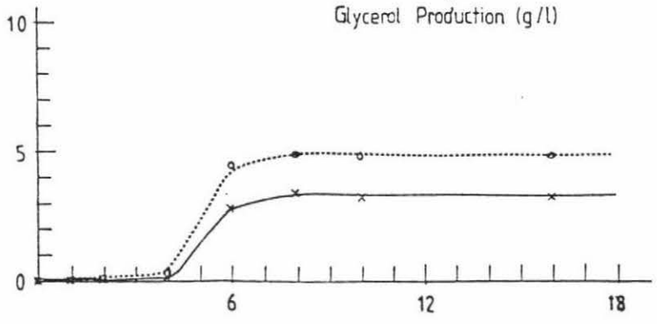


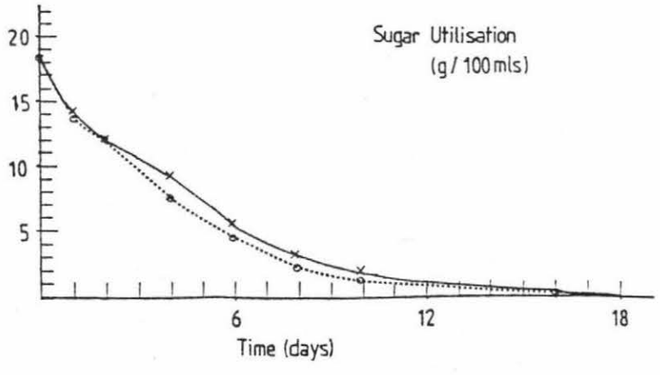


Muller Thurgau

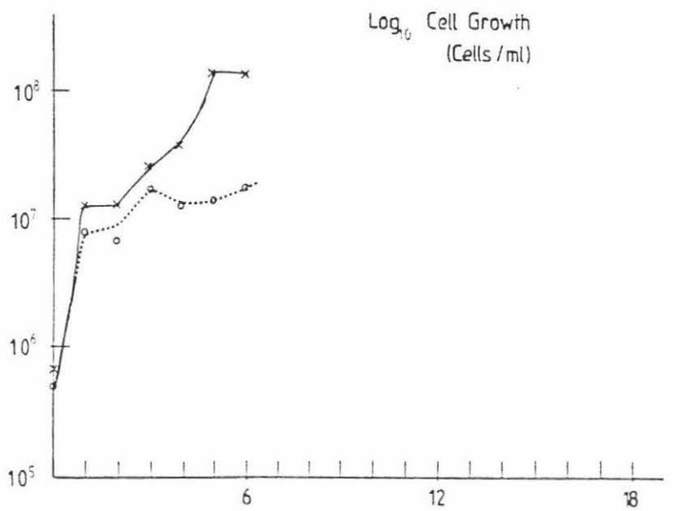


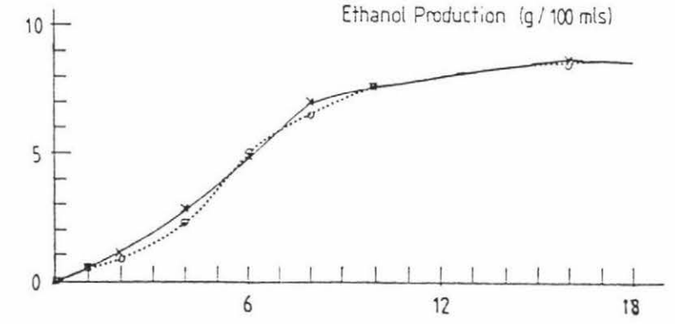


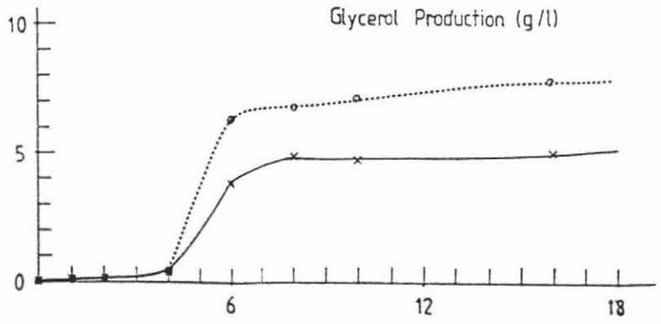




Chenin Blanc







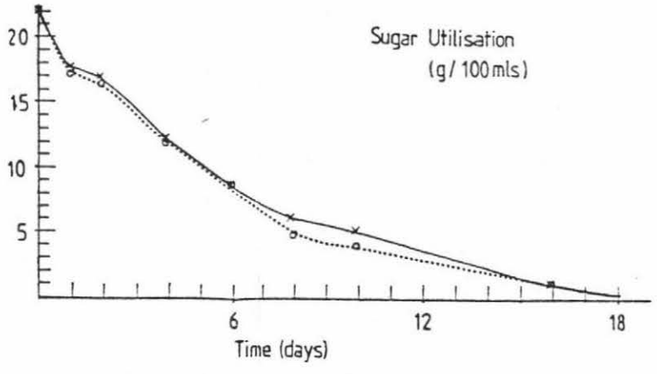


Table 2: Mean Glycerol Production (g/l) at a Constant Temperature of Fermentation (15°C) in Complete Defined Media [Std error ± 0.2g/l]

Day		0	1	2	4	6	8	10	16	25
Yeast	MD26	0	0	.045	.098	1.80	2.07	2.22	2.35	2.89
	AWI60	0	0	.065	.097	1.72	1.88	2.31	2.22	2.52
	AWI80	0	0	.073	.134	2.04	2.41	2.49	2.33	8.22
	XGL74	0	0	.076	.107	2.02	2.81	2.46	3.31	3.92
	XGL78	0	0	.022	.107	.49	2.21	2.63	2.62	2.94
	XGL81	0	0	.063	.140	1.29	3.02	3.21	3.20	3.60

Table 3: Mean Glycerol Production (g/l) at a Constant Temperature of Fermentation (15°C) in Muller Thurgau [Std error ± 0.2g/l]

Day	0	1	2	4	4	8	10	16	25
Yeast MD26	0	0.076	.127	.219	2.69	3.34	3.2	2.01	3.65
AWI60	0	.068	.104	.20	2.79	3.46	3.25	3.20	3.34
AWI80	0	.089	.158	.278	2.73	4.53	4.38	4.11	4.62
XGL74	0	.081	.134	.266	3.73	4.08	4.07	3.84	4.43
XGL78	0	.084	.115	.217	3.01	3.70	4.02	4.23	5.24
XGL81	0	.103	.156	.33	4.48	4.95	4.80	4.85	5.01

Table 4: Mean Glycerol Production (g/l) at a Constant Temperature of Fermentation (15°C) in Chenin Blanc [Std error ± 0.2g/l]

Day	0	1	2	4	6	8	10	16	25
Yeast MD26	0	.123	.177	.352	4.64	5.57	4.94	4.63	5.32
AWI60	0	.109	.175	.298	3.8	4.87	4.72	4.94	5.43
AWI80	0	.122	.172	.333	4.66	5.44	5.31	4.19	5.87
XGL74	0	.130	.222	.526	9.11	8.02	7.74	7.03	8.05
XGL78	0	.121	.180	.341	5.81	5.41	6.05	8.01	7.77
XGL81	0	.141	.124	.363	6.38	6.79	7.17	7.84	7.87

Table 5: Mean Ethanol Production (g/100ml) at a Constant Temperature of Fermentation (15°C) in Complete Defined Media [Std error ± 0.1g/100ml]

Day	0	1	2	4	6	8	10	16	25
Yeast MD26	0	0	.0869	1.10	2.11	2.99	3.42	4.63	4.63
AWI60	0	0	.499	1.14	2.15	2.99	3.88	4.68	4.43
AWI80	0	.016	.625	1.49	2.60	3.91	4.39	4.87	5.21
XGL74	0	0	.587	1.33	1.95	3.24	3.65	4.76	4.48
XGL78	0	0	.297	1.17	2.02	2.98	3.67	4.80	4.42
XGL81	0	0	.454	1.13	2.03	3.26	3.86	4.72	4.35

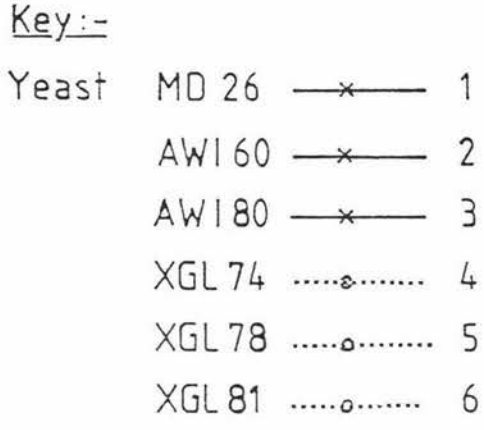
Table 6: Mean Ethanol Production (g/100ml) at a Constant Temperature of Fermentation (15°C) in Muller Thurgau [Std error ± 0.1g/100ml]

ay	0	1	2	4	6	8	10	16	25
east MD26	0	.828	1.64	3.28	5.76	7.41	7.37	7.66	7.59
AWI60	0	.745	1.38	3.16	5.26	7.02	7.40	7.77	7.28
AWI80	0	.811	1.9	3.44	5.63	7.53	7.55	5.96	7.19
XGL74	0	.839	1.54	3.21	5.28	6.79	7.26	7.72	7.22
XGL78	0	.729	1.35	2.35	3.5	4.70	5.40	7.38	7.12
XGL81	0	.898	1.79	3.85	5.98	7.54	7.57	7.46	6.98

Table 7: Mean Ethanol Production (g/100ml) at a Constant Temperature of Fermentation (15°C) in Chenin Blanc [Std error ± 0.1g/100ml]

Day		0	1	2	4	6	8	10	16	25
Yeast	MD26	0	.528	1.29	3.97	6.29	8.71	8.51	8.35	8.43
	AWI60	0	.510	1.15	2.92	4.97	7.05	7.68	8.78	8.24
	AWI80	0	.320	1.15	3.05	5.41	7.46	8.18	8.90	8.07
	XGL74	0	.493	1.30	3.70	7.71	8.27	8.59	8.95	8.23
	XGL78	0	.444	0.93	2.26	4.59	4.99	6.17	8.54	7.93
	XGL81	0	.515	0.89	2.35	5.02	6.53	7.76	8.50	8.04

FIGURE 3 : Glycerol Production in Complete Defined Media, Fermented at a Constant Temperature of Fermentation.



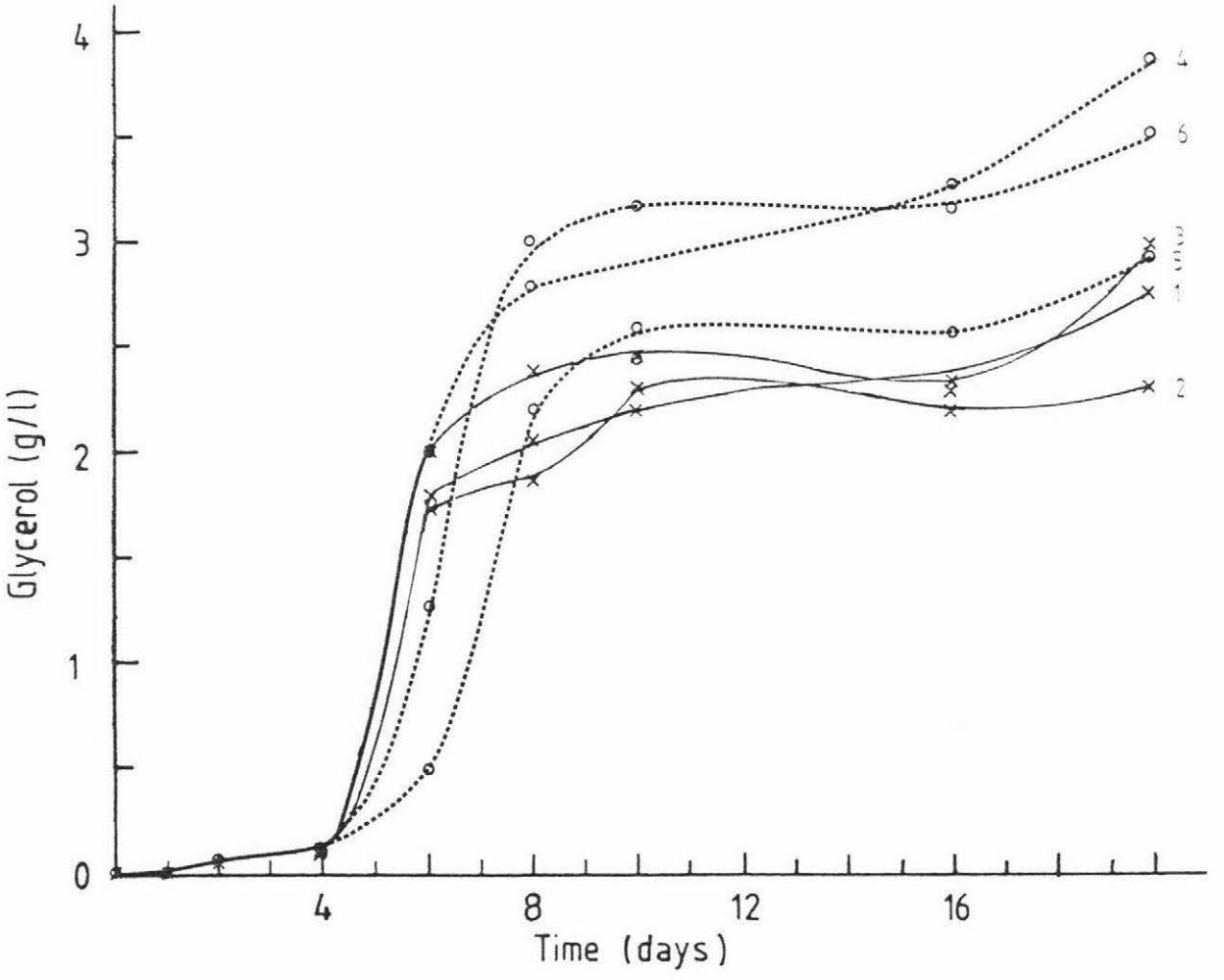
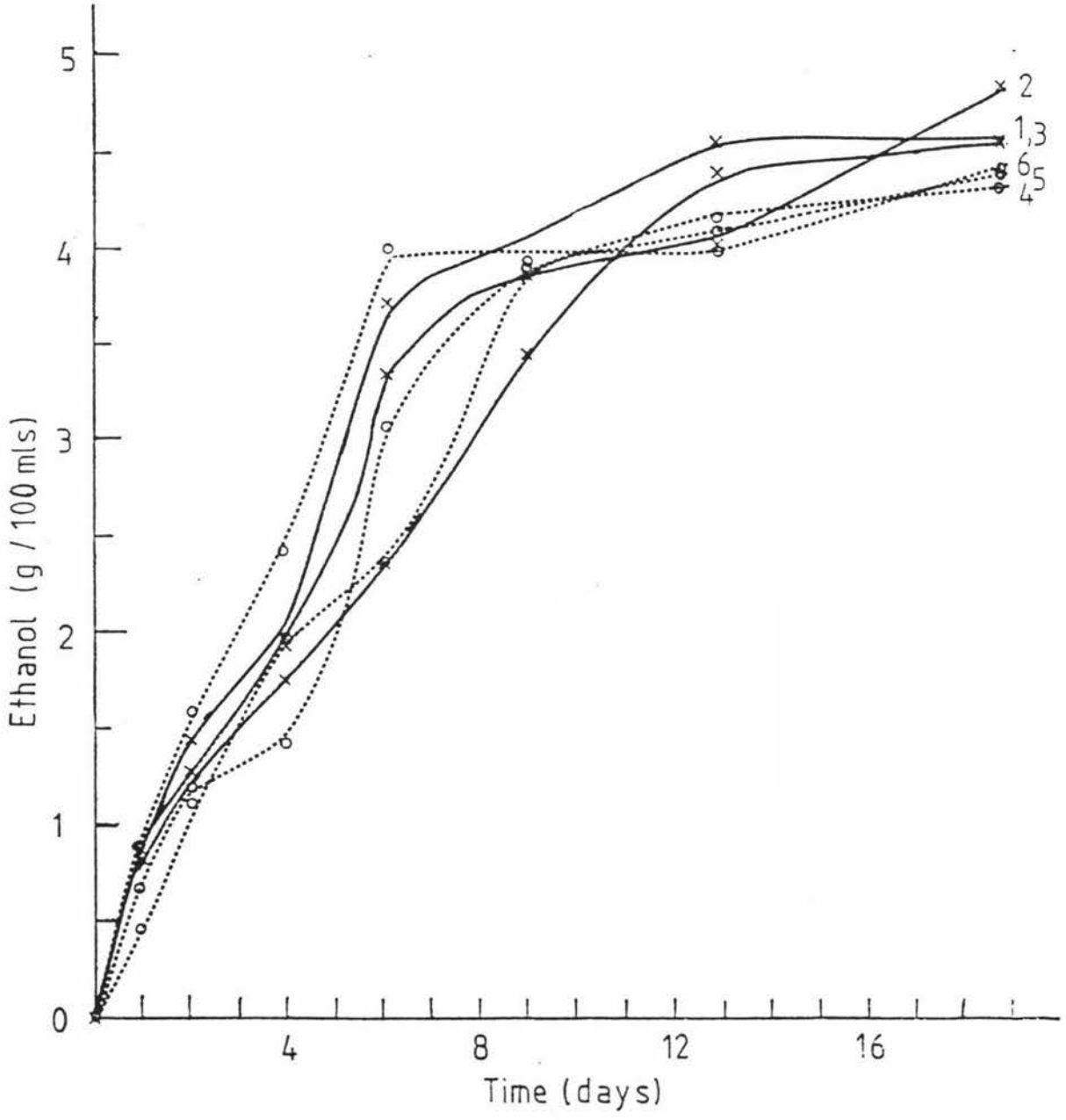

Glycerol (g/l)
0
1
2
3
4
4
8
12
16
Time (days)
4
5
2

FIGURE 4 : Ethanol Production in Complete Defined Media, Fermented at a Constant Temperature of Fermentation.


5
4
3
2
1
0
Ethanol (g / 100 mls)
4
8
12
16
Time (days)
2
1,3
6
5
4

Detailed analyses for sugar utilization, glycerol and ethanol production and viable cell number for all six yeasts in the three media are presented in Appendix A tables (I) - (XII).

Glycerol production in Complete Defined Medium by the six yeasts is shown in Figure 3. Two of the hybrid yeast strains produced significantly more glycerol than the parent strains. Glycerol production apparently commenced at the start of fermentation and continued throughout the process, although the rate of production declined in the latter stages.

Ethanol production under the same conditions followed a similar pattern (Figure 4). However, the final yields of ethanol for all six yeasts were very similar.

The same trends for all parameters were observed during the fermentation of the grape juices by the six yeasts (Appendix A tables (I) - (XII)).

Standard error of measurement is recorded with the appropriate Appendix table.

1.3 Fermentation trials with constant sugar utilization

Sugar utilization, glycerol production, ethanol production and viable cell number were also followed during the fermentation of three media by six yeast strains. This was carried out at a constant rate of sugar utilization and the procedures utilized have been previously described in the Methods, sections 2.2, 2.4 and 3.1.

Figure 5 presents results obtained from two of the yeast strains used in the three media. The mean values for glycerol and ethanol production are presented in Table 8-13 nd the viable cell number for all six yeasts in the three media are presented in Appendix B tables (I) - (XII).

Figure 6 shows glycerol production by the six yeasts. The three hybrid yeast strains produced significantly more glycerol than the parent strains. Glycerol production again appeared to commence at the start of fermentation and showed a rapid decline after 6-8 days.

Ethanol production under the same conditions is shown in Figure 7. The six yeasts produced similar levels of ethanol.

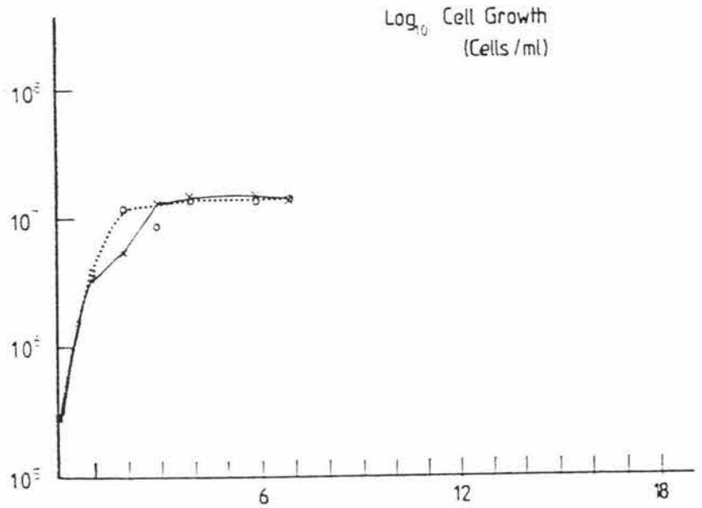
FIGURE 5 : Fermentation at Constant Sugar Utilisation
(Variable Temperature)

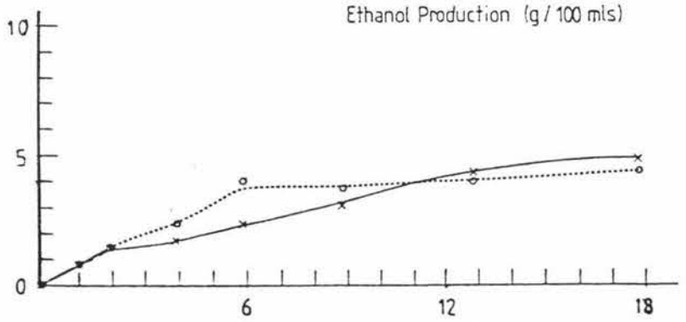
Key:-

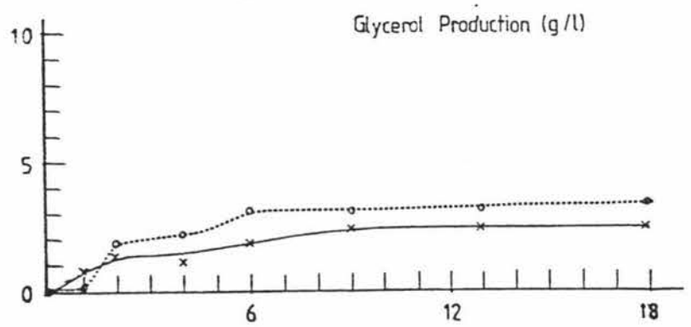
Yeast AWI 60 —*—

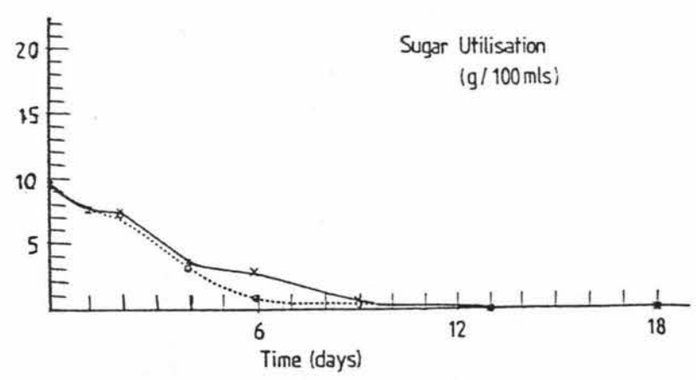
XGL 81 ..o....

Complete Defined Media

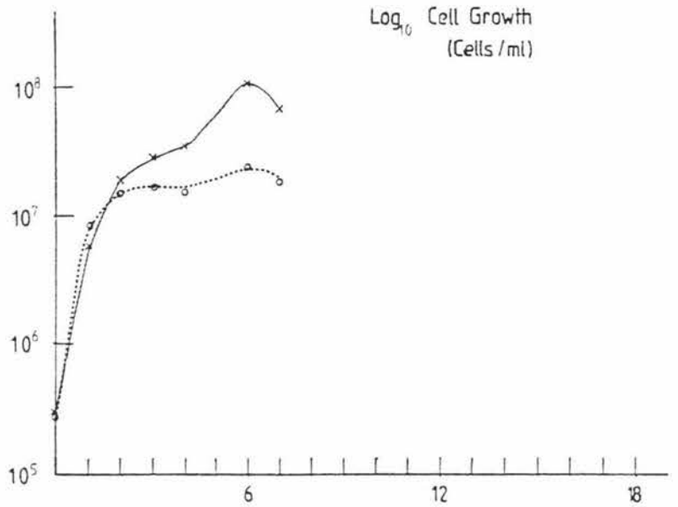


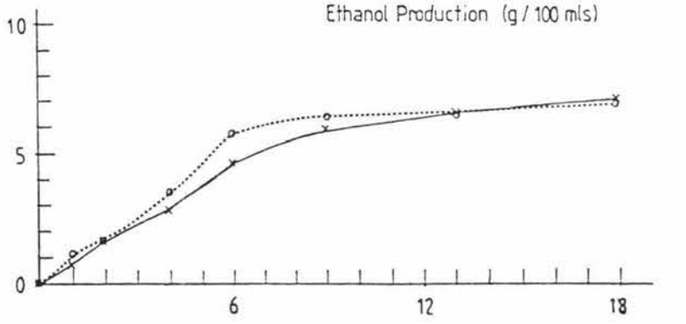


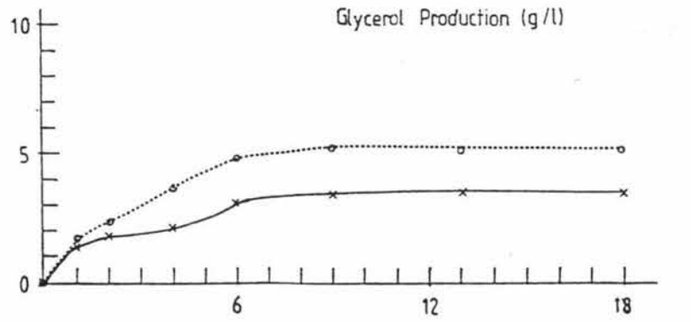


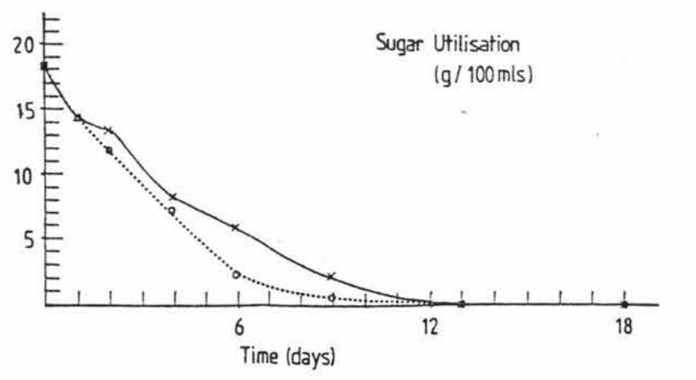


Muller Thurgau

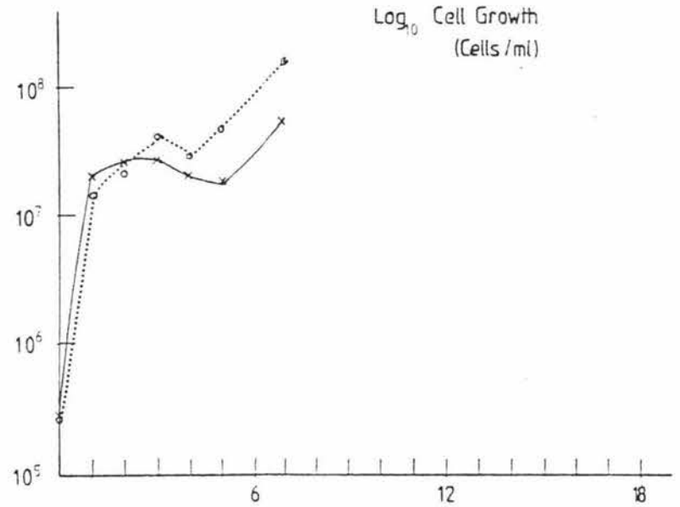


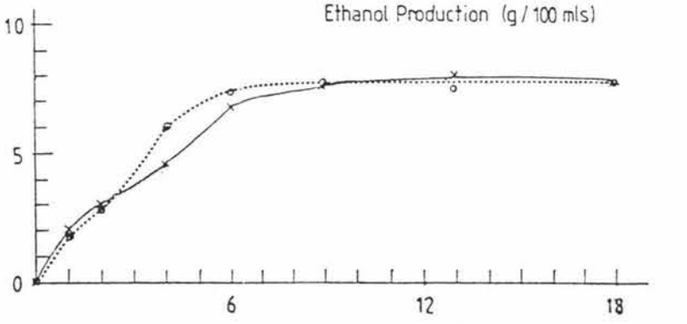


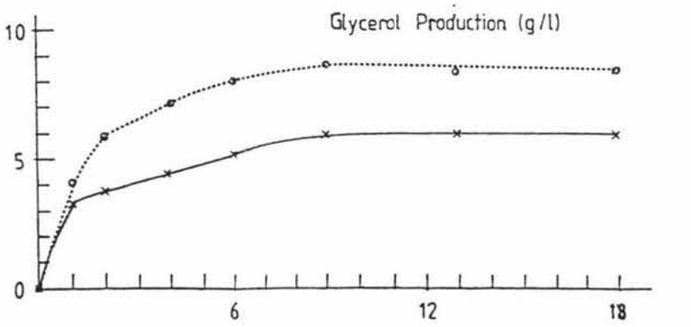




Chenin Blanc







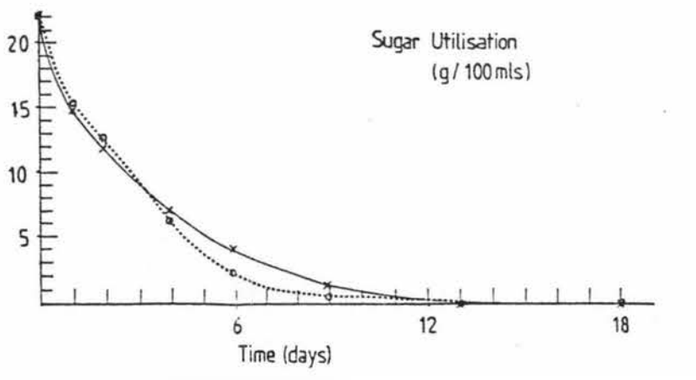


Table 8: Mean Glycerol Production (g/l) Fermented at a Constant Rate of Sugar Utilisation in Complete Defined Media [Std error ± 0.2g/l]

