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Significance of Cell Surface Charge on Microbial Susceptibility to Chitosan

LaQuita Mai Michael
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by LaQuita Mai Michael entitled "Significance of Cell Surface Charge on Microbial Susceptibility to Chitosan." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

David A. Golden, Major Professor

We have read this thesis and recommend its acceptance:

Svetlana Zivanovic, F. Ann Draughon

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Vice Chancellor for Academic
Affairs and Dean of Graduate Studies

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Significance of cell surface charge on microbial susceptibility to chitosan

A Thesis Presented
for the Master of Science Degree
The University of Tennessee, Knoxville

LaQuita Mai Michael
August, 2005

Dedication

This thesis is dedicated to the loving memory of my grandparents and late father

Dr. Charlene B. Michael
1918 - 1999

and

Mr. Joseph M. Michael Sr.
1916 - 1990

and

Mr. Joseph M. Michael Jr.
1942 - 1992

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I would like to show my appreciation toward my major professor, Dr. David Golden for his guidance and alliance. I would also like to extend my sincerest gratitude toward my committee members, Dr. F. Ann Draughon and Dr. Svetlana Zivanovic for their assistance and support.

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Last but definitely not least, I would like to express my utmost thanks to my caring mother, Mrs. Betty G. Michael, you are an essential person to me, thank you for being the devoted, and loyal woman that you are because without your presence in my life I would have not made it this far. I also would like to give thanks to my cousins, and brother, Charles (Chi) Michael, as well as the rest of my family; I will always remember all of the support you have all given to me regarding all of my accomplishments.

Without your love and the blessings from the Lord above none of this would have been possible.

Abstract

A study was conducted to determine the importance of cellular surface charge on susceptibility of yeasts to the natural biopolymer chitosan. The test organisms utilized were *Saccharomyces cerevisiae*, *Candida krusei*, and *Zygosaccharomyces bailii*. Surface charge was determined at various culture ages and under selected environmental conditions. Bovine serum albumin (BSA) was used as a protein standard to ensure an accurate method to measure microbial surface charge. Yeasts cells were grown to the early stationary phase, washed and suspended in potassium chloride with absorbance value (A_{600nm}) of 0.1 to 0.2, and charge was measured using a phase analysis light scattering (Zeta PALS) apparatus. The chosen absorbance was predetermined using BSA, which had minimal standard deviation within surface charge measurements.

Surface charge of *S. cerevisiae* cells was measured after growth in yeast-mold (YM) broth for 12, 18, 24, 36, 48, and 72 hr to determine changes in charge as a function of growth phase. The effect of short term exposure to various pH on surface charge was determined by suspending *S. cerevisiae* cells in acetate buffer adjusted to pH 3-11 using 0.1 N NaOH or 0.1 N HCl. Additionally, *S. cerevisiae* cells were adapted over time to pH 3, 4, and 8 to evaluate prolonged effects of growth pH on yeast surface charge.

Flocculation and viability of the three yeasts were also evaluated. Cells were washed in sodium chloride and resuspended in acetate buffer (pH 4.0) to achieve an absorbance (600 nm) of 3.0. Chitosan was added to the yeast suspensions to achieve concentrations of 0.00001-0.001%. The test concentrations were relatively low due to the increase in viscosity of suspensions with higher chitosan concentrations. Cells were

observed using a phase contrast microscope to detect morphological differences between species, at selected pH, and when chitosan was added.

Surface charge data for bovine serum albumin corresponded with previously published literature. Surface charge of yeasts cells was shown to be influenced by growth phase, species, environmental pH, and adaptation to non-optimal pH. After 48 hr, the surface charge of *S. cerevisiae* cells showed a significant increase or decrease ($p < 0.05$), and there were overall surface charge differences observed among the various pH values. However, pH adapted cells developed resistance to non-optimal pH due to adaptation, and only showed differences in pH between pH 3 and 8 and pH 4 and 8. This study showed that there were significant differences ($p<0.05$) in surface charge depending on the yeast species utilized. The average surface charge of *S. cerevisiae* cells was -19.6 mV, -12.07 mV for *C. krusei* and -25.82 mV for *Z. bailii*. *Candida krusei* had the least negative surface charge. This yeast was least affected by the antimicrobial affects of chitosan. *Z. bailii* had the most negative charge, which may be the reason chitosan was more effective against *Z. bailii*. This study demonstrated that chitosan, which is positively charged, may be more effective as an antimicrobial agent against microbial cells with more negative charges.

