INVESTIGATION OF MICROBIOLOGICAL AND CHEMICAL STABILIZATION OF BEVERAGE SYSTEMS BY IRON REMOVAL

A Thesis

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by

Tracy Lynn Brenner

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ABSTRACT

Iron is a ubiquitous element that is necessary for microbial nutrition and growth and, thus, potential food spoilage. In addition, iron has an extremely high affinity for oxygen and, as such, it may promote oxidative processes in beverages and foods, thereby requiring additional preservative considerations. Specific removal of iron by chelators may constitute a method to replace or synergistically enhance the action of traditional preservatives and antioxidants in many food applications.

Prior to studying the effects of iron removal on microbial growth and oxidative color changes, iron removal efficacy was first evaluated in a chemically defined medium using an insoluble iron chelating resin (FEC-1) in a column extraction process. The resin displayed superior selectivity and affinity for iron, significantly reducing iron levels by 87% to a final concentration of <2.0 ppb. In this medium, the resin reduced copper levels by 99.9%.

The extracted chemically defined medium was used to study the effects of iron removal on microbial growth. The removal of iron from the chemically defined medium had a negative effect on growth, reducing growth of most microorganisms by 25-80%; however, the concentration of iron removed from the medium was not sufficient to fully inhibit yeast growth. *Candida albicans* exhibited little growth reduction (<25%), and although thought to produce hydroxamate-type siderophores to aid with iron-limited growth, siderophores were not detected.

Select food preservatives (sodium benzoate, potassium sorbate) were added to extracted and non-extracted chemically defined medium to evaluate a possible synergistic effect on the growth reduction of *Hansenula anomala*. This organism

exhibited greater tolerance to potassium sorbate (350 ppm) than to sodium benzoate (200 ppm) and no differences were found between growth in extracted versus non-extracted media.

Iron removal was surveyed in various complex media. The majority of samples were batch extracted and the resin was found to decrease iron concentrations by >90% in seven samples out of 13 samples. Copper was also reduced in some samples although not to the same extent as the removal in chemically defined medium. The resin removed iron in Sauvignon Blanc wine by 99.7%, thereby, significantly reducing oxidative color changes.

Significant iron leaching was observed in the extracted chemically defined medium after submersion of food grade stainless steel ferrules. The initial iron concentration of the chemically defined medium was ~2.3 ppb and was increased to 166.4 ppb after one hour of exposure to the ferrules at room temperature. The ferrules were not pre-treated to remove fugitive surface iron from the ferrules and likely it was this fugitive iron that was detected in the samples.

Metabolic changes were observed during growth of *Candida vini* and *Candida albicans* in iron-extracted and non-extracted chemically defined media. Both organisms excreted citrate, succinate, and acetate. Acetate production by *C. vini* and *C. albicans* was delayed in extracted medium. *C. vini* produced significantly less succinate and more citrate in extracted medium compared to growth in non-extracted medium implicating aconitase as the limiting factor in the metabolic pathway. *C. albicans* produced negligible quantities of citrate in extracted medium suggesting the use of an alternative pathway in which aconitase is not the limiting factor.

BIOGRAPHICAL SKETCH

Tracy Brenner grew up in Guelph, Ontario, Canada and attended the University of Guelph to pursue a Bachelor of Science degree in Food Science. While attending college, Tracy earned several scholarships from external sponsors as well as annual academic scholarships through the Food Science Department and the College of Agriculture. Tracy completed two independent projects as an undergraduate research assistant in the Food Science Department. The first project was conducted at the Canadian Research Institute for Food Safety where she worked under the supervision of Dr. Mansel Griffiths to explore susceptibility of various food pathogens to organic sanitizers. The second project was in the Wine Microbiology Laboratory of Dr. Ramón Mira de Orduña where she assisted with characterizing the microbiological and physiological properties of the spoilage organism *Moniliella acetoabutans.*

Upon completion of her undergraduate studies, Tracy was accepted into the Food Science and Technology program at Cornell University and was stationed at the New York Agricultural Experiment Station in Geneva, NY. Tracy began her graduate program in January 2007 under the guidance of Dr. Ramón Mira de Orduña. She pursued a Master's degree in food microbiology with a focus on wine and beer spoilage microorganisms. This thesis entitled Investigation of microbiological and chemical stabilization of beverage systems by iron removal is submitted to the graduate faculty of Cornell University in partial completion for the degree of Master of Science in the spring of 2009.

This thesis is dedicated to my friends and family for their unconditional love and support.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Iron is an essential element of microbial nutrition and growth and, thus, is a factor in food spoilage. Iron may also contribute to oxidative processes in beverages and foods, thereby, creating a need for the addition of preservatives with antioxidant properties. Specific removal of iron by chelators may constitute a method to replace or synergistically enhance the action of traditional preservatives and antioxidants in many food applications. Iron removal may also have applications in areas outside of Food Science such as inclusion in products that require antimicrobial and/or antioxidant protection.

1.2. Iron and its Global Relevance

Iron constitutes about 6% of the Earth's crust and 35% of the mass of the Earth as a whole, making it the most abundant element on earth (Girard, 2004).

Iron is a biologically important element because of its chemical nature at near neutral pH values in aqueous environments, supporting microbial growth where it is available in two ionic states: ferric (Fe^{3+}) iron and ferrous (Fe^{2+}) iron. The corresponding salts and complexes of ferrous iron are soluble at neutral pH and can be present in water at pH 7 in concentrations of 10^{-2} M; if a reduced (low oxygen) state is maintained, the ferrous iron is sufficiently available for uptake in cells. However, in most aerobic environments, ferrous iron does not remain stable, but rather it reacts with oxygen to become oxidized, thus forming ferric iron (Miethke and Marahiel, 2007; Van Ho *et*

al., 2002b; Winkelmann, 1991; Dancis *et al.,* 1990). Ferric iron is highly insoluble at neutral pH and the maximum concentration of ferric iron in water at pH 7 is only 10^{-17} M lending to its poor bioavailability when in the ferric state (Dancis *et al.,* 1990).

Iron in its reduced elemental state is an important material for the manufacture of industrial items such as bars, strips, sheets, steel nails, spikes, wire, rods, pipes, and ferroalloy products. The two largest steel consuming industries have typically included automotive manufacturers and construction companies as well as use in structural engineering applications and general industrial applications (United States Environmental Protection Agency, 1995). According to the 2005 *Annual Survey of Manufacturers* (U.S. Census Bureau, 2005), shipment values in the iron and steel mills and ferroalloy manufacturing sector and the steel product manufacturing from purchased steel sector totaled 94.6 and 102.4 billion dollars in 2004 and 2005, respectively.

1.3. Iron as a Cofactor in Oxidation Reactions

Iron can easily gain and lose electrons which makes it essential as a cofactor in redox reactions. Iron also has a high affinity for oxygen, thus being involved in various redox reactions catalyzed by enzymes (Van Ho *et al.,* 2002a). Because iron can react with oxygen-containing compounds to form toxic oxygen radicals (ie. superoxide and hydroxyl radicals), it may contribute to oxidative damage of cells, aging, carcinogenesis, radiation injury, and even cell death (Matzanke, 1991; Yiakouvaki *et al.,* 2006). As such, most organisms have developed mechanisms to regulate the amount of iron present in biological fluids and cells (Van Ho *et al.,* 2002a). Regulation of iron is especially important considering there is currently no known mechanism for

excretion of iron from a cell (Neilands, 1991). In the following paragraphs, some examples of the relevance of iron in metabolism, food systems, and microbial nutrition will be discussed.

1.4. Iron in Metabolism and Nutrition

Iron is required in many biologically important reactions including participation in ATP and DNA biosynthesis, transport of oxygen and electrons, metabolism of natural and xenobiotic compounds, and participation in cell growth and regulation (Kontoghiorghes, 1995). More specifically, iron acts as a cofactor for enzymes including cytochrome P450, xanthine oxidase, catalase, NADH dehydrogenase, ribonucleotide reductase, and aconitase (Wessling-Resnick, 2000). Thus, if the availability of iron is limited, important biological processes may be impaired. For example, iron deficiency in the human diet can lead to disorders such as anemia which may affect immune function, cognitive development, work performance, and reproductive functions (Walter, 1989; Dallman, 1989).

Alternatively, excess iron in the body may lead to potentially fatal iron overload diseases such as thalassemia major (via red blood cell transfusions), idiopathic hemochromatosis (increased iron absorption due to genetic defect), and Bantu siderosis (due to increased absorption of dietary iron). These diseases are less common and lead to more severe side effects than iron deficiency diseases. Most individuals with iron overload diseases must participate in iron chelation therapy which typically includes the use of chelating drugs to remove iron from the blood and provide an excretory route (Kontoghiorghes, 1995).

1.5. Iron in Foods

1.5.1. Oxidation

Iron concentrations are sufficiently high in most foods to contribute to undesired oxidative spoilage. Of major importance in the food industry is lipid oxidation because of its impact on food spoilage in high fat foods. Typically, lipid oxidation leads to oxidative rancidity which is characterized by the development of off-flavors and may potentially render products unacceptable. Oxidation may also decrease the nutritional value of some foods, as well as creating potentially toxic by-products during reaction (Nawar, 1996). Iron is a key factor in the promotion of lipid oxidation in foods and is known to catalyze the initiation and propagation stages of this reaction (Miller, 1996). Specifically, ferrous iron (Fe^{2+}) reduces dioxygen (O_2) by one electron resulting in the Haber-Weiss-Fenton reaction sequence leading to hydroxyl radical ($OH^{\bullet}$) formation (Aisen *et al.,* 2001):

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-\bullet}$$

$$2O_2^{-\bullet} + 2H^{+} \rightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH^{-} + Fe^{3+}$$

The hydroxyl radical is a powerful oxidant and when exposed to unsaturated fatty acids, may remove hydrogen atoms thereby generating lipid free radicals and initiating lipid peroxidation (Miller, 1996).

Iron also plays a role in the oxidation of beverages, for example, wines. In wines, uptake of oxygen can lead to color changes, production of oxidized flavors, and possible precipitations. Besides iron, copper and manganese may also play a role as catalysts during oxidation because they can form complexes with oxygen, thereby leading to color changes and off-flavors (Cacho *et al.,* 1995).

1.6. Iron in Microbial Food Spoilage

Iron is prevalent in food systems, thereby contributing to oxidation reactions. Since iron is also essential for the growth of most microorganisms, iron plays a significant role in food spoilage. The one possible exception may be certain lactobacilli which have been shown to survive in low-iron environments (ie. dairy products in which much of the available iron is bound to lactoferrin, making the iron unavailable to many microorganisms) (Neilands, 1991; Archibald, 1983). Since most organisms require iron, they must be able to transport, store, and utilize available iron sources, especially during times of iron deprivation.

1.6.1. Transport of Iron

Saccharomyces cerevisiae is widely regarded as the model organism used to study transport of metals in eukaryotic cells because its genome has been sequenced. As such, a significant amount of information is available regarding metal transport in this microorganism. Studies focused on iron deficiency in *S. cerevisiae* have led to the identification of two distinct transport systems shown to mediate uptake of ionic Fe in yeast cells, a low affinity and high affinity system. Each transport system requires ferrous iron and, as such, the iron must first be reduced extracellularly before uptake into the cell (Andrews *et al.,* 1999; Dancis *et al.,* 1990; Lesuisse *et al.,* 1987; Georgatsou and Alexandraki, 1994). Cells grown in media with a high iron content will exhibit the low affinity system, while the high affinity system is used when cells are grown in iron-restricted medium (Eide *et al.,* 1992). The high affinity system requires oxygen and, thus, cannot be used under anaerobic conditions (Hassett *et al.,* 1998).

1.6.2. Siderophore Use

At neutral pH, iron is primarily present in the ferric form. Because of this oxidation state and the extremely low solubility of ferric iron, very little free iron is available for uptake by microorganisms (Ismail *et al.,* 1985b). To compensate for this phenomenon, some microorganisms utilize siderophores, ie. low molecular weight organic compounds that are produced by the cells during periods of iron deprivation (Matzanke, 1991). Siderophores have an extremely high affinity for ferric iron (Van Ho *et al.,* 2002a) and are capable of extracellular solubilization of ferric iron from organic substrates or even mineral forms of Fe (Matzanke, 1991).

The main function of the siderophore is to supply iron to the cell and, as such, the siderophores are excreted from the cells to retrieve available extracellular iron (Van Ho *et al.,* 2002a). The bound iron may then be reduced from the ferric form to the ferrous form at the cell membrane, or, specific transporters may be used for the internalization of the entire iron-siderophore complex (Lesuisse and Labbe, 1989).

The physical and chemical properties of siderophores vary widely and, accordingly, these compounds are categorized into two main chemical groups – catecholates/ phenolates and hydroxamates (Matzanke, 1991). Each of these groups have a very high affinity for ferric iron with a formation constant of 10^{30} and higher (Neilands, 1981; Matzanke, 1991).

Siderophores are synthesized by microorganisms including bacteria, as well as fungi such as yeasts. This review will specifically consider yeast that are relevant to food spoilage and human pathogenesis. Examples of food spoilage organisms include strains of *Rhodoturula* which have been shown to produce the dihydroxamate type

siderophore, rhodoturulic acid. Another example includes *S. cerevisiae,* an organism that cannot synthesize siderophores but can utilize various hydroxamate-type siderophores synthesized by other microorganisms. An example of a human pathogen is *C. albicans* which has been reported to produce hydroxamate-type siderophores (Holzberg and Artis, 1983; Ismail *et al.,* 1985b; Ismail *et al.,* 1985a; Sweet and Douglas, 1991). The ability to synthesize and use siderophores has been recognized as one of the virulence factors of *C. albicans* (Holzberg and Artis, 1983).

1.6.3. Storage and Utilization of Iron

As iron is transported into microbial cells, any iron not required for immediate use may be stored by cellular components for use at a later time. Free cellular iron is toxic to cells and must be sequestered to prevent toxicity. Ferritin, a protein involved in iron metabolism, is used for iron storage by mammals and many bacteria, however, *S. cerevisiae* lacks ferritin and utilizes cellular vacuoles as the main iron storage vessel (Aisen *et al.,* 2001; De Freitas *et al.,* 2008; Raguzzi *et al.,* 1988; Bode *et al.,* 1995; Li *et al.,* 2001). Raguzzi *et al*., (1988) showed that vacuolar iron concentration increased proportionally with the extracellular iron concentration while cytosolic iron remained stable. Bode *et al*., (1995) and Raguzzi *et al*., (1988) showed that even under reduced extracellular iron concentrations, vacuoles of *S. cerevisiae* continued to accumulate iron for use in the mitochondria.

1.7. Preservatives

A cornerstone for the food industry is the need for food preservation in order to maintain food quality and safety for human nutrition. Most foods are intrinsically microbiologically unstable and require a preservation technique to increase shelf-life. Traditional preservation techniques include heat treatment, fermentations with microorganisms (to render food more stable by formation of acid or ethanol), and

addition of preservatives (e.g. organic acids). Among the more common preservatives applied in beverage systems are sulfur dioxide, sorbic acid, and benzoic acid.