Day	0(25°)	1	2	4	6	9	13	18
Yeast MD26	0	1.06	1.16	1.29	2.25	2.28	2.3	2.38
AWI60	0	0.89	1.33	1.16	1.97	2.3	2.28	2.41
AWI80	0	0.89	1.16	1.46	2.08	2.16	2.38	2.44
XGL74	0	0.83	1.59	1.41	2.71	3.18	3.21	3.06
XGL78	0	0.75	1.2	1.60	2.51	2.77	2.77	2.88
XGL81	0	0.22	1.94	2.23	3.18	3.12	3.15	3.36

Table 9: Mean Glycerol Production (g/l) Fermented at a Constant Rate of Sugar Utilisation in Muller Thurgau [Std error ± 0.2g/l]

Day	0	1	2	4	6	9	13	18
Yeast MD26	0	1.06	1.95	2.16	3.49	3.53	3.52	3.54
AWI80	0	1.32	1.76	2.11	3.09	3.35	3.34	3.48
AWI80	0	1.38	2.08	2.3	3.59	3.87	4.0	3.92
XGL74	0	1.79	2.74	3.30	4.44	5.0	4.98	5.0
XGL78	0	1.24	1.89	2.91	4.12	4.44	4.9	4.8
XGL81	0	1.71	2.38	3.68	4.86	5.2	5.1	5.16

Table 10: Mean Glycerol Production (g/l) Fermented at a Constant Rate of Sugar Utilisation in Chenin Blanc [Std error ± 0.2g/l]

Day	0	1	2	4	6	9	13	18
Yeast MD26	0	2.56	3.14	3.94	4.96	5.61	5.95	5.94
AWI60	0	3.29	3.89	4.28	5.25	5.7	5.55	5.54
AWI80	0	2.48	4.47	4.77	5.59	6.56	6.02	5.9
XGL74	0	3.83	6.31	7.0	7.86	9.09	8.8	8.76
XGL78	0	2.48	3.76	4.50	5.9	7.62	7.91	7.88
XGL81	0	4.04	5.92	7.16	8.01	8.67	8.39	8.26

Table 11: Mean Ethanol Production (g/100ml) Fermented at a Constant Rate of Sugar Utilisation in Complete Defined Media [Std error ± 0.1g/100ml]

Day	0	1	2	4	6	9	13	18
Yeast MD26	0	0.88	1.29	1.99	3.73	3.90	4.58	4.56
AWI60	0	0.88	1.43	1.75	2.38	3.90	4.41	4.85
AWI80	0	0.80	1.13	1.93	3.36	3.59	4.04	4.55
XGL74	0	0.67	1.19	1.44	3.10	3.93	4.18	4.33
XGL78	0	0.47	1.10	1.98	2.36	3.96	4.11	4.38
XGL81	0	0.88	1.59	2.44	4.01	3.88	4.0	4.41

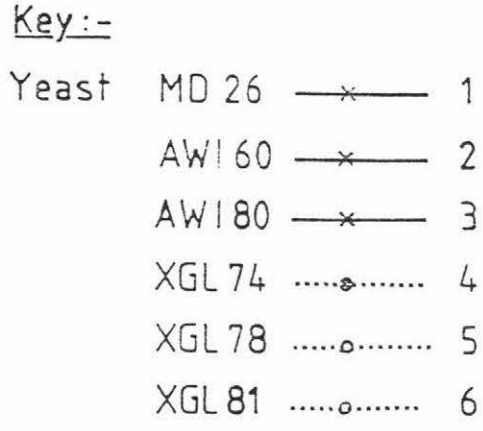
Table 12: Mean Ethanol Production (g/100ml) Fermented at a Constant Rate of Sugar Utilisation in Muller Thurgau [Std ± 0.1g/100ml]

Day	0	1	2	4	6	9	13	18
Yeast MD26	0	0.96	2.01	3.58	6.28	5.76	6.69	7.31
AWI60	0	0.72	1.59	2.82	4.63	5.98	6.55	7.04
AWI80	0	1.07	1.63	3.04	5.27	5.80	6.98	6.78
XGL74	0	1.21	1.67	3.11	4.81	5.88	6.62	6.75
XGL78	0	0.92	1.22	3.26	5.56	5.98	6.81	6.75
XGL81	0	1.14	1.63	3.51	5.82	6.48	6.48	6.88

Table 13: Mean Ethanol Production (g/100ml) Fermented at a Constant Rate of Sugar Utilisation in Chenin Blanc [Std error ± 0.1g/100ml]

Day	0	1	2	4	6	9	13	18
Yeast MD26	0	1.44	2.29	3.97	5.89	7.21	8.16	8.45
AWI60	0	2.01	3.02	4.54	6.76	7.53	8.0	7.70
AWI80	0	1.12	2.59	4.55	6.86	7.78	8.10	7.80
XGL74	0	1.46	2.72	4.51	6.12	7.28	7.78	7.78
SGL78	0	1.23	1.67	2.81	5.01	6.30	7.53	8.0
XGL81	0	1.74	2.78	6.08	7.40	7.77	7.51	5.88

FIGURE 6 : Glycerol Production in Complete Defined Media, Fermented at a Constant Rate of Sugar Utilisation.



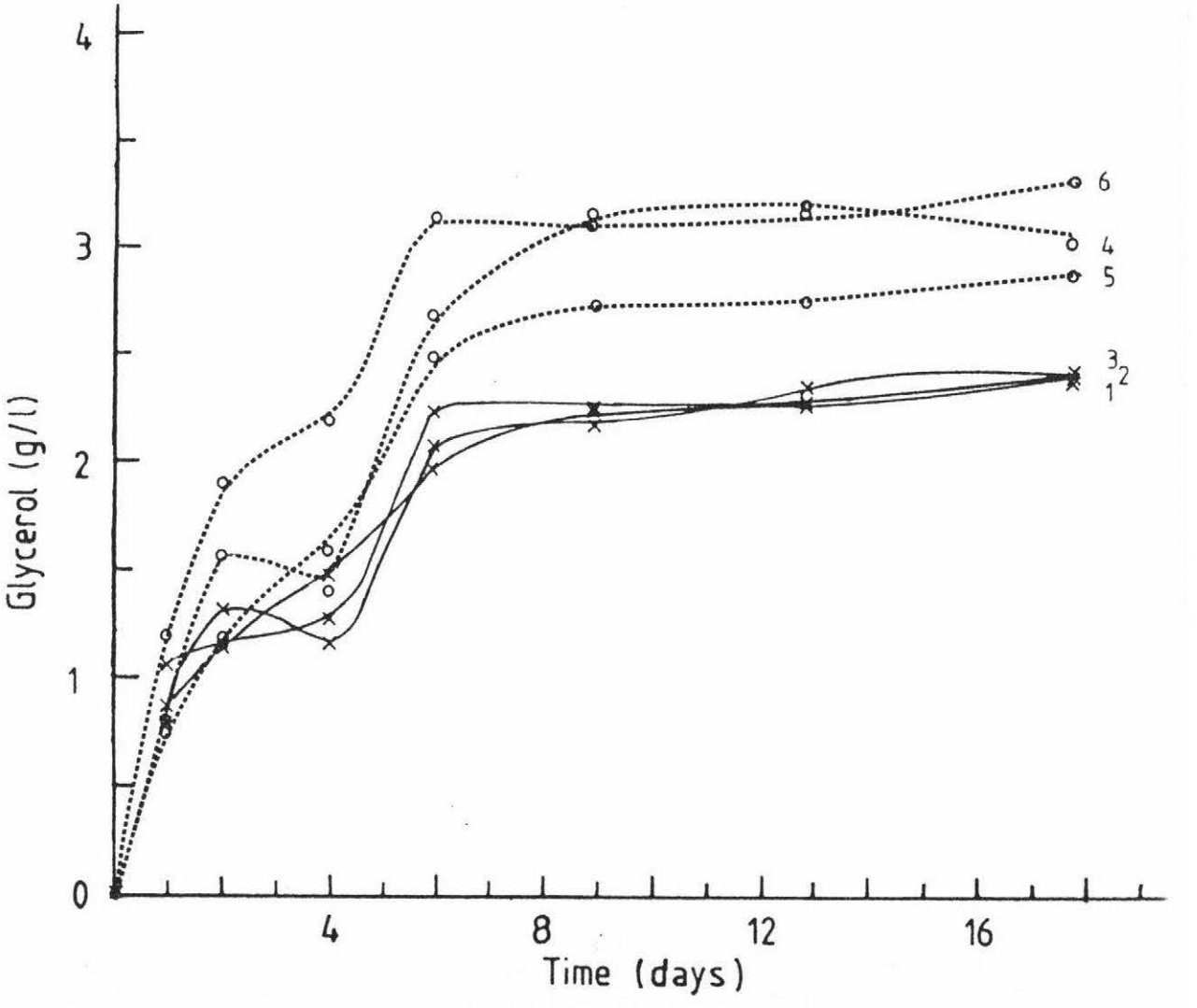
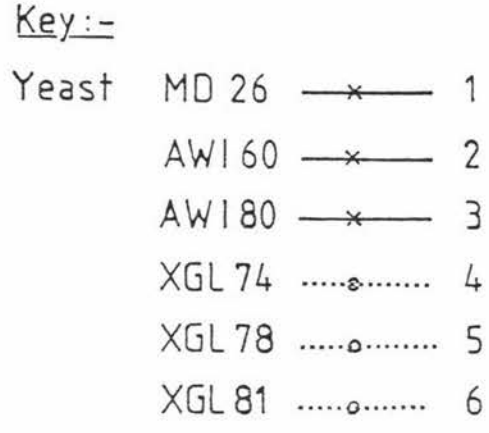
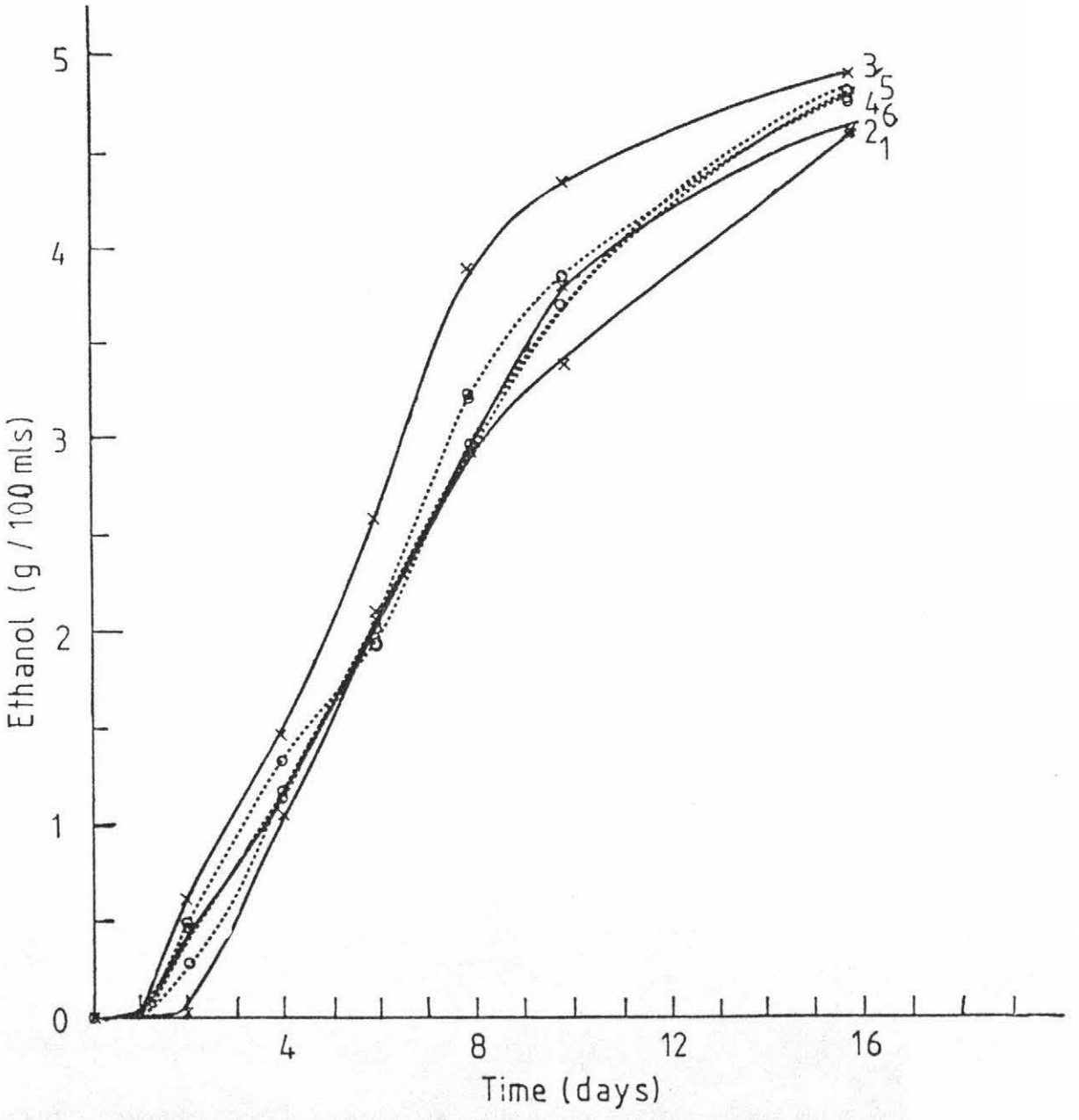

Glycerol (g/l)
Time (days)
0
1
2
3
4
4
8
12
16
6
4
5
3
2
1

FIGURE 7 : Ethanol Production in Complete Defined Media, Fermented at a Constant Rate of Sugar Utilisation.




Ethanol (g / 100 mls)
0
1
2
3
4
5
4
8
12
16
Time (days)
3
5
4
6
2
1

Appendix B tables (I) - (XII) illustrates the trends found for all the observed parameters during the fermentation of the grape juices by the six yeasts.

Standard error of measurement is recorded with the appropriate Appendix Table.

1.4 Effect of yeast type, medium and fermentation conditions on the efficiency of sugar utilization and the production of glycerol and ethanol

A common basis was required for the meaningful comparison of the fermentation abilities of different yeast strains under different conditions of fermentation. In the experiments reported in sections 1.2 and 1.3, the three fermentation media had different initial sugar concentrations. The efficiency of each fermentation, in terms of conversion of 1 gram of sugar into (capital X) grams of glycerol or ethanol, was calculated and analysed for statistical significance.

1.4.1 Efficiency of converting sugar to glycerol

Table 14 illustrates all six yeasts under conditions of different media and fermentation processes. Initial sugar levels and final glycerol levels are also displayed as grams of glycerol produced per 1 gram of sugar utilized. Experimental error is also shown. Data has been recorded for fermentation in each medium, i.e. Complete Defined Media (CDM), Muller Thurgau (MT), Chenin Blanc (CB); at a constant temperature of fermentation and at a constant rate of sugar utilization (variable temperature).

That glycerol production was influenced by the fermentation environment is apparent in this table. Glycerol production appeared to be poor in a fermentation medium of Muller Thurgau (which is of an intermediate initial sugar level), yet highest in Complete Defined Media or Chenin Blanc (depending on the yeast strain and the fermentation procedure).

1.4.2 Efficiency of converting sugar to ethanol

Table 15 illustrates the efficiency of ethanol production by the six yeasts. Initial sugar levels and final ethanol levels are shown, as are grams of ethanol produced per 1 gram of sugar utilized.

TABLE 14: EFFICIENCY OF CONVERSION (BY WEIGHT) OF SUGAR TO GLYCEROL.

Yeast	Media	Fermentation Temperature	Initial Sugar (g/l)	Final Glycerol (g/l)	Glycerol/Sugar
MD26	CDM	Constant	95.8	2.89	0.030 ± 0.003
	MT		184.2	3.65	0.020 ± 0.001
	CB		220.2	5.32	0.024 ± 0.001
	CDM	Variable	95.8	2.38	0.025 ± 0.003
	MT		184.2	3.54	1.019 ± 0.001
	CB		220.2	5.94	0.027 ± 0.001
AWI60	CDM	Constant	95.8	2.52	0.026 ± 0.003
	MT		184.2	3.34	0.018 ± 0.001
	CB		220.2	5.43	0.025 ± 0.001
	CDM	Variable	95.8	2.41	0.025 ± 0.003
	MT		184.2	3.48	0.019 ± 0.001
	CB		220.2	5.54	0.025 ± 0.001
AWI80	CDM	Constant	95.8	3.22	0.034 ± 0.003
	MT		184.2	4.62	0.025 ± 0.001
	CB		220.2	5.87	0.027 ± 0.001
	CDM	Variable	95.8	2.44	0.026 ± 0.003
	MT		184.2	3.92	0.021 ± 0.001
	CB		220.2	5.90	0.027 ± 0.001
XGL74	CDM	Constant	95.8	3.92	0.041 ± 0.003
	MT		184.2	4.43	0.024 ± 0.001
	CB		220.2	8.05	0.037 ± 0.001
	CDM	Variable	95.8	3.06	0.032 ± 0.003
	MT		184.2	5.0	0.027 ± 0.001
	CB		220.2	8.76	0.040 ± 0.001
XGL78	CDM	Constant	95.8	2.94	0.031 ± 0.003
	MT		184.2	5.24	0.028 ± 0.001
	CB		220.2	7.77	0.035 ± 0.001
	CDM	Variable	95.8	2.88	0.030 ± 0.003
	MT		184.2	4.80	0.026 ± 0.001
	CB		220.2	7.88	0.036 ± 0.001
XGL81	CDM	Constant	95.8	3.60	0.038 ± 0.003
	MT		184.2	5.01	0.027 ± 0.001
	CB		220.2	7.27	0.036 ± 0.001
	CDM	Variable	95.8	3.36	0.035 ± 0.003
	MT		184.2	5.16	0.028 ± 0.001
	CB		220.2	8.26	0.038 ± 0.001

TABLE 15: EFFICIENCY OF CONVERSION (BY WEIGHT) OF SUGAR TO ETHANOL.

Yeast	Media	Fermentation Temperature	Initial Sugar (g/l)	Final Ethanol (g/l)	Ethanol/Sugar
MD26	CDM	Constant	95.8	46.3	0.48 ± 0.03
	MT		184.2	75.9	0.41 ± 0.01
	CB		220.2	84.3	0.38 ± 0.01
	CDM	Variable	95.8	45.6	0.47 1 0.03
	MT		184.2	73.1	0.40 ± 0.02
	CB		220.2	84.5	0.38 1 0.01
AWI60	CDM	Constant	95.8	44.3	0.43 ± 0.03
	MT		184.2	72.8	0.40 ± 0.01
	CB		220.2	82.4	0.37 ± 0.01
	CDM	Variable	95.8	48.5	0.51 ± 0.03
	MT		184.2	70.4	0.38 ± 0.02
	CB		220.2	77.0	0.35 ± 0.01
AWI80	CDM	Constant	95.8	52.1	0.54 ± 0.03
	MT		184.2	71.9	0.39 ± 0.01
	CB		220.2	80.7	0.37 ± 0.01
	CDM	Variable	95.8	45.5	0.47 ± 0.03
	MT		184.2	67.7	0.37 ± 0.02
	CB		220.2	78.0	0.35 ± 0.01
XGL74	CDM	Constant	95.8	44.8	0.47 ± 0.03
	MT		184.2	72.2	0.39 ± 0.01
	CB		220.2	82.3	0.37 ± 0.01
	CDM	Variable	95.8	43.3	0.45 ± 0.03
	MT		184.2	67.6	0.37 ± 0.02
	CB		220.2	77.8	0.35 ± 0.01
XGL78	CDM	Constant	95.8	44.2	0.46 ± 0.03
	MT		184.2	71.2	0.39 ± 0.01
	CB		220.2	79.3	0.36 ± 0.01
	CDM	Variable	95.8	43.8	0.46 ± 0.03
	MT		184.2	67.5	0.37 ± 0.02
	CB		220.2	80.0	0.36 ± 0.01
XGL81	CDM	Constant	95.8	43.5	0.45 ± 0.03
	MT		184.2	69.8	0.38 ± 0.01
	CB		220.2	80.4	0.37 ± 0.01
	CDM	Variable	95.8	44.1	0.46 ± 0.03
	MT		184.2	68.8	0.37 ± 0.02
	CB		220.2	78.8	0.36 ± 0.01

The influence of the yeast's environment on ethanol production and the tendancy for ethanol production to be most efficient in completely defined media, yet poorest in Chenin Blanc grape juice, can be observed.

The efficiencies for AWI60 in complete defined media under variable temperature conditions and AWI80 in complete defined media under constant temperature conditions must be regarded with suspicion. This is because the former equals and the latter exceeds the maximum theoretical yield of ethanol from glucose by fermentation.

1.4.3 Effect of altering the media and fermentation process on glycerol and ethanol production

To compare the effects of altering the fermentation environment on production of ethanol and glycerol by each yeast, Figure 8 a-f illustrates the efficiency of ethanol production from sugar (by weight) plotted against the efficiency of glycerol production from sugar (by weight). Each graph illustrates production by all six yeasts for a given set of fermentation conditions, i.e. comparison of the three types of media following fermentation at constant temperature and constant sugar utilization (variable temperature).

These graphs illustrate that yeasts may produce ethanol and/or glycerol more efficiently depending on the type of medium and the type of fermentation process used. Conditions of maximum yield can be selected for each yeast strain.

1.5 Influence of the yeast strain on the efficiency of glycerol and ethanol production

For a given set of fermentation conditions (i.e. medium and fermentation proces) Figure 9 illustrates the efficiency of ethanol production from sugar (by weight) plotted against the efficiency of gycerol procduction from sugar (by weight) for each of the six yeasts. The ability for some yeasts to produce high levels of glycerol or ethanol or both, due to their genetic makeup, regardless of the environmental conditions is displayed.

The three high glycerol-producing yeasts (d, e and f) are readily distinguished from the parent strains (a, b and c) in terms of glycerol production. These graphs suggest a tendency for high glycerol producing

FIGURE 8 : Effect of Altering the Fermentation Conditions on the Efficiency of the Conversion of 1g Sugar into Glycerol and Ethanol.

Key :-

Fermentation Conditions: Constant Temperature

1 - Complete Defined Media

2- Muller Thurgau

3- Chenin Blanc

Variable Temperature

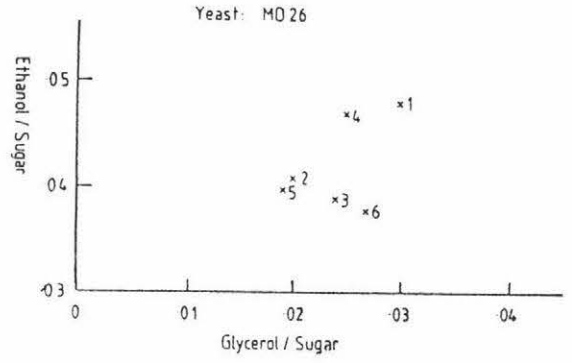
4- Complete Defined Media

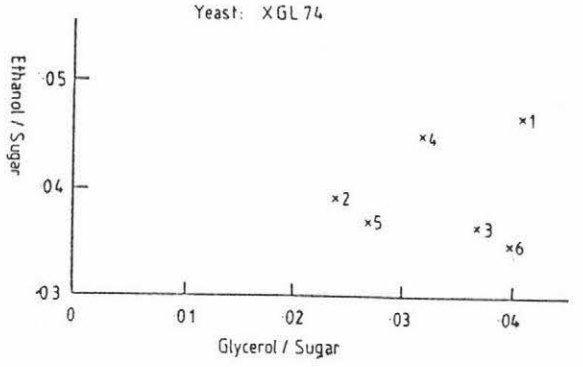
5- Muller Thurgau

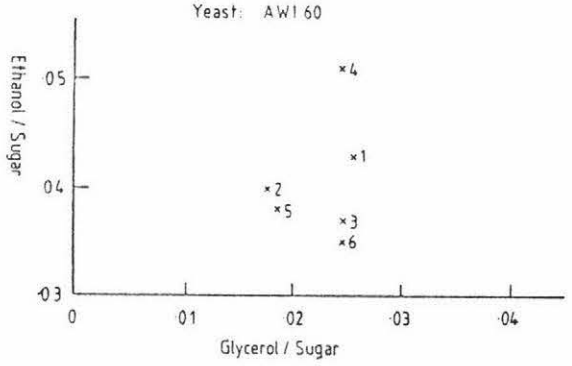
6- Chenin Blanc

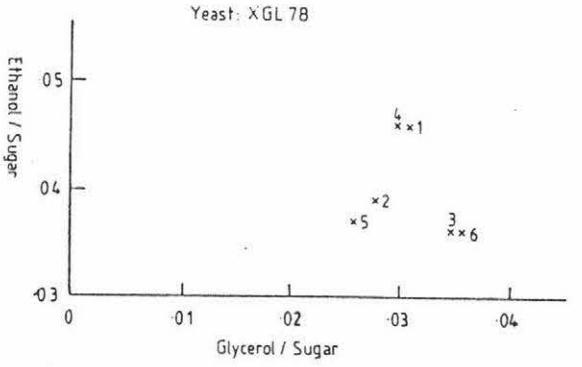
Units : Glycerol / Sugar (by weight)

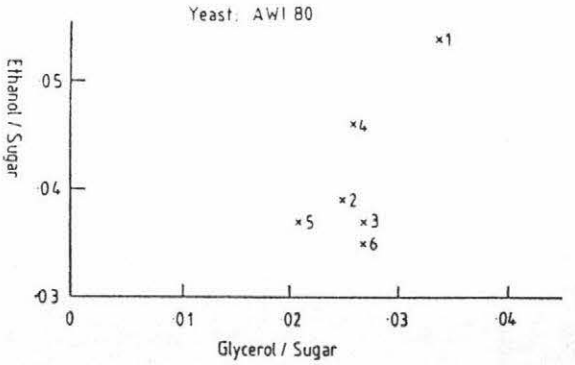
Ethanol / Sugar (by weight)


Yeast: MO 26
Ethanol / Sugar
·05
·04
·03
0
·01
·02
·03
·04
Glycerol / Sugar
×1
×2
×3
×4
×5
×6


Yeast: XGL 74
Ethanol / Sugar
·05
·04
·03
0
·01
·02
·03
·04
Glycerol / Sugar
×1
×2
×3
×4
×5
×6


Yeast: AWI 60
Ethanol / Sugar
·05
·04
·03
0
·01
·02
·03
·04
Glycerol / Sugar
×1
×2
×3
×4
×5
×6


Yeast: XGL 78
Ethanol / Sugar
·05
·04
·03
0
·01
·02
·03
·04
Glycerol / Sugar
×1
×2
×3
×4
×5
×6


Yeast: AWI 80
Ethanol / Sugar
·05
·04
·03
0
·01
·02
·03
·04
Glycerol / Sugar
×1
×2
×3
×4
×5
×6

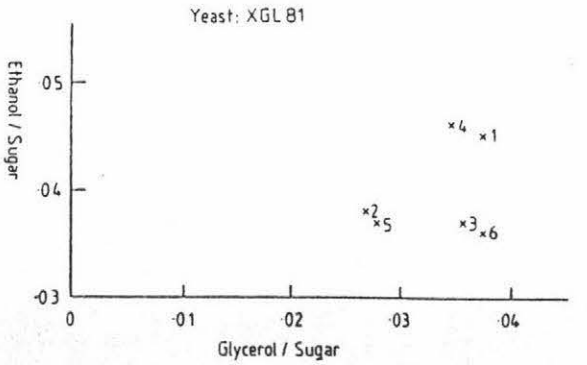
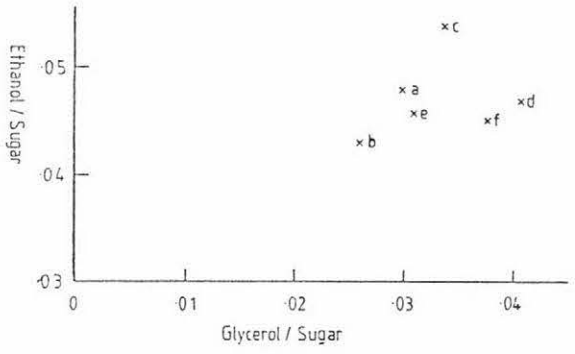
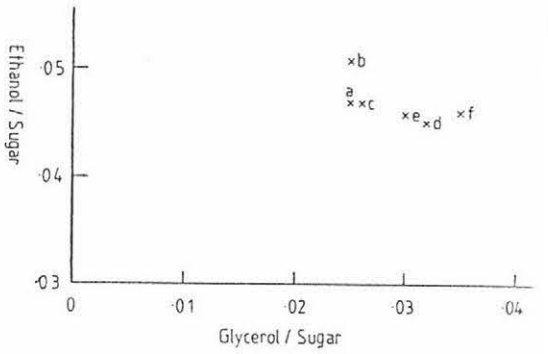

Yeast: XGL 81
Ethanol / Sugar
·05
·04
·03
0
·01
·02
·03
·04
Glycerol / Sugar
×1
×2
×3
×4
×5
×6

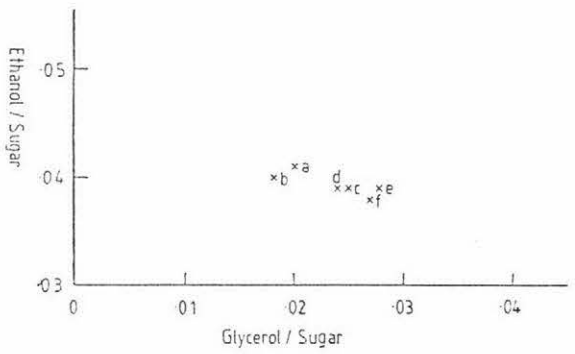
FIGURE 9 : Influence of the Yeast Strain on the Efficiency of Conversion of 1g Sugar into Glycerol and Ethanol.