Flocculation patterns of yeasts cells were altered by chitosan and lower log counts of some yeasts were observed when chitosan was added to suspensions. Chitosan was shown to inhibit growth of yeast species differently which may be partially explained by the surface charge differences of the cells. Yeasts cells were observed microscopically to identify changes in overall appearance and morphology when cells were exposed to chitosan. When chitosan was added to *S. cerevisiae* cell suspensions, cells appeared less

dense, and more rounded, as compared to *S. cerevisiae* control cells. Clustering, or clumping, of cells was also noticed when chitosan was present.

The surface charge of yeasts was shown to be affected by environmental pH, age, and species. These influential factors are important when determining the most desirable conditions for chitosan to serve as a natural food antimicrobial. Chitosan is currently approved as a dietary supplement by the Food and Drug Administration, and it has the potential to be used as an antimicrobial agent and inhibit microbial growth in foods.

Table of contents

List of Tables

List of Figures

Chapter I

Literature Review

Introduction

The environment is important when attempting to control microbial growth; temperature, pH, oxygen, and moisture content are attributes which are critical factors relative to controlling yeast growth in foods. Although yeasts are commonly present on raw fruits and vegetables and many processed foods, only a few yeast species are recognized as pathogenic to humans, and none of them are known to cause foodborne illness (Hurley et al., 1987). Nevertheless, a tremendous amount of food spoilage is caused by yeast growth, creating a significant economic problem. Controlling yeast proliferation in foods would reduce spoilage and increase profitability.

Yeasts are classified as fungi at the level of family; all yeasts are non-photosynthetic higher protists with rigid cell walls and exist as either unicellular organisms or mycelia (Jin et al., 1998). A characteristic of most yeasts, such as *Saccharomyces cerevisiae,* is that they divide by budding, instead of binary fission observed in bacteria. Yeasts can be differentiated from bacteria by their larger cell size and their oval, elongate, elliptical, or spherical cell shapes. Typical yeast cells range from 5 to 11 micrometers in diameter, some cells being even larger. Older yeast cultures tend to have smaller cells (Jay, 2000). Yeast cells are surrounded by a tough, rigid cell

wall that surrounds the periplasmic space, spans 100-200 nm and represents 26 to 32% of the dry weight of the cell (Nguyen et al., 1998; Stratford, 1994). Studies on the chemistry and structure of yeast cell walls have focused mainly on *S. cerevisiae* and *Candida albicans*. For these species, and most other yeasts that have been examined, the cell wall consists of about 85 to 90% polysaccharide and 10 to 15% protein. The cell wall of *S. cerevisiae* consists of four macromolecular classes, specifically cell wall proteins (CWPs), accounting for 30-50% of the dry weight of the cell. β1,6-glucan, β1,3-glucan (reserves more than half of the cell wall), and chitin accounts for only 1-2% in wild yeast cells (Kapteyn et al., 1996).

The polysaccharide component consists of a mixture of water-soluble mannan, alkali-soluble glucan, alkali-insoluble glucan, and small amounts of chitin. Constituents are covalently connected as determined by the resistance to extraction using hot detergents such as sodium dodecyl sufate (SDS) and Tween-80. β1,3-glucan is the predominant structural component, and together with chitin, it is responsible for cell wall rigidity, in turn determining its shape and strength. Proportions of the different fractions within the cell walls vary with yeast species and strain. In *S. cerevisiae* for example, there are approximately equal proportions of mannan and glucan, and within the glucan fraction, approximately equal amounts of alkali-soluble glucan and alkali-insoluble glucan exist (Kapteyn et al., 1999; Nguyen et al., 1998).

