# GLYCEROL PRODUCTION BY FOUR COMMON GRAPE MOULDS

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

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## 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.

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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).

# MATERIALS AND METHODS

## 1. GRAPES, MEDIA AND MOULD STRAINS

### 1.1 Grapes and juice preparation

#### 1.1.1 Grapes

Sound Black Hamburg grapes were used to produce juice from red grapes, and Chasselas grapes were used for the production of white juice.

#### 1.1.2 Juice Preparation

The red and white grapes were de-stemmed and then washed in distilled water. Each grape variety was then divided into two lots: half the grapes were pressed lightly by hand to provide free-run juice, and the other half was Waring-blended for one minute to provide homogenized juice. The juices were filtered via cheese-cloth, diatomaceous earth, 5 $\mu$ filters, and were finally filter sterilized using 0.45 $\mu$ filters. A problem was encountered during filtration due to polymerization of various components in the free-run juice. This was overcome by repeating diatomaceous earth filtration and immediately filtering with 8 $\mu$ filters (see Appendix I). The four types of juice were analysed by HPLC for initial levels of glucose, fructose and glycerol (see Appendix IV).

## 1.2 Media and Mould Strains

### 1.2.1 Media (PDA)

Potato Dextrose Agar (PDA, OXOID)

| Composition | (g/L) |
|---|---|
| Potato infusion (from 200 g of potatoes) | 4.0 |
| Bacto Dextrose | 20.0 |
| Bacto Agar | 15.0 |

Suspend 39.0 g in 1 L of distilled water and heat to boiling to completely dissolve.

Sterilize in autoclave at 15 psi (121°C) for 15 minutes.

Cool medium to 45-50°C and dispense into sterile petri dishes.

### 1.2.2 Moulds

Four grape moulds were used: Aspergillus niger, Penicillium italicum (M Baxter, Massey University, Palmerston North, New Zealand); Rhizopus nigricans AKA IFA 5781 (I S Maddox, Massey University, Palmerston North, New Zealand); Botrytis cinerea 7518 (J M Young, Plant Diseases Division, Dept of Scientific and Industrial Research (DSIR), Auckland, New Zealand).

# 2. ANALYTICAL TECHNIQUES

## 2.1 High Performance Liquid Chromatography (HPLC)

### 2.1.1 HPLC System

A Shimadzu HPLC LC-4A was used which included:

Refractive index detector, RID-2AS

LC sample injector, SIL-1A

Automatic sample injector, SIL-2AS

Column oven, CTO-2AS

Data processor chromatopac, C-$R_3$A

Analytical column, HPX-87 H (Bio Rad Laboratories)

300 x 7.8 mm column packed with Aminex, a cation exchange resin in hydrogen form

Guard column, Micro-guard cartridges (Bio-Rad Laboratories)

4.6 mm ID x 4 cm long (internal bed 3 cm) packed with Aminex

### 2.1.2 Operating Conditions

The mobile phase was 0.004M $H_2SO_4$, the column temperature was 65°C, the flow rate was 0.8 mL/min, the injection volume was 5 $\mu$L and the RI detector setting was $8 \times 10^{-5}$ RIUFS. The chart speed was 0.5 cm/min.

2.1.3 HPLC Calibration

Quantitation of glucose, fructose and glycerol was obtained by integration of peak area.

A BASIC program which combined a one-point calibration using the low standard and a two-point calibration using the low and the high standards was used. The following standards (either $A_1$, $A_2$; $B_1$, $B_2$ or $C_1$, $C_2$) were used to prepare calibration curves:

| Standard | Glucose | Fructose | Glycerol | Internal* Standard |
|---|---|---|---|---|
| | g/100mL | g/100 mL | g/100 mL | g/100 mL |
| Standards: | | | | |
| Low $A_1$ | 5.00 | 5.00 | 0.02 | - |
| High $A_2$ | 10.00 | 10.00 | 0.20 | - |
| Low $B_1$ | 5.00 | 5.00 | 0.20 | - |
| High $B_2$ | 10.00 | 10.00 | 2.00 | - |
| Low $C_1$ | 0.50 | 0.50 | 0.03 | 0.15 |
| High $C_2$ | 3.00 | 3.00 | 0.95 | 0.30 |

* 5% (w/v) propane-1,2-diol

Standards $A_1$, $A_2$ were used with samples from day 0 to day 10, while standards $B_1$, $B_2$ were used with samples from day 10 to day 27. Standards $C_1$, $C_2$ were used for samples treated with Polyclar or fractionated on Bio-Rex 5 columns.

Before samples were analysed, the appropriate low and high standards were analysed and response factors for glucose, fructose and glycerol were calculated. These response factors were subsequently used in calculating the glucose, fructose and glycerol concentrations in the samples.

### 2.1.4 Sample Clean-up

To avoid "poisoning" of the analytical column by phenolics, the samples from the lot of homogenized Chasselas juice and all juice samples obtained from red grapes were treated to remove phenolics. An 0.5 mL sample of juice was mixed with 0.2 mL of the internal standard, 5%(w/v) propane-1,2-diol and applied to a column containing "Polyclar-AT" or PVPP (polyvinylpolypyrrolidone) (see Appendix II). The sample was eluted with water and collected in a 5mL volumetric flask.

PVPP is a polymeric resin containing polyamide linkages. It reacts primarily with tannins and has a great affinity for catechins (Amerine et al., 1979).

Initial trials with PVPP columns indicated that PVPP did not absorb glucose, fructose or glycerol. Standards containing glucose, fructose and glycerol were passed through the columns and percentage recoveries were 98.2%, 97.9% and 97.5% respectively. All free-fun Chasselas juice required no sample clean-up and was sterile filtered via a 0.45$\mu$ filter and injected into the analytical column.

### 2.1.5 Fractionation of R. nigricans samples

A shoulder was detected on the glycerol peak in the R. nigricans samples. These samples were fractionated into acid and neutral fractions using Bio-Rex 5 resin (Bio Rad Laboratories). An 0.5 mL sample of grape juice was mixed with 0.2 mL of the internal standard, 5%(w/v) propane-1,2-diol, and 0.2 mL of 8.75%(v/v) ammonium hydroxide and applied to an Econocolumn (Bio Rad Laboratories) containing 0.6 mL of Bio-Rex 5 resin. Water was used to elute the neutral compounds from the resin, and the 5 mL fraction was collected using a volumetric flask. No shoulder was detected on the glycerol peak in the resulting neutral fraction; the coeluting compound was demonstrated in acid fraction.

The limit of detection of glycerol was > 0.1% in samples treated with Polyclar-AT and Bio-Rex 5 resin because of a 1:10 dilution of the original sample during treatment.

All standards and samples were filtered via an 0.45 $\mu$ filter prior to injection onto the analytical column.

## 2.2 Enzymatic Glycerol Assay

Selected samples from R. nigricans cultures in free-run white juice under semi-anaerobic conditions were enzymatically assayed to confirm the presence of glycerol, and to ascertain the variation between HPLC and enzymatic methods of glycerol analyses. The enzymatic glycerol assay method used is referenced from (Boehringer Mannheim, 1984).

The principle behind this enzymatic glycerol assay is the conversion of glycerol to glycerol-3-phosphate by the catalysing action of the enzyme glycerokinase (GK), and the phosphorylating action of Adenosine-triphosphate (ATP):

$$\text{GLYCEROL} + \text{ATP} \rightleftharpoons \text{GLYCEROL-3-PHOSPHATE} + \text{ADP}$$

Another enzyme, pyruvate kinase (PK) reconverts ADP to ATP while forming pyruvate:

$$\text{ADP} + \text{PEP} \rightleftharpoons \text{ATP} + \text{PYRUVATE}$$

A further enzyme, lactate dehydrogenase (LDH) will reduce pyruvate to lactate, while oxidizing nicotinamide adenine dinucleotide (NADH to $NAD^+$):

$$\text{PYRUVATE} + \text{NADH} + H^+ \rightleftharpoons \text{LACTATE} + NAD^+$$

The amount of NADH oxidized due to these three reactions is equivalent to the amount of glycerol present in the sample. This decrease was determined by measuring the change in absorbance by 372 Cecil spectrophotometer at wavelength set at 340 nanometers.

### 2.2.1 Reagents Used and Their Preparation

Reagents used:

5N Sodium hydroxide

Triethanolamine hydrochloride

Magnesium sulphate ($MgSO_4.7H_2O$)

Sodium bicarbonate ($NaHCO_3$)

Reduced Nicotinamide adenine dinucleotide ($NADH\text{-}Na_2$)

Adenosine 5′ Triphosphate ($ATP\text{-}Na_2$)

Phosphenol pyruvate (PEP-Na)

Pyruvate kinase/Lactate dehydrogenase (PK/LDH)

Glycerokinase (GK)

Preparation of the reagents (to analyse 50 samples):

(a) Triethanolamine hydrochloride buffer, pH = 7.6:

| | | |
|---|---|---|
| Triethanolamine hydrochloride | 11.2g | Adjust pH to 7.6 with 5 N NaOH and make up to 200mL with distilled $H_2O$. |
| $MgSO_4.7H_2O$ | 0.2g | |
| Distilled $H_2O$ | 150mL | |

(b) Reduced NAD/ATP/PEP solution:

| | | |
|---|---|---|
| $NADH\text{-}Na_2$ | 25 mg | Add to 5 mLs of |
| $ATP\text{-}Na_2$ | 100 mg | distilled water |
| PEP-Na | 50 mg | |
| $NaHCO_3$ | 250 mg | |

(c) Pyruvate kinase/Lactate dehydrogenase solution:

3 mg PK/mL, 1 mg LDH/mL Use suspension undiluted

(d) Glycerokinase solution:

(1 mg GK/mL) Use suspension undiluted

2.2.2 Sample Preparation

Samples containing more than 0.10 g/L glycerol require diluting with distilled deionized water:

| Estimated glycerol (g/L) | Dilution Required |
|---|---|
| < 0.10 g | No dilution required |
| 0.10 - 1.0 g | One-tenth |
| 1.0 - 10.0 g | One-hundredth |
| > 10.0 g | One-thousandth |

### 2.2.3 Assay Procedure

A 372 Cecil spectrophotometer set at a wavelength of 340 nanometer was used. Reaction mixtures were assayed in quartz cuvettes of 1 cm light path.

Glycerokinase may contain traces of glycerol. Therefore a sample blank containing distilled deionized water instead of sample was used to detect any traces of glycerol contained in the glycerokinase. Any optical density difference in the blank is substracted from the optical density difference of the samples.

Per cuvette, add 2.5 mL buffer, 0.1 mL NADH/ATP/PEP solution, 0.2 mL sample and 0.01 mL PK/LDH. The solutions were mixed thoroughly, and left to stand 3-6 mins until reaction had stopped. The optical density was then read = E1.

Then 0.01 mL of GK was added to each cuvette, and mixed thoroughly. The solutions were left a further 10-15 mins until the reaction had finished. The optical density was then read = E2.

### 2.2.4 Calculations

Glycerol concentration g/l = E x 0.209 x F

where E =(E1 - E2) - E (blank)

0.209 = conversion factor at 340 nanometers

F = dilution factor

## EXPERIMENTAL TECHNIQUES

### 1. GLYCEROL PRODUCTION BY FOUR GRAPE MOULDS

These experiments were designed to study the production of glycerol by four grape moulds grown on juices obtained from Chasselas and Black Hamburg grapes over a period of time.

#### 1.1 Mould Strains

Four mould strains were used in the experiment (Materials and Methods, 1.2.2):

| | |
|---|---|
| *Aspergillus niger* | *Penicillium italicum* |
| *Rhizopus nigricans* | *Botrytis cinerea* |

#### 1.2 Preparation of mould inoculum

The moulds were grown on plates of Potato Dextrose Agar (Oxoid, pH 5.6) for a week at 25°C. The mould spores were harvested using moistened sterile cotton swabs and suspended in 0.85%(w/v) NaCl and 0.05%(v/v) Tween 80 solution. The spore concentrations were calculated using a haemocytometer and were adjusted to approximately $10^6$ spores per mL. From these suspensions, the juice lots were inoculated.

1.3 Inoculation and Incubation of the Juice Lots

The four sterile juice lots (Materials and Methods, 1.1.2)

(a) Free-run juice from Chasselas grapes
(b) Homogenized juice from Chasselas grapes
(c) Free-run juice from Black Hamburg grapes
(b) Homogenized juice from Black Hamburg grapes

were inoculated with 0.1-0.2 mL of spore suspension which contained $1x10^6$ spores per mL. The final spore concentration in the grape juice was 1000 spores per mL.

For "semi-aerobic" growth, glass Petri dishes (1.5 x 9.0 cm) filled with 40 mL of Chasselas juice and glass Petri dishes (2 x 14 cm) filled with 100 mL of Black Hamburg juice were used.

For "semi-anaerobic" growth, screw-cap bottles (50 mL capacity, 4 cm diameter) filled with 30 mL of Chasselas juice and screw-cap bottles (100 mL capacity, 5 cm diameter) filled with 85 mL Black Hamburg juice were used.

The experiments were all done in triplicate. The moulds were incubated at 25°C for 26-29 days.

## 1.4 Sampling Procedure

During the 26-29 day incubation period, samples were aseptically taken every four days. Samples of 1 mL were taken aseptically using sterile pasteur pipettes for storage in sterile 5 mL glass vials at (-20°C) prior to analysis. Samples were then prepared and analysed for fructose, glucose and glycerol by HPLC as described in Materials and Method, 2.1.

## 1.5 Harvesting of Fungal Biomass

After 26-29 days, the fungi were harvested by filtration, so that dry weights could be obtained. Petri dishes containing the fungal biomass were dried in an oven at 60°C to constant weight.

## EXPERIMENTAL RESULTS

### 1. GLYCEROL PRODUCTION AND SUGAR UTILIZATION BY FOUR COMMON GRAPE MOULDS

Glycerol production and sugar utilization by four moulds were examined. Black Hamburg and Chasselas grapes were used to produce four juice types:

(a) Free-run Black Hamburg juice

(b) Homogenized Black Hamburg juice

(c) Free-run Chasselas juice

(d) Homogenized Chasselas juice

Four mould strains were separately grown on each juice type as a pure culture. Glycerol production and sugar utilization over the 26-29 day incubation period is illustrated in:

Figures 1.1 - 1.8, juice inoculated with A. niger

Figures 2.1 - 2.8, juice inoculated with P. italicum

Figures 3.1 - 3.8, juice inoculated with R. nigricans

Figures 4.1 - 4.8, juice inoculated with B. cinerea

## Graphical presentation of experimental results

Glycerol and sugar concentrations of the triplicates have been averaged and plotted against time (days). For initial glycerol, glucose and fructose concentrations including initial pH's of juices used, refer to Appendix IV. Each page contains four graphs comparing glycerol production and sugar utilization of a particular mould grown in Black Hamburg or Chasselas juice over a 26-29 day incubation period. The graphical presentation of results is achieved by using one standard sugar scale while incorporating one of five different glycerol scales. The text relevant to the graphs precedes the graphs.

Figures 1.1-1.8, juice inoculated with A. niger

Figures 2.1-2.8, juice inoculated with P. italicum

Figures 3.1-3.8, juice inoculated with R. nigricans

Figures 4.1-4.8, juice inoculated with B. cinerea

1.1 Glycerol production and sugar utilization by A. niger grown in free-run and homogenized Chasselas juice under semi-aerobic and semi-anaerobic conditions.

Glycerol was detected in four cultures during the first four days of incubation (Figs.1.1-1.4). The highest glycerol concentration 0.45%(w/v) was produced by A. niger in homogenized juice under semi-aerobic conditions (Fig.1.2).
Under semi-anaerobic conditions A. niger metabolized some of the glycerol it had produced in earlier stages of incubation (Figs.1.3 and 1.4).
A. niger cultures under semi-anaerobic conditions utilized both glucose and fructose throughout the 27 day incubation period.
Under semi-aerobic conditions there was rapid utilization of fructose and glucose during the first ten days, after which time utilization practically ceased.

1.2 Glycerol production and sugar utilization by A. niger grown in free-run and homogenized Black Hamburg juice under semi-aerobic and semi-anaerobic conditions.

Glycerol was not detected in all four cultures of A. niger (Figs.1.5 - 1.8). The pattern of sugar utilization by A. niger was similar under all cultivation conditions. Rapid utilization of glucose and fructose took place during the first three days of incubation, with little sugar utilization occurring after day five (Figs.1.5-1.8). The proportion of available sugar utilized was greater in Black Hamburg juice than Chasselas juice. In Black Hamburg juice A. niger utilized

approximately 60% of the available sugar under all conditions of juice preparation and incubation (Table 6). Sugar utilization in Chasselas juice varied depending on the method of juice preparation and incubation. Sugar utilization in Chasselas juice was 31% (free-run, semi-aerobic) ,51% (homogenized, semi-aerobic),45% (free-run, semi-anaerobic) and 58% (homogenized, semi-anaerobic) (Table 6).

Figures 1.1 - 1.4:

Glycerol production and utilization of glucose and fructose

A. niger in:

Figure 1.1: semi-aerobic free-run Chasselas juice

Figure 1.2: semi-aerobic homogenized Chasselas juice

Figure 1.3: semi-anaerobic free-run Chasselas juice

Figure 1.4: semi-anaerobic homogenized Chasselas juice

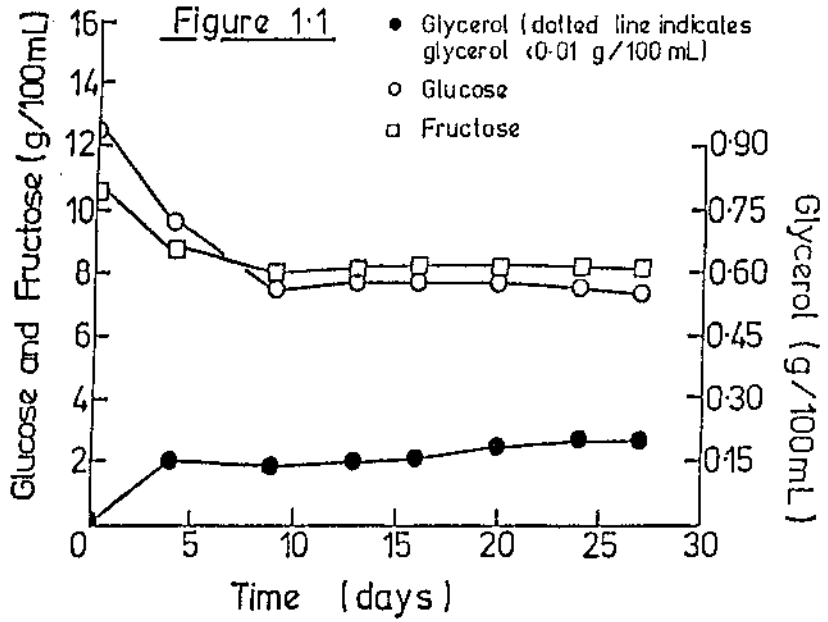


Figure 1·1
Glycerol (dotted line indicates glycerol <0·01 g/100 mL)
Glucose
Fructose
Glucose and Fructose (g/100mL)
Glycerol (g/100mL)
Time (days)

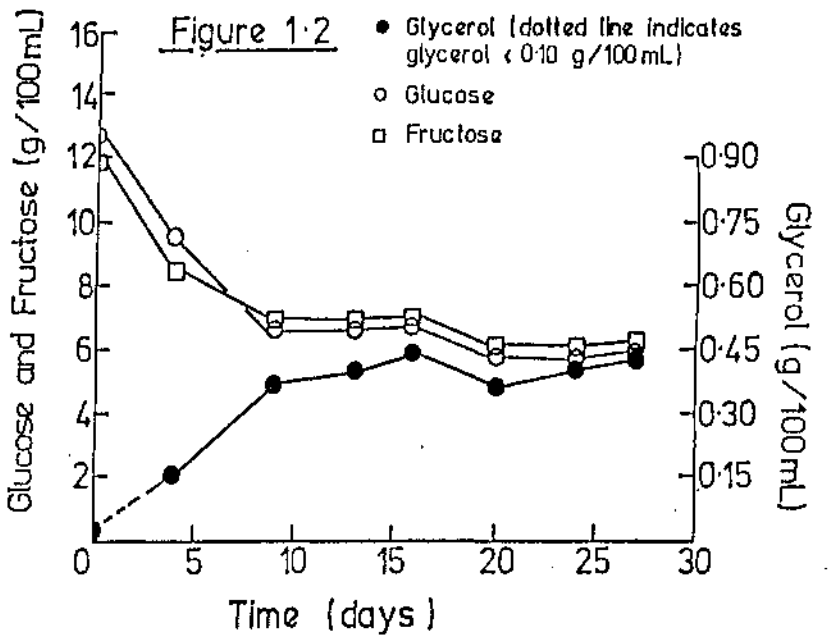


Figure 1·2
Glycerol (dotted line indicates glycerol < 0·10 g/100mL)
Glucose
Fructose
Glucose and Fructose (g/100mL)
Glycerol (g/100mL)
Time (days)