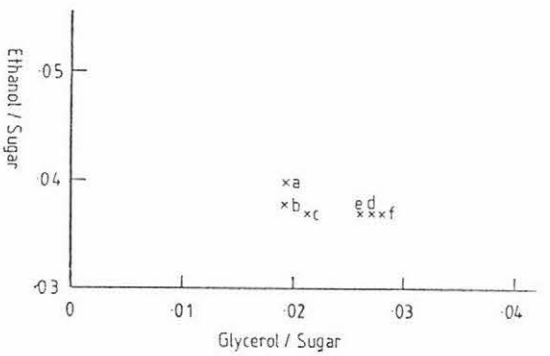
Key:-

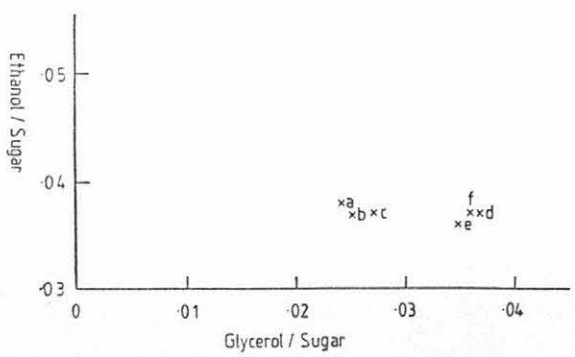
Yeast
a - MD 26
b - AWI 60
c - AWI 80
d - XGL 74
e - XGL 78
f - XGL 81

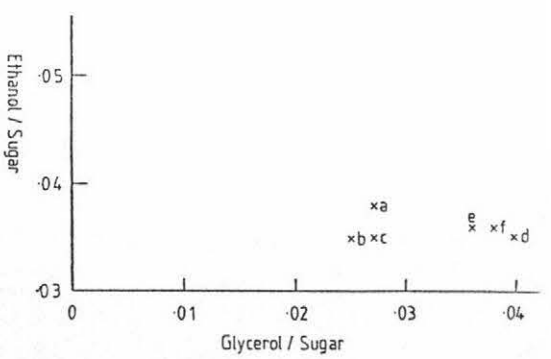

Ethanol / Sugar
·05
·04
·03
0
·01
·02
·03
·04
Glycerol / Sugar
×c
×a
×e
×d
×f
×b


Ethanol / Sugar
·05
·04
·03
0
·01
·02
·03
·04
Glycerol / Sugar
×b
a
×c
×e
×d
×f


Ethanol / Sugar
·05
·04
·03
0
·01
·02
·03
·04
Glycerol / Sugar
×b
×a
d
×c
×f
×e


Ethanol / Sugar
·05
·04
·03
0
·01
·02
·03
·04
Glycerol / Sugar
×a
×b
×c
e
d
×
×f


Ethanol / Sugar
·05
·04
·03
0
·01
·02
·03
·04
Glycerol / Sugar
×a
×b
×c
f
×
×d
×e


Ethanol / Sugar
·05
·04
·03
0
·01
·02
·03
·04
Glycerol / Sugar
×a
×b
×c
e
×
×f
×d

yeasts to produce less ethanol than low glycerol producing yeasts. However, results for each yeast vary greatly with the fermentation conditions.

It should be noted that glycerol production is plotted here on a scale ten times more sensitive than for ethanol production.

1.6 Testing for significant differences in the levels of glycerol and ethanol production

The 'Student's' t-distribution can be used to indicate significant differences between means of small population samples. In this instance it was used to analyse data on glycerol and ethanol production by the six strains of S. cerevisiae under different conditions of fermentation.

1.6.1 Comparing the efficiency of glycerol production between the parent wine-making yeasts and the high glycerol producing hybrids

In Table 16 the t-test has been used to compare mean efficiencies of glycerol produced between the parent strain (MD26, AWI60, AWI80) and the hybrid strains (XGL74, XGL78, XGL81) collectively. Conditions of fermentation are the same for each comparative test. Results shown in this table indicate that the hybrids produced significantly more glycerol than the parent strains regardless of the fermentation conditions involved. The data analysed was from fermentations carried out on completely defined media (CDM) and on the two complex media - Muller Thurgau (MT) and Chenin Blanc (CB) (following fermentation at constant temperature and at constant sugar utilization (variable temperature).

Table 17 compares the mean efficiencies of glycerol production by the hybrid and parent strains for a given set of fermentation conditions. The result of the comparison between duplicate samples of each hybrid strain and all three parent strains shows the ability of each hybrid strain to produce significantly more glycerol than the parent strains from which they were derived.

1.6.2 Comparing the efficiency of ethanol production between the parent winemaking yeasts and the high glycerol producing hybrids

Table 18 uses the t-test to compare mean levels of ethanol produced between the parent strain (MD26, AWI60, AWI80) collectively and the

TABLE 16

STUDENT T-TEST FOR EFFICIENCY OF GLYCEROL PRODUCTION

Glycerol production from 1g sugar by 3 parent yeast strains (MD26, AWI60, AWI80) compared against 3 high-glycerol producting hybrid yeast strains (XGL74, XGL78, XGL81) under conditions of varying the media and the fermentation process.

Comparison of Glycerol (g/l) Produced per 1g/l sugar:-

	Yeast	Media	Fermentation Temperature	Mean ($\bar{x}$) (g/l)	S D	t-value	% certainty
	Parent	CDM	Constant Temp	0.030	0.003	11.9	99.9%
*	Hybrid	"	" "	0.037	0.004		
	Parent	"	Variable "	0.025	0.002	21.0	99.9%
*	Hybrid	"	" "	0.032	0.002		
	Parent	MT	Constant Temp	0.021	0.003	12.79	99.9%
*	Hybrid	"	" "	0.026	0.002		
	Parent	"	Variable "	0.020	0.001	46.4	99.9%
*	Hybrid	"	" "	0.027	0.0008		
	Parent	CB	Constant Temp	0.025	0.001	81.2	99.9%
*	Hybrid	"	" "	0.036	0.0008		
	Parent	"	Variable "	0.026	0.001	45.5	99.9%
*	Hybrid	"	" "	0.038	0.002		

NB:
S D = standard deviation
n = results from 3 parent + 3 hybrid strains
n-2 = 4 degrees of freedom
* = 99.9% degree of certainty the difference is significant

TABLE 17

STUDENT T-TEST FOR EFFICIENCY OF GLYCEROL PRODUCTION

Glycerol production per lg sugar by parent strains (MD26, AWI60, AWI80) and individual hybrid strains (XGL74, XGL78, XGL81) sampled in duplicate.

Comparison of Glycerol (g/l) produced per lg of sugar:-

	Yeast	Media	Fermentation Temperature	Glycerol (g/l)	S.D.	t-value	% certainty
	Parent	CDM	Constant Temp.	0.030	0.003		
*	XGL74	"	"	0.041	0.007	24.2	99.9%
	XGL78	"	"	0.031	0.001	1.68	NSD
*	XGL81	"	"	0.038	0.004	10.08	99.9%
	Parent	CDM	Variable Temp.	0.025	0.0005		
*	XGL74	"	"	0.032	0.0005	59.4	99.9%
*	XGL78	"	"	0.030	0.002	17.3	99.9%
*	XGL81	"	"	0.035	0.002	34.6	99.9%
	Parent	MT	Constant Temp.	0.021	0.003		
	XGL74	"	"	0.024	0.0007	5.13	98%
*	XGL78	"	"	0.028	0.0007	11.96	99.9%
*	XGL81	"	"	0.027	0.0008	10.21	99.9%
	Parent	MT	Variable Temp	0.020	0.0009		
*	XGL74	"	"	0.027	0.0003	39.3	99.9%
*	XGL78	"	"	0.026	0.002	18.6	99.9%
*	XGL71	"	"	0.028	0.0004	40	99.9%
	Parent	CB	Constant Temp.	0.025	0.001		
*	XGL74	"	"	0.037	0.001	50.9	99.9%
*	XGL78	"	"	0.035	0.001	42.4	99.9%
*	XGL81	"	"	0.036	0.002	19.1	99.9%
	Parent	CB	Variable Temp.	0.026	0.0009		
*	XGL74	"	"	0.040	0.0	80.8	99.9%
*	XGL78	"	"	0.036	0.0007	43.0	99.9%
*	XGL81	"	"	0.038	0.0007	51.6	99.9%

NB:

S.D. = Standard Deviation
n = results from 3 parent strains + duplicate result from 1 hybrid strain
n-2 = 3 degrees of freedom
NSD = no significant difference
* = 99.9% degree of certainty the difference is significant

hybrid strains (XGL74, XGL78, XGL81) collectively. Comparisons have been made between the two groups for each set of fermentation conditions (i.e. for all three media following each of the two fermentation processes - at constant temperature and at constant sugar utilization (variable temperature). The parent strains produced (to 99.9% significance) more ethanol than the hybrid strains when fermented at a constant rate of sugar utilization in complete defined media, yet in Muller Thurgau and Chenin Blanc grape juices, this difference was observed in the fermentations runs at a constant temperature (marked * in Table 18).

Table 19 statistically compares ethanol production between the parent strains and the hybrid strains for a given set of fermentation conditions. The results of the comparison between duplicate samples of each hybrid strain and all three parent strains shows differences in ethanol production to be dependant on the media and fermentation process.

1.6.3 Influence of the fermentation process on glycerol production

Table 20 illustrates how the type of fermentation process influences on glycerol production from 1 gram of sugar. Mean production by the three parent strains, when fermented at a constant temperature of 15°C, was compared with production at constant sugar utilization (i.e. variable temperature). Data for the hybrid strains was treated in a similar manner. The t-test compared the mean glycerol levels produced following fermentation by each method. The effect of the fermentation process on glycerol production appeared to be influenced by the media used - (differences in glycerol production following the two fermentation processes were significant to a degree of 99.9% in the case of both the parent strains and the hybrids on complete defined media). In both cases more glycerol was produced in the fermentations run at a constant temperature. Smaller differences were observed following fermentation in the two grape juices.

Table 21 compares data for ethanol production by treating it in a similar manner to that for Table 20. As for glycerol production, the effect of the fermentation process on ethanol production was influenced by the type of media used - both the parent and the hybrid strains appear to have produced more ethanol (to a degree of 99.9% significance)

TABLE 18

STUDENT T-TEST FOR EFFICIENCY OF ETHANOL PRODUCTION

- Ethanol production from 1g of sugar by 3 parent strains (MD26, AWI60, AWI80) compared against 3 high glycerol producting hybrid yeast strains (XGL74, XGL78, XGL81) under conditions of varying the media and the fermentation process.

Comparison of Ethanol (g/l) Produced per 1g of sugar:-

	Yeast	Media	Fermentation Temperature	Mean ($\bar{x}$) (g/l)	S.D.	t-value	% certainty
	Parent	CDM	Constant Temp.	0.48	0.04)	2.11	NSD
	Hybrid	"	" "	0.46	0.008)		
	Parent	"	Variable Temp.	0.49	0.02)	12.3	99.9%
*	Hybrid	"	" "	0.46	0.005)		
	Parent	MT	Constant Temp.	0.40	0.008)	8.99	99.9%
*	Hybrid	"	" "	0.39	0.005)		
	Parent	"	Variable Temp.	0.38	0.01)	8.49	99.8%
	Hybrid	"	" "	0.37	0.0)		
	Parent	CB	Constant Temp.	0.38	0.009)	13.3	99.9%
*	Hybrid	"	" "	0.36	0.009)		
	Parent	"	Variable Temp.	0.36	0.02)	4.12	98%
	Hybrid	"	" "	0.35	0.005)		

NB: S.D. = Standard Deviation
n = results from 3 parent + 3 hybrid strains
n-2 = 4 degrees of freedom
NSD = No significant difference
* = 99.9% degree of certainty the difference is significant

TABLE 19

STUDENT T-TEST FOR EFFICIENCY OF ETHANOL PRODUCTION

Ethanol production per lg of sugar by parent strains (MD26, AWI60, AWI80) and individual hybrid strains (XGL74, XGL78, XGL81) sampled in duplicate.

Comparison of Ethanol (g/l) per lg of sugar:-

	Yeast	Media	Fermentation Temperature	Mean ($\bar{x}$) (g/l)	S.D.	t-value	% certainty
	Parent	CDM	Constant Temp.	0.48	0.04		
	XGL74	"	"	0.47	0.007	1.29	NSD
	XGL78	"	"	0.46	0.0	1.3	NSD
	XGL81	"	"	0.45	0.01	5.09	99%
	Parent	CDM	Variable Temp.	0.48	0.02		
	xgl74	"	"	0.45	0.0	6.36	99%
	XGL78	"	"	0.46	0.004	6.86	99%
	XGL81	"	"	0.46	0.003	6.89	99%
	Parent	MT	Constant Temp.	0.40	0.008		
	XGL74	"	"	0.39	0.003	6.28	99%
	XGL78	"	"	0.39	0.003	6.28	99%
*	XGL81	"	"	0.38	0.0008	17.3	99.9%
	Parent	MT	Variable Temp.	0.38	0.01		
	XGL74	"	"	0.37	0.002	5.14	98%
	XGL78	"	"	0.37	0.02	3.0	95%
	XGL81	"	"	0.37	0.004	5.0	98%
	Parent	CB	Constant Temp.	0.38	0.05		
	XGL74	"	"	0.37	0.006	7.92	99%
*	XGL78	"	"	0.35	0.009	19.3	99.9%
	XGL81	"	"	0.37	0.01	6.0	99%
	Parent	CB	Variable Temp.	0.36	0.01		
	XGL74	"	"	0.35	0.0	2.598	90%
	XGL78	"	"	0.35	0.02	3.0	98%
	XGL81	"	"	0.36	0.02	0	NSD

NB: S.D. = Standard Deviation
n = results from 3 parent strains + duplicate results of 1 hybrid strain
n-2 = 3 degrees of freedom
NSD = No significant difference
* = 99.9% degree of certainty the difference is significant

TABLE 20

Student t-test to Compare Glycerol Production at Constant Temperature Fermentation with Constant Sugar Utilisation (Variable Temp) Fermentation:

	Yeast	Media	Fermentation Temperature	Glycerol ($\bar{x}$) (g/l)	S.D.	t-value	% certainty
	Parent	CDM	Constant Temp.	0.030	0.003	11.8	99.9%
*	Parent	"	Variable "	0.025	0.002		
	Hybrid	"	Constant "	0.037	0.004	9.49	99.9%
*	Hybrid	"	Variable "	0.032	0.002		
	Parent	MT	Constant "	0.021	0.003	2.68	90%
	Parent	"	Variable "	0.020	0.001		
	Hybrid	"	Constant "	0.026	0.002	3.94	98%
	Hybrid	"	Variable "	0.027	0.0008		
	Parent	CB	Constant "	0.025	0.001	6.0	99%
	Parent	"	Variable "	0.026	0.001		
	Hybrid	"	Constant "	0.036	0.0008	7.88	99.8%
	Hybrid	"	Variable "	0.038	0.002		

TABLE 21

Student t-test to Compare Ethanol Production at a Constant Temperature Fermentation with a Constant Sugar Utilisation (Variable Temp) Fermentation:

	Yeast	Media	Fermentation Temperature	Ethanol ($\bar{x}$) (g/l)	S.D.	t-value	% certainty
	Parent	CDM	Constant Temp	0.48	0.04	1.897	NSD
	Parent	"	Variable "	0.49	0.02		
	Hybrid	"	Constant "	0.46	0.008	0.0	NSD
	Hybrid	"	Variable "	0.46	0.005		
*	Parent	MT	Constant "	0.40	0.008	13.3	99.9%
	Parent	"	Variable "	0.38	0.01		
*	Hybrid	"	Constant "	0.39	0.005	33.9	99.9%
	Hybrid	"	Variable "	0.37	0.0		
	Parent	CB	Constant "	0.38	0.009	7.74	99.8%
	Parent	"	Variable "	0.36	0.02		
	Hybrid	"	Constant "	0.36	0.009	8.24	99.8%
	Hybrid	"	Variable "	0.35	0.005		

NB: S.D. = standard deviation
n = results from 3 parent + 3 hybrid strains
n-2 = 4 degrees of freedom
NSD = no significant difference
* = 99.9% degree of certainty the difference is significant.

FIGURE 10: Growth Curves Showing Cell Growth of Parent and Hybrid Yeast Strains.

Key :- Fermentation Conditions

Constant Temperature —×— Parent

—o— Hybrid

Constant Sugar Utilisation ·····×····· Parent

·····o····· Hybrid

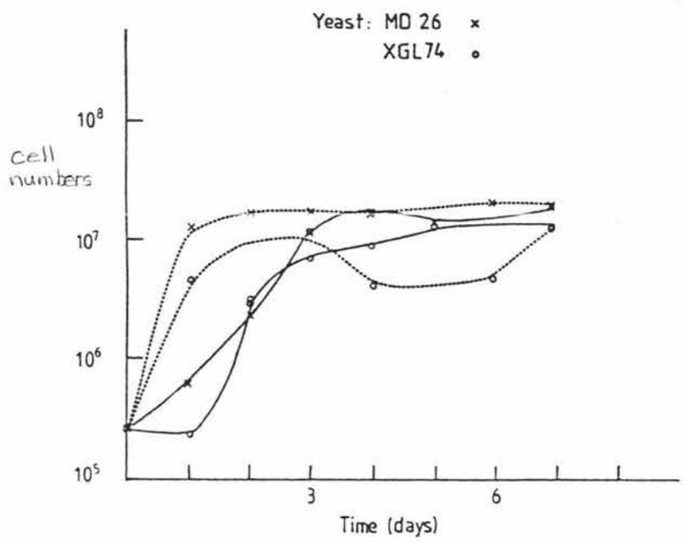
a - c : Yeast Growth on Complete Defined Media (CDM)

d - f : Yeast Growth on Muller Thurgau (MT)

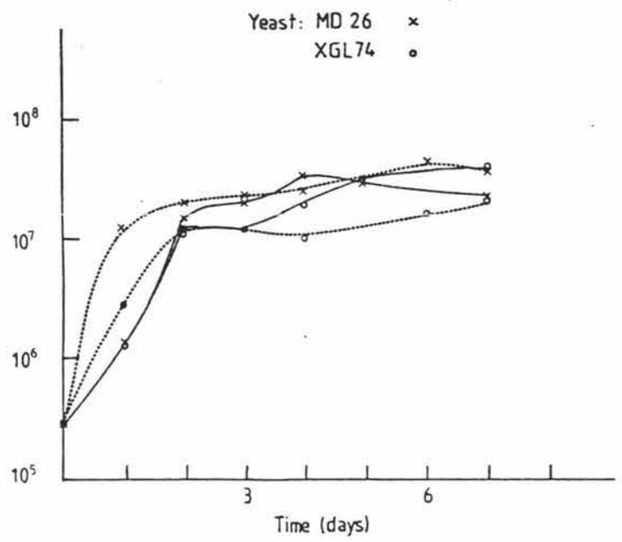
g - i : Yeast Growth on Chenin Blanc (CB)

Log Cell Growth (Cells / ml)

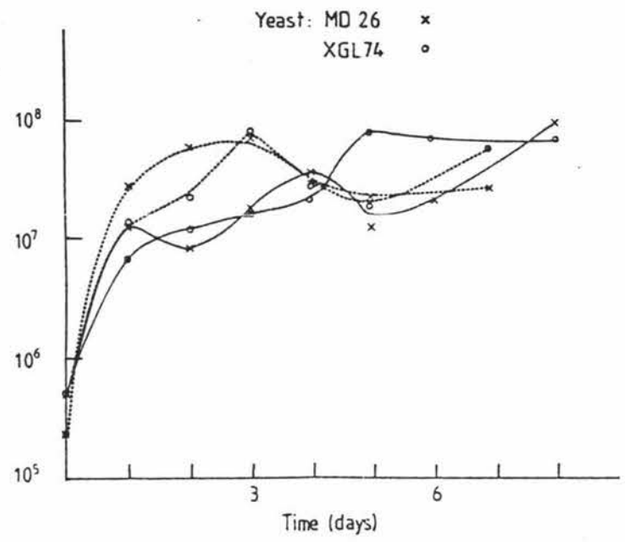
Complete Defined Media

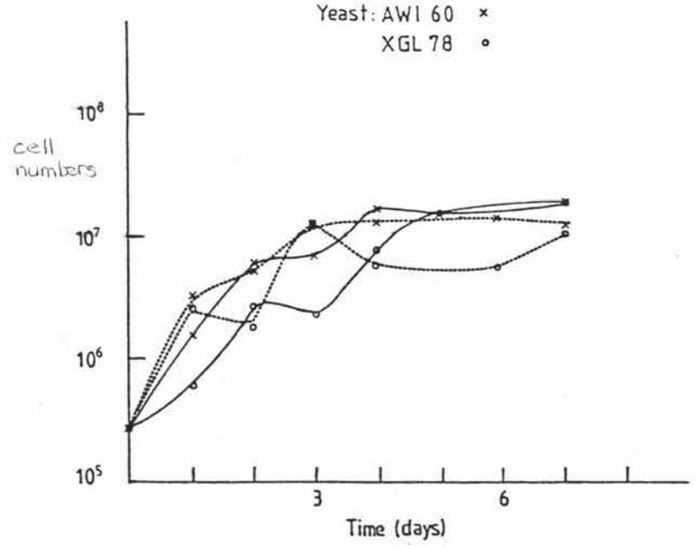


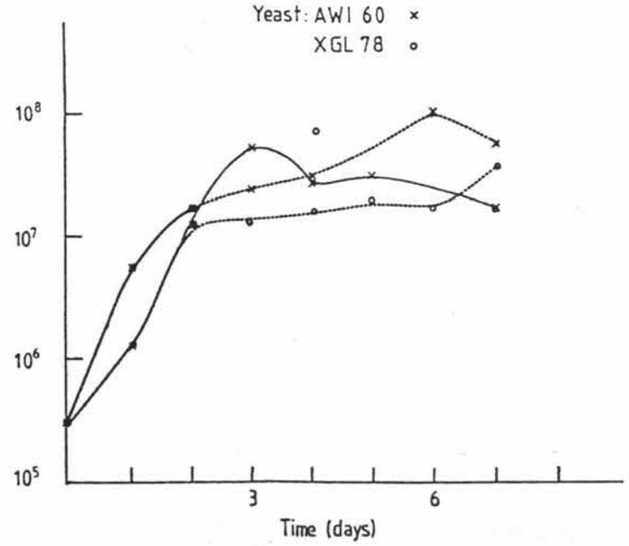
Muller Thurgau

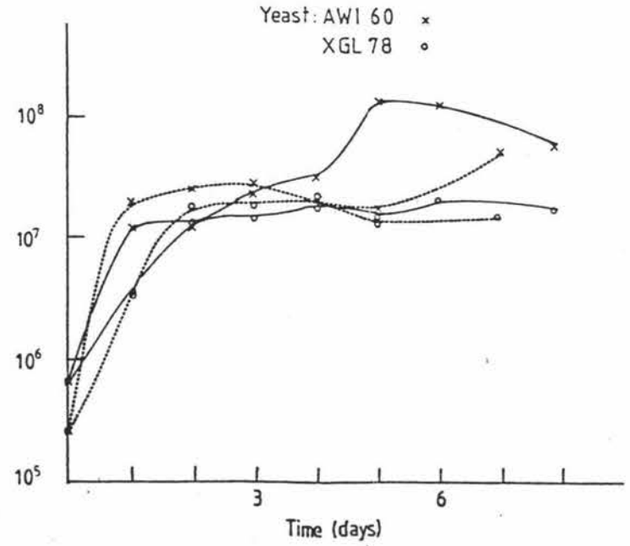


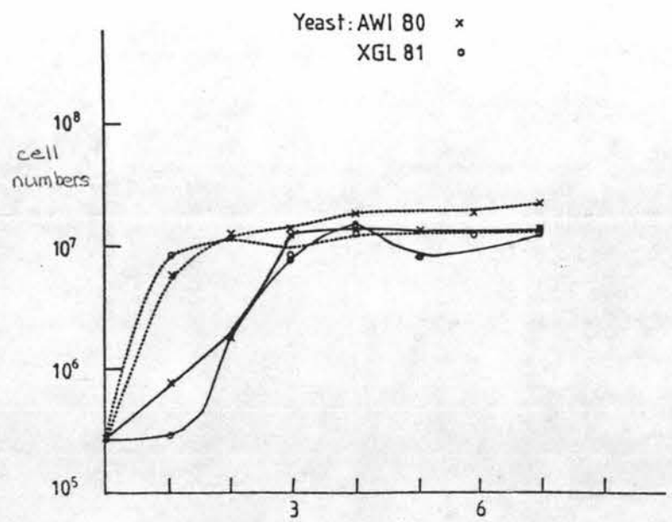
Chenin Blanc

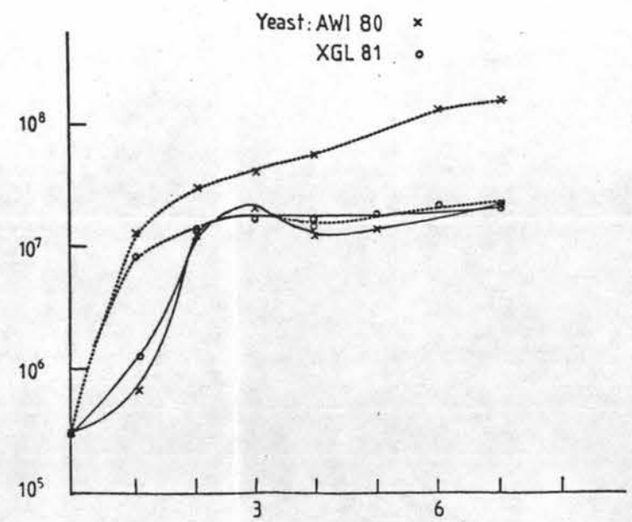


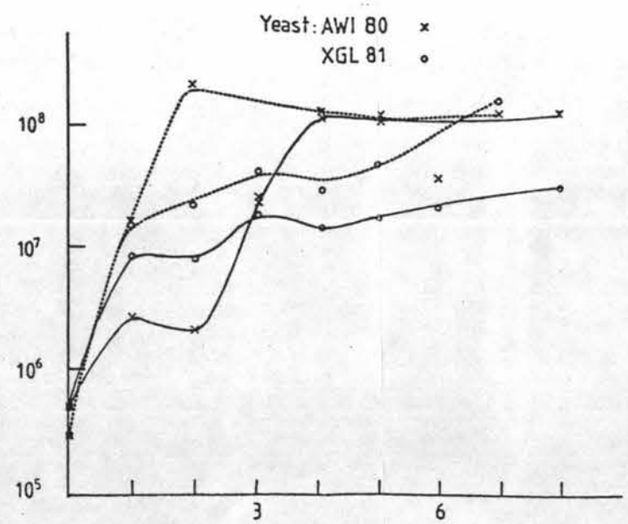












at the constant temperature of fermentation in Muller Thurgau grape juice. (Smaller differences were observed in the Chenin Blanc grape juice).

