GLYCEROL PRODUCTION BY FOUR COMMON GRAPE MOULDS

Aspergillus, *Botrytis*, *Penicillium* and *Rhizopus*

A Thesis Presented in Fulfilment of the Requirements

for Master of Science in Microbiology

at Massey University

RAJESH GOVIND RAVJI

1989

ABSTRACT

The production of glycerol by the grape moulds *Aspergillus niger*, *Penicillium italicum*, *Rhizopus nigricans* and *Botrytis cinerea* growing in juice from Chasselas and Black Hamburg grapes was examined. Juice from both free-run and homogenized whole grapes was filter sterilized and inoculated with single pure cultures of the moulds above. The four juice types were incubated at 25° C for 26 to 29 days. The inoculated juices were incubated in different air relations and during the 26 to 29 day incubation period , samples were taken periodically for the analysis of glycerol, glucose and fructose by HPLC. After 26 to 29 days, the moulds were harvested by filtration so that dry mycelial weights could be obtained.

Large differences in glycerol production were noted among the grape moulds. Under similar conditions of cultivation in Chasselas juice, *R. nigricans* and *B. cinerea* produced significantly more glycerol than *A. niger* and *P. italicum*. The levels of glycerol never exceeded 0.5g/100mL, whereas all cultures of *R. nigricans* and *B. cinerea* exceeded this level after 15 to 18 days of incubation. In Black Hamburg juice glycerol was not detected in cultures of *A. niger* and *P. italicum*. The levels of glycerol produced by all the four moulds were lower in Black Hamburg than in Chasselas juice.

Overall more sugar was utilized in Black Hamburg juice than in Chasselas juice under similar conditions. *B. cinerea* utilized the most total sugar in Chasselas juice than all the other moulds, while *R.*

nigricans utilized the most total sugar in Black Hamburg juice than all the other moulds. In Chasselas juice B. cinerea and R. nigricans displayed a preference for glucose over fructose, while in Black Hamburg juice no preference was evident. The pattern of sugar utilization over the incubation period between Chasselas and Black Hamburg juice was markedly different. In Chasselas juice under most cultivation conditions the four moulds utilized glucose and fructose throughout the incubation period, while in Black Hamburg juice there was rapid utilization during the first three days followed by a reduced rate of sugar utilization in the latter stages of incubation.

The four moulds differed in their production of mycelial dry weight. These differences were most marked in Chasselas juice where B. cinerea, depending on air relations, produced five to seven times more mycelial dry weight than R. nigricans and more than twice the mycelial dry weight produced by A. niger and P. italicum. In Black Hamburg juice B. cinerea produced two to three times more mycelial mass than the other three moulds.

At present in the Californian wine industry an HPLC method is currently under investigation, where the level of glycerol in the grape juice is used as an indicator of fungal rot of the grapes. This study has demonstrated that certain grape moulds do not produce the same amount of glycerol and that the level of glycerol is not related to the mycelial growth.

Thus this investigation has established that glycerol may not be used as a suitable indicator of fungal rot.

ACKNOWLEDGEMENTS

I wish to thank the Department of Microbiology and Genetics, Massey University, for the facilities and the opportunity for this research project.

In particular, I would like to thank Dr Roy Thornton and Dr Susan B Rodriguez for their advice, criticism and constant encouragement throughout this project.

I would like to acknowledge the assistance of Dr M.R.McLellan in the statistical analysis of my experimental data.

Also I would like to acknowledge the support of grant numbers 84/151 and 85/151 from the New Zealand Grants Committee.

I would also like to thank my family especially Mum and Dad for the support and motivation during the project and thesis write-up.

To my colleagues, for their support and companionship throughout the year.

Thanks must also go to Miss Sheralee Studholme for typing the thesis manuscript.

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INTRODUCTION

1. FUNGAL METABOLISM

Fungi have a heterotrophic mode of nutrition. The glycolytic pathways in fungi provide energy, precursors and reducing power in the form of NADH and NADPH which are used in degradative and biosynthetic reactions.

The two major glycolytic pathways that operate in most fungal cells are the Embden-Meyerhof (EM) pathway and the pentose-phosphate (PP) pathway.

The EM pathway is functional during aerobic and anaerobic growth in many fungi, including species of *Aspergillus*, *Penicillium*, *Rhizopus* and *Botrytis*. Glycerol is a by-product of a glycolytic reaction in the EM pathway. As much as 10% of the glucose utilized can be converted to glycerol.

In the first three reactions of the EM pathway, glucose is converted to fructose-1,6-diphosphate which is then cleaved into dihydroxyacetone phosphate (DHA-P) and glyceraldehyde-3-phosphate (G-3-P). DHA-P and G-3-P are interconverted by the enzyme triose phosphate isomerase. The glycolytic pathway proceeds through G-3-P. Alternatively, G-3-P can be converted to glycerol by the enzymes, glycerol-3-phosphate dehydrogenase and glycerol-1-phosphatase.