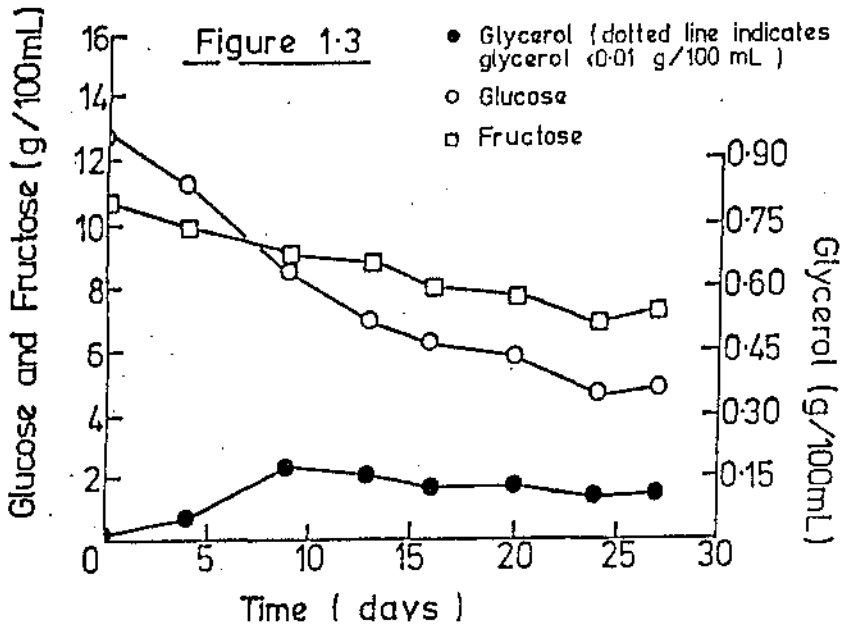


Figure 1·3
Glycerol (dotted line indicates glycerol <0·01 g/100 mL)
Glucose
Fructose
Glucose and Fructose (g/100mL)
Glycerol (g/100mL)
Time (days)

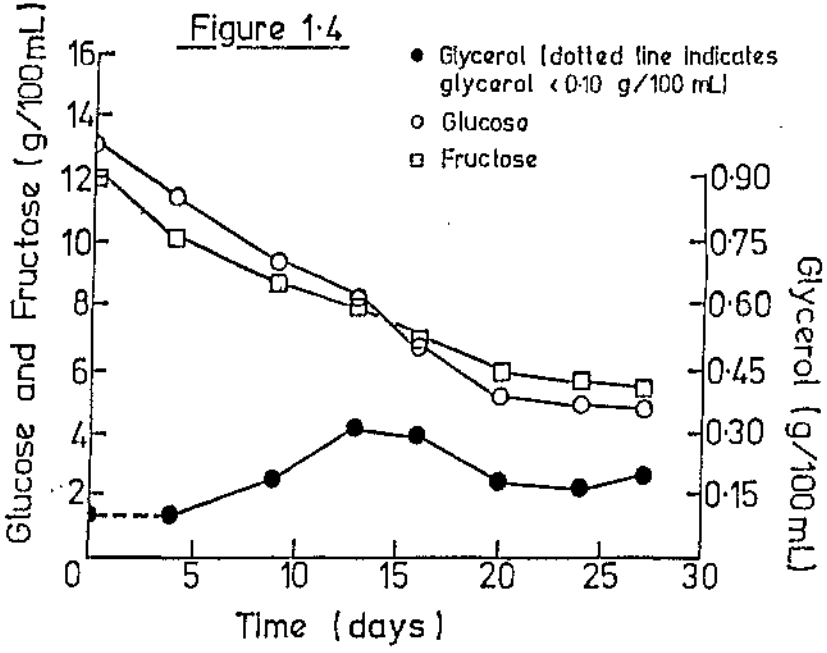


Figure 1·4
Glycerol (dotted line indicates glycerol < 0·10 g/100 mL)
Glucose
Fructose
Glucose and Fructose (g/100mL)
Glycerol (g/100mL)
Time (days)

Figures 1.5 - 1.8:

Glycerol production and utilization of glucose and fructose by *A. niger* in:

Figure 1.5: semi-aerobic free-run Black Hamburg juice

Figure 1.6: semi-aerobic homogenized Black Hamburg juice

Figure 1.7: semi-anaerobic free-run Black Hamburg juice

Figure 1.8: semi-anaerobic homogenized Black Hamburg juice

Figure 1·5

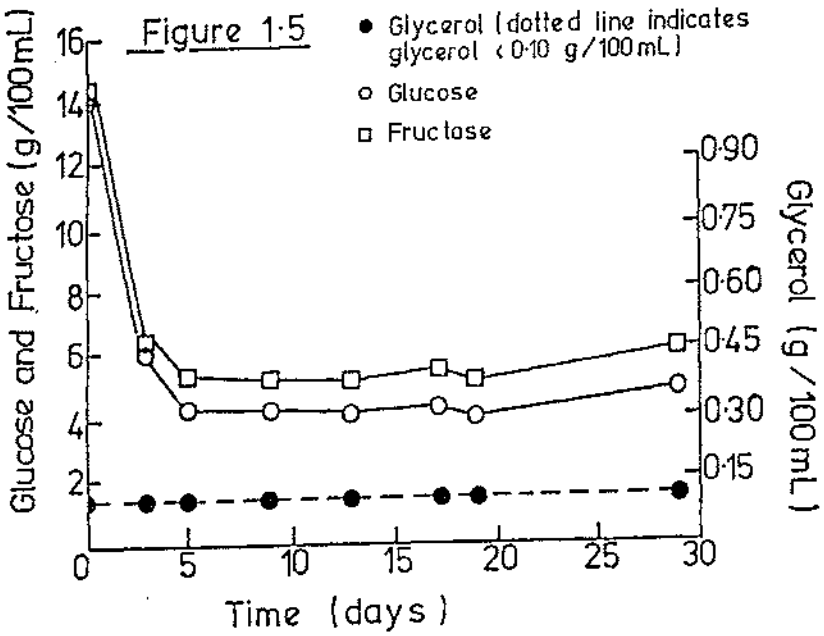



Figure 1·6

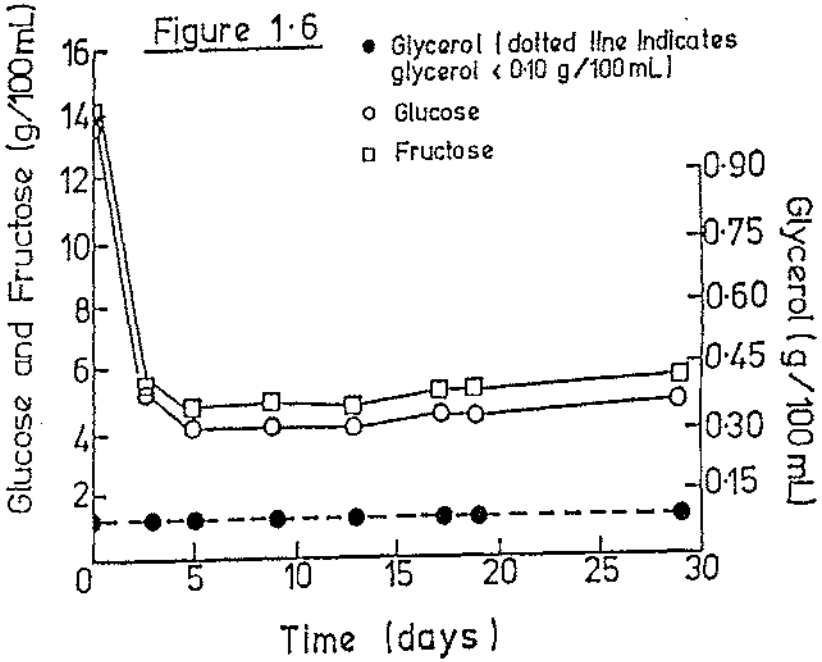



Figure 1·7

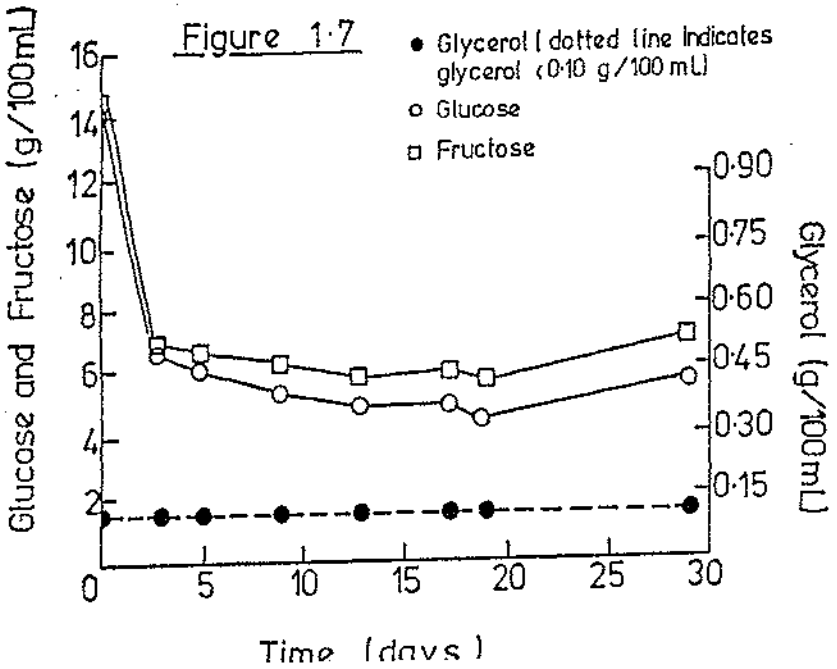
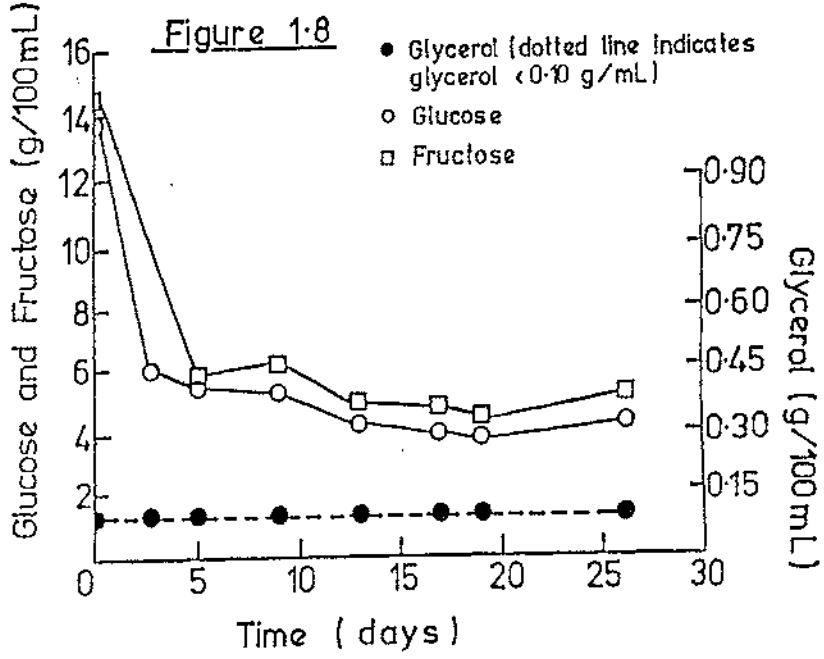



Figure 1·8



1.3 Glycerol production and sugar utilization by P. italicum grown in free-run and homogenized Chasselas juice under semi-aerobic and semi-anaerobic conditions.

Glycerol was detected in all four cultures after four to eight days incubation (Figs. 2.1-2.4). A maximum concentration of 0.48% w/v was produced by P. italicum grown in homogenized juice under semi-aerobic conditions (Fig.2.2).

P. italicum metabolized much of the glycerol that it produced (Figs. 2.1-2.4). In free-run juice cultures, the glycerol level dropped below the limit of detection (0.01g/100mL) after approximately 20 days (Fig. 2.1 and 2.3).

Under all culture conditions, glucose and fructose were utilized throughout the incubation period, with more glucose than fructose been utilized in all cultures (ie) glucophilic.

1.4 Glycerol production and sugar utilization by P. italicum grown in free-run and homogenized Black Hamburg juice under semi-aerobic and semi-anaerobic conditions.

Glycerol was not detected in any of the four cultures of P. italicum throughout the whole 26-29 day incubation period (Figs.2.5 - 2.8).

Sugar utilization by P. italicum was similar under all conditions of cultivation. Rapid utilization of glucose and fructose took place during the first three days of incubation, with very little sugar utilized after the fifth day of incubation (Figs.2.5 - 2.8). The proportion of available sugar utilized was greater in Black Hamburg juice than in Chasselas juice. In Black Hamburg juice approximately

58% of the total sugar was utilized by P. italicum, whereas only approximately 46% of the total sugar was utilized in Chasselas juice under all conditions of juice preparation and incubation (Table 6).

Figures 2.1 - 2.4:

Glycerol production and utilization of glucose and fructose by *P. italicum* in:

Figure 2.1: semi-aerobic free-run Chasselas juice

Figure 2.2: semi-aerobic homogenized Chasselas juice

Figure 2.3: semi-anaerobic free-run Chasselas juice

Figure 2.4: semi-anaerobic homogenized Chasselas juice

Figure 2·1

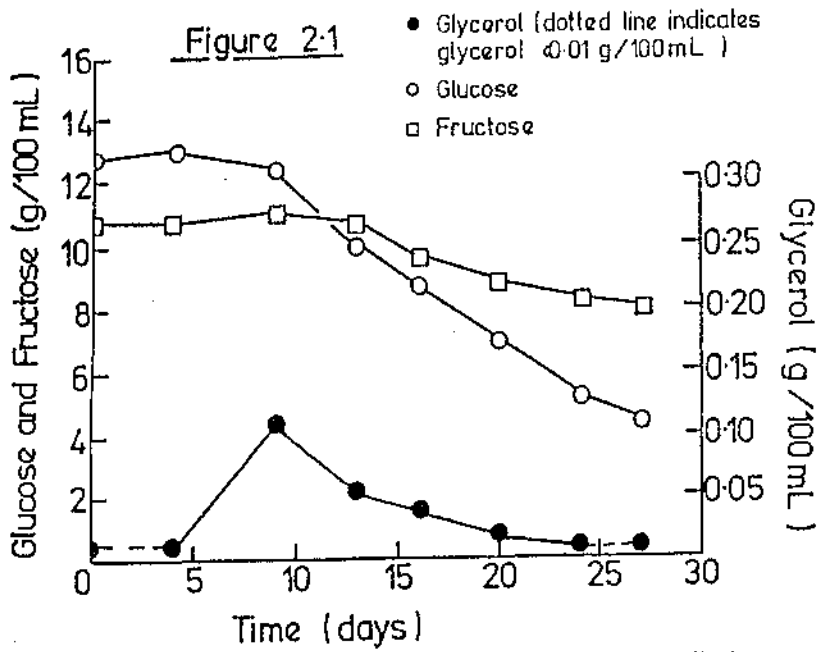



Figure 2·2

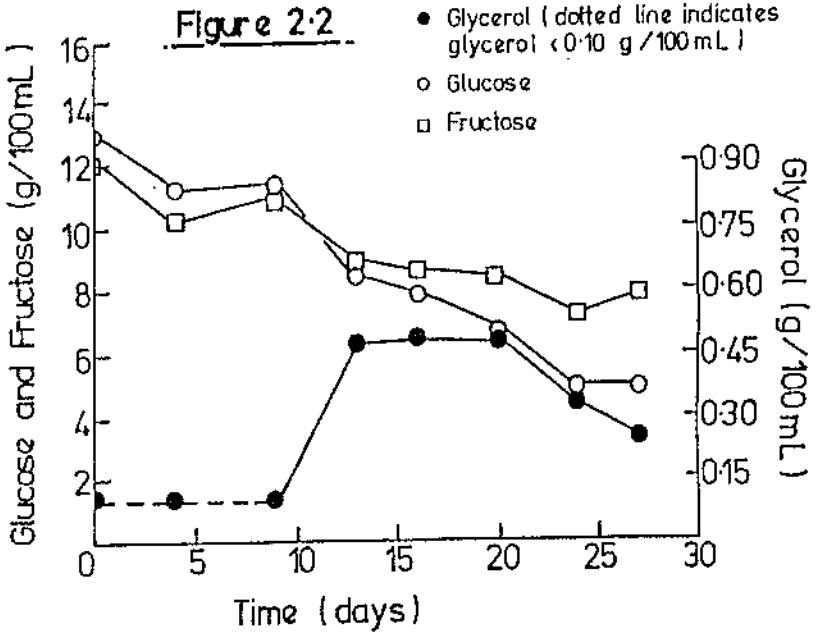



Figure 2·3

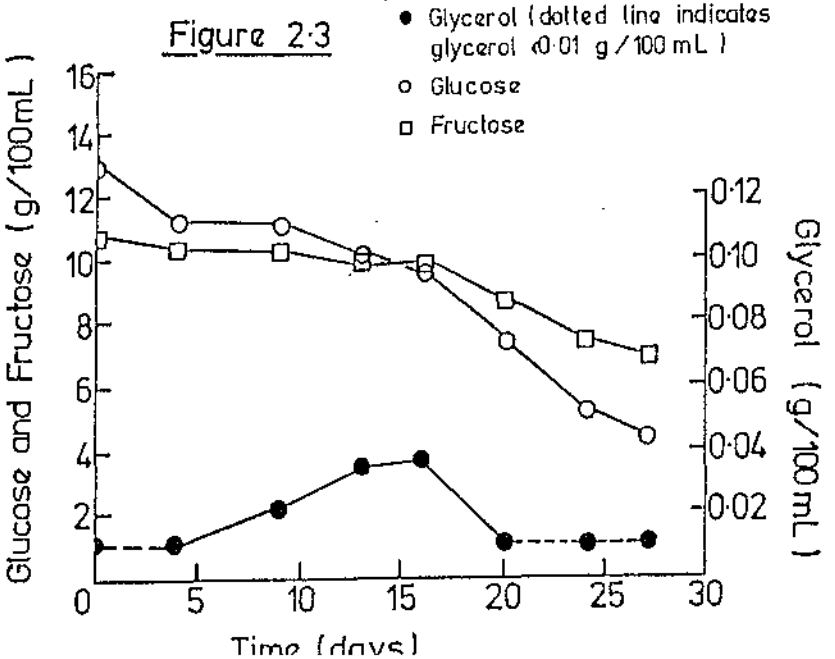
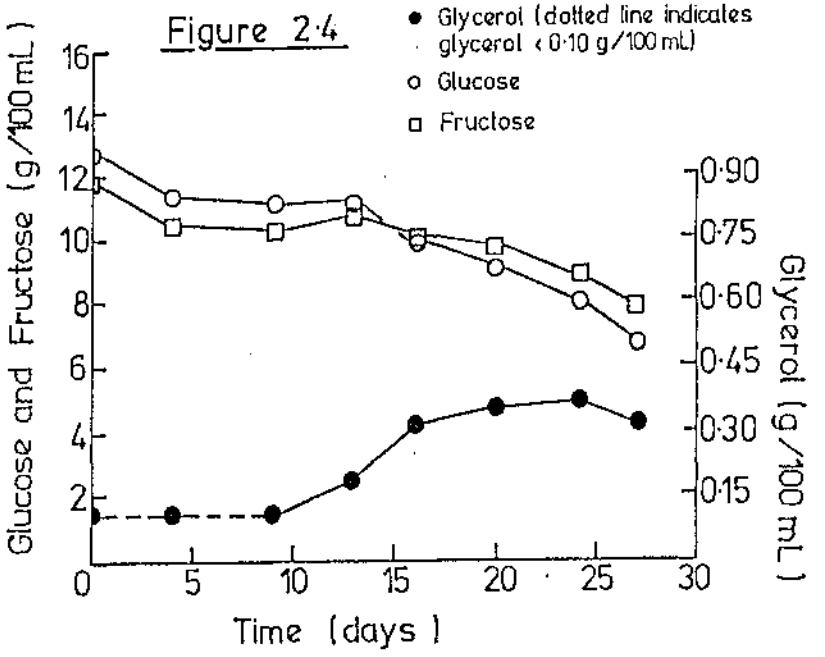



Figure 2·4



Figures 2.5 - 2.8:

Glycerol production and utilization of glucose and fructose by *P. italicum* in:

Figure 2.5: semi-aerobic free-run Black Hamburg juice

Figure 2.6: semi-aerobic homogenized Black Hamburg juice

Figure 2.7: semi-anaerobic free-run Black Hamburg juice

Figure 2.8: semi-anaerobic homogenized Black Hamburg juice

Figure 2·5

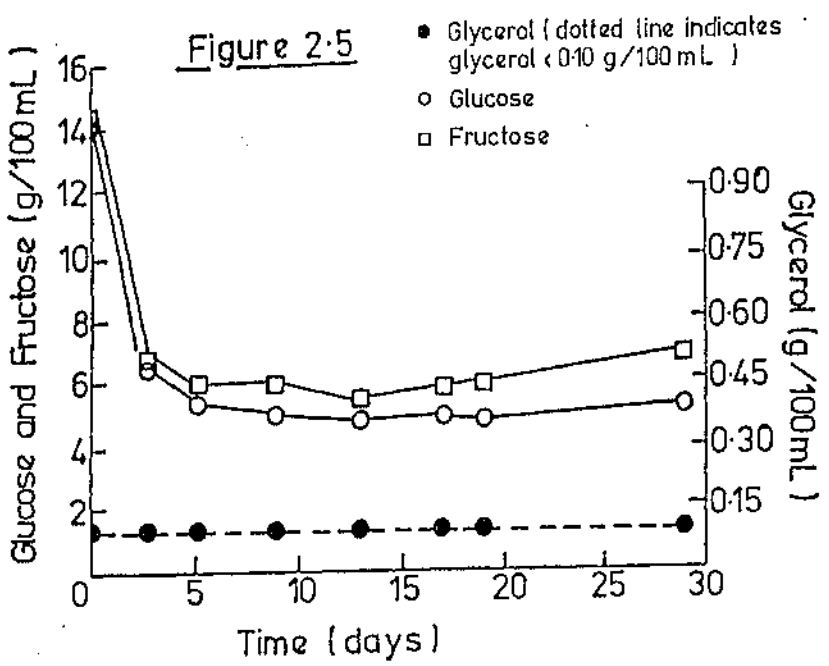



Figure 2·6

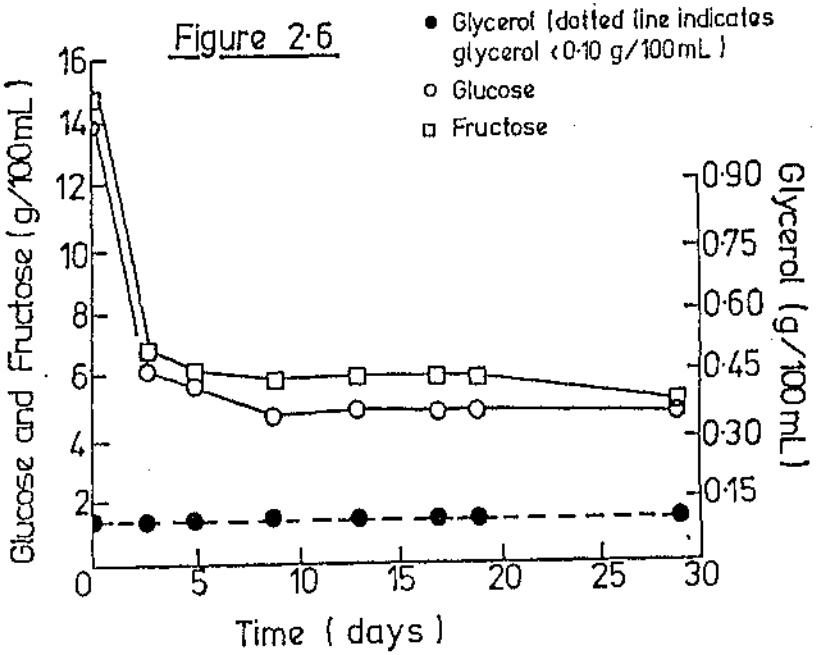



Figure 2·7

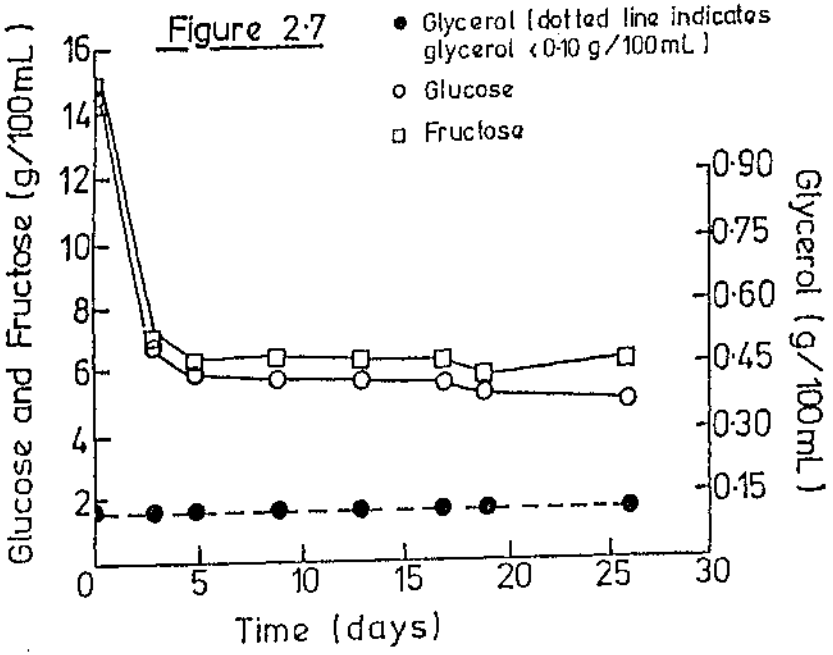
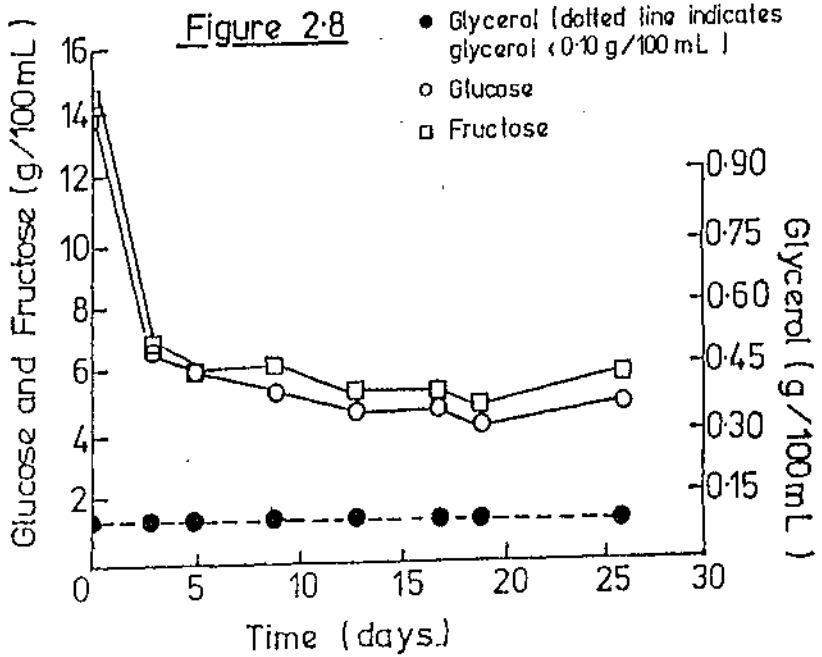



Figure 2·8



1.5 Glycerol production and sugar utilization by R. nigricans grown in free-run and homogenized Chasselas juice under semi-aerobic and semi- anaerobic conditions.

Glycerol was detected in all four cultures (Figs.3.1-3.4), with the maximum concentration of 2.09% w/v produced in free-run juice under semi-aerobic conditions (Fig.3.1). R. nigricans under semi-aerobic conditions produced more glycerol with a shorter lag phase than R. nigricans cultures under semi-anaerobic conditions.

R. nigricans cultures under semi-anaerobic conditions utilized both glucose and fructose throughout the 27 day incubation period (Fig.3.3 and 3.4). In semi-aerobic conditions there was a decline in the rate of glucose and fructose utilization in the latter stages of incubation (Figs.3.1 and 3.2).

A glucophilic response was evident in all four R. nigricans cultures, i.e. preferential utilization of glucose over fructose (Figs.3.1 and 3.2).

Insufficient liquid remained for sampling in two of the three semi-aerobic free-run cultures of R. nigricans by day 20. Thus, the values for glycerol, glucose and fructose for days 20 and 27 are based on the analysis of one culture.

1.6 Glycerol production and sugar utilization by R. nigricans grown in free-run and homogenized Black Hamburg juice under semi-aerobic and semi-anaerobic conditions.

Glycerol was detected after five days in cultures of R. nigricans grown under semi-aerobic conditions (Figs.3.5 and 3.6), but was not detected in free-run and homogenized juice under semi-anaerobic conditions until days 13 and 17 respectively (Figs.3.7 and 3.8).
In Black Hamburg juice R. nigricans produced similar amounts of glycerol except in free-run juice under semi-aerobic conditions (fig 3.5) where R. nigricans produced 2 - 4 times more glycerol than the other three Rhizopus cultures. A maximum glycerol accumulation of 0.83% w/v was observed in free-run juice under semi-aerobic conditions by day 19 (fig 3.5)

Rhizopus nigricans produced significantly more glycerol in Chasselas juice than in Black Hamburg juice. The average glycerol concentration produced by Rhizopus nigricans cultures in Black Hamburg juice was 0.42% w/v, while in Chasselas juice the average glycerol concentration was 1.64% w/v.
In both Black Hamburg and Chasselas juice the maximum glycerol produced by R. nigricans cultures was observed in free-run juice under semi-aerobic conditions. In Chasselas and Black Hamburg juice R. nigricans produced a maximum glycerol concentration of 2.10% w/v and 0.83% w/v respectively.

In Black Hamburg juice all four R. nigricans cultures displayed a rapid utilization of sugars during the first three days of incubation, after which sugar utilization practically ceased except in free-run juice under semi-aerobic conditions (Fig.3.5) in which the sugar utilization continued throughout the 29 day incubation period.

As illustrated in figures 3.1-3.8 and Table 6, R. nigricans utilized similar amounts of glucose in both Chasselas and Black Hamburg juices. An average of 73% of the glucose was utilized by the four R. nigricans cultures in Chasselas juice while an average of 80% was utilized in Black Hamburg juice. The maximum individual glucose utilization of 99.5% was observed in R. nigricans cultures grown in free-run juice under semi-aerobic condition.

Glucose utilization by R. nigricans was similar in Chasselas and Black Hamburg juices but fructose utilization was markedly different between the two juice types (Table 6).

In Chasselas juice the % fructose utilized by the four R. nigricans cultures were as follows:

| | |
|---|---|
| free-run, semi-aerobic | 1.58% |
| free-run, semi-anaerobic | 26.7% |
| homogenized, semi-aerobic | 22.5% |
| homogenized, semi-anaerobic | 34.8% |

As illustrated above R. nigricans utilized similar amounts of fructose except in free-run juice under semi-aerobic conditions where only 1.6% of the fructose was utilized. The average % fructose utilized by the four R. nigricans cultures in Chasselas juice was approximately 20%, the figure of 1.6% has a marked effect on the average.

The average % fructose utilized by the four R. nigricans cultures in Black Hamburg juice was 67% with an individual maximum fructose utilization of 86% observed in free-run juice under semi-aerobic conditions (Table 6). Rhizopus nigricans utilized 2 - 3 times more fructose in Black Hamburg juice than in Chasselas juice.

Rhizopus nigricans utilized more total sugar in Black Hamburg juice than in Chasselas juice. In Chasselas juice Rhizopus nigricans utilized similar amounts of total sugar available under different conditions, with an average of 49% of the total sugar being utilized by the end of the 26-29 day incubation period. In Black Hamburg juice R. nigricans utilized similar amounts of the total sugar available except in free-run juice under semi-aerobic conditions where 92.5% of the total sugar available was utilized. The average total sugar utilized by the four R. nigricans cultures in Black Hamburg juice was 73%.

Overall similar amounts of glucose were utilized in both Black Hamburg and Chasselas juice, but in Chasselas juice there was limited utilization of the available fructose, thus resulting in a lower total sugar utilization. The glucophilic response, so evident in Chasselas juice, was much less marked in Black Hamburg juice.

Figures 3.1 - 3.4:

Glycerol production and utilization of glucose and fructose by *R. nigricans* in:

Figure 3.1: semi-aerobic free-run Chasselas juice

Figure 3.2: semi-aerobic homogenized Chasselas juice

Figure 3.3: semi-anaerobic free-run Chasselas juice

Figure 3.4: semi-anaerobic homogenized Chasselas juice

Figure 3·1

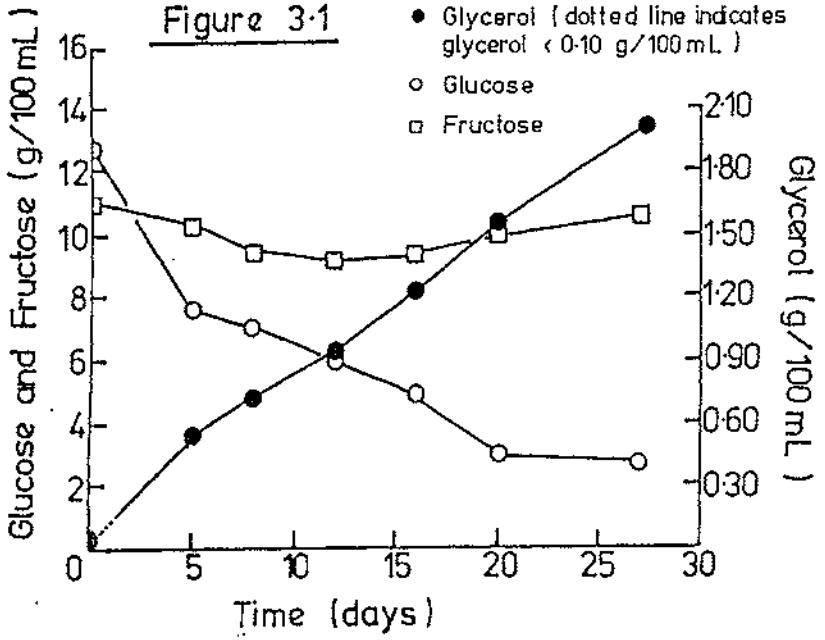



Figure 3·2

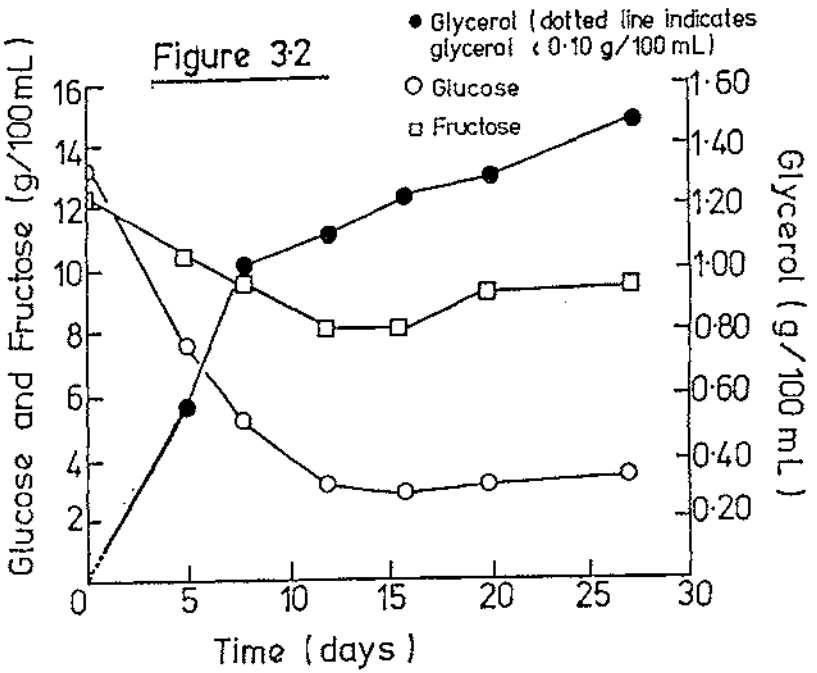



Figure 3·3

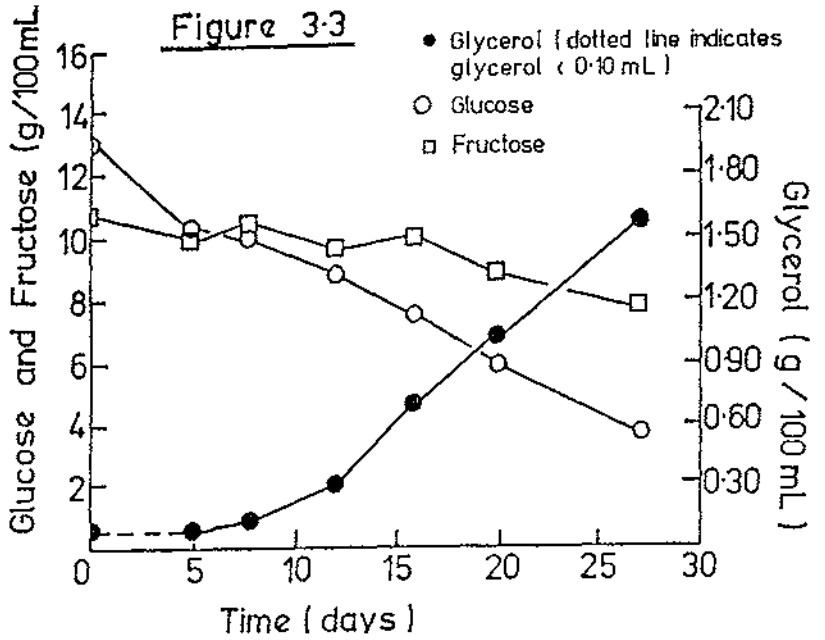
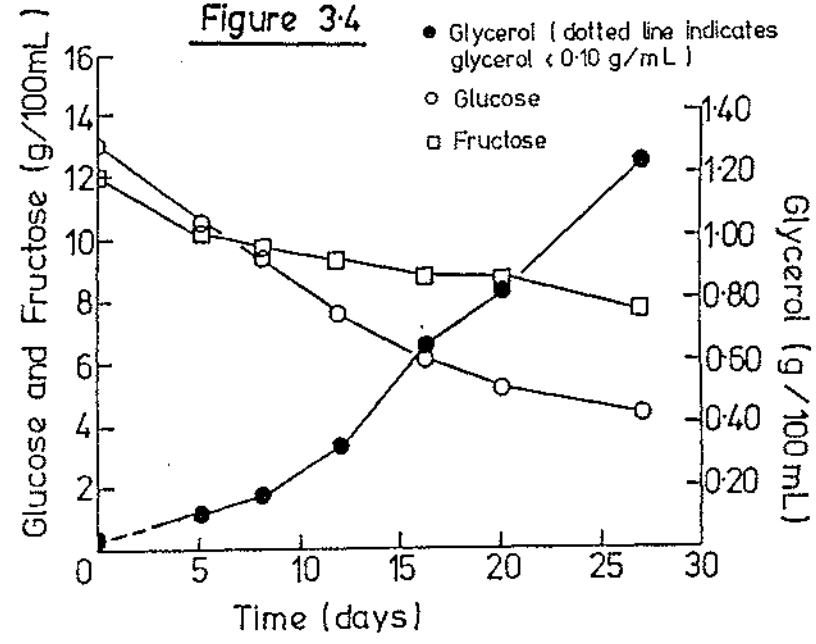



Figure 3·4



Figures 3.5 - 3.8:

Glycerol production and utilization of glucose and fructose by *R. nigricans* in:

Figure 3.5: semi-aerobic free-run Black Hamburg juice

Figure 3.6: semi-aerobic homogenized Black Hamburg juice

Figure 3.7: semi-anaerobic free-run Black Hamburg juice

Figure 3.8: semi-anaerobic homogenized Black Hamburg juice

Figure 3·5

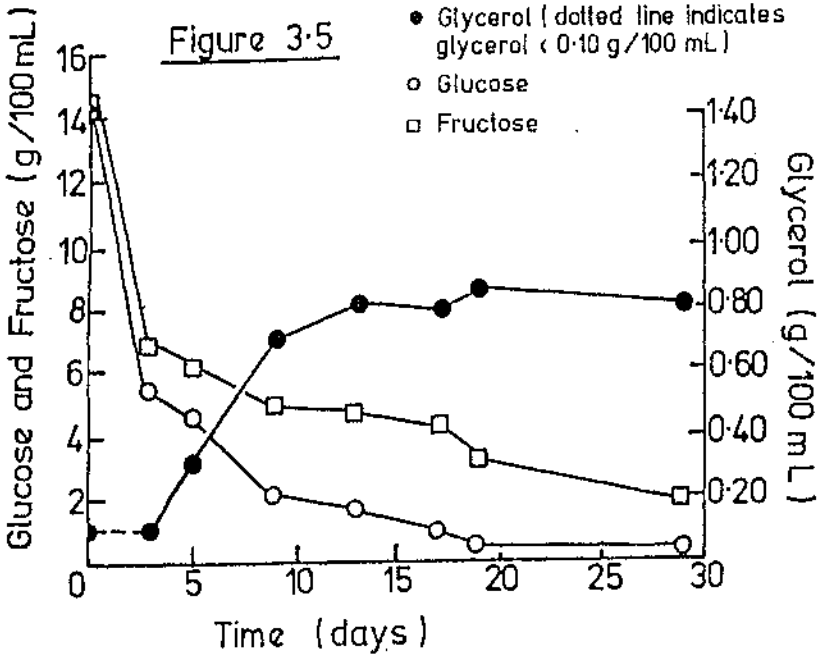



Figure 3·6

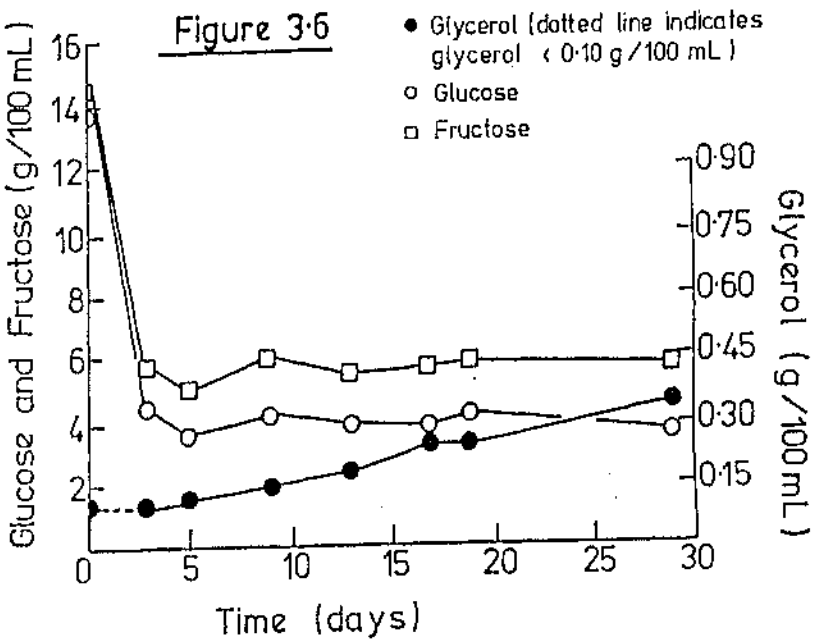



Figure 3·7

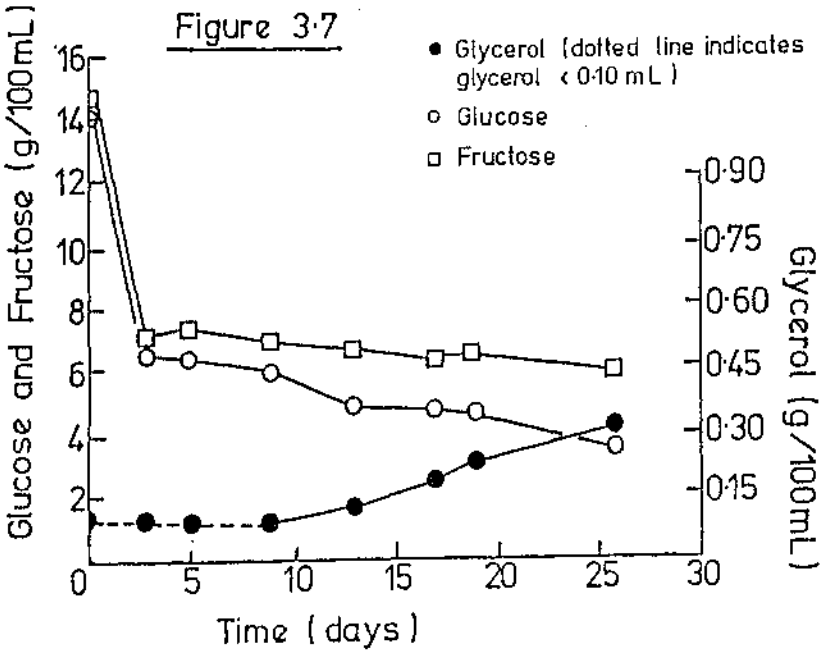
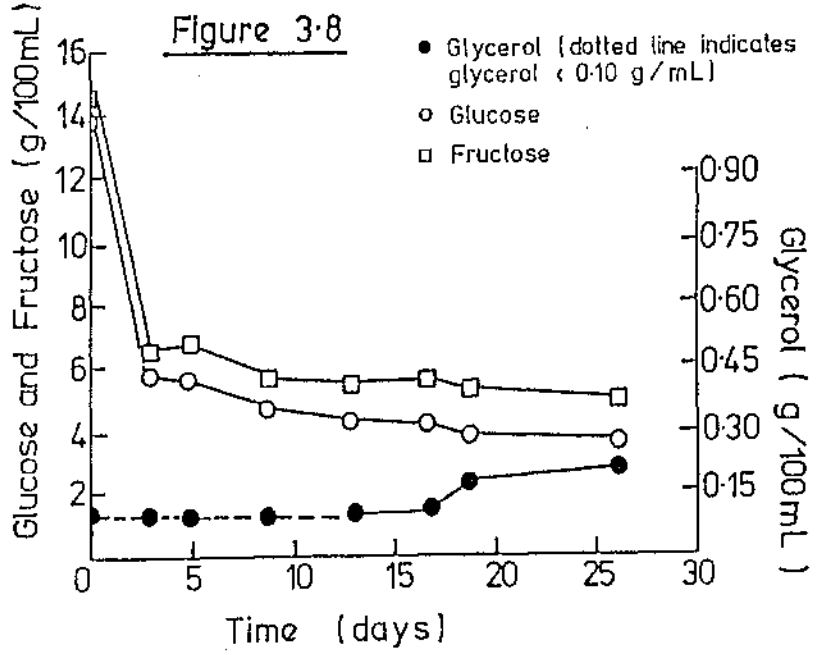



Figure 3·8



1.7 Glycerol production and sugar utilization by B. cinerea grown in free- run and homogenized Chasselas juice under semi-aerobic and semi-anaerobic conditions.

All B. cinerea cultures produced 1.0 - 1.2% w/v of glycerol under all cultivation conditions (Figs.4.1 - 4.4).
Sugar utilization by B. cinerea cultures underwent an initial lag phase of four to five days, during which there was very little sugar utilization. However, this was followed by rapid utilization of glucose and fructose. A glucophilic response was evident in all four cultures (Figs.4.1 - 4.4).

1.8 Glycerol production and sugar utilization by B. cinerea grown in free-run and homogenized Black Hamburg juice under semi-aerobic and semi- anaerobic conditions.

B. cinerea produced glycerol under all cultivation conditions (Figs.4.5 - 4.8). B. cinerea cultures showed an increase in glycerol concentration with time, except in the homogenized juice under semi-aerobic conditions (Fig.4.6).Glycerol production by B. cinerea in Black Hamburg juice varied depending on the method of juice preparation and incubation (Figs.4.5-4.8).
Glycerol production in Black Hamburg juice was approximately

0.6% w/v free-run, semi-aerobic

0.2% w/v homogenized, semi-aerobic

0.4% w/v free-run, semi-anaerobic

0.3% w/v homogenized, semi-anaerobic

B. cinerea produced more glycerol in Chasselas juice than in Black Hamburg juice. In Chasselas juice B. cinerea produced similar amounts of glycerol with an average of 1.1% being produced by the end of the incubation period.

In Black Hamburg juice rapid utilization of sugar took place during the first three days of incubation, with reduced utilization after day five.

Fungal growth was so dense in B. cinerea cultures in free-run juice under semi-aerobic conditions that no liquid remained for sampling after 17 days (Fig.4.5).

In Chasselas juice, glucose and fructose utilization by B. cinerea underwent an initial lag phase followed by rapid utilization over the remaining incubation period, the pattern of sugar utilization in Black Hamburg was different in that sugar utilization took place during the first three days after which there was little or no further utilization.

In Chasselas and Black Hamburg juices B. cinerea utilized similar amounts of glucose with an average of 84% and 82% respectively (Table 6).

Fructose utilization in Black Hamburg juice varied depending on the method of juice preparation and incubation. Fructose utilization in Black Hamburg juice was

62% free-run, semi aerobic

70% homogenized, semi-aerobic

50% free-run, semi-anaerobic

60% homogenized, semi-anaerobic

Fructose utilization in Chasselas juice was also varied depending on the method of juice preparation and incubation. Fructose utilization in Chasselas juice was

52% free-run, semi-aerobic

41% homogenized, semi-aerobic

35% free-run, semi-anaerobic

42% homogenized, semi-anaerobic

While glucose utilization in Chasselas and Black Hamburg juice were similar, fructose utilization in Chasselas juice was less than in Black Hamburg juice. A glucophilic response was evident in all four B. cinerea cultures grown in Chasselas juice (figures 4.1 - 4.4).

In Chasselas and Black Hamburg juices, Botrytis cinerea utilized similar amounts of total sugar available. In Chasselas juice an average of 64% of the total sugar was utilized and in Black Hamburg juice an average of 71% of the total sugar available was utilized under all conditions of juice preparation and incubation (table 6).

Figures 4.1 - 4.4:

Glycerol production and utilization of glucose and fructose by *B. cinerea* in:

Figure 4.1: semi-aerobic free-run Chasselas juice

Figure 4.2: semi-aerobic homogenized Chasselas juice

Figure 4.3: semi-aerobic free-run Chasselas juice

Figure 4.4: semi-aerobic homogenized Chasselas juice

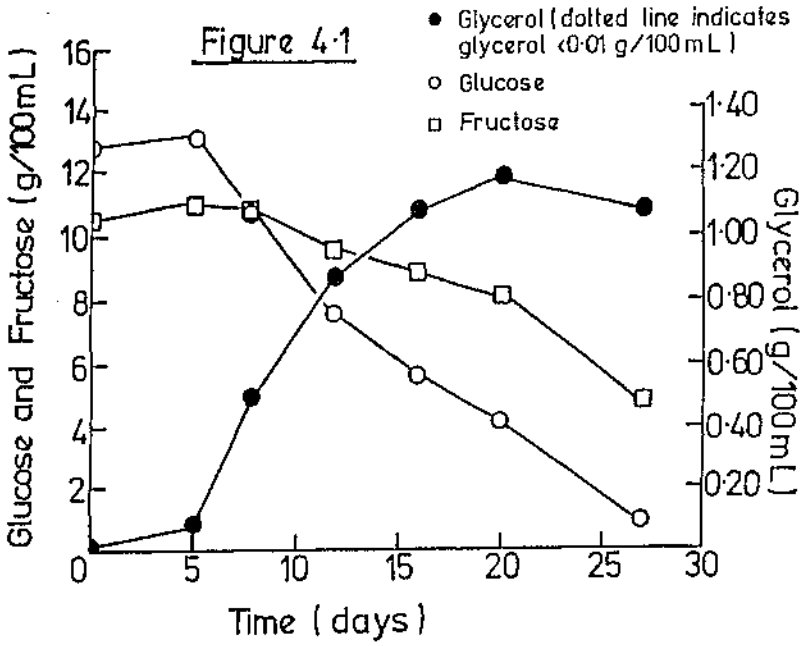


Figure 4·1
Glycerol (dotted line indicates glycerol <0·01 g/100 mL)
Glucose
Fructose
Glucose and Fructose (g/100mL)
Glycerol (g/100mL)
Time (days)

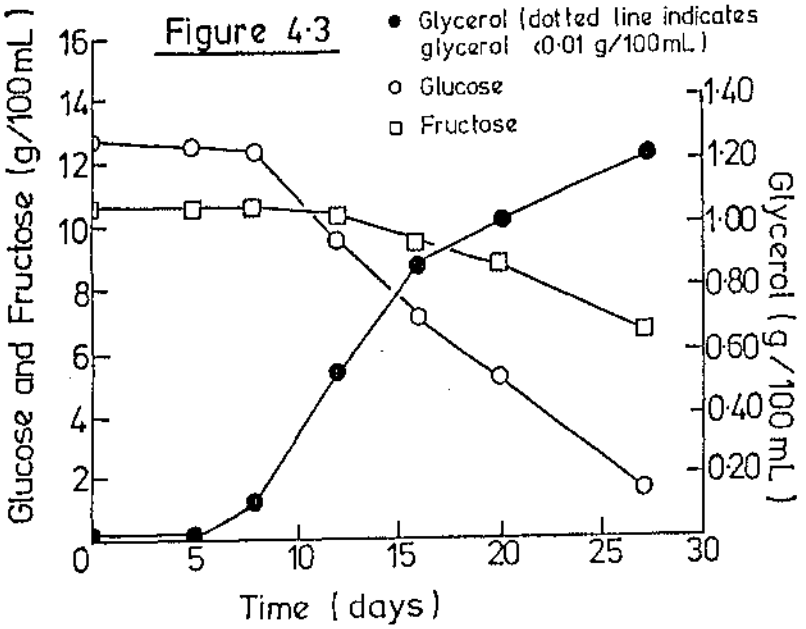


Figure 4·3
Glycerol (dotted line indicates glycerol <0·01 g/100mL)
Glucose
Fructose
Glucose and Fructose (g/100mL)
Glycerol (g/100mL)
Time (days)

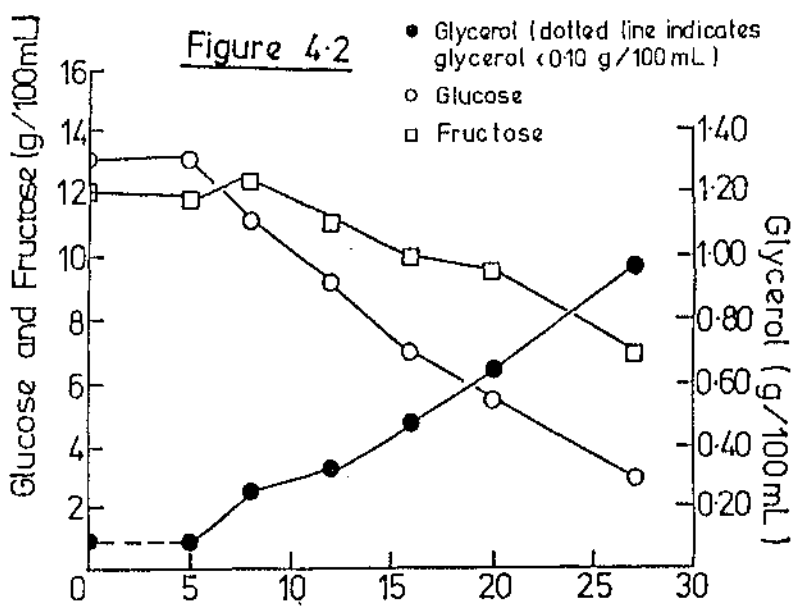


Figure 4·2
Glycerol (dotted line indicates glycerol <0·10 g/100mL)
Glucose
Fructose
Glucose and Fructose (g/100mL)
Glycerol (g/100mL)

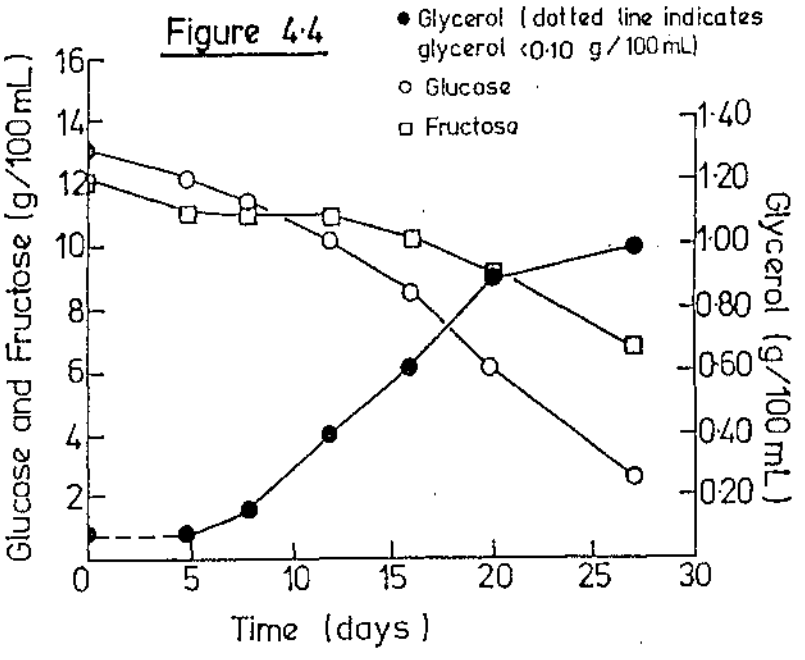


Figure 4·4
Glycerol (dotted line indicates glycerol <0·10 g/100mL)
Glucose
Fructose
Glucose and Fructose (g/100mL)
Glycerol (g/100mL)
Time (days)

Figures 4.5 - 4.8:

Glycerol production and utilization of glucose and fructose by *B. cinerea* in:

Figure 4.5: semi-aerobic free-run Black Hamburg juice

Figure 4.6: semi-aerobic homogenized Black Hamburg juice

Figure 4.7: semi-anaerobic free-run Black Hamburg juice

Figure 4.8: semi-anaerobic homogenized Black Hamburg juice

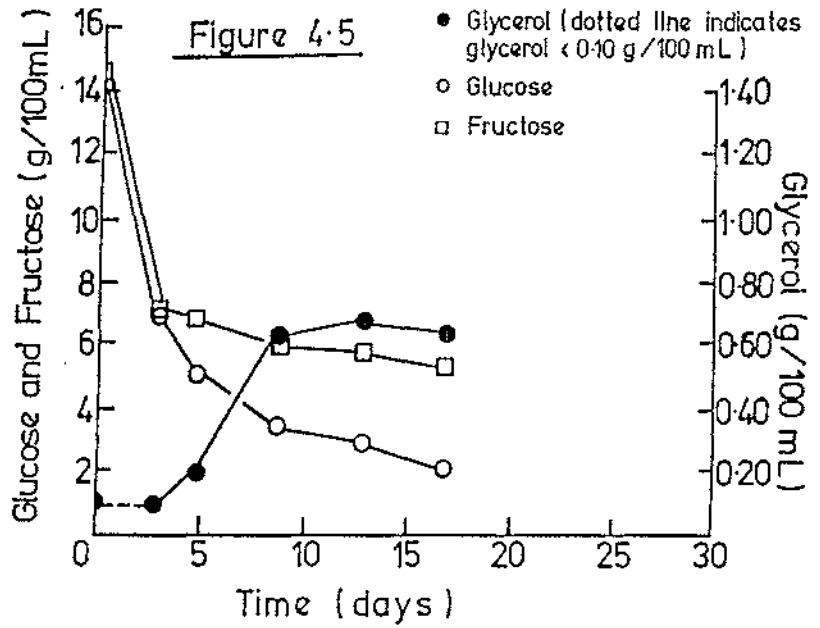


Figure 4·5
Glycerol (dotted line indicates glycerol < 0·10 g/100 mL)
Glucose
Fructose
Glucose and Fructose (g/100mL)
Glycerol (g/100 mL)
Time (days)

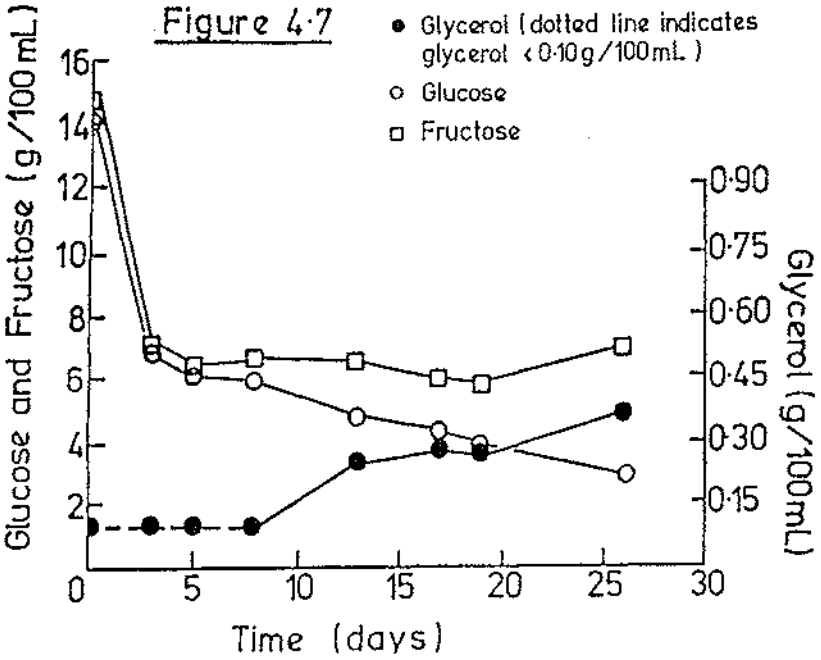


Figure 4·7
Glycerol (dotted line indicates glycerol < 0·10 g/100 mL)
Glucose
Fructose
Glucose and Fructose (g/100 mL)
Glycerol (g/100mL)
Time (days)

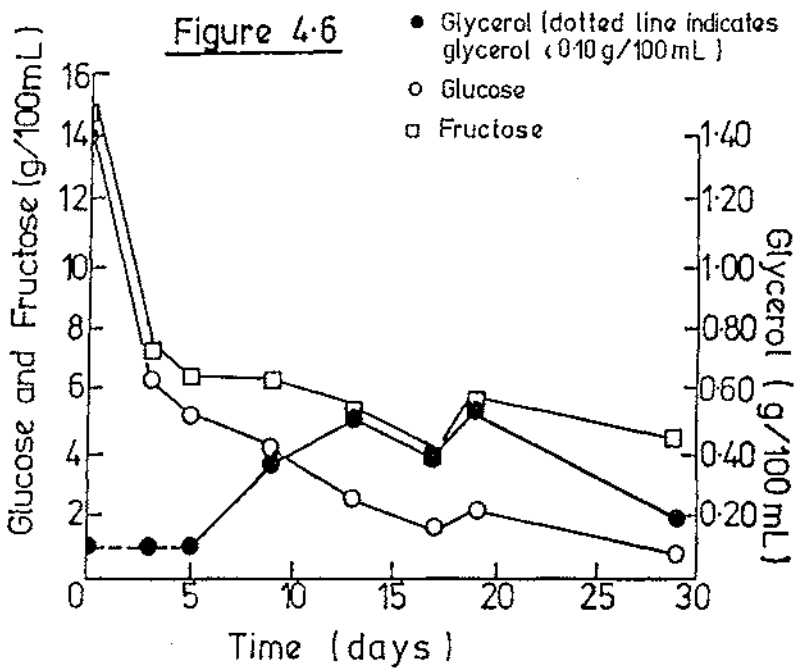


Figure 4·6
Glycerol (dotted line indicates glycerol < 0·10 g/100 mL)
Glucose
Fructose
Glucose and Fructose (g/100mL)
Glycerol (g/100 mL)
Time (days)

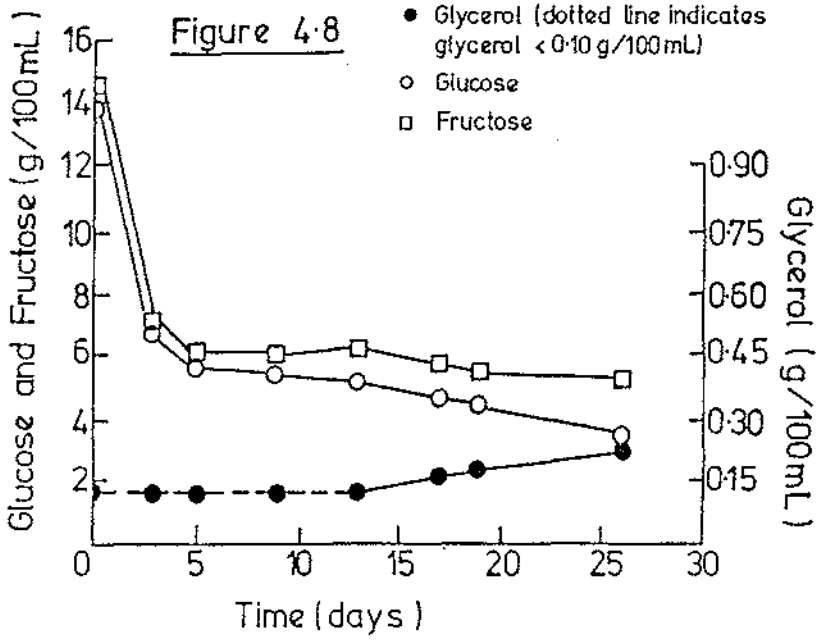


Figure 4·8
Glycerol (dotted line indicates glycerol < 0·10 g/100 mL)
Glucose
Fructose
Glucose and Fructose (g/100 mL)
Glycerol (g/100mL)
Time (days)

## 2. MYCELIAL DRY WEIGHTS PRODUCED BY FOUR GRAPE MOULDS.

### 2.1 Mycelial dry weights produced by four moulds in Black Hamburg juice.

The mycelial dry weights of four grape moulds grown in free-run and homogenized Black Hamburg juice after a 26-29 day incubation period are given in Tables 1 and 2 respectively.