1.7.1. Sulfur Dioxide

Sulfur dioxide is used in various food systems. Surface treatment with SO_2 is commonly used to prevent browning and growth of spoilage organisms on fruits and vegetables, while in beverages it can be added to prevent oxidations and microbial growth (Ough and Were, 2005). Sulfur dioxide is essential in conventional winemaking because of its antimicrobial and antioxidant properties. To prevent oxidation and microbial growth, SO_2 is typically added to crushed grapes in the amount of 0-50 mg/L; however, fruit in poor condition may require the addition of up to 120 mg/L (Ough and Amerine, 1988). Many wine bacteria (ie. malolactic bacteria) and some yeast (ie. *Pichia* spp., *Rhodoturula* spp.) are susceptible to the effects of SO_2; however, other yeast, such as *Zygosaccharomyces* spp., are more resistant (Goto, 1980; Rhem and Wittman, 1962).

In both Canada and the United States, the maximum concentration of total SO_2 in wines and musts cannot exceed 350 mg/L (Canadian Food Inspection Agency, 2007; Alcohol and Tobacco Tax and Trade Bureau, 2006). Sulfur dioxide levels in bottled wines for consumption are typically maintained at 60-130 mg/L (Ough and Amerine, 1988).

The Food and Drug Administration (FDA) allows for the use of SO_2 in fruit juices, fruit concentrates, and dehydrated fruits and vegetables (Ough and Were, 2005). Concentrations of SO_2 can range from 10 to 100 mg/kg SO_2 in fruit juices, to as high as 500 mg/kg SO_2 in fruit pulps, purees, and fillings (Gould, 2000).

1.7.2. Sorbic Acid & Benzoic Acid

Sorbic acid/sorbate salts and benzoic acid/benzoate salts are used for preservations in foods, beverages, cosmetics, and pharmaceuticals (Stopforth *et al.,* 2005; Chipley, 2005). These preservatives are especially efficient when applied in foods with low pH values because the undissociated acids formed under these conditions are responsible for antimicrobial activity (Chipley, 2005; Stopforth *et al.,* 2005). However, sorbates and benzoates may be used in foods with pH values as high as 7.0 and 5.5, respectively (Sofos and Busta, 1981; Chung and Lee, 1982).

Both preservatives are Generally Recognized As Safe (GRAS) by Health Canada and the U.S. Food and Drug Agency (USFDA) if concentrations of the preservative are within the maximum permitted level (Chipley, 2005; Stopforth *et al.,* 2005; Health Canada, 2006). In the United States, benzoic acid/benzoate salts are GRAS if below 0.1% in foods and beverages (Chipley, 2005), while sorbic acid/sorbate salts are generally encountered in foods and beverages in concentrations from 0.02% to 0.3% (Stopforth *et al.,* 2005). Typically, these concentrations do not influence the organoleptic properties of foods, however, in some foods, even the lowest concentrations of preservatives may change the taste of a product (Stopforth *et al.,* 2005).

Although benzoic and sorbic acids have GRAS status in North America, studies have shown that under certain conditions, the addition of benzoic acids to beverages may lead to formation of unwanted, potentially dangerous compounds. In the early 1990's, benzene, a known carcinogen, was shown to form in carbonated beverages from the reaction of benzoate salts/benzoic acid and ascorbic acid (Page *et al.,* 1992). At this time, benzoic and ascorbic acids were being added to soft drinks as preservatives and

antioxidant/vitamin supplements, respectively. Trace metals such as Cu (II) and Fe (II) catalyze the reduction of oxygen (O_2) by reacting with ascorbic acid to form free radicals. Consequently, these free radicals were shown to produce benzene from reaction with benzoic acid (Gardner and Lawrence, 1993).

Studies have shown that various yeast species are tolerant to sorbate. Yeast that have shown sorbate tolerance include *Saccharomyces* spp. (Bills *et al.,* 1982; Splittstoesser *et al.,*), *Candida* spp., *Brettanomyces* spp., and *Zygosaccharomyces* spp. (Warth, 1977). Furthermore, Warth (1985) suggested that yeast species which were tolerant to one preservative, specifically benzoic acid, would be tolerant to other preservatives (ie. sorbic acid, acetic acid). This observation holds true for many species of the genera *Zygosaccharomyces* which tend to be resilient to both benzoic and sorbic acids up to concentrations of 1300 ppm and 800 ppm, respectively (Neves *et al.,* 1994; Warth, 1989).

1.7.3. Preservatives and Health Effects

While the application of preservatives such as SO_2 and benzoic and sorbic acids may assure microbiological stability, their application has been scrutinized because of potentially negative impacts on human health, thus, resulting in a more negative public perception.

In general, most humans are tolerant to SO_2 at reasonable concentrations ($\leq$ 0.7 mg SO_2 per kg body weight), however, SO_2 has been known to provoke allergic reactions in some individuals, particularly those suffering from asthma (Ough and Were, 2005; Dahl *et al.,* 1986; Vally and Thompson, 2001). Monitoring of SO_2 sensitivity and allergic reactions began in the 1980's by the U.S. Food and Drug Administration

(FDA) Center for Food Safety and Applied Nutrition (CFSAN). As of 1999, 1132 complaints of adverse reactions to SO_2 have been reported; of those with accurate information, 48.6% were recorded as severe (Warner *et al.,* 2000).

Oral benzoic acid consumption has reportedly lead to various allergic reactions in humans, including one account of severe anaphylaxis (Parke and Lewis, 1992; Schaubschlager *et al.,* 1991; Michils *et al.,* 1991). Furthermore, in a review on the toxicology of sorbic acid and sorbates, Walker (1990) describes several reports in which sorbic acid has been associated with idiosyncratic intolerances in humans. Included in this list are associations with non-immunological contact urticaria, chronic urticaria, and burning mouth syndrome. Sorbic acid has also been cited as a contact allergen (Parke and Lewis, 1992).

1.7.4. Alternate Approaches to Preservative Use

Thus far, this literature review has introduced two key points to consider. First, iron is required for many important reactions including those that occur in the microbial cell, as well as oxidative reactions in both biological and food systems. Second, within the overall goal to reduce preservative utilization while assuring control of food spoilage microorganisms, the availability of alternative methods for preservation would be ideal. Typically, a combination of physical methods (ie. heat treatment, ultraviolet radiation, refrigeration) and chemical methods (ie. addition of preservatives, decrease in pH) are used as a "hurdle technology" approach to improve the quality and shelflife of food products (Chipley, 2005). As a new or additional approach to food preservations, removal of iron could be used in an attempt to limit oxidative and microbial spoilage, thereby limiting the requirement for addition of preservatives.

1.8. Iron Chelation Techniques

Various chelation techniques have been used in beverages and laboratory growth media to reduce the concentration of metals (ie. iron, copper, and manganese) responsible for initiating oxidative processes and essential for microbial growth. Such techniques include the use of cation exchange resins (Benitez *et al.,* 2002) and iron removal via soluble or insoluble metal chelators (Feng *et al.,* 1995a; Holbein *et al.,* 2007; Feng *et al.,* 1997b; Feng *et al.,* 1997a).

Insoluble and soluble chelators have been studied for use in grape juice, white wines, milk, and water (Feng *et al.,* 1997a; Feng *et al.,* 1997b; Benitez *et al.,* 2002; Feng *et al.,* 1995b; Korngold, 1994b; Palacios *et al.,* 2001). Most notable are the studies on iron removal in white grape juice and white wine in which the chelating resins removed iron, copper, and manganese to levels low enough to reduce microbial growth (≥ 90 %) (Feng *et al.,* 1997a; Feng *et al.,* 1997b; Benitez *et al.,* 2002).

Of particular interest is the insoluble FEC-1 iron(III) chelating resin, which is largely iron specific (Feng *et al.,* 1997b). Due to the high iron specificity of this chelator and the observed decrease in microbial growth, the FEC-1 resin was the primary iron chelator used throughout the studies reported in this thesis.

1.9. Summary and Objectives of the Thesis

This introduction has presented previous research relevant to the importance of iron as an essential cofactor in many important reactions, including those that may lead to food quality degradation. Many food products require additions of chemical preservatives; however, even additions of small amounts of preservatives may lead to flavor/aroma defects in foods as well as possible adverse health reactions in some

individuals. Thus, the aim of this thesis project was to investigate a possible alternative to current preservation methods, with emphasis on reducing the addition of chemical preservatives. Specifically, this thesis focussed on removal of iron from growth media and beverage systems in an effort to reduce both microbial growth and oxidative reactions.

The first objective of this study was to physically extract iron from a chemically defined yeast culture medium in order to create an iron-limited environment for growth studies. As such, iron-limiting techniques were employed to develop iron-extracted media which were subsequently analysed for trace iron concentrations using ICP-MS (Chapter 3).

Following the preparation of iron-extracted media, growth studies were conducted to investigate the iron requirements of various food spoilage yeast. These studies were conducted using iron-sufficient or iron-deficient yeast cultures in an iron-extracted chemically defined medium (Chapter 4).

Chapter 5 evaluates the possible synergism between common food and beverage preservatives (benzoic and sorbic acid) and low-iron systems. A screening study was conducted using various preservative concentrations in iron-deficient chemically defined medium and growth was monitored qualitatively.

Iron removal strategies were evaluated in complex food matrices including fruit, vegetable, and grain-based systems. Additionally, oxidative stability of white wine after iron removal was investigated, as was the feasibility of iron removal applications in industry (Chapter 6).

In Chapter 7 experiments were conducted to determine potential metabolic targets of iron deprivation in selected yeast cultures.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1. General

2.1.1. Water

Water used throughout the study was obtained from a Milli-Q water purification system (Millipore; Bedford, MA, USA) that provided a minimum resistance of 18 megohm cm^{-1}.

2.1.2. Media Sterilization

Media were sterilized by autoclaving (121°C for 15 minutes) or by sterile filtration using either a 0.22 µm mixed cellulose membrane filter (Millipore, USA) or a 0.20 µm nylon syringe filter unit (Fisher Scientific, Fairlawn, NY, USA).

2.1.3. Origin of Chemicals and Media

Growth media were provided by Difco (Sparks, MD, USA). Amino acids were provided by Alfa Aesar (Heysham, Lancaster, UK) with a purity of ≥ 99%. Organic acids were obtained from Sigma Aldrich (St. Louis, MO, USA) or Acros Organics (NJ, USA) with purity of >98%. All other chemicals (minerals, vitamins, salts, etc.) were ACS grade and provided by Fisher Scientific unless otherwise stated.

2.1.4. Glass and Plasticware

Glassware was used only for autoclaving and storage of complex culture media. Due to the nature of this project (ie. low iron conditions), platicware was used for all preparation, filtration, and storage of chemically defined media, buffers, water, and chemical stock solutions. Sterile, disposable, 16 ml polystyrene tubes (BD Falcon;

Franklin Lakes, NJ, USA) were used during growth of all cultures, and sterile 15 or 50 ml centrifuge tubes (Corning; Corning, NY, USA) were used for storage of cultures.

With the exception of pre-sterilized disposable culture tubes, all plasticware was soaked in 3% iron-chelating solution (Citronox; Jersey City, NJ, USA) for 24 hours and then rinsed thoroughly with ultra pure water.

2.2. Growth Media and Stock Solutions

2.2.1. Media Preparation

2.2.1.1. YPD Medium and Agar (Yeast Peptone Dextrose Medium)

YPD medium was prepared by dissolving 50 g YPD powder (Difco, Sparks, MD, USA) in purified water (adjusted to 1 L) followed by autoclaving. Solid media for viable cell counts and culture conservation were prepared by adding 15 g of agar (Difco) prior to autoclaving.

2.2.1.2. Chemically Defined Media

The media employed was a modified version of the GPP medium first described by Kulkarni and Nickerson (1981). This medium does not include Fe salts; residual iron from other medium components is the only source of iron in this defined medium.

The chemically defined medium was composed of the following (per liter distilled water): 0.5 g $MgSO_4 \cdot 7H_2O$, 13.6 g Na_2HPO_4, 1.32 g $(NH_4)_3PO_4$, 2.0 g L-asparagine, and 20 g glucose. To this base medium, 1.0 ml of vitamin solution and 0.25 ml of mineral solution were added. The vitamin solution consisted of 2.0 mg biotin, 20 mg pyridoxine-HCl, and 20 mg thiamine-HCl per 100 ml of 20% ethanol. The mineral

solution contained 0.5 g $CuSO_4 \cdot 5H_2O$, 0.54 g $MnSO_4 \cdot H_2O$, and 0.5 g $ZnSO_4 \cdot 7H_2O$ per 100 ml 0.1 N HCl. For growth of *S. cerevisiae*, the base medium included 0.1 g/L $CaCl_2$, 0.2 mg/l $Na_2MoO_4 \cdot 2H_2O$, and 1 g/l glutamine in place of L-asparagine. Both the vitamin and mineral solutions were filter sterilized with 0.22 μm pore size polyethersulfone filter units (Sterivex; Millipore, USA) and stored at 4°C until needed. The pH of the medium was adjusted to 4.5 using concentrated phosphoric acid and then filtered (0.45 μm cellulosic membrane; Fisher Scientific, USA).

2.2.2. Complex Media

The complex media in this study included a pure French Chardonnay must (International Trading Company, Guelph, Ontario), a sparkling white grape juice without additives (Appletiser Ltd., South Africa), and Sauvignon Blanc wine made from pure Sauvignon Blanc must (International Trading Company, Guelph, Ontario).

2.2.3. Stock Solutions

2.2.3.1. Peptone Water

Peptone water (0.1%) was prepared by dissolving 0.1 g of Bacto Peptone (Difco, USA) in deionized water and adjusting to 100 ml. Peptone water was distributed into 1.5 ml microcentrifuge tubes (Fisher Scientific, USA) in 0.9 ml aliquots and then autoclaved. Peptone water was used for cell culture dilutions.

2.2.3.2. Iron Citrate

A 500 μM iron-citrate stock solution was prepared by dissolving 0.135 g of $FeCl \cdot 6H_2O$ and 0.441 g of sodium citrate in deionized water and adjusting to 1 liter of Fe-extracted, ultra pure water. This stock solution was used to prepare a 5 μM iron-excess medium by adding 10 ml to 1 liter of chemically defined extracted medium.

2.2.3.3. Soluble Chelator Stock Solution

A 31.25 mg/ml iron-chelation stock solution was prepared by using a soluble iron chelator (DIBI; Chelation Partners, Guelph, Ontario). The solution was sterile filtered using a 0.20 µm nylon syringe filter unit (Fisher Scientific, USA) and stored at 4°C until needed.

2.2.3.4. Sorbate and Benzoate Stock Solutions

Potassium sorbate and sodium benzoate salts were dissolved in extracted chemically defined medium (10 g/l), sterile filtered through 0.20 µm nylon syringe filter units (Fisher Scientific, USA) and stored at 4°C until needed. Maximum storage time was 7 days.

2.3. Removal of Iron from Media

2.3.1. Iron-chelation Resin

The iron chelation resin, FEC-1, was kindly provided by Chelation Partners (Guelph, ON, Canada); the resin has an iron binding capacity of 70 µmol/g resin. Before use in column extraction/separation processes, the resin was hydrated overnight in ultra pure water.

2.3.2. Iron Removal by Column Separation

2.3.2.1. Removal of Iron from Chemically Defined Medium

Iron was extracted from the chemically defined medium by column separation. A 30 X Ø2.5 cm glass column (Pharmacia, New York, NY, USA) was half-filled with distilled water and then carefully filled with hydrated FEC-1 resin. The column was packed under constant elution to create a homogenous resin bed. A porous PTFE disc was placed on the surface of the resin bed to prevent disturbance of the bed surface

from medium addition. The resin in the column remained completely hydrated at all times. The column was loaded with medium using a peristaltic pump (Masterflex L/S model 7518-10; Cole Palmer Instrument Co., Vernon Hills, IL) and iron-extracted medium was collected in polypropylene containers. The extracted medium was filter sterilized through a 0.22 μm mixed cellulose membrane filter (Millipore, USA) and then transferred into sterile, polypropylene containers. The medium was stored at 4°C until needed.