In the pentose phosphate (PP) pathway, NADPH is generated as glucose-6-phosphate is oxidised to ribose-5-phosphate. The function of the PP pathway is to produce reducing power in the form of NADPH and to provide precursors for the synthesis of nucleotides and amino acids. Pentose sugars such as xylose and arabinose can also be utilized by fungi via the PP pathway (Cochrane, 1976).

Another glycolytic pathway may be present in a few fungal species. A system analogous to the Entner-Doudoroff (ED) pathway has been demonstrated in *A. niger* (Elzainy *et al.*, 1973).

2. CELLULAR FUNCTIONS OF GLYCEROL

Glycerol in the fungal cell is utilized in the formation of phospholipids, triglycerides, and it may also play an important role in osmoregulation.

Lipids, especially triglycerides and phospholipids, play an important role in fungal physiology. Glycerol-containing intermediates are used in the biosynthesis of triglycerides which often function as storage products and which are important during spore germination.

Glycerol in the form of glycerol-3-phosphate is usually derived from the glycolytic intermediate dihydroxyacetone phosphate (DHA-P).

$$NADH + DHA\text{-}P \rightleftharpoons NAD^{+} + GLYCEROL\text{-}3\text{-}PHOSPATE$$

The triglyceride molecule is formed by glycerol-3-phosphate condensing the Co-enzyme A derivatives of three fatty acids.

Phospholipids, because of their amphipathic character, are found primarily in the plasma membrane and membranes of cellular organelles. Phospholipids derived from glycerol are called phosphoglycerides. Of the phosphoglycerides group, phosphatidyl-choline and phosphatidylethanolamine are the most common, with phosphatidylserine and phosphatidylinositol found in smaller amounts (Garraway and Evans, 1984).

Glycerol may be produced by various fungal species to modify the osmotic balance of the cell. Various fungal species grown in increasing concentrations of NaCl show corresponding increases in glycerol production. The glycerol reduces the effect of increased NaCl concentration, thus contributing to the osmotic stability of the cell (Gustafsson and Norkrans, 1976).

3. FUNGAL DISEASES OF GRAPEVINES

Fungal diseases can affect most parts of the grapevine and at different stages of maturity of the vine.

Four common moulds found on grapes are *Aspergillus niger*, *Penicillium italicum*, *Rhizopus nigricans* and *Botrytis cinerea*. Each of these moulds have individual conditions of infection, symptoms and associated methods of control.

Rhizopus species are widely distributed in nature. *Rhizopus nigricans* is the causative agent for *Rhizopus* rot in grapes (Winkler *et al*., 1974). *Rhizopus nigricans* is found in warm regions and is frequently found associated with damaged grapes. The fungus enters via cracks or punctures in the skin of the berries caused by excess pressure of berry contact during enlargement, just before and during ripening. Infection leads to breakdown of the grape tissue, causing the grape to soften and leak juice from the infected clusters (Winkler *et al*., 1974).

Penicillium species are also widely distributed. The group is characterized by a wide temperature growth range, with an optimum of 15-24°C. *Penicillium* species may be a problem in packed grapes. Infection may begin with grape injury, due to rough handling, or too tight packing. Under conditions of high humidity, as in a packed box, the fungus, once established, may spread rapidly from decayed to sound grapes.

If thinning berry clusters is carried out properly, *Penicillium* species are of minor importance as a field disease of table grapes. In areas of raisin or table grape production, the temperatures are too high for their optimum development. But in cooler regions, where wine grape varieties with compact clusters are grown, they may cause serious fungal infection. Infection usually imparts unpleasant organoleptic properties to the must and resulting wine.

Aspergillus niger is known to cause black-mould rot in some grape varieties, and is mainly confined to ripening grapes in the vineyard (Winkler *et al.*, 1974). This species infects grapes via skin breaks, punctures or when the ripened grapes are wet. In the San Joaquin Valley (California), *Aspergillus* species grow abundantly on rain damaged grapes (Amerine *et al.*, 1979). High temperatures of 21-37°C favour the development of this fungus (Winkler *et al.*, 1974), which sometimes is termed a hot weather mould.

The symptoms of black-mould rot are characterized by a watery, odorous decay, associated with masses of black dust-like conidia.
Black-mould rot may spread to adjoining berries only when berries are in contact or when the juice of a decaying berry makes contact with skins of sound berries. Compact grape clusters are susceptible to fungal attack.

Another common fungus found in vineyards is Botrytis cinerea. Whether a Botrytis cinerea infection is beneficial or not depends primarily on the initial conditions of infection. The ideal conditions for grape infection by Botrytis cinerea are high humidity (90-100% R.H.) and warm temperatures (20-25°C). Once the initial infection has taken place, the ideal growth conditions are humid mornings, followed by fine days (Mushet, 1984).