1.6.7 Relating production of glycerol and ethanol to rate of cell growth

Cell growth was determined using the viable counting technique described in method 2.4.

Figure 10 graphically compares cellular growth rates (numerical data is shown in Appendix tables A(X) - (XII) and B(X) - (XII). Each graph individually compares one parent strain (at both a constant temperature fermentation and at a constant sugar utilization fermentation) with one hybrid strain (also following the same fermentation procedures). Rates of cell growth were only visually compared.

Figure 10 suggests little difference in growth between the parent and hybrid strains when the fermentation conditions were similar. The fermentation process appeared to influence the growth rate (the higher initial temperature of the constant sugar utilization fermentation provided for rapid cell growth), and the fermentation media appeared to influence the cell propulation at stationary phase.

2. THE EFFECT OF SULPHUR DIOXIDE ON THE PRODUCTION OF GLYCEROL AND ETHANOL

Sulphur dioxide levels were determined using the method developed by Rankine and Pocock (1972) (Method 2.3) and adjusted accordingly by the addition of sodium metabisulphite or hydrogen peroxide as required. Free and total levels of sulphur dioxide (ppm) and the pH of each set of flasks are recorded in Appendix C. The differences in pH, 3.44-3.73 in complete defined media and 2.93-3.06 in Chenin Blanc, were not considered significant.

Fermentation trials were carried out using yeast strains AWI60 (a low glycerol producing wine yeast) and XGL81 (a high glycerol producing hybrid yeast) in Chenin Blanc grape juice (CB) and in the synthetic complete defined media (CDM) at the appropriate levels of sulphur dioxide. When the fermentations had proceeded to dryness (length of time to ferment to dryness increased as the sulphur dioxide level was raised), samples were taken and filtered for analysis by HPLC (method 2.2). Yeast strain AWI60 was fermented in duplicate at each sulphur dioxide level in both media. While strain XGL81 was fermented in triplicate in a similar manner.

Glycerol and ethanol production for each set of conditions is displayed in Appendix C Tables CIII and IV. Mean values are presented in Table 22 and a graphical presentation of this data is displayed in Figures 11 and 12. As the sulphur dioxide level was raised, AWI60 showed an increase in glycerol production and a corresponding decrease in ethanol production, while XGL81 exhibited the converse, i.e. decreased glycerol production and increased ethanol production.

The range for each set of data is displayed on the graphs to substantiate that these trends do exist and differences in glycerol and/or ethanol production may occur, as the sulphur dioxide level is raised, beyond the range of experimental variation.

Time taken to ferment to dryness increased as the sulphur dioxide level was raised (especially >100 ppm). This delay was more pronounced with XGL81, which took 7 weeks to ferment to dryness at 300 ppm SO_2, whereas AWI60, at this level, completed fermentation at 5 weeks. At low levels of sulphur dioxide (0-100 ppm), both yeasts fermented to dryness within 4 weeks.

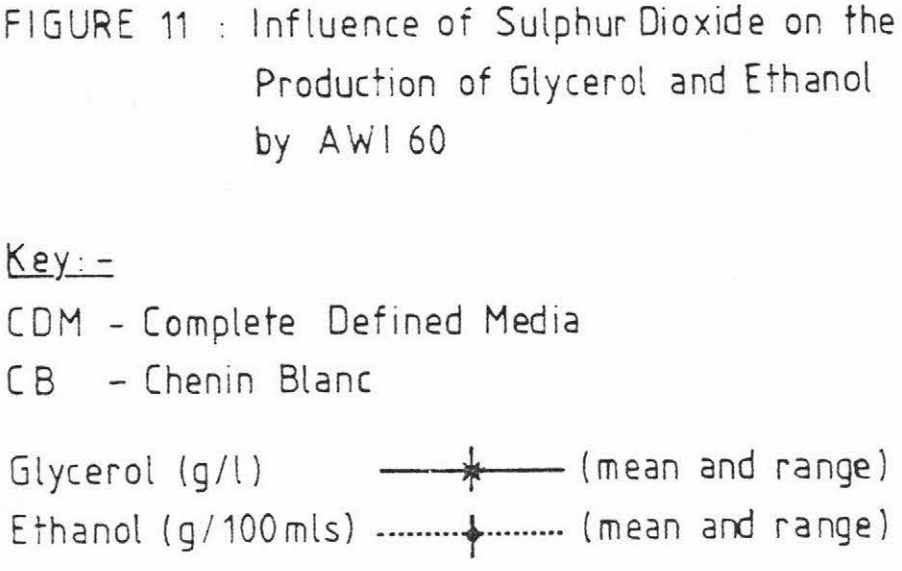
TABLE 22: Production of Glycerol and Ethanol in the Presence of Different Levels of Sulphur Dioxide

Yeast	Approx SO_2 level (ppm)	Media	Glycerol (g/l)	Ethanol (g/100ml)
AWI60	0	CDM	2.5	4.6
	50		2.7	4.6
	100		2.5	4.6
	150		2.7	4.3
	200		2.7	4.4
	250		2.6	4.2
	300		2.7	4.2
	0	CB	5.9	9.0
	50		6.2	9.0
	100		5.9	9.0
	150		6.2	9.1
	200		6.5	8.8
	250		6.3	8.9
	300		6.4	8.7
XGL81	0	CDM	3.6	4.0
	50		3.9	3.8
	100		4.2	3.9
	150		4.0	4.2
	200		3.9	4.1
	250		3.9	4.4
	300		3.8	4.4
	0	CB	8.2	7.0
	50		8.7	7.1
	100		8.2	7.2
	150		8.0	7.5
	200		-	-
	250		7.7	8.4
	300		7.3	8.5

NB Glycerol = $\pm$ 0.2g/l

Ethanol = $\pm$ 0.1g/100ml

FIGURE 11 : Influence of Sulphur Dioxide on the Production of Glycerol and Ethanol by AWI 60



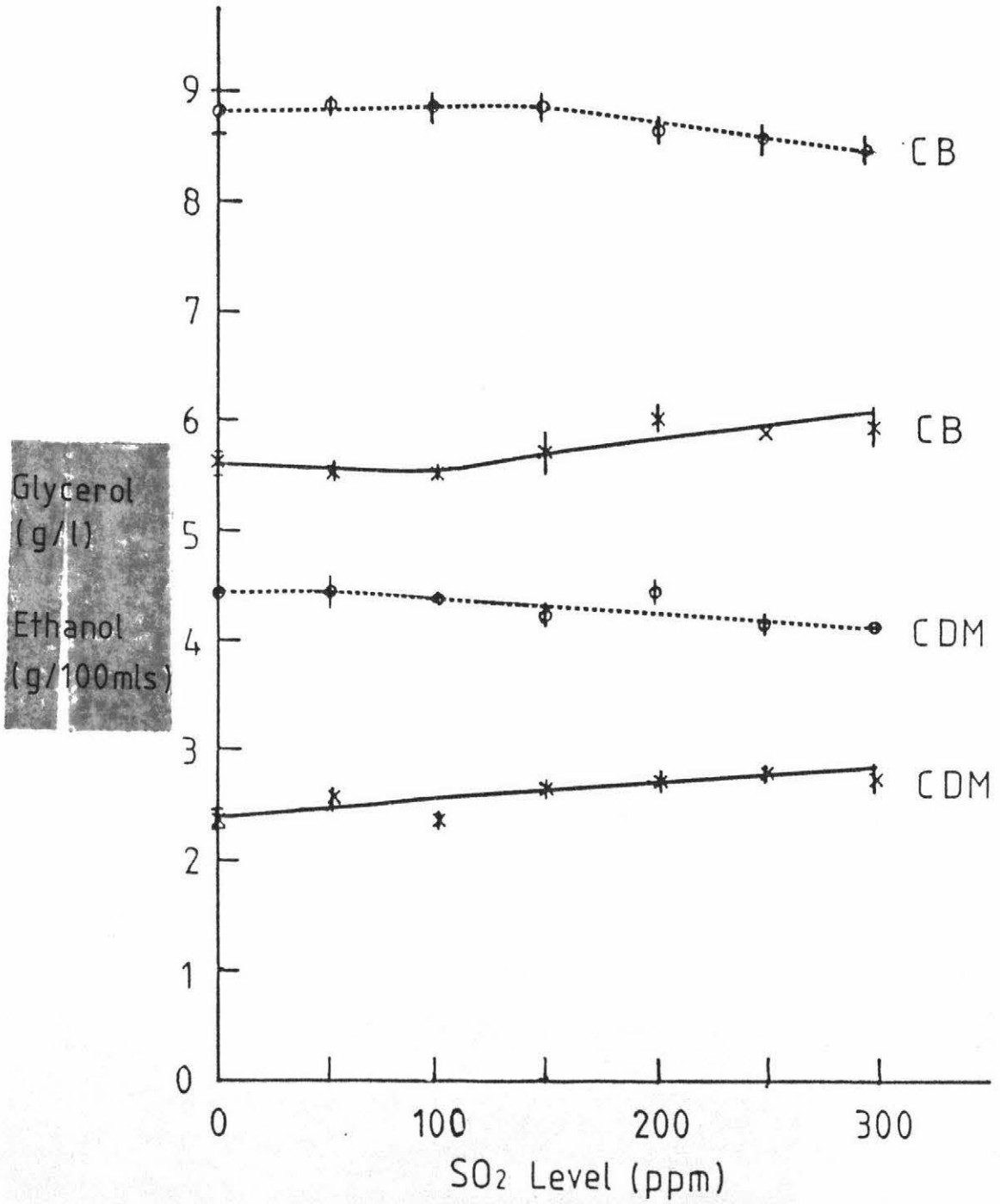
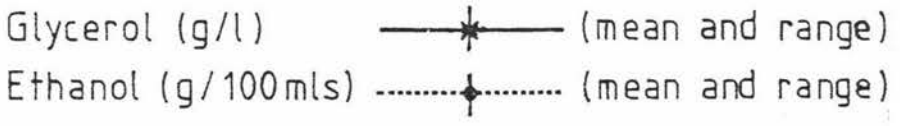

Glycerol (g/l)
Ethanol (g/100mls)
9
8
7
6
5
4
3
2
1
0
CB
CB
CDM
CDM
0
100
200
300
SO2 Level (ppm)

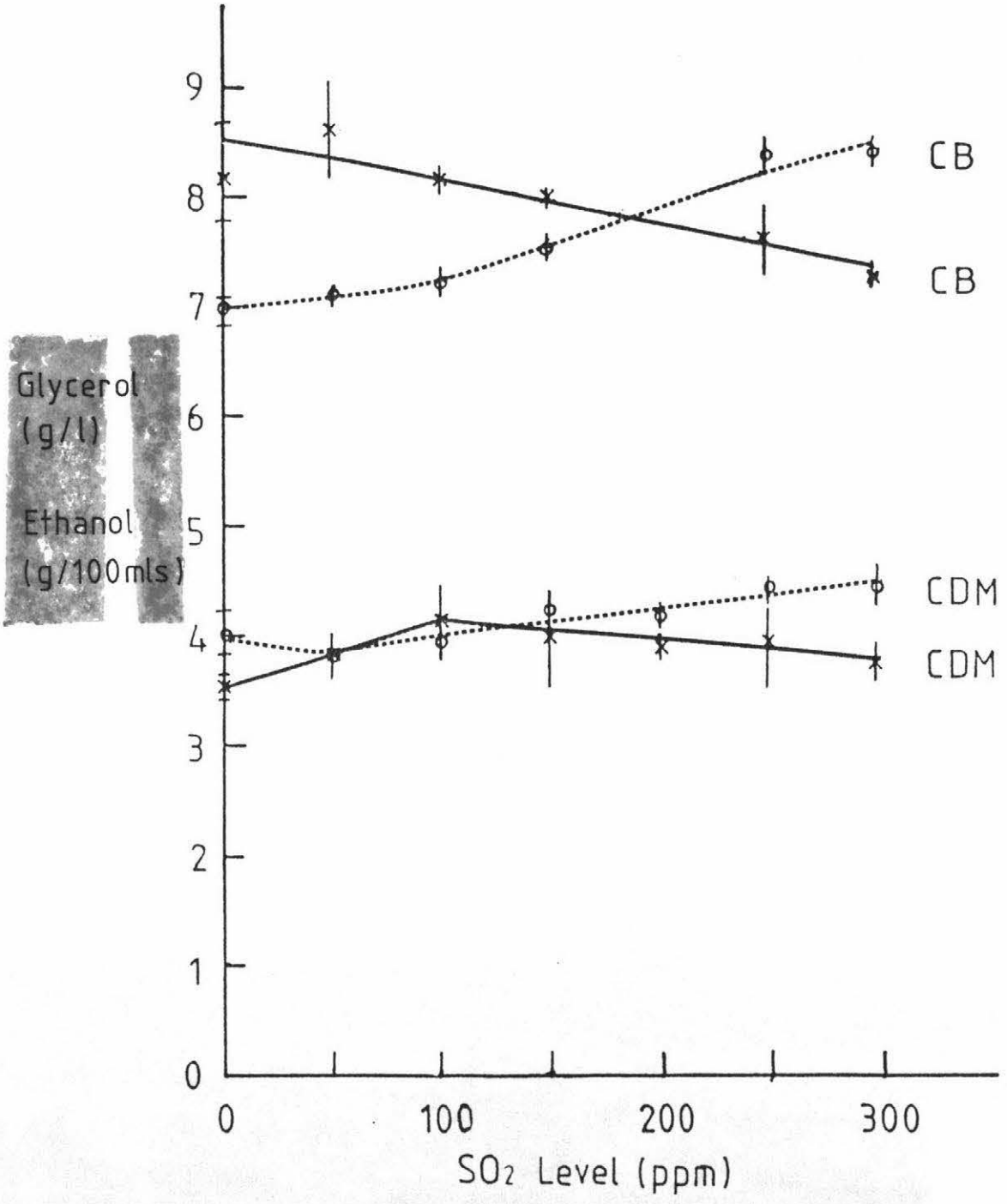
FIGURE 12 : Influence of Sulphur Dioxide on the Production of Glycerol and Ethanol by XGL 81

Key: -

CDM - Complete Defined Media

CB - Chenin Blanc




Glycerol (g/l)
Ethanol (g/100mls)
9
8
7
6
5
4
3
2
1
0
CB
CB
CDM
CDM
0
100
200
300
SO2 Level (ppm)

3 INFLUENCE OF GLYCEROL IN THE MEDIA PRIOR TO FERMENTATION ON THE PRODUCTION OF GLYCEROL AND ETHANOL

Glycerol was added in increments of approximately 5 g to the fermentation media prior to inoculation. Samples of both media (Chenin Blanc and complete defined media) at each glycerol level were taken and analysed by HPLC (Method 2.3) for the initial glycerol content. Results are shown in Table 23.

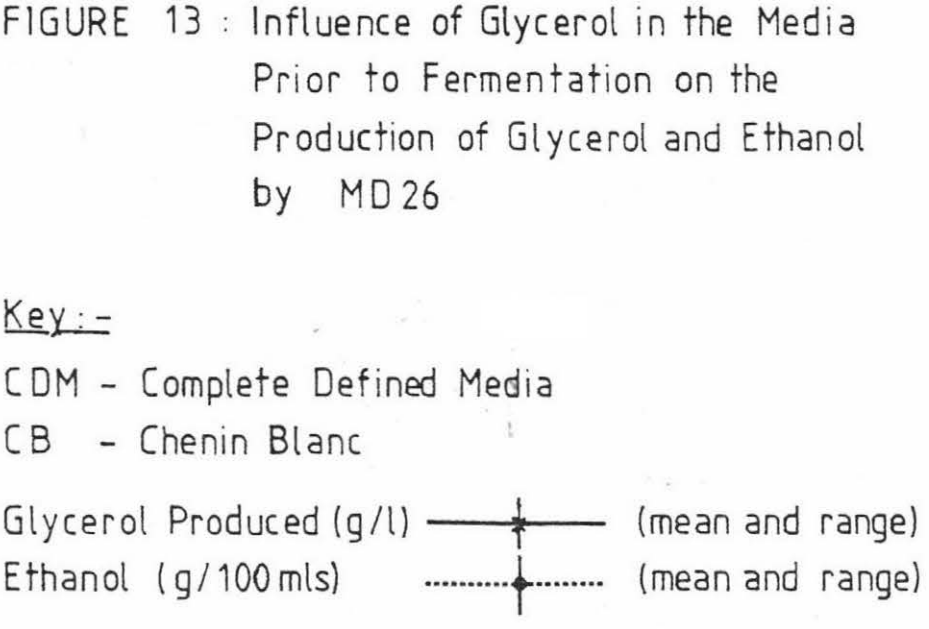
Fermentations were performed aerobically at 15°C as detailed in Method 3.3. Two yeast strains were used in these fermentation trials - MD26 (a low glycerol-producing wine yeast) and XGL81 (a high glycerol-producing hybrid yeast). Samples were taken once the fermentations had reached dryness, and analysed for glycerol and ethanol production by HPLC. Glycerol production by each yeast was assumed to be the initial glycerol level (as determined by HPLC analysis) subtracted from the level of glycerol detected once fermentation was complete. The results are displayed in Table 23.

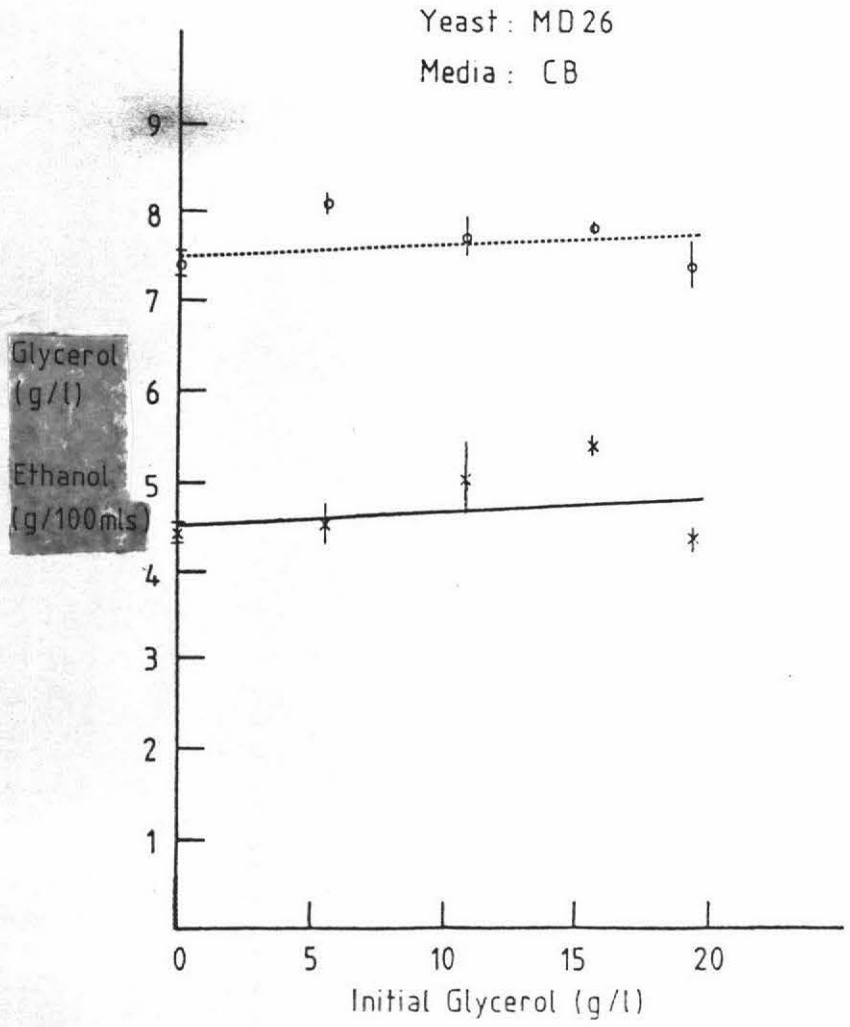
Figures 13 and 14 graphically display glycerol and ethanol production, as a function of glycerol added to the media prior to fermentation.

TABLE 23: Influence of Glycerol in the Media Prior to Fermentation on the Production of Glycerol and Ethanol

Yeast	Media	Initial Glycerol (g/l)	Final Glycerol (g/l)	Glycerol Production (g/l) x_1, x_2	Glycerol Production (g/l) $\bar{x}$	Ethanol Production (g/100 ml) x_1, x_2	Ethanol Production (g/100 ml) $\bar{x}$
MD26	CDM	0.00	3.20 2.97	3.20 2.97	3.09	4.12 4.22	4.17
		4.26	7.51 7.41	3.25 3.15	3.20	4.17 4.11	4.14
		9.55	12.52 12.19	2.97 2.64	2.81	3.86 2.96	3.41
		13.47	17.35 16.91	3.88 3.44	3.66	4.14 4.05	4.10
		19.00	21.92 23.04	2.92 4.04	3.58	4.00 4.06	4.03
	CB	0.15	4.51 4.74	4.36 4.59	4.48	7.31 7.60	7.46
		6.11	10.47 10.85	4.36 4.74	4.55	8.21 8.09	8.15
		11.60	16.27 17.01	4.67 5.41	5.04	7.92 7.53	7.73
		16.07	21.56 21.43	5.49 5.35	5.43	7.86 7.80	7.83
		22.56	27.02 26.85	4.46 4.29	4.35	7.66 7.11	7.39
XGL81	CDM	0.00	4.15 4.18	4.15 4.18	4.17	4.26 4.02	4.14
		4.26	8.07 8.35	3.81 4.09	3.95	4.12 3.45	3.79
		9.55	13.43 13.69	3.88 4.14	4.01	3.93 3.95	3.94
		13.47	18.36 17.84	4.89 4.37	4.63	3.77 4.03	3.90
		19.00	28.56 23.00	4.56 4.00	4.28	3.93 3.79	3.86
	CB	0.15	7.71 7.35	7.56 7.20	7.38	7.63 7.45	7.54
		6.11	13.89 14.03	7.78 7.92	7.85	8.06 7.74	7.90
		11.60	19.96 19.70	8.36 8.10	8.23	8.01 8.07	8.04
		16.07	23.59 23.72	7.52 7.65	7.59	7.24 7.56	7.40
		22.56	29.74 30.02	7.18 7.46	7.32	7.80 7.65	7.73

FIGURE 13 : Influence of Glycerol in the Media Prior to Fermentation on the Production of Glycerol and Ethanol by MD 26




Yeast : MD 26
Media : CB
Glycerol (g/l)
Ethanol (g/100mls)
9
8
7
6
5
4
3
2
1
0
5
10
15
20
Initial Glycerol (g/l)

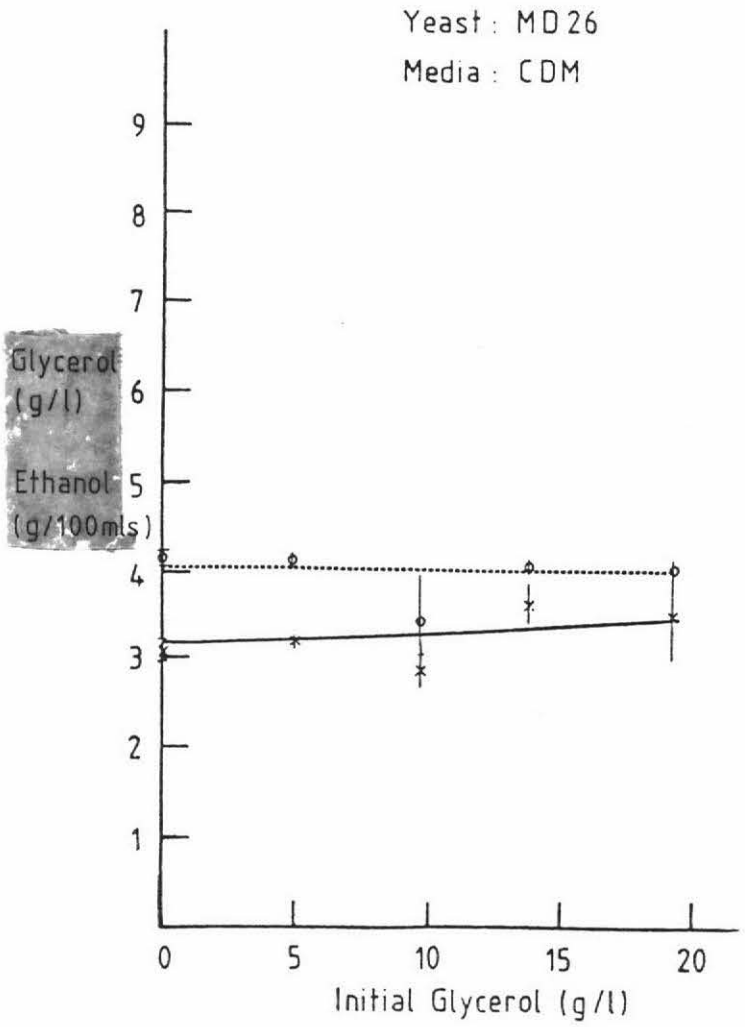
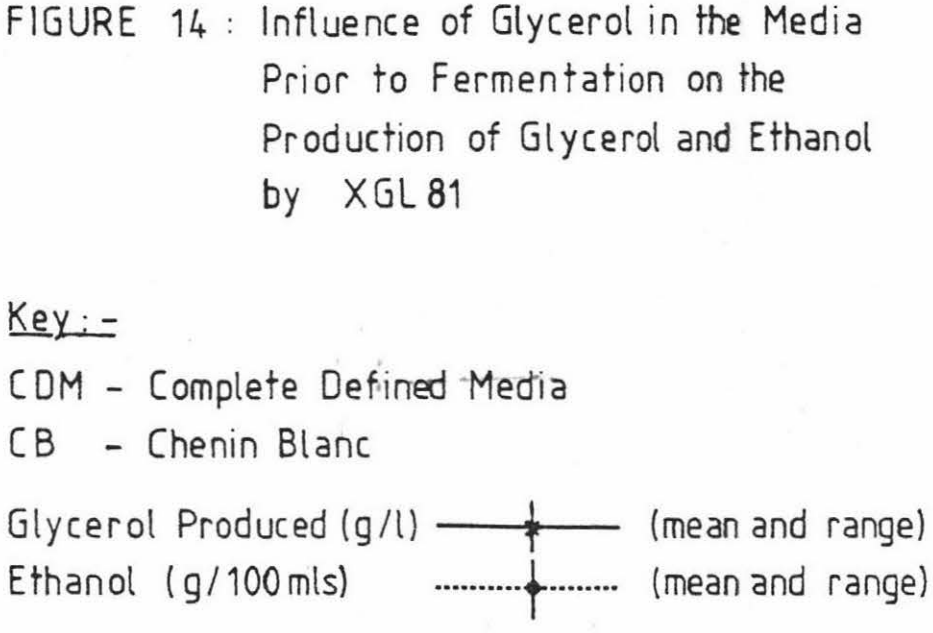
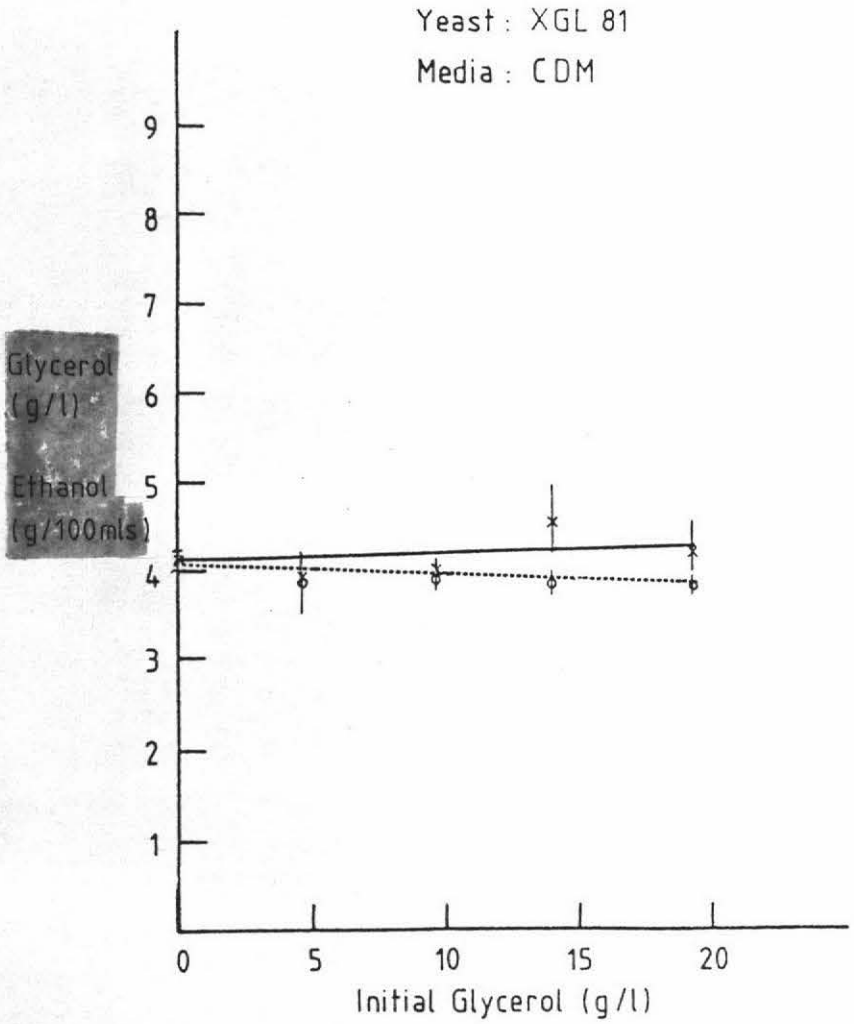
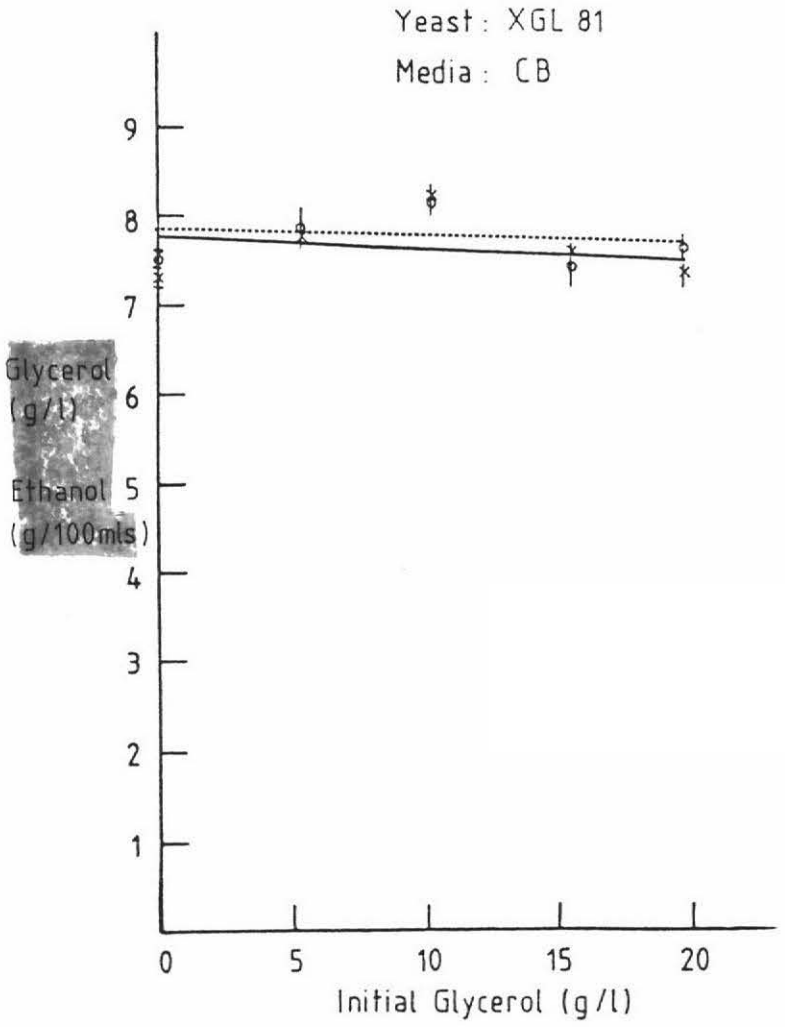