Following iron extraction, resins were regenerated with five bed volumes (BV) of 1.0 M trace metal HCl (Fisher Scientific, USA) followed by flushing with ultra pure water until the effluent reached a neutral pH (approximately 10 BV of water).

2.3.2.2. Removal of Iron from Complex Media

The Chardonnay must was first pre-treated with diatomaceous earth (Perma-Guard; Albuquerque, New Mexico), and then filtered using a 0.45 μm mixed cellulose membrane filter (Millipore, U.S.A). The must was extracted using the column method and then filtered with a 0.22 μm mixed cellulose membrane filter (Millipore, USA). Samples were stored at 4°C until needed.

2.3.3. Iron Removal by Batch Separation

Prior to extraction, the carbonated media were placed in a sonnicator (Aquasonic Model 150D; VWR Scientific, USA) for 15 minutes to remove CO_2 and all samples were filtered through a 0.45 μm mixed cellulose membrane filter (Millipore, USA) for sterility. FEC-1 resin (3 g) was added to 100 ml of sample and shaken at room temperature for 24 h (Feng *et al.,* 1997b). Following extraction, samples were filtered from the resin using a plastic Büchner funnel with a Whatman No.2 11.0 cm filter

paper (Whatman Ltd; Maidstone, England). The extracted samples were filtered sterilized through a 0.20 μm nylon syringe filter unit (Fisher Scientific, USA). All samples were stored at 4°C until needed.

2.4. Microorganisms and Culture Conditions

2.4.1. Microorganisms

The yeast cultures used in this study included food spoilage yeasts *Candida vini*, *Pichia fermentans*, *Hansenula anomala*, *Metschnikowia pulcherrima*, *Brettanomyces intermedius*, *Rhodoturula glutinis*, *Zygosaccharomyces bailii*, *Candida stellata*, and *Saccharomyces cerevisiae* and opportunistic human pathogen *Candida albicans*. These cultures were provided by the Wine Microbiology Lab at the New York State Agricultural Experiment Station at Cornell University. All cultures were maintained on YPD agar slants at 4°C.

2.4.2. Culture Conditions

2.4.2.1. Iron Sufficient Cultures

Iron-sufficient cultures were prepared by transferring stock yeast cultures into extracted chemically defined medium to initiate growth. These starter cultures were grown for 24 hours at 27°C and then centrifuged (ThermoIEC Centra CL12 Centrifuge, Thermo Fisher Scientific) at 1940 x *g* for 5 minutes and re-suspended in 5.0 ml of chemically defined medium base salts solution (chemically defined medium devoid of vitamins, minerals and glucose). The iron-sufficient yeast cell suspensions were stored at 4°C until needed.

2.4.2.2. Iron Limited Cultures

Iron-limited yeast cultures were prepared by sub-cultivating yeast cultures four times over a total time period of 10 to 12 weeks. All inoculations were carried out at a rate of 0.1% (v/v). First, starter cultures were prepared by transferring stock yeast cultures into fresh extracted chemically defined medium. The starter cultures were grown for 24 hours at 27°C and then transferred to fresh chemically defined medium; this is considered the first cultivation. At stationary phase, the yeast cultures were sub-cultivated into fresh extracted chemically defined medium; this is considered the first sub-cultivation. After four sub-cultivations, the yeast cultures were centrifuged (ThermoIEC Centra CL12 Centrifuge, Thermo Fisher Scientific) at 1940 x *g* for 5 minutes and then re-suspended in 5.0 ml of chemically defined medium base salts solution (chemically defined medium devoid of vitamins, minerals and glucose); the iron-deficient yeast cell suspensions were stored at 4°C until needed.

2.4.3. Iron-limited Growth

Inoculations were carried out at a rate of 0.1% (v/v) with the exception of inoculation during the first cultivation in which the inoculum was ~$1x10^6$ cells/ml (iron sufficient cells) or ~$1x10^4$ cells/ml (iron deficient cells). Starter cultures (24 hr) were prepared as above, using either iron-deficient or iron-sufficient yeast cell suspensions. Actively growing cultures were inoculated into chemically defined medium and incubated as static cultures. At stationary phase growth, the yeast cultures were sub-cultivated into fresh media.

2.4.4. Growth Evaluation

Direct cell counts were made using a Neubauer-ruled haemocytometer (Fisher Scientific, USA) and, if needed, cultures were diluted with chemically defined or complex medium to achieve a $1x10^4$ cells/ml inoculum. Growth was measured

spectrophotometrically at OD 600 nm (Pharmacia Novaspec II). Viable cell counts (CFU/ml) were determined at t=0 for each cultivation.

2.5. Analytical Methods

2.5.1. General Analytical Apparatus

2.5.1.1. Determination of pH

All pH measurements were determined using an Accumet Research pH meter (Model AR50; Fisher Scientific, USA) which was calibrated with buffer solutions prior to use (pH 2, 4, 7; Fisher Scientific, USA).

2.5.2. Trace Metal Analysis of Chemically Defined and Complex Media

Trace metal analysis of all media was determined using inductively coupled plasma-high resolution mass spectrophotometry (ICP-MS). All samples were sterile filtered and then acidified to pH <2 with HCl (Trace Metal Grade, Fisher). Testing was carried out at the Wisconsin State Laboratory of Hygiene (WSLH) at the University of Wisconsin Madison.

2.5.3. Cell Enumeration and Estimation of Biomass

2.5.3.1. Total Cell Counts

Yeast cells were enumerated microscopically using a Neubauer counting chamber. Budding cells were considered individual cells only when daughter cells were greater than 50% the size of the parent cell. Viability was evaluated by adding methylene blue solution (0.1% methylene blue in 2% sodium citrate) to cultures prior to counting; cells that remained blue in color were not considered in final viable cell counts.

2.5.3.2. Viable Cell Counts

Viable cells counts (VBC) were conducted at t=0 and stationary phase during growth studies. VBC were also conducted to ensure purity of cultures. Depending on cell concentrations, yeast cultures were diluted with 0.1% peptone water (2.2.3.1) and then 100 µl was spread onto YPD agar plates (2.2.1.1). Plates were incubated at 27°C and quantification of colonies was completed after 48 hours.

2.5.3.3. Cell Biomass Yield

The dry weight (biomass) of yeast cultures was determined by distributing yeast culture samples into 1.5 ml microcentrifuge tubes (Fisher Scientific, USA) and centrifuging at 13000 x *g* for 10 minutes. The supernatant was removed and deionized water was subsequently added to the tube to wash the culture into pre-weighed disposable aluminum dishes (Fisherbrand; Fisher Scientific, USA). The dishes were placed in an oven at 100°C and dried to reach a constant weight (normally 24 hours).

2.5.3.4. Optical Density (OD) Measurements

Optical density measurements were used to monitor growth of yeast cultures over time. The 600 nm wavelength was chosen to prevent interference from medium color.

2.5.4. Siderophore Analysis

2.5.4.1. Catcholate/Phenolate Assay

The presence of catecholate/phenolates in samples was determined by using a modified version of Arnow's (1937) method. Samples were distributed into disposable culture tubes (Fisherbrand; Fisher Scientific, USA) and to each 0.5 ml sample, the following solutions were added in sequence: 0.5 ml 0.5 N HCl, 0.5 ml molybdate-nitrite solution (10% (w/v) sodium nitrite and 10% (w/v) sodium

molybdate in water), 0.5 ml 1.0 N NaOH, and 0.5 ml deionized water. After addition of each solution, the culture tubes were gently mixed. A 200 μM catechol (Sigma; St. Louise, MO, USA) standard and a 200 μM desferrioxamine B (Deferoxamine mesylate salt; Sigma; St. Louise, USA) standard were used as positive and negative controls, respectively. Within two hours of sample preparation, the absorbance (510 nm) was read using a UV/VIS spectrophotometer (Ultraspec 3100; Biochrom, Cambridge, UK).

2.5.4.2. Hydroxamate Assay

The presence of hydroxamate siderophores in samples was determined by using a modified version of Emery and Neilands' (1962) method. A 0.4 ml aliquot of each sample was distributed into disposable culture tubes to which the following solutions were added in sequence: 1.0 ml 2.19 mM periodic acid (Sigma; St. Louis, USA), 2.6 ml deionized water, and 2 drops of glycerol; the culture tubes were mixed gently after each addition. A 200 μM catechol (Pyrocatechol; Sigma; St. Louise, USA) standard and a 200 μM desferrioxamine B (Deferoxamine mesylate salt; Sigma; St. Louis, MO, USA) standard were used as negative and positive controls, respectively. Within two hours of sample preparation, the absorbance was read at 264 nm.

2.5.5. Oxidation Tests

Oxidative color change was evaluated by measuring browning products spectrophotometrically at 420 nm.

2.5.6. Iron Leaching Tests

Food grade stainless steel ferrules (316 grade, 1" OD; Swageguard, Solon, Ohio) were submersed in extracted chemically defined medium (Sections 2.2.1.2, 2.3.2.1) and

incubated at different time intervals and temperatures. After incubation, samples were analyzed for trace metal concentrations.

2.5.7. Preservative Assay

Chemical preservatives (potassium sorbate, sodium benzoate) were added to chemically defined medium in varying concentrations. Concentrations of each chemical preservative were transferred to sterile, 96-well microtiter plates and then inoculated with specific yeast cultures to achieve 10^3 cells/ml final concentration. Growth was monitored qualitatively.

2.5.8. Chemical Analysis of Metabolites

2.5.8.1. Organic Acids

Concentrations of organic acids were determined using a high performance liquid chromatography (HPLC) system (Shimadzu, USA) equipped with an Aminex HPX-87H, 300 mm x 7.8 mm, organic acid analysis column (BioRad Laboratories, Richmond, CA) and a photo diode array detector (SPD-M20A; Shimadzu).

CHAPTER 3: IRON REMOVAL FROM CHEMICALLY DEFINED MEDIA USING A SELECTIVE IRON CHELATING RESIN

3.1. Introduction

The focus of this thesis was to study the effect of removing iron from growth media and beverage systems in an effort to reduce both microbial growth and oxidative reactions, hence, a powerful iron chelator was required. Previous studies have shown the effects of insoluble iron chelators on iron removal from media such as water, milk, and wine (Feng *et al.,* 1997a; Feng *et al.,* 1995a; Feng *et al.,* 1995a); however, these chelators were shown to have relatively low selectivity for specific metals, such as iron. One resin, FEC-1, demonstrated selectivity and affinity for iron, reducing iron concentrations in sparkling white wine samples to levels below 1 ppb using a column separation processes (Feng *et al.,* 1997b). For this reason, the FEC-1 insoluble iron chelator was employed during the current study in order to evaluate its performance in a chemically defined medium.

3.2. Experimental Conditions

3.2.1. Media Preparation

The media employed was a modified version of the GPP medium first described by Kulkarni and Nickerson (1981) and is described in section 2.2.1.2.

Three versions of chemically defined media were prepared. The non-extracted chemically defined medium will be referred to as “non-extracted” medium while the iron-extracted chemically defined medium will be referred to as “extracted” medium.

Extracted chemically defined medium with the addition of 5.0 μM Fe (2.2.3.2) will be referred to as "iron-added" medium.

3.2.2. Iron Removal

Iron was extracted from the chemically defined medium by column separation, as described in section 2.3.2.1.

3.3. Results

Full trace metal analysis by ICP-MS was found to be accurate and reproducible. Table 3.1 shows the repeatability results from iron determination in extracted and non-extracted media, as well as the recovery results for the iron-added samples used as controls to measure accuracy. Control samples were prepared by adding 5 μM (279.35 μg/l) Fe to extracted chemically defined medium and subsequently diluting the samples 1:1.

Table 3.1: Precision of the ICP-MS method for iron determination and iron recovery in chemically defined media including extracted, non-extracted and the iron-spiked media, and a 1:1 mixture of the iron-spike with the extracted chemically defined medium.

Sample	*n*	Mean (μg/l)	%CV[a]	Average Recovery (%)
Extracted	12	2.3	15.5	-
Non-extracted	14	19.6	4.2	-
Iron-added	4	321.9	0.52	-
1:1	4	159.8	1.6	103.7

[a] coefficient of variation

Table 3.2 illustrates average trace metal concentrations in chemically defined media. The % removal represents the calculated difference in metal concentrations between non-extracted and extracted media. Major differences in metal concentrations were detected for Fe and Cu, each of which was reduced by > 88%.

Table 3.2: Average total trace metal concentrations in chemically defined media extracted using a column separation process.

	Trace Metal Concentration		
Metal	**Initial (ppb)**	**Final (ppb)**	**Removal (%)**
Fe	19.60 ± 3.25	2.31 ± 1.24	88.21
Mg	50.33 ± 3.22	49.34 ± 0.64	1.96
Mn	434.47 ± 16.91	422.71 ± 11.47	2.71
Cu	327.21 ± 31.63	0.18 ± 0.15	99.95
Zn	292.37 ± 29.87	275.97 ± 48.91	5.61

Figure 3.1 illustrates the removal efficiency of the FEC-1 chelator in the chemically defined medium using a column process for several biologically important metal cations. Compared to initial iron concentrations, the resin removed an average of > 88% iron. The resin also removed significant quantities of copper from the chemically defined medium, with average removal of > 99%. Other metals such as Mn, Zn, and Mg were not appreciably affected.

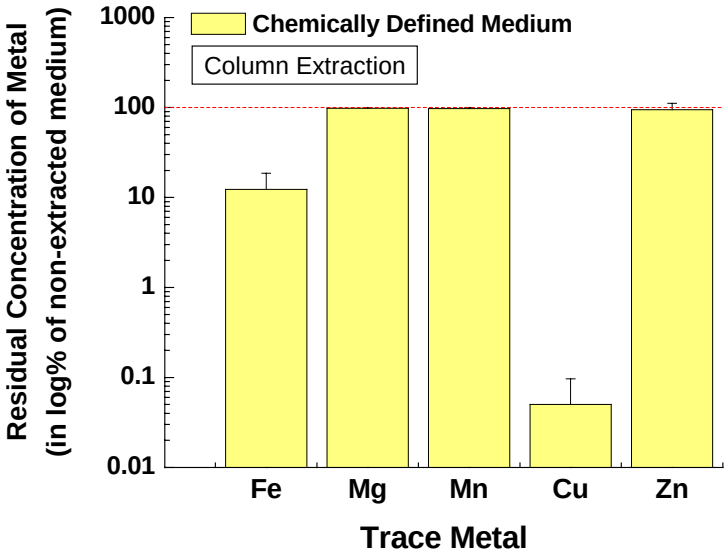

Figure 3.1: Log percent remaining trace metals in chemically defined medium after extraction with FEC-1 resin in column process; expressed as percent remaining metal compared to initial metal concentrations in non-extracted chemically defined medium. The red dotted line represents 100% initial concentration of metals in non-extracted medium.