In free-run Black Hamburg juice, B. cinerea under semi-aerobic conditions produced more mycelial dry weight than any other mould (Table 1). All four moulds grown semi-aerobically produced more mycelial dry weight than moulds grown semi-anaerobically (Tables 1 and 2).

In Black Hamburg juice R. nigricans produced less mycelial dry weight than the other three moulds, except in homogenized juice under semi-aerobic conditions where P. italicum produced less mycelial dry weight (Table 2). B. cinerea produced approximately 3-5 times more mycelial dry weight than R. nigricans and 2-4 times more mycelial dry weight than A. niger and P. italicum (Tables 1 and 2).

### 2.2 Mycelial dry weights produced by four moulds in Chasselas juice.

The mycelial dry weights of four grape moulds grown in free-run and homogenized Chasselas juice after a 26-29 day incubation period are given in Tables 3 and 4 respectively.

In Chasselas juice B. cinerea illustrated a similar pattern to that in Black Hamburg juice, in that it produced predominantly more mycelial dry weight than any other mould (Tables 3 and 4). All four moulds produced more mycelial dry weight semi-aerobically than semi-anaerobically except for R. nigricans which produced more mycelial dry weight in homogenized Chasselas juice under semi-anaerobic conditions than under semi-aerobic conditions (Table 4). In Chasselas juice, B. cinerea produced approximately 5-10 times more mycelial dry weight than R. nigricans and 2-4 times more mycelial dry weight than A. niger and P. italicum (Tables 3 and 4).

3. AVERAGE DRY MYCELIAL DRY WEIGHTS/mL OF JUICE.

The average mycelial dry weight/mL of juice, produced by four grape moulds grown in Black Hamburg and Chasselas juice under semi-aerobic and semi-anaerobic conditions are given in Table 5.

B. cinerea produced more mycelial dry weight/mL juice than the other three moulds under all conditions. In free-run Black Hamburg juice under semi-aerobic conditions, B. cinerea produced four times more mycelial dry weight/mL juice than A. niger and P. italicum and five times more than R. nigricans. B. cinerea produced significantly less mycelial dry weight/mL juice in homogenized juice than free-run juice under semi-aerobic conditions.

In Black Hamburg juice each same mould species produced similar mycelial dry weights/mL juice in both free-run and homogenized under semi-anaerobic conditions. All four moulds in Black Hamburg juice produced more mycelial dry weight/mL juice under semi-aerobic conditions than semi-anaerobic conditions.

Table 1 : Average mycelial dry weights of moulds grown in free-run Black Hamburg juice.

| | Mould | Average mycelial dry weight (grams) |
|---|---|---|
| Semi-anaerobic | B. cinerea | 1.529 |
| | A. niger | 0.649 |
| | P. italicum | 0.821 |
| | R. nigricans | 0.356 |
| Semi-aerobic | B. cinerea | 4.878 |
| | A. niger | 1.299 |
| | P. italicum | 1.181 |
| | R. nigricans | 0.964 |

Table 2 : Average mycelial dry weights of moulds grown in homogenized Black Hamburg juice.

| | Mould | Average mycelial dry weight (grams) |
|---|---|---|
| Semi-anaerobic | B. cinerea | 1.511 |
| | A. niger | 0.717 |
| | P. italicum | 0.728 |
| | R. nigricans | 0.498 |
| Semi-aerobic | B. cinerea | 3.406 |
| | A. niger | 1.490 |
| | P. italicum | 0.964 |
| | R. nigricans | 1.327 |

Table 3 : Average mycelial dry weights of moulds grown in free-run Chasselas juice.

| | Mould | Average mycelial dry weight (grams) |
|---|---|---|
| Semi-anaerobic | B. cinerea | 2.424 |
| | A. niger | 0.598 |
| | P. italicum | 0.558 |
| | R. nigricans | 0.403 |
| Semi-aerobic | B. cinerea | 3.112 |
| | A. niger | 0.864 |
| | P. italicum | 0.964 |
| | R. nigricans | 0.491 |

Table 4 : Average mycelial dry weights of moulds grown in homogenized Chasselas juice.

| | Mould | Average mycelial dry weight (grams) |
|---|---|---|
| Semi-anaerobic | B. cinerea | 2.722 |
| | A. niger | 0.802 |
| | P. italicum | 0.926 |
| | R. nigricans | 0.513 |
| Semi-aerobic | B. cinerea | 2.903 |
| | A. niger | 1.389 |
| | P. italicum | 1.700 |
| | R. nigricans | 0.284 |

Table:5 Average dry mycelial weight per mL of juice, produced by four moulds after a 26-29 day incubation period.

| Juice | Mould | Semi-aerobic | | Semi-anaerobic | |
|---|---|---|---|---|---|
| | | Average gram dry weight per container | Average mg dry weight per mL juice | Average gram dry weight per container | Average mg dry weight per mL juice |
| Free-run Chasselas | A. niger | 0.864 [a] | 21.6 | 0.598 [b] | 19.9 |
| | R. nigricans | 0.491 [a] | 12.3 | 0.403 [b] | 13.4 |
| | P. italicum | 0.964 [a] | 24.1 | 0.558 [b] | 18.6 |
| | B. cinerea | 3.112 [a] | 77.8 | 2.424 [b] | 80.8 |
| Homogenized Chasselas | A. niger | 1.389 [a] | 34.7 | 0.802 [b] | 26.7 |
| | R. nigricans | 0.284 [a] | 7.10 | 0.513 [b] | 17.1 |
| | P. italicum | 1.700 [a] | 42.5 | 0.926 [b] | 30.9 |
| | B. cinerea | 2.903 [a] | 72.6 | 2.722 [b] | 90.7 |
| Free-run Black Hamburg | A. niger | 1.299 [c] | 13.0 | 0.649 [d] | 7.64 |
| | R. nigricans | 0.964 [c] | 9.64 | 0.356 [d] | 4.19 |
| | P. italicum | 1.181 [c] | 11.8 | 0.821 [d] | 9.66 |
| | B. cinerea | 4.878 [c] | 48.8 | 1.529 [d] | 18.0 |
| Homogenized Black Hamburg | A. niger | 1.490 [c] | 14.9 | 0.717 [d] | 8.44 |
| | R. nigricans | 1.327 [c] | 13.3 | 0.498 [d] | 5.86 |
| | P. italicum | 0.964 [c] | 9.64 | 0.728 [d] | 8.56 |
| | B. cinerea | 3.406 [c] | 34.1 | 1.511 [d] | 17.8 |

[a] 40 mL juice container
[b] 30 mL juice container
[c] 100 mL juice container
[d] 85 mL juice container

## 4. PERCENTAGE SUGAR UTILIZATION BY FOUR GRAPE MOULDS GROWN IN CHASSELAS AND BLACK HAMBURG JUICE AFTER 26 - 29 DAY INCUBATION PERIOD.

The sugar utilization by the four grape moulds grown in Chasselas and Black Hamburg juice after a 26-29 day incubation period are given in Table 6.

### 4.1 Sugar utilization by four moulds grown in Chasselas juice after a 26-29 day incubation period.

In Chasselas juice B. cinerea utilized more total sugar under all cultivation conditions than any other mould (Table 6). A marked preference for glucose over fructose was evident in all cultures of B. cinerea (Figs.4.1 — 4.4). Total sugar utilization by B. cinerea (except in free-run juice under semi-aerobic conditions) and R. nigricans was relatively constant, 60-63% and 44-52% respectively.

### 4.2 Sugar utilization by four moulds grown in Black Hamburg juice after a 26-29 day incubation period.

In Black Hamburg juice R. nigricans under semi-aerobic conditions utilized more total sugar than any other mould (Table 6). While R. nigricans utilized significantly more in semi-aerobic conditions, the other conditions of cultivation had similar total sugar utilization. In free-run Black Hamburg juice A. niger, R. nigricans, and B. cinerea utilized more total sugar under semi-aerobic conditions than under the semi-anaerobic conditions, while the reverse was observed with P.

italicum. In homogenized Black Hamburg juice A. niger, R. nigricans, and P. italicum utilized similar amounts of total sugar whether grown under semi-aerobic conditions or semi-anaerobic conditions, while B. cinerea utilized more total sugar under semi-aerobic conditions than semi-anaerobic conditions.

Table 6 : % Sugar utilized by four moulds after 26-29 days incubation

| Juice | Mould | Semi-aerobic | | | Semi-anaerobic | | |
|---|---|---|---|---|---|---|---|
| | | Glucose % | Fructose % | Total % | Glucose % | Fructose % | Total % |
| Free-run Chasselas | A. niger | 40.9 | 18.6 | 30.8 | 60.0 | 27.7 | 45.3 |
| | R. nigricans | 79.1 | 1.58 | 43.9 | 70.0 | 26.7 | 50.3 |
| | P. italicum | 65.2 | 22.7 | 45.9 | 64.8 | 33.8 | 50.7 |
| | B. cinerea | 89.3 | 51.6 | 72.2 | 85.7 | 35.1 | 62.7 |
| Homogenized Chasselas | A. niger | 53.1 | 48.6 | 51.0 | 63.6 | 52.8 | 58.4 |
| | R. nigricans | 73.2 | 22.5 | 49.1 | 68.5 | 34.8 | 52.4 |
| | P. italicum | 61.0 | 33.9 | 48.1 | 47.2 | 33.0 | 40.4 |
| | B. cinerea | 77.8 | 41.3 | 60.4 | 81.2 | 41.7 | 62.4 |
| Free-run Black Hamburg | A. niger | 65.2 | 54.9 | 60.0 | 59.8 | 49.3 | 54.5 |
| | R. nigicans | 99.5 | 85.6 | 92.5 | 73.9 | 59.4 | 66.6 |
| | P. italicum | 61.5 [a] | 50.2 [a] | 55.8 [a] | 65.5 | 57.0 | 61.2 |
| | B. cinerea | 83.8 | 62.3 | 73.0 | 77.8 | 50.4 | 64.0 |
| Homogenized Black Hamburg | A. niger | 65.3 | 60.1 | 62.7 | 67.3 | 61.3 | 64.2 |
| | R. nigicans | 72.9 | 58.8 | 65.7 | 74.6 | 62.4 | 68.4 |
| | P. italicum | 59.7 | 52.4 | 56.0 | 61.4 | 57.0 | 59.1 |
| | B. cinerea | 93.4 | 69.9 | 81.5 | 74.0 | 60.4 | 67.1 |

[a] Sugar utilized after 17 days

## 5. GROWTH YIELDS OF MOULDS IN CHASSELAS AND BLACK HAMBURG JUICE.

The growth yields of the four moulds grown in Chasselas and Black Hamburg juice are given in Table 7. The units are displayed as micrograms of mycelial dry weight per gram of sugar utilized. The grams of sugar utilized are calculated from the final day; sugar = fructose + glucose.

### 5.1 Growth yields of Moulds in Chasselas Juice.

B. cinerea in all conditions produced significantly more mycelial dry weight per gram sugar utilized than any other mould. B. cinerea produced more mycelial dry weight per gram of sugar utilized under semi-anaerobic conditions than under semi-aerobic conditions. R. nigricans produced less mycelial dry weight per gram of sugar utilized than any other mould, while A. niger and P. italicum produced similar growth yields in all conditions except in homogenized juice under semi-aerobic conditions. A. niger and P. italicum produced more mycelial dry weight per gram of sugar utilized in semi-aerobic conditions than in semi-anaerobic conditions.

### 5.2 Growth yield of moulds in Black Hamburg juice.

B. cinerea in all conditions produced more mycelial dry weight per gram of sugar utilized than any other mould. The value for B. cinerea growing in free-run Black Hamburg juice under semi-aerobic conditions

must be treated with some caution. All four moulds produced higher growth yields under semi-aerobic conditions than semi-anaerobic conditions. Each mould produced similar growth yields whether grown on free-run or homogenized, while under semi-aerobic conditions there were differences in growth yields of B. cinerea and R. nigricans (Table 7).

All four moulds produced more mycelial dry weight/gram sugar utilized in Chasselas than in Black Hamburg juice.

Table 7 : Growth yield of moulds in grape juice

| Juice | Mould | mg mycelial dry wt/g sugar utilized[a] | |
|---|---|---|---|
| | | Semi-aerobic | Semi-anaerobic |
| Free-run Chasselas | A. niger | 297.1 | 186.3 |
| | R. nigricans | 118.4 | 113.0 |
| | P. italicum | 222.4 | 155.3 |
| | B. cinerea | 456.1 | 545.2 |
| Homogenized Chasselas | A. niger | 266.3 | 178.8 |
| | R. nigricans | 56.6 | 127.5 |
| | P. italicum | 345.3 | 298.4 |
| | B. cinerea | 469.9 | 568.7 |
| Free-run Black Hamburg | A. niger | 76.4 | 49.5 |
| | R. nigricans | 36.8 | 22.2 |
| | P. italicum | 74.7 | 55.7 |
| | B. cinerea | 236.0[b] | 99.2 |
| Homogenized Black Hamburg | A. niger | 83.8 | 46.3 |
| | R. nigricans | 71.2 | 30.2 |
| | P. italicum | 60.7 | 51.1 |
| | B. cinerea | 147.4 | 93.4 |

[a] Calculated from final day samples; sugar = glucose + fructose

[b] Calculated from dry weight after 27 days incubation and sugar utilization at 17 days

## 6. COMPARISON BETWEEN H.P.L.C AND ENZYMATIC METHODS OF GLYCEROL ANALYSIS.

A comparison between H.P.L.C and enzymatic methods of glycerol analysis on selected samples from R. nigricans cultures grown in free-run Chasselas juice under semi-anaerobic conditions is given in Table 8.

In R. nigricans samples a shoulder was detected on the glycerol peak. These samples were fractionated into an acid and a neutral fraction (materials and methods 2.1.5). The fractionated samples were analysed by H.P.L.C (materials and methods 2.1) and by an enzymatic glycerol assay referenced from Boehringer Mannheim, 1984.
The enzymatic glycerol assay was used to confirm the amount of glycerol detected in the fractionated samples as determined by the H.P.L.C method.

The results of the two methods of analysis were tabulated in table 10 and graphically illustrated in figures 5.1 and 5.2. Table 10 and figures 5.1 and 5.2 illustrate a close correlation between the H.P.L.C and enzymatic methods of glycerol analysis. The close correlation between the two methods confirmed that glycerol detected in the fractionated samples by H.P.L.C was glycerol and that the coeluting substance had been removed during the fractionation of the R. nigricans samples.

Table 8 :A comparison between HPLC and enzymatic methods of glycerol analysis on selected samples from Rhizopus nigricans cultures in free-run Chasselas juice under semi-anaerobic conditions

| Incubation Day | Glycerol Concn. via HPLC analysis g/100 mL | Average g/100 mL | Glycerol Concn. via enzymatic analysis g/100 mL | Average g/100 mL |
|---|---|---|---|---|
| 5 | < 0.1 ) | < 0.1 | 0.105) | 0.091 |
| 5 | < 0.1 ) | | 0.114) | |
| 5 | < 0.1 ) | | 0.054) | |
| 8 | 0.181) | 0.091 | 0.191) | 0.132 |
| 8 | < 0.1 ) | | 0.072) | |
| 12 | 0.504) | 0.311 | 0.523) | 0.403 |
| 12 | 0.429) | | 0.460) | |
| 12 | < 0.1 ) | | 0.226) | |
| 16 | 0.906) | 0.899 | 0.961) | 0.925 |
| 16 | 0.891) | | 0.888) | |
| 20 | 1.300) | 1.066 | 1.442) | 1.115 |
| 20 | 1.248) | | 1.275) | |
| 20 | 0.650) | | 0.627) | |
| 27 | 2.300) | 1.670 | 2.508) | 1.756 |
| 27 | 1.943) | | 1.986) | |

**Figures 5.1 and 5.2:** Glycerol production by R. nigricans grown in free-run Chasselas juice under semi-anaerobic conditions during a 27 day incubation period. Figures 5.1 and 5.2 illustrate the same samples analysed by different methods.

**Figure 5.1**

Glycerol analysed by H.P.L.C method.

**Figure 5.2**

Glycerol analysed by enzymatic assay method.