Yeast : MD 26
Media : CDM
Glycerol (g/l)
Ethanol (g/100mls)
9
8
7
6
5
4
3
2
1
0
5
10
15
20
Initial Glycerol (g/l)

FIGURE 14 : Influence of Glycerol in the Media Prior to Fermentation on the Production of Glycerol and Ethanol by XGL 81




Yeast : XGL 81
Media : CDM
Glycerol (g/l)
Ethanol (g/100mls)
9
8
7
6
5
4
3
2
1
0
5
10
15
20
Initial Glycerol (g/l)


Yeast : XGL 81
Media : CB
Glycerol (g/l)
Ethanol (g/100mls)
9
8
7
6
5
4
3
2
1
0
5
10
15
20
Initial Glycerol (g/l)

4. HYBRIDISATION PROGRAMME TO SELECT FOR HIGH GLYCEROL PRODUCING STRAINS OF SACCHAROMYCES CEREVISIAE

4.1 General outline

Two hybrid diploid strains of S. cerevisiae, XGL74 and XGL81, were chosen to initiate this hybridisation programme as they are able to produce relatively high levels of glycerol compared to many wine yeasts.

4.2 Selection for the first generation of haploids yeasts

The micromanipulation procedure used throughout this hybridisation programme is described in Method Section 3.4.

On sporulation of yeast XGL74 strains and XGL 81 (Method 3.4.1), the asci were dissected out and, the haploid spores released and isolated by micromanipulation. Twenty haploid segregants from each of yeast strains XGL74 and XGL81 were grown up into colonies on MYGP agar. The mating types of these segregants were determined (Method 3.4.1.1). The 40 haploid segregants were tested for production of glycerol and ethanol in fermentations of both Muller Thurgau and Chenin Blanc grape juices. (Methods 3.1 and 3.4.2).

Appendix D Tables D(I) and (II) show mating types, glycerol production (g/l) and ethanol production (g/100 ml) from Muller Thurgau for 20 haploid colonies derived from each of the diploid yeast strains XGL74 and XGL81 respectively. Appendix D Tables D (III) and (IV) display data for the same 40 haploid yeasts and the two parent yeast strains XGL74 and XGL81 fermented on Chenin Blanc.

Samples were taken from each culture, following fermentation to dryness and analysed by HPLC for glycerol and ethanol (Method 2.2).

The best haploid segregants of yeast strains XGL74 and XGL81 (of compatible mating type) were selected in each of the following categories:

(1) highest levels of glycerol production in Muller Thurgau

(2) high levels of glycerol and ethanol production in Muller Thurgau

(3) highest levels of glycerol production in Chenin Blanc

(4) high levels of glycerol and ethanol production in Chenin Blanc

The results of this selection from the first generation of haploid segregants are shown in Table 24.

4.2.1 Selection for the first generation of diploid yeasts

Each of the four haploid pairs were mated (Method 3.4.1.1). Approximately ten zygotes from each mating were isolated by micromanipulation (Method 3.4).

Fermentation trials were carried out with these diploid strains and the fermentation end products analysed by HPLC (Appendix D, Tables (V) to (VIII).

The strains chosen for categories (1) and (2) were both derived from haploid segregants selected only for high levels of glycerol production (category 1). Results of this selection for the first generation of diploid yeasts are displayed in Table 25.

For convenience:

XGA = XGL74(18) x XGL81(18) (v)
XGB = XGL74(18) x XGL81(18) (ix)
XGC = XGL74(3) x XGL81(4) (vi)

Yeast strain XGA was the superior strain in Categories 1 and 3.

4.3 Selection for the second generation of haploid yeasts

The diploid yeast strains XGA, XGB and XGC selected from the first generation cross were sporulated (Method 3.41) and approximately twenty haploid spores were isolated from each cross by micromanipulation. Fermentation trials were carried out using these haploid segregants and their fermentation end-products analysed by HPLC. (Appendix D, Tables D(IX) to (XII)).

Table 26 shows the glycerol and ethanol produced by those yeast strains selected for each of the four categories. Note that in the case of Muller Thurgau, those strains selected were from category (2) only (i.e. high glycerol and ethanol production in Muller Thurgau), and the strains selected for Chenin Blanc were selected from category (3) only (i.e. high glycerol production Chenin Blanc).

TABLE 24: SELECTION FROM THE FIRST GENERATION OF HAPLOID YEASTS

	Yeast	Mating type	Glycerol Production (g/l)	Ethanol Production (g/100 ml)
Category 1)	Muller Thurgau - high glycerol production			
	XGL74 (18)	α	5.85	6.88
	XGL81 (18)	a	5.26	7.30
2)	Muller Thurgau - high glycerol and ethanol production			
	XGL74 (19)	α	4.96	7.17
	XGL81 (15)	a	5.21	7.41
3)	Chenin Blanc - high glycerol production			
	XGL73 (3)	a	8.97	8.80
	XGL81 (4)	α	10.75	8.14
4)	Chenin Blanc - high glycerol and ethanol production			
	XGL74 (18)	a	8.07	9.16
	XGL81 (18)	α	8.55	8.45

TABLE 25: SELECTION FROM THE FIRST GENERATION OF DIPLOID YEASTS

	Yeast	Mating Type	Glycerol Production (g/l)	Ethanol Production (g/100 ml)
Category 1)	Muller Thurgau - high glycerol production			
	XGA (XGL74(18) x XGL81(18)(v)		5.57	6.99
2)	Muller Thurgau - high glycerol and ethanol production			
	XGB (XGL74(18) x XGL81(18)(ix)		5.18	7.26
3)	Chenin Blanc - high glycerol production			
	XGA (XGL74(18) x XGL81(18)(v)		8.94	7.11
4)	Chenin Blanc - high glygerol and ethanol production			
	XGC (XGL74(3) x XGL81(4)(vi)		8.04	7.93

4.3.1 Selection for the second generation of diploid yeasts

Each of the four haploid pairs were mated (Method 3.4.1.1) and approximately ten zygotes from each pair isolated by micromanipulation. These were tested in fermentation trials and analysed by HPLC for production of glycerol and ethanol. (Appendix D, Tables D (XIII) to (XVI)). Those diploids selected for the hybridisation programme are shown in Table 27.

For convenience:

XGD = XGA(8) x XGA(13) (vii)
XGE = XGA(8) x XGA(13) (v)
XGF = XGC(13) x XGC(15) (xi)
XGG = XGC(13) x XGC(15) (ix)

(NOTE that the selection of diploid strains for the hybridisation programme was dependent on the levels of glycerol (and ethanol) produced following fermentation and also on their ability to produce a high percentage of wellformed, viable spores).

4.4 Selection for the third generation of haploid yeasts

The four diploid yeast strains, XGD, XGE, XGF and XGG were sporulated and approximately twenty haploid spores were isolated from each cross by micromanipulation. Suitable haploid segregants were selected for the four categories following fermentation in the appropriate grape juice and analysed by HPLC. (Appendix D, Tables D (XVII) to (XX)).

Table 28 shows glycerol and ethanol produced by those yeast strains selected for the four categories of the hybridisation programme.

4.4.1 Selection for the third generation of diploid yeasts

Fermentation trials were carried out with approximately ten zygotes from each of the above diploid matings, (Appendix D, Tables D (XXI) to (XXIV)).

Table 29 shows glycerol and ethanol production of the yeasts selected for the four categories of the hybridisation programme.

TABLE 26: SELECTION FROM THE SECOND GENERATION OF HAPLOID YEASTS

	Yeast	Mating Type	Glycerol Production (g/l)	Ethanol Production (g/100ml)
Category 1)	Muller Thurgau - high glycerol production			
	XGA (8)	α	5.18	6.51
	XGB(13)	a	4.95	6.15
2)	Muller Thurgau - high glycerol and ethanol production			
	XGA (1)	α	4.46	6.92
	XGA (7)	a	4.29	6.71
3)	Chenin Blanc - high glycerol production			
	XGC(13)	a	10.60	6.95
	XGC(15)	α	10.60	6.97
4)	Chenin Blanc - high glycerol and ethanol production			
	XGC (4)	a	9.83	7.76
	XGC(11)	α	9.75	7.50

TABLE 27: SELECTION FROM THE SECOND GENERATION OF DIPLOID YEASTS

	Yeast	Mating Type	Glycerol Production (g/l)	Ethanol Production (g/100ml)
Category 1)	Muller Thurgau - high glycerol production			
	XGD (XGA(8) x XGA(13)(vii))		6.41	6.71
2)	Muller Thurgau - high glycerol and ethanol production			
	XGE (XGA(8) x XGA(13)(v))		5.74	6.99
3)	Chenin Blanc - high glycerol production			
	XGF (XGC(13) x XGC(15)(xi))		10.17	7.36
4)	Chenin Blanc - high glycerol and ethanol production			
	XGG (XGC(13) x XGC(15)(ix))		9.98	7.59

TABLE 28: SELECTION FROM THE THIRD GENERATION OF HAPLOID YEASTS

Yeast	Mating Type	Glycerol Production [g/l]	Ethanol Production [g/100ml]
Category (1) Muller Thurgau - high glycerol production			
XGD(12)	α	5.77	6.87
XGE(7)	a	5.72	7.36
(2) Muller Thurgau - high glycerol and ethanol production			
XGE(7)	a	5.72	7.36
XGE(14)	α	5.49	7.38
(3) Chenin Blanc - high glycerol production			
XGF(6)	α	10.00	7.83
XGG(4)	a	11.14	7.60
(4) Chenin Blanc - high glycerol and ethanol production			
XGF(12)	a	9.50	8.19
XGG(1)	α	9.84	8.00

TABLE 29: SELECTION FROM THE THIRD GENERATION OF DIPLOID YEASTS

Yeast	Mating Type	Glycerol Production [g/l]	Ethanol Production [g/100ml]
Category (1) Muller Thurgau - high glycerol production			
XGH [XGE(7) x XGE(14)(ix)]		6.42	6.80
(2) Muller Thurgau - high glycerol and ethanol production			
XGI [XGD(12) x XGE(7)(iv)]		6.28	7.33
(3) Chenin Blanc - high glycerol production			
XGJ [XGF(12) x XGG(1)(viii)]		9.82	6.23
(4) Chenin Blanc - high glycerol and ethanol production			
XGK [XGF(12) x XGG(i)(vii)]		9.49	6.83

For convenience:

XGH = XGE(7) x XGE(14) (ix)
XGI = XGD(12) x XGE(7) (iv)
XGJ = XGF(12) x XGG(1) (viii)
XGK = XGF(12) x XGG(1) (vii)

These selected diploid strains were the final group of yeasts involved in this hybridisation programme. Changes in glycerol and ethanol yield can be observed in Table 30 and Figure 15 and 16.

4.5 Progress in selecting for hybrids over three successive generations

Selection for Category 1 yeast strains (high glycerol production in Muller Thurgau) displayed in Figure 15 showed an increase in glycerol production by those diploid hybrids selected from the second generation. The third generation suggested an increase in the average yield of glycerol by the hybrids developed (Table 30) but no increase in the maximum yield achieved.

Category 2 (high glycerol production with minimal loss of ethanol production in Muller Thurgau) displayed in Figure 15 showed an increase in glycerol production was achieved in the third generation of hybrids, with no loss in ethanol production. The average yields of glycerol and ethanol (Table 30) showed a similar increase.

Category 3 (high glycerol production in Chenin Blanc) displayed in Figure 16 showed a sharp increase in the production of glycerol until the second haploid generation. There was a slight decrease in glycerol production thereafter. A slight, but consistent, decline in ethanol production was observed over the three generations of hybridisation.

Category 4 (high glycerol production with minimal loss of ethanol production in Chenin Blanc) displayed in Figure 16 showed the production of ethanol remaining constant during the hybridisation programme. However, glycerol production increased markedly from the first haploid generation through to the second haploid generation and remained steady thereafter.

TABLE 30: PROGRESS THROUGH THREE GENERATIONS OF SELECTION FOR HIGH GLYCEROL AND ETHANOL PRODUCING YEASTS (Improvements in Average Yield also listed)

Generation	Category	Glycerol Prodn (g/l) average	Glycerol Prodn (g/l) highest	Ethanol Prodn (g/100ml) average	Ethanol Prodn (g/100ml) highest
Parent	1	-	5.81/5.76	-	7.08/7.03
1H		4.32	-	6.91	-
"		4.76	5.85/5.26	7.09	6.88/7.30
1D		4.96	5.57	7.13	6.99
2H		4.33	5.18/4.95	6.48	6.51/6.15
2D		5.67	6.41	6.74	6.71
3H		5.33	5.77/5.72	7.01	6.87/7.36
3D		6.10	6.42	6.73	6.80
Parent	2	-	5.81/5.76	-	7.08/7.03
1H		4.32	-	6.91	-
"		4.76	4.96/5.21	7.09	7.17/7.41
1D		4.85	5.18	7.03	7.26
2H		4.47	4.46/4.29	6.11	6.92/6.71
2D		4.73	5.74	6.59	6.99
3H		5.46	5.49/5.72	7.29	7.38/7.36
3D		6.43	6.28	7.14	7.33
Parent	3	-	8.47/8.35	-	8.03/7.97
1H		8.03	-	8.92	-
"		8.13	8.97/10.75	8.07	8.80/8.14
1D		8.42	8.94	6.97	7.11
2H		9.53	10.60/10.60	7.32	6.95/6.97
2D		9.96	10.17	7.43	7.36
3H		9.58	10.00/11.14	7.87	7.83/7.60
3D		8.88	9.82	6.44	6.23
Parent	4	-	8.47/8.35	-	8.03/7.97
1H		8.03	-	8.92	-
"		8.13	8.07/8.55	8.07	9.16/8.45
1D		7.91	8.04	7.61	7.93
2H		9.52	9.83/9.75	6.86	7.76/7.50
2D		9.89	9.98	7.38	7.59
3H		9.45	9.50/9.84	7.92	8.19/8.00
3D		8.88	9.49	6.44	6.83

FIGURE 15 : Glycerol and Ethanol Production by Three Successive Generations of Selected Hybrids for Fermentation in Muller Thurgau

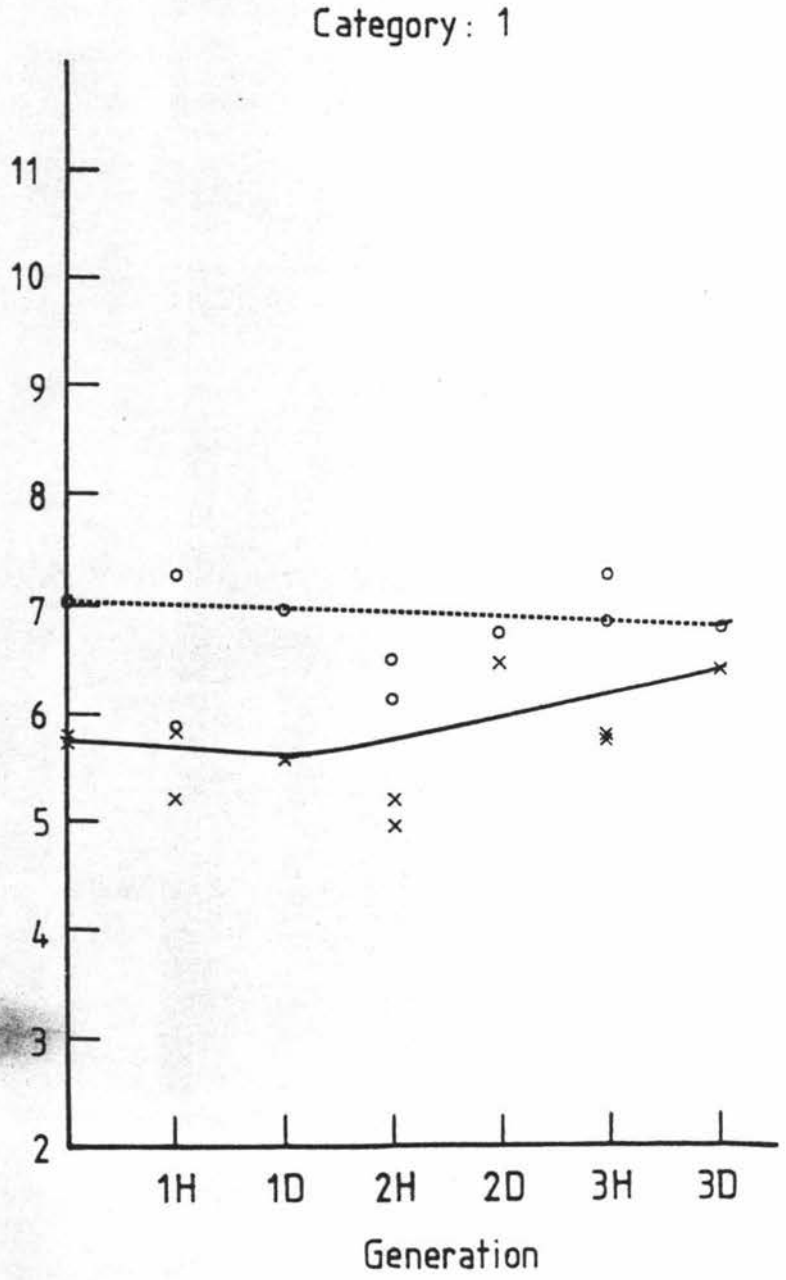
Key :-

1 H - First Haploid Generation Selection

1 D - First Diploid Generation Selection

Glycerol (g/l) ——×——

Ethanol (g/100 mls) ·····o·····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1H
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2H
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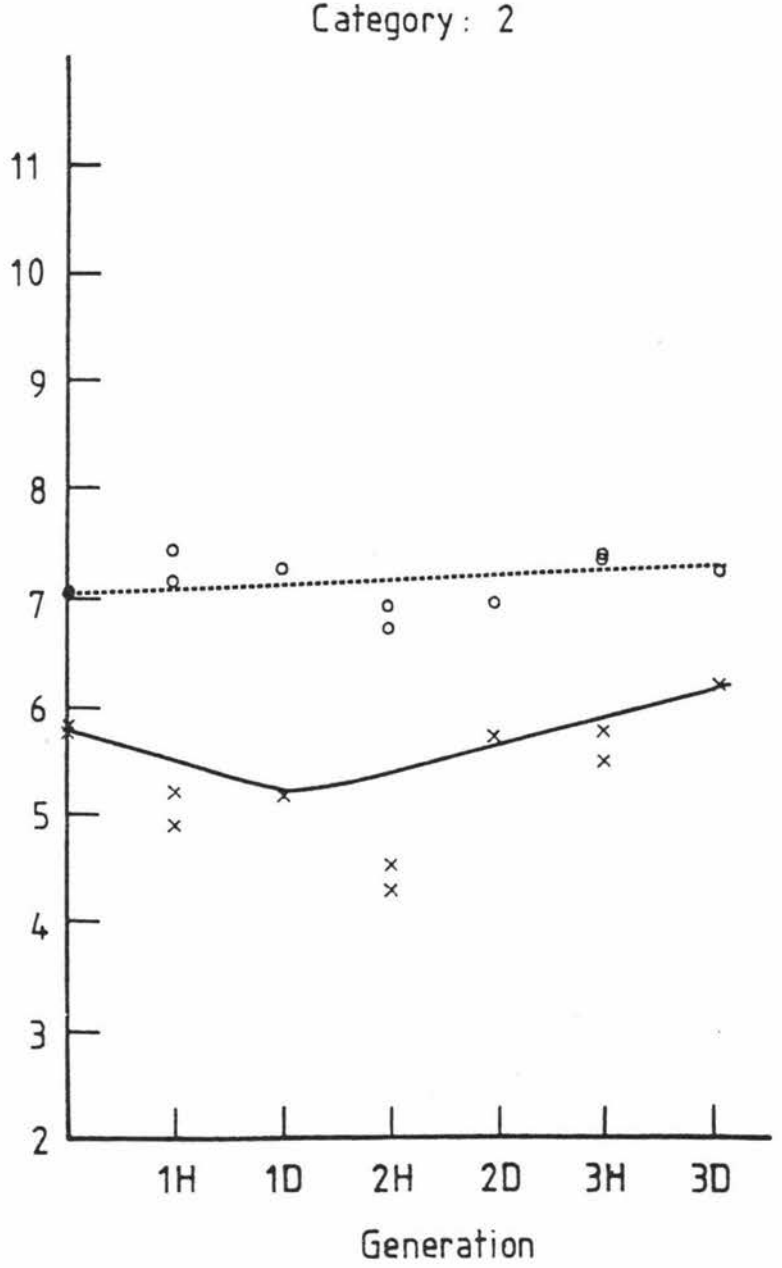
3H
3D
Generation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1H
1D
2H
2D
3H
3D
Generation

FIGURE 16 : Glycerol and Ethanol Production by Three Successive Generations of Selected Hybrids for Fermentation in Chenin Blanc

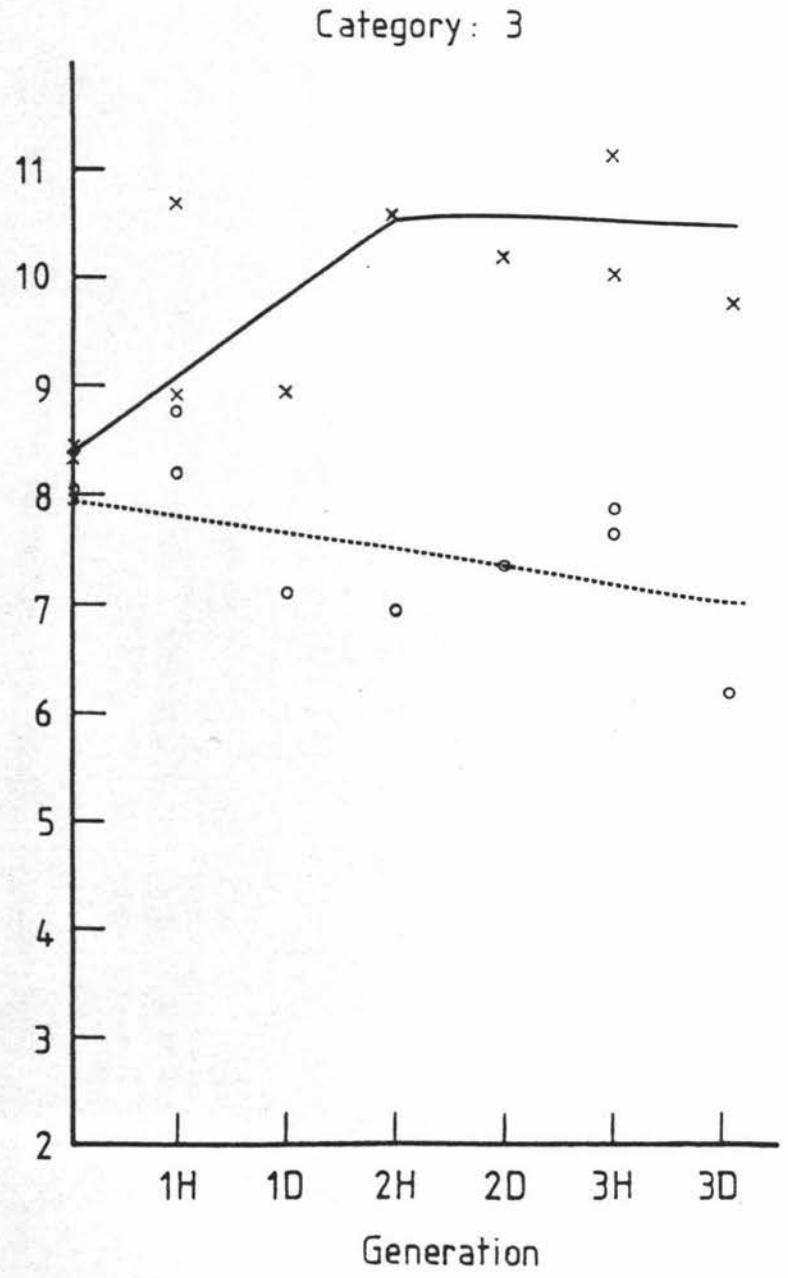
Key :-

1 H - First Haploid Generation Selection

1 D - First Diploid Generation Selection

Glycerol (g/l) ——×——

Ethanol (g/100 mls) .....o.....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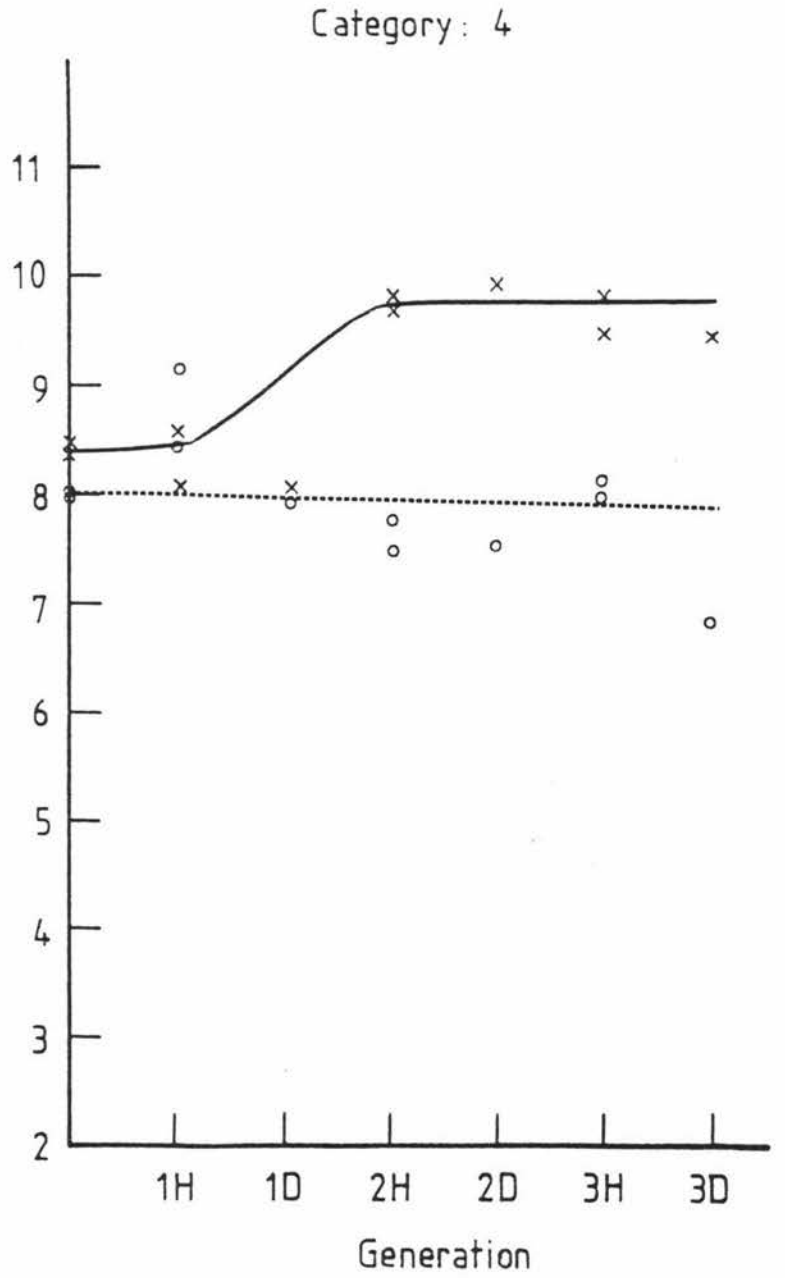
1D
2H
2D
3H
3D
Generation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1H
1D
2H
2D
3H
3D
Generation

DISCUSSION AND CONCLUSIONS

1 GLYCEROL PRODUCTION DURING FERMENTATION TO DRYNESS BY STRAINS OF SACCHAROMYCES CEREVISIAE

Glycerol production was influenced by both the yeast strain and the fermentation environment (Figure 2). The two fermentation factors were found to affect the rate and timing of glycerol production (Figure 3, 4). The constant temperature fermentations (15°C) showed that the rates of glycerol production for all six yeasts in the three media were very slow during the initial stages of the fermentation process and greatest between days 4 and 10. The fermentations with constant sugar utilization produced glycerol most rapidly between days 1 and 8 although the reduction in rate between days 3 and 4 have been a result of dropping the temperature at day 1 from 25°C to 15°C.