3.4. Discussion

The FEC-1 insoluble iron chelating resin was evaluated for iron removal from chemically defined medium. Previous studies have shown insoluble chelation resins to remove several metals (Fe, Mn, Cu, Zn, and Mg) from various solutions (Feng *et al.,* 1997a; Benitez *et al.,* 2002; Garg *et al.,* 1999). Using sparkling white grape juice, Feng *et al*., (1997b) showed that the FEC-1 resin was largely selective for Fe and partially selective for Cu and did not remove other metals such as Mn, Zn, or Mg. Feng *et al*., (1997b) also showed that the resin had a high affinity for iron and moderate affinity for copper. The results of the present study showed similar results in a chemically defined medium with the resin removing both Fe and Cu, and having little, if any, effect on the reduction of Mn, Mg, and Zn (Figure 3.1). Furthermore, the

current study showed a high affinity of the resin for both iron and copper, significantly reducing the concentrations of both metals in the medium.

3.5. Conclusions

The insoluble FEC-1 iron chelating resin significantly reduced the iron concentrations in chemically defined medium, thus, demonstrating its high affinity for Fe^{3+}. This resin also displayed superior selectivity for iron, exhibiting little to no effect on most other metals in solution. The resin demonstrated selectivity for copper in this particular medium and further testing is needed to determine FEC-1 selectivity for copper in complex media (Chapter 6).

Because iron extraction of the chemically defined medium reduced iron levels to low ppb iron concentrations, this medium was selected for use in growth studies of various wine and beer spoilage yeast (Chapter 4).

CHAPTER 4: MICROBIAL GROWTH STUDIES IN IRON-EXTRACTED CHEMICALLY DEFINED MEDIUM

4.1. Introduction

The previous chapter showed that the insoluble chelation resin, FEC-1, was able to reduce iron levels in a chemically defined medium to 2.3 ppb. These levels are low compared with previous research published in literature (Feng *et al.,* 1995a; Korngold, 1994a; Benitez *et al.,* 2002; Feng *et al.,* 1994). Because the FEC-1 resin was shown to perform well in the chemically defined medium in Chapter 3, the same chelation technique was used to assess the effects of iron removal on the growth of specific food spoilage microorganisms as discussed in the current chapter.

The microorganisms used for this study were chosen from among important production and spoilage organisms in the food industry. Of interest is *S. cerevisiae*, one of the most important organisms used for the production of bread, wine, beer, and other fermented products, and is considered a model organism used to study and compare the growth of many other yeast. Also chosen was *Z. bailii*, an osmotolerant organism that exhibits high tolerance to food preservatives and is a problem in the beverage industry. In addition, *C. albicans* was included because it has been shown to produce hydroxamate-type siderophores in iron-limited conditions (Holzberg and Artis, 1983; Ismail *et al.,* 1985b; Ismail *et al.,* 1985a; Sweet and Douglas, 1991).

Although iron requirements have been evaluated in select yeast cultures (Jones and Greenfield, 1984; Sweet and Douglas, 1991), to the author's knowledge, iron requirements of the wine and beer spoilage yeast used in this study have yet to be

assessed. As such, this chapter will evaluate maximum growth of several yeast cultures in extracted chemically defined medium compared to growth in iron-excess medium. Positive and negative controls were implemented throughout the study. Negative controls consisted of the inclusion of DIBI (Holbein *et al.,* 2007), a strong soluble iron chelator that would assure iron chelation beyond what was extracted from the medium. Finally, this chapter will discuss possible siderophore production in *C. albicans*.

4.2. Experimental Conditions

4.2.1. Media Preparation and Extraction

Preparation of the chemically defined media employed for this study is described in section 2.2.1.2 and extraction is described in section 2.3.2.1.

Each cultivation included the following chemically defined media: extracted chemically defined medium, extracted chemically defined medium + 2.5 mg/ml soluble iron chelator DIBI (2.2.3.3), non-extracted chemically defined medium, and 5 μM iron-excess chemically defined medium. Chemically defined medium plus iron was used as the positive control (100% growth) while extracted chemically defined medium plus DIBI was considered the negative control.

4.2.2. Organisms and Culture Conditions

The yeast cultures used in this study included food spoilage yeasts *C. vini*, *P. fermentans*, *H. anomala*, *M. pulcherrima*, *B. intermedius*, *R. glutinis*, *Z. bailii*, *C. stellata*, and *S. cerevisiae* and opportunistic human pathogen *C. albicans*. These cultures were provided by the Enology Lab at the New York State Agricultural Experiment Station, Cornell University and were maintained for purity on YPD agar

slants at 4°C. Iron-sufficient or iron-limited yeast cultures (2.4.2.1, 2.4.2.2) were used during the study, and cells were measured as described in sections 2.5.3.1 and 2.5.3.2. Iron-limited growth was conducted as per section 2.4.3.

4.2.3. Siderophore Analysis

Supernatants produced by *C. albicans* were collected at various time intervals and were stored at -10°C until needed. Potential production of siderophores was analyzed by subjecting supernatant to the method described in section 2.5.4.

4.3. Results

4.3.1. Yeast Cultures

The yeast cultures chosen for this study are shown in Table 4.1. These organisms are commonly associated with spoilage and degradations of beverages, primarily wine and beer.

4.3.2. Growth of Iron-Sufficient and Iron-limited Cultures

Growth yield of the iron-sufficient and iron-limited cultures was determined by measuring turbidity (OD_{600}) of the cultures over time and then expressing the maximum growth values as a percentage of 100% growth obtained from cultures grown in the iron-excess controls. To evaluate the effectiveness of maximum growth yield measured by turbidity, viable cell counts (CFU/ml) were completed at each cultivation.

Maximum growth of each iron-sufficient culture at each cultivation is shown in Figure 4.1 a. With the exceptions of *C. albicans* and *Z. bailii*, iron-limited growth of the initially iron-sufficient cultures showed a 25-50% decline in percent maximum specific growth yield (% Y_{max}) over cultivations 1 to 3 (Figure 4.1 a). To force severe

Table 4.1: Common wine and beer spoilage organisms.

Microorganism	**Where found**[1]	**Relevance**[1]
C. vini	Wine, beer, cherries, plums	Permanent, ubiquitous microflora on cherries and plums; bottled wine contaminant and contaminant in top fermented beers
P. fermentans	Must, wine, beer, soft drinks, apples	Beer contaminant; found in high-sugar foods
H. anomala	Must, wine, cider, beer, soft drinks, fruit juice, cherries	Osmotolerant; xerotolerant; can grow in vitamin free media
M. pulcherrima	Must, wine, beer, grapes, apples, cherries, plums	One of the predominant yeast strains found at early to mid stages of must fermentation
B. intermedius	Wine, cider, beer, soft drinks	May lead to "bret" fault in wine
R. glutinis	Must, beer, grapes, apples,	Rarely associated with food spoilage; tolerant of low temperatures and low pH; one of the most common yeasts in foods
C. albicans	Gastrointestinal tract, human mouth	Opportunistic human pathogen; may produce hydroxamate-type siderophores
Z. bailii	Must, wine, cider, soft drinks, conc. juice, grapes, apples	Osmotolerant; resistant to many food preservatives; bottled wine contaminant
C. stellata	Must, wine, soft drinks, conc. juices, grapes, apples, cherries	Associated with sour rot disease of grapes; one of the predominant yeast strains found at early to mid stages of must fermentation
S. cerevisiae	Must, wine, cider, sake, beer, grapes, apples, fermented products	Essential for production of many fermented products; considered a contaminant in some products

[1](Deak and Beuchat, 1996)

nutritional deficiencies during growth, vitamins and minerals were omitted from the chemically defined medium during cultivations 4 through 6, and consequently, maximum growth was reduced by > 75% for all yeast except *H. anomala*. Growth increased after re-addition of vitamins and minerals to the medium in cultivations 7 and 8.

Viable cell counts for the iron-sufficient cultures are shown in Figure 4.1 b. Similar to the maximum growth yield results, viable cell counts show a decline in growth of up to 2 log reductions over cultivations 1 to 3 and an additional 2 to 3 log reductions with vitamin and mineral omission.

Percent maximum growth yield of each iron-limited culture at each cultivation is shown in Figure 4.2a. Compared to the maximum growth yield achieved in the iron-excess controls, growth of the iron-limited cultures in extracted chemically defined medium showed an initial of decrease in $\%Y_{max}$ of approximately 38-86%. The exceptions to this growth inhibition include *S. cerevisiae* and *C. albicans* which only achieved a decreased $\%Y_{max}$ of < 20%. Maximum growth remained fairly consistent over 7 cultivations, with the exception of *B. intermedius*. Viable cell counts of the iron-limited yeast cultures (Figure 4.2b) confirmed that maximum growth of most cultures remained fairly stable over 7 cultivations. Table 4.2 uses data obtained in both growth studies to group organisms according to their growth performance in iron extracted medium. Specifically, growth of *C. albicans* and *S. cerevisiae* was less affected by iron removal while the growth of *R. glutinis* and *P. fermentans* was most affected by iron removal.

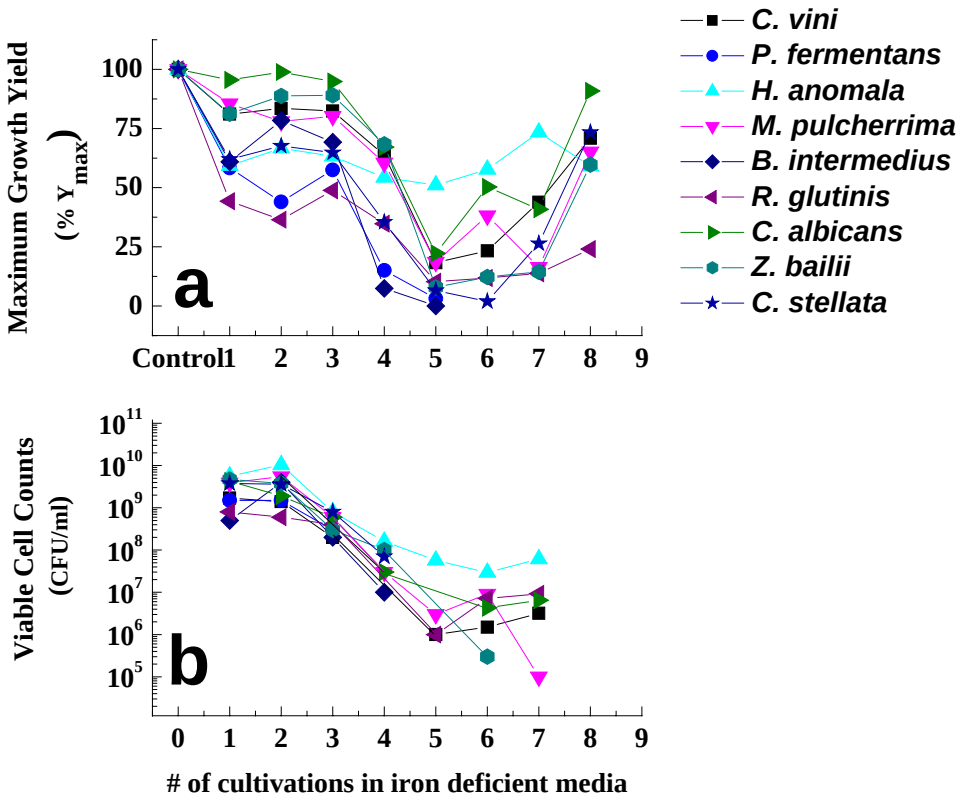

Figure 4.1: Percent maximum growth yield (%Y_{max}) and viable cell counts (average CFU/ml) of iron-sufficient yeast cultures over 8 cultivations. Maximum growth (Y_{max}) of yeast sub-cultivations was measured at OD600 to a maximum 336 hours and expressed as % of 100% growth obtained from control cultures grown in extracted chemically defined medium with 5 μM added iron. The "Control" label represents the maximum growth obtained from iron-added chemically defined media 100% control. Viable cell counts were recorded at stationary phase for each cultivation. Error bars were omitted for the sake of clarity.

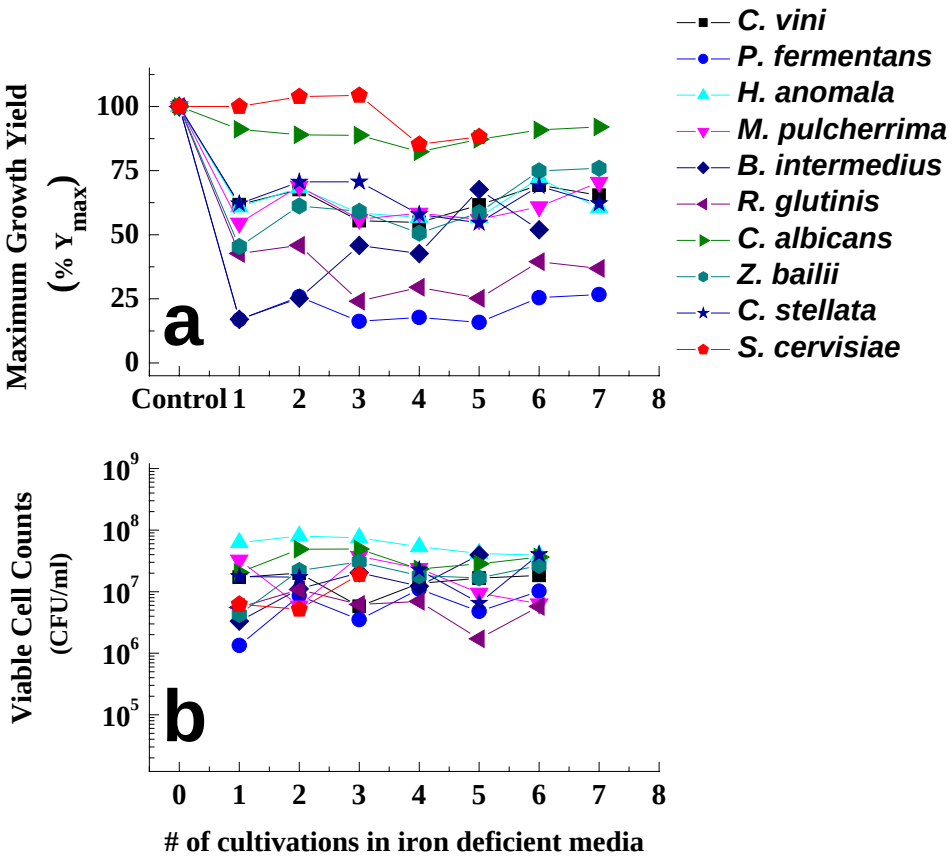

Figure 4.2: Percent maximum growth yield (%Y_{max}) and viable cell counts (average CFU/ml) of iron-limited yeast cultures over 7 cultivations. Maximum growth (Y_{max}) of yeast sub-cultivations was measured at OD600 to a maximum 336 hours and expressed as % of 100% growth obtained from control cultures grown in extracted chemically defined medium with 5 μM added iron. The "Control" label represents the maximum growth obtained from iron-added chemically defined media 100% control. Viable cell counts were recorded at stationary phase for each cultivation. Error bars were omitted for the sake of clarity.

Table 4.2: Growth inhibition of iron-limited yeast cultures in extracted chemically defined medium as measured by turbidity**.**

Least Growth Reduction 0 – 25%	**Intermediate Growth Reduction 25 – 50%**	**Most Growth Reduction 50 – 100%**
C. albicans	*H. anomala*	*R. glutinis*
S. cerevisiae	*C. stellata*	*P. fermentans*
	B. intermedius[a]	
	M. pulcherrima	
	C. vini	
	Z. bailii	

[a]: displayed inconsistent growth

4.3.3. Siderophore Analysis

Supernatants produced by *C. albicans* cultures were analyzed for catecholate and hydroxamate-type siderophores. Table 4.3 show that this organism produced little to no catecholate or hydroxamate-type siderophores after four sub-cultivations in extracted chemically defined medium. Absorbance values obtained for extracted medium were not significantly different from values obtained for iron-excess medium for either siderophore type.

