THE INHIBITION OF YEAST SPOILAGE OF BLUEBERRIES DURING MODIFIED ATMOSPHERE PACKAGING STORAGE

By

NGOC BICH DAY

B.Sc., The University of British Columbia, 1985

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Department of Food Science

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 1988

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Food Science

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date April 19, 1988

ABSTRACT

Modified atmosphere packaging storage combines an atmosphere of higher carbon dioxide and lower oxygen levels than air, with chilling temperatures to extend shelf-life of fresh fruits.

In three modified atmosphere packaging storage trials, blueberries were packaged in film bags with different gas permeabilities, and stored at about 4°C. Storage of blueberries in packages of a film with intermediate gas permeability produced an aerobic atmosphere and a relatively low carbon dioxide level, resulting in rapid growth of yeast and molds on blueberries. Packaging blueberries in a film with very low gas permeability created a high carbon dioxide almost anaerobic atmosphere, which successfully inhibited yeast and mold growth on blueberries for up to eight weeks.

The possibility of yeast inhibition by antifungal compounds accumulated in blueberries stored under modified atmosphere packaging conditions was investigated by using the disk diffusion assay. The results of these assays showed the absence of antifungal activity against two Rhodotorula species, a Zygosaccharomyces species, a Cryptococcus species, a Debaryomyces species, and indicated that the inhibition of yeast growth was due to low temperature, high carbon dioxide level and anaerobic

conditions. The effects of temperature and atmosphere composition were investigated by using natural flora of blueberry juice and two yeast isolates grown in sterilized juice. At 21°C, yeast growth was slow in the presence of carbon dioxide and absence of oxygen. At low temperature, yeast growth was slow in the presence of oxygen, but was inhibited in the anaerobic, high carbon dioxide environment.

It is proposed that the micro-aerobic environment of modified atmosphere packaging storage might have allowed slow desaturation of yeast membrane fatty acids which enabled yeasts to maintain membrane fluidity and function at low temperature. Furthermore, yeast growth during storage of modified atmosphere packaged blueberries may be affected by low temperature and high carbon dioxide conditions.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I am grateful to Drs. W.D. Powrie, S. Nakai and P. Townsley who commented on ideas throughout the study and carefully reviewed the manuscript. Dr. C.H. Wu provided guidance in the MAP storage trials and gas chromatography. Dr. B.J. Skura supervised thesis research with patience and gave much-needed encouragement.

V. Skura and S. Yee assisted when technical problems were encountered. D. Yan and W. Kam helped with photography.

Figures were drafted by Stephen who also gave much understanding and inspiration.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION

The shelf-life of fresh blueberries in the distribution system is limited primarily by fungal spoilage (Ballinger and Kushman, 1970; Ceponis and Cappellini, 1978). Postharvest deterioration not only causes economic loss to producers but also influences the availability and cost of blueberries to consumers.

The use of Modified Atmosphere Packaging (MAP) storage to extend the shelf-life of fresh fruits is currently being studied in the Department of Food Science, University of British Columbia. MAP implies the packaging of foods in film bags with selective permeabilities, and blanketing the product with an atmosphere of gas having higher carbon dioxide and lower oxygen levels compared with those in air. MAP storage combines modified atmosphere with chilling temperature to retard decay and delay senescence. Growth of molds is inhibited in MAP storage due to their inability to grow in low oxygen atmosphere and sensitivity to high carbon dioxide. Under anaerobic atmosphere, many yeast species can generate energy via fermentation of sugars. However, yeast spoilage of blueberries has been found to be absent in a low oxygen-high carbon dioxide environment. The mechanism of yeast inhibition in blueberries under MAP storage

conditions demanded attention.

This study approached the problem in three stages:

1. The growth trend of yeasts and molds on blueberries with different gas storage conditions was followed as an indication of their inhibition under MAP storage.

2. The possible synthesis of antifungal compound(s) by blueberry fruit in response to temperature and atmospheric stresses of MAP storage was investigated.

3. The effects of low temperature and anaerobic atmospheres on yeasts isolated from blueberries were studied.

1.1 LITERATURE REVIEW

1.1.1 Important blueberry diseases and application of fungicides

Spoilage level of blueberries varies from year to year, with decay incidence possibly being as high as 18% (Ceponis et al., 1973). Anthracnose, gray mold rot and black rot are the most common types of spoilage affecting blueberries (Cappellini et al., 1972; Ceponis et al., 1973).

Anthracnose is a defect with black or dark brown spots covering tissue. Pink spore masses of Colletotricum gloeosporioides (the imperfect stage of Glomerella cingulata) may appear on the spots.

Gray mold rot is evidenced by grayish-brown spots covered with mycelia of Botritis cinerea.

Black rot of blueberries is due to Alternaria tenuis. The decay areas on the blueberries are brown, soft and watery.

Application of chemicals to reduce postharvest decay of blueberries has been suggested. Ballinger (1983) reported reduction of anthracnose decay by dipping blueberries in 100 ppm solution of captafol. However, this treatment left visible white residues on the berries. Anthracnose, gray mold rot and black rot were decreased by dipping blueberries in a solution of 5000 ppm of 2-aminobutane and 100 ppm sodium hypochlorite, but the natural bloom of the berries was removed by sodium hypochlorite (Ceponis and Cappellini, 1978). When application of fungicides is considered, the possible toxicity of their residues to mammals must be extensively studied.

1.1.2 Effects of MAP storage on mold growth

The presence of carbon dioxide and low oxygen level in the atmosphere together with low temperature of MAP storage generally retards mold growth.

El-Kazzas et al. (1983) reported that under 21% oxygen and 15% carbon dioxide, fewer strawberries were infected by B. cinerea than those under air. Diameter of A. tenuis

and B. cinerea colonies decreased with decreasing oxygen levels to 1% (Follstad, 1966; Adair, 1971). Colony diameter on solid media is not a very accurate measurement of growth, since molds often produce aerial mycelia in addition to diffuse surface growth. Wells and Uota (1970) used mass of mycelia grown in liquid medium as a growth index. These authors found that at 4% oxygen, mycelial mass of A. tenuis was 31%, of Fusarium roseum was 38%, of B. cinerea was 45%, and of Cladosporium herbarum was 50% of the mass in air. Carbon dioxide greatly inhibited mycelial growth even in the presence of 21% oxygen: growth of B. cinerea and C. herbarum was completely abolished at a 45% carbon dioxide level, growth of A. tenuis and Rhizopus stolonifer were about 20% compared to growth in air. At 1% oxygen, spore germination of B. cinerea was inhibited by 16% carbon dioxide, while germination of C. herbarum and R. stolonifer were inhibited by 32% carbon dioxide. Svircev et al. (1984) noted that carbon dioxide caused distortion of fungi germ tubes, and interfered with the normal germination process.

1.1.3 Yeast metabolism in relation to MAP storage conditions

1.1.3.1 Anaerobic growth of yeasts

In contrast to molds which are obligate aerobes, many yeasts can grow under anaerobic conditions.

Fermentation of sugars supplies energy and metabolic precursors for yeast growth in anaerobic environments. Fermentation is an oxidation-reduction process where molecular oxygen is replaced by organic compounds as terminal electron acceptors. When fermentative ability exists, glucose is always fermented; other sugars may also be fermented by some yeast species. The biochemical pathway for the fermentation of glucose can be divided into three major parts (Figure 1-1). The first part is a series of reactions leading to production of glyceraldehyde-3-phosphate. In the second part, pyruvate and adenosine triphosphate (ATP) molecules carrying the high-energy phosphate bond are produced. In the third part, ethanol and carbon dioxide are released. The series of reactions leading to pyruvate formation is called glycolysis.

Ethanol and carbon dioxide are the most common end products of glucose fermentation, although other products also appear in minor proportions. In one glycolytic step, the oxidation of glyceraldehyde-3-phosphate to 1,3-

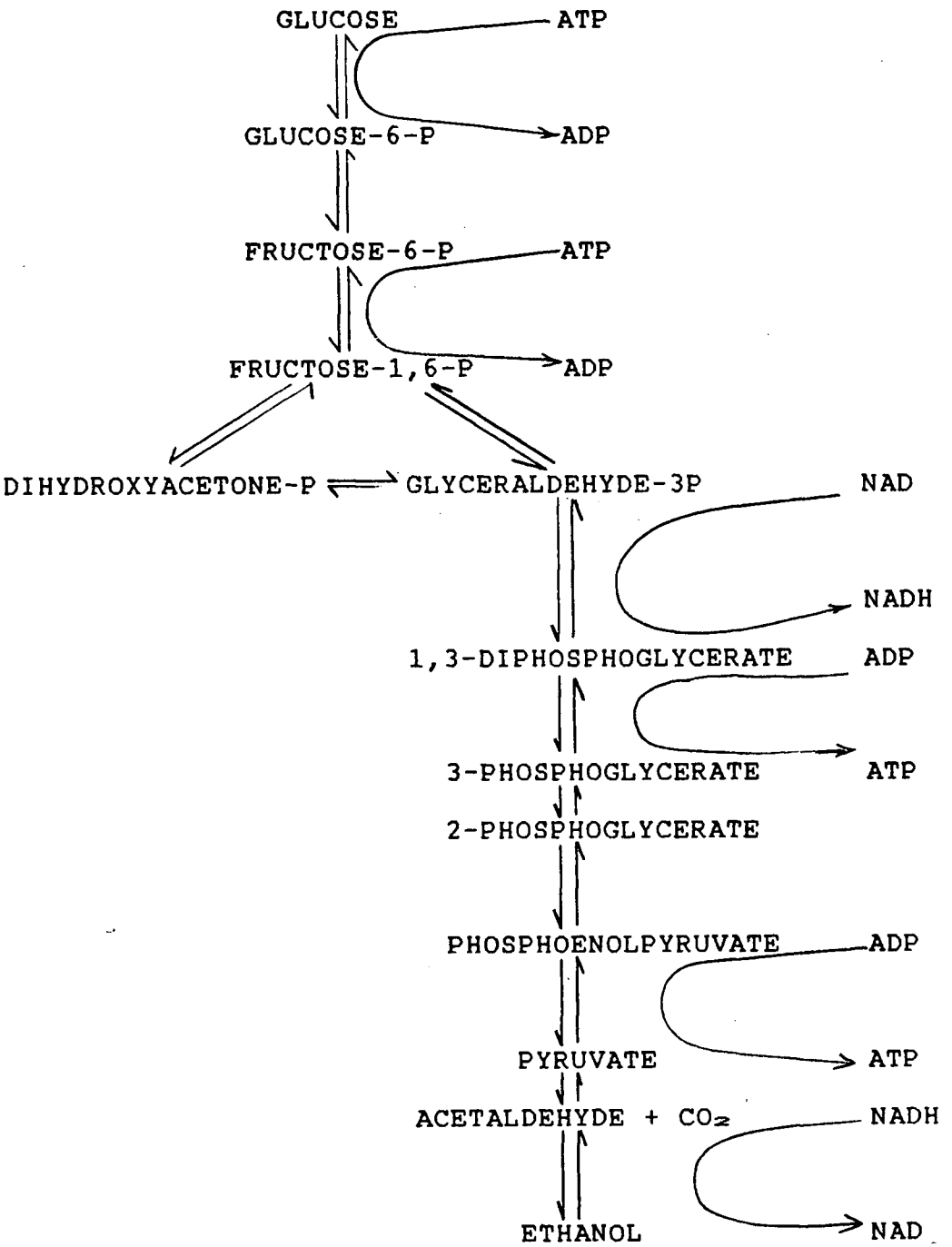

Figure 1-1: Fermentation of glucose by yeasts (Brock, 1979).

diphosphoglycerate, an oxidized coenzyme nicotinamide adenine dinucleotide (NAD) acts as an acceptor to the hydrogen atom removed from glyceraldehyde-3-phosphate and becomes reduced (NADH). The cell has only a limited supply of NAD, so NAD must be regenerated for glycolysis to proceed. The last step in fermentation, reduction of acetaldehyde to ethanol, fulfills this objective: NADH donates a hydrogen atom to acetaldehyde leading to formation of ethanol and NAD. Regeneration of NAD can be carried out by an alternative route, namely the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate which is then hydrolyzed to glycerol.

When the growth medium is very alkaline, another type of fermentation occurs to produce ethanol, acetic acid, glycerol and carbon dioxide. The biochemical explanation of this fermentation route is that in alkaline media, an aldehyde dehydrogenase with an alkaline pH optimum, converts acetaldehyde to acetic acid. This enzyme requires NAD, so that the cells must utilize the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate to re-oxidize NADH. The role of this pathway is to bring medium pH within the range favourable for growth (Sols et al., 1971).

When oxygen is available, glucose is oxidized completely in respiration process. The early step in

glucose oxidation follows glycolysis, but pyruvate is oxidized to carbon dioxide, through a series of reactions known as the tricarboxylic acid cycle. NADH molecules produced during both glycolysis and the tricarboxylic acid cycle can be reoxidized by the electron-transport system, with oxygen as the terminal electron acceptor. The electron-transport system is coupled with ATP synthesis so that 3 ATP molecules are produced per NADH, and a total of 38 ATP molecules are produced by aerobic utilization of glucose. The relationship of glycolysis, tricarboxylic acid cycle and electron-transport system is illustrated in Figure 1-2.

A comparison of ATP yield from aerobic respiration to ATP yield from fermentation shows that aerobic utilization is much more favourable energetically than fermentation. The pathways involved in aerobic utilization of glucose are similar in aerobic molds, yeasts and bacteria. Molds generally lack the ability to ferment glucose, and fail to grow under anaerobic conditions.

1.1.3.2 Influence of low temperature on yeast growth

Temperature can exert a profound effect on growth and survival of yeasts. Most yeasts can grow slowly at or near 0°C. The optimum temperature, at which growth rate is maximum, occurs in the range of 20°C to 30°C for most

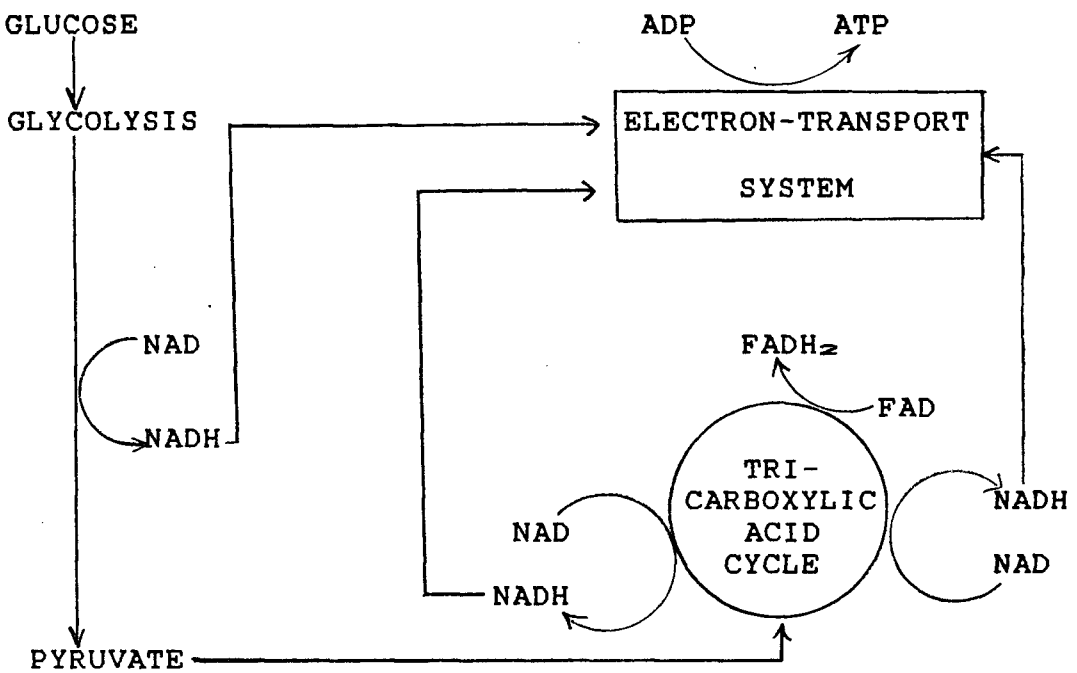

Figure 1-2: Relationship between glycolysis, tricarboxylic acid cycle and electron-transport system.

yeasts, except the psychrophilic species. Maximum temperature for growth of most yeasts, with a few exceptions, is in the 30°C to 40°C range (Stokes, 1971). The biochemical processes affected by temperature are complex and numerous and include enzyme activity, substrate transport, and protein synthesis.

One factor which determines the upper temperature limit is thermal resistance of enzymes. A Cryptococcus species failed to grow at 30°C unless α-ketoglutarate, citrate or isocitrate were added to the growth medium, which indicated that several enzymes of the tricarboxylic acid cycle were inactivated at this temperature (Hagen and Rose, 1962). A psychrophilic Candida nivalis suffered severe membrane damage at 25°C, with leakage of soluble phosphates, amino acids and small peptides (Nash and Sinclair, 1968). These results suggested that enzyme inactivation and membrane damage set the upper temperature limit for yeast growth.

Baxter and Gibbons (1962) reported that a psychrophilic Candida species could oxidize glucose at 0°C, whereas another Candida species was inactive at this temperature. At 10°C and 15°C, psychrophilic Candida gelida exhibited more rapid fermentation rates than those for Saccharomyces cerevisiae. Amino acid uptake by Candida utilis ceased

below 4°C (Quetsch and Danforth, 1964), but uptake remained operative in Rhodotorula glutinis at -4°C (Clinton, 1968). These data indicate that enzyme activity and substrate transport are crucial factors governing the minimum growth temperature of yeasts.

Preliminary MAP storage experiments with fresh fruits showed that both mold rots and yeast spoilage were absent in a low oxygen-high carbon dioxide atmosphere. The inhibition of mold rots was not surprising, but inhibition of yeast spoilage was unexpected. When a population of natural flora present on berry surfaces is subjected to chilling temperature and anaerobic atmosphere, some yeast species will be unable to grow, but some species which can grow at low temperature and possess fermentative ability will continue to proliferate. In a survey of yeasts of strawberry, Candida sake, Kloeckera apiculata, Torulopsis candida, and Torulopsis fragaria were found to have both the ability to ferment sugars and grow at 5°C (Buhagiar and Barnett, 1971). Fermentation of sugars by yeasts associated with blueberries at low temperature were investigated in this study.

1.1.4 Background on phytoalexins

The mechanism of yeast inhibition in MAP storage of fruits demanded attention. A possible explanation is the

accumulation of antifungal compounds in fruits in response to a chilling temperature and an anaerobic environment.

1.1.4.1 Structure and distribution

Müller and Börger (1940) proposed the concept of plant resistance. These authors used a potato cultivar which was resistant to one species of Phytophthora infestans, but susceptible to another species. The virulent species rapidly colonized tuber slices but growth of the avirulent species was restricted to dead tuber cells. The virulent species, however, was unable to infect tissues previously inoculated with spores of the avirulent species. Müller and Börger (1940) concluded that protection was caused by production of an antifungal compound by potato cells in response to the initial inoculation. They called this substance a phytoalexin. Phytoalexins are defined as "antimicrobial compounds that are synthesized by and accumulated in plants which have been exposed to microorganisms" (Mansfield and Bailey, 1982).

Phytoalexin accumulation may be triggered not only by exposure to microorganisms but also by a number of stresses such as cut injury, ultraviolet light, temperature adversity, and ethylene (Haard and Cody, 1978; Cheema and Haard, 1978; Currier and Kuc, 1975). The term stress metabolite has been coined to include compounds

formed in response to injury, physiological stress and microbial infection (Haard and Cody, 1978). In contrast to the term phytoalexin, stress metabolite does not imply a functional role of these compounds.

Table 1-1 lists several antimicrobial compounds known to accumulate in plants in response to both fungal and non-fungal illicitors. Their structures are presented in Figure 1-3.

A stimulus which illicits production of a metabolite type does not necessarily promote accumulation of other metabolites in a tissue. In sweet potato roots, ethylene stimulated accumulation of phenolic compounds, terpenes accumulated in response to chilling storage, cut injury and UV radiation promoted coumarin formation. Accumulation of stress metabolites also depends on the physiological condition of tissues: mercuric acetate readily caused formation of rishitin in potato tubers after cold storage, but was ineffective on freshly harvested tubers (Cheema and Haard, 1978). In general, the formation of stress metabolites in response to a wide array of stimuli may be part of a general repair mechanism operating in damaged tissues (Kúc and Shain, 1977).

Table 1-1: Examples of antimicrobial compounds isolated from plant tissues (adapted from Haard and Cody, 1978).

Compounds	Plant sources	Fungal Illicitors	Non-fungal Illicitors
Pisatin	Garden pea	Monilia fructicola	UV radiation, DNA-intercalators
Phaseollin	Green bean	Colletotricum lindemuthiamum	Heavy metal ions, transcriptional inhibitors.
Rishitin	Irish potato	Phytophthora infestans	$AgNO_3$
6-methoxy-mellein	Carrot	Ceratocystis fimbriata	Chilling, $HgCl_2$
Ipomeamarone	Sweet potato	Ceratocystis fimbriata	Chilling, SDS, $HgCl_2$

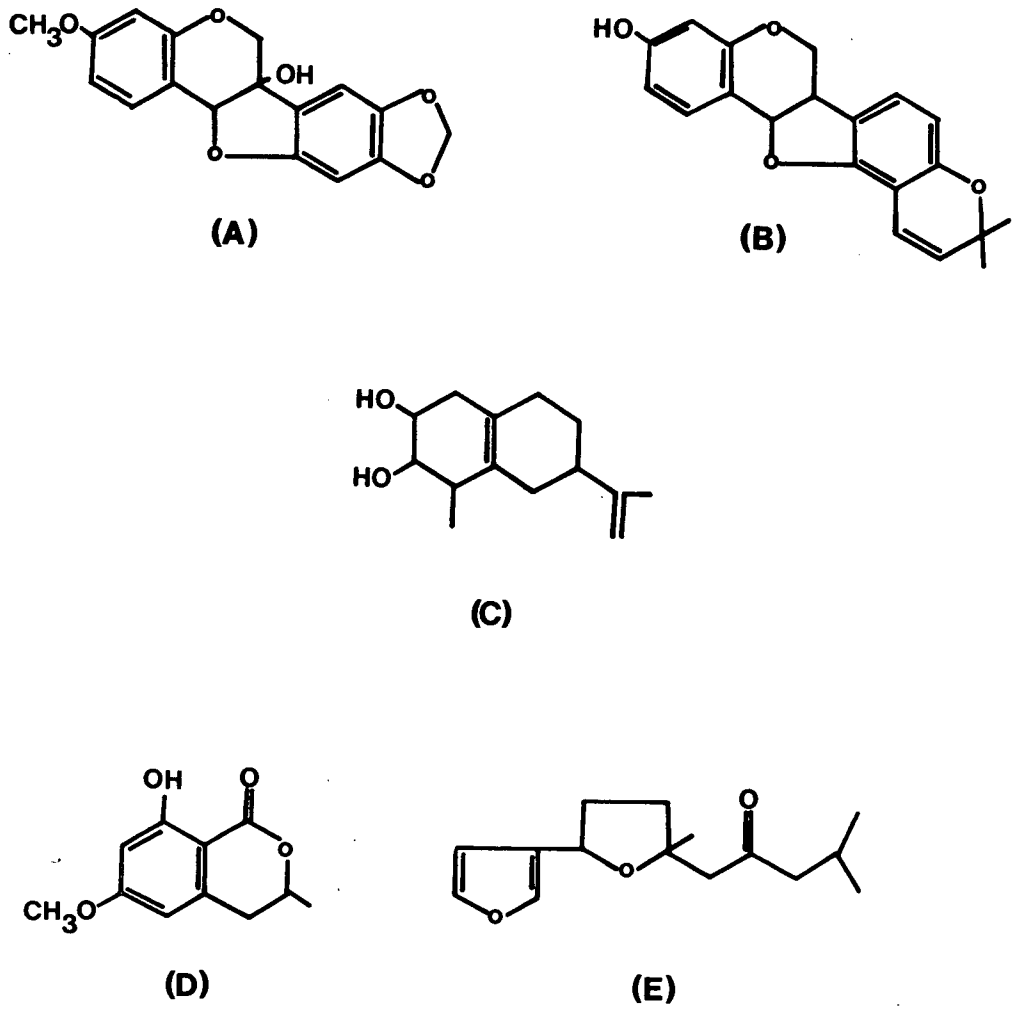

Figure 1-3: Structures of several phytoalexins (Kuc and Shain, 1977). (a) Pisatin, (b) phaseollin, (c) rishitin, (d) 6-methoxy-mellein, and (e) Ipomeamarone.

