GLYCEROL PRODUCTION BY VARIOUS STRAINS OF SACCHAROMYCES CEREVISIAE

A Thesis presented in Partial Fulfilment
of the Requirements for the Degree of
Master of Science in Microbiology at
Massey University

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1987

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.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr R J Thornton for his guidance, support and encouragement to complete the experimental requirements for my degree. I would also like to thank Dr S B Rodriguez for her timely assistance and motivation in the laboratory.

I would also like to thank Professor D Bacon [and Dr B Jarvis] for his assistance and ideas for beginning my thesis write-up.

Also my family for the much needed support and criticism which was of much value to my write-up.

And to Dr H Larson and G Fisher for their help with the graphical presentation of parts of my thesis.

Also to Mr A Hooper for his assistance with statistical analysis for my data.

Credit is also due to my colleagues for their assistance during my research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

APPENDIX TABLES

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.

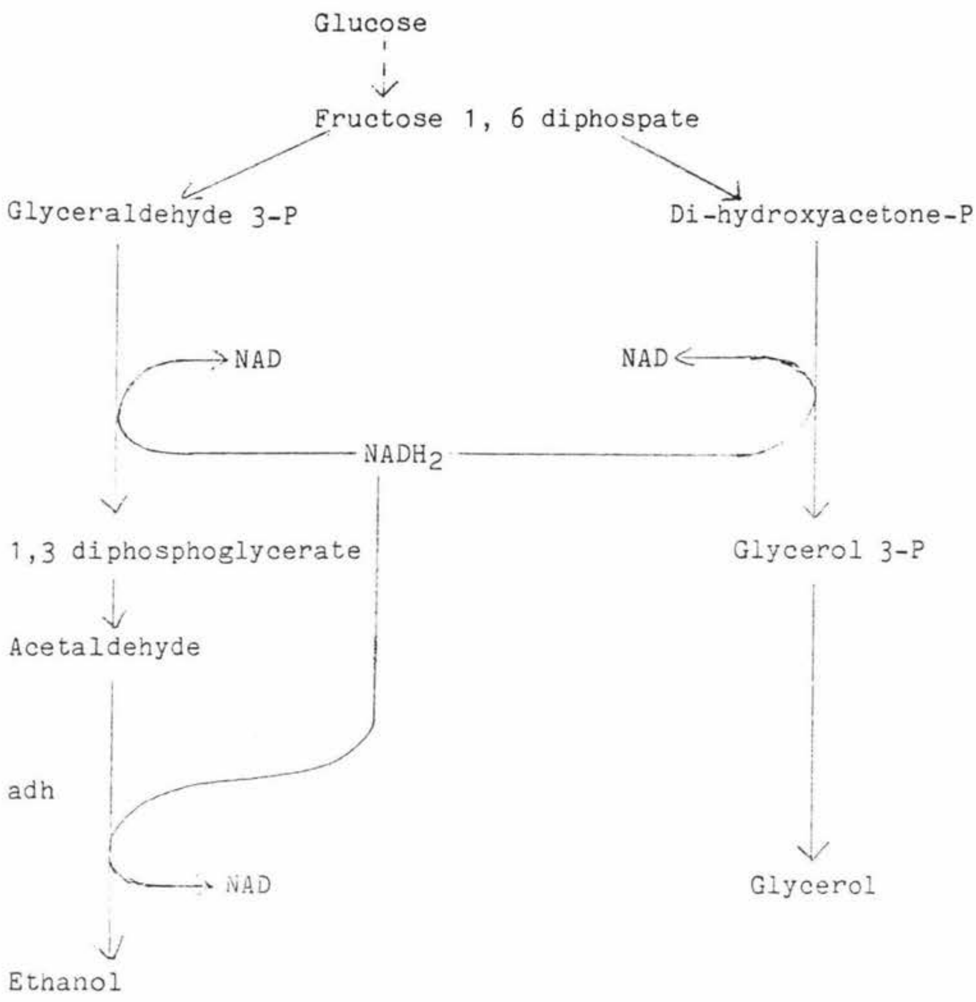
2. GLYCEROL PRODUCTION - ENZYMATIC PATHWAYS

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

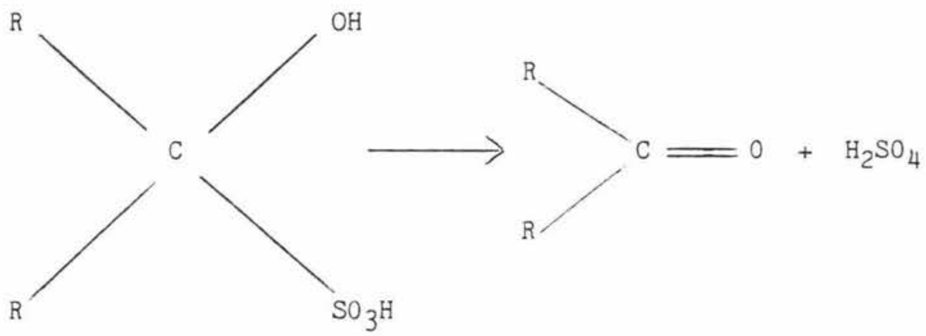
These functions require the presence of free sulphur dioxide as much of the sulphur dioxide added is bound to substances such as acetaldehyde, pyruvate and sugars present in grape juice. Since there are legal limitations* on the total sulphur dioxide content (i.e. free + bound SO_2) of wines, the nature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