Table 4.3: Analysis of culture supernatants produced by *C. albicans* grown in extracted and iron-added chemically defined media. Supernatants were analyzed for the potential production of catecholate and hydroxamate-type siderophores. All cultures were grown without shaking.

	Siderophore Chemical Type	
	Catecholate	Hydroxamate
Growth Condition	A_{510} nm	A_{264} nm
Extracted	0.019	0.056
5 μM added	0.018	0.067
Hydroxamate Control (200 μM)	0.012	0.176
Catecholate Control (200 μM)	0.476	0.027

Because of the negative findings in the first siderophore analysis (Table 4.3), a second experiment was conducted in order to re-test the potential production of siderophores by *C. albicans*; results are shown in Table 4.4. Samples were taken at three time intervals and results indicated presence of hydroxamates in extracted medium and in iron-added medium after 192 h. A significant difference was not found for the presence of hydroxamates between *C. albicans* grown in extracted medium versus growth in iron-added medium.

Table 4.4: Analysis of culture supernatants produced by *C. albicans* grown in extracted and iron-added chemically defined media. Supernatants were analyzed for the potential production of hydroxamate-type siderophores. All cultures were grown without shaking.

	Siderophore Chemical Type		
	Hydroxamate		
	A_{264} nm		
Growth Condition	24 hr	72 hr	192 hr
Extracted	0.0060	0.024	0.089
5 μM added	0.0065	0.020	0.092
Hydroxamate Control (200 μM)	0.160		
Catecholate Control (200 μM)	0.034		

4.4. Discussion

4.4.1. Growth of Iron-Sufficient and Iron-limited Cultures

This chapter investigated the effects of iron removal on the growth of several microorganisms. Previous works have explored the effects of iron removal on microbial growth or oxidation and have shown promising results (Feng *et al.,* 1997b; Feng *et al.,* 1997a; Benitez *et al.,* 2002); however, select studies investigating microbial iron uptake have shown weaknesses in their methodologies when using what

they considered "iron-deficient" medium. In some studies, the simple omission of iron salt was assumed to result in the absence of iron from the medium (Elli *et al.,* 2000; Georgatsou and Alexandraki, 1994), while in other works, final iron concentrations were not reported at all (Imbert and Blondeau, 1998; Lesuisse *et al.,* 1987; Dancis *et al.,* 1990; Georgatsou and Alexandraki, 1994). The present study showed that non-extracted chemically defined medium had approximately 20 ppb endogenous iron (Table 3.2) indicating significant iron contaminations from other constituents in the medium. Even after iron removal with a powerful chelating resin, the final iron content of the extracted medium was approximately 2.3 ppb. Thus, it is important not to assume that a medium is iron-limited because of simple iron omission.

Despite iron removal to extremely low levels, complete inhibition of microorganisms was not found in this work. In iron-extracted medium, iron-sufficient cultures exhibited < 1 log reduction before the omission of vitamins and minerals, while the iron-limited cultures exhibited < 1 log reduction over five sub-cultivations. Feng *et al.,* (1997b) reported similar results with *S. cerevisiae* grown in grape juice that was extracted using the FEC-1 resin. Alternatively, some studies have found complete inhibition of microbial growth after iron extraction of growth media, however, the chelators were either insoluble and removed other metals in addition to iron (Feng *et al.,* 1997a), or the chelators were soluble, rendering iron unavailable to microorganisms but not actually removing the iron (Feng *et al.,* 1995a). Because some insoluble chelators remove other metals important for microbial growth, iron removal alone cannot be considered the main factor in growth inhibition. Also, soluble iron chelators imply that iron is still present in solution but is unavailable for uptake by microorganisms. Therefore, if a siderophore-producing organism is grown in the presence of these soluble chelators, it is possible that the affinity of the siderophore for

iron is actually stronger than the affinity of the chelator, hence, making available previously bound iron.

As shown in Figure 4.1, iron removal alone was not enough to fully inhibit the microorganisms; hence, vitamins and minerals were omitted from the extracted medium to force additional nutritional deficiencies. The omission of the vitamins and minerals lead to an additional 2 log reduction in $\%Y_{max}$ in the majority of yeast cultures, with the exception of *H. anomala* which has been shown to grow in vitamin-free media (Burkholder *et al.,* 1944). Additional studies will be needed to determine if there is a possible compound effect due to omission of vitamins and minerals in addition to iron removal, or if omission of vitamins and minerals alone can lead to a reduction in growth.

The viable cell counts for all iron-limited organisms followed a similar trend as the results obtained from turbidity measurements. The $\%Y_{max}$ values remained constant over six sub-cultivations in extracted medium for most yeast cultures. Reasons for this behavior may include the fact that sub-cultivations were not effective at reducing iron carryover, or that the yeast cultures had sufficient residual intracellular iron to survive seven cultivations in low-iron medium. Additionally, the yeast may require far less iron than was present in the extracted chemically defined medium in this study.

The majority of iron-limited yeast cultures displayed an intermediate growth response in iron-extracted medium, exhibiting $\%Y_{max}$ reductions between 25–50%. *C. albicans* and *S. cerevisiae* exhibited very little growth reduction indicating that they may have developed mechanisms to withstand low-iron environments. *C. albicans* has been

shown to produce siderophores (Ismail *et al.,* 1985b) and this may explain why it was capable of growing at near-maximum rates throughout the study.

The microorganisms in this study were cultured under static conditions. Although the cultures were grown aerobically, the oxygen was likely consumed, thus limiting oxidation of Fe^{2+} to Fe^{3+}. Without additional aeration, iron remained in ferrous (Fe^{2+}) form and was more available for uptake by the yeast cultures. Thus, additional studies will be needed to investigate Y_{max} differences of cultures grown with constant aeration versus those grown without aeration.

4.4.2. Siderophore Analysis

C. albicans has been reported to produce both catecholate and hydroxamate-type siderophores (Ismail *et al.,* 1985b); however, in this study, siderophores were not detected at levels found in previous studies (Holzberg and Artis, 1983; Ismail *et al.,* 1985b; Ismail *et al.,* 1985a; Sweet and Douglas, 1991).

Although Ismail *et al.,* (1985a) reported detection of catecholate-type siderophores by *C. albicans*, only 40% of their *C. albicans* strains produced this type of siderophore. Catecholate-type siderophores are predominantly produced by bacteria while both bacteria and fungi produce hydroxamate-types siderophores (Neilands, 1981). Because few *C. albicans* strains have been reported to produce catecholate-type siderophores, it is not surprising that the strain of *C. albicans* used for the present study was not found to produce the catecholate-type siderophore.

Hydroxamate-type siderophores have been detected spectrophotmetrically in the supernatants of some strains of *C. albicans* (Ismail *et al.,* 1985b). In the present study, absorbance values corresponding to hydroxamate-type siderophore production by *C. albicans* in extracted chemically defined medium indicated negligible siderophore production compared to the hydroxamate control (Table 4.3, Table 4.4). Additionally, the hydroxamate assay results indicated that *C. albicans* produces a low concentration of hydroxamates after 192 hours (~134 μM) in extracted medium (Table 4.4); however, comparison with ongoing research within a different project revealed that the same strain of *C. albicans* grown for 144 h, produced a much greater concentration of hydroxamates (~330 μM) under similar conditions (B.E.Holbein, personal communication). One important difference between the two experiments was that, in the latter, the cultures were grown in flasks with shaking, while the cultures from the present study were grown in plastic test tubes under static conditions. Ismail *et al.,* (1985a) suggested that culture growth under static conditions favors iron in the ferrous form, rendering it more available for use by *C. albicans*, thereby reducing the need for production of siderophores. Thus, it is possible that the static growth conditions achieved for the *C. albicans* cultures in the present study were sufficient to maintain iron in the ferrous state, thereby satisfying the immediate needs of the cultures and consequently limiting siderophore production. The possible increased availability of Fe to yeasts as Fe^{2+} in static cultures with reduced oxygen availability could have implications for Fe limited growth as well. It would be interesting to compare maximal growth rates of the yeasts tested here in Fe deficient media under static, low oxygenation conditions versus shaken, highly oxygenated cultural conditions. Further studies will be required to test this hypothesis and these could include comparative studies at different oxygen levels and the use of a soluble iron chelator to supplement iron removal by the FEC-1 resin.

4.5. Conclusions

Results indicated that the removal of iron from chemically defined medium reduced growth of most cultures; however, the concentration of iron removed from the medium was not sufficient to fully inhibit the growth of all yeasts in this study. Utilizing this iron-removal method in combination with other preservation techniques may be beneficial to the beverage industry such that we may be able to reduce the concentration of preservatives used in beverages (ie. benzoic acid, sorbic acid, SO_2) by reducing the amount of iron present in the beverage. A hurdle technology approach may prevent both oxidative changes and microbial growth (Chapter 5).

CHAPTER 5: HURDLE EFFECT COMBINING FE-LIMITED MEDIA WITH THE ADDITION OF TRADITIONAL FOOD PRESERVATIVES

5.1. Introduction

Chapter 4 studied the effect of iron chelation on microbial growth inhibition. Although iron removal did not lead to full inhibition of microbial growth, considerable growth reductions were found in some organisms. As such, since iron removal to low ppb levels was not sufficient to establish complete microbiological stability, the application of this technology in conjunction with traditional preservatives may constitute a method to enhance the action of these preservatives, especially with regards to microorganisms that are particularly resistant to specific food preservatives. Thus, the effectiveness of iron removal in combination with specific preservatives on growth inhibition of *H. anomala* was assessed. A screening study was conducted using a combination of treatments including iron-extracted or non-extracted media with additions of potassium sorbate or sodium benzoate; growth was evaluated qualitatively. Preliminary work had indicated that *H. anomala* was resistant to potassium sorbate and sodium benzoate at concentrations below 100 ppm, hence, concentrations between 100-500 ppm were used. Additional previous work by the author illustrated that the addition of the soluble iron chelator DIBI can reduce growth of *H. anomala* to non-detectable levels in extracted chemically defined medium. As such, this chelator was used as a negative growth control during the study. Potassium sorbate and sodium benzoate are commonly used preservatives in the food industry, hence, they were chosen for this study.

5.2. Experimental Conditions

5.2.1. Media Preparation

Preparation of the chemically defined medium used in this study is described in section 2.2.1.2 and extraction is described in section 2.3.2.1. Extracted and non-extracted chemically defined medium were used for culture growth, while extracted chemically defined medium + 2.5 mg/ml soluble chelator (DIBI) was used as a negative growth control.

Preparation of sodium benzoate and potassium sorbate stock solutions is described in section 2.2.3.4. The preservatives were added to the chemically defined media in varying concentrations in the range of 0-500 ppm.

5.2.2. Organisms and Inoculation

The microorganism used for study was an iron-limited culture of *H. anomala* (Section 2.4.2.2). The assay was prepared using the method in section 2.5.7. After inoculation of the culture into the 96-well plate, a sterile, breathable, rayon adhesive film (VWR, USA) was placed over the plate to prevent contamination, and then the plates were incubated aerobically at 27°C. Growth was monitored over a 14 day period. All incubations were performed in triplicate.

5.3. Results

Table 5.1 and Table 5.2 illustrate growth of *H. anomala* in extracted and non-extracted chemically defined media with additions of sodium benzoate or potassium sorbate at concentrations of 0-500 ppm. Growth of *H. anomala* was observed in all positive control wells (no added preservatives) and was inhibited by additions of either 200 ppm sodium benzoate or 350 ppm potassium sorbate in both extracted and

non-extracted chemically defined media. Growth in non-extracted medium lead to slightly greater cell density than growth in extracted medium. DIBI addition fully inhibited growth in all wells to which it was added, including wells filled with extracted and non-extracted media, with or without added preservatives.

Table 5.1: Growth of *H. anomala* in extracted (EX) and non-extracted (NE) chemically defined medium (pH 4.5) with added sodium benzoate at varying concentrations. 2.5 mg/ml DIBI soluble iron chelator was added to one set of wells as a negative growth control.

		Sodium Benzoate Concentrations (ppm)					
Day	Media	0	100	200	300	400	500
2	EX	XXX	-	-	-	-	-
	NE	XXX	-	-	-	-	-
4	EX	XXX	X	-	-	-	-
	NE	XXXX	X	-	-	-	-
6	EX	XXX	XX	½	-	-	-
	NE	XXXX	XXX	½	-	-	-
9	EX	XXX	XX ½	XX	-	-	-
	NE	XXXX	XXX	XX	-	-	-
11	EX	XXX	XX ½	XX	-	-	-
	NE	XXXX	XXX	XX	-	-	-
13	EX	XXX	XX	XX	-	-	-
	NE	XXXX	XXX	XX	-	-	-
18	EX	XXX	XX	XX	-	-	-
	NE	XXXX	XXX	XXX	-	-	-
DIBI 0-18	EX	-	-	-	-	-	-
	NE	-	-	-	-	-	-

(X): Degree of growth (observed cloudiness in wells). "XXXX" = maximum growth

(-): No growth (observed transparent solution in wells)

Table 5.2: Growth of *H. anomala* in extracted (EX) and non-extracted (NE) chemically defined medium (pH 4.5) with added potassium sorbate at varying concentrations. 2.5 mg/ml DIBI soluble iron chelator was added to one set of wells as a negative growth control.

		Potassium Sorbate Concentrations (ppm)						
Day	Media	0	100	200	300	350	400	500
2	EX	XXX	-	-	-	-	-	-
	NE	XXX	-	-	-	-	-	-
4	EX	XXX	X ½	X	½	-	-	-
	NE	XXXX	XX	X	½	-	-	-
6	EX	XXX	X ½	X	X	½	-	-
	NE	XXXX	XX	X	½	-	-	-
9	EX	XXX	X ½	X	X	½	-	-
	NE	XXXX	XX	XX	X	-	-	-
11	EX	XXX	X ½	X	X	X	-	-
	NE	XXXX	XX	XX	X ½	X	-	-
13	EX	XXX	X ½	X ½	X	X	-	-
	NE	XXXX	XX	XX	X ½	X	-	-
18	EX	XXX	XX	X ½	X ½	X	-	-
	NE	XXXX	XX	XX	X ½	X	-	-
DIBI 0-18	EX	-	-	-	-	-	-	-
	NE	-	-	-	-	-	-	-

(X): Degree of growth (observed cloudiness in wells). "XXXX" = maximum growth

(-): No growth (observed transparent solution in wells)

Table 5.3: Minimum concentrations of preservatives required to inhibit growth of *H. anomala* under aerobic conditions in chemically defined medium at pH 4.5.

Species	**Media**	**Potassium Sorbate (ppm)**	**Sodium Benzoate (ppm)**
H. anomala	Extracted	350	200
	Non-extracted	350	200

5.4. Discussion

H. anomala was the yeast of choice for this study because previous results (Chapter 4) indicated that this organism exhibited resilience to iron removal and vitamin and mineral removal in extracted chemically defined medium.

H. anomala exhibited tolerance to sorbates and benzoates to a similar degree as other yeast species studied (Bills *et al.,* 1982; Warth, 1977; Splittstoesser *et al.,*). In the present study, the efficiency of the two preservatives differed, with *H. anomala* exhibiting a greater tolerance to potassium sorbate (350 ppm) than to sodium benzoate (200 ppm). Although under anaerobic conditions, Warth (1985) showed a similar trend with *H. anomala* which also exhibited greater tolerance to sorbic acid (168 ppm) than to benzoic acid (122 ppm). In a later study, Warth (1989) found the minimum inhibitory concentration of sorbic acid for *H. anomala* to be 223 ppm, which is considerably lower than what was observed in this study.