1.1.4.2 Biosynthesis of phytoalexins

The interactions between primary and secondary metabolism in phytoalexin synthesis, and the role of key intermediates such as phenylalanine, acetyl-Coenzyme A (CoA), malonyl-CoA, and mevalonic acid are outlined in Figure 1-4.

Development of an understanding of metabolic controls of phytoalexin synthesis requires studies on the properties and activities of enzymes involved. Enzymes studied have included those catalyzing synthesis of early precursors, as well as those directly responsible for formation of phytoalexins.

Recent studies indicated that phytoalexin formation may involve activation of the biosynthetic pathways by de novo enzyme synthesis (Manfield, 1983). Stoessl (1982) proposed two possible mechanisms which lead to diversion of normal metabolic processes to phytoalexin synthesis: (1) the imposition or removal of metabolic blocks on the normal metabolic route, or (2) increased synthesis of general biosynthetic precursors.

Both mechanisms may be operating in different plants for synthesis of different compounds.

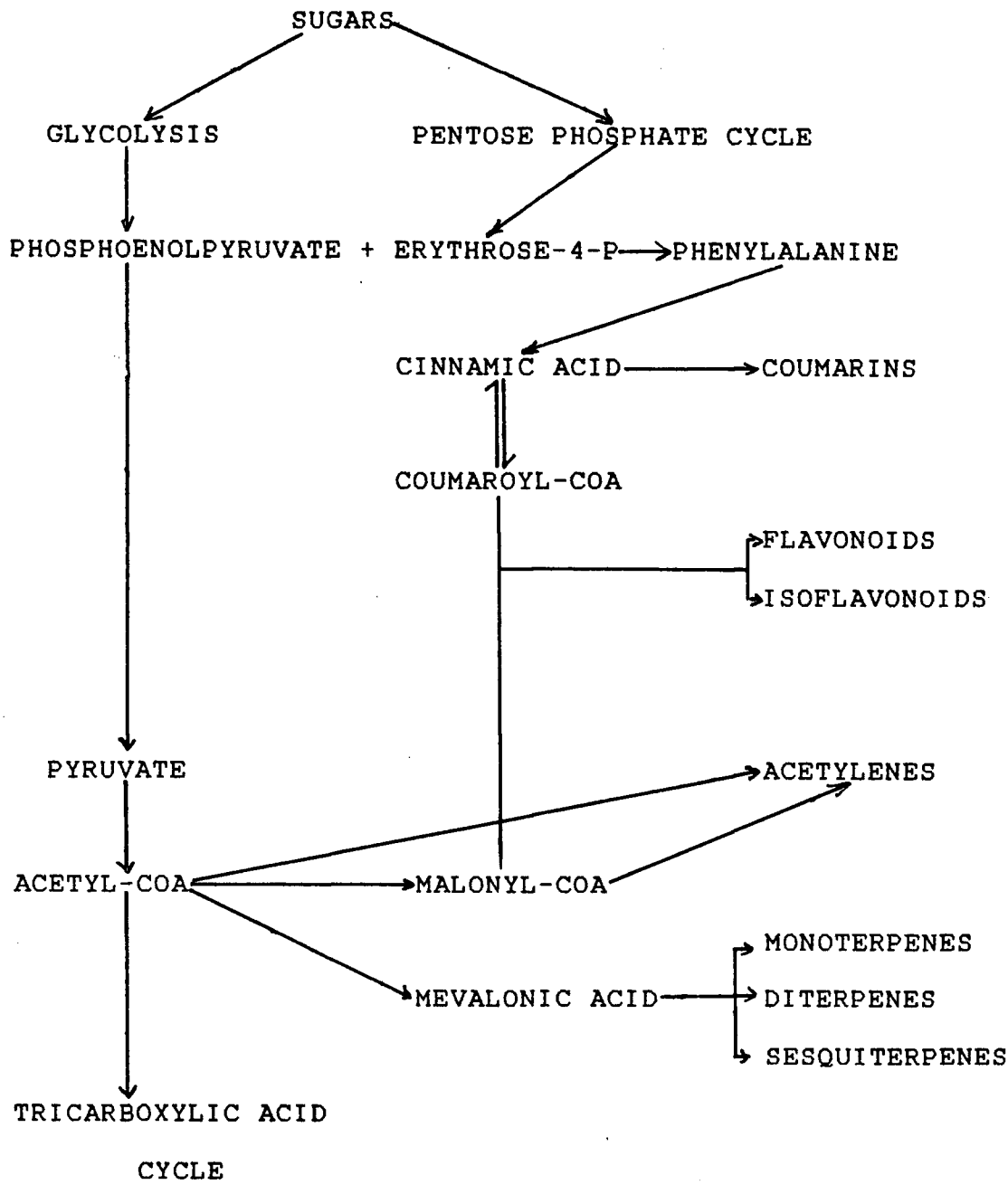

Figure 1-4: Relationship between respiration and synthesis of phytoalexins (adapted from Mansfield, 1983).

1.1.4.3 Structure-activity relationship

Little can be stated definitely concerning the relationship between molecular structure and activity of phytoalexins. A common feature is the hydrophobic nature of phytoalexins, which probably enhances effective penetration of fungal membranes (Smith, 1982). A hydrophobic side chain is essential for antifungal activities of wighteone (Ingram et al., 1977), and kievitone (Smith, 1978).

In the investigation of structure-activity patterns of vignafuran analogues, Carter et al. (1978) demonstrated that presence of one phenolic hydroxyl in the molecule was vital for activity, since fully methylated analogues were inactive. The toxicity of some isoflavonoid phytoalexins was postulated to be dependent on steric and compositional requirements: the two aromatic rings must be almost perpendicular to one another and small oxygen-containing substituents must be present (Perrin and Cruickshank, 1969). However, these results were disputed by subsequent research (VanEtten, 1976). The relationship between structure and toxicity of antifungal compounds requires clarification.

1.1.4.4 Modes of action

Among many studies of phytoalexins, there have been few

attempts to determine the modes of action. The precise mode of action of any phytoalexin remains to be defined. The available information suggests two possible modes of activity: phytoalexins are multi-site toxicants, and damage of membrane systems is instrumental in their activity.

Multi-site activity of phytoalexins offers advantages to plants. Site-specific compounds are more easily countered by fungi as small mutations may be sufficient to induce a change at the site of action and confer resistance (Smith, 1982). Phytoalexins could cause such damage as disorganization of cellular components in fungi after a few minutes' treatment (Harris and Dennis, 1977). Selective inhibition of one particular metabolic process seems unlikely, since cellular damage would not be apparent in a short time after treatment.

Evidence of toxicological effects of phytoalexins indicate that membrane dysfunction plays an important role in activity. Harris and Dennis (1977) reported that treatment of three Phytophthora species with terpenoids resulted in swelling of the cell cytoplasmic granulation, bursting of the cell membrane and loss of cellular contents. These cytological changes were comparable to changes observed after treatment of fungal cells with the membranolytic agent Triton X-100. Membrane damage is

reflected in leakage of electrolytes and metabolites, which inevitably leads to loss of mycelial dry weight (VanEtten and Bateman, 1971). Membrane damage adversely affected substrate intake: glucose uptake by germinating spores of Stemphylium botryosum was inhibited after phytoalexin treatment (Higgins, 1978).

For many phytoalexins, there may not be only one site of action but many targets. A compound which induces membrane damage may also affect other reactions, resulting in gross fungistatic or fungicidal effects.

1.1.5 Methodology in yeast susceptibility testing with antifungal compounds

When investigating the inhibition of yeast growth by phytoalexin(s) which may accumulate in blueberries during MAP storage, an assay which can provide meaningful information is required.

Since the phytoalexin concept evolved from plant resistance to mold infections, studies on phytoalexin toxicity have been carried out almost exclusively with molds. These assays included mycelial growth on agar surfaces (Skipp and Bailey, 1977), growth of mycelia in liquid media (VanEtten and Bateman, 1971), growth of germ tubes (Harris and Dennis, 1977; Higgins, 1978).

Methodology for testing susceptibility of yeasts is based mainly on clinical tests with antifungal drugs used

to treat human infections. Three procedures, broth dilution, agar dilution and disk diffusion have been recommended for yeast susceptibility testing (Shadomy and Espinel-Ingroff, 1980). At present, there is no standardized method of testing the susceptibility of yeasts to antifungal agents.

1.1.5.1 Broth dilution method

In broth dilution method, antifungal compounds are incorporated into a series of tubes containing a liquid medium to achieve a range of concentrations. After inoculation and incubation, the tubes are examined for the minimum inhibitory concentration (MIC). MIC is defined as the lowest concentration of drug which inhibits visible turbidity. This visual end-point has the advantage of simplicity, however, it entails some serious problems. Visual end point determination requires a subjective judgement, and variation between observers is an obvious problem. In addition, attempts to distinguish "almost no visible growth" to "complete absence of visible growth" can be difficult and erroneous. Another drawback is dependence of MIC on inoculum size: MIC for some drugs increased with heavy inocula (Galgiani and Stevens, 1976 and 1978). The inoculum-dependence of visual end point method is illustrated in Figure 1-5. The basis of this

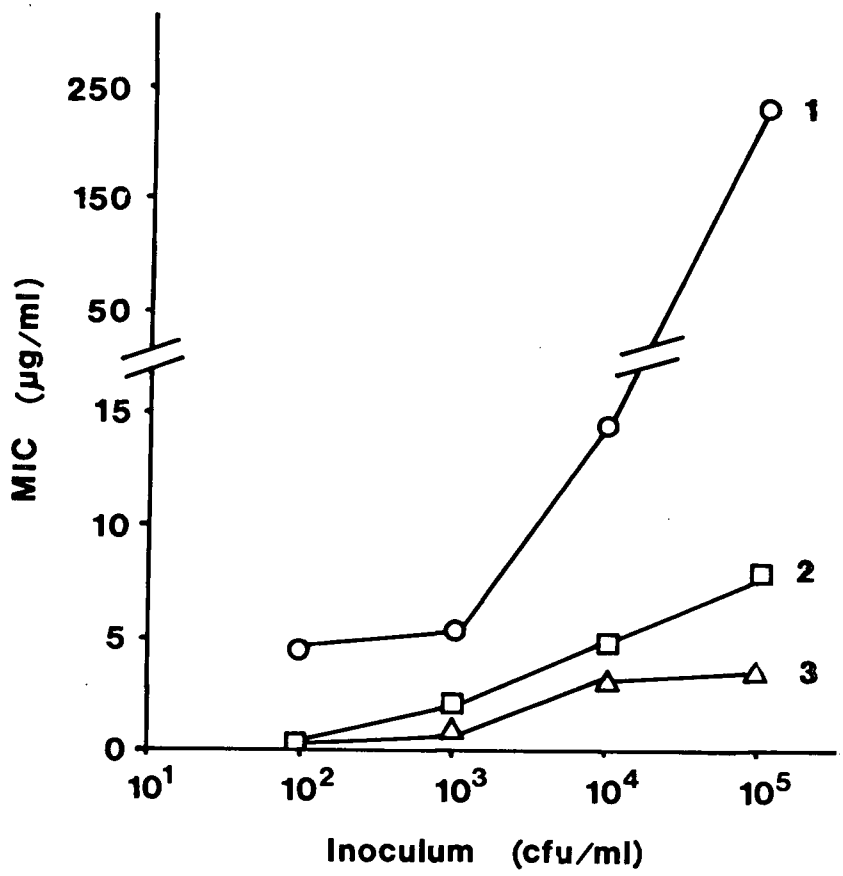

Figure 1-5: MIC of 5-fluorocytosine at different inoculum sizes for three yeast strains (Galgiani and Stevens, 1976). Curves 1: Candida albicans, 2: Candida tropicalis, 3: Torulopsis glabrata.

problem is the use of visual turbidity as an index of growth. At the time set for reading the end point, tests with large starting inocula will have more tubes in the dilution series reaching visible turbidity than tests using the same drug concentrations but starting with smaller inocula. This is because fewer doublings are required for a large inoculum to become turbid. At time of test reading, a small inoculum would have fewer turbid tubes, and the first clear tube would occur at a lower drug concentration than in the tubes with a large inoculum. Therefore, a high drug concentration is necessary to keep tubes with a large inoculum from reaching visual turbidity within a fixed time of end point reading. Galgiani and Stevens (1976) replaced visual turbidity by spectrophotometric measurements to determine growth inhibition in a broth dilution method. They calculated the inhibitory concentration (IC) as the lowest drug concentration that met the criterion:

$$\% T \geq \% T_{control} + n(100 - \% T_{control})$$

where control = drug-free tube

% T = percent transmission

n = a selected fraction less than 1

This formula defines a fraction of inhibition (set by

n) as a function of turbidity in drug-free controls. This end point determination is independent of inoculum size, it eliminates subjectivity, and variation among observers.

1.1.5.2 Agar dilution method

Broth dilution tests can be adapted for use with agar media. In this method, a yeast isolate is inoculated into a series of agar plates containing different dilutions of an antifungal drug. Inoculated drug-free plates serve as controls. MIC is defined as the lowest concentration of drug preventing macroscopic formation of colonies.

Dependence of MIC on inoculum size is also a problem in this method (Stevens, 1984). MIC increases with heavy inocula. Tests with large starting inocula have more plates showing visible colonies than tests with small inocula, at time of reading results. This is because a colony starting with a small number of cells will not become visible at the same time as a colony starting with a large cell number. A solution to this problem would be to define MIC as the drug concentration which reduces the number of colony forming units on test plates to a preset fraction of the number of colony forming units on drug-free control plate. The application of this calculation has not been reported.

1.1.5.3 Disk diffusion method

In this method, a high inoculum of a yeast isolate is spread on surface of agar plates to form a lawn of dense growth. Antifungal compounds are impregnated into assay disks, which are then placed on the agar surface. Growth inhibition by an antifungal compound is shown by a clear zone around the corresponding disk. The diameter of inhibition zone is influenced by diffusion and concentration of antifungal compounds and sensitivity of yeast isolates.

The disk diffusion method has the advantage of rapidity, the ease of performance, and economy. Several disks containing different concentrations of a compound or drug-free control could be tested on one plate. Dependence of test results on inoculum size is largely eliminated, because a high starting inoculum is required to achieve a lawn of dense growth.

Clinical use of the disk diffusion method for yeast susceptibility testing has become popular. Commercially-prepared disks of antifungal drugs are now available in Europe (Kostiala and Kostiala, 1984).

1.2 SUMMARY OF LITERATURE REVIEW AND OBJECTIVES OF THE STUDY

1. The shelf-life of fresh blueberries is limited primarily by mold decay. C. gloeosporioides, B. cinerea, and A. tenuis are common spoilage organisms of blueberries. MAP storage may be used to inhibit mold decay and extend the shelf-life of blueberries. In previous MAP storage experiments, yeast spoilage of blueberries was also absent, although many yeast species can grow at low temperature and also have the ability to ferment sugars in anaerobic condition.

The growth trend of yeasts, molds and bacteria, the headspace atmosphere of packages of modified atmosphere packaged blueberries, and berry pH, soluble solids content will be followed during MAP storage.

2. Yeast inhibition may be attributed to the production of antifungal compound(s) by blueberry fruit in response to low temperature and anaerobic environment. A variety of stress metabolites are known to accumulate in plants exposed to stresses such as chilling and cut injury, UV radiation, and fungal infection. Some stress metabolites exhibit fungicidal or fungistatic activities to protect plants against microbial invasion. Extracts of

blueberries held under MAP storage will be tested for antifungal activity.

3. Difficulties involved in *in vitro* assay arise because there are no standardized procedures for testing yeast susceptibility, and the structure of any antifungal compound, if present, is unknown.

The disk diffusion method will be used because it is rapid, and results of this method are relatively independent of starting inoculum size. In addition, this method is more easily adapted for use with modified atmospheres to mimic MAP storage than the broth dilution method, and it is less time and labour consuming than the agar dilution method.

CHAPTER 2

EXPERIMENTATION, RESULTS AND DISCUSSION: MODIFIED ATMOSPHERE PACKAGING STORAGE OF BLUEBERRIES.

2.1 ANALYSES OF BLUEBERRIES IN MAP STORAGE

In initial studies, yeast and mold, and total plate counts were followed with time during storage of blueberries, to establish the microbial growth trends. Finally, headspace composition, fruit pH, and soluble solid contents were determined in addition to enumeration of microorganisms on blueberries.

2.1.1 MATERIALS AND METHODS

In 1986, two blueberry varieties Bluecrop (mid-season) and Jersey (late season), were used in separate storage trials. Blueberries were purchased from the B.C. Blueberry Co-op (Richmond, B.C.). In each trial, all berries were pooled to form one population before packaging. Approximately 50 g of berries were packaged in each 20 cm x 12 cm bag. Two storage conditions of low-oxygen and intermediate-oxygen were established using packaging films whose transmission properties are shown in Table 2-1. All MA packages were heat sealed; in the control (air) condition, berries were stored in unsealed plastic bags of the type frequently used for fruit and vegetable packaging at homes and retail stores. After packaging, the bags were stored at 4°C.

Two samples from each MAP storage condition and control

Table 2-1: Properties of the packaging films.

Film	Transmission rates[1]			M.V.T.R.[2]
	Nitrogen	Oxygen	Carbon dioxide	
High Barrier	0.015	0.05	0.3	0.3
Intermediate Barrier	22	83	420	0.6

[1] expressed as cm^3 / $100in^2$ / 24 hours / atm
[2] Moisture Vapor Transmission Rate, g/100 in^2 / 24 hours at 95% Relative Humidity.

were used to determine the total aerobic plate count, anaerobic plate count, and yeast and mold counts every week for six weeks and every two weeks for six more weeks. Twenty-five grams of berries were stomached in 225 ml of (0.1%) peptone water with a Colworth Stomacher; 1 ml-samples of appropriate dilutions were filtered through the Hydrophobic Grid Membrane Filter (HGMF), which was housed in the Isogrid Filtration units (QA Laboratories, Toronto, Ont). The HGMF is a polysulfone membrane filter (0.45 µm), on which is printed a grid of hydrophobic material. The hydrophobic grid prevents spreading of mold colonies. After filtration, the filters were placed on appropriate media for incubation. Enumeration media included: Plate Count agar (PCA) with 0.005% Congo Red dye for aerobic or anaerobic plate counts, PCA supplemented with 0.01% chloramphenicol and 0.01% chlortetracycline HCl for yeast and mold counts, PCA supplemented with 0.01% chloramphenicol, 0.01% chlortetracycline HCl and 0.5% sodium pyruvate for yeast and mold counts. The latter medium was used because pyruvate might have a beneficial effect on yeast and mold enumeration (Koburger, 1986). The two media for yeast and mold counts contained 0.01% Trypan Blue so that colonies appeared blue against the black and white background of filters. PCA was purchased from Difco Co., Detroit, MI, while all antibiotics and

dyes were obtained from Sigma Chemicals, St. Louis, MO. Plates for the determination of anaerobic plate counts were incubated in an anaerobic jar (Oxoid, Nepean, Ont), and anaerobic environment was achieved using the Gas-Generating kit and anaerobic catalyst (Oxoid, Nepean, Ont). All plates were incubated at 21°C for 4 days, except plates incubated anaerobically (7 days, 21°C).

Most probable numbers (MPN) were obtained by the formula (Sharpe and Michaud, 1975):

$$MPN = 1600 \times \ln [1600 / (1600 - X)]$$

where X = total number of squares containing colonies in a filter which has a maximum of 1600 squares.

Geometric means of transformed (log base 10) MPN from antibiotic-supplemented PCA and antibiotic-pyruvate-supplemented PCA were compared by paired t-test.

In 1987, one MAP storage trial was set up. Bluecrop blueberries were stored in two conditions: intermediate-oxygen and low-oxygen content; air storage was omitted. The packaging films, procedure and storage temperature were identical to those used in the previous trials. Aerobic and anaerobic plate counts, yeast and mold counts, fruit pH, soluble solid content, and headspace gas

analysis were carried out at two-week intervals for 12 weeks.

Four samples from each storage condition were used for microbial counts. The media and plating procedure were identical to those used in the previous trials, except for the elimination of antibiotic-pyruvate-supplemented medium. For anaerobic plate counts, the Gas-Generating kit was replaced by air evacuation followed by flushing of the anaerobic jars with a gas mixture (10% carbon dioxide, 10% hydrogen, 80% nitrogen).

Composition of headspace atmosphere was obtained from four samples of each storage condition. The analyses were carried out using a gas chromatograph (Shimadzu GC-9A) equipped with a thermal conductivity detector, and a Carbosieve S-II column (Supelco, Oakville, Ont), with helium carrier gas (30 ml/min). The temperature was programmed to hold for 7 minutes at 35°C, then increased to 225°C at 32°C/min. Peak areas were integrated by a Shimadzu C-R 3A Chromatopac integrator.

Four bags from each storage condition were sampled, and internal pH of 10 berries from each bag were measured using a surface electrode (Fisher Scientific, Vancouver, B.C.). When berry exudates were present, their pH was also measured.

Four bags from each storage condition were sampled for

soluble solids. Berries were homogenized with a Waring blender (half speed, 1 min), and centrifuged (4,080 x g, 15 min, 20°C) to clarify juice. Soluble solid contents were determined with a refractometer (Bausch and Lomb, Rochester, NY) operated at 20°C.

2.1.2 RESULTS AND DISCUSSION

2.1.2.1 Headspace atmosphere of packages with low gas permeability

Changes in headspace atmosphere of low-permeability packages are presented in Table 2-2.

Since the packaging film had a very low transmission rates for oxygen, nitrogen and carbon dioxide, diffusion of these gases across the film can be considered negligible relative to berry respiration rates, and changes in the headspace composition are the result of fruit respiration.

During the process of energy utilization, carbohydrates provide readily available energy and are preferred starting materials. Glucose catabolism begins with glycolysis, a sequence of reactions which results in the conversion of one glucose molecule to two molecules of pyruvate (Section 1.1.3.1). The overall process of glycolysis occurring in plant tissues is similar to glycolytic breakdown of glucose by yeasts and other organisms.

Table 2-2: Headspace gas composition of blueberry packages. The high barrier film was used.[1]

Time (weeks)	Headspace composition (%)		
	Oxygen	Nitrogen	Carbon dioxide
0	21.28 (0.17)	78.72 (0.17)	0
2	0.82 (0.19)	52.87 (5.32)	46.30 (5.49)
4	0.65 (0.18)	42.80 (3.69)	56.55 (3.76)
6	0.41 (0.10)	38.45 (4.11)	61.14 (4.62)
8	0.63 (0.16)	34.58 (2.14)	64.79 (1.87)
10	0.42 (0.11)	32.68 (1.37)	66.93 (1.39)
12	0.48 (0.14)	30.56 (1.32)	68.96 (1.55)

[1] Data represent the means of four replicates and the sample standard deviations are shown in brackets.

The fate of pyruvate depends primarily on the availability of oxygen. In the presence of oxygen as an electron acceptor, pyruvate is channelled in the direction of aerobic respiration. The distinguishing characteristics of aerobic respiration are: (1) oxygen is the ultimate electron acceptor, (2) the complete oxidation of pyruvate to carbon dioxide and water, and (3) the efficient conservation of free energy as ATP.

Oxygen serves as the terminal electron acceptor to provide for the continuous reoxidation of reduced coenzyme molecules. These coenzyme molecules (in their oxidized form) carry out the stepwise oxidation of intermediates derived from pyruvate. The reoxidation of these reduced coenzymes by transfer of electrons to oxygen is accompanied by the generation of ATP. The overall equation for aerobic respiration can be written as:

$$C_6H_{12}O_6 + 6\ O_2 + 38\ ADP + 38P_i = 6\ CO_2 + 6\ H_2O + 38\ ATP$$

The low permeability of the packaging film for oxygen restricted its diffusion into the storage bags from the atmospheric air, so that oxygen consumed by aerobic respiration could not be replenished. Oxygen level became limiting after two weeks of storage, and thus berries must have switched to anaerobic respiration. Ethanol is the

most common end-product of anaerobic respiration in plants (Devlin and Witham, 1983). Pyruvate is decarboxylated to acetaldehyde which acts as an acceptor molecule in the re-oxidation of NADH to regenerate NAD. The overall equation for anaerobic respiration of glucose by plants is written:

$$C_6H_{12}O_6 + 2\ ADP + 2P_i = 2\ C_2H_5OH + 2\ CO_2 + 2\ H_2O + 2\ ATP$$

During anaerobic respiration of plants, lactic acid could also appear as end-product. This mechanism for anaerobic reoxidation of NADH involves reduction of the carbonyl group of pyruvate to form lactate. Lactic acid formation may protect plants from ethanol poisoning (Zemlianukhin and Ivanov, 1978):

$$C_6H_{12}O_6 + 2\ ADP + 2P_i = 2\ C_3H_6O_3 + 2\ H_2O + 2\ ATP$$

The continuous increase of carbon dioxide content in storage packages over time (Table 2-2) indicated that ethanol formation might be the predominant mode of anaerobic respiration by berries, since lactic acid production involves no net gain of carbon dioxide.