**FIGURE 5.1**

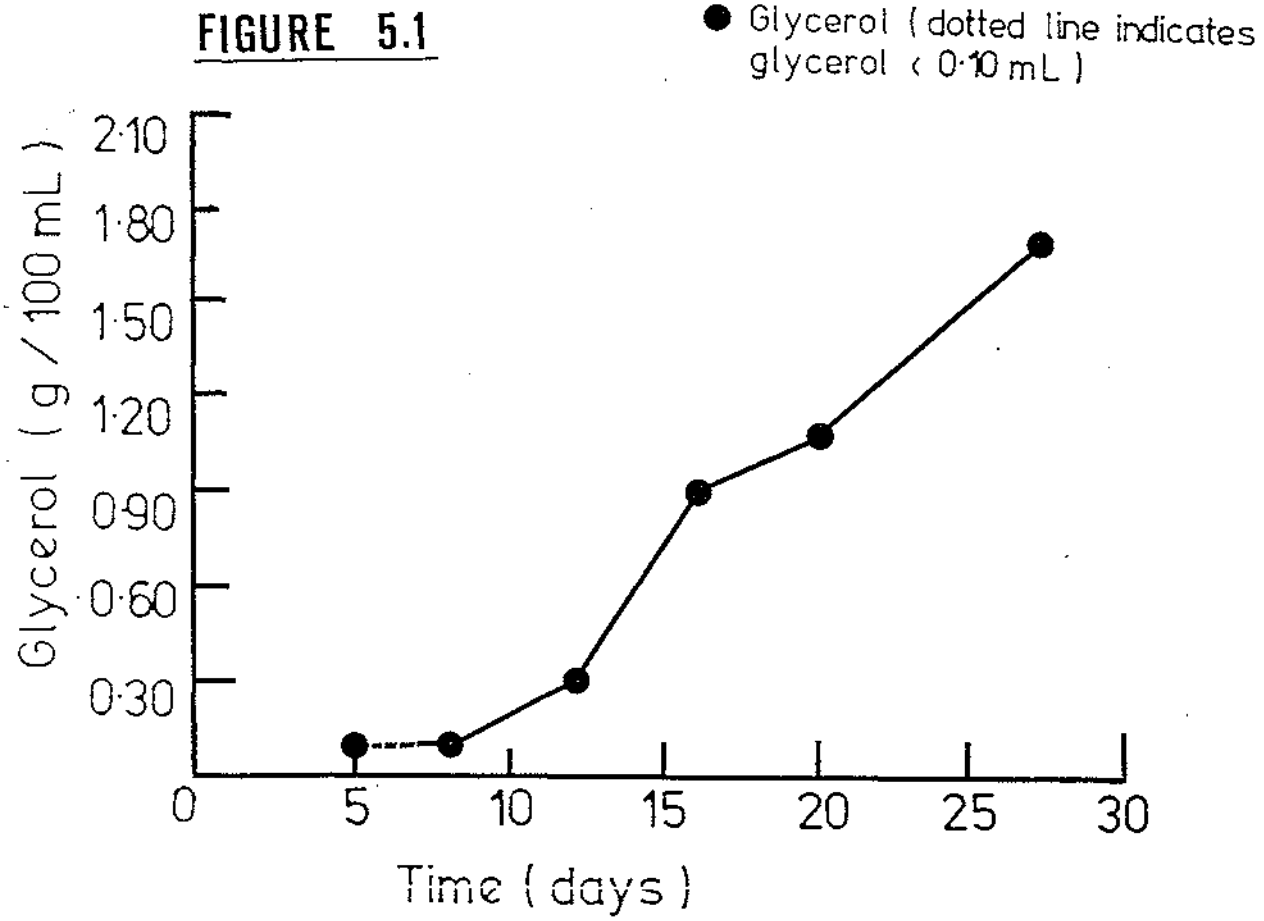
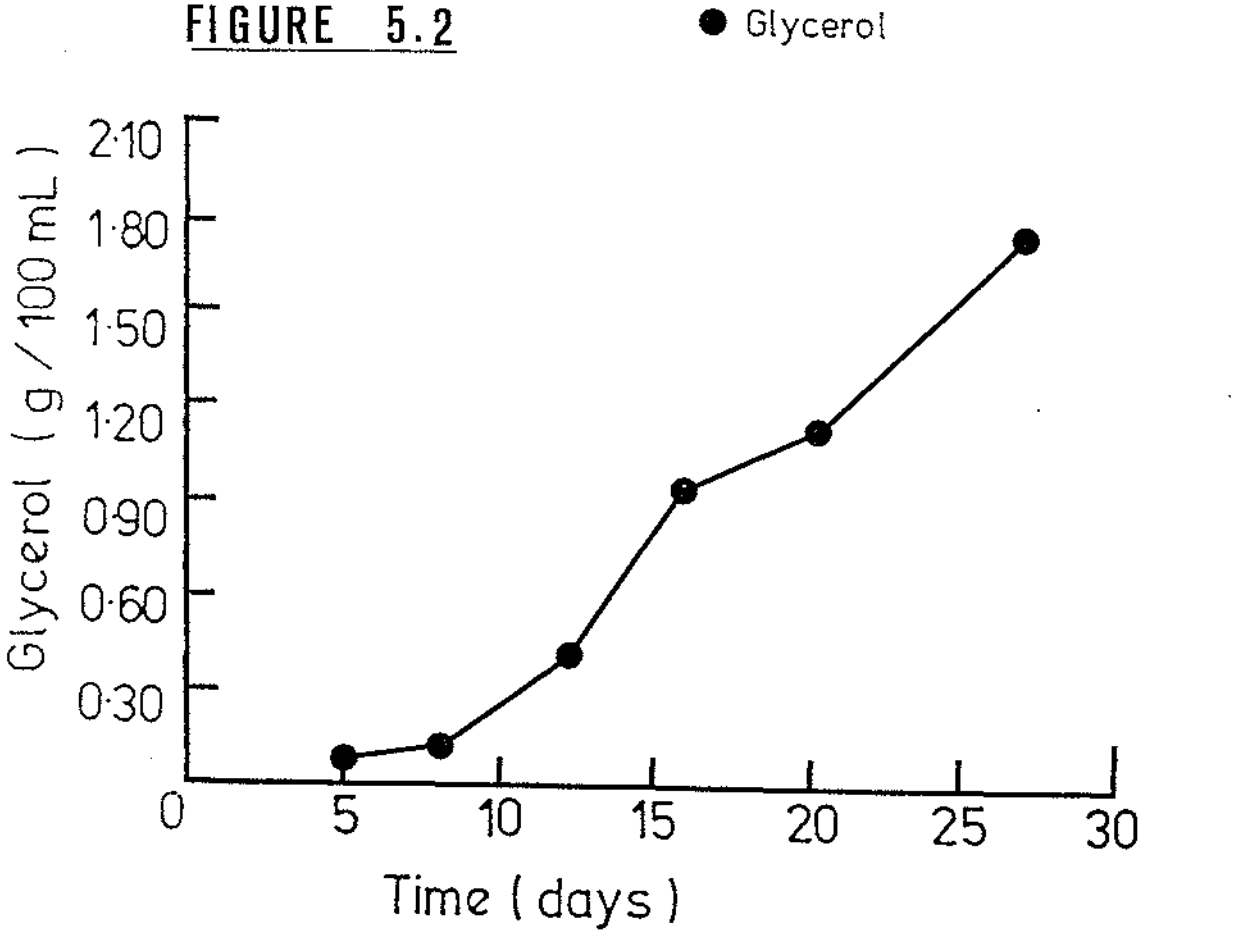



**FIGURE 5.2**



## 7. STATISTICAL ANALYSIS OF EXPERIMENTAL DATA.

The final day data recorded previously in the results section was subjected to statistical analysis to determine whether the observed differences in the raw data were statistically significant.

The experimental design was based on a three factor, balanced, factorial design with three replicates per treatment combination. Analysis of variance (ANOVA) was used to determine significance of main effects and interactions. The statistical package, SAS,(SAS Users Guide; Statistics, Version 5 Edition. SAS Institute Inc., Cary, NC. 1985), was used for analysis of variance. The Tukey's H.S.D (Honestly Significant Difference) Test was used to do all pairwise comparisons on treatment combination means as well as main effect means where appropriate (Gill,1978).

For Tables 9 to 13, at the bottom of each table there is the M.S.D (Mean Significant Difference) which indicate which figures are significantly different from each other.

### 7.1 Statistical analysis of experimental data from experiment using Chasselas Juice.

The method of juice preparation, i.e. free-run or homogenized, did not influence the production of glycerol by any individual mould (Table 9). The amount of glycerol produced by each mould was statistically similar whether it was grown in free-run or homogenized juice. Statistical

differences were detected when glycerol production by the different mould were compared. A. niger and P. italicum produced significantly less glycerol than R. nigricans and B. cinerea. The ANOVA for mould x preparation relative to glycerol production demonstrated that air relations did not significantly affect glycerol production by the four moulds in Chasselas juice.

Air relations caused a significant difference in the production of mycelial dry weight by B. cinerea but not in any of the other three moulds. B. cinerea produced significantly more mycelial dry weight than A. niger and P. italicum under semi-aerobic conditions (Table 10). The method of juice preparation did not significantly affect the production of mycelial dry weight by the four moulds in Chasselas juice.

7.2 Statistical analysis of experimental data from experiment using Black Hamburg juice.

By contrast air relations did influence glycerol production by the four moulds in the Black Hamburg (Table 11). R. nigricans produced significantly more glycerol under semi-aerobic than semi-anaerobic conditions and the reverse was true for B. cinerea. R. nigricans produced significantly more glycerol than any of the other three moulds under semi-aerobic conditions. R. nigricans and B. cinerea produced similar amounts of glycerol under semi-anaerobic conditions.

The method of juice preparation also influenced glycerol production by the four moulds in Black Hamburg juice (Table 12).

R. nigricans produced significantly more glycerol in free-run than in homogenized juice whereas B. cinerea produced similar amounts of glycerol. In free-run juice R. nigricans produced significantly more glycerol than any of the other three moulds in any condition of juice preparation.

A three way interaction between mould, juice preparation and air relations relative to mycelial dry weight production was detected in Black Hamburg juice (Table 13). The mycelial dry weight produced by each individual mould did not differ significantly whether grown under semi-aerobic or semi-anaerobic conditions, or in free-run or homogenized juice except in the instance of B. cinerea. B. cinerea produced more mycelial dry weight under semi-aerobic than under semi-anaerobic conditions in both free-run and homogenized juice.

Furthermore, B. cinerea produced more mycelial dry weight in free-run than in homogenized juice under semi-aerobic conditions. Similar amounts of mycelial dry weight were produced by B. cinerea under semi-anaerobic conditions in both free-run and homogenized juice. Under semi-aerobic conditions B. cinerea produced significantly more mycelial dry weight than any of the other three moulds under any condition of cultivation.

Table 9 : Significant interactions of mould x juice preparation relative to glycerol production in Chasselas juice

| Mould | Glycerol* (g/100 mL) | |
|---|---|---|
| | Free-run | Homogenized |
| *A. niger* | $0.163^{a}$ | $0.348^{ab}$ |
| *P. italicum* | $0.00^{a}$ | $0.296^{ab}$ |
| *R. nigricans* | $1.777^{d}$ | $1.384^{cd}$ |
| *B. cinerea* | $1.176^{cd}$ | $1.007^{c}$ |

The mean significant difference (MSD) = 0.627

* Significant differences between means at the 95% confidence level or above are noted with different letters

Table 10: Significant interactions of mould x air relations relative to mycelial dry weight production in Chasselas juice

| Mould | Mycelial dry weight* (mg/mL juice) | |
|---|---|---|
| | Semi-Aerobic | Semi-Anaerobic |
| *A. niger* | $28.1^{cd}$ | $23.3^{bc}$ |
| *P. italicum* | $33.1^{d}$ | $24.7^{bcd}$ |
| *R. nigricans* | $9.7^{a}$ | $15.2^{ab}$ |
| *B. cinerea* | $75.3^{e}$ | $85.8^{f}$ |

The mean significant difference (MSD) = 9.59

* Significant differences between means at the 95% confidence level or above are noted with different letters

Table II: Significant interactions of mould x air relations relative to glycerol production in Black Hamburg juice

| Mould | Glycerol* (g/100 mL) | |
|---|---|---|
| | Semi-Aerobic | Semi-Anaerobic |
| A. niger | $0^{a}$ | $0^{a}$ |
| P. italicum | $0^{a}$ | $0^{a}$ |
| R. nigricans | $0.563^{c}$ | $0.275^{b}$ |
| B. cinerea | $0.181^{a}$ | $0.322^{b}$ |

The mean significant difference (MSD) = 0.189

* Significant differences between means at the 95% confidence level or above are noted with different letters.

Table 12: Significant interactions of mould x juice preparation relative to glycerol production in Black Hamburg juice

| Mould | Glycerol* (g/100 mL) | |
|---|---|---|
| | Free-run | Homogenized |
| A. niger | 0[a] | 0[a] |
| P. italicum | 0[a] | 0[a] |
| R. nigricans | 0.565[c] | 0.274[b] |
| B. cinerea | 0.370[b] | 0.228[b] |

The mean significant difference (MSD) = 0.189

* Significant differences between means at the 95% confidence level or above are noted with different letters

Table 13: Significant interactions of mould x juice preparation x air relations relative to mycelial dry weight production in Black Hamburg juice

| Juice | Mould | Mean Dry Weight* (mg/mL) | |
|---|---|---|---|
| | | Semi-Aerobic | Semi-Anaerobic |
| Free-run | A. niger | $13.0^{abc}$ | $7.6^{abc}$ |
| | P. italicum | $11.8^{abc}$ | $9.6^{abc}$ |
| | R. nigricans | $9.7^{abc}$ | $4.2^{a}$ |
| | B. cinerea | $48.8^{e}$ | $18.0^{bc}$ |
| Homogenized | A. niger | $14.9^{bc}$ | $8.4^{abc}$ |
| | P. italicum | $9.6^{abc}$ | $8.6^{abc}$ |
| | R. nigricans | $13.3^{ab}$ | $5.8^{ab}$ |
| | B. cinerea | $34.1^{d}$ | $17.8^{bc}$ |

The mean significant difference (MSD) = 10.0

* Significant differences between means at the 95% confidence level or above are noted with different letters

# DISCUSSION

## 1. SUGAR UTILIZATION BY FOUR GRAPE MOULDS

In Chasselas juice, B. cinerea utilized more total sugar under all cultivation conditions than any other mould (table 6). A marked preference for glucose over fructose was evident in cultures of B. cinerea and R. nigricans. Under most cultivation conditions all four moulds utilized glucose and fructose throughout the incubation period.

In free-run Black Hamburg juice, R. nigricans under semi-aerobic conditions utilized more total sugar than any other mould (table 6). The pattern of sugar utilization by the four moulds in Black Hamburg juice was similar under most cultivation conditions. There was rapid utilization of glucose and fructose during the first three days of incubation, with little sugar utilization occurring after day five. The four moulds displayed no preference for glucose over fructose.

These results suggest that the grape cultivar influences the quantity and pattern of sugar utilization by the four moulds. The observed differences in sugar utilization may be caused by differences in the chemical composition of the juices from the two grape cultivars e.g. a higher phenolic content in Black Hamburg juice. A different chemical composition may influence the metabolism of the different moulds e.g. glucophlic utilization by B. cinerea and R. nigricans in Chasselas juice. The precise nature of these differences remain to be elucidated.

## 2. DRY WEIGHT YIELDS OF THE MOULDS

The four moulds differed in the mycelial dry weights produced in Chasselas and Black Hamburg juice. B. cinerea produced more mycelial dry weight than any other mould in both juices. The differences were most marked in Chasselas juice where B. cinerea, depending on the degree of aerobiosis, produced 5 - 10 times more mycelial dry weight than R. nigricans, while in Black Hamburg juice B. cinerea produced 3 - 5 times more mycelial dry weight than R. nigricans. In both Chasselas and Black Hamburg juice B. cinerea produced 2 - 4 times more mycelial dry weight than A. niger and P. italicum cultures.

Statistical analysis showed that there were significant interactions of mould and air relations relative to mycelial dry weight production in Chasselas juice (Table 10). Only B. cinerea produced significantly different quantities of mycelial dry weight under semi-aerobic and semi-anaerobic conditions. Under semi-aerobic conditions R. nigricans produced significantly less and B. cinerea significantly more mycelial dry weight than A. niger and P. italicum. No significant difference was detected in mycelial dry weight production by A. niger, P. italicum and R. nigricans under semi-anaerobic conditions, but B. cinerea produced significantly more than these three moulds.

In Black Hamburg juice statistical analysis confirmed a three way interaction between mould, juice preparation and air relations relative to mycelial dry weight production (Table 13). In free-run

juice under semi-aerobic conditions B. cinerea produced significantly more mycelial dry weight than the other three moulds in any cultural conditions. A. niger, P. italicum and R. nigricans produced similar quantities of mycelial dry weight in free-run juice under both semi-aerobic and semi-anaerobic conditions.

B. cinerea produced significantly more mycelial dry weight than R. nigricans but not A. niger or P. italicum under semi-anaerobic conditions in free-run juice. A similar pattern was observed in homogenized Black Hamburg juice. Comparison of mycelial dry weight yields in free-run and homogenized juice showed that B. cinerea produced significantly more mycelial dry weight in free-run that homogenized juice under semi-aerobic conditions. The other significant difference was between R. nigricans in free-run juice semi-anaerobically and A. niger in homogenized juice semi-anaerobically.

The complex interactions confirmed by statistical analysis demonstrate that mycelial dry weight production by the four moulds is dependent on many factors.

## 3. GLYCEROL PRODUCTION BY FOUR GRAPE MOULDS.

In Black Hamburg juice R. nigricans and B. cinerea produced glycerol under all conditions of cultivation, whereas glycerol, above the limits of detection (0.1g/100mL), was not found in A. niger or P. italicum cultures at any time during the period of incubation.
Higher levels of glycerol were produced in free-run than in homogenized Black Hamburg juice. The maximum glycerol accumulation of 0.83%(w/v) was observed in free-run juice under semi-aerobic conditions by day nineteen.

In Chasselas juice the four moulds had different patterns of glycerol production with respect to time. The highest glycerol producer was R. nigricans in free-run juice under semi-aerobic conditions. In Chasselas juice R. nigricans and B. cinerea produced significantly more glycerol than A. niger and P. italicum. Glycerol was produced rapidly in A. niger cultures over the first 12-15 days of incubation. A small gradual increase was detected in the glycerol level after day 15 in the semi-aerobic A. niger cultures, whereas glycerol levels decreased in the semi-anaerobic cultures.
The production of glycerol occurred later in P. italicum cultures (4-9 days). Higher levels of glycerol were detected in homogenized than in free-run juice cultures of P. italicum and glycerol levels peaked and then declined in all P. italicum cultures.

Statistical analysis showed that juice preparation in Chasselas juice (Table 9) and air relations in Black Hamburg juice (Table 11) influenced glycerol production by the four moulds. R. nigricans and B. cinerea produced significantly more glycerol than A. niger and P. italicum in both free-run and homogenized Chasselas juice. R. nigricans also produced significantly more glycerol in free-run juice than B. cinerea in homogenized juice.

R. nigricans produced significantly more glycerol semi-aerobically than semi-anaerobically in Black Hamburg juice and more glycerol semi-aerobically than B. cinerea semi-aerobically or semi-anaerobically (Table 11). B. cinerea produced significantly less glycerol semi-aerobically than semi-anaerobically. R. nigricans and B. cinerea did not differ significantly in glycerol production under semi-anaerobic conditions in Black Hamburg juice.

These results demonstrate unequivocally the effect of grape cultivar on glycerol production by the four moulds. Glycerol production is inhibited in Black Hamburg juice to such an extent that it was never detected in A. niger and P. italicum cultures. Furthermore the quantities of glycerol produced by R. nigricans and B. cinerea in Black Hamburg juice were less than half those produced by the same moulds in Chasselas juice.

## 4. EFFICIENCY OF GLYCEROL PRODUCTION.

The efficiency of glycerol production by four moulds grown in Chasselas and Black Hamburg juice are given in Table 14. The units of Table 14 are displayed as micrograms of glycerol produced per gram of sugar utilized. The grams of sugar utilized are calculated from final day; sugar = fructose + glucose.

### 4.1 The efficiency of glycerol production by four moulds grown in Chasselas juice.

In Chasselas juice R. nigricans produced more glycerol per gram of sugar utilized than the other three moulds under all conditions. By day 27, the levels of glycerol per gram of sugar utilized in P. italicum cultures were lower than the other three moulds, but higher levels of glycerol had been produced and then metabolised during the period of incubation (Figures 2.1 and 2.4) In P. italicum grown in free-run juice cultures, the glycerol level had dropped below the limit of detection (0.01g/100mL) after approximately twenty days (Figures 2.1 and 2.3) Under all conditions P. italicum utilized fructose and glucose throughout the incubation period. A. niger and R. nigricans produced more glycerol per gram of sugar utilized under semi-aerobic conditions than under semi-anaerobic conditions. B. cinerea produced similar efficiency of glycerol production figures under all conditions except free-run juice under semi-anaerobic conditions.

4.2 The efficiency of glycerol production by four moulds grown in Black Hamburg juice.

A. niger and P. italicum produced no detectable amounts of glycerol throughout the 26-29 day incubation period, though there was rapid utilization of glucose and fructose during the first three days of incubation.

Comparisons between the efficiency of glycerol production by R. nigricans and B. cinerea under semi-aerobic conditions must be made cautiously: in free-run juice, no liquid remained in B. cinerea cultures after day 17 and in homogenized juice the amount of glycerol detected in B. cinerea cultures declined after day 19. In semi-anaerobic cultures, R. nigricans and B. cinerea produced glycerol more efficiently when grown on free-run juice than on homogenized juice.

The differences in the yield of glycerol/g sugar utilized in the two juices emphasize the different responses of the four moulds to the different enviromental parameters.

Table 14: Efficiency of glycerol production

| Juice | Mould | mg glycerol/g sugar utilized[a] | |
|---|---|---|---|
| | | Semi-aerobic | Semi-anaerobic |
| Free-run | A. niger | 32.3 | 11.2 |
| Chasselas | R. nigricans | 204.4 | 137.3 |
| | P. italicum | * * | * * |
| | B. cinerea | 64.8 | 83.9 |
| Homogenized | A. niger | 33.4 | 16.8 |
| Chasselas | R. nigricans | 120.3 | 93.2 |
| | P. italicum | 21.1 | 32.8 |
| | B. cinerea | 64.3[b] | 64.9[b] |
| Free-run | A. niger | * | * |
| Black Hamburg | R. nigricans | 30.8 | 17.5 |
| | P. italicum | * | * |
| | B. cinerea | 30.5[c] | 20.6 |
| Homogenized | A. niger | * | * |
| Black Hamburg | R. nigricans | 17.6 | 11.4 |
| | P. italicum | * | * |
| | B. cinerea | 7.8[b] | 15.0 |

[a] Calculated from final day samples; sugar = glucose + fructose

[b] Glycerol concentration peaked at day 19 and decreased thereafter

[c] Calculated using day 17 data

* Glycerol below limits of detection

* * Glycerol was produced, but was entirely metabolized by day 27

## 5. GLYCEROL PRODUCTION AS A FUNCTION OF MYCELIAL DRY WEIGHT.

Glycerol production as function of mycelial dry weight of four moulds grown in Chasselas and Black Hamburg juice after a 26-29 day incubation period are given in Table 15. The units of table 15 as displayed as micrograms of glycerol produced per gram dry weight. Glycerol is calculated from final day samples (Appendices V, VI, VII and VIII).