The iron reduction from extraction using the chelator FEC-1 was not efficient enough to inhibit the growth of *H. anomala* even with the addition of preservatives as visible from the similar growth response obtained in extracted and non-extracted medium. Yet, more efficient iron chelation was shown to lead to growth inhibition since addition of the soluble chelator DIBI (2.5 mg/ml) led to complete growth inhibition even in the absence of added preservatives. Studies with *C. albicans* (Holbein *et al.,* 2007) have shown that addition of DIBI at concentrations not inhibitory for growth (0.0125 or 0.025 mg/ml) substantially lowered the MIC for both potassium sorbate and methyl parabenzoate as well as fluconazole when tested in a static tube grown culture system similar to that used in the present study. It might be useful to examine addition of lower concentrations of DIBI, i.e., at sub-inhibitory concentrations, in

order to examine the possibility of synergistic hurdle activity of DIBI with the preservatives tested.

Slight cell density differences were observed in extracted medium versus non-extracted medium, where growth in non-extracted medium led to slightly higher cell densities. However, this suspected effect was minimal and was not quantified. In addition, minimum preservative concentrations required to completely inhibit growth of *H. anomala* did not differ in cultivations with extracted and non-extracted medium. One possible explanation may be that the cultures were grown under static conditions in 96-well microtiter plates. Although grown aerobically, the lack of shaking reduced aeration of the media and iron may have been present in the reduced ferrous (Fe^{2+}) form, which has higher bioavailability. Under the low oxygen conditions in the wells, the ferrous iron was more available for uptake by the yeast, thus, promoting growth of the yeast cultures. Results are similar to those discussed in section 4.4 in which growth of the yeast cultures was achieved in extracted chemically defined medium under static conditions.

Concentrations of preservatives increased in relatively large increments (50-100 ppm increments) to a maximum of 500 ppm. Smaller increments (1-10 ppm increments) were not used, thus more work will be needed to determine specific minimal inhibitory concentrations of sodium benzoate and potassium sorbate on *H. anomala* at pH 4.5. Furthermore, this study focused on one specific food spoilage organism and further research will be needed to determine possible synergistic effects of iron removal and preservative addition on other microorganisms, particularly those shown to be resistant to traditional preservatives.

5.5. Conclusions

This study indicated that *H. anomala* was relatively resistant to both sodium benzoate and potassium sorbate preservatives. Negligible differences in growth were detected between extracted versus non-extracted medium at pH 4.5. The addition of preservatives in addition to iron removal does not show a synergistic effect for growth inhibition of *H. anomala* and, as such, it can be reasonably concluded that this method would not be particularly efficient in industry without the possible addition of a soluble iron chelator, such as DIBI.

CHAPTER 6: APPLICATION OF IRON REMOVAL STRATEGIES IN OTHER FOOD MATRICES AND ITS EFFECT ON OXIDATIVE STABILITY

6.1. Introduction

Thus far, this thesis has evaluated the reduction of iron in chemically defined medium, the effects or iron reduction on microbial growth, as well as the possible application of this technology in a combined hurdle approach. In this chapter, iron removal from other food matrices will be considered.

The purpose of this study was to survey iron removal in a selection of matrices that included liquid food products, both alcoholic and non-alcoholic, with different pH values, different compositions, and from different origins (ie. of vegetable, fruit, grain, and animal origin).

A primary concern in the food industry is the oxidative stability of foods. Hence, this chapter also describes a study carried out to investigate the effect of iron removal on oxidation. Sauvignon Blanc wine was chosen as the model system for this study.

Finally, regardless of the efficiency of iron removal, it was important to consider the applied significance of iron removal in the food industry. Accordingly, a preliminary study was carried out to imitate the extent of iron leaching from food equipment surfaces into a model solution after iron extraction.

6.2. Experimental Conditions

6.2.1. Iron Extraction from Complex Media

The column method was used to extract iron from Chardonnay must as described in section 2.3.2.2. Iron removal in the white grape juice was achieved using a single batch extraction as explained in section 2.3.3. Before shaking, the headspace was flushed with CO_2 and the container closed to prevent oxidation.

A multiple batch separation process was used to extract Sauvignon Blanc wine and ten other food liquids shown in Table 6.1.

Table 6.1: Complex liquid food matrices to be extracted using a batch process.

Food Liquid	pH	Origin	Manufacturer
White Grape Juice	2.74	Fruit	Grapetiser
Sauvignon Blanc Wine	3.59	Fruit	Winecellar, Cornell
Pickle Brine	3.70	Vegetable	Wegmans
Cherry Juice	3.37	Fruit	Cherry Pharm
Pineapple Juice	3.75	Fruit	Libby's
Chardonnay Must	3.25	Fruit	International Trading Company
Olive Brine	6.82	Vegetable	Musco Family Olive Co
Green Bean Juice	5.25	Vegetable	Wegmans
Iced Tea	3.01	Grain	Lipton
Chicken Broth	6.35	Animal	Wegmans
Coconut Water	5.08	Fruit	Goya
Vegetable Broth	4.85	Vegetable	Wegmans
Beer	4.38	Grain	Becks

For the multiple batch separations, the filtrate was collected from each sample after the first batch extraction (2.3.3) and then 3 g of fresh resin was added to each sample and shaken at room temperature for an additional 2 h. The resin was removed from each sample by filtration through a Whatman filter paper (No.2, 11.0 cm) centered in a plastic Büchner funnel and then each sample was filter sterilized through a 0.20 μm nylon syringe filter unit. Samples of non-extracted and extracted food liquids were stored at 4°C until needed.

6.2.2. Oxidation in Sauvignon Blanc Wine

Sauvignon Blanc wine was extracted using the multiple batch process as described in section 6.2.1. Two sets of samples were prepared: Fe-added wine (positive controls) and iron-extracted wine. Positive controls were prepared by adding 55 μg iron (section 2.2.3.2) to 10 ml of extracted wine. Caffeic acid (100 mg/l) was added to all samples. The headspace of each sample was measured and then samples were gently mixed and then incubated aerobically at 30°C. Oxidative color change was evaluated spectrophotometrically at 420 nm daily. Samples were prepared in triplicate.

6.2.3. Iron Leaching Assay

Preparation of the iron leaching assay is described in section 2.5.6. Samples were gently shaken during incubation. Samples were prepared in duplicate.

6.3. Results

6.3.1. Food Liquid Matrices

Iron was extracted from twelve different food liquids using batch extraction, and from Chardonnay must using column extraction. The trace iron analysis (Table 6.2) illustrated considerable removal of iron from all food liquids, with iron reduction of > 85% in ten out of twelve samples. A clear trend between iron reduction and pH was not observed ($R^2 = -0.31$) (Figure 6.1).

Table 6.2: Average trace iron concentrations in food liquids after extraction by a multiple batch separation with FEC-1 resin.

Food Liquid	Initial pH	Final pH	[Fe] initial (ppb)	[Fe] final (ppb)	Reduction (%)
White Grape Juice[a]	2.74	2.68	2128.2 ± 2.3	4.91 ± 0.3	99.8
Sauvignon Blanc Wine	3.59	3.62	2096.4 ± 11.8	5.26 ± 1.5	99.7
Pickle Brine	3.70	3.81	840.4 ± 3.1	14.8 ± 1.1	98.2
Cherry Juice	3.37	3.46	1995.4 ± 37.6	35.0 ± 1.2	98.2
Pineapple Juice	3.75	3.76	675.6 ± 9.9	22.8 ± 1.1	96.6
Chardonnay Must[b]	3.25	3.19	4897.0 ± 16.0	464.03 ± 1.7	90.5
Olive Brine	6.82	6.80	5965.9 ± 118.7	579.1 ± 28.1	90.3
Green Bean Juice	5.25	5.01	4965.0 ± 76.7	623.1 ± 4.8	87.4
Iced Tea	3.01	3.60	164.5 ± 2.2	21.6 ± 0.2	86.9
Chicken Broth	6.35	6.01	167.6 ± 3.3	23.8 ± 1.1	85.8
Coconut Water	5.08	5.00	261.9 ± 2.8	49.8 ± 1.1	81.0
Vegetable Broth	4.85	4.78	87.5 ± 0.7	33.9 ± 0.6	61.2
Beer	4.38	4.48	15.6 ± 1.1	7.5 ± 1.1	52.1

[a]: single batch extraction

[b]: column extraction

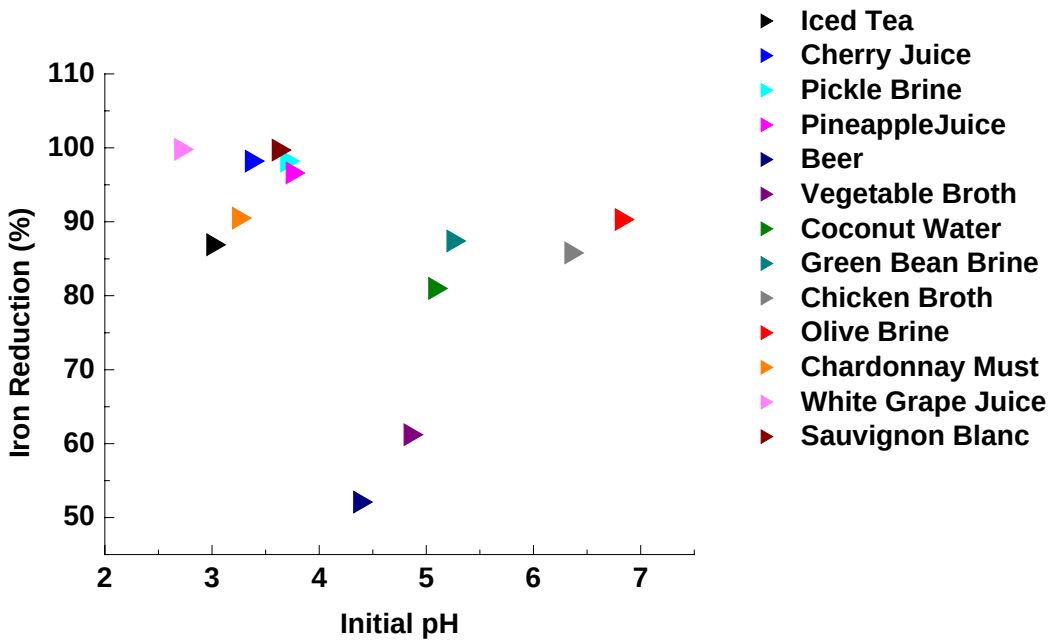

Figure 6.1: Comparison of percent iron removal in complex media and initial pH of each food liquid sample**.**

Full trace metal analyses were obtained for white grape juice, Chardonnay must, and Sauvignon Blanc wine (Table 6.3). Batch iron removal from the white grape juice and Sauvignon Blanc wine (Figure 6.2 & Figure 6.3) showed iron reductions of > 99% to 4.91 ppb and 5.26 ppb, respectively. The resin also removed Cu by > 33% from both media. Column iron removal reduced iron concentrations in the Chardonnay (Figure 6.4) by 90.5% while Cu was reduced by 59%. Reduction of Mg, Mn, and Zn in the white grape juice and Sauvignon Blanc wine was not observed, although there was slight reduction of these trace metals observed in the Chardonnay must.

Table 6.3: Average total trace metal concentrations in complex media extracted using the column or batch separation process.

		Chardonnay Must	White Grape Juice	Sauvignon Blanc Wine
Metal	**Concentration**	**Column**	**Single Batch**	**Multiple Batch**
Fe	Initial (ppb)	4896.95 ± 15.97	2128.18 ± 2.33	2096.38 ± 11.83
	Final (ppb)	464.03 ± 1.70	4.91 ± 0.27	5.26 ± 1.46
	Removal (%)	90.52	99.77	99.74
Mg	Initial (ppm)	76.5 ± 0.46	47.03 ± 0.45	98.92 ± 0.79
	Final (ppm)	57.75 ± 0.12	50.24 ± 3.65	108.59 ± 3.25
	Removal (%)	11.46	-6.83	-9.78
Mn	Initial (ppb)	1090.19 ± 6.54	472.96 ± 3.27	1961.87 ± 4.66
	Final (ppb)	990.43 ± 23.54	505.75 ± 14.45	2172.37 ± 119.74
	Removal (%)	9.15	-6.93	-10.73
Cu	Initial (ppb)	1964.76 ± 10.09	52.36 ± 2.26	325.08 ± 8.27
	Final (ppb)	806.58 ± 8.50	35.09 ± 5.04	199.23 ± 11.14
	Removal (%)	58.95	33.0	38.71
Zn	Initial (ppb)	1034.35 ± 20.28	531.70 ± 20.06	2822.03 ± 4.20
	Final (ppb)	874.90 ± 20.62	591.60 ± 43.85	2823.43 ± 131.31
	Removal (%)	15.42	-11.27	-0.05

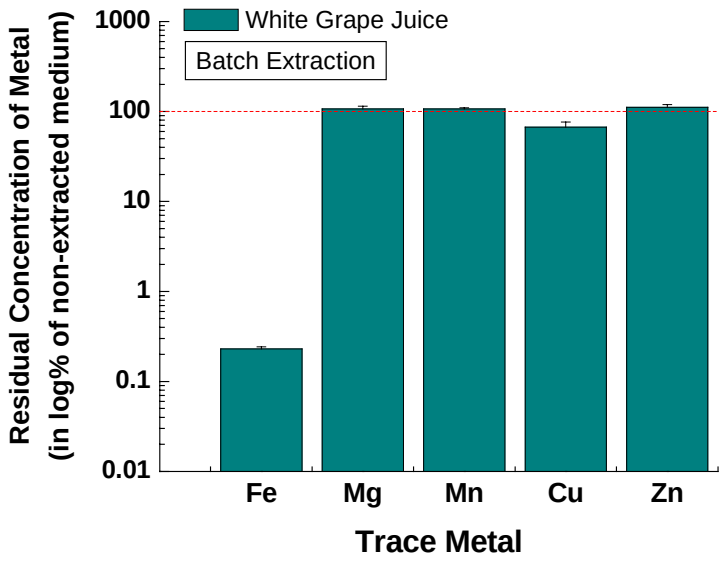

Figure 6.2: Log percent remaining trace metals in white grape juice after single batch extraction with FEC-1 resin and expressed as percent remaining metal compared to initial metal concentrations in non-extracted white grape juice. The red dotted line represents 100% initial concentration of metals in non-extracted medium.

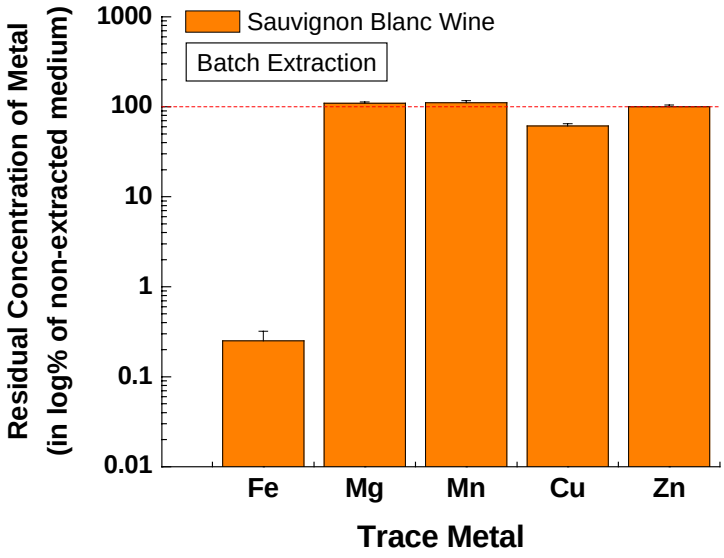

Figure 6.3: Log percent remaining trace metals in Sauvignon Blanc wine after double batch extraction with FEC-1 resin and expressed as percent remaining metal compared to initial metal concentrations in non-extracted Sauvignon Blanc wine. The red dotted line represents 100% initial concentration of metals in non-extracted medium.