Members of the genus *Saccharomyces* are ascosporogenous yeasts that multiply by multilateral budding and produce spherical spores in asci. They are diploid and do not ferment lactose. All baker's, brewer's, wine, and champagne yeasts are strains of *S. cerevisiae*. They are found in Kefir grains and can be isolated from a wide range of foods,

such as dry-cured salami and numerous fruits, although *S. cerevisiae* rarely causes spoilage (Jay, 2000).

Zygosaccharomyces bailii is a fermentative ascomycete yeast that can grow at low pH and in the presence of maximum permitted levels of lipophilic organic acid preservatives (Thomas and Davenport, 1985). *Z. bailii* is a commercially important spoilage yeast due to its capability of surviving low pH (pH 1.8) in the presence of weak organic preservatives, such as benzoic acid, a common yeast inhibitory additive used in the food industry. This preservative resistance causes great loses to the food and beverage industries due to spoilage.

Candida is an ascomycete yeast that is the most common cause of opportunistic mycoses worldwide. *Candida krusei* colonies are typically dry, dull, and often, a mycelial border is observed on Sabouraud dextrose agar. The colonies are cream colored and their shape is often lenticular (Larone, 1995).

Intrinsic parameters of yeasts

Intrinsic parameters are the parameters of plant, animal, and microbial tissues that are inherent components of the tissue. These parameters include pH, moisture content, and nutrient content, and with respect to yeasts, the most important intrinsic factors are water activity (Aw), nutrients, and acidity. The majority of yeasts are less sensitive to decreases in Aw than most bacteria, and usually are quite capable of growth at Aw values less than 0.90 (Deak, 1991). The most important nutrients for yeasts are carbohydrates, which serve as the primary energy sources (Rose, 1987).

The majority of microorganisms grow best at pH around neutrality (6.6-7.5), whereas few grow below pH 4.0. Bacteria tend to be more sensitive to non-optimal pH than molds and yeasts (Jay, 2000). Most yeasts tolerate a wide pH range and grow easily at pH between 3 and 8. Yeasts show an incredible tolerance to pH and many species are able to grow at pH as low as 1.3-1.7. This tolerance strongly depends on the type of acidulant, such as acetic or propionic acids (Deak, 1991; Pitt, 1974).

Fungi often exist in harsh living environments to which they must adapt in order to remain viable. Yeast cells are capable of making necessary adjustments to the components and structure of their cell membranes and walls in response to environmental alterations such as pH, temperature, oxygen, and/or nutrient accessibility (Jay 2000). When the cell is damaged or stressed, changes may affect cell wall organization, mainly due to the existence of cell wall repair mechanisms that compensate for cell destruction. The primary function of the cell wall is to provide shape, mechanical strength and protection of the cell and to keep desired components inside the cell and foreign invaders out of the cell.

Yeast adaptation

Single-celled microorganisms that freely reside in nature, such as yeasts, are challenged with large variations in their natural environments. Rapidly activated mechanisms are crucial to maintaining the capacity of yeasts to proliferate. Environmental changes may be of a physical or chemical nature, such as osmotic pressure, radiation, temperature, solute concentration and water activity, toxic chemical agents, nutrient availability, ion presence, and pH. As cells respond to unexpected stress,

they do so in different phases. During the primary phase, known as the stress-responsive phase, cell changes, such as shrinkage of cells, occurs; defense processes are triggered in the second phase (adaptation phase), and adapted cells resume proliferation, by regaining there ability to reproduce.