### 5.1 Glycerol production as a function of mycelial dry weight of four moulds grown in Chasselas juice.

R. nigricans produced significantly more glycerol per gram of mycelial dry weight than any of the other three moulds (table 15).
In free run juice under semi-aerobic and semi-anaerobic conditions P. italicum produced glycerol but had metabolised it to below the limits of detection by the final day. Similarly P. italicum in homogenized Chasselas juice produced and then metabolised much of the glycerol it produced but a detectable amount remained on the final day. In homogenized juice under semi-anaerobic conditions A. niger, P. italicum, and B. cinerea produced approximately the same concentrations of glycerol per gram dry weight. B. cinerea produced similar amounts of glycerol under all cultivation conditions. Glycerol production by A. niger and R. nigricans was greater in semi-aerobic than semi-anaerobic conditions.

5.2 Glycerol production as a function of mycelial dry weight of four moulds grown in Black Hamburg juice.

R. nigricans produced more glycerol per gram mycelial dry weight than any of the other three moulds (table 15). R. nigricans produced significantly more glycerol in free-run juice than homogenized juice and under semi-anaerobic than semi-aerobic conditions in homogenized juice. Comparisons of glycerol production as a function of mycelial dry weight by R. nigricans and B. cinerea must be made cautiously in free-run juice, no liquid remained in B. cinerea cultures after day 17 and in homogenized juice the amount of glycerol detected in B. cinerea cultures declined after day 19.

Under semi-anaerobic conditions, R. nigricans produced almost four times more glycerol than B. cinerea in free-run juice and over two times more in homogenized juice. In Black Hamburg juice glycerol production by A. niger and P. italicum was not detected in any culture.

In general, B. cinerea produced similar amounts of glycerol/mycelial dry weight in both juices under all conditions of cultivation, whereas production by R. nigricans was greater in Chasselas than in Black Hamburg juice.

Table 15: Glycerol production as a function of mycelial dry weight

| Juice | Mould | mg glycerol/g dry weight | |
|---|---|---|---|
| | | Semi-aerobic | Semi-anaerobic |
| Free-run | A. niger | 94 | 61 |
| Chasselas | R. nigricans | 1365 | 1194 |
| | P. italicum | * | * |
| | B. cinerea | 143 | 154 |
| Homogenized | A. niger | 126 | 95 |
| Chasselas | R. nigricans | 2149 | 799 |
| | P. italicum | 60[a] | 111[a] |
| | B. cinerea | 140 | 113 |
| Free-run | A. niger | * | * |
| Black Hamburg | R. nigricans | 836 | 918 |
| | P. italicum | * | * |
| | B. cinerea | 132[b] | 208 |
| Homogenized | A. niger | * | * |
| | R. nigricans | 252 | 374 |
| | P. italicum | * | * |
| | B. cinerea | 55[a] | 165 |

[a] Glycerol produced and then metabolized

[b] Glycerol concentration from day 17

* Glycerol below limits of detection

SUMMARY

The production of glycerol by moulds during growth in grape juice is influenced by many factors. These data have demonstrated that grape cultivar, method of juice preparation, air relations, length of incubation and genus of mould all influence the quantity of glycerol detected in the grape juice.

(1) Higher levels of glycerol were detected in Chasselas than Black Hamburg juice cultures of the four moulds.

(2) Homogenization resulted in different levels of glycerol production by the four moulds in Chasselas juice.

(3) More glycerol was produced by R. nigricans under aerobic than anaerobic conditions in Black Hamburg juice.

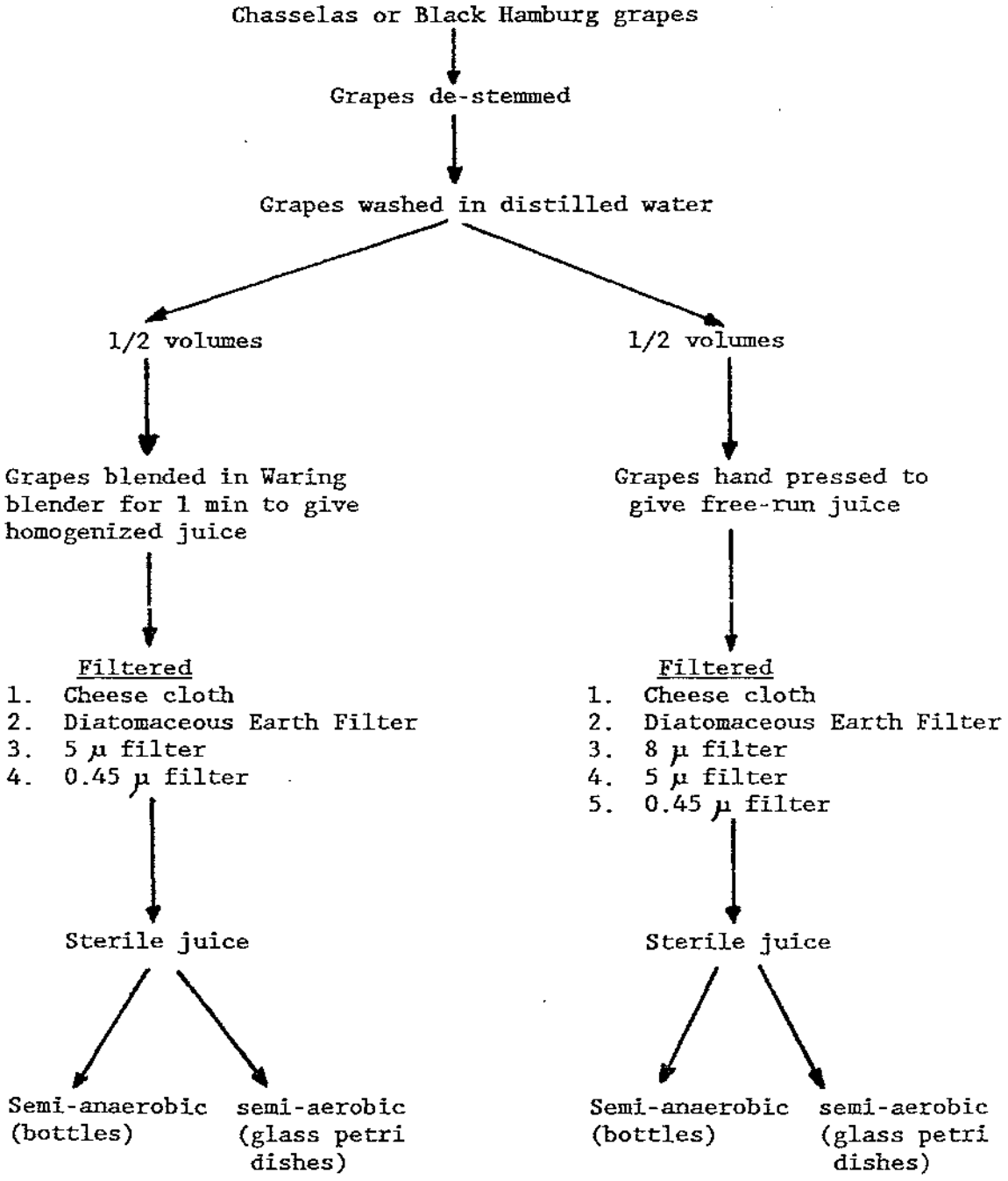
(4) Glycerol accumulated with time during growth of the moulds in Chasselas juice except in P. italicum cultures where production was followed by utilization. Glycerol accumulation was not detected in Black Hamburg juice cultures of R. nigricans and B. cinerea under anaerobic conditions until 8-12 days of incubation.

It is evident from these data that the glycerol level of grape juice cannot be used as the only indicator of the fungal rot of grapes. One can conceive of the situation in which complete fungal rot of Black Hamburg by A. niger or P. italicum could be described as "without rot" by using the glycerol level as the sole indicator.

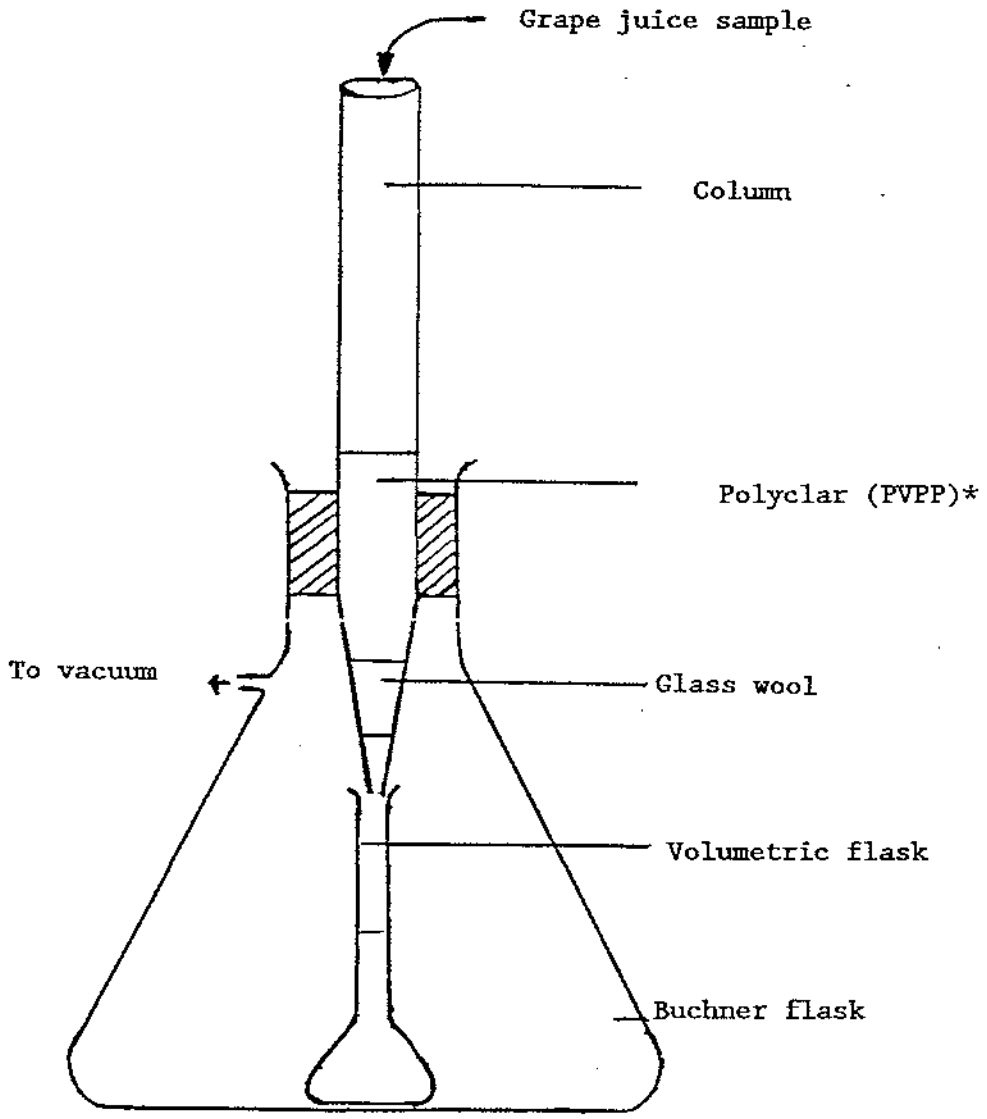
These data also reflect the limitations of visual inspection for the determination of fungal rot. R. nigricans produces little mycelial dry weight in comparison with other moulds but utilizes similar quantities of sugar. Thus the visual appearance is of less rot but the consumption of sugar and the production of metabolic end products is similar and, in the case of glycerol, greater than that of the other three moulds.

There is much interest in the food industry to find objective methods for the detection and quantitation of moulds in foods. Recent advances in enzyme-linked immunosorbent assay (ELISA) techniques suggest that they may offer an objective method for the detection and quantitation fungal rot in grapes (Notermans and Heuvelman,1985; Notermans et al.,1986; Notermans and Soentoro,1986).

Appendix I: Juice preparation outline



Appendix II: Apparatus to remove phenolics from grape juice




* Polyvinylpolypyrolidone

Appendix III: Juice volumes used in experiment

Black Hamburg juice

100 mL of juice per petri dish* (semi-aerobic)

85 mL of juice per bottle* (semi-anaerobic)

Chasselas juice

40 mL of juice per petri dish* (semi-aerobic)

30 mL of juice per bottle* (semi-anaerobic)

*for petri dish and bottle dimensions, refer Experimental Techniques 1.3

Appendix IV: Initial glycerol, glucose and fructose concentrations, including initial pHs of the various juices are tabulated below

| Juice | Glycerol (g/100 mL) | Glucose (g/100 mL) | Fructose (g/100 mL) | pH |
|---|---|---|---|---|
| Black Hamburg: | | | | |
| Free-run | < 0.10 | 14.02 | 14.31 | 3.00 |
| Homogenized | < 0.10 | 13.95 | 14.41 | 3.56 |
| Chasselas: | | | | |
| Free-run | < 0.01 | 12.89 | 10.74 | 3.15 |
| Homogenized | < 0.10 | 13.40 | 12.18 | 3.61 |

Appendix V: Glycerol concentration in final day free-run Chasselas juice samples.

| Juice | Mould | Glycerol Concentration g/100mL* | |
|---|---|---|---|
| | | Semi-aerobic | Semi-anaerobic |
| Free-run Chasselas juice | A. niger | 0.206 | 0.120 |
| | R. nigricans | 2.099 | 1.670 |
| | P .italicum | <0.01 | <0.01 |
| | B. cinerea | 1.107 | 1.243 |

* Average glycerol concentrations in free-run Chasselas juice after 26-29 day incubation period at 25°C.

Appendix VI:Glycerol concentration in final day homogenized Chasselas juice samples.

| Juice | Mould | Glycerol Concentration g/100mL* | |
|---|---|---|---|
| | | Semi-aerobic | Semi-anaerobic |
| Homogenized Chasselas juice | A. niger | 0.445 | 0.252 |
| | R. nigricans | 1.480 | 1.288 |
| | P .italicum | 0.252 | 0.339 |
| | B. cinerea | 0.988 | 1.024 |

* Average glycerol concentrations in homogenized Chasselas juice after 26-29 day incubation period at 25°C.

Appendix VII: Glycerol concentration in final day free-run Black Hamburg juice samples.

| Juice | Mould | Glycerol Concentration g/100mL* | |
|---|---|---|---|
| | | Semi-aerobic | Semi-anaerobic |
| Free-run Black Hamburg juice | *A. niger* | <0.1 | <0.1 |
| | *R. nigricans* | 0.800 | .331 |
| | *P .italicum* | <0.1 | <0.1 |
| | *B. cinerea* | 0.630 | 0.370 |

* Average glycerol concentrations in free-run Black Hamburg juice after 26-29 day incubation period at 25°C.

Appendix VIII: Glycerol concentration in final day homogenized Black Hamburg juice samples.

| Juice | Mould | Glycerol Concentration g/100mL* | |
|---|---|---|---|
| | | Semi-aerobic | Semi-anaerobic |
| Homogenized Black Hamburg juice | A. niger | <0.1 | <0.1 |
| | R. nigricans | 0.327 | 0.220 |
| | P .italicum | <0.1 | <0.1 |
| | B. cinerea | 0.181 | 0.276 |

* Average glycerol concentrations in homogenized Black Hamburg juice after 26-29 day incubation period at 25°C.

Appendix IX :Reprint of publication concerning work described in this thesis.

# Glycerol Production by Four Common Grape Molds

R. G. RAVJI[1], S. B. RODRIGUEZ[2*], and R. J. THORNTON[3]

The production of glycerol by the grape molds *Aspergillus niger*, *Penicillium italicum*, *Rhizopus nigricans*, and *Botrytis cinerea* growing in juice from Golden Chasselas and Black Hamburg grapes was examined. Juice from both free-run and homogenized whole berries was used together with different air relations and an incubation temperature of 25°C. Samples were taken periodically for analysis of glycerol by HPLC, and the mycelial dry weights of all cultures were determined after 26- to 29-day incubations. Large differences in glycerol production were observed among the molds. *R. nigricans* and *B. cinerea* produced more glycerol than *A. niger* and *P. italicum* under all conditions of cultivation. *B. cinerea* produced more mycelial mass than the other three molds under all conditions except in Black Hamburg juice under "anaerobic" conditions.

*KEY WORDS:* grapes, molds, glycerol, HPLC

Fungal rot is a major grape defect. In the California wine industry, grape defects are assessed by a visual inspection method. This method is subject to variability, and an alternative and objective method is being sought (1,2,4,5). Attention is currently focused on a high performance liquid chromatography (HPLC) method which quantifies glycerol, acetic acid, and ethanol as indicators of microbial activity (1,2,4,5). Glycerol has been related to mold growth, acetic acid to the growth of acetic acid bacteria, and ethanol to yeast growth (4). However, for this HPLC method to be acceptable, it must first be established that different molds produce the same amount of glycerol from the same amount of mycelial growth in different grapes under all conditions of cultivation.

In the present study, the production of glycerol by four molds which commonly infect grapes, *Aspergillus niger* (black-mold rot), *Penicillium italicum* (blue-mold rot), *Rhizopus nigricans* (Rhizopus rot), and *Botrytis cinerea* (gray-mold rot), was examined in two juices, Golden Chasselas and Black Hamburg. These rots have a variety of symptoms ranging from leakage of juice from the clusters (Rhizopus rot) to dehydration of berries leaving only shells (black-mold and gray-mold rots) (11). Grape varieties with compact clusters are prone to infection by these molds, but loose-clustered varieties are also susceptible. In this study, juice was prepared in two ways to encompass the potential range of microenvironments present in the grape clusters. In addition, mold 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 grape surface versus grape interior.

## Materials and Methods

**Juice preparation:** Sound Golden Chasselas and Black Hamburg grapes were used to prepare the juice. The grapes were destemmed and washed in distilled water before dividing them into two lots. Half of the grapes were pressed lightly by hand to provide free-run juice; the remaining grapes were homogenized in a Waring Blendor™ for one minute. The juices were filtered via cheesecloth, diatomaceous earth (DE), 5-μm filters, and finally sterile 0.45-μm filters. Difficulty was experienced in filtering the free-run juices but not the homogenized juices. The problem was overcome by DE filtration immediately followed by 8-μm filtration prior to 5-μm and 0.45-μm filtration.

**Molds:** Four molds were used: *A. niger* and *P. italicum* (M. Baxter, Massey Univ., Palmerston North, New Zealand); *R. nigricans* IFA 5781 (I. Maddox, Massey Univ., Palmerston North, New Zealand); and *B. cinerea* (J. M. Young, Plant Diseases Div., Dept. Scientific and Industrial Res., Auckland, New Zealand).

The molds were grown on potato dextrose agar, pH 5.6 (Oxoid), at 25°C. After one week, the mold spores were harvested using moistened sterile swabs and suspended in sterile 0.85% NaCl containing 0.05% Tween 80. The spore concentrations were calculated using a hemacytometer. The juice lots were inoculated in triplicate for each set of conditions using 0.1 to 0.2 mL of the appropriately diluted spore suspensions. The final spore concentration in the juice was 1000 spores/mL.

For "aerobic" growth, glass petri dishes (1.5 cm × 9 cm) filled with 40 mL Golden Chasselas juice and glass petri dishes (2 cm × 14 cm) filled with 100 mL Black Hamburg juice were used. For "anaerobic" growth, screw-capped bottles (50-mL capacity, 4 cm d) filled with 30 mL Chasselas juice and screw-capped bottles (100-mL capacity, 5 cm d) filled with 85 mL Black Hamburg juice were used.

The cultures were incubated at 25°C for 26 to 29 days. Samples (1.0 - 1.5 mL) were taken aseptically every four days for analysis of glycerol by HPLC.

After 26 to 29 days, the fungi were harvested by filtration so that dry weights could be obtained. Petri dishes containing the fungal biomass were dried in an oven (60°C) until there was no further decrease in weight.

**HPLC methods:** A Shimadzu HPLC LC-4A system equipped with a refractive index detector was used. An Aminex HPX-87H organic acid analysis column (300 mm × 7.8 mm, Bio Rad Laboratories), and a Micro-Guard

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Refill Cartridge Cation-H guard column (Bio Rad Laboratories) were used under the following conditions: 0.004 $M$ $H_2SO_4$, mobile phase, 0.8 mL/minute, 65°C, and 8 × $10^{-5}$ RIUFS. All standards and samples were filtered (0.45 μm) prior to injection.

Quantitation of glycerol was obtained by integration of peak area. Calibration curves were prepared using a BASIC program which combined a one-point calibration using the low standard and a two-point calibration using the low and the high standards. The following glycerol standards were used to prepare calibration curves: 0.02 and 0.20 g/100 mL glycerol for day 0 to 10 samples; 0.20 and 2.00 g/100 mL for day 11 to 29 samples; and 0.03 and 0.95 g/100 mL for Polyclar-treated or fractionated samples.

**Fractionation of *R. nigricans* samples:** A shoulder was detected on the glycerol peak in the *R. nigricans* samples. These samples were fractionated into acid and neutral fractions using Bio-Rex 5 resin (Bio Rad Laboratories) (6). A 0.5-mL sample of grape juice was mixed with 0.2 mL of 8.75% ammonium hydroxide and applied to an Econocolumn (Bio Rad Laboratories) containing 0.6 mL of Bio-Rex 5 resin. Water was used to elute the neutral compounds from the resin, and the 5-mL fraction was collected using a volumetric flask. No shoulder was detected on the glycerol peak in the resulting neutral fraction. The coeluting compound was demonstrated in the acid fraction.