The changes in oxygen and carbon dioxide contents in packages are expected, because these gases participate in berry metabolism. The change in nitrogen content was

unexpected since berries can not metabolize this inert gas. The model presented in Figure 2-1 helps to explain the observed change in nitrogen.

Figure 2-1 (a) shows a hypothetical atmosphere of a closed system where there is no gas exchange with the environment. When carbon dioxide is introduced into the system (Figure 2-1 b), nitrogen content expressed as percent of total volume is decreased although its volume was not changed. In Figure 2-1 (c), more carbon dioxide is added and further depression of nitrogen percent is observed. In both Figure 2-1 (b) and (c), oxygen volume was small relative to the other gases so its depression caused by carbon dioxide was not dramatic.

The changes in headspace composition of packages with low gas permeability were similar to the situation demonstrated in Figure 2-1. Carbon dioxide produced by berry respiration was retained in the storage bags, which led to an increase in total headspace volume. The increase of carbon dioxide quantity caused an apparent decrease in percent nitrogen in the headspace, even though the actual quantity of nitrogen remained unchanged with time.

2.1.2.2 Headspace atmosphere of packages with intermediate gas permeability

In these packages, diffusion of permanent gases across the packaging film must be considered in relation to

(a)	(b)	(c)
80 ml N_2 (80%)	80 ml N_2 (72%)	80 ml N_2 (66%)
20 ml O_2 (20%)	1 ml O_2 (0.9%)	1 ml O_2 (0.8%)
	30 ml CO_2 (27%)	40 ml CO_2 (33%)

Figure 2-1: A model of headspace composition in the high barrier film packages. (a) a closed system with starting headspace composition; (b) carbon dioxide is added, and oxygen is taken out; (c) more carbon dioxide is added with no change to other gases.

changes in the headspace atmosphere.

Aerobic respiration of berries caused the decrease of oxygen content and an increase of carbon dioxide during storage (Table 2-3). The decrease in oxygen level reflected the shortfall between oxygen consumption by berries and inward oxygen diffusion through the packaging film. If berry respiration was considered alone, then one mole of carbon dioxide would be produced per mole of oxygen consumed. However, at low storage temperature, solubility of carbon dioxide in aqueous solutions within the berry tissues increased and a portion of carbon dioxide produced by respiration would be dissolved in tissues, while a part would be liberated into the package headspace. The increase of carbon dioxide quantity in the headspace created a gradient of higher carbon dioxide concentration inside the packages than in air. Carbon dioxide inside the bags then had a tendency to diffuse outward according to this gradient. The rate of its diffusion was influenced by the permeability of the packaging film. Therefore, the quantity of carbon dioxide observed in the package headspace was the net difference between total carbon dioxide produced by respiration and the sum of dissolved carbon dioxide in fruit tissue and the quantity lost by diffusion through the packaging film.

Total pressure of permanent gases in fruit tissues,

Table 2-3: Headspace gas content of blueberry packages. The intermediate barrier film was used [1].

Time (weeks)	Headspace composition (%)		
	Oxygen	Nitrogen	Carbon dioxide
0	21.28 (0.17)	78.72 (0.17)	0
2	18.04 (2.23)	80.92 (2.33)	1.04 (0.23)
4	12.52 (3.03)	84.91 (1.97)	2.57 (0.77)
6	9.48 (3.06)	86.94 (3.29)	3.54 (0.64)
8	7.77 (1.53)	89.16 (1.12)	3.07 (0.44)
10	7.38 (1.12)	88.33 (2.80)	4.29 (1.60)
12	6.32 (1.10)	89.42 (2.60)	4.26 (1.53)

[1] Data represent the means of four replicates and the sample standard deviation are shown in brackets.

which is the sum of partial pressures of oxygen, nitrogen and carbon dioxide, must be equal to the total pressure of gases in the headspace (Figure 2-2).

As shown in Table 2-3, the change (decrease) in oxygen content was larger than the change (increase) of carbon dioxide in the headspace, due to outward diffusion of carbon dioxide and its increased solubility in berry tissue. This difference created an imbalance in total pressure of gases between fruit tissue and headspace. Nitrogen would be exchanged from intercellular spaces of the fruit tissue, and possibly dissolved nitrogen, to gaseous nitrogen in the headspace, in order to balance total pressure between fruit tissue and headspace. This exchange resulted in an increase of nitrogen content in headspace.

2.1.3 pH of blueberries in MAP storage

Growth of bacteria is profoundly influenced by pH: there usually is a maximum and minimum limit for growth and an optimum range for each species. Yeasts and molds are generally less sensitive to low pH than bacteria (Koburger and Marth, 1984). pH also affects the activity of antimicrobial compounds. This may be an effect on stability or binding to target sites (Stevens, 1984).

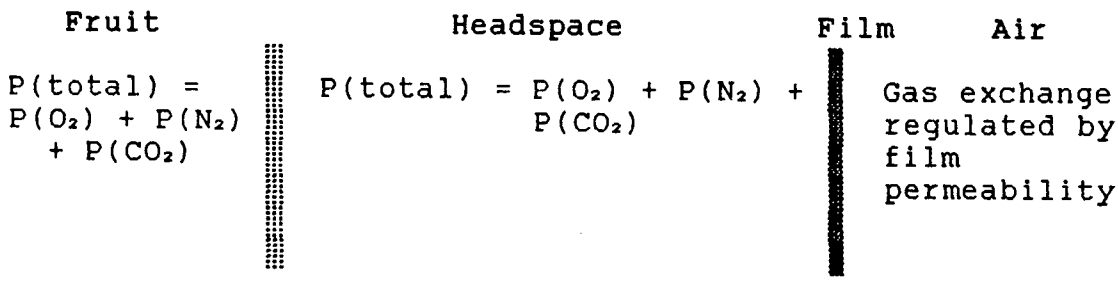

Figure 2-2: Gas exchange between fruit tissues, headspace of package and environment.

In a low oxygen environment (packages of low gas permeability), pH of the berries remained stable over 12 weeks (Table 2-4). Anaerobic respiration of berries led to production of ethanol, carbon dioxide, and possibly a small amount of lactic acid. A part of the carbon dioxide produced was released into the package headspace, and a small part dissolved in berry tissues as carbonic acid. The amount of protons produced by dissociation of lactic acid and carbonic acid would be small since these are weak acids. Weak dissociation of these acids could not cause a pH decrease in berry tissue, because berry juice had an ability to resist pH changes. This buffer capacity is attributed to organic salts, and acid phosphates because of their ability to bind protons.

Berries stored in an intermediate oxygen environment (packages of intermediate gas permeability) also exhibited a stable pH. A portion of carbon dioxide produced by aerobic respiration of berries existed in tissue as carbonic acid, which weakly dissociated to release protons. The buffering agents in berry tissue were able to maintain a stable pH.

Berry exudate was observed after four weeks in intermediate oxygen storage, and no exudate was present in the low oxygen packages. Exudate is probably a result of

Table 2-4: pH of blueberries and exudates during MAP storage.

Time (weeks)	pH (cut berries)[1]		pH (exudate)[2]
	Low O_2	Intermediate O_2	Intermediate O_2
0	3.49 (0.38)	3.49 (0.38)	no exudate
2	3.69 (0.49)	3.61 (0.53)	no exudate
4	3.68 (0.41)	3.55 (0.47)	no exudate
6	3.68 (0.47)	3.54 (0.36)	3.24 (0.20)
8	3.50 (0.40)	3.43 (0.35)	3.05 (0.14)
10	3.54 (0.53)	3.49 (0.21)	2.97 (0.17)
12	3.49 (0.48)	3.47 (0.22)	2.99 (0.15)

[1] Data represent the means of pH taken from 40 berries, and the sample standard deviations are shown in brackets.
[2] no exudate was present in low oxygen samples throughout storage time. Data represent the means of exudates in four bags and the sample standard deviations are shown in brackets.

juice leakage from berries due to fruit senescence and autolysis as well as microbial penetration. In intact berries and other aerial organs of plants, the cuticle constitutes the first barrier against invasion of fungi. Plant cuticle consists of the insoluble polymer cutin, embedded in a mixture of waxy materials. Cutin is a polyester composed of C_{16} and C_{18} hydroxy and epoxy fatty acids (Kolattukudy, 1985). Since cutin is the main barrier in the cuticle, it was proposed that penetrating fungi secrete a cutin degrading enzyme, cutinase. Cutinase isolated from B.cinerea and other fungi can hydrolyze cutin into oligomers and monomers (Soliday and Kolattukudy, 1976; Dickman et al., 1982). After breaking the first barrier with cutinase, fungi encounter the cell wall. Therefore, successful penetration requires both cutinase and cell wall degrading enzymes such as cellulase and pectinase. Pectinolytic activity has been found in many yeast genera: Kluyveromyces, Candida, Torulopsis, and Saccharomyces (Luh and Phaff, 1951). Pectin hydrolysis by species of Debaryomyces, Candida, and Rhodotorula which leads to softening of cucumbers and olives has been reported (Bell and Etchells, 1956; Vaughn et al., 1969). The hydrolytic activity of cutinase, pectinase and cellulase on cutin and cell wall might contribute to

leakage of juice from berries.

In contrast to intact berries where microbial growth is largely confined to berry surface with limited nutrient supply, the pool of exudate provided a rich growth medium. Utilization of exudate sugars by microorganisms would proceed initially via aerobic oxidation pathway, until rapid respiration led to depletion of oxygen, which has a low solubility in non-aerated solutions. When oxygen tension is low, yeasts and bacteria which have the ability to ferment sugars would utilize this pathway to generate energy and metabolic precursors. Fermentation of exudate sugars by microorganisms produced ethanol, carbon dioxide and other compounds such as glycerol, acetic acid and lactic acid. Many yeasts could also switch to fermentative metabolism when a large concentration of sugars is available in the medium, even under aerobic conditions (Sols et al., 1971). This impairment of respiratory capability is known as the "Crabtree effect"; its mechanism is not well understood. The "Crabtree effect" might operate in some yeast species, and contribute to fermentation of exudate sugars. If the buffering salts of exudate were exhausted to balance protons dissociated from carbonic acid produced by berry metabolism, then further dissociation of acid products of microbial fermentation would cause a decrease of exudate

pH.

2.1.4 Soluble solid contents of blueberry fruit

Table 2-5 shows refractive index values expressed as soluble solid contents of blueberries during MAP storage. Soluble solid contents of blueberries remained at the same level throughout storage in packages with low oxygen or intermediate oxygen atmosphere. El-Kazzaz et al. (1983) also reported no significant difference in soluble solid contents among strawberries in several controlled-atmosphere treatments.

During berry metabolism, carbohydrates are not always broken down completely for synthesis of energy-rich ATP molecules, instead they can serve as precursors to intermediates in the synthesis of amino acids, lipids, and pigments (Devlin and Witham, 1983). The compounds derived from oxidation of carbohydrates supply the building blocks for other compounds. Soluble solid contents of berries remained at the same level during storage most likely as a result of the dynamic interaction between anabolism and catabolism in tissues.

During infection, microorganisms produced enzymes which hydrolyzed the components of berry cell walls. Evidence for this process was the presence of berry exudates in the intermediate-oxygen packages (see Table 2-4). Hydrolysis of cell wall polysaccharides could result in release of

Table 2-5: Soluble solid contents of blueberry fruit in MAP storage.

Time (weeks)	Soluble solids (as % sucrose)[1]	
	Low O_2	Intermediate O_2
0	12.3 (0.4)	12.3 (0.4)
2	12.0 (0.2)	12.0 (0.3)
4	11.6 (0.3)	11.8 (0.2)
6	11.8 (0.3)	11.4 (0.2)
8	11.8 (0.2)	11.2 (0.3)
10	12.0 (0.4)	11.4 (0.1)
12	12.0 (0.4)	11.6 (0.2)

[1] Data represent the means of four replicates and the sample standard deviations are shown in brackets.

sugars which might cause an increase in soluble solid contents of blueberries. The soluble solid contents of berries taken from the intermediate-oxygen packages did not increase, probably because sugars of berry cell wall were utilized by microorganisms.

2.1.5 Growth of microorganisms on blueberry during MAP storage

2.1.5.1 Anaerobic plate counts

Anaerobic plate counts represented the most probable number of bacteria capable of growing under anaerobic conditions.

In the two storage trials of 1986, anaerobic plate counts showed large fluctuations due to inconsistent performance of the Gas-Generating kit (Oxoid, Nepean, Ont), which produced hydrogen and carbon dioxide. In the presence of palladium catalyst, hydrogen released by the Gas-Generating kit reacts with oxygen in the anaerobic jar to form water; this reaction depletes oxygen and creates an anaerobic atmosphere. This reaction which depends on the rate of hydrogen release and activity of catalyst could sometimes proceed slowly, and result in delay of oxygen depletion. When anaerobic atmosphere was not promptly established in the anaerobic jars, growth of aerobic organisms probably occurred on the Hydrophobic

Grid Membrane filter, causing erratically high MPN values. This problem was overcome in the 1987 storage trial by the use of air evacuation and subsequent flushing of incubating jars with a gas mixture (10% carbon dioxide, 10% hydrogen, 80% nitrogen).

Figure 2-3 shows anaerobic plate counts for blueberries stored in low and intermediate oxygen environments. The increasing growth trend in intermediate oxygen storage, combined with the ability to grow in anaerobic incubation identified these bacteria as facultative aerobes. Facultative aerobes are able to obtain energy by either aerobic respiration or fermentation, and do not require oxygen for biosynthesis (Brock, 1979). These organisms grow better with oxygen because aerobic respiration is far more efficient than fermentation in energy production.

In low oxygen storage, growth increase during the first two weeks was followed by a plateau where counts remained at the same level for 10 weeks. This shift corresponded to the increase in carbon dioxide level of packages. The inhibitory effects of carbon dioxide on anaerobically grown Bacillus cereus and Streptococcus cremoris have been reported by Enfors and Molin (1980). At present there are two proposed mechanisms of carbon dioxide inhibition to bacteria: (1) Carbon dioxide inhibits enzymatic reactions such as carboxylation, decarboxylation which are critical

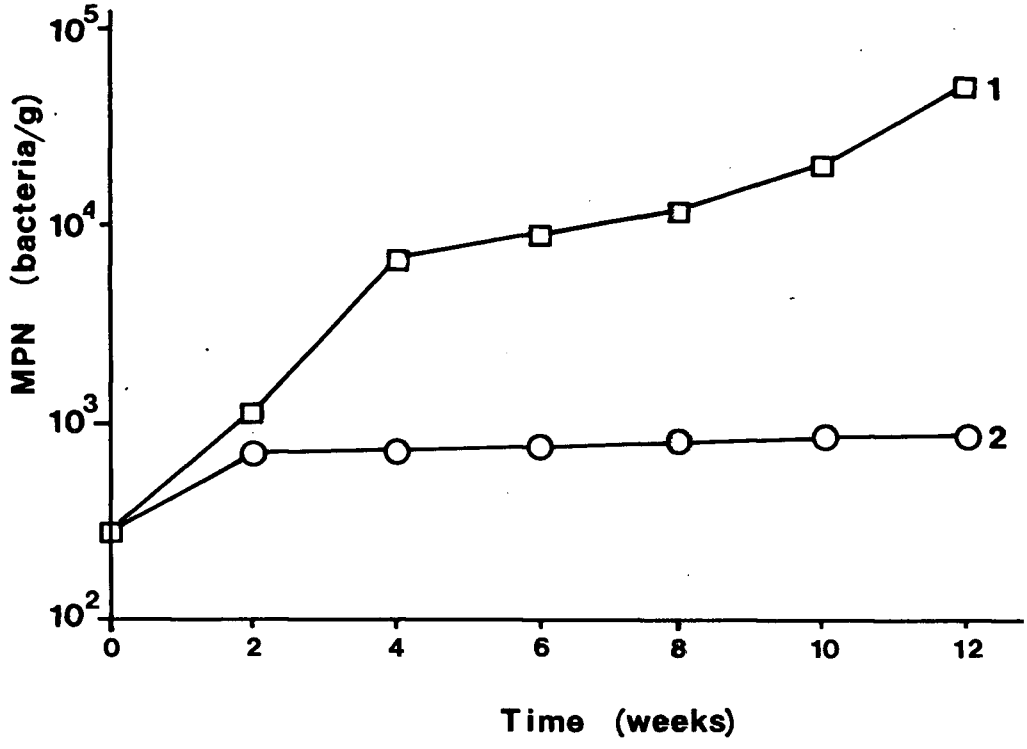

Figure 2-3: Anaerobic plate counts of Bluecrop blueberries in two MAP storage conditions (1987). Curves 1: intermediate oxygen, 2: low oxygen storage. Points represent means of four samples. Geometric means of two storage conditions were significantly different only during 4-12 weeks by Student's t-test.

for growth (King and Nagel, 1975), and (2) Carbon dioxide affects spore germination (Enfors and Molin, 1978). Both mechanisms may operate in different organisms.

2.1.5.2 Aerobic plate counts of blueberries

Aerobic plate counts represented populations of obligate and facultative aerobic bacteria on blueberries. Because of their slow growth and poor competitive ability, yeasts and molds can only develop in media which is unfavorable for bacteria.

Aerobic plate counts of samples from air and intermediate oxygen storage increased up to 8 weeks of storage, followed by a plateau between 8-12 weeks (Figures 2-4, 2-5, 2-6). The growth increase indicated spoilage of the berries, and rapid growth of microorganisms. The plateau showed exhaustion of nutrients, and competition by large numbers of bacteria. Aerobic bacteria in the intermediate oxygen atmosphere reached the plateau at a lower population than those in air. This is probably a result of oxygen limitation in the last six weeks of intermediate oxygen storage (Table 2-3).

Aerobic plate counts of samples from low oxygen atmosphere showed no increase over time. Under the very low oxygen atmosphere, obligate aerobes could not develop because they require oxygen for energy generation and

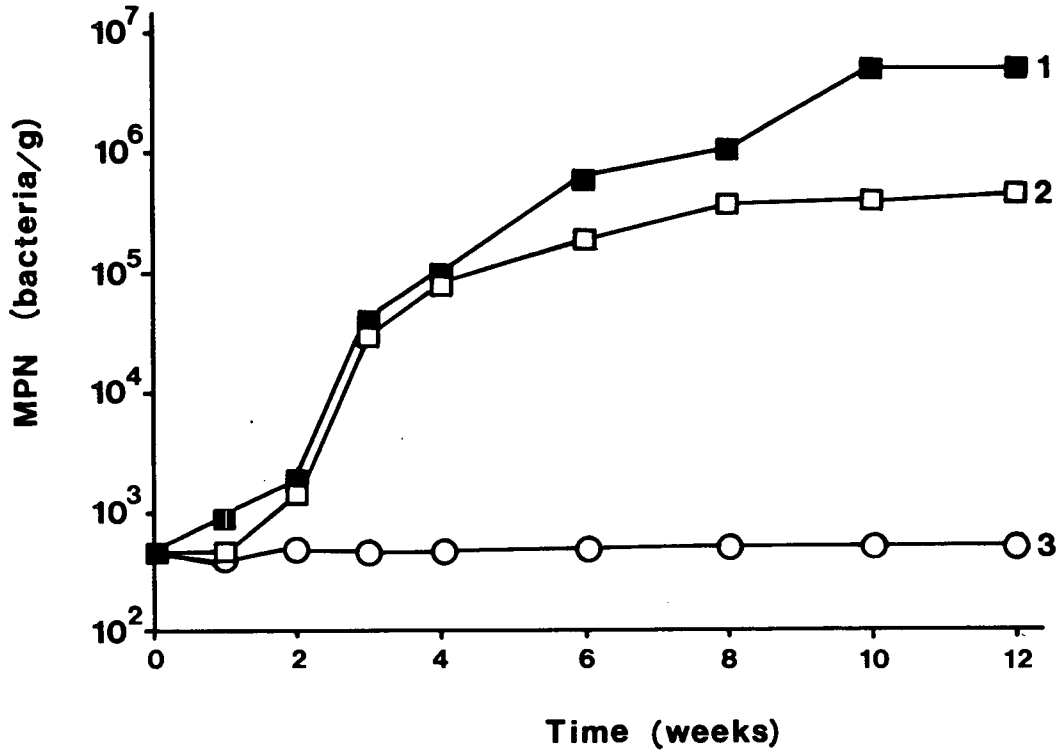

Figure 2-4: Aerobic plate counts of Bluecrop blueberries in three storage conditions (1986). Curves 1: air, 2: intermediate oxygen, 3: low oxygen atmosphere. Points represented means of 2 samples. Geometric means of air and intermediate oxygen samples were significantly different only at 10-12 weeks by Student's t-test ($\alpha = 0.05$). Means of intermediate and air samples were significantly different from means of low oxygen samples at 3-12 weeks by Student's t-test ($\alpha = 0.05$).

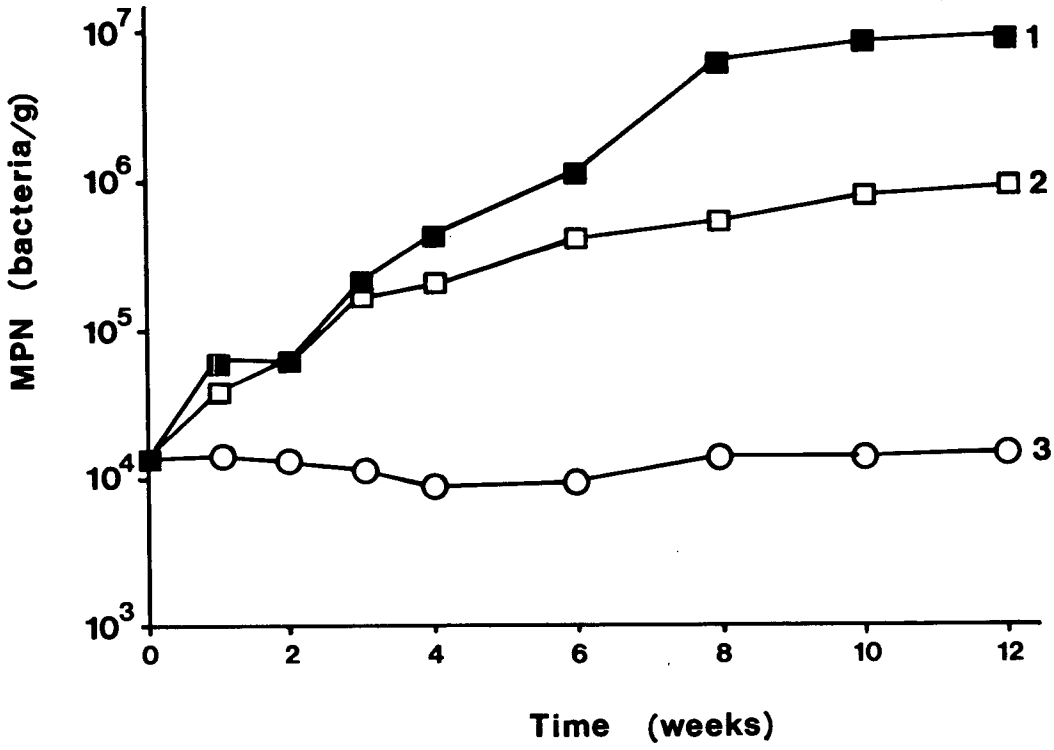

Figure 2-5: Aerobic plate counts of Jersey blueberries in three storage conditions (1986). Curves 1: air, 2: intermediate oxygen, 3: low oxygen atmosphere. Points represented means of 2 samples. Geometric means of air and intermediate oxygen samples were significantly different only at 8-12 weeks by Student's t-test ($\alpha = 0.05$). Means of intermediate and air samples were significantly different from means of low oxygen samples at 3-12 weeks by Student's t-test ($\alpha = 0.05$).