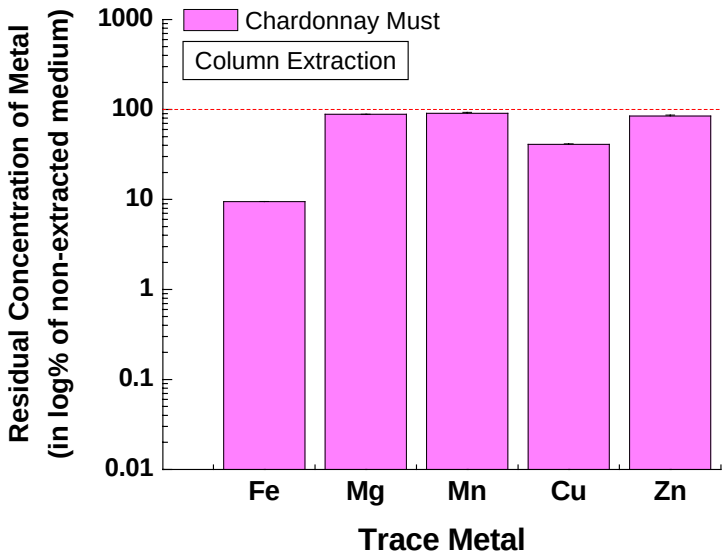

Figure 6.4: Log percent remaining trace metals in Chardonnay must after extraction with FEC-1 resin in column process and expressed as percent remaining metal compared to initial metal concentrations in non-extracted Chardonnay must. The red dotted line represents 100% initial concentration of metals in non-extracted must.

6.3.2. Oxidation Study in Sauvignon Blanc Wine

To maintain consistency, all wine samples were first batch extracted and then essential components were added back if necessary. Caffeic acid was added to all samples because considerable color loss was noted after extraction. Also, 5.5 mg/l of iron was added back to half of the wine samples to mimic iron concentrations found in white wine; these samples will be considered "Fe-control" wines.

Figure 6.5 shows color change in Fe-extracted and Fe-control Sauvignon Blanc wine as measured over 15 days. The rate of color change was linear for both the Fe-control and Fe-extracted wine samples (R^2 value 0.999 and 0.995, respectively). The color change of the Fe-control wines increased at an average rate of 0.0008 nm/d while the color change in the Fe-extracted wines increased at an average rate of 0.0003 nm/d.

Maximum oxidative color changes were found to be significantly different between the Fe-control and the Fe-extracted wine samples (at $\alpha = 0.01$).

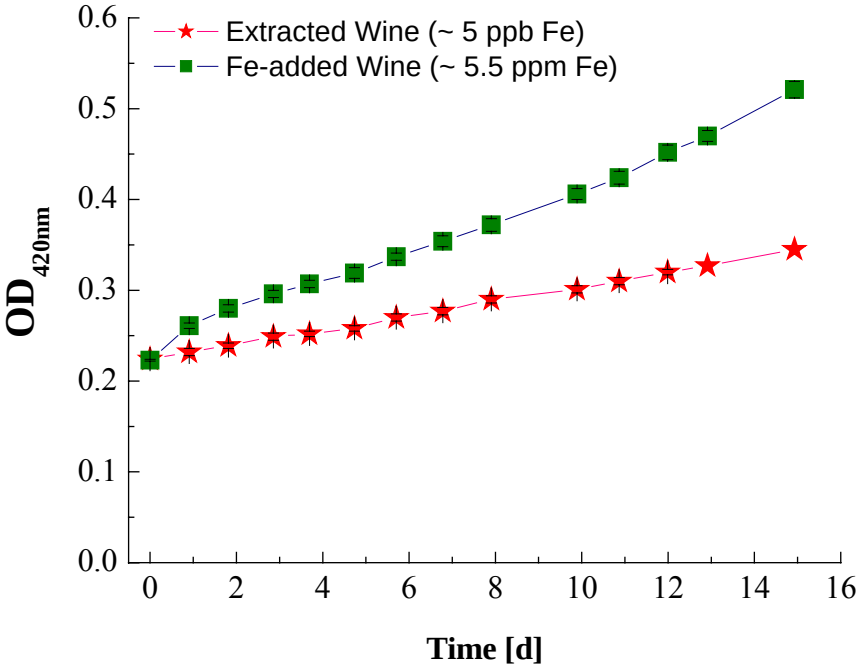

Figure 6.5: Color change of iron-extracted and iron-control (Fe-added) Sauvignon Blanc wine measured spectrophotometrically at 420 nm and stored under aerobic conditions over a period of 15 days.

6.3.3. Iron Leaching Assay

Leaching of iron from stainless steel ferrules into extracted chemically defined medium is illustrated in Table 6.4. Compared to the extracted control medium, significant leaching of iron was found for all time intervals and temperatures, with the most leaching occurring after one hour at room temperature. Statistically significant differences between iron concentrations in the control samples and ferrule-added samples were found at a confidence interval of 0.05 after one hour, and 0.01 after one week.

Table 6.4: Iron concentrations in extracted chemically defined medium measured after exposure to stainless steel ferrules at different temperatures and time intervals and compared to iron concentration in extracted chemically defined medium at t=0.

Time	Temperature (°C)	Iron Concentration (ppb)
T = 0	n/a	2.3 ± 1.2
1 hour	22	166.4 ± 7.8[a]
1 hour	50	118 ± 5.7[a]
1 week	22	94 ± 4.3[b]
1 week	50	85 ± 3.9[b]

[a]: statistical difference at α=0.05

[b]: statistical difference at α=0.01

6.4. Discussion

6.4.1. Food Liquid Matrices

In Chapter 3, the FEC-1 insoluble iron chelating resin was evaluated for iron removal from chemically defined medium. This resin was shown to have high affinity and high selectivity for Fe. Although evaluated previously in white grape juice (Feng *et al.*, 1997b), the efficacy of this resin had not been evaluated in wine or other food liquids.

In this chapter, iron removal was surveyed for a wide array of food products, both at higher and lower pH, and in both alcoholic and non-alcoholic beverages, including fruit, vegetable, grain, and animal based liquids. The results of iron removal from the complex food liquid matrices (Table 6.2) have further shown that the FEC-1 iron chelator has a high affinity for iron. With the exception of two samples (vegetable broth, beer), the FEC-1 resin reduced iron concentrations by ≥ 81%.

The least iron reduction was found in vegetable broth and beer (61.2 and 52.1%, respectively), both of which had the lowest initial iron concentrations. As initial iron concentrations increased, percent iron reduction also increased. Accordingly, these data suggest possible associations between initial iron concentration and iron extraction efficiency. It is also possible that sample matrices contained components that competed for iron, e.g. polyphenols, proteins, fat, and heme-complexed Fe; thus, if the sample had a low initial iron concentration and a matrix that competed strongly for iron, the efficiency of the chelator could have been reduced.

Evaluation of iron removal was carried out with complex media using both the column and batch separation processes. Iron removal from Chardonnay must using the column process (Figure 3.1) achieved similar average percentages of iron removal (< 90%) compared to iron removal from chemically defined medium as presented in Chapter 3; however, final iron concentrations in the extracted must averaged 464 ppb which was considerably greater than the 2.31 ppb iron levels in the extracted chemically defined medium. Because the medium was chemically defined, the components of the medium (ie. Fe, Cu) may have been more readily available for extraction by the resin, whereas the Fe and Cu in the complex Chardonnay medium may have been bound to other components in the wine, thereby limiting removal by the resin in the column process. The Chardonnay wine was not extracted via batch process and additional studies will be needed to determine if the batch process will reduce iron levels by a greater amount than the column method.

Compared to the column extraction method, results from the single and multiple batch extraction process showed a much greater reduction in iron (> 90% iron reduction in seven of 13 complex media). Batch extraction of complex media, e.g. white grape

juice and Sauvignon Blanc wine, showed only a slight difference in percent removal of iron compared to column extraction of complex media, e.g. Chardonnay must. However, the final concentrations of iron extracted by batch method (iron reduced to 4.91 and 5.26 ppb in white grape juice and wine, respectively) were considerably lower than the iron extracted by column method (iron reduced to 464 ppb in Chardonnay must). Possible reasons behind the more effective batch process may include that there was greater surface area exposure of the resin to wine, and that there was a longer contact time (24 h batch process compared to 5 minute column process). Additionally, the batch extraction involved continuous shaking of samples which allowed for constant aeration of media, likely oxidizing Fe^{2+} into Fe^{3+}, which the resin is selective for. However, because the same complex media were not extracted using both the batch and column separation process, more data will be needed to verify efficiency of the batch versus column method.

Feng *et al*., (1997b) suggested that the use of a multi-batch process (versus single batch) improved iron removal efficiency because each fresh batch of resin (iron free) would be more effective than partially iron-loaded resin, establishing a new equilibrium with the remaining iron complexes. However, the findings of the present study did not show a specific relationship between use of a multi-batch process and amount of iron removal.

6.4.2. Oxidation Study

In addition to the iron removal survey, white grape juice, must and wine were subjected to an extended analysis with regards to non-iron metals removed during Fe-extraction. These beverages, specifically white wine, are highly susceptible to

oxidation, making them a model system in which to study the effects of iron removal on oxidative damage.

The results from the complete metal analysis showed that besides iron, copper was also significantly reduced in the white grape juice, Chardonnay must, and Sauvignon Blanc wine. While this indicated reduced selectivity of FEC-1 for iron, this removal could be useful to further stabilize foods, since copper has also been shown to contribute to oxidative reactions (Danilewicz *et al.*, 2003). Mn, Mg, and Zn, may also be needed as trace metals for the nutrition of some microorganisms.

Following extraction, color change was noted in each of the grape beverages. The color of beverage became paler while the resin retained a burgundy color even after regeneration with strong acid. One hypothesis is that the resin was able to remove polyphenols as well as Fe and Cu. Benitez *et al.*, (2002) noted similar losses of color in white wines after extraction with a chelating resin and this loss of color was associated with reduced susceptibility to browning, loss in organoleptic quality, and loss of polyphenolic compounds. To compensate for this potential polyphenol loss during the oxidation study presented in this chapter, and to ensure that iron removal (and to some extent copper removal) were solely responsible for reduced oxidative color changes, polyphenols (100 mg/l caffeic acid) were added to all Sauvignon Blanc wine samples after extraction.

Results of the oxidation study showed statistically significant differences between the maximum color change in the wines that were re-adjusted to common Fe levels, and the maximum color change in Fe-extracted wines. The Fe-extracted wines exhibited less susceptibility to oxidation over 15 days, although, under the experimental

conditions, there was still a statistically significant increase in color change of the extracted wine over time (at $\alpha = 0.01$).

The experimental conditions of this oxidation study (ie. initial aeration, aerobic environment, 30°C) were designed to promote the greatest oxidative color changes in the least amount of time. Further studies will be needed to determine efficacy of the FEC-1 resin on a larger scale (ie. bottled wines) for longer periods of time and under anaerobic conditions. Sensory evaluation will also be needed to determine if iron/copper removal and the hypothesized polyphenol removal has any effect on organoleptic properties of wines.

6.4.3. Iron Leaching Assay

To consider the applied significance of iron removal in industry, extracted chemically defined medium was exposed to food grade stainless steel ferrules in a controlled setting. The ferrules were representative of iron piping or holding tanks used in an industrial setting for transfer or storage of liquid matrices. The results indicate an initial rapid leaching from the ferrules into the medium that leveled off over time. Likely, the initial rapid release of iron was due to fugitive iron on the surface of the ferrules because the ferrules were not passivated as pre-treatment to remove surface oxidized fugitive iron prior to testing. The decrease in the rate of iron leaching over time may be attributed to possible adsorption of iron into the ferrules, or adsorption of the iron into the plastic sample tubes.

Because the ferrules were not treated to remove weakly bound surface iron prior to submersion in the medium, further work will be needed to determine if the application of iron extraction in industry would be suitable. Further work may involve various

washing techniques to remove residual surface iron of the ferrules, a broader range of times and temperatures to better examine rate of iron release, possible use of soluble metal chelating agents, and a larger scale study involving stainless steel tanks and/or piping used in industry.

6.5. Conclusions

The FEC-1 resin used in this work was shown to have a high affinity and high selectivity for iron in complex media, reducing iron concentrations by >81% in most media and exhibiting little to no effect on most other metals in solution. The resin displayed some selectivity for copper, too, and because copper has also been found to catalyze oxidative reactions, its removal next to iron may further reduce unwanted oxidations in beverage systems. The FEC-1 resin removed enough iron to significantly reduce color change in the wine. However, larger scale studies will be necessary to determine the effects of iron removal on shelf-life and organoleptic properties of white wines. Finally, further testing will be required to determine if iron removal would be suitable in industries that utilize stainless steel equipment during manufacturing of food products.

CHAPTER 7: EFFECTS OF IRON REMOVAL ON METABOLISM OF *C. VINI* AND *C. ALBICANS*

7.1. Introduction

This work has shown the efficacy of the FEC-1 chelating resin for removal of iron from chemically defined medium, as well as the iron requirements of specific spoilage yeast. Although the degree of iron removal was not found to be sufficient enough to completely inhibit growth of the yeast cultures studied here, the reduction of iron levels may still lead to modified metabolic capacities. This study investigated whether low but non-inhibitory iron concentrations led to physiological changes in the cell resulting in compositional changes of the medium.

7.2. Experimental Conditions

7.2.1. Medium and Culture Conditions

Extracted or non-extracted chemically defined medium (150 ml) was dispensed into sterile 250 ml polycarbonate flasks (2.3.2.1). Iron-limited cultures (2.4.2.2) of *C. vini* or *C. albicans* were inoculated into the media at a final concentration of approximately $1x10^3$ cells/ml. Culture flasks were placed in an incubated shaker (27°C, 120 RPM; New Brunswick Scientific Classic Series C24KC) and samples were taken over a period of 14 days.

Sample aliquots were aseptically dispensed into sterile 1.5 ml microcentrifuge tubes and centrifuged at 13000 RPM for 10 minutes (Eppendorf 5417C Microcentrifuge).

Sample supernatant was removed after centrifugation and dispensed into sterile microcentrifuge tubes. Samples were stored at –18°C until needed.

7.2.2. HPLC Analysis of Metabolites

7.2.2.1. Standard Solutions

Standard solutions of analytical grade organic acids were prepared (Table 7.1) in ultra pure water and membrane-filtered (0.45 μm). Fresh standard solutions were prepared daily.

Table 7.1: Final concentrations of organic acid standard solutions.

Acid	Final Concentration (g/l)
Tartrate	6.4
Malate	6.4
Lactate	6.4
Citrate	2.0
Pyruvate	2.0
Acetate	2.0
Succinate	2.0
Fumarate	0.75
Formate (internal standard)	2.0

7.2.2.2. Sample Preparation

Samples and organic acid standards were gently mixed and then dispensed into syringeless polyproylene filter vials housing a 0.2 μm nylon filter (Whatman; Florham Park, NJ). Formic acid (2 g/L) was added as an internal standard in samples and standards.