HPLC analysis of the unfractionated and fractionated samples showed that an acidic compound was accumulating in the free-run aerobic *R. nigricans* culture in Golden Chasselas juice (Fig. 1). All results reported for *R. nigricans* are of fractionated samples.

**Sample clean-up:** The samples from the lot of homogenized Chasselas juice and all red juice samples were treated to remove phenolics. A 0.5-mL sample of juice was mixed with 0.2 mL of the internal standard (5% (w/v) propane-1,2-diol) and applied to a column containing "Polyclar-AT" or PVPP (polyvinylpolypyrrolidone). The sample was eluted with water and collected in a 5-mL volumetric flask. The clean-up procedure did not affect recovery of glycerol. Complete recovery of known quantities of glycerol was achieved.

**Statistical analyses:** The experimental design was based on a three-factor balanced, factorial design with three replicates per treatment combination. Analysis of variance (AOV) was used to determine significance of main effects and interactions. The statistical package, SAS, was used for analysis of variance (10). Tukey's HSD (Honestly Significant Difference) test was used to do all pairwise comparisons on treatment combination means, as well as main effect means where appropriate (3).

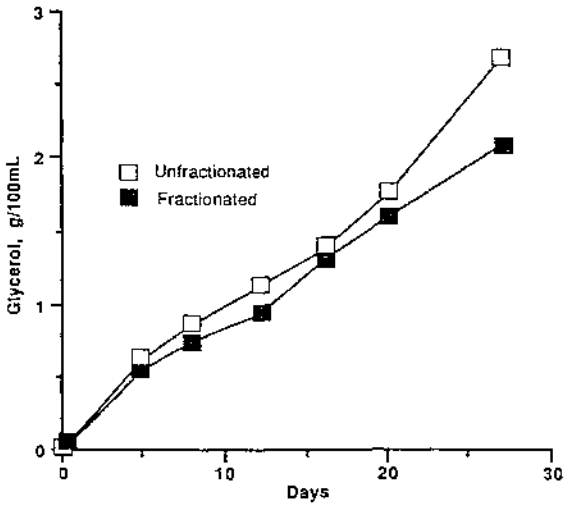


Fig. 1. Glycerol levels in unfractionated and fractionated samples of *R. nigricans* cultures in free-run Golden Chasselas juice under aerobic conditions.

## Results

Glycerol was not detected (< 0.1 g/100 mL) in Golden Chasselas or Black Hamburg juice prior to inoculation. The pH of free-run juice was 3.1 (±0.1), while that of homogenized berries was 3.6. Sugar content (% w/v) was 23.3 and 25.3 for free-run and homogenized Golden Chasselas juice, respectively, and 28.3 and 28.4 for free-run and homogenized Black Hamburg juice, respectively. The analysis of variance (AOV) of glycerol production by the four molds after a 27-day incubation in Golden Chasselas juice is shown in Table 1. *A. niger* and *P. italicum* produced significantly less glycerol than *R. nigricans* and *B. cinerea*. Although the AOV indicated that juice preparation (homogenized *vs.* free-run) significantly affected the quantity of glycerol produced, the conservative nature of Tukey's HSD test at the 95% level failed to identify these differences. The air relations did not significantly affect the production of glycerol by individual molds in Chasselas juice; however, the air relations did have a significant effect on the production of

Table 1. Significant interactions of mold × juice preparation relative to glycerol production in Golden Chasselas juice.

| Mold | Glycerol* (g/100 mL) | |
|---|---|---|
| | Free-run | Homogenized |
| *A. niger* | 0.163[a] | 0.348[a] |
| *P. italicum* | 0.00 [a] | 0.296[a] |
| *R. nigricans* | 1.777[c] | 1.384[bc] |
| *B. cinerea* | 1.176[bc] | 1.007[b] |

* Significant differences between means at the 95% confidence level or above are noted with different letters.

Table 2. Significant interactions of mold × air relations relative to mycelial dry weight production in Golden Chasselas juice.

| Mold | Mycelial dry weight* (mg/mL juice) | |
|---|---|---|
| | Aerobic | Anaerobic |
| *A. niger* | 28.1[cd] | 23.3[bc] |
| *P. italicum* | 33.1 [d] | 24.7[bcd] |
| *R. nigricans* | 9.7[a] | 15.2[ab] |
| *B. cinerea* | 75.3[e] | 85.8[f] |

* Significant differences between means at the 95% confidence level or above are noted with different letters.

mycelial mass by *B. cinerea* (Table 2). *R. nigricans* produced significantly less and *B. cinerea* significantly more mycelial mass under aerobic conditions than did *A. niger* and *P. italicum*. Furthermore, the AOV of the mycelial dry weight data indicated that, collectively, the four molds produced significantly more mycelial mass in the homogenized than in the free-run juice.

The AOV of glycerol production by the four molds after a 26- to 29-day incubation in Black Hamburg juice under different conditions of juice preparation and air relations are shown in Tables 3 and 4. Comparisons of glycerol production by *B. cinerea* in Black Hamburg juice must be treated cautiously; dehydration prevented sampling for HPLC analysis from the free-run aerobic *B. cinerea* cultures after day 17. Thus, the day 17 level of glycerol for *B. cinerea* was used to compute the AOV. *R. nigricans* produced significantly more glycerol under the aerobic than under the anaerobic conditions and significantly more glycerol than the other three molds (Table 3). Glycerol was not detected in the final day samples of *A. niger* or *P. italicum* cultures nor at any time during the period of incubation (see below). In Black Hamburg juice, *R. nigricans* produced significantly more glycerol in free-run than in homogenized juice and significantly more glycerol than the other three molds (Table 4).

Table 3. Significant interactions of mold × air relations relative to glycerol production in Black Hamburg Juice.

| Mold | Glycerol (g/100 mL) | |
|---|---|---|
| | Aerobic | Anaerobic |
| *A. niger* | 0[a] | 0[a] |
| *P. italicum* | 0[a] | 0[a] |
| *R. nigricans* | 0.563[c] | 0.275[b] |
| *B. cinerea* | 0.181[a] | 0.322[b] |

* Significant differences between means at the 95% confidence level or above are noted with different letters.

Table 4. Significant interactions of mold × juice preparation relative to glycerol production in Black Hamburg juice.

| Mold | Glycerol* (g/100 mL) | |
|---|---|---|
| | Free-run | Homogenized |
| *A. niger* | 0[a] | 0[a] |
| *P. italicum* | 0[a] | 0[a] |
| *R. nigricans* | 0.565[c] | 0.274[b] |
| *B. cinerea* | 0.370[b] | 0.228[b] |

* Significant differences between means at the 95% confidence level or above are noted with different letters.

The juice preparation and the level of air relations significantly affected the production of mycelial mass by *B. cinerea* in Black Hamburg juice (Table 5). *B. cinerea* produced significantly more mycelial mass under the aerobic than the anaerobic conditions in both homogenized and free-run juice. In addition, *B. cinerea* produced more mycelial mass in free-run than in homogenized juice under the aerobic conditions. Furthermore, under the aerobic conditions, *B. cinerea* produced significantly more mycelial mass than the other three molds. *R. nigricans* produced significantly less mycelial mass than *B. cinerea* under all conditions except for homogenized juice under aerobic conditions.

Table 5. Significant interactions of mold × juice preparation × air relations relative to mycelial dry weight production in Black Hamburg juice.

| Juice | Mold | Mean dry weight* (mg/mL) | |
|---|---|---|---|
| | | Aerobic | Anaerobic |
| Free-run | *A. niger* | 13[abc] | 7.6[abc] |
| | *P. italicum* | 11.8[abc] | 9.6[abc] |
| | *R. nigricans* | 9.7[abc] | 4.2[a] |
| | *B. cinerea* | 48.8[e] | 18.0[bc] |
| Homogenized | *A. niger* | 14.9[bc] | 8.4[abc] |
| | *P. italicum* | 9.6[abc] | 8.6[abc] |
| | *R. nigricans* | 13.3[ab] | 5.8[ab] |
| | *B. cinerea* | 34.1[d] | 17.8[bc] |

* Significant differences between means at the 95% confidence level or above are noted with different letters.

The four molds had different patterns of glycerol production in Golden Chasselas juice with respect to time. Glycerol was produced rapidly in *A. niger* cultures over the first 12 to 15 days of incubation (Fig. 2). A small increase was detected in glycerol levels after day 15 in the aerobic *A. niger* cultures, whereas glycerol levels decreased in the anaerobic cultures. Higher levels of glycerol were detected in homogenized than in free-run juice cultures of *P. italicum* (Fig. 3), and glycerol levels peaked and then declined in all *P. italicum* cultures. The production of glycerol occurred later in *P. italicum* cultures (4 - 9 days) than in any of the other three molds. Glycerol was produced throughout the period of incubation by cultures of *R. nigricans* and *B. cinerea* in Golden Chasselas juice (Fig. 4, 5). Large standard deviations of replicates of the final day anaerobic cultures of *R. nigricans* have been omitted for clarity.

Glycerol production by the four molds in Black Hamburg juice differed from that in Golden Chasselas juice. Glycerol, above the limit of detection of 0.1 g/100 mL, was not found in *A. niger* or *P. italicum* cultures at any time during the period of incubation. The production of

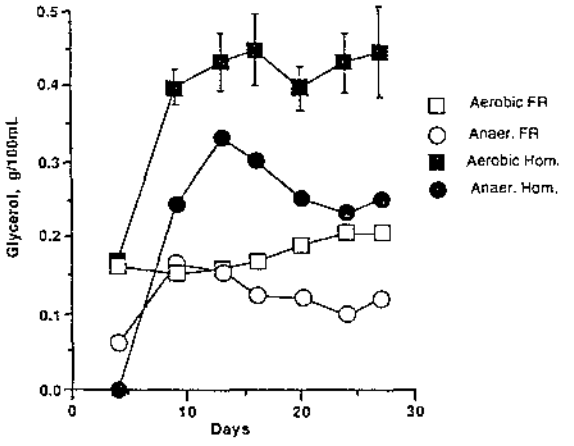


Fig. 2. Production of glycerol by *A. niger* with respect to time under different conditions of cultivation in Golden Chasselas juice. Standard deviations of the replicates are indicated by error bars. Where error bars cannot be discerned, the standard deviations are less than the width of the symbol.

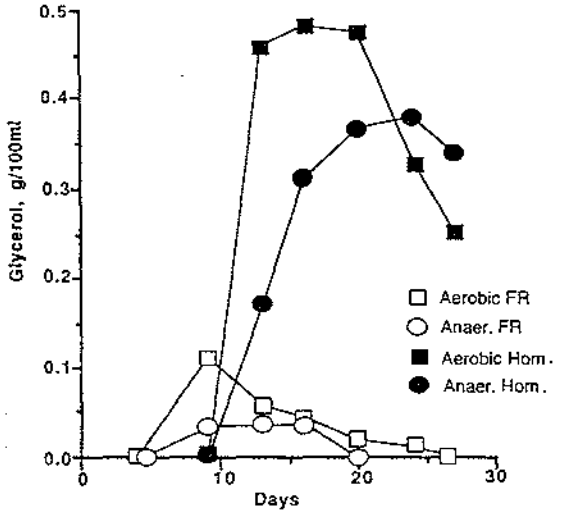


Fig. 3. Production of glycerol by *P. italicum* with respect to time under different conditions of cultivation in Golden Chasselas juice. Standard deviations of the replicates are indicated by error bars. Where error bars cannot be discerned, the standard deviations are less than the width of the symbol.

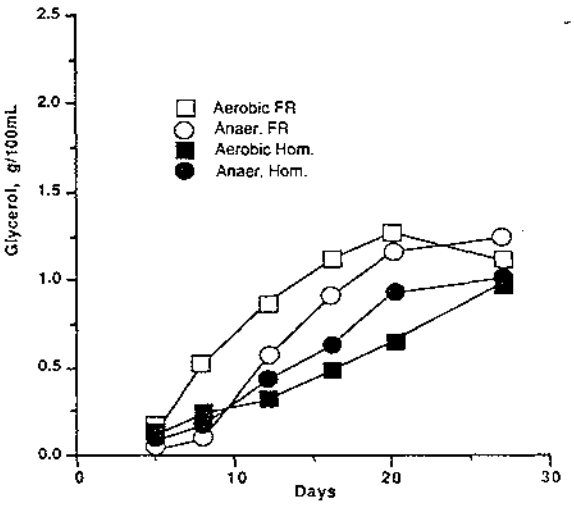


Fig. 5. Production of glycerol by *B. cinerea* with respect to time under different conditions of cultivation in Golden Chasselas juice. Standard deviations of the replicates are indicated by error bars. Where error bars cannot be discerned, the standard deviations are less than the width of the symbol.

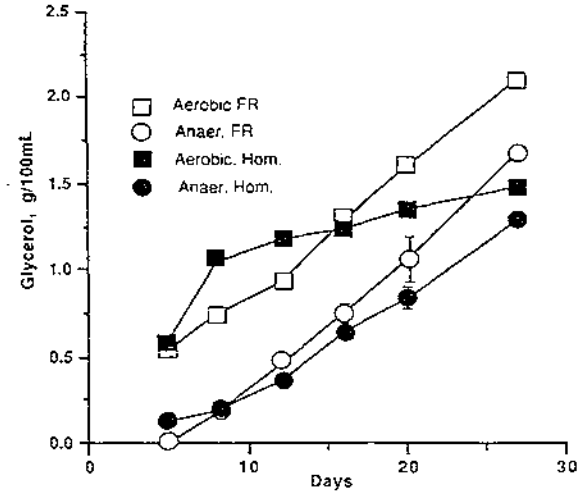


Fig. 4. Production of glycerol by *R. nigricans* with respect to time under different conditions of cultivation in Golden Chasselas juice. Standard deviations of the replicates are indicated by error bars. Where error bars cannot be discerned, the standard deviations are less than the width of the symbol.

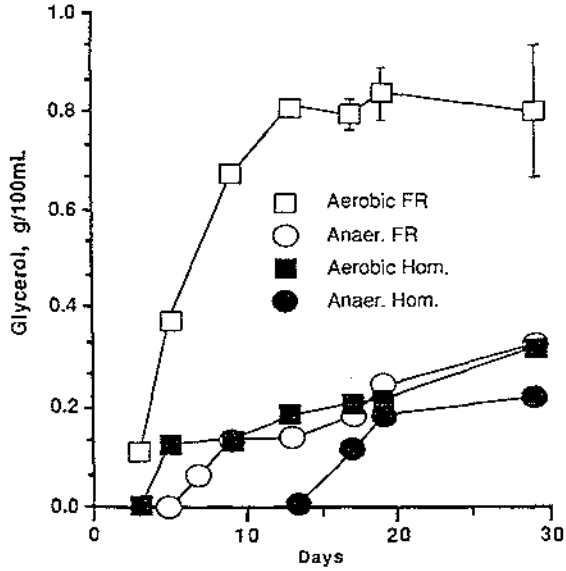


Fig. 6. Production of glycerol by *R. nigricans* with respect to time under different conditions of cultivation in Black Hamburg juice. Standard deviations of the replicates are indicated by error bars. Where error bars cannot be discerned, the standard deviations are less than the width of the symbol.

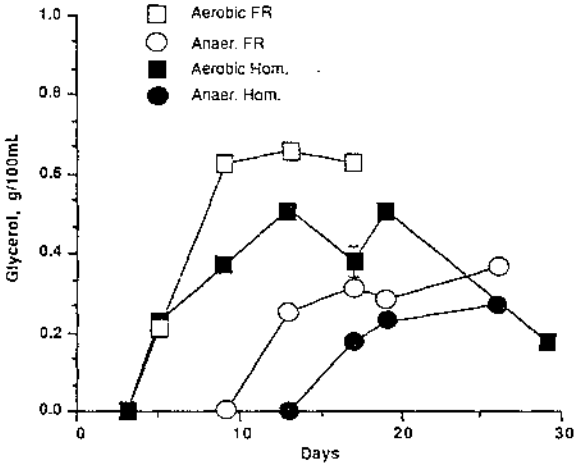


Fig. 7. Production of glycerol by *B. cinerea* with respect to time under different conditions of cultivation in Black Hamburg juice. Standard deviations of the replicates are indicated by error bars. Where error bars cannot be discerned, the standard deviations are less than the width of the symbol.

glycerol by *R. nigricans* and *B. cinerea* occurred later than in Golden Chasselas juice, and levels were lower (Fig. 6, 7).

## Discussion

The suggestion has been made that the level of glycerol in grape juice can be used as an indication of fungal rot of grapes (1,2,4,5). This suggestion implies that all grape molds produce the same amount of glycerol under the same condition of cultivation. This suggestion also implies that the production of glycerol is an indicator of fungal mycelial growth and that glycerol production is related to the "moldy" or off-flavors detected in wines made from grapes with high levels of fungal rot. This paper addresses the first two implications, *i.e.*, that similar amounts of glycerol are produced by all grape molds and that the level of glycerol is related to mycelial growth.

This study demonstrates that, under similar conditions of cultivation in Golden Chasselas juice, *R. nigricans* and *B. cinerea* produce significantly more glycerol than *A. niger* and *P. italicum*. The levels of glycerol in *A. niger* and *P. italicum* cultures never exceed 0.5 g/100 mL, whereas all cultures of *R. nigricans* and *B. cinerea* exceed this level after 15 to 18 days of incubation. Furthermore, glycerol appears to be a transient metabolic end-product of *P. italicum*. Glycerol was not detected in cultures of *A. niger* and *P. italicum* grown in Black Hamburg juice. Comparisons between glycerol production in Black Hamburg juice by *R. nigricans* and *B. cinerea* must be treated cautiously for the reason stated previously. The levels of glycerol produced by all four molds were lower in Black Hamburg than in Golden Chasselas juice. These observations suggest that glycerol production by the four molds is either inhibited by the presence of some factor(s) in Black Hamburg juice or, conversely, promoted by the presence of some factor(s) in Golden Chasselas juice.

The method of juice preparation and the air relations during incubation influence the production of glycerol by the four molds. Glycerol was not detected in *P. italicum* cultures after a 27-day incubation in free-run Golden Chasselas juice compared with 0.296 g/100 mL in homogenized juice. Furthermore, the AOV of glycerol production in Golden Chasselas juice indicated that, overall, there was a significant difference between free-run and homogenized cultures. A statistically significant difference was observed in the production of glycerol by *R. nigricans* in free-run and homogenized Black Hamburg juice. Furthermore, the air relations affected the production of glycerol by *R. nigricans* in Black Hamburg juice although the same was not true in Golden Chasselas juice.

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

In both Black Hamburg and Golden Chasselas juice,

Table 6. Glycerol production as a function of mycelial dry weight.

| Juice | Mold | mg glycerol/g dry wt | | | |
|---|---|---|---|---|---|
| | | Aerobic | | Anaerobic | |
| | | Golden Chasselas | Black Hamburg | Golden Chasselas | Black Hamburg |
| Free-run | *A. niger* | 97 | * | 60 | * |
| | *P. italicum* | * | * | * | * |
| | *R. nigricans* | 1711 | 830 | 1243 | 788 |
| | *B. cinerea* | 143 | 129[b] | 154 | 206 |
| Homogenized | *A. niger* | 127 | * | 93 | * |
| | *P. italicum* | 59[a] | * | 110[a] | * |
| | *R. nigricans* | 2084 | 249 | 754 | 376 |
| | *B. cinerea* | 136 | 53[a] | 112 | 158 |

[a] Glycerol produced and then metabolized.
[b] Glycerol concentration from day 17.
* Glycerol below limits of detection.

the method of juice preparation and air relations during incubation influenced the production of mycelial mass by *B. cinerea* but not by the other three molds. *B. cinerea* produced significantly more mycelial mass under the anaerobic than aerobic conditions in Golden Chasselas juice and *vice versa* in Black Hamburg juice. In addition, *B. cinerea* produced significantly more mycelial mass in the free-run than in the homogenized Black Hamburg juice under the aerobic conditions.

The production of glycerol by the four molds during the time span of the experiment emphasizes the problems involved in using glycerol as an indicator of fungal rot (Fig. 2, 3, 4, 5, 6, 7). In some instances, glycerol was detected in cultures within three days of inoculation, whereas in other instances glycerol was not detected until 10 to 12 days of incubation. The extreme situation was Black Hamburg juice in which glycerol was not detected at any time in *A. niger* and *P. italicum* cultures. Although culture volume depletion due to sampling was greater in Golden Chasselas than in Black Hamburg cultures, the authors believe that culture conditions were not significantly affected by the sampling.

Thus far, it has been demonstrated that individual molds produce different amounts of glycerol and different amounts of mycelial mass dependent upon the type of grape, the method of juice preparation, and air relations during incubation. These differences are highlighted when the production of glycerol is expressed as a function of mycelial dry weight after 27 to 29 days incubation (Table 6).

There is much interest in the food industry in methods for the detection and quantitation of molds in foods. Recent advances in enzyme-linked immunosorbent assay (ELISA) techniques suggest that they may offer a realistic method for the quantitative determination of fungal rot of grapes (7,8,9).

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