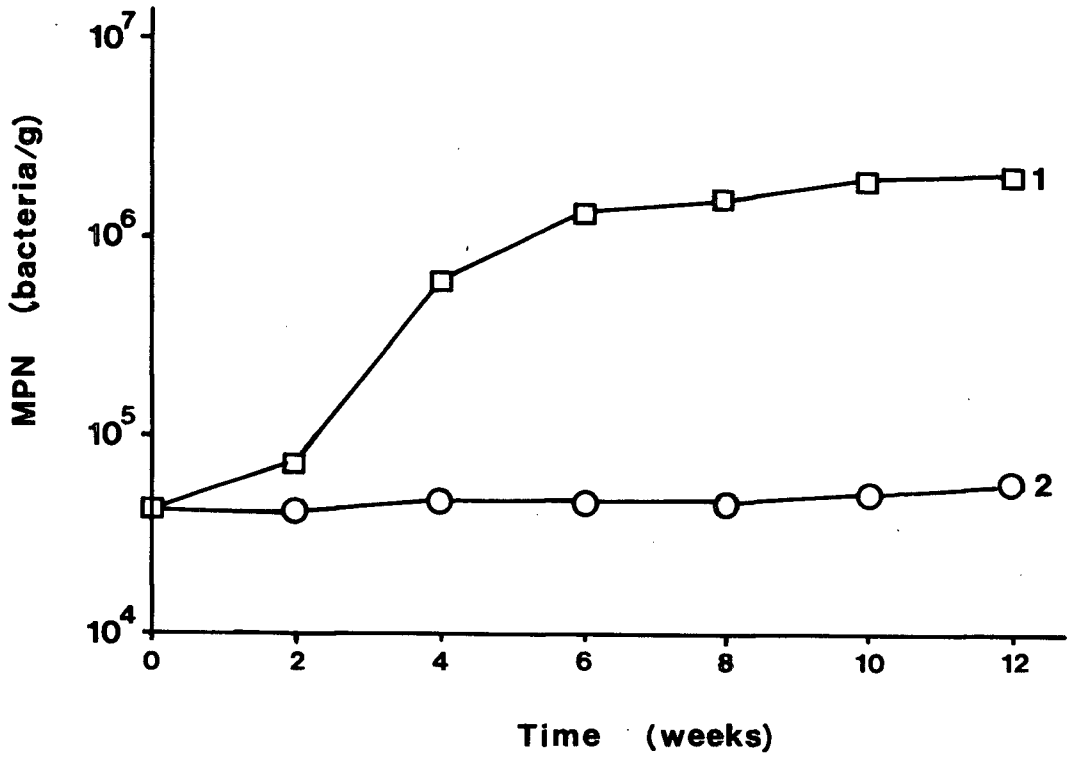

Figure 2-6: Aerobic plate counts of Bluecrop blueberries in two storage conditions (1987). Curves 1: intermediate oxygen, 2: low oxygen atmosphere. Points represented means of 4 samples. Means of intermediate oxygen samples were significantly different from means of low oxygen samples at 4-12 weeks by Student's t-test ($\alpha = 0.05$).

biosynthesis of cellular components. Facultative aerobic bacteria were inhibited by the high carbon dioxide level of this storage condition.

2.1.5.3 Yeast and mold counts

The use of pyruvate-antibiotic-supplemented medium for yeast and mold counts was discontinued after 8 weeks of the 1986 trials, because no significant benefit over the standard antibiotic-supplemented medium was found by paired t-test at the 95% confidence level.

Yeast and mold counts of blueberries from the intermediate oxygen packages reached a plateau at lower counts than counts of samples in air (Figures 2-7 and 2-8), due to the sensitivity of molds to carbon dioxide and low oxygen atmosphere. Svircev et al. (1984) reported that at 7.5% carbon dioxide (17.5% oxygen) germ tubes of B. cinerea were considerably distorted and shorter than germ tubes developed in air. At 4% oxygen, mycelial growth of B. cinerea was only 45%, and that of A. tenuis was 31% compared with growth in air (Wells and Uota, 1970). Under carbon dioxide and the low oxygen atmosphere of intermediate oxygen storage, mycelial growth and sporulation of molds would be less than growth and sporulation in air. This difference explained the smaller

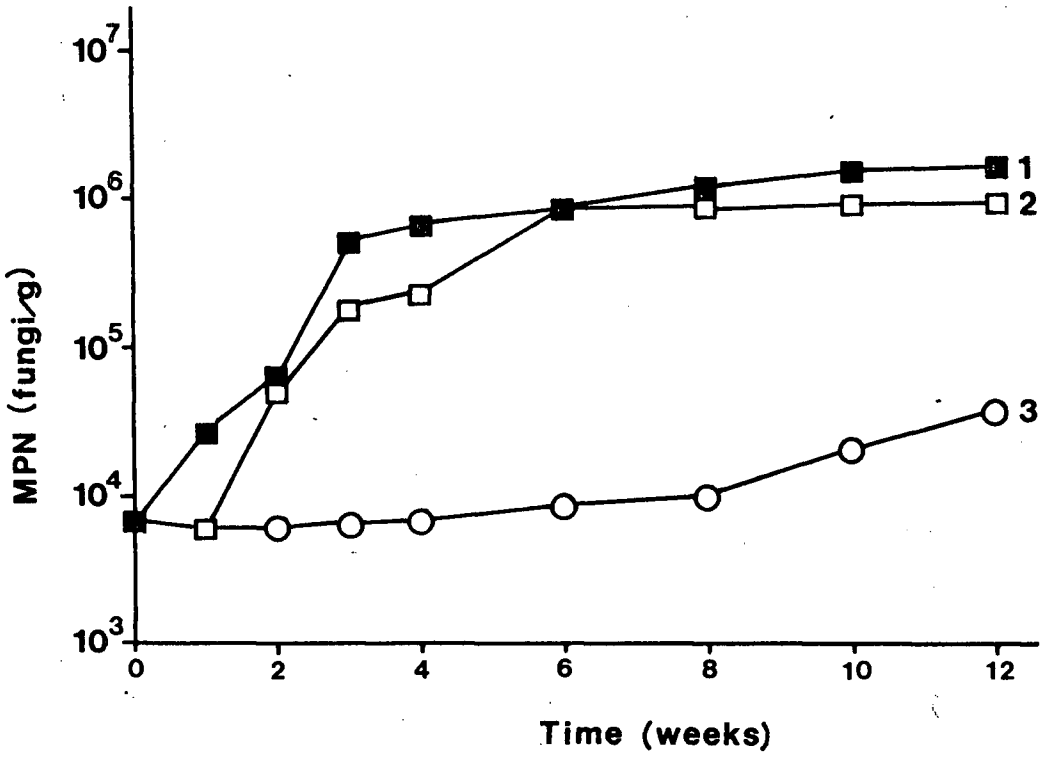

Figure 2-7: Yeast and mold counts of Bluecrop blueberries in three storage conditions (1986). Curves 1: air, 2: intermediate oxygen, 3: low oxygen atmosphere. Points represent means of two samples. Geometric means of air and intermediate oxygen samples are not different by Student's t-test ($\alpha = 0.05$). Means of air and intermediate oxygen samples were different from means of low oxygen samples during 2-12 weeks by Student's t-test ($\alpha = 0.05$).

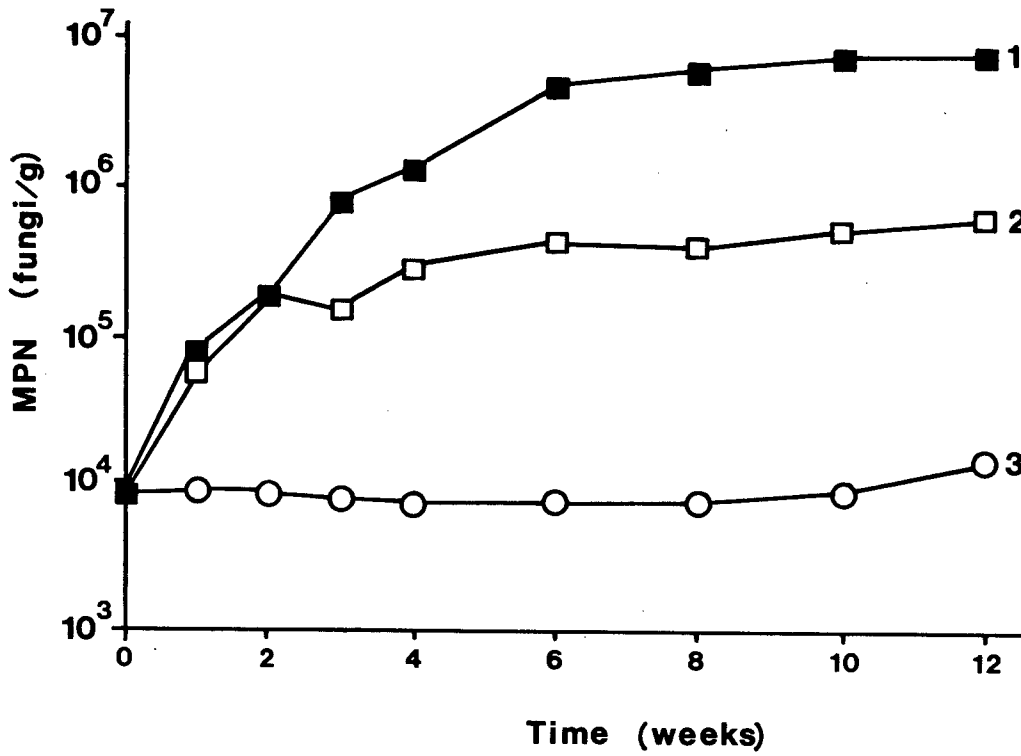

Figure 2-8: Yeast and mold counts of Jersey blueberries in three storage conditions (1986). Curves 1: air, 2: intermediate oxygen, 3: low oxygen atmosphere. Points represent means of two samples. Geometric means of air and intermediate oxygen samples are significantly different between 4-12 weeks by Student's t-test ($\alpha = 0.05$). Means of air and intermediate oxygen samples were different from means of low oxygen samples during 1-12 weeks by Student's t-test ($\alpha = 0.05$).

yeast and mold counts from blueberries stored in the intermediate oxygen environment than from blueberries stored in air. However, there was no difference between these counts in a trial with Bluecrop (mid-season) berries (Figure 2-7). In the trial where differences of yeast and mold counts existed between air and intermediate oxygen storage, Jersey (late season) variety was used. The discrepancy between two trials may be due to different mold species making up the flora present on the surface of blueberries. C. gloeosporioides, B. cinerea, and A. tenuis were the predominant molds isolated from blueberries grown in New Jersey, U.S.A (Cappellini et al., 1972). Those authors reported an interesting relationship between these organisms: infections by these molds occurred at almost equal frequencies in berries picked early in the season, but during late season the occurring frequency of C. gloeosporioides was much higher than B. cinerea and A. tenuis. A direct comparison between results obtained from blueberries grown in New Jersey and local blueberries is inappropriate; nevertheless, a similar shift of predominant species might alter respective species numbers and contribute to different growth trends of fungi observed for Bluecrop and Jersey blueberries.

Yeast and mold counts from low oxygen blueberry samples were low compared to counts from blueberries stored in intermediate oxygen environment (Figure 2-9). Mycelial growth and sporulation of molds were inhibited by high carbon dioxide and low oxygen environment (Svircev et al., 1984; Wells and Uota, 1970; Follstad, 1966). In all three storage trials, blueberries in the low oxygen atmosphere remained free of mold infection throughout 12 weeks. Yeast growth was expected since some yeast species have the ability to ferment sugars under anaerobic condition. However, no increase was observed in yeast and mold counts up to 6-8 weeks of storage. This observation suggested the presence of an inhibitory mechanism. Since berry pH remained stable during storage, growth inhibition by pH is not applicable. A small increase of yeast growth was observed after 8 weeks. This increase showed a recovery process, and must be considered in relation to the mechanism of yeast inhibition in low oxygen storage.

2.2 IDENTIFICATION OF YEASTS ISOLATED FROM BLUEBERRY

Five yeast species were isolated from local blueberries. Identification to the genus level was carried out using macroscopic and microscopic morphology, ability to ferment sugars, ability to utilize various compounds as sole source of carbon, the mode of vegetative

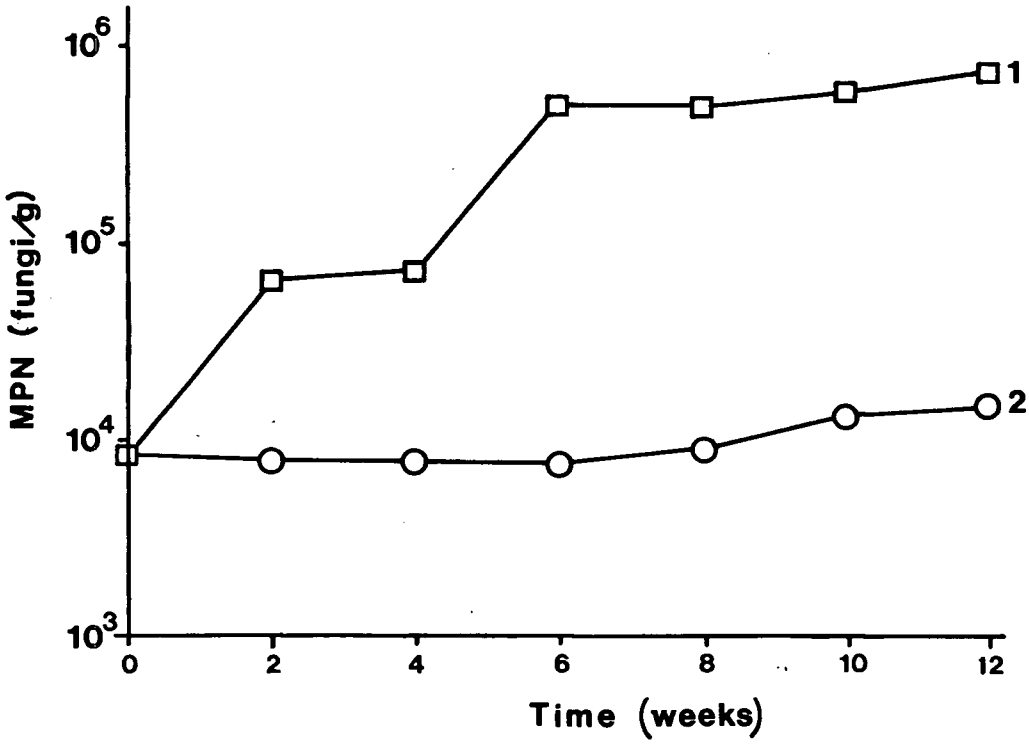

Figure 2-9: Yeast and mold counts of Bluecrop blueberries in two storage conditions (1987). Curves 1: intermediate oxygen, 2: low oxygen atmosphere. Points represent means of 4 samples. Geometric means of intermediate oxygen samples were different from means of low oxygen samples during 2-12 weeks by Student's t-test ($\alpha = 0.05$).

and sexual reproduction, production of ammonia from urea, production of extracellular amyloid compounds, and growth at 37°C (Van der Walt and Yarrow, 1984). The procedures and results of these tests are presented in the Appendix.

Identification was based on genus characteristics reported in the literature (Kregger Van Rij, 1984; Barnett et al., 1983). The yeast species were identified as two Rhodotorula species, a Zygosaccharomyces species, a Cryptococcus species, and a Debaryomyces species.

2.3 CONCLUSIONS

1. The packaging film with intermediate gas permeability allowed some outward diffusion of carbon dioxide produced by berry respiration. As a result, the atmosphere within the storage packages remained aerobic with a relatively low carbon dioxide level.

Yeast, mold and bacterial growth were vigorous in the early part of storage, and were somewhat inhibited by oxygen limitation after 6 weeks of storage. This MAP storage condition could not suppress spoilage of berries, especially mold rot.

2. Packaging blueberries in a film with very low gas permeability created a high carbon dioxide and almost

anaerobic atmosphere.

Oxygen was rapidly consumed by aerobic respiration of berries within two weeks of storage. In an almost anaerobic atmosphere, blueberries began anaerobic respiration to generate energy with ethanol, carbon dioxide, and possibly lactic acid as end-products. Anaerobic respiration raised the carbon dioxide level to about 70% of the headspace atmosphere at the end of the storage trial. This packaging film and storage temperature successfully inhibited blueberry spoilage. Molds, obligate and facultative aerobic bacteria could not grow in an almost anaerobic atmosphere with a high carbon dioxide content. Yeast growth was also retarded, even though some species can generate energy via fermentation. Inhibition by low pH is not applicable because berry pH remained stable and within the growth range throughout storage. The mechanism of yeast inhibition in MAP storage merits further research.

3. Five yeast species isolated from blueberry were identified as two Rhodotorula species, a Zygosaccharomyces species, a Cryptococcus species, and a Debaryomyces species.

CHAPTER 3:

EXPERIMENTATION, RESULTS AND DISCUSSION: DETECTION OF ANTIFUNGAL ACTIVITY IN BLUEBERRIES STORED UNDER A MODIFIED ATMOSPHERE. EFFECTS OF TEMPERATURE AND CARBON DIOXIDE ON YEAST GROWTH.

3.0 PURPOSE OF EXPERIMENTS

This research phase clarified the possible presence of phytoalexins in blueberries, and the effects of low temperature, high carbon dioxide on yeast growth. The disk diffusion method was used to detect presence of phytoalexins in blueberries held in MAP storage. Yeast growth in blueberry juice under different temperatures and atmosphere compositions was studied as a model of yeast behaviour during MAP storage.

3.1 DISK DIFFUSION ASSAYS

3.1.1 MATERIALS AND METHODS

Blueberries from low-oxygen packages were used after two, three and ten weeks of MAP storage. All berries from a storage package (approximately 50 g) were homogenized in a Waring blender (half speed, 1 min), and centrifuged (4,080 x g, 15 min, 4°C) to separate juice and pulp. Juice and pulp were freeze-dried and extracted with a series of solvents: hexanes, ethanol, and water. Water extraction was not necessary for the pulp portion, because water-soluble compounds would be present in berry juice. In each extraction, 2 ml of a solvent was added to freeze-dried residue (juice or pulp) and stirred with a glass rod for five minutes. Three successive extractions yielded

6 ml of each solvent extract. When extractions with a solvent were completed, berry residues were dried under flowing nitrogen at room temperature before addition of another solvent. Freeze-dried residue of blueberry juice (from ≈ 50 g of berries) was also reconstituted with 6 ml of water, and subsequently used in the assays as unfractionated juice.

Berries from replicate storage packages were extracted separately. Each solvent extract from berries of each package was tested independently and also as a pooled sample. The pooled sample was made up by pooling extracts from berries from two or four replicated packages, and the final volume was reduced under flowing nitrogen to the volume of a single sample. If phytoalexin concentrations in extracts from an individual package were below levels necessary for yeast inhibition, then pooling of extracts from replicate packages increased their concentrations, so that yeast inhibition could be detected. Blueberries which had not undergone MAP storage were treated by the same procedure, and their extracts served as controls in the assays.

The yeast strains isolated from blueberries were test organisms. Yeast cultures were grown in a 250 ml-Erlenmeyer flask containing 100 ml of Trypticase Soy broth (Difco, Detroit, MI). The culture flasks were incubated

overnight at 21°C, in a shaking incubator (80 rpm, New Brunswick Scientific Co., Edison, NJ). The overnight cultures were then collected by centrifugation (7,710 x g, 15 min, 21°C), and resuspended in sterile 0.1% peptone water. The yeast inocula were adjusted to contain approximately 10^6 colony-forming units (cfu)/ml using standard curves (see Appendix).

The test medium was prepared by acidifying Yeast Morphology agar (Shadomy and Espinel-Ingroff, 1980) to pH 3.5 with citric acid to achieve the average pH of blueberries. Citric acid was reported to be the major organic acid in blueberries (Meyer, 1968; Markakis et al., 1963). A sterile cotton swab dipped in a yeast inoculum was used to inoculate agar plates. Swabbing of the agar surface was repeated three times, with a 60-degree rotation of the test plate each time, to ensure an even distribution of inoculum.

All solvent extracts and unfractionated juice were filter sterilized. Pectinase (Sigma, St. Louis, MO) was added to unfractionated juice and the water extract at 2% (w/v) final concentration (10 min, room temperature) to facilitate filter sterilization. Filters made of polyvinylidene difluoride polymer (0.45 µm, Millipore Corporation, Bedford, MA) were used. High absorbency paper disks (6.4 mm diameter, Schleicher & Schnell, Keene, NH)

were dipped in extracts, and solvents were allowed to evaporate before the disks were placed on the inoculated agar surface. The procedures of berry extraction and disk diffusion assay are outlined in Figure 3-1.

Each test plate contained disks of pulp extracts (hexanes, ethanol), juice extracts (hexanes, ethanol, water), and unfractionated juice. Extracts of blueberries which had not undergone MAP storage were included on the control plates; disks containing sterile hexanes and ethanol were used as blanks. The plates were incubated under four conditions: 21°C-air; 4°C-air; 21°C-carbon dioxide; 4°C-carbon dioxide. The last two conditions were established by packaging plates in the high-barrier film used in MAP storage (see Table 2-1) with air evacuation and carbon dioxide backflush. pH of the agar medium did not decrease upon exposure to the carbon dioxide atmosphere.

3.1.2 RESULTS AND DISCUSSION

The *in vitro* assays were designed to approximate *in vivo* conditions of blueberries held in MAP storage. The agar medium was adjusted to the average pH of blueberries (see Table 2-4). A high carbon dioxide, low temperature incubation was also included. Since the structures and characteristics of potential phytoalexins in blueberries

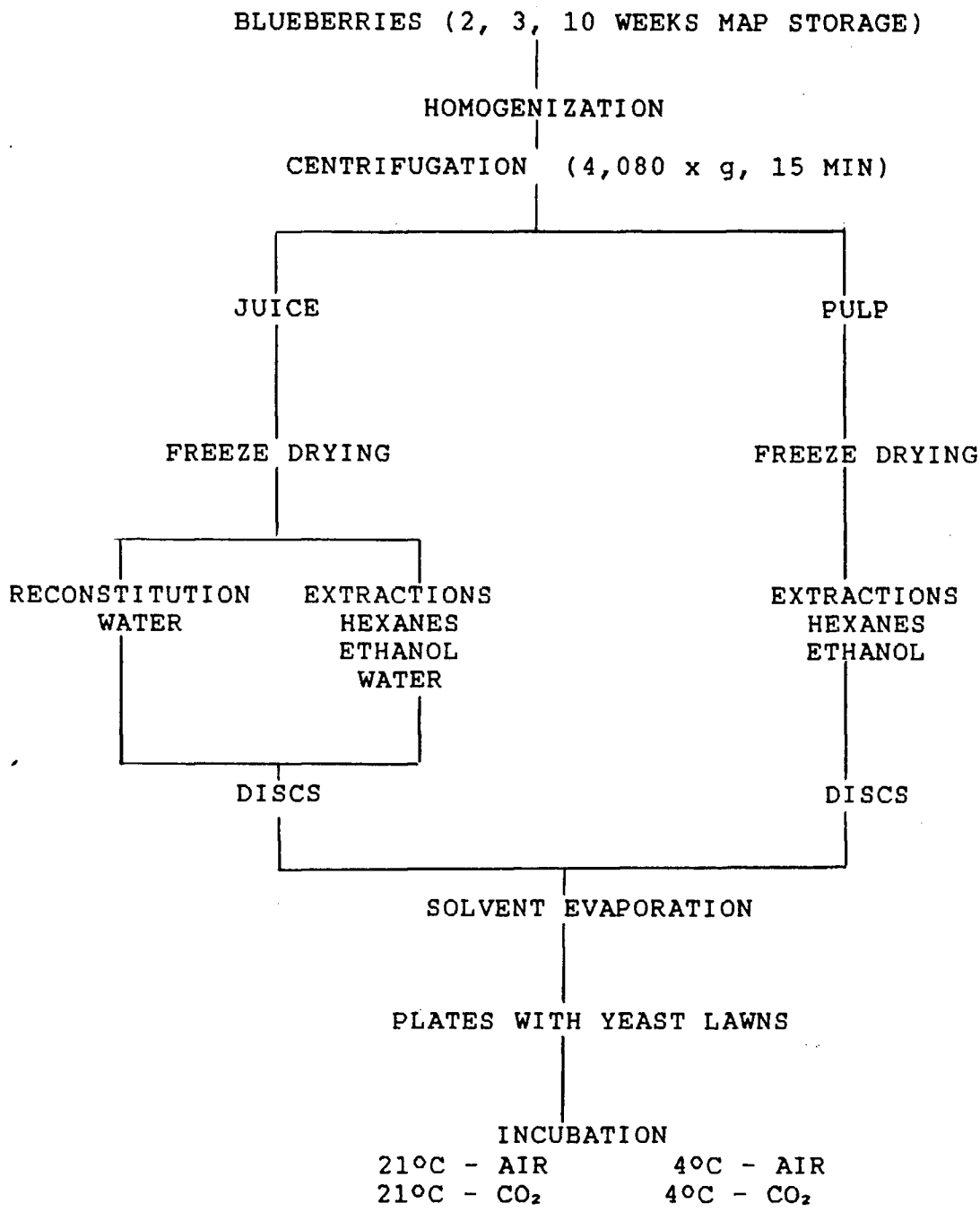

Figure 3-1: General procedures of blueberry extraction and disk diffusion assay.

were unknown, the use of different solvents for their extraction and concentration was necessary. All solvents were evaporated before deposition of disks on agar, to eliminate growth inhibition by solvents. Remaining solutes would be somewhat soluble in the agar medium since they existed in the aqueous environment of berry tissues.