7.2.2.3. HPLC

Organic acid analysis was conducted using a modified HPLC method by Schneider et al. (1987). The HPLC system was equipped with a photo diode array detector (adjusted to 210 nm) and an Aminex HPX-87H organic acid column (section 2.5.8.1.). Separation was conducted using isocratic conditions with a mobile phase of 6.5 mM H_2SO_4 at a flow rate of 0.6 ml/min and temperature of 60°C.

7.3. Results

Figure 7.1 shows the course of organic acid production by *C. albicans* during cultivations in extracted and non-extracted chemically defined media grown with shaking at 28°C. As visible in this graph, growth occurred over a time period of 338 hours, as indicated by increasing biomass. In the non-extracted medium, several metabolites were excreted during the initial growth phase; acetate was reabsorbed later during growth. In order to efficiently compare these fermentations, the maximum and final levels of specific metabolites were compared and data from this analysis can be found in Table 7.2.

For *C. albicans,* differences were observed with regards to the time course of acetate production between non-extracted and extracted media. Maximum concentrations of acetate were produced at 164 hours in non-extracted medium (424 mg/l), while acetate production was still increasing after 338 hours growth in extracted medium (459 mg/l). The initial increase in acetate production could be seen at ~50 hours in non-extracted medium and at ~100 hours in extracted medium.

Negligible citrate and succinate concentrations were found in the extracted medium of *C. albicans* (max 21 mg/l and 24mg/l, respectively), however both citrate and succinate were found in non-extracted medium showing maximum concentrations of 166 mg/l and 87.0 mg/l, respectively.

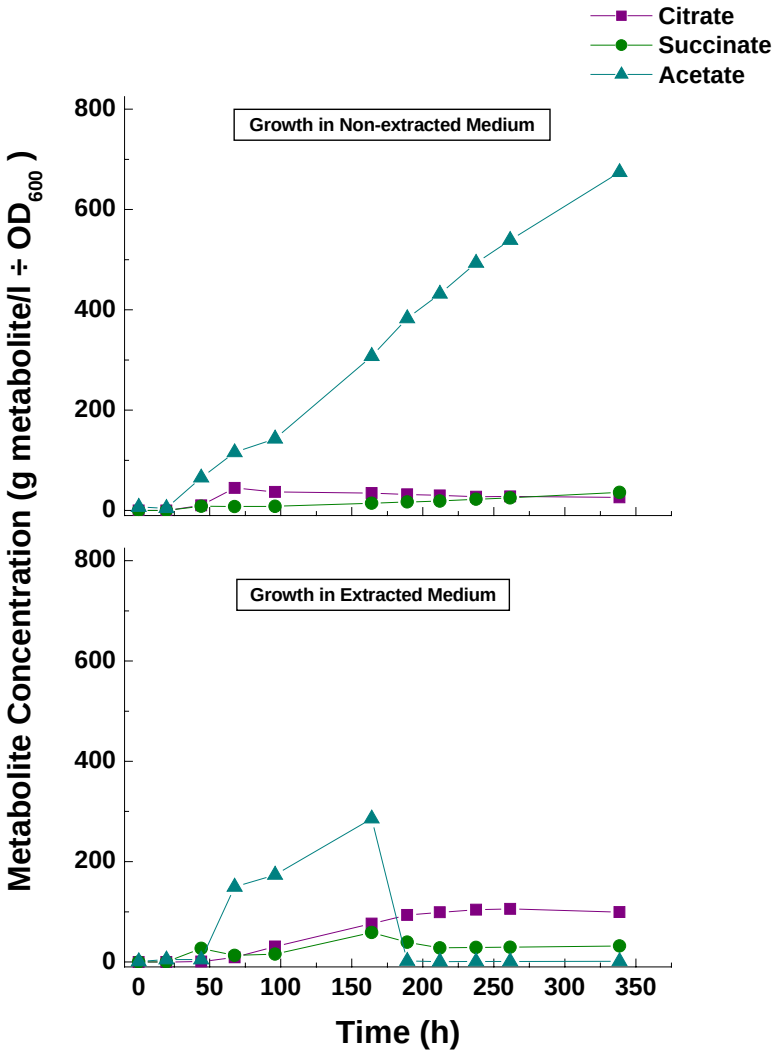

Figure 7.1: Production profiles of organic acids by *C. albicans* during cultivations in iron-extracted and non-extracted chemically defined media. Cultures were grown with shaking at 28°C. Concentrations of metabolites were normalized using growth data measured by OD_{600}.

Table 7.2: Maximum concentrations of organic acids produced by *C. albicans* during growth in extracted or non-extracted media.

Media	Organic Acid	Maximum Concentration (mg/l)	Time of Max. Conc. (hours)	Final Concentration (mg/l)
Extracted	Citrate	45.0	49	26.2
	Acetate	674.1	338	674.1
	Succinate	36.0	338	36.0
Non-extracted	Citrate	105.7	260	99.5
	Acetate	285.7	162	1.3
	Succinate	58.6	162	31.9

C. vini was cultivated in extracted and non-extracted chemically defined media at 28°C with shaking for a period of 338 hours and several metabolites were excreted during this time, as shown in Figure 7.2. The maximum and final organic acid concentrations produced by *C. vini* are compared in Table 7.3.

Clear differences were found with regards to the production of acetate by *C. vini*. In non-extracted medium, initial production and final re-adsorption of acetate by *C. vini* started earlier during growth than seen in the extracted medium.

Citrate and succinate were produced at very low concentrations in extracted medium, and reached maximum concentrations of 189 mg/l and 131 mg/l, respectively. However, succinate was produced at far greater concentrations in the non-extracted medium, while citrate production was negligible.

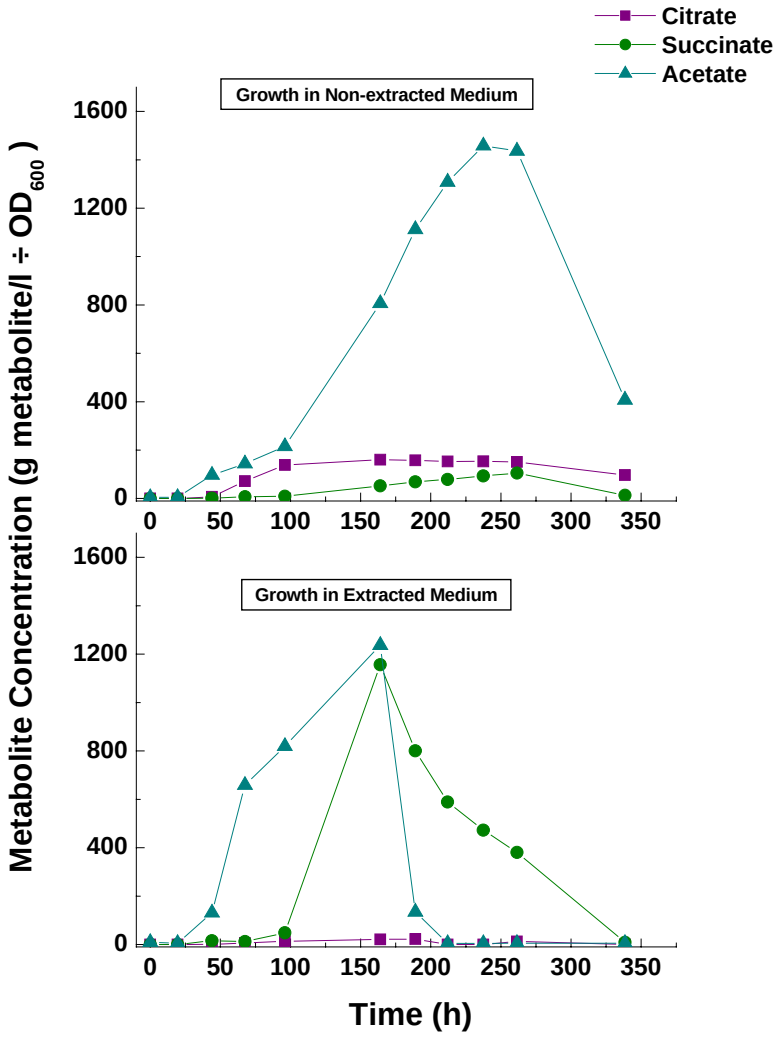

Figure 7.2: Production profiles of organic acids by *C. vini* during cultivations in iron-extracted and non-extracted chemically defined media. Cultures were grown with shaking at 28°C. Concentrations of metabolites were normalized using growth data measured by OD_{600}.

Table 7.3: Maximum concentrations of organic acids produced by *C. vini* during growth in extracted or non-extracted media.

Media	Organic Acid	Maximum Concentration (mg/l)	Time of Max. Conc. (hours)	Final Concentration (mg/l)
Extracted	Citrate	160.3	162	96.9
	Acetate	1458.1	236	407.5
	Succinate	104.9	260	13.2
Non-extracted	Citrate	22.2	187	0
	Acetate	1237.0	162	8.5
	Succinate	1156.1	162	5.5

7.4. Discussion

Iron has been shown as a cofactor for several enzymes that assist with cell growth and regulation (Wessling-Resnick, 2000). Specifically, iron is known as a cofactor for enzymes in the tricarboxcylic acid cycle (TCA) during production of organic acids such as succinate (Michal, 1999). If the availability of iron is limited, important biological processes may be altered, and production of specific metabolites may increase or decrease. For example, iron is a cofactor of aconitase, an enzyme that degrades citric acid. If iron supply is sufficiently limited, citrate might be over-produced or accumulate because it cannot be broken down. This is the case for *Aspergillus niger* which has been shown to produce considerable quantities of citric acid when grown in iron-limited conditions (Grewal and Kalra, 1995).

The aim of the present study was to investigate whether low but non-inhibitory iron concentrations may lead to changes in production or degradation of specific metabolites during the growth of *C. vini* and *C. albicans* in iron-extracted or non-extracted chemically defined media.

Differences were observed with respect to the production profile of acetate by *C. vini* and *C. albicans*. Both organisms exhibited delayed production of acetate in iron-extracted medium, however, the concentrations accumulated in the medium were higher than those found for non-extracted medium. These data are interesting because acetate (acetic acid) is often an unwanted by-product in fermented products such as wine, cheese, or yogurt, hence, the metabolic production and accumulation profile of this metabolite is worth investigating in more detail.

The fact that iron is required for the degradation of citric acid may explain the increase in citric acid production and the decrease in succinate production by *C. vini* in extracted medium. Following citrate production, aconitase degrades this compound into various other compounds necessary in the TCA cycle. By limiting iron in the medium, the enzyme would have reduced activity, thereby potentially decreasing the rate at which succinate was produced. The growth response of *C. vini* in iron-limited conditions was consistent with the requirement of iron by aconitase as shown by the significant reduction in the production or accumulation of succinate in iron-extracted medium.

The negligible production of citrate by *C. albicans* in iron-extracted medium does not support the hypothesis that iron removal results in loss of aconitase activity or induces citrate over-production or accumulation. Furthermore, the results are not in agreement with what is known about *A. niger* with regards to citrate over-production when this organism is grown in iron-limited conditions. It is possible that *C. albicans* utilizes a less sensitive or differently controlled metabolic pathway that is less sensitive or does not require iron as a cofactor. Even if iron was required in the metabolic pathways

used by *C. albicans*, the limiting step would appear not to be aconitase activity because iron limitation did not lead to citrate over-production or accumulation in the present study.

As shown in Table 3.2, the FEC-1 resin removed significant quantities of copper from the extracted chemically defined medium. In *C. albicans*, copper has been shown as a cofactor in superoxide dismutase (SOD) (Lamarre *et al.,* 2001), an enzyme required to prevent oxidative damage within the cell. Removal of copper in the extracted chemically defined medium may reduce the activity of SOD, thereby potentially increasing the risk of oxidative damage to the cell and possibly reducing cell viability. It is possible that the removal of copper from the medium also played a role in metabolic differences in extracted and non-extracted media, however, additional studies would be needed for verification (ie. copper added back to medium).

7.5. Conclusions

Differences in production profiles and concentrations of specific metabolites were observed during growth of *C. vini* and *C. albicans* in iron-deprived environments. The over-production or accumulation of citrate by *C. vini* in iron-extracted medium was consistent with the role of iron as a cofactor for aconitase as was demonstrated. However, comparable over-production or accumulation of citrate by *C. albicans* in extracted medium was not observed suggesting that this organism utilizes less sensitive or alternative pathways for citrate metabolism.

CHAPTER 8: GENERAL CONCLUSIONS

The chapters in this thesis have presented research on iron removal in chemically defined and complex food and beverage systems with an emphasis on determining the iron requirements of several microorganisms and the potential for reductions in preservative use in beverages. The following discussion aims to contextualize the findings and provides ideas for future research.

Many food and non-food systems require some method of preservation to protect against microbial and/or oxidative degradations of the product. Preservation techniques can interfere with product qualities, as is the case for foods in terms of adding chemical preservatives which may lead to organoleptic degradation of the product or allergic reactions in some consumers. Because preservation strategies may interfere with the wholesomeness and organoleptic qualities of the product, there is an ongoing interest in finding alternative preservation methods.

The research presented in this thesis investigated an innovative approach that relied not on the addition of a compound, but on the removal of a compound. Iron is important as an element in nutrition, but because many foods are naturally low in iron, these foods do not constitute a significant source of iron. Thus, iron removal from these food products should not have any negative effects.

Highly specific and efficient iron chelators are now available and may be used for iron removal strategies. In this research, a synthetic iron chelating resin, FEC-1, reduced iron to such low levels that very sensitive analytical equipment was required for

detection; however, this iron removal was not fully sufficient to significantly inhibit yeasts under the testing conditions chosen. Interestingly, the hurdle approach studied in this thesis was not conclusive, which may have been due to the experimental design. However, based on the reduction in growth with low Fe and the sensitivity of the yeast to the Fe chelator DIBI, it would be expected that this approach could work, maybe in more applied systems or for certain food systems.

Although iron removal from a chemically defined medium was not sufficient to completely inhibit microbial growth, production and degradation of specific metabolites was altered. Specifically, iron-limited conditions led to an increase in the production of citrate, a decrease in succinate production, and a delay in the time course for production of acetate. Organic acids make up just a small subset of compounds in microbial metabolism, hence, it would be interesting to study the effect of metal concentrations on the production or degradation of many other metabolites in industrial fermentations such as the production of cheese, wine, or beer.

In addition to a microbial preservation strategy, iron removal also has the potential to improve oxidative stability. Removal of iron from white wine was sufficient to significantly reduce oxidative color change and might have application in the wine industry as long as organoleptic qualities are not affected.

Finally, it was found that the applicability of iron removal in the food industry might suffer due to the common use of stainless steel equipment, as well as the presence of compounds in foods which a) may compete for iron, or b) limit the amount of ferrous iron present in the food. However, if contactable stainless steel surfaces are passivated or, if iron removal is the last step in the manufacturing process, e.g. directly before

bottling of beverages, then this technique might find application in the food industry. Other non-food products may not suffer from these problems because they are often formulated with defined chemical ingredients. Thus, there may be a wide field of application for iron removal beyond the field of food science. Overall, the present results for food materials did not indicate a significant improvement that could lead to a practical application for this approach at this time. However, the results have shown that iron removal is successful and leads to inhibition of microorganisms, thus, demonstrating proof of concept.

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