The slower production of glycerol in the constant temperature fermentations appeared to coincide with the period of time during which the cell growth rate was exponential, i.e. when cells were using their energy sources for the production of cell biomass. This is in agreement with the findings of Radler and Schutz (1982) who state that they found "no indication that glycerol is primarily formed at the beginning of fermentation, although the amounts of glycerol formed at the early stages are slightly higher than during the later stages".

This is in contrast to the findings of Rankine and Bridson (1971) and Nordstrom (1966, 1968) all of whom suggested that glycerol production during alcoholic fermentation was the means by which the yeast cell retained its redox balance under anaerobic conditions. Cell growth is an oxidative process counterbalanced in part by the reductive process of glycerol formation. Rankine and Bridson (1971) also claimed that "it has been established that glycerol is formed largely during the early stages of fermentation".

The maximum rates of glycerol production by all six yeasts covered a similar time span for the three media used. This time span was influenced by the temperature of fermentation. The final levels of glycerol produced appeared to depend on the rate of production during this high glycerol producing stage. Marked differences occurred between rate of production by the parent strains and the hybrid yeasts (Figures 3 and 6). This suggests that the hybrids have a more efficient enzyme activity for glycerol production than the parent wine-making yeast

strains. Radler and Schutz (1982) have studied differences in enzyme activities between different yeast strains, with results supporting this hypothesis.

1.1 Relationship between Cell Growth and Glycerol Production

Data on cell growth is presented in Appendix A and B tables A (X) - (XII) B(X) - (XII) and displayed graphically in Figure 10 (graphs a to i). From these results it is apparent the conditions of fermentation influence the growth rate. The higher rates of cell growth in the constant sugar utilization fermentations were probably the result of the higher incubation temperatures than those experienced in the constant temperature fermentations (15°C). The cell numbers reached for each yeast in stationary phase were similar for both types of fermentation.

The cell growth rate appeared to influence the rate of glycerol production but not the final yield of glycerol (Figure 2). Glycerol production was most rapid soon after exponential growth has ceased.

Nordstrom (1966 and 1968) and Rankine and Bridson (1971) suggested that glycerol production is a necessary byproduct of cell growth which balances the oxidative reactions of cell growth. This would suggest high glycerol producing yeasts produce more cell biomass than low glycerol producing yeasts. In these experiments no such correlation was observed. To support Nordstrom's theory one would expect cell numbers at stationary phase and/or the cell growth rate at the exponential growth phase to be higher for the hybrid yeasts especially strains XGL74 and XGL81. However, the cell populations and growth rates of yeast strains XL74 and XGL81 were not dependant on the fermentation conditions (Figure 10 a-i). In the complete defined media a distinct lag period of growth in the case of these two hybrid yeasts was apparent.

The lag between exponential cell growth and glycerol production (Figure 2) more clearly seen at the slower constant temperature fermentation, may be a result of energy being channelled toward rapid cell growth during the aerobic growth phase of the fermentation. Once growth slows some of this energy becomes available for the formation of fermentation end-products such as glycerol. It has been proposed that differences in the amount of glycerol produced between yeast strains is due to differences in activities of the enzymes associated with glycerol production. (Radler and Schutz, 1982) claim that the activities of the ethanol producing enzymes can influence glycerol production).

It is clear from these data that the level of glycerol produced depends on the yeast strain used. However, the hypothesis that glycerol is produced to balance the oxidative processes of growth is questionable.

Fermentation trials in a variety of grape juices covering a wide range of conditions of temperature which are sampled every 6 to 12 hours for cell growth and glycerol production during the fermentation, would be of value in establishing a relationship between glycerol production and cell growth. However, aeration of the fermentation is a problem if sampling is frequent, especially if the yeast strain is flocculent and the flask must be shaken prior to sampling, as this can affect cell growth and raise the glycerol yield.

1.2 Influence of the fermentation conditions on glycerol production

The fermentation conditions influenced the rate of glycerol production. The higher temperatures used for the constant sugar utilization fermentation shortened the time taken for fermentation to proceed to dryness. This effect was probably the result of an increased cell growth rate at the start of the fermentation. The cell numbers reached at stationary phase were not significantly greater than the numbers reached by the yeasts in the constant temperature fermentations (Figure 10).

The differences between glycerol and ethanol levels produced by the two fermentation processes have been compared and tested for significance to a level of 99.9% certainty that the differences are significant (Tables 20 and 21). Those media in which glycerol production was most efficient show significant differences in production. Higher levels of glycerol production were seen in complete defined media in the constant temperature fermentations, whereas more glycerol was produced in Chenin Blanc during constant sugar utlization fermentations.

Comparisons of ethanol production indicate that it was significantly higher in Muller Thurgau constant temperture fermentations. (Table 21).

1.3 Influence of the fermentation media on glycerol production

The relationship between the fermentation conditions, i.e. temperature, and glycerol production appears to depend partly on the yeast strain involved. It does, in addition, depend to a large extent on the media used. The influence of the fermentation temperature on the yield of glycerol has been the subject of debate in the past (as noted by Amerine et al (1980) and Wootton, Weekes and Lee (1983)). Some research reports indicate findings that glycerol yield increases if the fermentation temperature is raised (Ough, Fong and Amerine, 1972; Rankine and Bridson, 1971; Eshenbruch and Fisher, 1983). Other papers suggest glycerol production decreases at higher fermentation temperature - (Hickenbotham and Ryan 1948).

The yeast strain, the fermentation process and the medium influenced the efficiency of conversion of sugar to ethanol and glycerol (Table 14, 15; Figure 8). These six groups suggest that the fermentation conditions for maximum efficiency of glycerol and ethanol production from sugar is unique to each yeast strain. This could explain the discrepancies reported relating to the influence of the fermentation temperature on the yeast strain. Further investigation of the fermentation process, involving a wide range of fermentation temperatures, yeast strains and grape juices will be necessary before any conclusion can be reached.

Glycerol yield in Muller Thurgau juice of an intermediate sugar level, was relatively low yet a high efficiency of conversion to ethanol was achieved for all six yeasts. Glycerol production was higher in Chenin Blanc juice, of a high initial sugar level, while ethanol production per gram of sugar was relatively low. The production of both glycerol and ethanol per gram of sugar was more efficient in complete defined medium, of low initial sugar level, than in the grape juices. Lower cell numbers were detected in complete defined medium at the stationary phase than in the other two media, which reached comparable cell numbers. This could, in part, explain the differences in efficiency of production of ethanol and glycerol.

The variations in efficiency observed in each medium does not appear to depend on the sugar levels. Rankine and Bridson (1971) showed that different grape juices of the same initial sugar level and fermented in similar conditiosn yielded different levels of glycerol.

The differences between glycerol and ethanol yields following fermentation of the three media may have been caused by substances present in some grape juices which inhibit glycerol and/or ethanol production. The Muller Thurgau grape juice may have contained substances (native or additives) inhibitory to glycerol production thus promoting the formation of ethanol. The Chenin Blanc grape juice may have contained substances which inhibited ethanol production and promoted the formation of glycerol.

The levels of certain micronutrients in the media has been shown by Ralder and Schutz (1981) to influence glycerol production. They have also shown that the requirements by the yeasts for growth factors varies considerably depending on the strain. These findings may in part explain the variations in the efficiency of glycerol production between different yeasts and different media observed as shown in Table 14 and illustrated in Figures 8 and 9. Glycerol production declines before all the sugar is utilized (Figure 2). This may have been influenced by the age of the cells, i.e. production may only occur during a certain stage of the cells' life. Alternatively, it may have been due to the levels of micronutrients present in the media - when they are used up, glycerol production declines. More studies on the effects of different micronutrients are necessary before any conclusion can be drawn.

The observation that Muller Thurgau, of intermediate initial sugar level, was a poor medium for glycerol production yet the other media were suitable for yielding high levels of glycerol may be related to work by Radler and Schutz (1982). They found that the glycerol : ethanol ratio (expressed as g glycerol formed per 100 g ethanol) varies with the concentration of glucose for the same type of medium. This ratio was found to be a minimum at 150 g/l glucose, which coincides with the sugar level of the Muller Thurgau grape juice. The ratio was found to increase at higher or lower sugar concentrations for both yeasts used in their experiment. This sugar effect may also be responsible for the results observed for all six yeasts in Muller Thurgau. No satisfactory explanations have been proposed for this phenomena. It would be necessary to carry out further investigations, e.g. varying both the sugar level and levels of micronutrients for a range of media, before any conclusion could be reached.

1.4 Influence of the Yeast Strain on Glycerol Production

The yeast strain appeared to exert a strong influence on the levels of glycerol produced. Significant differences were seen in the efficiencies of glycerol and ethanol production of the six yeast strains (Figure 9). The hybrids were clearly more efficient glycerol producers than are the parent wine-making strains MD26, AWI60, and AWI80. The differences in efficiency of ethanol production was less clear, though under some of the fermentation conditions, the high glycerol-producing hybrids did produce significantly less ethanol.

In contrast to the hypothesis proposed by Radler & Schutz (1981), Eustace & Thornton (in press) proposed that the high glycerol yields achieved by some yeast strains were not necessarily achieved at the expense of ethanol. They offered two alternative hypotheses to support their observations:

(i) that high glycerol yields may be a result of selecting for genes capable of a high efficiency of glycerol production and/or of a high efficiency of glycerol production and/or the elimination of genes responsible for a low efficiency of glycerol formation.

(ii) a reduction in phospholipid biosynthesis, for which glycerol-3-phosphate is a major precursor in yeast, by selecting for enzymes with a low substrate affinity. This may result in the accumulation of glycerol.

Eustace and Thornton (in press) found that during their hybridisation programme, the glycerol : ethanol ratio actually increased. This is not supportive of the hypothesis proposed by Radler & Schutz (1981) that glycerol is proposed at the expense of ethanol. However, that some limitation of glycerol (or ethanol) production may occur due to enzymatic competition for NAD cannot be discounted. Johansson & Sjoestrom (1984) found that a partial alcohol dehydrogenase deficient mutant (whereby the activity of ADH-1 was reduced 15-fold) produced glycerol levels 6-7 times that of the wild-type strain.

2 THE EFFECT OF SULPHUR DIOXIDE ON THE PRODUCTION OF GLYCEROL AND ETHANOL

The addition of sulphur dioxide to the fermentation media increased the yield of glycerol while inhibiting ethanol production (Rose & Harrison, 1969).

Glycerol and ethanol production by a winemaking yeast (AWI60) and a high glycerol producing hybrid yeast (XGL81) when fermented in media of increasing sulphur dioxide content was examined - Appendix C, Table (IV).

An increase of sulphur dioxide level in the fermentation media was associated with an increase in glycerol production and a corresponding decrease in ethanol production by the wine yeast AWI60. These trends were observed both in Chenin Blanc grape juice and in the synthetic complete defined media at sulphur dioxide levels greater than 100 ppm (Figure 11). These results agree with previous reports and support the hypothesis that sulphur dioxide acts by binding to certain substances such as acetaldehyde, thus preventing its reduction to ethanol. This makes available any associated NADH for the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate, which is then dephosphorylated to glycerol.

Eschenbruch and Fisher (1984) suggested that the low levels of pH and sulphur dioxide used for winemaking do not significantly influence glycerol production. Analysis of fermentations by yeast strain AWI60 supported this statement as little change in the production of ethanol and glycerol was observed in either the Chenin Blanc or the complete defined media, until the sulphur dioxide level was greater than 100 ppm (Table 22).

The high glycerol producing hybrid yeast XGL81 was fermented in both the synthetic complete defined media and in the Chenin Blanc grape juice (Table 22 and Figure 12).

Contrary to previous findings, glycerol production was reduced and ethanol production correspondingly enhanced as the level of sulphur dioxide in the media was raised. Glycerol production by yeast strain XGL81 appeared to increase at low sulphur dioxide levels, i.e. raising the sulphur dioxide from 0 to 100 ppm in complete defined media and from 0-50 ppm in Chenin Blanc (Table 22). However, a substantial decrease in glycerol production was apparent as the sulphur dioxide level was raised to 300 ppm. A corresponding increase in ethanol production was noted as the sulphur dioxide level was raised.

The anomalous trend shown by yeast strain XGL81 in the presence of high levels of sulphur dioxide may have been a result of a low tolerance of the hybrid to sulphur dioxide which slowed its growth and metabolic processes. The result is supported by the longer time required for yeast strain XGL81 to ferment to dryness at high sulphur dioxide levels when compared with yeast strain AW160. This could change the kinetics of the fermentation altering the balance of end-products formed. Figures 2 and 5 suggest glycerol production followed exponential growth, therefore any restriction on growth rate is likely to influence the glycerol yield.

In the presence of sulphur dioxide, acetaldehyde builds up and is diverted to glycerol production because its reduction to ethanol is blocked. However, this will only take place in actively metabolising cells. Sulphur dioxide inhibits enzyme reactions in general (its addition to grape juice inhibits browning reactions, which are enzymatic, and kills bacteria and wild yeasts by blocking their enzyme systems).

The addition of sulphur dioxide to yeast strain XGL81 at levels greater than 100 ppm may

(1) be lethal to a major portion of the population, therefore slowing fermentation until sufficient numbers of sulphur dioxide tolerant cells are reached to complete the fermentation

or

(2) prevent cell metabolism and growth until all the sulphur dioxide is "mopped-up" by the cells, then permitting any survivors to grow.

Either explanation would change the kinetics of the fermentation process and influence the yields of end-products formed, including the glycerol : ethanol ratio.

Radler and Schutz (1981) observed that sugar levels of the range 150 g/l seemed least favourable for glycerol formation. The effect of sulphur dioxide on yeast strain XGL81 was more pronounced in Chenin Blanc than in complete defined media. A further possible explanation

for the effect of sulphur dioxide on XGL81 could be that the sulphur dioxide sensitivity of yeast strain yeast strain XGL81 slows its growth and may result in increased sugar requirements for cell growth. Since glycerol production appeared to occur once cell growth has passed the exponential phase, the level of sugar present in the media at this stage may be such that it does not favour the formation of glycerol.

To test this hypothesis, further fermentation trials comparing yeasts of a wide range of glycerol producing capabilities would be necessary, covering a range of sulphur dioxide levels from 0 ppm to 1000 ppm (or as high a sulphur dioxide level as the yeasts can tolerate). These trials would be carried out in media of different sugar levels. Daily sampling for glycerol, ethanol, free and bound pyruvate and acetaldehyde may also provide useful information regarding glycerol production.

3 INFLUENCE OF GLYCEROL IN THE MEDIA PRIOR TO FERMENTATION ON THE PRODUCTION OF GLYCEROL AND ETHANOL

In an attempt to simulate the condition of botrytised graspe, glycerol was added to the medium prior to the fermentation in order to determine if high levels of glycerol in the fermentation media had any influence on the cells metabolism. Conceivably, glycerol production might be reduced by a feedback inhibition mechanism, or stimulated by a low ratio of intracellular : extracellular glycerol levels.

Gancedo et al (1968) suggested that S. cerevisiae had a low cellular permeability to glycerol, and that during the fermentation of glucose, the intracellular glycerol concentration could be as high as 0.1M, with an intracellular : extracellular glycerol concentration of up to 150. They suggested that glycerol accumulates in S. cerevisiae until a concentration is reached that allows excretion into the medium at a rate equal to its formation.

A low glycerol producing wine yeast MD26 and a high glycerol producing hybrid yeast XGL81 were fermented in complete defined media and in Chenin Blanc.

Utilization of glycerol did not occur during the fermentation. This would have appeared as a drop in glycerol production when all fermentation trials containing glycerol before inoculation were compared

against a glycerol-free control media (Tables 23, Figures 13 and 14). However, daily sampling of the fermenting medium would be necessary to confirm this observation. It is possible that utilization may occur during the early growth stage, but is unlikely since high sugar concentrations suppress respiration and glycerol is a non-fermentable carbon source.

In view of the work by Gancedo et al (1968) it would also be of interest to sample daily for glycerol to determine if glycerol production, i.e. release into the media, occurs at a later stage if high levels of glycerol are already present in the media.

The presence of glycerol in the media prior to fermentation did not significantly inhibit or stimulate glycerol production (Figure 14). Glycerol production in Chenin Blanc was reduced at 22.56 g/l of initial glycerol after a slight increase at 10 g/l and 15 g/l initial glycerol. Fermentation trials run anaerobically at initial glycerol levels of up to 35 g/l might be more informative but these would represent very severe instances of botryitised grapes.

4 HYBRIDISATION PROGRAMME TO SELECT FOR HIGH GLYCEROL PRODUCING STRAINS OF SACCHAROMYCES CEREVISIAE

Selective hybridisation of pure culture wine yeasts has been shown to be effective in improving the efficiency of conversion of grape sugar to ethanol (Thornton, 1982) and to glycerol (Eustace and Thornton, in press). Gene transfer has a lower probability of success owing to the multigenic nature of glycerol and ethanol production

The aims of this programme were to determine:

(1) Whether selective hybridisation for increased glycerol yield would cause a decline in the yield of ethanol

(2) Whether selective hybridisation for increased glycerol and ethanol yields would produce strains of yeast which had increased efficiency of conversion of sugar to ethanol and glycerol

(3) Whether different grape juices influence the glycerol and ethanol yields of the yeast strains selected for the above properties

The yeast strains XGL74 and XGL81 used to initiate this hybridisation programme were diploid heterothallic hybrids, developed for their ability to produce high levels of glycerol.

4.1 Selection in Muller Thurgau Grape Juice

Category 1. Selection for high glycerol yield (Table 30, Figure 15).

The yield of glycerol increased from 5.81 g/l (yeast strain XGL74) and 5.76 g/l (yeast strain XGL81) to 6.42 g/l, which was produced by the highest yielding strain in the final generation of diploid hybrid strains. This represented a 10.8% increase in glycerol yield. The average yield of glycerol increased from 4.32 g/l to 6.10 g/l of the final generation of diploid hybrid strains, i.e. an increase of 41.2% in the average glycerol yield. The average yield of glycerol increased in each successive diploid generation. However, the glycerol yields of the most productive diploid strains, in generations two and three, were similar. The ethanol yield of each generation of selected strains did not differ significantly.

Category 2. Selection for high glycerol yield without a decrease in ethanol yield (Table 30, Figure 15).

Slight increases in glycerol yield from 5.8 g/l to 6.2 g/l, and ethanol yield from 7.05 g/100 ml to 7.33 g/100 ml were observed as an increase in yields of 10.7% and 4% respectively for glycerol and ethanol.

It is not clear from these data whether the maximum yields of glycerol and ethanol were achieved by this hybridisation programme. Glycerol yield did not increase significantly between the second and third diploid generation in the Category 1 selection. This could be because:

(a) maximum efficiency of sugar conversion to glycerol and ethanol had already been achieved in the development of hybrid yeast strains XGL74 and XGL81

or

(b) the media composition was limiting any increase in glycerol production - i.e. the yeasts may not have been near maximum efficiency of glycerol production, instead a limiting factor in the media may have prevented any improvement in glycerol yield.

Hypothesis (a) could be examined by a continuation of the hybridisation programme for a further two or three generations. The absence of a significant improvement in glycerol yield would support this hypothesis but would not negate hypothesis (b). The micronutrient experiements described previously would discriminate between these alternatives.

4.2 Selection in Chenin Blanc Grape Juice

Category 3. Selection for high glycerol yield (Table 30, Figure 16).

The glycerol yield increased from 8.47 g/l (yeast strain XGL74) and 8.35 g/l (yeast strain XGL81) to 9.82 g/l for the highest gylcerol producing strain of the final diploid generation. This represented an increase in the glycerol yield of 16.8%. The ethanol yield declined from an average of 8.0 g/100 ml produced by the parent strains to 6.44 g/100 ml average yield of the third generation of diploid hybrid strains.

Category 4. Selection for high glycerol yield with no loss in ethanol yield (Table 30, Figure 16).

The glycerol yield increased from 8.47 g/l (yeast strain XGL74) and 8.35 g/l (yeast strain XGL81) to 9.49 g/l by the highest glycerol producing strain of the final diploid generation. This represented an increase in glcerol yield of 12.8%. A marked decrease was observed in the ethanol yield of the final diploid generation, although the average yield was similar to that of the parent strains for most of the hybridisation programme. In both category 3 and 4, selection for the individual highest glycerol yielding strains were isolated in the second diploid generation.

Strains of yeast which produced higher yields of glycerol were developed through the hybridisation programme in categories 3 and 4.

However, in both categories the increased glycerol yield seemed to be at the expense of ethanol.

It has already been shown (Discussion Section 1.3) that Chenin Blanc grape juice is a suitable medium for the high efficiency of conversion of grape sugar to glycerol but is a relatively poor medium for ethanol production. Due to the nature of the Chenin Blanc, it may be easier to select for improved glycerol yield. This improvement may occur at the expense of ethanol production, which supports the relevant hypothesis by Radler and Schutz (1982).

4.3 Consequences of the hybridisation programme

The data suggests that those yeasts which produced high levels of glycerol and/or ethanol in one grape juice, tended to be only slightly above average in the other. This was clear in the first generation selection and for yeast strain XGA of the second generation selection, where the same yeasts were fermented in the two types of grape juice.

These results support the proposal by Schenbruch et al (1981) and Thornton (1982) that wine yeasts may be tailored to ferment efficiently in specific grape juices, i.e. that wine yeasts have different winemaking capabilities in different grape juices.

SUMMARY

The effects of the fermentation conditions and the fermentation media on the production of glycerol and ethanol by six strains of the yeast Saccharomyces cerevisiae were studied.

Fermentation conditions

Two types of fermentation conditions were used: constant temperature fermentations (15°C) and constant sugar utilization fermentations, in which the temperature was altered during the course of the fermentation to maintain a constant rate of sugar utilization (1% sugar/day).

Glycerol production

The higher rate of glycerol production and cell growth observed in the constant sugar utilization fermentation were probably the consequence of higher fermentation temperatures. However, the final yields of glycerol were similar in both the constant temperature and the constant sugar utilization fermentations. The maximum rate of glycerol production correlated with the end of the exponential phase of cell growth in both types of fermentation.

The production of glycerol and ethanol was investigated in three media: complete defined media, Muller Thurgau and Chenin Blanc grape juices.

Efficiency in glycerol and ethanol production

Differences in the efficiency of glycerol and ethanol produced per gram of sugar utilized may have been due to variations in the levels of essential micronutrients in the different media, or the presence of inhibiting substances. The highest yields of glycerol were found in the fermentations of the complete defined media and the lowest in the Muller Thurgau grape juice.

Influence of yeast strain

The strain of yeast influenced glycerol production. Differences in glycerol production by the six yeasts were probably the consequence of different levels of activity of the enzymes involved in glycerol and ethanol formation.

Comparison of the two fermentation conditions and the three media suggested that each yeast required unique conditions for maximum production of glycerol.

Influence of Sulphur Dioxide

The addition of sulphur dioxide at levels of greater than 100 ppm to the fermentation medium stimulated glycerol production and inhibited ethanol production by a wine yeast. The opposite was observed when a hybrid yeast, selected for high glycerol production, carried out the fermentation. However, the wine yeast strain was sulphur dioxide tolerant whereas the hybrid yeast was sulphur dioxide sensitive and thus its metabolic activities were modified.

Glycerol addition

The addition of glycerol to the fermentation medium, prior to fermentation, did not cause feedback inhibition or stimulation of glycerol production by the yeast strains tested.

The Effect of Hybridisation

A selective hybridisation programme for high glycerol yield and for high glycerol and constant ethanol yield in two grape juice media was carried out.

Glycerol production by the hybrid yeast strains selected in Muller Thurgau reached a maximum in the second generation of hybridisation, whereas in Chenin Blanc the glycerol yield increased until the third generation of hybridisation. The increased glycerol yield was achieved at the expense of the ethanol yield.

Little increase in the glycerol yield was achieved when selection for constant ethanol yield was also a factor. It was suggested that increases in glycerol yield may have been limited by the lack of micronutrients, the presence of inhibiting substances or that the most active forms of the enzymes involved in glycerol production have been selected.

The hybrid yeast strains selected for high glycerol yield in one grape juice were not necessarily suited to high glycerol yield in

another grape juice. This supported the suggestion that specific yeast strains could be selectively hybridised for their fermentation properties in specific grape juices.