The desirable development of Botrytis cinerea is known as noble rot, and leads to the evaporation of water from the berry, which results in the concentration of the sugars present. Glycerol, which is a product of mould metabolism, may be present in concentrations up to 2.0% in the must obtained from botrytised grapes.

Early trials using Botrytis cinerea spores to infect healthy harvested grapes have been successful in producing sweet wines (Nelson and Nightingale, 1959).

In the Boredeaux region of France and the Rhine region of Germany, botrytised grapes are used to produce quality wines with a distinct botrytised flavour and aroma.

In New Zealand, naturally botrytised vineyard grapes have been used to produce sweet wines (Mushet, 1984).

If the initial conditions of infection are cold and humid, a _Botrytis cinerea_ infection will cause grey mould. Grey mould is an infection characterized by conidiophores which protrude from the wound the covering the berry while the mycelium rapidly spreads through the parenchyma within the berry (Ribereau-Gayon _et al._, 1980). Berry splitting, bird and insect damage will expose the grape to secondary yeast, bacterial and mould infections. Berry splitting occurs after a significant rainfall.

4. INHIBITORY COMPOUNDS PRESENT IN GRAPES

The natural phenolic compounds found in wine include flavonoids, pigments and larger tannins. Most of these phenols originate directly from the grape.

Practically speaking, every phenol derivative possesses some ability to inhibit micro-organisms (Jenkins _et al._, 1957). Phenols seem to inhibit by a surface-adsorption mechanism. However, Bosund (1962) indicated that phenols can act specifically by uncoupling certain oxidative phosphorylation reactions.

The total phenol content of _Vitis vinifera_ wine grapes vary significantly between red and white grape varieties. The total phenol content of red grape varieties is approximately 5600 mg/kg berries, measured as gallic or tannic acid equivalents. The total phenol content of white grape varieties is approximately 3900 mg/kg berries (Singleton and Esau, 1969).

The following table illustrates the percentage distribution of phenol in *Vitis vinifera* wine grapes:

Total phenol distribution as a percentage in *Vitis vinifera* wine grapes

Grape part	Red varieties	White varieties
Skin	33.01%	23.22%
Pressed pulp	0.73%	0.90%
Juice	3.66%	4.52%
Seeds	62.60%	71.35%
TOTAL	100.00%	100.00%
	(5600 mg/kg)	(3900 mg/kg)

Source: (Singleton and Esau, 1969)

In both red and white grape varieties, a high percentage of the phenolics are found in the seeds and skins. Red grape varieties have a higher phenol content in the skin, due to the presence of anthocyanins.

5. THE USE OF HPLC FOR GLYCEROL DETECTION IN THE VINEYARD

Glycerol is a product of sugar metabolism in micro-organisms. The presence of high concentrations of glycerol in grape juice traditionally has been related to the growth of the mould, Botrytis cinerea, in the vineyard. Depending on the climatic conditions, B. cinerea can be a desirable development in the vineyard, or it can cause disease like the moulds Aspergillus, Penicillium and Rhizopus (Amerine et al., 1979; Winkler et al., 1974).

Workers in the past have used high performance liquid chromatography (HPLC) to quantitate glycerol in musts and wines (Rapp and Ziegler, 1979; Goiffan et al., 1980; Flak, 1981). More recently in the Californian wine industry, the search for an alternative to the subjective method of determining the percent rot in grapes by visual inspection has led to the development of an HPLC method which quantitates glycerol, acetic acid and ethanol (Kupina, 1984; Marcley, 1984; Dorschel, 1986). The concentration of glycerol in the juice from grape samples have been related to the degree of mould growth; the concentration of acetic acid to the growth of acetic acid bacteria (Acetobacter and Gluconobacter) and the concentration of ethanol to the growth of yeast (Kupina, 1984).

In this present study, the production of glycerol by four moulds which commonly infect grapes - Aspergillus niger (Black-mould rot), Penicillium italicum (Blue-mould rot), Rhizopus nigricans (Rhizopus rot) and Botrytis cinerea (grey-mould rot) was examined.

The moulds were grown on free-run and homogenized juice obtained from red and white grapes. The juice was prepared in two ways, finger pressed (free-run) and homogenized, to encompass the potential range of micro-environments present in grape clusters. For example, juice produced by homogenizing whole grapes was used to observe the effect that inhibitory substances in the grape skins and seeds, such as phenolic compounds, might have on the glycerol production of these moulds.

The mould cultures were incubated in containers of different size, giving different surface areas, to allow for the different air relations which exist in rots of compact and loose-clustered grape varieties, as well as growth on grape surfaces (semi-aerobic) vs grape interiors (semi-anaerobic).