Growth of yeast species varied under different temperatures and atmosphere compositions. No inhibition was observed with any yeast species and any extract tested. Good growth of all isolates occurred in 21°C-air and 4°C-air incubations to produce confluent lawns on agar plates (Figures 3-2, 3-3). Two Rhodotorula species and a Cryptococcus species failed to grow under carbon dioxide incubation (Figure 3-4), probably because they could only utilize sugars aerobically. Since these assays were carried out before identification of yeast isolates, their inability to ferment sugars was unknown. Only the Zygosaccharomyces strain and the Debaryomyces strain grew during 21°C-carbon dioxide incubation, because these isolates could ferment sugars under anaerobic conditions. Growth of all yeast isolates was inhibited in the 4°C-carbon dioxide incubation.

The results of disk diffusion assays showed an absence of antifungal activity in blueberries as tested against

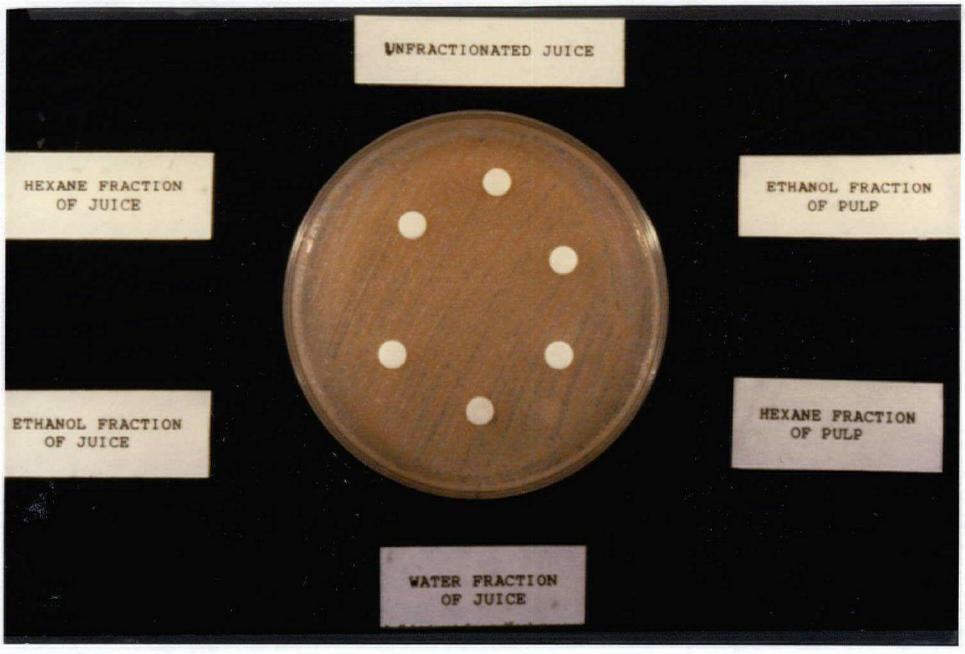

Figure 3-2: Disk diffusion assay using a Rhodotorula species in 21°C-air incubation. The agar surface was covered with yeast growth. An inhibition zone was absent around all disks containing berry extracts.

Figure 3-3: Disk diffusion assay using a Rhodotorula species in 4°C-air incubation. The agar surface was covered with yeast growth. An inhibition zone was absent around all disks containing berry extracts.

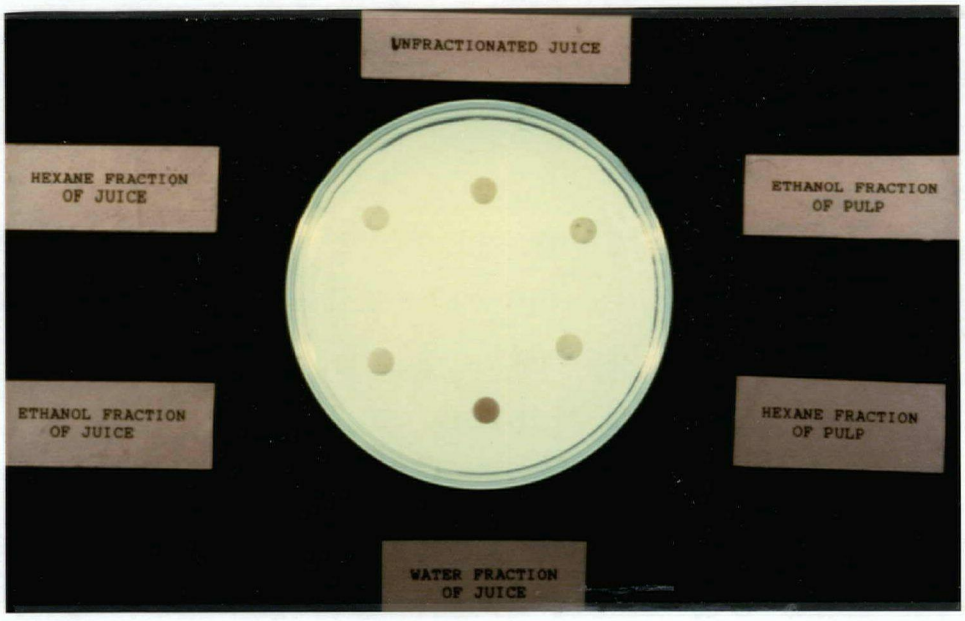

Figure 3-4: Disk diffusion assay using a Rhodotorula species in 4°C-carbon dioxide incubation. Lawn of growth was absent on the agar surface.

the five yeast strains, and indicated that inhibition of yeast growth was due to low temperature, high carbon dioxide level and anaerobic conditions.

3.2 EFFECTS OF CARBON DIOXIDE AND LOW TEMPERATURE ON YEAST GROWTH

The disk diffusion assays indicated inhibition of yeast growth under carbon dioxide and low temperature. This inhibitory effect was studied using natural flora of blueberry juice and yeast isolates grown in sterilized juice at different temperatures and atmospheres.

3.2.1 MATERIALS AND METHODS

3.2.1.1 Studies using cultures of yeast isolates

Two yeast isolates Zygosaccharomyces sp. and Debaryomyces sp. were selected for this study because they can ferment sugars when oxygen is absent. Each yeast were grown in a 250 ml-Erlenmeyer flask containing 100 ml of Trypticase Soy broth (Difco, Detroit, MI). The culture flasks were incubated (21°C, 48 hours) in a shaking incubator (80 rpm, New Brunswick Scientific Co., Edison, NJ). Cells were harvested by centrifugation (7,710 x g, 15 min, 21°C), and washed by suspending in 0.1% peptone water. Dilutions were prepared to obtain inocula of approximately 10^6 cfu/ml.

The test medium was sterilized blueberry juice.

Homogenized blueberries were centrifuged (4080 x g, 15 min, 21°C) for juice collection. Pectinase (Sigma Chemicals, St. Louis, MO) was added to juice at the 2% (w/v) final concentration to facilitate sterilizing filtration. Pectinase treatment was carried out at room temperature, for 15 minutes with occasional stirring. Juice was further clarified by a series of filtrations using Whatman No.4, No.2, and No.5 filters, successively. This filtration series enhanced the speed of subsequent filter sterilization (0.45 µm, mixed esters of cellulose, Millipore Corporation, Bedford, MA). Fifty milliliter-aliquots of sterilized juice were aseptically dispensed to sterilized Square-Pak flasks (100 ml, American Sterilizer Co., Erie, PA), which were covered with gas-tight rubber caps. The headspace of each flask (ca 50 ml) was flushed with a gas mixture (25% carbon dioxide 75% nitrogen, or 100% carbon dioxide) for 15 minutes at the rate of 50 ml/min. The gas mixtures were sterilized by hydrophobic gas filters (0.3 µm, Gelman Sciences Inc., Ann Arbor, MI) before entering into culture flasks. This procedure is illustrated in Figure 3-5. The control flasks were not flushed to maintain air in the headspace. Flasks were incubated at either 4°C or 21°C to achieve the following conditions:

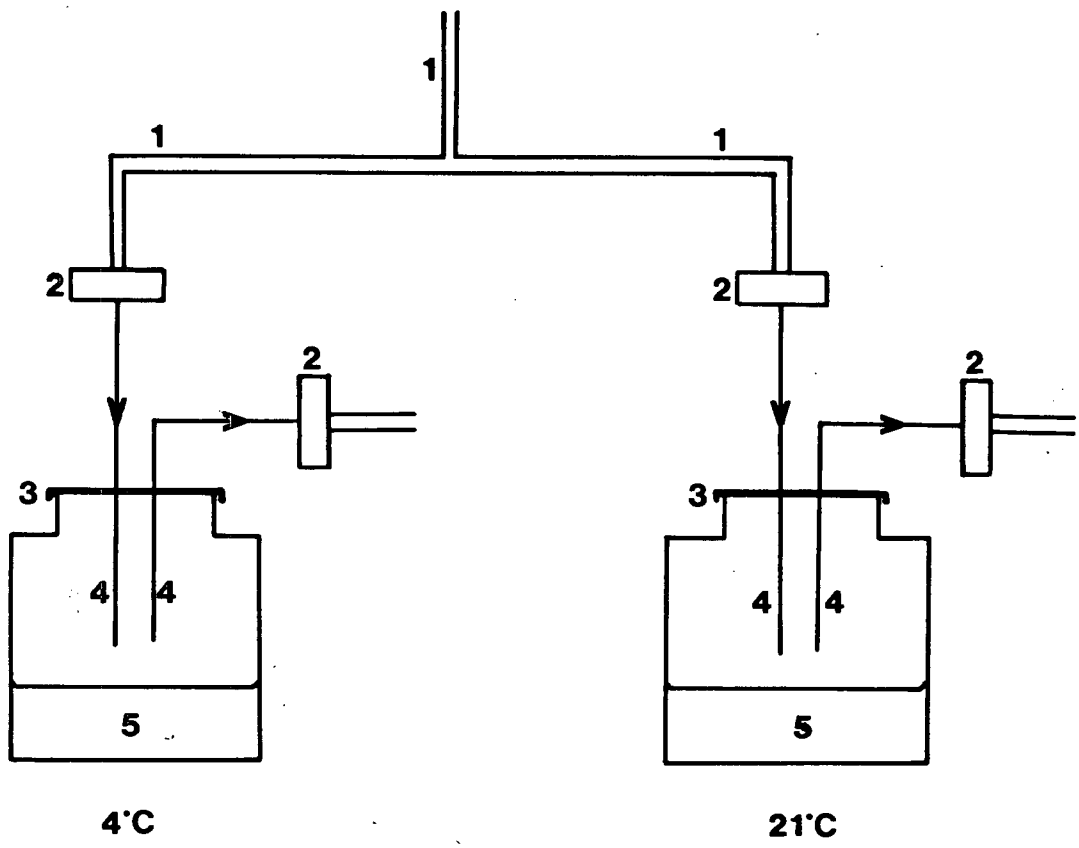

Figure 3-5: Flushing of culture flasks with a gas mixture. Numbers 1: gas tubes, 2: sterilizing filters, 3: air-tight caps, 4: sterilized needles, 5: blueberry juice. Arrow heads point to direction of gas flow.

21°C: air	4°C: air
25% carbon dioxide	25% carbon dioxide
100% carbon dioxide	100% carbon dioxide

For the 21°C condition, flasks were incubated in a orbital shaking incubator (80 rpm, New Brunswick Scientific Co., Edison, NJ), and for the 4°C incubation, an orbital shaking bath (80 rpm, Lab Line Instrument Inc., Melrose Park, IL) was used.

After a short incubation period (30 min) to establish desired juice temperatures, 0.5 ml of yeast inoculum was injected into each flask using sterile needles and syringes (Becton and Dickson, Rutherford, NJ). After inoculation, duplicate samples were taken from each flask (time 0). Sampling was carried out every 24 hours for three days. All samples were taken at a point well below the juice surface. Juice samples were plated using the Hydrophobic Grid Membrane filter (QA Laboratories, Toronto, Ont.) and Plate Count agar (Difco, Detroit, MI) which contained 0.01% Trypan Blue dye (Sigma Chemicals, St. Louis, MO). Use of antibiotics in this medium to suppress bacterial growth was not necessary when working with pure yeast cultures.

Uninoculated juice in "dummy" flasks (100% carbon dioxide, 21°C or 4°C) did not show any change in pH during

incubation.

3.2.1.2 Studies using natural yeast and mold flora of blueberries

Juice was prepared by the procedure described in the preceding section, except for a few minor changes. Since juice sterility was not desired, pectinase and filter sterilization were omitted, and only Whatman No.4 filter was used for juice clarification. The sampling and plating procedures were identical to those used in studies with pure cultures. However, the plating medium trypan blue-PCA was supplemented with 0.01% chloramphenicol (Sigma Chemicals, St. Louis, MO) and 0.01% chlortetracycline HCl (Sigma Chemicals, St. Louis, MO) to suppress bacterial growth on the HGMF.

3.2.2 RESULTS AND DISCUSSION

3.2.2.1 Studies with natural flora

In a mixed population of yeast and mold flora, different temperatures likely inhibit growth of some species, therefore, the growth data from 4°C and 21°C should be treated separately. At 21°C (Figure 3-6), mold growth was inhibited under carbon dioxide atmosphere, so that counts of samples in 25% and 100% carbon dioxide incubations represented yeast growth only. Under anaerobic condition, yeasts could ferment sugars to supply

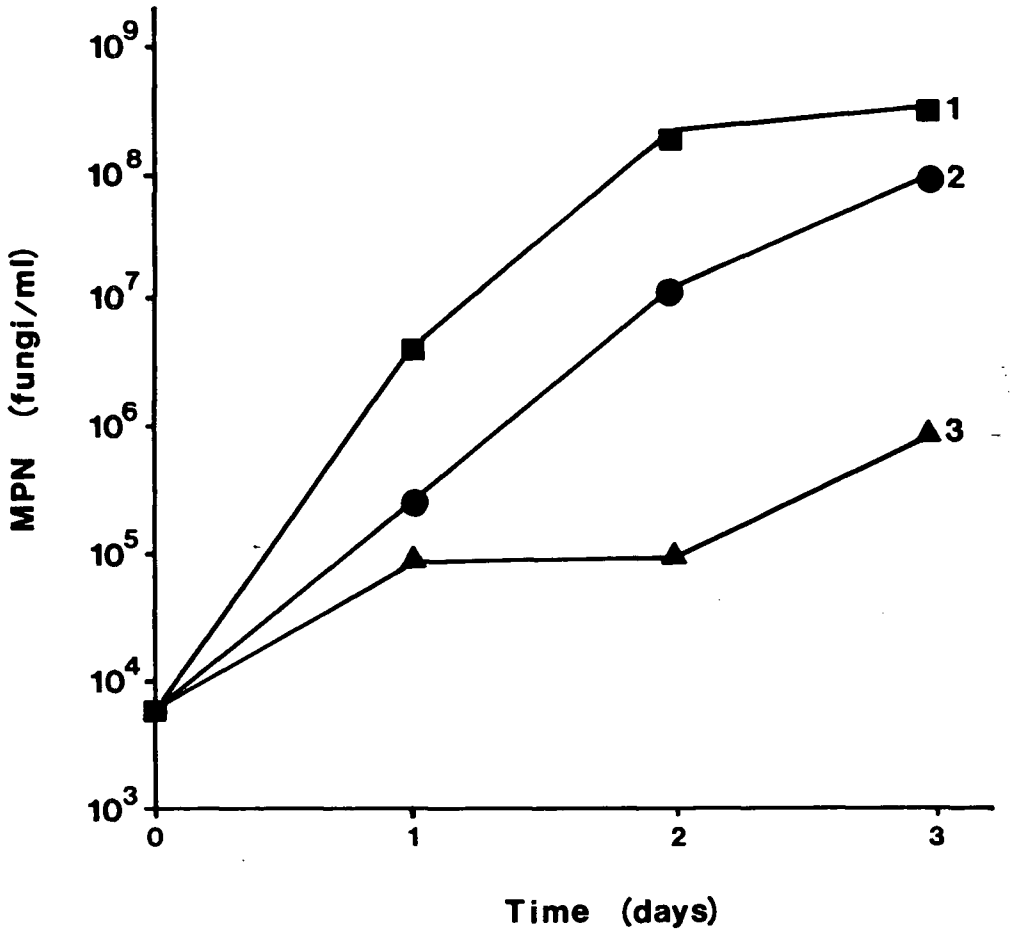

Figure 3-6: Yeast and mold population in blueberry juice at 21°C. Curves 1: air, 2: 25% CO_2, 3: 100% CO_2. Points are means of two counts. Counts of samples in air were significantly different from counts of 25% CO_2 or 100% CO_2 samples by Student's t-test ($\alpha = 0.05$). Counts of samples in 25% CO_2 were significantly different from counts of samples in 100% CO_2 by Student's t-test ($\alpha = 0.05$).

energy and metabolic precursors. An increase of carbon dioxide level to 100% apparently caused a decrease in yeast growth, compared to growth in 25% carbon dioxide. These results indicated that some yeast strains in the blueberry microflora were sensitive but some strains were resistant to a high carbon dioxide atmosphere, since growth of flora was not completely inhibited in the 21°C-100% carbon dioxide condition. Several reports have been published on the effect of carbon dioxide on yeast growth, but the data were obtained under conditions such as high carbon dioxide pressures (Kunkee and Ough, 1966; Perigo et al., 1964), high sugar contents in media (Witter et al., 1958), and limiting-substrate concentrations (Chen and Gutmans, 1976), so that no comparison with this study could be made. At 4°C and under 25% or 100% carbon dioxide atmosphere, yeast and mold counts showed no increase from initial counts (Figure 3-7), which confirmed earlier results of low yeast and mold counts during low-oxygen storage of blueberries. Results of this study (4°C) indicated that the high carbon dioxide level at 12 weeks of low-oxygen storage (68.96%) may not have greater effect on yeast and mold flora than its effect at two weeks (46.30%, see Table 2-2). Inhibition of yeast and mold flora in the 4°C-25% CO_2 and 4°C-100% CO_2

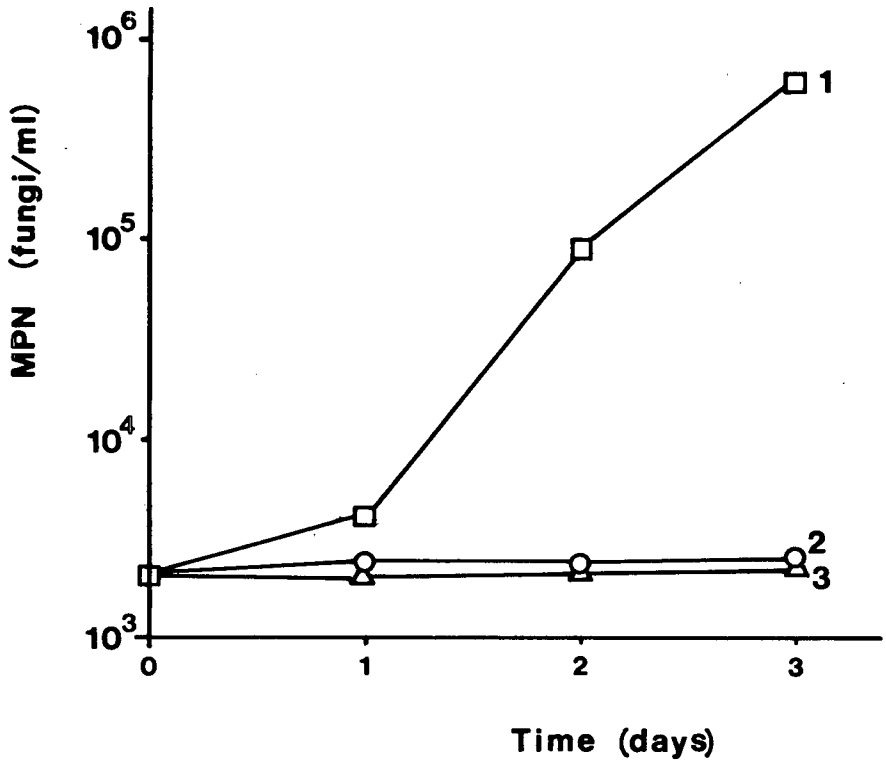

Figure 3-7: Yeast and mold population in blueberry juice at 4°C. Curves 1: air, 2: 25% CO_2, 3: 100% CO_2. Points represent means of two counts. Counts of the sample in air were significantly different from counts of samples in 25% CO_2 or 100% CO_2 by Student's t-test ($\alpha = 0.05$).

environments was probably the combined effects of low temperature, high carbon dioxide and anaerobic atmosphere.

3.2.2.2 Studies with yeast isolates

Growth of Zygosaccharomyces sp. and Debaryomyces sp. under different temperature and atmosphere combinations are presented in Figures 3-8 and 3-9. At 21°C, a growth lag occurred in the 25% and 100% carbon dioxide environments, as compared to growth in air. In the absence of oxygen, yeasts can derive less energy via fermentation than by aerobic utilization of sugars. Since carbon dioxide is a product of decarboxylation reactions, high carbon dioxide concentrations will affect reaction equilibria, and the rates of enzymatic decarboxylation. When this occurred, the decarboxylation reactions such as those involved in the tricarboxylic acid cycle would become rate-limiting steps in cellular metabolism, if other enzymes were operating at normal rates, and the effect of high carbon dioxide would be slow growth (King and Nagel, 1975; Kritzman et al., 1977). Slow growth of Zygosaccharomyces sp. and Debaryomyces sp. under 25% or 100% carbon dioxide atmosphere was probably the result of both high carbon dioxide and inefficient energy generation in absence of oxygen. These two effects

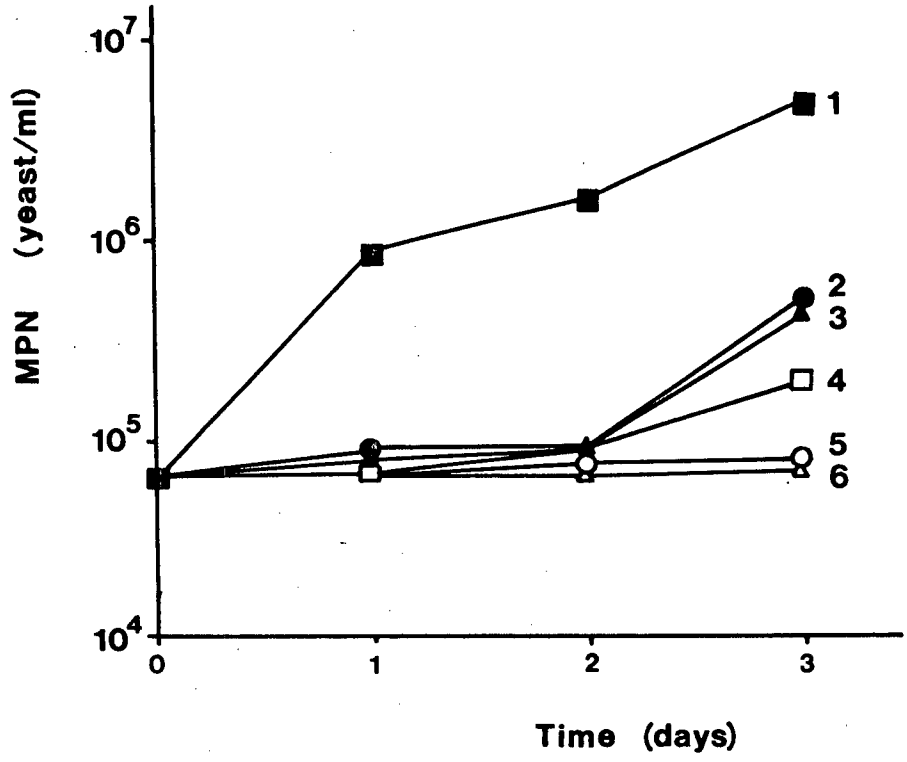

Figure 3-8: Growth of a Zygosaccharomyces species in blueberry juice. Curves 1: 21°C-air, 2: 21°C-25% CO_2, 3: 21°C-100% CO_2, 4: 4°C-air, 5: 4°C-25% CO_2, 6: 4°C-100% CO_2. Points are means of two counts. Sample means were compared by Duncan's Multiple Range test ($\alpha = 0.05$): counts of 21°C-air sample were significantly different from counts of other samples; counts of 21°C-25% CO_2 sample, 21°C-100% CO_2 sample and 4°C-air sample were not different from one another, but they were significantly different from counts of 4°C-25% CO_2 and 4°C-100% CO_2 samples at 72 hours.