APPENDICES

APPENDIX A

TABLE AI: Sugar Utilisation [g/100ml] at a Constant Temperature of Fermentation (15°C) in Complete Defined Media [Std error ± 0.5g/100ml]

Day	0	1	2	4	6	8	10	16	25
Yeast MD26	9.68	9.7 9.4 9.6	8.9 9.4 9.2	6.4 8.0 7.2	4.1 7.4 5.8	2.6 6.3 4.4	0.7 5.0 2.9	0.1 0.5 0.3	0.0 0.0 0.0
AWI60	9.86	9.1 8.9 9.0	9.2 8.7 8.9	7.4 6.9 7.2	5.6 5.2 5.4	3.8 3.0 3.4	2.1 1.5 1.8	0.1 0.0 0.1	0.0 0.0 0.0
AWI80	9.74	8.5 9.0 8.8	8.6 8.6 8.6	6.3 6.3 6.3	4.0 4.5 4.3	1.9 2.8 2.4	0.3 0.9 0.6	0.0 0.0 0.0	0.0 0.0 0.0
XGL74	9.53	9.4 8.8 9.1	9.2 8.2 8.7	7.8 5.5 6.7	6.2 3.0 4.6	5.1 1.6 3.4	3.7 0.3 2.0	0.2 0.0 0.1	0.0 0.0 0.0
XGL78	9.62	9.2 9.2 9.2	8.9 9.9 9.4	7.0 9.2 8.6	5.6 2.9 7.3	4.0 - 4.0	1.9 - 1.9	0.0 - 0.0	0.0 0.0 0.0
XGL81	9.71	9.2 9.4 9.3	8.5 9.2 8.8	6.3 7.6 7.0	4.8 5.2 5.0	2.3 5.0 3.7	0.4 2.7 1.5	0.2 0.0 0.1	0.0 0.0 0.0

1. Sample x_1 Sample x_2 $\bar{x}$
2. - Indicates no data available for sample x
3. Single readings only taken for day 0

APPENDIX A

TABLE AII: Sugar Utilisation [g/100ml] at a Constant Temperature of Fermentation [15°C] in Muller Thurgau [Std Error ± 0.5g/100ml]

Day	0	1		2		4		6		8		10		16		25	
Yeast MD26	13.3	13.6 13.5	13.6	13.2 12.8	13.0	9.6 8.9	9.2	6.7 4.1	5.4	3.5 2.0	2.8	1.8 1.2	1.5	0.2 0.2	0.2	0.0 0.0	0.0
AWI60	14.3	14.2 14.2	14.2	11.5 13.3	12.4	8.2 10.6	9.4	3.1 8.0	5.6	0.8 6.2	3.5	0.5 3.6	2.0	0.2 0.4	0.3	0.0 0.0	0.0
AWI80	14.5	13.9 14.2	14.1	12.6 11.6	12.1	8.9 8.3	8.6	5.9 4.4	5.1	5.6 1.8	3.7	1.2 0.9	1.1	0.3 0.3	0.3	0.0 0.0	0.0
XGL74	14.1	14.0 14.0	14.0	12.8 12.6	12.7	10.4 9.0	9.7	7.7 5.3	6.5	4.7 2.5	3.6	2.9 -	2.9	0.4 0.3	0.4	0.0 0.0	0.0
XGL78	14.7	14.3 14.3	14.3	12.6 13.0	12.8	11.8 11.0	11.4	9.3 8.9	9.1	8.3 7.0	7.7	7.5 6.4	7.0	0.2 2.0	1.1	0.0 0.0	0.0
XGL81	14.3	14.2 13.8	14.0	12.4 11.9	12.2	7.6 7.4	7.5	6.3 2.8	4.5	3.2 1.1	2.1	1.8 0.7	1.3	1.5 0.3	0.9	0.0 0.0	0.0

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TABLE AIII: Sugar Utilisation [g/100ml] at a Constant Temperature of Fermentation [15°C] in Chenin Blanc [Std Error ± 0.5g/100ml]

Day		0	1		2		4		6		8		10		16		25	
Yeast	MD26	22.7	17.5 17.3	17.4	15.9 16.6	16.3	10.9 10.5	10.7	6.5 4.6	5.6	4.0 2.5	3.2	2.9 1.9	2.4	0.2 0.7	0.5	0.0 0.0	0.0
	AWI60	24.9	17.5 17.8	17.7	17.1 17.5	17.3	12.2 12.4	12.3	8.9 8.3	8.6	7.1 5.4	6.3	6.4 4.2	5.3	0.8 1.8	1.3	0.0 0.0	0.0
	AWI80	20.8	17.3 17.9	17.6	16.9 16.7	16.8	13.0 11.8	12.4	8.8 7.3	8.0	5.5 3.5	4.5	4.1 2.5	3.3	1.2 1.0	1.1	0.1 0.1	0.1
	XGL74	22.0	17.9 17.7	17.8	16.9 17.3	16.3	12.0 9.8	10.9	7.9 5.6	6.7	3.1 1.8	2.4	2.1 1.4	1.7	0.8 0.8	0.8	0.1 0.1	0.1
	XGL78	21.6	17.8 17.6	17.7	17.3 16.8	17.1	15.1 14.0	14.6	12.8 13.3	13.1	9.1 7.2	8.2	9.5 6.5	8.2	0.7 3.6	2.1	0.1 0.1	0.1
	XGL81	20.0	17.3 17.4	17.4	16.4 17.3	16.8	12.9 15.7	14.3	8.0 9.8	8.9	3.0 6.8	4.9	2.2 5.9	4.1	1.9 0.8	1.3	0.0 0.0	0.0

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TABLE AIV: Glycerol Production [g/l] at a Constant Temperature of Fermentation [15°C] in Complete Defined Media [Std Error ± 0.2g/l]

Day	0	1		2		4		6		8		10		16		25	
Yeast MD26	0.0	0.0 0.0	0.0	.05 .04	.05	.13 .07	.10	2.1 1.5	1.8	2.5 1.7	2.1	2.2 2.2	2.2	2.3 2.4	2.4	2.7 3.1	2.9
AWI60	0.0	0.0 0.0	0.0	.05 .08	.07	.10 .09	.10	1.8 1.6	1.7	1.9 1.8	1.9	2.2 2.4	2.3	2.3 2.1	2.2	2.5 2.5	2.5
AWI80	0.0	0.0 0.0	0.0	.07 .07	.07	.14 .13	.14	2.1 2.0	2.1	2.4 2.4	2.4	2.4 2.6	2.5	2.2 2.4	2.3	3.2 -	3.2
XGL74	0.0	0.0 0.0	0.0	.05 .09	.07	.11 .11	.11	2.0 2.0	2.0	2.6 3.0	2.8	3.2 2.7	2.5	3.7 2.9	3.3	4.6 3.2	3.9
XGL78	0.0	0.0 0.0	0.0	.00 .04	.02	.11 -	.11	0.4 0.8	0.6	2.2 -	2.2	2.6 -	2.6	2.6 -	2.6	3.0 2.8	2.9
XGL81	0.0	0.0 0.0	0.0	.09 .04	.06	.17 .11	.14	2.8 1.8	1.3	3.2 2.9	3.1	3.2 3.2	3.2	3.0 3.4	3.2	3.2 4.0	3.6

1. Sample x_1 Sample x_2 $\bar{x}$
2. - Indicates no data available for sample x
3. Single readings only taken for day 0

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TABLE AV: Glycerol Production [g/l] at a Constant Temperature of Fermentation [15°C] in Muller Thurgau [Std error ± 0.2g/l]

Day		0	1		2		4		6		8		10		16		25	
Yeast	MD 26	0.0	.08 .08	.08	.12 .13	.13	.21 .22	.22	2.8 2.6	2.7	3.2 3.5	3.4	3.1 3.3	3.2	2.9 2.9	2.9	7.67 7.51	7.59
	AWI60	0.0	.08 .06	.07	.10 .11	.11	.22 .18	.20	3.4 2.2	2.8	3.7 3.2	3.5	3.1 3.4	3.3	3.3 3.1	3.2	7.15 7.40	7.28
	AWI80	0.0	.09 .09	.09	.15 .17	.16	.27 .28	.28	3.9 3.6	3.8	4.9 4.4	4.6	4.7 4.0	4.4	4.4 3.8	4.1	7.25 7.14	7.19
	XGL74	0.0	.07 .09	.08	.13 .14	.14	.26 .27	.27	3.7 3.8	3.8	3.9 4.2	4.1	3.9 4.3	4.1	4.0 3.7	3.9	7.27 7.16	7.22
	XGL78	0.0	.07 .09	.08	.11 .12	.12	.20 .24	.22	2.7 3.3	3.0	3.5 3.9	3.7	4.0 4.0	4.0	4.1 4.3	4.2	7.18 7.06	7.12
	XGL81	0.0	.1 .1	.1	.16 .15	.16	.32 .34	.33	4.8 4.1	4.5	4.8 5.1	5.0	4.7 4.9	4.8	4.7 5.0	4.9	6.96 6.99	6.98

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TABLE AVI: Glycerol Production [g/l] at a Constant Temperature of Fermentation [15°C] in Chenin Blanc [Std error ± 0.2g/l]

Day		0	1		2		4		6		8		10		16		25	
Yeast	MD26	0.0	.13 .12	.13	.21 .15	.18	.36 .34	.33	4.4 4.8	4.6	5.4 5.8	5.6	4.6 5.2	4.9	4.4 4.8	4.6	5.2 5.4	5.3
	AWI60	0.0	.13 .09	.11	.18 .17	.18	.30 .30	.30	3.6 4.0	3.8	4.8 5.0	4.9	4.8 4.6	4.7	5.2 4.7	4.9	5.5 5.3	5.4
	AWI80	0.0	.12 .12	.12	.16 .18	.17	.34 .32	.33	4.5 4.8	4.7	5.4 5.4	5.4	5.3 5.3	5.3	4.7 5.7	5.2	6.0 5.8	5.9
	XGL74	0.0	.12 .14	.13	.22 .22	.22	.52 .53	.53	8.8 9.4	9.1	8.0 8.0	8.0	7.7 7.7	7.7	5.7 8.4	7.0	8.2 7.9	8.1
	XGL78	0.0	.12 .12	.12	.17 .19	.18	.34 .36	.34	5.5 6.1	5.8	5.1 5.7	5.4	6.3 5.8	6.0	8.0 8.0	8.0	8.0 7.6	7.8
	XGL81	0.0	.16 .12	.14	.19 .13	.16	.44 .29	.36	8.2 4.6	6.4	7.4 6.2	6.8	7.7 6.6	7.2	7.4 8.2	7.8	7.1 7.5	7.3

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TABLE AVII: Ethanol Production [g/100ml] at a Constant Temperature of Fermentation [15°C] in Complete Defined Media [Std error ± 0.1g/100ml]

Day		0	1		2		4		6		8		10		16		25	
Yeast	MD26	0.0	0.0		0.48 0.26	0.37	1.4 0.8	1.1	2.7 1.5	2.1	3.9 2.1	3.0	4.3 2.5	3.4	4.7 4.5	4.6	4.6 4.6	4.6
	AWI60	0.0	0.0		0.32 0.68	0.50	1.0 1.2	1.1	2.1 2.2	2.2	3.1 2.9	3.0	3.9 3.8	3.9	4.7 4.7	4.7	4.4 4.4	4.4
	AWI80	0.0	0.16 0.16	0.16	0.61 0.65	0.63	1.5 1.5	1.5	2.7 2.5	2.6	4.1 3.7	3.9	4.5 4.3	4.4	4.7 5.0	4.9	5.2 -	5.2
	XGL74	0.0	0.0		0.38 0.80	0.59	0.8 1.8	1.3	1.4 2.5	1.9	2.2 4.3	3.2	2.9 4.4	3.7	4.7 4.9	4.8	4.4 4.5	4.5
	XGL78	0.0	0.0		0.51 0.09	0.30	1.2 -	1.2	2.0 -	2.0	3.0 -	3.0	3.7 -	3.7	4.8 -	4.8	4.4 -	4.4
	XGL81	0.0	0.0		0.60 0.31	0.45	1.4 0.8	1.1	2.6 1.4	2.0	3.8 2.7	3.3	4.4 3.3	3.9	4.8 4.6	4.7	4.2 4.5	4.4

1. Sample x_1 Sample x_2 $\bar{x}$
2. - Indicates no data available for sample x
3. Single readings only taken for day 0

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TABLE AVIII: Ethanol Production [g/100ml] at a Constant Temperature of Fermentation [15°C] in Muller Thurgau [Std error ± 0.1g/100ml]

Day		0	1		2		4		6		8		10		16		25	
Yeast	MD26	0.0	0.82 0.84	0.83	1.6 1.7	1.7	3.0 3.6	3.3	5.2 6.3	5.8	7.2 7.6	7.4	7.2 7.6	7.4	7.7 7.6	7.7	7.7 7.5	7.6
	AWI60	0.0	0.75 0.74	0.75	1.3 1.4	1.4	3.7 2.6	3.2	6.5 4.1	5.3	8.0 6.0	7.0	7.7 7.1	7.4	7.8 7.7	7.8	7.2 7.4	7.3
	AWI80	0.0	0.77 0.85	0.81	1.7 2.1	1.9	3.2 3.7	3.5	5.3 6.0	5.7	7.3 7.7	7.5	7.3 7.8	7.5	7.9 8.0	8.0	7.3 7.1	7.2
	XGL74	0.0	0.81 0.87	0.84	1.5 1.6	1.6	3.0 3.4	3.2	4.9 5.6	5.3	6.2 7.4	6.8	7.0 7.5	7.3	7.8 7.6	7.7	7.3 7.1	7.2
	XGL78	0.0	0.72 0.74	0.73	1.2 1.5	1.4	2.2 2.5	2.4	3.3 3.7	3.5	4.5 4.9	4.7	5.3 5.5	5.4	7.7 7.0	7.4	7.2 7.0	7.1
	XGL81	0.0	0.92 0.88	0.90	1.8 1.8	1.8	3.6 4.1	3.8	5.6 6.2	5.9	7.0 8.1	7.6	7.4 7.8	7.6	7.2 7.7	7.5	7.0 7.0	7.0

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TABLE AIX: Ethanol Production [g/100ml] at a Constant Temperature Fermentation [15°C] in Chenin Blanc [Std error ± 0.1g/100ml]

Day		0	1		2		4		6		8		10		16		25	
Yeast	MD26	0.0	0.52 0.54	0.53	1.5 1.1	1.3	4.0 4.0	4.0	6.2 6.3	6.3	8.6 8.8	8.7	8.3 8.7	8.5	7.8 8.9	8.4	8.7 8.1	8.4
	AWI60	0.0	0.54 0.46	0.51	1.3 1.0	1.2	2.9 2.9	2.9	4.7 5.2	5.0	6.6 7.5	7.1	7.7 7.6	7.7	8.9 8.6	8.8	8.2 8.3	8.3
	AWI80	0.0	0.50 0.51	0.52	1.2 1.1	1.2	2.9 3.2	3.1	5.0 5.8	5.4	7.1 7.8	7.5	7.8 8.5	8.2	8.8 9.0	8.9	8.1 8.0	8.1
	XGL74	0.0	0.42 0.57	0.30	1.1 1.5	1.3	3.2 4.2	3.7	7.0 8.4	7.7	8.1 8.5	8.3	8.5 8.7	8.6	9.2 8.8	9.0	8.4 8.1	8.3
	XGL78	0.0	0.43 0.46	0.45	0.8 1.0	0.9	2.0 2.4	2.2	4.0 5.1	4.6	4.6 5.4	5.0	6.0 6.3	6.2	8.8 8.3	8.5	7.7 8.1	7.9
	XGL81	0.0	0.56 0.47	0.51	1.2 0.6	0.9	3.0 1.7	2.4	6.5 3.5	5.0	7.5 5.6	6.5	8.2 7.3	7.8	8.3 8.7	8.5	7.7 8.3	8.0

TABLE AX: Viable Cell Counts [Cells/Ml] at a Constant Temperature of Fermentation [15°C] in Complete Defined Media

DAY	0	1	2	3	4	5	7
Yeast MD26	4.12×10^5	8.0×10^5	4.0×10^6	1.1×10^7	2.96×10^7	1.92×10^7	1.32×10^7
AW160	4.31×10^5	2.1×10^5	8.1×10^6	8.18×10^6	2.89×10^7	2.12×10^7	2.33×10^7
AW180	4.22×10^5	9.0×10^5	2.63×10^6	1.3×10^7	1.63×10^7	1.46×10^7	1.43×10^7
XGL74	4.19×10^5	3.9×10^5	5.1×10^6	8.78×10^6	9.75×10^7	1.26×10^7	1.13×10^7
XGL78	4.2×10^5	7.8×10^5	4.7×10^6	3.98×10^6	9.13×10^6	2.43×10^7	2.78×10^7
XGL81	4.18×10^5	4.35×10^5	2.94×10^6	9.15×10^6	2.38×10^7	9.15×10^6	1.14×10^7

TABLE AXI: Viable Cell Counts [Cells/Ml] at a Constant Temperature of Fermentation [15°C] in Muller Thurgau

Day		0	1	2	3	4	5	7
Yeast	MD26	4.62×10^5	1.65×10^6	2.38×10^7	3.55×10^7	5.97×10^7	5.0×10^7	4.01×10^7
	AWI60	4.4×10^5	1.15×10^6	1.64×10^7	7.51×10^7	4.89×10^7	5.03×10^7	2.91×10^7
	AWI80	4.4×10^5	8.27×10^5	1.66×10^7	3.08×10^7	1.28×10^7	1.71×10^7	3.36×10^7
	XGL74	4.6×10^5	1.13×10^6	1.57×10^7	1.3×10^7	3.41×10^7	5.24×10^7	6.18×10^7
	XGL78	4.61×10^5	1.25×10^6	2.42×10^7	1.23×10^7	9.15×10^7	2.79×10^7	1.01×10^7
	XGL81	4.16×10^5	1.23×10^6	1.73×10^7	2.09×10^7	2.2×10^7	2.66×10^7	3.26×10^7

TABLE AXII: Viable Cell Counts [Cells/Ml] at a Constant Temperature of Fermentation [15°C] in Chenin Blanc

Day	0	1	2	3	4	5	6	8
Yeast MD26	7.21×10^5	1.16×10^7	9.3×10^6	2.88×10^7	5.67×10^7	1.17×10^7	3.23×10^7	9.07×10^7
AW160	8.45×10^5	1.23×10^7	1.15×10^7	4.28×10^7	5.63×10^7	1.57×10^8	1.23×10^8	7.74×10^7
AW180	7.06×10^5	4.9×10^6	3.6×10^6	4.5×10^7	1.11×10^5	1.13×10^8	5.74×10^7	1.04×10^8
XGL74	7.08×10^5	7.7×10^6	1.1×10^7	2.04×10^7	3.11×10^7	9.39×10^7	8.59×10^7	5.18×10^7
XGL78	7.17×10^5	5.7×10^6	1.7×10^7	1.88×10^7	2.66×10^7	1.82×10^7	3.06×10^7	2.21×10^7
XGL81	7.02×10^5	9.4×10^6	8.7×10^6	2.54×10^7	1.19×10^7	1.73×10^7	2.94×10^7	4.99×10^7

APPENDIX B

TABLE BI: Sugar Utilisation [g/100ml] Fermented at a Constant Rate of Sugar Utilisation in Complete Defined Media [Std Error ± 0.5g/100ml]

Day		0	1		2		4		6		9		13		18	
Yeast	MD26	9.68	7.7 8.0	7.8	6.0 6.4	6.3	3.4 3.6	3.5	2.2 2.3	2.3	0.0 0.0	0.0	0.0 0.0	0.0	0.0 0.0	0.0
	AWI60	9.86	7.9 8.0	8.0	7.5 8.9	8.2	4.2 3.2	3.7	3.3 2.5	2.9	1.0 0.6	0.8	0.0 0.0	0.0	0.0 0.0	0.0
	AWI80	9.74	8.0 8.3	8.2	7.6 7.0	7.3	4.6 3.6	4.1	2.6 2.2	2.4	0.0 0.0	0.0	0.0 0.0	0.0	0.0 0.0	0.0
	XGL74	9.53	8.3 8.4	8.4	9.3 10.4	9.8	4.1 3.7	3.9	3.2 3.0	3.1	1.0 0.8	0.9	0.0 0.0	0.0	0.0 0.0	0.0
	XGL78	9.62	8.4 8.4	8.4	9.0 8.7	8.9	6.1 3.9	5.0	3.9 2.7	3.3	0.8 0.4	0.6	0.0 0.0	0.0	0.0 0.0	0.0
	XGL81	9.71	7.8 7.9	7.9	7.1 7.3	7.2	3.0 3.0	3.0	0.8 1.0	0.9	0.5 0.0	0.3	0.0 0.0	0.0	0.0 0.0	0.0

APPENDIX B

TABLE BII: Sugar Utilisation [g/100ml] Fermented at a Constant Rate of Sugar Utilisation in Muller Thurgau [Std Error ± 0.5g/100ml]

Day		0	1		2		4		6		9		13		18	
Yeast	MD26	13.8	- 13.5	13.5	14.7 11.8	13.2	5.8 6.0	5.9	2.7 2.9	2.8	0.4 0.4	0.4	0.0 0.0	0.0	0.0 0.0	0.0
	AWI60	14.2	14.3 14.2	14.3	14.4 12.4	13.4	8.1 8.9	8.5	5.3 6.6	5.9	0.7 1.5	1.1	0.0 0.0	0.0	0.0 0.0	0.0
	AWI80	14.2	14.5 14.2	14.4	11.6 15.0	13.3	7.0 7.2	7.1	5.8 3.6	4.7	1.0 1.0	1.0	0.0 0.0	0.0	0.0 0.0	0.0
	XGL74	14.0	14.1 14.0	14.1	13.6 12.3	12.9	6.9 9.3	8.1	5.0 5.8	5.4	0.9 1.4	1.2	0.0 0.1	0.1	0.0 0.0	0.0
	XGL78	14.7	- 14.7	14.7	11.7 -	11.7	8.6 7.5	8.1	4.7 4.7	4.7	0.6 0.7	0.7	0.0 0.0	0.0	0.0 0.0	0.0
	XGL81	14.2	14.3 14.2	14.3	11.3 12.4	11.8	5.6 8.4	7.0	2.0 4.7	2.4	0.3 0.8	0.5	0.0 0.0	0.0	0.0 0.0	0.0

APPENDIX B

TABLE BIII: Sugar Utilisation [g/100ml] Fermented at a Constant Rate of Sugar Utilisation in Chenin Blanc [Std Error ± 0.5g/100ml]

Day		0	1		2		4		6		9		13		18	
Yeast	MD26	22.7	16.6 15.8	16.2	14.5 13.1	13.8	8.6 8.5	8.6	5.7 4.7	4.2	1.8 0.6	1.2	0.0 0.0	0.0	0.0 0.0	0.0
	AWI60	24.9	14.9 14.3	14.6	12.8 11.0	11.9	8.3 5.3	6.8	5.5 2.9	4.2	2.3 0.9	1.5	0.0 0.0	0.0	0.0 0.0	0.0
	AWI80	20.8	16.9 16.8	16.9	- 18.9	18.9	9.1 8.4	8.8	3.8 3.7	3.8	0.4 0.9	0.6	0.0 0.0	0.0	0.0 0.0	0.0
	XGL74	22.0	16.0 -	16.0	16.6 15.2	15.9	10.5 7.6	9.0	6.0 3.3	3.7	2.3 0.8	1.6	0.0 0.0	0.0	0.0 0.0	0.0
	XGL78	21.6	17.5 17.3	17.4	18.5 16.2	17.3	11.7 11.0	11.4	8.8 8.7	8.8	3.9 2.8	3.4	0.0 0.0	0.0	0.0 0.0	0.0
	XGL81	20.0	15.3 15.6	15.5	11.8 13.5	12.7	5.4 7.0	6.2	2.0 2.6	2.3	0.3 0.5	0.4	0.0 0.0	0.0	0.0	0.0

APPENDIX B

TABLE BIV: Glycerol Production [g/l] Fermented at a Constant Rate of Sugar Utilisation in Complete Defined Media [Std Error ± 0.2g/l]

Day		0	1		2		4		6		9		13		18	
Yeast	MD26	0.0	1.2 0.9	1.1	1.2 1.1	1.2	1.3 1.3	1.3	2.3 2.2	2.3	2.3 2.3	2.3	2.4 2.2	2.3	2.5 2.2	2.4
	AWI60	0.0	0.9 0.9	0.9	1.2 1.4	1.3	1.3 1.0	1.2	1.9 2.0	2.0	2.2 2.4	2.3	2.3 2.2	2.3	2.8 2.2	2.4
	AWI80	0.0	0.8 0.9	0.9	1.2 1.1	1.2	1.5 1.4	1.5	2.1 2.1	2.1	2.2 2.2	2.2	2.5 2.3	2.4	2.4 2.4	2.4
	XGL74	0.0	0.8 0.8	0.8	1.6 1.6	1.6	1.5 1.3	1.4	2.6 2.8	2.7	3.2 3.2	3.2	3.0 3.4	3.2	3.0 3.1	3.1
	XGL78	0.0	0.7 0.8	0.8	1.2 1.2	1.2	1.9 1.3	1.6	2.4 2.6	2.5	2.8 2.8	2.8	2.8 2.7	2.8	3.1 2.7	2.9
	XGL81	0.0	1.2 1.2	1.2	1.9 1.9	1.9	2.3 2.1	2.2	3.4 3.0	3.2	2.9 3.1	3.0	3.3 3.0	3.2	3.4 3.2	3.3

APPENDIX B

TABLE BV: Glycerol Production [g/l] Fermented at a Constant Rate of Sugar Utilisation in Muller Thurgau [Std Error ± 0.2g/l]

Day		0	1	2	4	6	9	13	18
Yeast	MD26	0.0	0.78 1.34 1.06	2.23 1.67 1.95	2.3 2.1 2.2	3.6 3.4 3.5	3.4 3.1 3.3	3.5 3.5 3.5	3.5 3.5 3.5
	AWI60	0.0	1.23 1.42 1.32	1.82 1.70 1.76	2.0 2.2 2.1	3.1 3.0 3.1	3.4 3.3 3.4	3.4 3.3 3.4	3.6 3.4 3.5
	AWI80	0.0	1.40 1.36 1.38	1.79 2.37 2.08	2.0 2.6 2.3	3.5 3.7 3.6	3.9 3.9 3.9	4.1 3.9 4.0	3.9 3.9 3.9
	XGL74	0.0	1.88 1.69 1.79	2.92 2.56 2.74	3.0 3.6 3.3	4.5 4.4 4.5	5.2 4.8 5.0	5.1 4.8 5.0	- 5.0 5.0
	XGL78	0.0	- 1.24 1.24	1.89 - 1.89	3.2 2.6 2.9	4.6 3.6 4.1	4.5 4.3 4.4	4.8 4.9 4.9	5.1 4.5 4.8
	XGL81	0.0	1.63 1.80 1.71	2.36 2.39 2.38	3.9 3.5 3.7	4.9 4.8 4.9	5.2 5.2 5.2	4.9 5.2 5.1	5.1 5.2 5.2

APPENDIX B

TABLE BVI: Glycerol Production [g/l] Fermented at a Constant Rate of Sugar Utilisation in Chenin Blanc [Std error ± 0.2g/l]

Day		0	1		2		4		6		9		13		19	
Yeast	MD26	0.0	2.4 2.7	2.6	3.0 3.2	3.1	3.6 4.2	3.9	4.9 5.0	5.0	5.4 5.7	5.6	5.8 5.9	5.9	5.9 5.8	5.9
	AWI60	0.0	3.1 3.5	3.3	3.7 4.1	3.9	4.0 4.6	4.3	5.0 5.5	5.3	5.4 6.0	5.7	5.3 5.7	5.5	5.4 5.6	5.5
	AWI80	0.0	2.4 2.6	2.5	- 4.5	4.5	4.9 4.7	4.8	5.6 5.6	5.6	6.6 6.6	6.6	6.0 6.0	6.0	5.9 -	5.9
	XGL74	0.0	3.8 -	3.8	5.9 6.7	6.3	6.8 7.2	7.0	7.3 8.4	7.9	8.8 9.3	9.1	8.7 8.8	8.8	8.9 -	8.9
	XGL78	0.0	2.7 2.2	2.5	4.2 3.4	3.8	4.6 4.4	4.5	5.6 6.2	5.9	7.2 8.0	7.6	7.7 8.1	7.9	7.7 8.1	7.9
	XGL81	0.0	4.2 3.9	4.0	5.9 5.9	5.9	7.0 7.3	7.2	8.3 7.7	8.0	8.6 8.8	8.7	8.3 8.5	8.4	8.1 8.4	8.3

APPENDIX B

TABLE BVII: Ethanol Production [g/100ml] Fermented at a Constant Rate of Sugar Utilisation in Complete Defined Media [Std error ± 0.1g/100ml]

Day		0	1		2		4		6		9		13		18	
Yeast	MD26	0.0	0.9 0.9	0.9	1.3 1.2	1.3	2.1 1.9	2.0	4.0 3.4	3.7	4.0 4.0	4.0	4.7 4.5	4.6	4.6 4.5	4.6
	AWI60	0.0	0.9 0.9	0.9	1.3 1.5	1.4	1.8 1.7	1.8	3.0 1.8	2.4	3.9 3.9	3.9	4.0 4.0	4.0	5.2 4.6	4.9
	AWI80	0.0	0.8 0.8	0.8	1.1 1.1	1.1	2.0 1.8	1.9	3.3 3.4	3.4	3.6 3.6	3.6	3.9 4.2	4.1	4.5 4.6	4.6
	XGL74	0.0	0.7 0.6	0.7	1.2 1.2	1.2	1.5 1.3	1.4	3.0 3.2	3.1	3.9 3.9	3.9	4.1 4.3	4.2	4.1 4.5	4.3
	XGL78	0.0	0.3 0.7	0.5	1.1 1.1	1.1	2.1 1.8	2.0	2.2 2.5	2.4	3.9 4.0	4.0	4.3 3.9	4.1	4.4 4.4	4.4
	XGL81	0.0	1.0 0.8	1.9	1.6 1.5	1.6	2.5 2.3	2.4	4.1 3.9	4.0	3.7 4.1	3.9	4.1 3.9	4.0	4.4 4.4	4.4