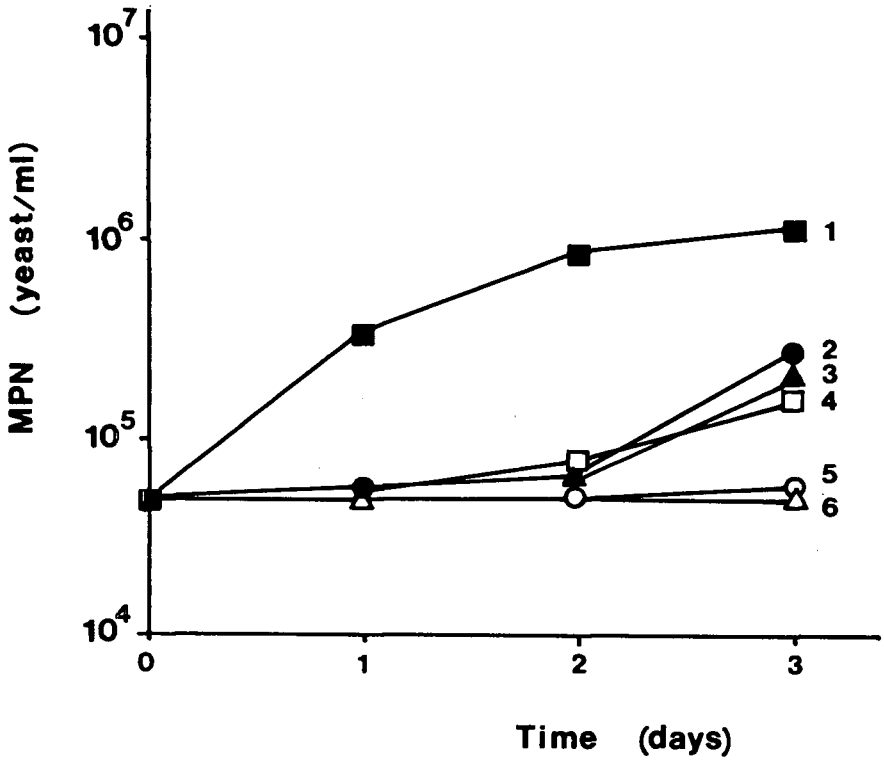

Figure 3-9: Growth of a Debaryomyces species in blueberry juice. Curves 1: 21°C-air, 2: 21°C-25% CO_2, 3: 21°C-100% CO_2, 4: 4°C-air, 5: 4°C-25% CO_2, 6: 4°C-100% CO_2. Points are means of two counts. Sample means were compared by Duncan's Multiple Range test ($\alpha = 0.05$): counts of 21°C-air sample were significantly different from counts of other samples; counts of 21°C-25% CO_2 sample, 21°C-100% CO_2 sample and 4°C-air sample were not different from one another, but they were significantly different from counts of 4°C-25% CO_2 and 4°C-100% CO_2 samples at 72 hours.

could not be distinguished under the conditions of this experiment.

Low temperature had a marked effect on yeast species: growth in air at 4°C was lower than at 21°C, and growth was inhibited under the 25% carbon dioxide atmosphere. Solubility of carbon dioxide increased at low temperature and contributed to growth inhibition. However, the composition and functionality of yeast membranes might be the key factor affected by low temperatures and might explain the inhibition of yeast growth in MAP storage.

Yeasts generally have a high proportion of unsaturated fatty acids in membrane lipids when grown at low temperatures (Kates and Baxter, 1962; Arthur and Watson, 1976). Phospholipid molecules, which have a glycerol-3-phosphate backbone acylated with fatty acids at carbons 1 and 2, form the bilayer structure of biological membranes. Some membrane phospholipids may be specifically associated with membrane proteins and are essential for their activities. The entire membrane structure is dynamic rather than static, with most components capable of lateral diffusion and rotational motion about an axis perpendicular to the bilayer plane. Rotation rate in a lipid bilayer is a function of both temperature and membrane composition. Depending on temperature, membranes

may pass from a gel phase, in which the fatty acyl chains of phospholipids are highly ordered, to a liquid phase in which the fatty acyl chains are more mobile. This phase transition process (illustrated in Figure 3-10a) is accompanied by increased rotational motion about the carbon-carbon bonds of the hydrocarbon chains of phospholipids, which allows the hydrocarbon chains to assume random conformations. In general, unsaturated fatty acyl chains undergo phase transition at lower temperatures than saturated fatty acids. This is because unsaturation produces a "kink" in the fatty acyl chains which leads to more disorder in the bilayer than saturated chains (Figure 3-10b). Therefore, the proportion of unsaturated fatty acids will affect the fluidity (defined as the inverse of viscosity) of membranes at a given temperature.

Arthur and Watson (1976) examined the membrane lipid composition of psychrophilic (Leucosporidium frigidum, growth temperature limit -2°C to 20°C), mesophilic (Candida lipolytica, temperature limit 5° to 35°C), and thermophilic (Torulopsis bovina, temperature limit 25° to 45°C) yeasts, and reported that saturated fatty acids composed 30-40% of membrane lipid of thermophilic yeasts, whereas the psychrophilic yeast contained approximately 90% unsaturated fatty acids, 53% of which was linolenic

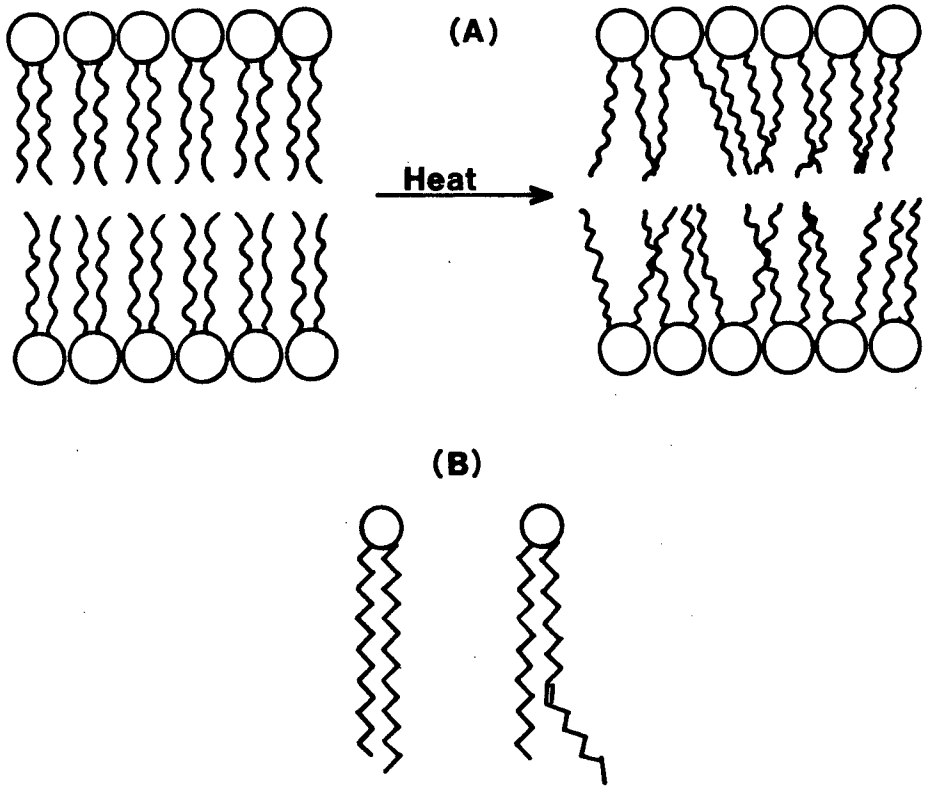

Figure 3-10: A model of phase transition of fatty acids. (a) Changes in phospholipid bilayer during phase transition. (b) Introduction of a double bond into a fatty acyl chain results in an inflexible kink in the phospholipid tail, compared with a phospholipid containing only saturated fatty acids (adapted from Jacobson and Saier, 1983).

acid (Table 3-1). These authors proposed that degree of membrane lipid unsaturation may be an important factor which determines yeast ability to grow at low temperatures. The high content of linoleic acid (midphase transition temperature -5°C) and linolenic acid (midphase transition temperature -11°C) would permit yeast membranes to be in a semi-fluid state and remain functional at low temperatures. Watson (1978) correlated the degree of lipid unsaturation of L. frigidum and T. bovina with their glucose uptake at different temperatures. In L. frigidum, glucose transport was most active at 2°C but negligible at 40°C; glucose transport of T. bovina was most rapid at 40°C but was minimal at 2°C. He suggested that at low temperatures membranes of T. bovina with a large amount of saturated fatty acids were too rigid, and the high degree of unsaturated fatty acids allowed membranes of L. frigidum to be functional.

Yeast adaptation to low temperatures via modification of lipid composition has been reported (Brown and Rose, 1969; Kates and Paradis, 1973; Pugh and Kates, 1975; Arthur and Watson, 1976; Ferrante et al., 1983). The linoleic acid content of mesophilic C. lipolytica grown at 10°C doubled compared to its content in cells grown at 25°C (Kates and Baxter, 1962). Kates and Paradis (1973)

Table 3-1: Fatty acid composition of yeast membranes (adapted from Arthur and Watson, 1976).

Yeast	Percentage of total fatty acids[1]					
	Saturated	Unsaturated				
	C_{12} - C_{18}	$C_{14:1}$	$C_{16:1}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
L. frigidum	9	Trace	Trace	11	27	53
C. lipolytica	11	3	20	44	21	Trace
T. bovina	28	3	42	23	ND	ND

ND: not detected

[1] subscript numbers preceding colons denote number of carbons in fatty acid chains, numbers following colons denoted number of double bonds in fatty acids.

noted that unsaturation of fatty acids in phospholipids of C. lipolytica was greater at 10°C than 25°C. An inverse relationship between contents of oleic and linoleic acids was observed in all phospholipids, which indicated desaturation of oleic acid to form linoleic acid. Yeast phospholipids are located almost exclusively in membranes. Changes in degree of unsaturation of fatty acyl chains of phospholipids would alter membrane fluidity and function. Activity of oleoyl-CoA desaturase, which catalyzes desaturation of CoA-ester of oleic acid, was higher in membranes of C. lipolytica grown at 10°C than in cells grown at 25°C; increased enzyme activity was correlated with a decrease in oleic acid content and increase in linoleic acid content at 10°C (Pugh and Kates, 1975; Ferrante et al., 1983). Increased activity of oleyl-CoA desaturase was also observed in Torulopsis utilis grown at suboptimum temperatures (Meyer and Bloch, 1963).

The inverse relationship between temperature and unsaturated fatty acid contents represents yeast efforts to maintain fluidity and function of membranes at a particular temperature. In the present study, growth of Zygosaccharomyces sp. and Debaryomyces sp. at 4°C in air was probably a result of their adaptation to low temperature. Since low temperature also caused a decrease

in enzyme activity, growth of two yeast isolates were slower in 4°C-air than in 21°C-air (Figures 3-8 and 3-9). Desaturation of fatty acids requires presence of molecular oxygen (Bloomfield and Bloch, 1960; Yaun and Bloch, 1961; Pugh and Kates, 1975). Two models of the electron transport chain involved in the desaturation of fatty acids have been summarized (Fulco, 1974). In both models, oxygen acts as the terminal acceptor of the hydrogen atoms removed from fatty acids during desaturation. An anaerobic environment then inhibits desaturation of fatty acids and prevents yeast adaptation to low temperatures. The inability to desaturate membrane fatty acids may explain inhibition of Zygosaccharomyces sp. and Debaryomyces sp. at 4°C, 25% or 100% carbon dioxide, although the inhibitory effect of carbon dioxide could not be entirely discounted.

3.3 YEAST GROWTH IN BLUEBERRIES DURING MAP STORAGE

When blueberries were packaged in a film with low gas permeability, inhibition of yeast growth lasted six to eight weeks which was followed by a growth increase (see Figures 2-7, 2-8, 2-9). In this storage condition, growth of yeast flora was probably influenced by three factors: high carbon dioxide, low temperature and low oxygen atmosphere.

Carbon dioxide is a product of cellular decarboxylation reactions. High carbon dioxide concentrations in MAP storage may affect reaction equilibrium and rates of enzymatic decarboxylation. Decarboxylation reactions then would become rate-limiting steps in cellular metabolism, if other enzymes were operating at normal rates. High carbon dioxide would result in slow growth (King and Nagel, 1975; Kritzman et al., 1977).

Low temperatures affect the fluidity and function of cell membranes. Membrane-associated processes rely on a semifluid environment for their operation. Yeasts have a remarkable ability to increase the proportion of membrane unsaturated fatty acids in response to a decrease in temperature. This ensures proper fluidity of membranes at low temperatures. Low temperatures may also affect activity of enzymes involved in cellular metabolism, and cause decreased metabolic rates and slow growth. Increased solubility of carbon dioxide at low temperature could contribute to growth inhibition.

Oxygen is essential for the synthesis of ergosterol which is an important component of mitochondrial membrane (Linnane et al., 1972). In the absence of oxygen, mitochondrial structures lack the folded inner membrane, and protein synthesizing activity. Pinto and Nes (1983) showed that 0.21% oxygen was sufficient for synthesis of

ergosterol, and promoted growth of Saccharomyces cerevisiae. During two to twelve weeks of MAP storage of blueberries in the present study, headspace of low-oxygen packages contained approximately 0.4% to 0.8% oxygen (see Table 2-2). Therefore, a deficiency of ergosterol in yeast flora was unlikely to occur during MAP storage.

The fungal counts of blueberries showed that yeast growth increased after six to eight weeks in MAP storage (see Figures 2-7, 2-8, 2-9), which indicated a recovery process. Microbial adaptation to carbon dioxide has not been studied extensively, and recovery usually only occurs when presence of carbon dioxide is removed (International Committee for Microbial Specifications for Foods, 1980), although Johnson and Ogrydziak (1984) have reported evidence of genetic adaptation to carbon dioxide by Pseudomonas-like bacteria. Yeast recovery in MAP storage of blueberries was observed even though the carbon dioxide contents of storage packages continued to increase. In view of this recovery process, yeast adaptation to low storage temperature via desaturation of fatty acyl chains of membrane phospholipids may be the key factor affecting yeast growth on blueberries during MAP storage.

Since oxygen is required for fatty acid desaturation, micro-aerobic condition of MAP storage may inhibit desaturation of fatty acids, which in turn, prevents yeast

adaptation to low temperatures. At 15°C, desaturation of fatty acids occurred in Candida utilis, grown under an oxygen tension of 1 mmHg, and its total fatty acids contained 34% linoleic acid and a small proportion of linolenic acid (Brown and Rose, 1969). During two to twelve weeks of MAP storage in this study, oxygen content of low-oxygen packages ranged approximately from 0.4% (760 mmHg x 0.4/100 = 3.04 mmHg) to 0.8% (6.28 mmHg). This range of headspace oxygen composition was higher than 1 mmHg required for synthesis of linoleic and linolenic acids in C. utilis. Since the yeast flora present on berry surfaces was made up of many species, a direct comparison with data from C. utilis is inappropriate. Nevertheless, small amounts of oxygen in the MAP packages of blueberries might have allowed a slow desaturation of fatty acids which led to yeast recovery and growth in the latter part of the storage trials (see Figures 2-7, 2-8, 2-9).

Since high carbon dioxide, low temperature and low oxygen conditions co-existed in MAP storage, the effects of these factors on yeast growth could not be separated. Therefore, when yeasts modified membranes to maintain proper functions at low storage temperature, their growth rates may have been slow due to the effects of carbon dioxide and low temperature on metabolic reactions. These

phenomena require further study.

3.4 CONCLUSIONS

1. Diffusion disk assays were set up to detect antifungal phytoalexin accumulation in blueberries during MAP storage. The results showed an absence of antifungal compounds as tested against five yeast strains, and indicated inhibition of yeast growth due to low temperature, high carbon dioxide level and anaerobic conditions.

2. The effects of temperature and atmosphere were investigated using natural flora of blueberry juice and yeast isolates grown in sterilized juice, under different temperatures and atmosphere compositions. At 21°C, yeast growth was slow in the presence of carbon dioxide and absence of oxygen. In air, slow growth accompanied exposure to low temperature. The responses of yeast and mold flora as well as yeast cultures in these experiments confirmed observations of low fungal populations on blueberries during MAP storage.

3. The following model may explain mechanism of yeast inhibition in MAP storage. Yeasts adapt to low temperatures via desaturation of fatty acyl chains of

phospholipids to maintain membrane fluidity and function. Since desaturation requires molecular oxygen, micro-aerobic environment of MAP storage might have allowed a slow rate of desaturation. Yeast growth may also be slow due to the effects of low temperature on rates of metabolic processes. The solubility of carbon dioxide increases at low temperature, and carbon dioxide may affect the rates of decarboxylation reactions, such as those involved in the tricarboxylic acid cycle, and cause slow growth.

REFERENCES

Adair, C.N. 1971. Influence of controlled-atmosphere storage conditions on cabbage postharvest decay fungi. Plant Dis. Reptr. 55:864.

Arthur, H. and Watson, K. 1976. Thermal adaptation in yeasts: growth temperatures, membrane lipid, and cytochrome composition of psychrophilic, mesophilic, and thermophilic yeasts. J. Bacteriol. 128:56.

Ballinger, W.E. 1983. Postharvest decay of blueberries as influenced by water dips and captafol. J. Amer. Soc. Hort. Sci. 108:422.

Ballinger, W.E. and Kushman, L.J. 1970. Relationship of stage of ripeness to composition and keeping quality of high bush blueberries. J. Amer. Soc. Hort. Sci. 95:239.

Barnett, J.A., Payne, R.W. and Yarrow, D. 1983. Yeasts, characteristics and identification. Cambridge University Press, Great Britain. 811 p.

Baxter, R.M. and Gibbons, N.E. 1962. Observations on the physiology of psychrophilism in a yeast. Can. J. Microbiol. 8:511.

Bell, T.A. and Etchells, J.L. 1956. Pectin hydrolysis by certain salt tolerant yeasts. Appl. Microbiol. 4:196.

Bloomfield, D.K. and Bloch, K. 1960. The formation of unsaturated fatty acids. J. Biol. Chem. 235:337.

Brock, T.D. 1979. Biology of microorganisms. Prentice-Hall, Inc., Englewood Cliffs. NJ. pp.98-99.

Brown, C.M. and Rose, A.H. 1969. Fatty-acid composition of Candida utilis as affected by growth temperature and dissolved oxygen tension. J. Bacteriol. 99:371.

Buhagiar, R.W.M. and Barnett, J.A. 1971. The yeasts of strawberries. J. Appl. Bacteriol. 34:729.

Cappellini, R.A., Stretch, A.W. and Maiello, J.M. 1972. Fungi associated with blueberries held at various storage times and temperatures. Phytopathol. 62:68.

Carter, G.A., Chamberlain, K. and Wain, R.L. 1978. Investigations of fungicides. XX. The fungitoxicity of analogues of the phytoalexin 2-(2'-methoxy-4'-hydroxyphenyl)-6-methoxy-benzofuran (vignafuran). Ann.

Appl. Biol. 88:57.

Ceponis, M.J. and Capellini, R.A. 1978. Relationship of chemical application time to postharvest disease control of blueberies. Plant Dis. Reptr. 62:1005.

Ceponis, M.J., Kaufman, J. and Tietjen, W.H. 1973. Decays of New Jersey blueberries in marketing channels. Plant Dis. Reptr. 57:487.

Cheema, A.S. and Haard, N.F. 1978. Induction of rishitin and lubinin in potato tuber discs by non-specific elicitors and the influence of storage conditions. Physiol. Plant Pathol. 13:233.

Chen, S.L. and Gutmans, F. 1976. Carbon dioxide inhibition of yeast growth in biomass production. Biotechnol. Bioeng. 18:1455.

Clinton, R.H. 1968. Effects of temperature on the uptake of glutamic acid and lysine in a psychrophilic yeast. Antonie van Leeuwenhoek. 34:99.

Currier, W.W. and Kuc, J. 1975. Effect of temperature on rishitin and steroid glycoalkaloid accumulation in potato tuber. Phytopathol. 65:1194.

Devlin, R.M. and Witham, F.H. 1983. Plant Physiology. PWS Publishers, Boston, MA. pp.316-342.

Dickman, M.B., Patil, S.S. and Kolattukudy, P.E. 1982. Purification and characterization of an extracellular cutinolytic enzyme from Colletotrichum gloeosporioides on Carica papaya. Physiol. Plant Pathol. 20:333.

El-Kazzaz, M.K. Sommer, N.F. and Fortlage, R.J. 1983. Effect of different atmospheres on postharvest decay and quality of fresh strawberries. Phytopathol. 73:282.

Enfors, S.O. and Molin, G. 1978. The influence of high concentrations of carbon dioxide on the germination of bacterial spores. J. Appl. Bacteriol. 45:279.

Enfors, S.O. and Molin, G. 1980. Effect of high concentrations of carbon dioxide on growth rate of Pseudomonas fragi, Bacillus cereus and Streptococcus cremoris. J. Appl. Bacteriol. 48:409.

Ferrante, G., Ohno, Y. and Kates, M. 1983. Influence of temperature and growth phase on desaturase activity of the mesophilic yeast Candida lipolytica. Can. J. Biochem. Cell Biol. 61:171.

Follstad, M.N. 1966. Mycelial growth rate and sporulation of Alternaria tenuis, botrytis cinerea, Cladosporium herbarum, and Rhizopus stolonifer in low-oxygen atmospheres. Phytopathol. 56:1088.

Fulco, A.J. 1974. Metabolic alterations of fatty acids. Ann. Rev. Biochem. 43:215.

Galgiani, J.N. and Stevens, D.A. 1976. Antimicrobial susceptibility testing of yeasts: a turbidimetric technique independent of inoculum size. Antimicrob. Agents Chemother. 10:721.

Galgiani, J.N. and Stevens, D.A. 1978. Turbimetric studies of growth inhibition of yeasts with three drugs: inquiry into inoculum-dependent susceptibility testing, time of onset of drug effect, and implications for current and newer methods. Antimicrob. Agents Chemother. 13:249.

Haard, N.F. and Cody, M. 1978. Stress metabolites in postharvest fruits and vegetables-Role of ethylene. In Postharvest Biology and Biotechnology, Hultin, H.O. and Milner, M. (eds.), Food and Nutrition Press Inc., Westport, Connecticut. pp.111-135.

Hagen, P.O. and Rose, A.H. 1962. Studies on the biochemical basis of the low maximum temperature in a psychrophilic Cryptococcus. J. Gen. Microbiol. 27:89.

Harris, J.E. and Dennis, C. 1977. The effect of post-infectional potato tuber metabolites and surfactants on zoospores of Oomycetes. Physiol. Plant Pathol. 11:163.

Higgins, V.J. 1978. The effect of some pterocarpanoid phytoalexins on germ tube elongation of Stemphylium botryosum. Phytopathol. 68:338.

Ingram,J.L., Keen, N.T. and Hymowitz, T. 1977. A new isoflavone phytoalexin from fungus-inoculated stems of Glycine wightii. Phytochem. 16:1943.

International Committee on Microbiological Specifications for Foods. 1980. Gases as Preservatives. In Microbial ecology of foods, vol.1: Factors affecting life and death

of microorganisms. Academic Press, New York, NY. pp. 170-192.

Jacobson, G.R. and Saier, M.H. 1983. Biological membranes: structure and assembly. In Biochemistry, Zubay, G. (ed.), Addison-Wesley, Reading, MA. pp.573-619.

Johnson, A.R. and Ogrydziak, D.M. 1984. Genetic adaptation to elevated carbon dioxide atmospheres by Pseudomonas-like bacteria isolated from rock cod (Sebastes spp.). Appl. Environ. Microbiol. 48:486.

Kates, M. and Baxter, R.M. 1962. Lipid composition of mesophilic and psychrophilic yeasts (Candida species) as influenced by environmental temperature. Can. J. Biochem. Physiol. 40:1213.

Kates, M. and Paradis, M. 1973. Phospholipid desaturation in Candida lipolytica as a function of temperature and growth. Can. J. Biochem. 51:184.

King, A.D. and Nagel, C.W. 1975. Influence of carbon dioxide upon the metabolism of Pseudomonas aeruginosa. J. Food Sci. 40:362.

Koburger, J.A. 1986. Effect of pyruvate on recovery of fungi from foods. J. Food Prot. 49:231.

Koburger, J.A. and Marth, E.H. 1984. Yeasts and molds. In Compendium of Methods for the Microbiological Examination of Foods. Speck, M.L. (ed.), American Public Health Association, Washington, D.C. pp.197-202.

Kolattukudy, P.E. 1985. Enzymatic penetration of the plant cuticle by fungal pathogens. Ann. Rev. Phytopathol. 23:223.

Kostiala, A.A.I. and Kostiala, I. 1984. Broth dilution and disc diffusion methods in the susceptibility testing of pathogenic Candida albicans against four antimycotics. Mycopathol. 87:121.

Kregger van Rij, N.J.W. 1984. The Yeasts. A taxonomic study. Elsevier Science Publishers, Amsterdam. 1082 p.

Kritzman, G., Chet, I. and Henis, Y. 1977. Effect of carbon dioxide on growth and carbohydrate metabolism in Sclerotium rolfsii. J. Gen. Microbiol. 100:167.

Kuc, J. and Shain, L. 1977. Antifungal compounds associated with disease resistance in plants. In Antifungal compounds, vol. 2: Interactions in biological and ecological systems. Sisler, H.D. and Siegel, M.R. (eds), Marcel Dekker Inc., New York, NY, pp. 497-535.

Kunkee, R.E. and Ough, C.S. 1966. Multiplication and fermentation of Saccharomyces cerevisiae under carbon dioxide pressure in wine. Appl. Microbiol. 14:643.

Linnane, A.W., Haslam, J.M., Lukins, H.B. and Nagley, P. 1972. The biogenesis of mitochondria in microorganisms. Ann. Rev. Microbiol. 26:163.

Luh, B.S. and Phaff, H.J. 1951. Studies on polygalacturonase of certain yeasts. Arch. Biochem. Biophys. 33:212.

Mansfield, J.W. 1983. Antimicrobial compounds. In Biochemical Plant Pathology, Callow, J.A. (ed.), John Wiley & Sons Ltd., pp.237-265.

Mansfield, J.W. and Bailey, J.A. 1982. Phytoalexins: current problems and future prospects. In Phytoalexins, Bailey, J.A. and Mansfield, J.A. (eds.), Blackie, Glasgow, pp. 319-322.

Markakis, P., Jarczyd, A. and Krishna, S.P. 1963. Nonvolatile acids of blueberries. J. Agric. Food Chem. 11:8.

Meyer, F. and Bloch, K. 1963. Effect of temperature on the enzymatic synthesis of unsaturated fatty acids in Torulopsis utilis. Biochim. Biophys. Acta 77:671.

Meyer, L.H. 1968. Food Chemistry. Reinhold Bood Corp., New York, NY. pp.275-276.

Muller, K.O. and Borger, H. 1940. Experimentelle Untersuchungen uber die Phytophthora-Resistenz der Kartoffel-zugleich ein Beitrag zum Problem der erworbenen Resistenz im Pflanzenreich. Arb. Biol. Anst. Reichsanst 23:189. [Mansfield, J.W. 1983. Antimicrobial compounds. In Biochemical Plant Pathology, Callow, J.A. (ed.), John Wiley & Sons Ltd. pp.237-265.

Nash, C.H. and Sinclair, N.A. 1968. Thermal injury and death in an obligately psychrophilic yeast, Candida nivalis. Can. J. Microbiol. 14:691.

Perigo, J.A., Gimbert, B.L. and Bashford, T.E. 1964. The effect of carbonation, benzoic acid and pH on the growth rate of a soft drink spoilage yeast as determined by a turbidostatic continuous culture apparatus. J. Appl. Bact. 27:315.

Perrin, D.R. and Cruickshank, I.A.M. 1969. The antifungal activity of pterocarpans towards Monilinia fructicola. Phytochem. 8:971.

Pinto, W.J. and Nes, W.R. 1983. Stereochemical specificity for sterols in Saccharomyces cerevisiae. J. Biol. Chem. 258:4472.

Pugh, E.L. and Kates, M. 1975. Characterization of a membrane-bound phospholipid desaturase system of Candida lipolytica. Biochim. Biophys. Acta 380:442.

Quetsch, M.F. and Danforth, W.F. 1964. Kinetics of accumulation of uric acid and xanthine by the yeast Candida utilis. J. Cell. Comp. Physiol. 64:123.

Shadomy, S. and Espinel-Ingroff, A. 1980. Susceptibility testing with antifungal drugs. In Manual of clinical microbiology, Lennette, E.H., Ballows, A., Hausler, W.J. and Truant, J.P. (eds.), American Society of Microbiologists, Washington DC, pp.647-653.

Sharp, A.N. and Michaud, G.L. 1975. Enumeration of high numbers of bacteria using hydrophobic grid membrane filters. Appl. Microbiol. 30:519.

Skipp, R.A. and Bailey, J.A. 1977. The fungitoxity of isoflavonoid phyotalexins measured using different types of bioassay. Physiol. Plant Pathol. 11:101.

Smith, D.A. 1978. Observations on the fungitoxicity of the phytoalexin, kievitone. Phytopathol. 58:81.

Smith, D.A. 1982. Toxicity of phytoalexins. In Phytoalexins, Bailey, J.A. and Mansfield, J.W.(eds.), Blackie, Glasgow, pp.218-252.

Soliday, C.L. and Kolattukudy, P.E. 1976. Isolation and characterization of a cutinase from Fusarium roseum culmorum and its immunological comparison with cutinases from F. solani pisi. Arch. Biochem. Biophys. 176:334.

Sols, A., Gancedo, C. and DelaFuente, G. 1971. Energy-

yielding metabolism in yeasts. In The Yeasts, vol. 2: Physiology and biochemistry of yeasts. Rose, A.H. and Harrison, J.S. (eds.), Academic Press, London, U.K. pp. 271-307.

Stevens, D.A. 1984. Antifungal drug susceptibility testing. Mycopathol. 87:135.

Stoessl, A. 1982. Biosynthesis of phytoalexins. In Phytoalexins, Bailey, J.A. and Mansfield, J.W. (eds.), Blackie, Glasgow, pp.133-180.

Stokes, T.D. 1979. Influence of temperature on the growth and metabolism of yeasts. In The Yeasts, vol. 2: Physiology and biochemistry of yeasts. Rose, A.H. and Harrison, J.S. (eds.), Academic Press, London, U.K. pp. 119-134.

Svircev, A.M., McKeen, W.E. and Berry, J.W. 1984. Sensitivity of Peronospora hyoscyami f.sp. tabacina to carbon dioxide, compared to that of Botrytis cinerea and Aspergillus niger. Phytopathol. 74:445.

Van der Walt, J.P. and Yarrow, D. 1984. Methods for the isolation, maintenance, classification and identification of yeasts. in The Yeasts. A taxonomic study, Kregger van Rij, N.J.W. (ed.), Elsevier Science Publishers, Amsterdam. pp.45-104.

VanEtten, H.D. 1976. Antifungal activity of pterocarpans and other selected isofalvonoids. Phytochem. 15:655.

VanEtten, H.D. and Bateman, D.F. 1971. Studies on the mode of action of the phytoalexin phaseollin. Phytopathol. 61:1363.

Vaughn, R.H., Jakubczyk, T., MacMillan, J.D., Higgins, T.E., Dave, B.A. and Crampton, V.M. 1969. Some pink yeasts associated with softening of olives. Appl. Microbiol. 18:771.

Watson, K. 1978. Thermal adaptation in yeasts: correlation of substrate transport with membrane lipid composition in psychrophilic and thermotolerant yeasts. Biochem. Soc. Trans. 6:293.

Wells, J.M. and Uota, M. 1970. Germination and growth of five fungi in low-oxygen and high-carbon dioxide atmospheres. Phytopathol. 60:50.

Witter, L.D., Berry, J.M. and Folinazzo, J.F. 1958. The viability of Escherichia coli and a spoilage yeast in carbonated beverages. Food Res. 23:133.

Yaun, C. and Bloch, K. 1961. Conversion of oleic acid to linoleic acid. J. Biol. Chem. 236:1277.

Zemlianukhin, A.A. and Ivanov, B.F. 1978. Metabolism of organic acids of plants in the conditions of hypoxia. In Plant life in anaerobic environment. Hook, D.D. and Crawford, R.M.M. (eds.), Ann Arbor Science Publisher Inc., MI. pp.203-268.

APPENDIX: IDENTIFICATION OF YEAST ISOLATES.

A1.1 Isolation of yeasts from blueberry

Blueberries purchased from the B.C. Blueberry Co-op (Richmond, B.C.) were used. Ten samples of 25 g each were stomached in 225 ml of 0.1% peptone water by a Colworth stomacher. Homogenates were then plated by the spiral plating method (Spiral System Instruments, Inc. Bethesda, Maryland), on PCA (Difco, Detroit, MI) supplemented with 0.01% chloramphenicol and 0.01% chlortetracycline HCl (Sigma Chemicals, St. Louis, MO). All plates were incubated at 21°C. Yeast colonies were picked from these plates and re-streaked on Yeast Morphology (YM) agar (Van der Walt and Yarrow, 1984). Only colonies with different morphology were picked to avoid excessive replications. Streaking of single colonies was repeated several times to obtain pure cultures.

Five yeast strains were isolated from blueberries and were tentatively labelled A,B,C,D,E. Cultures were maintained on YM agar slants.

A1.2 Identification of isolates

Identification of isolates to the genus level was based on microscopic and macroscopic morphology, sexual reproduction, pattern of sugar fermentation, ability to utilize various compounds as sole carbon source, formation

of extracellular amyloid compounds, production of ammonia from urea, and ability to grow at 37°C. Test procedures followed those described by Van der Walt and Yarrow (1984) and Barnett et al. (1983).

A1.2.1 Colonial morphology

Cultures were streaked on YM agar plates for isolated colonies (48 hours, 21°C). The macroscopic characteristics of isolates are summarized in Table A-1.

A1.2.2 Characteristics of vegetative cells

Yeasts were grown on YM agar for 48 hours (21°C). Single colonies were used to inoculate yeast nitrogen base containing 25 mM D-glucose, which was incubated for 20 hours in a shaking incubator (21°C, 80 rpm). After incubation, a sample of 10 µL was placed on a microscope slide and covered with a coverslip. For photography, volume of suspension was adjusted to avoid cell movements. Micrographs of yeast isolates are presented in Figures A-1 to A-5. All photomicrographs had a total magnification of 1000 x. A Zeiss Photomicroscope and Ilford XP1 400 film were used. Vegetative cells of all isolates lacked formation of pseudo or true mycelium. Vegetative reproduction of isolates was by multilateral budding.

Table A-1: Colonial morphology of yeast isolates grown on on YM agar plates for 48 hours, at 21°C.

Yeast	Description of colonies
A	Pink to orange-red colonies with even edge, convex, dull, smooth surface. Average diameter is 4 mm.
B	Light cream colonies with even edge, convex, shiny surface. Average diameter is 2 mm.
C	Light pink colonies with even edge, raised elevation, and very dull, dry surface. Average diameter is 3 mm.
D	Cream colonies with slimy appearance, even edge, convex elevation. Average diameter is 3 mm.
E	Light cream colonies with flat, dull surface. Average diameter is 2 mm.

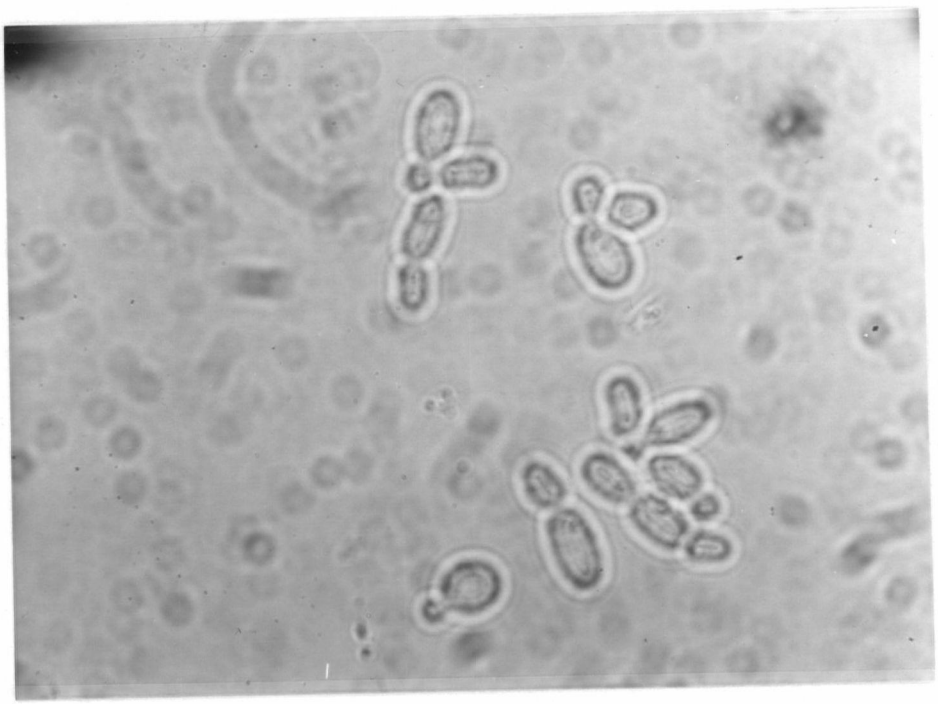

Figure A-1: Vegetative cells (1000 x) of yeast isolate A (later identified as a Rhodotorula species), grown on YNB-glucose for 20 hours at 21°C.

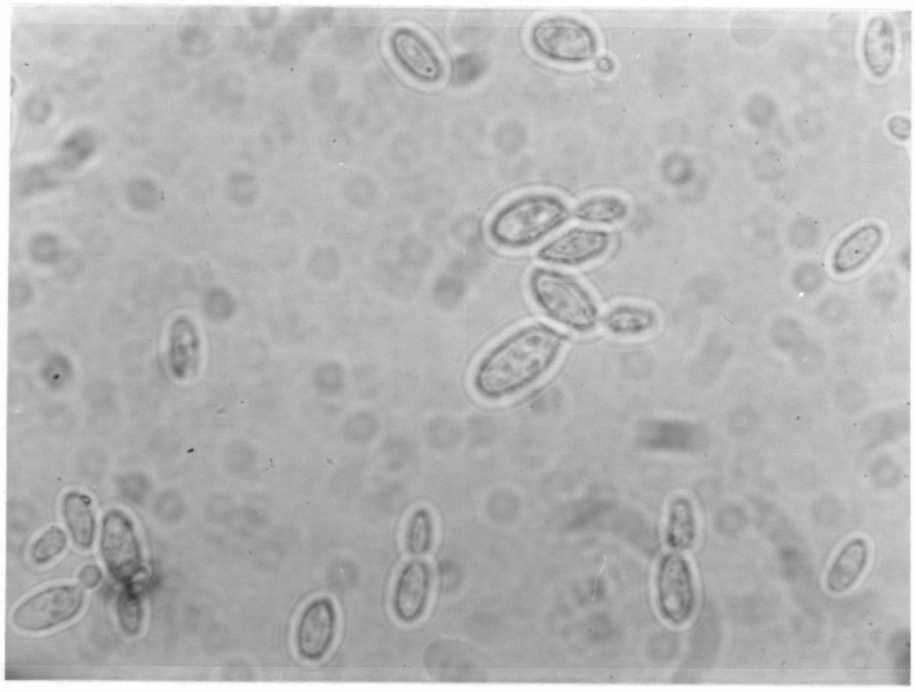

Figure A-2: Vegetative cells (1000 x) of yeast isolate B (later identified as a Zygosaccharomyces species), grown on YNB-glucose for 20 hours at 21°C.

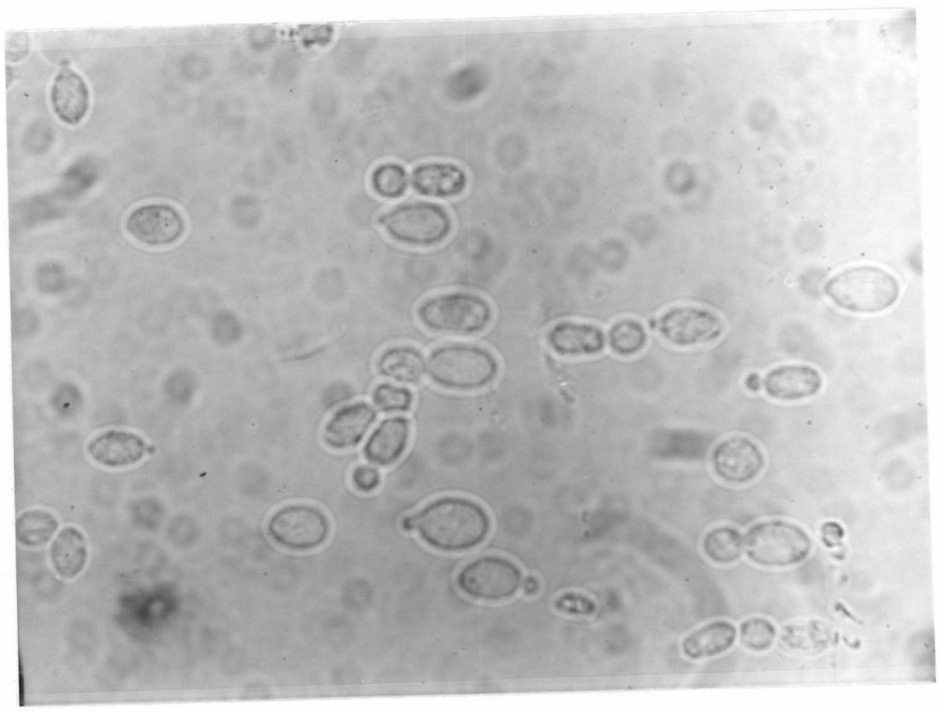

Figure A-3: Vegetative cells (1000 x) of yeast isolate C (later identified as a Rhodotorula species), grown on YNB-glucose for 20 hours at 21°C.

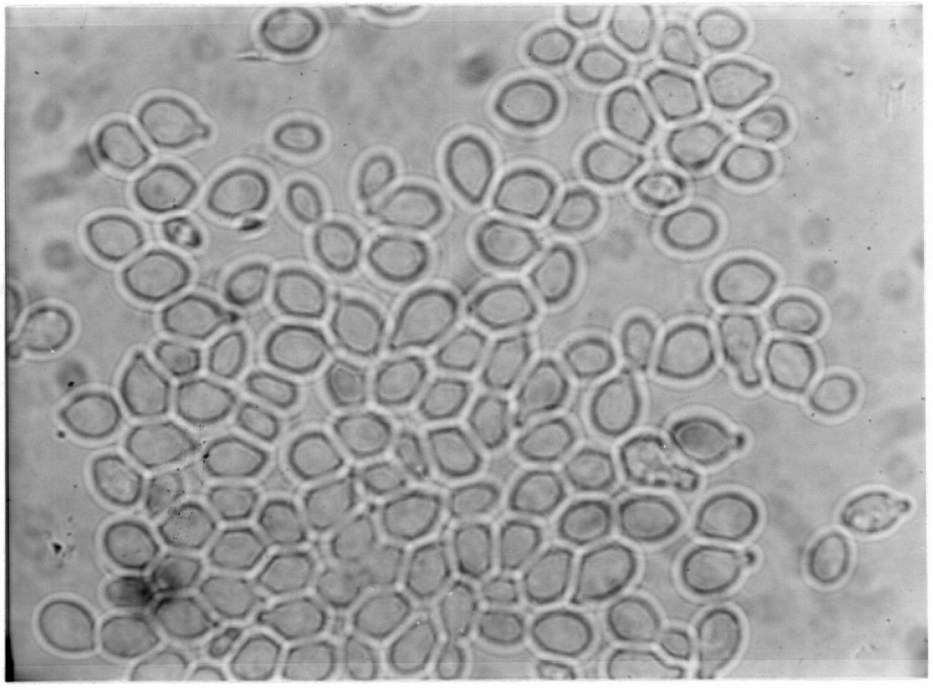

Figure A-4: Vegetative cells (1000 x) of yeast isolate D (later identified as a Cryptococcus species), grown on YNB-glucose for 20 hours at 21°C.

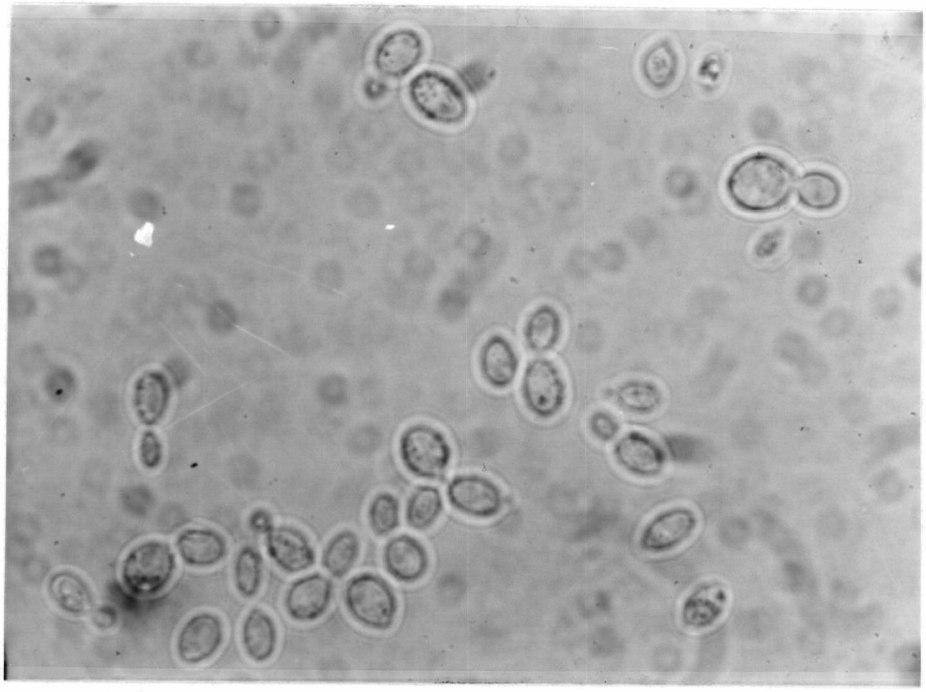

Figure A-5: Vegetative cells (1000 x) of yeast isolate E (later identified as a Debaryomyces species), grown on YNB-glucose for 20 hours at 21°C.

A1.2.3 Sexual reproduction

Growth from YM agar (48 hours, 21°C) were transferred to Potato Dextrose agar, Saboraud Dextrose agar and 2% agar (growth restrictive medium). The sporulation media were incubated at 21°C for 3 days before being examined microscopically. Since no growth occurred on 2% agar medium after three days, this medium was maintained at 21°C, and examined weekly for 4 weeks. All isolates failed to grow on 2% agar medium. Growth from Saboraud Dextrose and Potato Dextrose agar (3 days) were stained and examined microscopically for ascospores. Heat-fixed smears were flooded with 5% aqueous malachite green (Sigma Chemicals, St. Louis, MO) and heated to steaming (30 sec). The excess stain was washed off, and smears were counterstained with 0.5% Safranin (Sigma Chemicals, St. Louis, MO). The mature ascospores stained green and vegetative cells red under light microscopic examination (1000 x). The results are summarized in Table A-2.