APPENDIX B

TABLE BVIII: Ethanol Production [g/100ml] Fermented at a Constant Rate of Sugar Utilisation in Muller Thurgau [Std error ± 0.1g/100ml]

Day		0	1		2		4		6		9		13		18	
Yeast	MD26	0.0	0.62 1.32	0.96	2.4 1.6	2.0	3.6 3.6	3.6	6.1 6.5	6.3	5.9 5.6	5.8	6.7 6.7	6.7	7.3 7.3	7.3
	AWI60	0.0	1.13 0.31	0.72	1.8 1.4	1.6	2.8 2.8	2.8	5.1 4.1	5.6	6.1 5.9	6.0	6.7 6.4	6.6	7.1 6.9	7.0
	AWI80	0.0	1.02 1.11	1.07	1.4 1.8	1.6	2.7 3.3	3.0	4.5 6.1	5.3	5.9 5.7	5.8	7.0 7.0	7.0	6.7 -	6.7
	XGL74	0.0	1.27 1.15	1.21	1.8 1.6	1.7	2.9 3.3	3.1	4.7 4.9	4.8	5.9 5.9	5.9	6.5 6.7	6.6	6.8 6.7	6.8
	XGL78	0.0	- 0.92	0.92	1.2 -	1.2	3.6 2.9	3.3	6.1 5.0	5.6	6.1 5.9	6.0	6.7 6.9	6.8	7.1 6.4	6.8
	XGL81	0.0	1.14 1.13	1.14	1.7 1.5	1.6	4.2 2.9	3.5	6.6 5.0	5.8	6.4 6.5	6.5	6.5 -	6.5	7.0 6.8	6.9

APPENDIX B

TABLE BIX: Ethanol Production [g/100ml] Fermented at a Constant Rate of Sugar Utilisation in Chenin Blanc [Std error ± 0.1g/100ml]

Day		0	1		2		4		6		9		13		18	
Yeast	MD26	0.0	1.2 1.6	1.4	2.1 2.5	2.3	3.7 4.2	4.0	5.4 6.3	5.9	7.1 7.3	7.2	8.2 8.2	8.2	8.5 8.4	8.5
	AWI60	0.0	1.9 2.1	2.0	2.8 3.2	3.0	4.2 4.8	4.5	6.2 7 3	6.8	7.5 7.5	7.5	8.5 8.1	8.3	8.2 7.3	7.7
	AWI80	0.0	1.1 1.1	1.1	- 2.6	2.6	4.6 4.5	4.5	6.9 6.8	6.9	7.9 7.7	7.8	7.8 8.4	8.1	7.8 -	7.8
	XGL74	0.0	1.5 -	1.5	2.4 3.0	2.7	4.1 4.9	4.5	5.9 6.3	6.1	6.9 7.5	7.3	7.6 8.0	7.8	7.6 -	7.6
	XGL78	0.0	2.5 0.9	1.2	1.8 1.5	1.7	3.0 2.6	2.8	4.9 5.1	5.0	6.1 6.7	6.3	7.6 7.4	7.5	7.5 8.5	8.0
	XGL81	0.0	1.7 1.7	1.7	2.8 2.7	2.8	5.2 7.0	6.1	7.6 7.2	7.4	7.8 7.7	7.8	7.5 7.5	7.5	7.8 7.8	7.8

TABLE BX: Viable Cell Counts [Cells/Ml] at a Constant Sugar Utilisation Fermentation in Complete Defined Media

DAY		0 (25°C)	1 (25°C)	2 (15°C)	3 (15°C)	4 (15°C)	6 (16°C)	7 (16°C)
Yeast	MD26	4.43×10^5	1.4×10^7	2.05×10^7	2.1×10^7	2.2×10^7	3.57×10^7	3.39×10^7
	AW160	4.66×10^5	5.4×10^6	7.2×10^6	1.24×10^7	1.89×10^7	1.99×10^7	1.47×10^7
	AW180	4.20×10^5	7.8×10^6	1.41×10^7	1.66×10^7	2.7×10^7	2.7×10^7	3.93×10^7
	XGL74	4.38×10^5	6.9×10^6	4.9×10^6	1.08×10^7	6.25×10^6	6.25×10^6	1.2×10^7
	XGL78	4.48×10^5	4.5×10^6	2.8×10^6	1.45×10^7	7.63×10^6	7.63×10^6	1.04×10^7
	XGL81	4.65×10^5	5.9×10^6	1.09×10^7	9.39×10^6	1.4×10^7	1.4×10^7	1.5×10^7

TABLE BXI: Viable Cell Counts [Cells/Ml] at a Constant Sugar Utilisation Fermentation in Muller Thurgau

	Day	0 (25°C)	1 (25°C)	2 (15°C)	3 (15°C)	4 (15°C)	6 (16°C)	7 (16°C)
Yeast	MD26	4.64×10^5	1.14×10^7	3.18×10^7	3.91×10^7	4.25×10^7	6.26×10^7	5.36×10^7
	AW160	4.47×10^5	7.75×10^6	2.9×10^7	4.4×10^7	5.26×10^7	1.03×10^8	8.18×10^7
	AW180	4.72×10^5	1.9×10^7	4.91×10^7	5.65×10^7	7.43×10^7	1.24×10^8	2.08×10^8
	XGL74	4.49×10^5	4.75×10^6	1.45×10^7	1.30×10^7	1.05×10^7	2.7×10^7	3.76×10^7
	XGL78	4.36×10^5	7.75×10^6	2.95×10^7	1.54×10^7	2.49×10^7	2.5×10^7	6.37×10^7
	XGL81	4.26×10^5	9.13×10^6	1.91×10^7	2.13×10^7	1.96×10^7	3.89×10^7	2.59×10^7

Table BXII: Viable Cell Counts [Cells/Ml] at a Constant Sugar Utilisation Fermentation in Chenin Blanc

	Day	0 (25°C)	1 (25°C)	2 (15°C)	3 (15°C)	4 (15°C)	5 (15°C)	7 (16°C)
Yeast	MD26	3.91×10^5	4.54×10^7	7.52×10^7	8.24×10^7	4.91×10^7	3.66×10^7	4.4×10^7
	AW160	4.07×10^5	3.09×10^7	4.04×10^7	4.56×10^7	3.04×10^7	2.85×10^7	7.25×10^7
	AW180	4.1×10^5	2.25×10^7	1.39×10^8	4.34×10^7	1.88×10^8	1.06×10^8	1.61×10^8
	XGL74	4.01×10^5	1.68×10^7	3.73×10^7	9.16×10^7	4.23×10^7	2.93×10^7	7.78×10^7
	XGL78	4.08×10^5	5.25×10^6	2.84×10^7	3.2×10^7	3.96×10^7	1.08×10^7	1.78×10^7
	XGL81	4.06×10^5	1.55×10^7	3.41×10^7	6.42×10^7	4.88×10^7	6.95×10^7	2.07×10^8

APPENDIX C

APPENDIX TABLE C(I): Sulphur Dioxide Levels and Associated pH Changes in Complete Defined Media

Approximate Total SO_2 (ppm)	pH	Actual Free SO_2 (ppm)	Actual Total SO_2 (ppm)
0	3.57	0	0
50	3.53	15	51
100	3.49	42	109
150	3.47	63	163
200	3.44	80	196
250	3.75	110	253
300	3.50	150	326

APPENDIX TABLE C(II): Sulphur Dioxide Levels and Associated pH Changes in Chenin Blanc

Approximate Total SO_2 (ppm)	pH	Actual Free SO_2 (ppm)	Actual Total SO_2 (ppm)
0	2.97	0	0
50	2.94	2	56
100	2.93	6	100
150	2.97	25	146
200	3.04	42	190
250	3.06	64	255
300	3.07	93	315

APPENDIX TABLE C(III): Production of Glycerol and Ethanol by AWI60 in the Presence of Different Levels of Sulphur Dioxide

Yeast	Approx. SO_2 level (ppm)	Media	Glycerol (g/l) x_1 , x_2	$\bar{x}$	S.D.	Ethanol (g/100 ml) x_1 , x_2	$\bar{x}$	S.D.
AW160	0	CDM	2.40 2.54	2.47	0.07	4.48 4.68	4.58	0.10
	50		2.68 2.81	2.74	0.07	4.78 4.46	4.62	0.16
	100		2.49 2.55	2.52	0.03	4.56 4.54	4.55	0.01
	150		2.64 2.70	2.67	0.03	4.37 4.29	4.33	0.04
	200		2.60 2.74	2.67	0.07	4.49 4.47	4.48	0.01
	250		2.62 2.66	2.64	0.02	4.25 4.19	4.22	0.03
	300		2.61 2.83	2.72	0.11	4.22 4.22	4.22	0.00
	0	CB	5.80 5.96	5.88	0.08	9.16 8.74	8.95	0.21
	50		6.12 6.34	6.23	0.11	9.03 8.95	8.99	0.04
	100		5.94 5.84	5.89	0.05	8.87 9.13	9.00	0.13
	150		5.92 6.38	6.16	0.22	9.01 9.27	9.14	0.13
	200		6.58 6.34	6.46	0.12	8.63 8.97	8.80	0.17
	250		6.34 6.34	6.34	0.00	9.02 8.59	8.87	0.19
	300		6.29 6.55	6.42	0.13	8.86 8.48	8.67	0.19

APPENDIX TABLE C(IV): Production of Glycerol and Ethanol by XGL81 in the Presence of Different Levels of Sulphur Dioxide

Yeast	Approx. SO_2 level (ppm)	Media	Glycerol (g/l) x_1 , x_2	$\bar{x}$	S.D.	Ethanol (g/100 ml) x_1 , x_2	$\bar{x}$	S.D.
XGL81	0	CDM	3.63			3.87		
			3.38	3.55	0.12	3.87	4.00	0.19
			3.64			4.27		
	50		4.20			3.94		
			3.74	3.89	0.22	3.74	3.82	0.09
			3.73			3.79		
	100		3.92			4.05		
			4.75	4.18	0.40	3.89	3.92	0.09
			3.88			3.84		
	150		4.15			4.05		
			4.13	4.00	0.56	4.33	4.23	0.13
			3.72			4.30		
	200		3.95			4.20		
			3.79	3.88	0.06	4.12	4.14	0.04
			3.90			4.11		
	250		3.40			4.41		
			4.80	3.91	0.63	4.43	4.43	0.03
			3.52			4.46		
	300		3.65			4.54		
			3.78	3.79	0.12	4.49	4.42	0.14
			3.95			4.22		
	0	CB	8.36			7.06		
			7.63	8.24	0.45	7.05	6.98	0.11
			8.72			6.82		
	50		8.43			7.24		
			8.34	8.69	0.45	7.03	7.12	0.09
			9.32			7.10		
	100		8.21			7.18		
			7.92	8.19	0.20	7.33	7.24	0.07
			8.43			7.26		
	150		7.90			7.62		
			8.12	8.01	0.09	7.54	7.54	0.07
			7.99			7.45		
	200		-			-		
			-	-	-	-	-	-
			-			-		
	250		6.89			8.40		
			7.88	7.65	0.55	8.32	8.44	0.12
			8.18			8.60		
	300		7.28			8.34		
			7.38	7.29	0.07	8.41	8.46	0.12
			7.21			8.62		

NB S.D. = Standard deviation
CDM = Complete defined media
CB = Chenin Blanc

APPENDIX D

APPENDIX TABLES D(I) and (II): First Generation Haploids in Muller Thurgau

Table D(I): XGL74

Yeast	Mating Type	Glycerol (g/l)	Ethanol (g/100ml)
XGL74	Diploid Parent	5.81	7.08
Haploid 1	α	4.27	6.89
2	α	5.14	6.87
3	a	4.82	6.99
4	a	4.69	7.20
5	a	5.38	7.07
6	α	4.49	6.93
7	a	4.78	6.73
8	a	4.71	6.47
9	a	4.75	6.64
10	a	4.70	7.11
11	a	4.75	7.07
12	a	5.44	7.05
13	α	4.76	7.06
14	a	5.78	6.88
15	α	5.33	7.10
16	α	4.48	6.61
17	a	4.47	6.61
* 18	α	5.85	6.88
* 19	α	4.96	7.17
20	α	5.39	6.96
Average Yield		4.32	6.91

Table D(II): XGL81

Yeast	Mating Type	Glycerol (g/l)	Ethanol (g/100ml)
XGL81	Diploid Parent	5.76	7.03
Haploid 1	a	3.65	7.21
2	α	3.21	7.01
3	a	3.80	6.84
4	α	5.18	7.03
5	a	3.79	6.98
6	α	4.88	6.91
7	a	5.03	7.15
8	a	5.04	7.31
9	α	5.22	7.12
10	α	4.59	6.97
11	α	5.05	7.31
12	a	5.04	7.24
13	α	5.10	7.06
14	α	5.08	7.23
* 15	a	5.21	7.41
16	a	5.05	6.62
17	α	4.86	7.17
* 18	a	5.26	7.30
19	α	4.97	7.08
20	α	5.22	6.87
		4.76	7.09

Category (1) = Greatest yield of glycerol by - XGL74 haploid (18) - α
and XGL81 haploid (18) - a

Category (2) = Greatest yield of glycerol and ethanol by - XGL74 haploid (19) - α
and XGL81 haploid (15) - a

APPENDIX TABLES D(III) and (IV): First Generation Haploids in Chenin Blanc

Table D(III): XGL74

Yeast	Mating Type	Glycerol (g/l)	Ethanol (g/100ml)
XGL74	Diploid Parent	8.47	8.03
Haploid 1	α	8.06	8.89
2	α	7.61	8.75
* 3	a	8.97	8.80
4	a	7.88	9.04
5	a	8.18	9.06
6	α	7.54	8.90
7	a	7.85	8.57
8	a	7.97	9.02
9	a	8.15	9.10
10	a	8.59	8.98
11	a	7.75	8.97
12	a	7.89	8.90
13	α	7.76	9.00
14	a	8.03	8.95
15	α	7.90	8.80
16	α	7.93	8.94
17	a	7.98	8.69
* 18	α	8.07	9.16
19	α	7.98	9.01
20	a	8.72	8.80
Average Yield		8.03	8.92

Table D(IV): XGL81

Yeast	Mating Type	Glycerol (g/l)	Ethanol (g/100ml)
XGL81	Diploid Parent	8.35	7.97
Haploid 1	a	5.89	7.51
2	α	6.01	7.41
3	a	6.40	7.98
* 4	α	10.75	8.14
5	a	7.34	8.10
6	α	8.38	8.05
7	a	8.16	8.53
8	a	8.24	7.65
9	α	8.04	8.27
10	α	8.84	7.91
11	α	8.74	8.18
12	a	7.77	8.07
13	α	9.02	8.31
14	α	7.89	8.75
15	a	7.82	8.01
16	a	7.85	7.94
17	α	8.84	7.93
* 18	a	8.55	8.45
19	α	8.82	8.29
20	α	9.21	8.01
		8.13	8.07

Category (3) = Greatest yield of glycerol by XGL74 haploid (3) - a
and XGL81 haploid (4) - α

Category (4) = Greatest yield of glycerol and ethanol by XGL74 haploid (18) - α
and XGL81 haploid (18) - a

APPENDIX TABLE D(V) and (VI): First Generation Diploids in Muller Thurgau

Table D(V): XGL74(18) x XGL81(18)

Yeast	Glycerol (g/l)	Ethanol (g/100ml)
Diploid i	4.42	7.43
ii	4.87	7.32
iii	4.84	7.20
iv	5.16	7.11
* v	5.18	7.26
vi	4.69	6.83
vii	4.92	7.09
viii	4.97	6.96
* ix	5.57	6.99
Average Yield	4.96	7.13

Table D(VI): XGL74(19) x XGL81(15)

Yeast	Glycerol (g/l)	Ethanol (g/100ml)
Diploid i	4.92	7.05
ii	5.11	7.07
iii	4.61	7.07
iv	4.82	7.00
v	4.81	7.02
vi	4.99	7.12
vii	4.80	6.95
viii	4.76	6.96
	4.85	7.03

Category (1) = Greatest yield of glycerol by
XGL 74(18) x XGL 81(18)(ix) = XGA

Category (2) = Greatest yield of glycerol and ethanol by
XGL 74(18) x XGL 81(18)(v) = XGB

APPENDIX TABLE D(VII) and (VIII): First Generation Diploids in Chenin Blanc

Table D(VII): XGL74(3) x XGL81(4)

Yeast	Glycerol (g/l)	Ethanol (g/100ml)
Diploid i	8.23	6.58
ii	8.55	7.08
iii	8.60	7.03
iv	8.74	7.01
v	8.32	7.04
* vi	8.94	7.11
vii	7.55	6.93
Average Yield	8.42	6.97

Table D(VIII): XGL74(18) x XGL81(18)

Yeast	Glycerol (g/l)	Ethanol (g/100ml)
Diploid i	8.08	7.65
ii	8.09	7.60
iii	8.09	7.66
iv	7.48	7.84
* v	8.04	7.93
vi	8.07	7.46
vii	8.02	7.36
viii	6.90	7.58
ix	8.42	7.41
	7.91	7.61

Category (1) = Greatest yield of glycerol by
XGL 74(3) x XGL 81(4)(vi) = XGC

Category (2) = Greatest yield of glycerol and ethanol by
XGL 74(18) x XGL 81(18)(v) = XGA

APPENDIX TABLES D(IX) and (X): Second Generation Haploids in Muller Thurgau

Table D(IX): XGA

Yeast	Mating Type	Glycerol (g/l)	Ethanol (g/100ml)
XGA	Diploid Parent	5.18	7.26
*Haploid 1	α	4.46	6.92
2	a	4.60	6.87
3	α	4.45	6.39
4	α	4.03	6.44
5	a	4.26	6.31
6	α	4.21	6.56
* 7	a	4.29	6.71
* 8	α	5.18	6.51
9	a	4.13	6.34
10	a	4.12	6.62
11	a	4.09	6.13
12	α	4.72	6.96
13	α	4.65	6.66
14	a	4.15	6.02
15	a	4.17	6.31
16	a	4.09	6.44
17	α	4.10	6.22
18	a	4.29	6.15
Average Yield		4.33	6.48

Table D(X): XGB

Yeast	Mating Type	Glycerol (g/l)	Ethanol (g/100ml)
XGB	Parent Diploid	5.57	6.99
Haploid 1	a	4.40	5.98
2	a	4.79	6.08
3	α	4.60	6.03
4	a	4.32	6.53
5	a	3.92	6.22
6	a	4.01	6.10
7	α	4.50	6.07
8	α	4.04	6.09
9	a	4.64	6.20
10	α	4.69	6.12
11	α	4.43	6.02
12	a	4.91	6.14
* 13	a	4.95	6.15
14	a	4.05	6.40
15	α	4.62	5.78
16	a	4.46	6.08
17	α	4.51	5.98
18	α	4.58	6.05
		4.47	6.11

Category (1) = Greatest yield of glycerol by XGA haploid (8) - α
and XGB haploid (13) - a

(2) = Greatest yield of glycerol and ethanol by XGA haploid (1) - α
and XGB haploid (7) - a

APPENDIX TABLES D(XI) and (XII): Second Generation Haploids in Chenin Blanc

Table D(XI): XGC

Yeast	Mating Type	Glycerol (g/l)	Ethanol (g/100ml)
XGC	Diploid Parent	8.94	7.11
Haploid 1	a	8.94	7.20
2	a	10.21	7.42
3	α	9.07	7.76
* 4	a	9.83	7.76
5	α	8.79	7.75
6	a	9.21	7.74
7	α	8.97	7.61
8	α	9.74	7.51
9	a	8.62	7.22
10	α	9.56	7.33
* 11	α	9.75	7.50
12	α	9.18	7.33
* 13	a	10.60	6.95
14	a	9.41	7.26
* 15	α	10.60	6.97
16	α	10.05	6.99
17	a	8.99	7.12
18	α	9.72	7.02
19	α	9.89	6.94
20	a	9.44	7.06
Average Yield		9.53	7.32

Table D(XII): XGA

Yeast	Mating Type	Glycerol (g/l)	Ethanol (g/100ml)
XGA	Diploid Parent	8.04	7.93
Haploid 1	α	7.72	6.94
2	a	7.22	7.07
3	α	7.01	6.80
4	α	7.35	7.01
5	a	8.16	6.94
6	α	7.93	6.92
7	a	7.01	6.95
8	α	8.90	6.70
9	a	7.01	7.05
10	a	8.08	6.73
11	α	7.16	6.97
12	α	8.76	6.70
13	α	8.02	6.59
14	a	7.88	6.85
15	a	6.46	6.82
16	a	6.91	6.91
17	α	7.09	6.85
18	a	6.65	6.73
		7.52	6.86

Category (1) = Greatest yield of glycerol by XGC haploid (13) - a
and XGC haploid (15) - α

Category (2) = Greatest yield of glycerol and ethanol by XGC haploid (4) - a
and XGC haploid (11) - α

APPENDIX TABLE D(XIII) and (XIV): Second Generation Diploids in Muller Thurgau

Table D(XIII): XGA(8) x XGB(13)

Yeast	Glycerol (g/l)	Ethanol (g/100ml)
Diploid i	5.17	6.68
ii	5.48	6.63
iii	5.17	6.90
iv	5.86	6.73
* v	5.74	6.99
vi	5.85	6.53
* vii	6.41	6.71
Average Yield	5.67	6.74

Table D(XIV): XGA(1) x XGA(7)

Yeast	Glycerol (g/l)	Ethanol (g/100ml)
Diploid i	4.92	6.41
ii	4.40	6.70
iii	4.93	6.35
iv	4.88	6.75
v	4.62	6.59
vi	4.69	6.80
vii	4.66	6.53
	4.73	6.59

Category (1): Greatest yield of glycerol by XGA (8) x XGA (13)(vii) = XGD

Category (2): Greatest yield of glycerol and ethanol by XGA (8) x XGA (13)(v) = XGE

APPENDIX TABLE D(XV) and (XVI): Second Generation Diploids in Chenin Blanc

Table D(XV): XGC(13) x XGC(15)

Yeast	Glycerol (g/l)	Ethanol (g/100ml)
Diploid i	10.21	7.29
ii	10.15	7.45
iii	9.66	7.39
iv	10.12	7.48
v	9.48	7.43
vi	10.11	7.46
vii	9.83	7.50
viii	10.16	7.34
* ix	9.98	7.59
x	9.65	7.43
* xi	10.17	7.36
Average Yield	9.96	7.43

Table D(XVI): XGC(4) x XGC(11)

Yeast	Glycerol (g/l)	Ethanol (g/100ml)
Diploid i	8.87	7.39
ii	9.13	7.60
iii	10.18	7.30
iv	9.24	7.35
v	9.14	7.36
vi	9.49	7.35
vii	9.41	7.31
viii	9.02	7.40
ix	9.74	7.33
	9.39	7.38

Category (3) = Greatest yield of glycerol by XGC (13) x XGC (15) (xi) = XGF

Category (4) = Greatest yield of glycerol and ethanol by XGC (13) x XGC (15)(ix) = XGG

APPENDIX TABLE D(XVII) and (XVIII): Third Generation Haploids in Chenin Blanc

Table D(XVII): XGD

Yeast	Mating Type	Glycerol (g/l)	Ethanol (g/100ml)
XGD	Diploid Parent	6.41	6.71
Haploid 1	α	5.19	6.91
2	a	4.92	6.35
3	α	5.18	6.91
4	a	5.51	7.09
5	α	5.06	6.85
6	a	5.20	7.01
7	α	5.08	7.13
8	a	5.65	7.00
9	a	5.62	7.18
10	α	5.58	7.21
11	α	5.08	7.09
* 12	α	5.77	6.87
13	α	4.96	7.10
14	a	5.10	7.00
15	α	5.73	7.11
16	a	5.12	7.02
17	a	5.21	7.10
18	α	5.49	7.04
19	a	5.44	7.27
20	α	5.68	7.02
Average Yield		5.33	7.01

Table D(XVIII): XGE

Yeast	Mating Type	Glycerol (g/l)	Ethanol (g/100ml)
XGE	Diploid Parent	5.74	6.99
Haploid 1	a	5.53	7.20
2	a	5.43	7.33
3	a	5.65	7.31
4	α	5.37	7.37
5	α	5.29	7.40
6	a	5.62	7.12
* 7	a	5.72	7.36
8	α	5.04	7.25
9	a	5.40	7.35
10	a	5.69	7.36
11	α	5.44	7.40
12	α	5.58	7.18
13	a	5.50	7.38
* 14	α	5.49	7.38
15	α	5.15	7.43
16	a	5.59	7.28
17	α	5.26	7.15
18	a	5.74	7.19
19	a	5.17	7.17
20	α	5.56	7.26

Category (1) = Greatest yield of glycerol by XGD haploid (12) - α
and XGE haploid (7) - a

Category (2) = Greatest yield of glycerol and ethanol by XGE haploid (14) - α
and XGE haploid (7) - a

APPENDIX TABLE D(XIX) and (XX): THIRD GENERATION OF HAPLOIDS IN CHENIN BLANC

Table D(XIX): XGF

Yeast	Mating Type	Glycerol (g/l)	Ethanol (g/100ml)
XGF	Diploid Parent	10.17	7.36
Haploid 1	α	9.86	7.92
2	α	9.84	7.96
3	α	9.89	7.78
4	a	8.21	8.18
5	α	9.83	7.79
* 6	α	10.00	7.83
7	a	10.22	6.98
8	a	9.75	7.65
9	α	9.23	7.93
10	α	9.13	8.02
11	a	9.89	7.88
* 12	a	9.50	8.19
13	a	10.33	7.64
14	α	9.69	7.78
15	α	9.35	8.20
16	α	8.63	8.11
Average Yield		9.58	7.87

Table D(XX): XGG

Yeast	Mating Type	Glycerol (g/l)	Ethanol (g/100ml)
	Diploid Parent	9.98	7.59
* Haploid 1	α	9.84	8.00
2	α	9.15	8.19
3	a	9.56	8.06
* 4	a	11.14	7.60
5	α	9.32	7.80
6	a	8.84	8.06
7	a	10.06	7.87
8	a	9.62	7.51
9	a	9.18	7.73
10	α	9.62	7.90
11	a	8.56	7.88
12	a	10.65	7.83
13	a	9.65	7.95
14	α	9.43	7.96
15	α	9.46	8.00
16	a	9.55	8.11
17	a	9.86	8.00
18	a	7.92	7.95
19	α	8.80	7.97
20	a	8.73	8.08
		9.45	7.92

Category (3) = Greatest yield of glycerol by XGF (6) - α
and XGG (4) - a

Category (4) = Greatest yield of glycerol and ethanol by XGF (12) - a
and XGA (1) - α

APPENDIX TABLE DXXI and XXII: Third Generation Diploids in Muller Thurgau

Table DXXI: XGD(12) x XGE(7)

Yeast		Glycerol [g/l]	Ethanol [g/100ml]
Diploid	i	6.36	7.07
	ii	6.09	7.13
	iii	6.14	7.12
*	iv	6.28	7.33
	v	6.15	6.99
	vi	6.07	7.17
Average Yield		6.18	7.14

Table DXXII: XGE(7) x XGE(14)

Yeast		Glycerol [g/l]	Ethanol [g/100ml]
Diploid	i	6.39	7.10
	ii	5.90	6.58
	iii	5.20	6.84
	iv	6.23	6.99
	v	6.00	6.42
	vi	6.30	7.17
	vii	5.40	6.16
	viii	6.35	6.95
*	ix	6.42	6.80
		6.10	6.78

Category (1): Greatest Yield of Glycerol by XGE(7) x XGE(14)(ix) = XGH

Category (2): Greatest Yield of Glycerol and Ethanol by XGD(12) x XGE(7)(iv) = XGI

APPENDIX TABLE DXXIII and XXIV: Third Generation Diploids in Chenin Blanc

Table DXXIII:

XGF(6) x XGG(4)

Yeast		Glycerol [g/l]	Ethanol [g/100ml]
Diploid	i	8.08	6.60
	ii	8.59	6.63
	iii	7.39	5.56
	iv	8.16	6.05
	v	8.43	6.66
	vi	8.54	6.40
	vii	9.17	6.40
	viii	8.64	6.47
Average Yield		8.38	6.35

Table DXXIV:

XGF(12) x XGG(1)

Yeast		Glycerol [g/l]	Ethanol [g/100ml]
Diploid	i	8.30	6.82
	ii	8.51	6.37
	iii	8.23	6.09
	iv	8.38	6.07
	v	8.90	6.56
	vi	9.37	6.53
*	vii	9.49	8.83
*	viii	9.82	6.23
		8.88	6.44

Category (3): Greatest Yield of Glycerol by XFG(12) x XGG(1)(viii) = XGJ

Category (4): Greatest Yield of Glycerol and Ethanol by XGF(12) x XGG(1)(vii) = XGK

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