A1.2.4 Production of ammonia from urea

Yeasts differ in their ability to hydrolyze high concentrations of urea to ammonia in media containing an organic nitrogen source. The test medium was Christensen's urea agar. Two-percent agar solution was dispensed in 4.5 ml aliquots to test tubes. The tubes

Table A-2: Sexual reproduction of yeast isolates.

Yeast	Description
A	no sexual reproduction on any media tested.
B	Cylindrical ascus with obtuse ends (5 µm long) containing 3 spores.
C	no sexual reproduction on any media tested.
D	no sexual reproduction on any media tested.
E	Ellipsoid asci (5-7 µm long) with 2 ascospores each.

were autoclaved for 15 minutes at 121°C. After autoclaving, 0.5 ml of a filter-sterilized (20%) urea base (Difco, Detroit, MI) was added, and mixed; the medium was allowed to set in slanted position.

Yeast cultures grown in YM agar (48 h, 21°C) were used to inoculate urea slants. Tubes were observed daily up to three days on incubation (21°C), positive reactions were recorded after appearance of a deep red colour in slants. Isolates A,C, and D gave positive reactions, B and E responses were negative.

A1.2.5 Growth at 37°C

Yeasts were grown on YM agar at 37°C for 4 days. None of the isolates were able to grow at this temperature.

A1.2.6 Assimilation of carbon compounds

The ability of yeast isolates to utilize the following 13 compounds was tested: galactose (Difco, Detroit, MI), sucrose (BDH Chemicals, Vancouver, BC), maltose (Difco, Detroit, MI), cellobiose (Sigma Chemicals, St. Louis, MO), lactose (Difco, Detroit, MI), melibiose (Sigma Chemicals, St. Louis, MO), raffinose (Sigma Chemicals, St. Louis, MO), xylose (Sigma Chemicals, St. Louis, MO), arabinose (Sigma Chemicals, St. Louis, MO), adonitol (Sigma Chemicals, St. Louis, MO), inulin (Sigma Chemicals,

St. Louis, MO), myo-inositol, salicin (Difco, Detroit, MI). As glucose is utilized by all yeasts, it was included as a standard for comparing the rates at which other compounds were utilized.

The assimilation medium was made up by dissolving 0.67 g Yeast Nitrogen base (Difco, Detroit, MI) and an appropriate amount of carbon compound equivalent to 5 g glucose, to 100 ml distilled water. When raffinose was used as the carbon source, it was used at an amount equivalent to 10 g glucose. Media were sterilized by filtration (0.45 µm cellulose ester filter, Millipore Corporation, Bedford, MA), and dispensed in 10 ml-aliquots. Inulin was dissolved by gently heating before filtration since undissolved inulin may be lost during filtration.

Yeast isolates grown in YM agar (48 hours, 21°C) were inoculated into 5 ml of sterile distilled water. Two drops of this inoculum was added to each tubes containing assimilation medium. All tubes were incubated at 21°C, and examined every 7 days for 3 weeks. The degree of utilization was determined by placing tubes against a white card bearing lines drawn approximately 3/4 mm wide. If growth completely obliterated the lines, it was recorded as 3 +; if the lines appeared as diffuse bands, growth was rated as 2 +; if the clear lines appeared

growth was rated as 1 +. A 3 + or 2 + reaction within three weeks was considered positive, and a 1 + reaction as negative. The results of this test are presented in Table A-3.

A1.2.6 Production of extracellular amyloid compounds

After 21 days, the carbon assimilation test cultures were examined for presence of amyloid compounds. One drop of Gram's iodine solution was added per tube. Presence of amyloid compounds was indicated by blue to green colour in tubes. Only isolate D gave a positive reaction for this test.

A1.2.7 Fermentation of sugars

A wine yeast Saccharomyces cerevisiae was also tested in this procedure to check for efficacy of the media. The fermentation basal medium contained 4.5 g yeast extract and 7.5 g peptone in 1L distilled water. Sufficient amount of bromothymol blue was added as an indicator. The basal medium (4ml) was pipetted to Durham tubes (test tubes carrying insert tubes approximately 50 x 6mm), and the tubes were autoclaved for 15 minutes at 121°C. After sterilization, 2ml-aliquots of various sterile sugar solutions were added to the tubes. The sugars were dissolved in distilled water to make up 6% solutions, except raffinose which was made up in a 12% solution. All

Table A-3: Utilization of carbon compounds by yeast isolates.

Compounds	Yeasts				
	A	B	C	D	E
Galactose	+	+	+	+	+
Sucrose	+	+	+	+	+
Maltose	-	+	-	+	+
Cellobiose	+	+	+	+	+
Lactose	-	-	-	+	+
Mellibiose	-	+	-	+	-
Raffinose	+	+	+	+	+
Inuline	-	-	-	-	-
Xylose	+	+	+	+	+
Inositol	-	+	-	-	+
Arabinose	-	+	+	+	+
Salicin	+	+	+	+	+
Adonitol	-	+	+	+	+

sugar solutions were sterilized by filtration. The final concentration of sugars in fermentation tubes was 2%, except raffinose was tested at a 4% final concentration. The fermentation tubes were inoculated with 0.1ml of a yeast suspension made by dispersing growth on Yeast Morphology agar in water. After inoculation, all tubes were tightly capped and incubated (21°C) and observed every 24 hours for gas and acid production (Van der Walt & Yarrow, 1984).

The above procedure failed to detect any gas formation in fermentation tubes, because the inverted insert tubes can only trap gas produced at or near the bottom of test tubes. Carbon dioxide produced by yeast cultures fermenting near the surface of medium yielded an apparent negative reaction. A new procedure for carbon dioxide detection was adapted. In this procedure, the fermentation medium was prepared as described above, and dispensed into tubes capped by air-tight rubber stoppers. At 1 week and 2 week intervals, the headspace of tubes was analyzed by gas chromatography. The instrument and operating conditions were identical to those used in gas analysis of samples in MA storage. Continued production of carbon dioxide in headspace after oxygen was depleted, and acid production was recorded as positive fermentation. Isolates A, C and D lacked the ability to ferment any of

the sugars tested. The patterns of fermentation of isolates B and E are outlined in Table A-4.

The yeast isolates were identified using the characteristics of genera compiled by Kregger van Rij (1984) with cross-reference to the work of Barnett *et al.* (1983). Isolates were identified as follows:

A: a *Rhodotorula* species

B: a *Zygosaccharomyces* species

C: a *Rhodotorula* species

D: a *Cryptococcus* species

E: a *Debaryomyces* species

A1.3 Standard curves of yeast isolates

Yeast cultures were grown in YM broth for 48 hours, at 21°C. Yeast counts (colony-forming units/ml) were obtained by plating dilutions of yeast cultures on Plate Count agar using the spiral plating method (Spiral System Instrument Inc., Bethesda, Maryland). Absorbance (420 nm) of culture dilutions was obtained using a Shimadzu UV-160 spectrophotometer. Yeast counts were plotted against absorbance to prepare standard curves (Figures A-6 to A-10).

Table A-4: Fermentation of sugars by two isolates.

Sugars	Yeasts	
	B	E
Glucose	+	+
Galactose	-	-
Sucrose	+	+
Maltose	+	-
Lactose	-	-
Raffinose	+	+

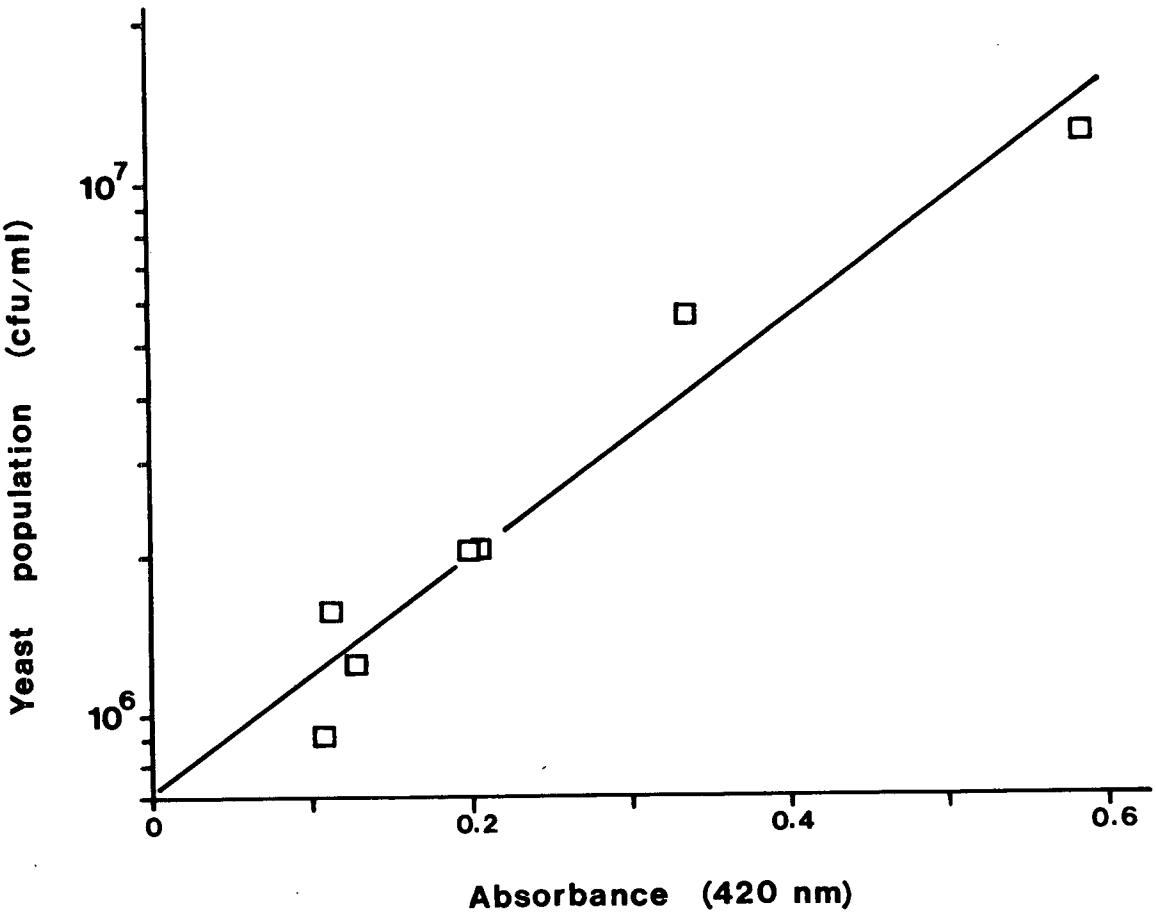

Figure A-6: Standard curve of a Rhodotorula species (isolate A). The curve was fitted by linear regression (r = 0.97).

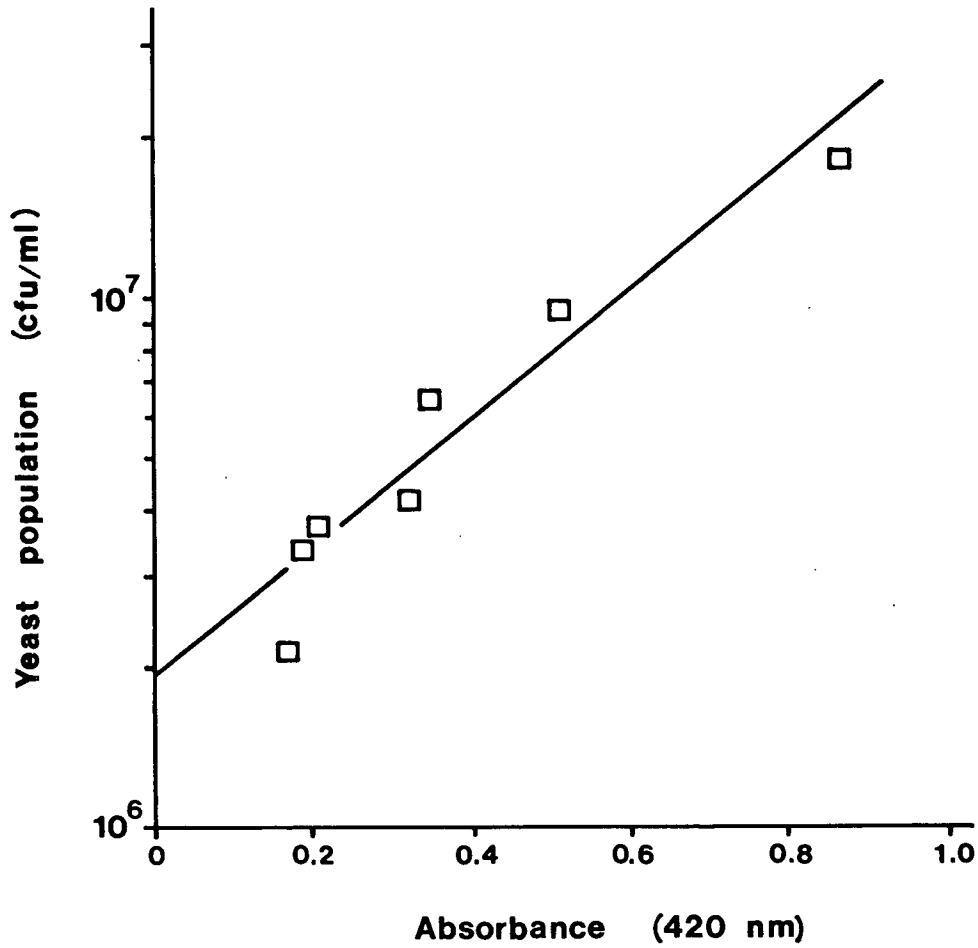

Figure A-7: Standard curve of a Zygosaccharomyces species (isolate B). The curve was fitted by linear regression ($r = 0.92$).

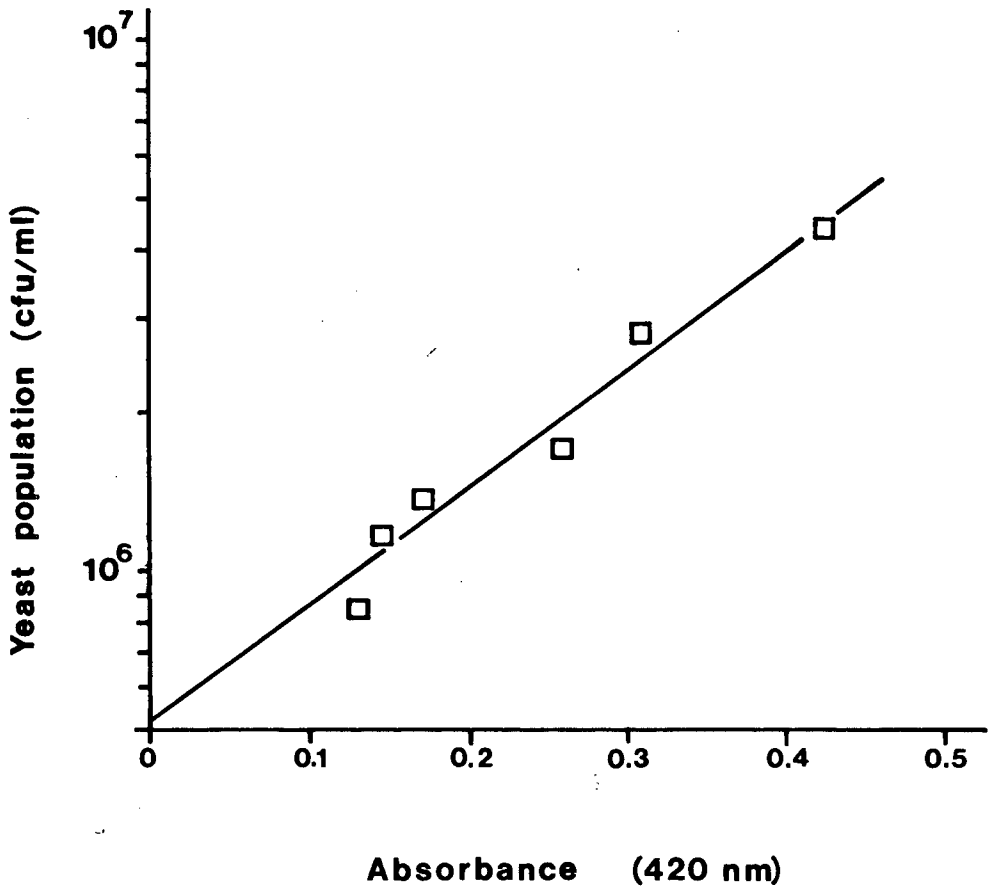

Figure A-8: Standard curve of a Rhodotorula species (isolate C). The curve was fitted by linear regression (r = 0.98).

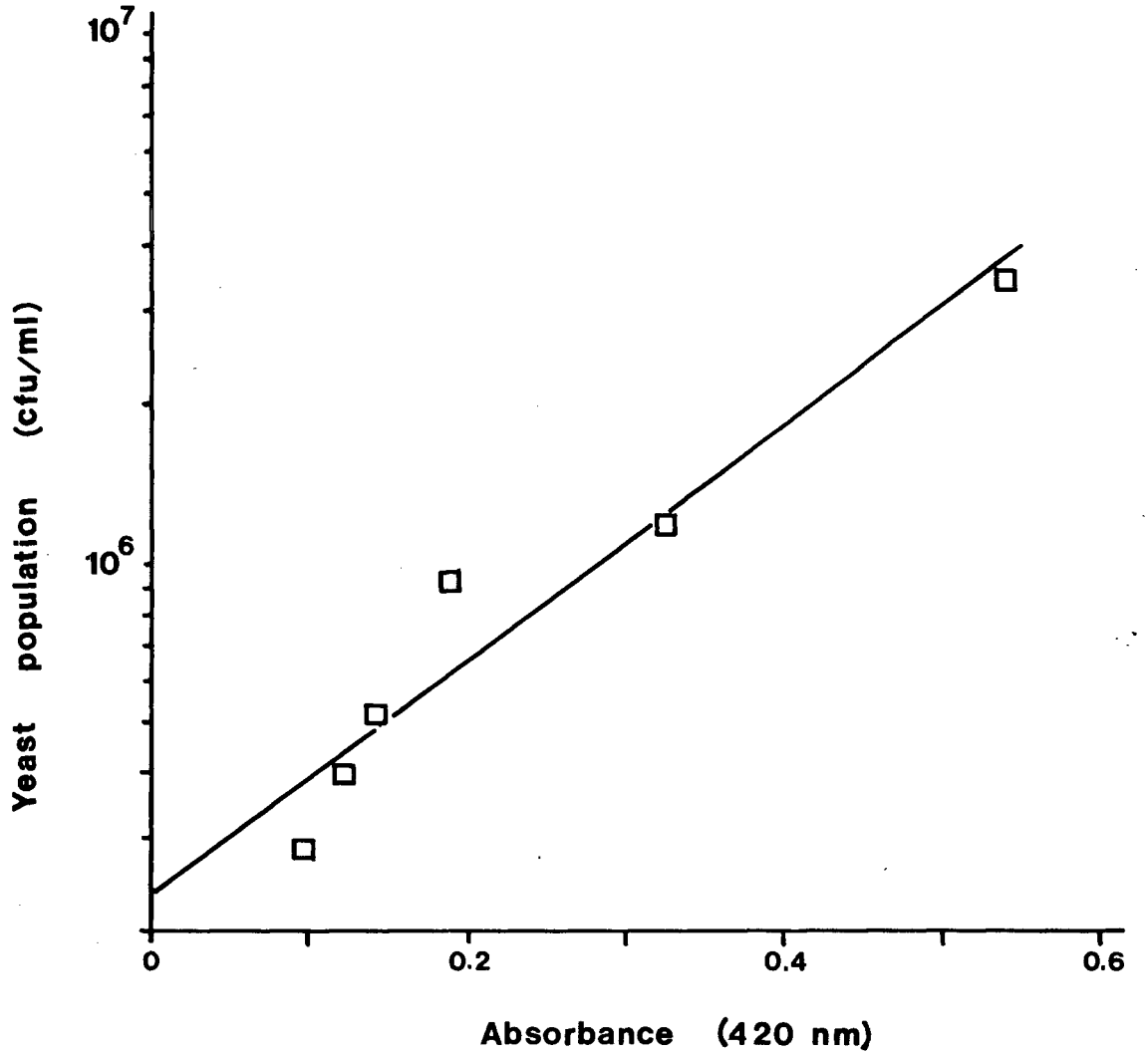

Figure A-9: Standard curve of a Cryptococcus species (isolate D). The curve was fitted by linear regression (r = 0.96).

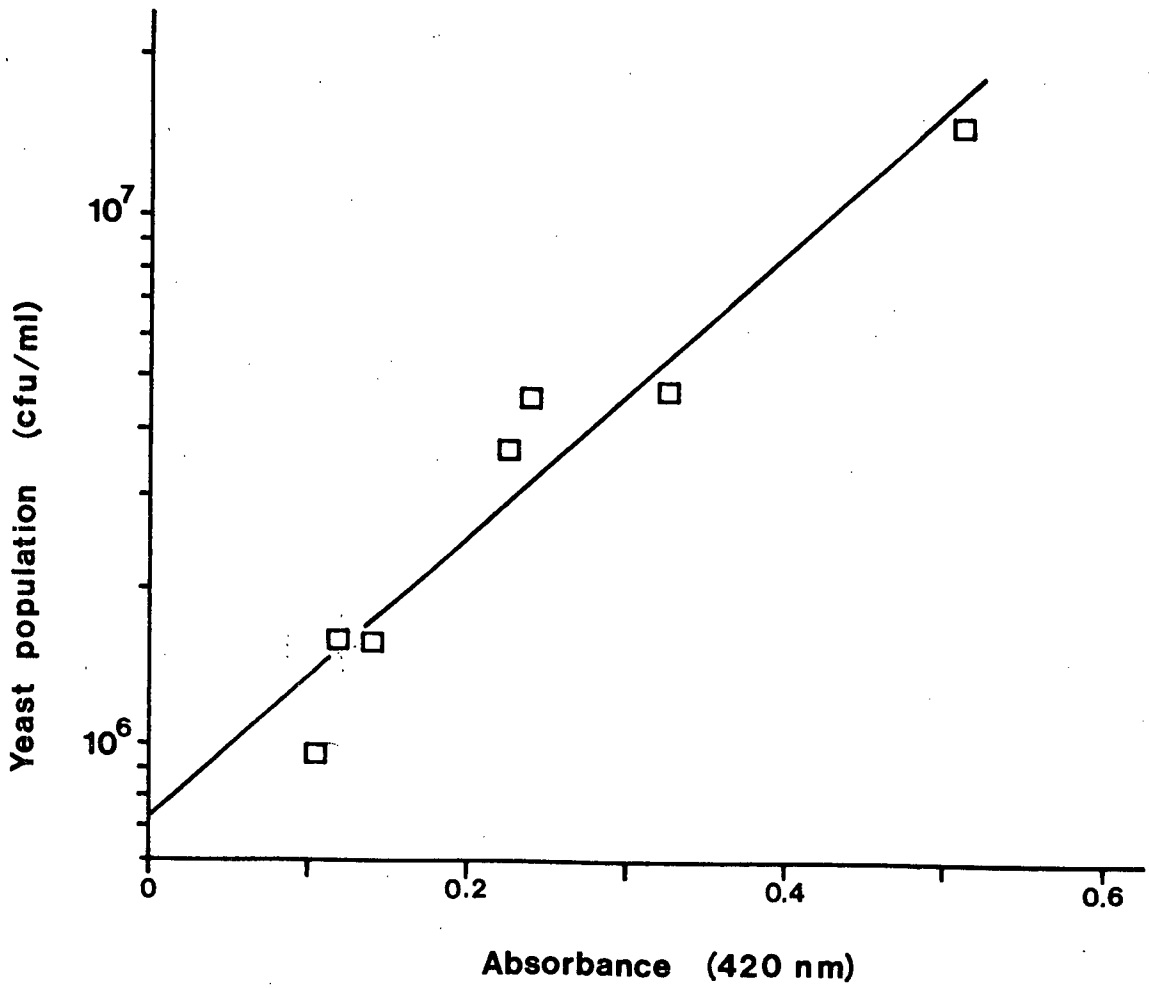

Figure A-10: Standard curve of a Debaryomyces species (isolate E). The curve was fitted by linear regression (r = 0.96).