Microorganisms must have specifically balanced internal conditions in order to obtain optimal growth and function. The internal condition of the cell is important for cell survival. However, alterations in the external environment can result in various cellular disruptions that may affect the internal milieu. These disruptions can cause destabilization of cell structures (Hahmann and Mager, 2003). Therefore, cells must be capable of protecting and maintaining internal homeostasis in response to changing external conditions. Adverse pH affects the functioning of microbial enzymes and the transport of nutrients into the cell. When microorganisms are exposed to environments below or above neutrality, their ability to proliferate depends on their ability to adapt or bring the intracellular pH to a more desirable range. The use of chemical preservatives, specifically weak organic acids, as antimicrobial agents is quite common. However, yeasts differ in their susceptibility to acidic environments. *Z. bailii* is a food spoilage yeast that can tolerate high acid concentrations and low pH. On the other hand, *S. cerevisiae* can not remain viable in such an environment (Jay, 2000).

Oftentimes, yeast cells must contend with changes in osmotic pressure, temperature, long periods of nutrient deprivation, and acidity of their environment. When these changes suddenly occur, the cell must promptly adjust its internal environment to that required for physiological growth by using their defense mechanism systems. Studies conducted regarding the mechanisms that *S. cerevisiae* uses to adapt to

new environments have emerged over the past years. Yeast cells gain cross protection against different stressful environments. That is, when cells are exposed to a small dose of one stress, they may become resistant to normally lethal doses of other unfavorable living conditions (Hohmann et al., 2003; Lewis et al., 1995).

One mechanism that yeasts use to protect the internal system from environmental changes is to initiate a common gene expression program that protects the cell during adverse encounters. In a study conducted by Gasch et al. (2000), DNA microassays were used to identify approximately 900 genes whose expression was altered in *S. cerevisiae* responding to a variety of stressful environment changes. (The complete list of the genes that participate in this response can be viewed at http://www-genome.stanford.edu/yeast_stress). The changes of these genes are a feature of responses to different environments, and initiation of this program begins when the environment becomes unfavorable.

In addition, stress plays an important role in applied biotechnology areas. Different industries benefit from studies involving yeast and stress-related research, such as, ethanol tolerance of wine yeast and protection of food from spoilage. These are a couple of reasons why yeast stress responses are an active research area and were utilized in this study.

Cell surface charge

Determination of surface charge of microbial cells and biopolymers is of prominent importance for understanding their behavior and functions under various environmental conditions. Surface properties have provided information about cell

surface composition, isoelectric point, rates of uptake of nutrients and antimicrobial drugs, as well as flocculation patters of organisms. Surface charge is important because in order to examine adaptation mechanism(s) in yeasts to acidic environments and to investigate mechanism(s) of the antimicrobial action of chitosan, measuring charge at the cell surface is relevant.

Most viable cells have fixed negative charges on the cell membrane surface, primarily due to cell wall components such as phosphates, proteins, and carboxylate groups (Chang et al., 2002) (Table 1, all tables are shown in the appendix). According to Becker et al. (1996), the bilayer assists with the membrane structure. The hydrophobic tails are on the inside and the hydrophilic heads point toward the aqueous environment at the membrane surface. Every biological membrane has such a lipid bilayer as its basic structure. The cell membrane phospholipid bilayer plays a role in the surface charge of the cell. The nature of the interface between the outer layers of the cell wall and microbial environment plays a considerable role in cell physiology. The cell wall, combined with the cell membrane, is crucial for exchange of nutrients and waste molecules between the microbial cell and its surroundings. The cell membrane consists of proteins and phospholipids; cell wall composition significantly differs between genera and species. Nevertheless, regardless on taxonomic classification, all microbial cells contain carboxyl, phosphoric, and amino groups in their outer membranes. These groups can easily be ionized as a function of environmental pH and contribute to the net charge of the cell surface. At physiological pH, most microbial cells posses a net negative surface charge, due to the cell membrane phospholipid bilayer (Harkes et al., 1992). Microbes are also negatively charged due to the presence of polysaccharides within the

wall and cell membrane macromolecules, such as, peptidoglycans and phospholipids. The degree of this charge can be determined based on electrostatic mobility of cells in an electric field, similar to electrophoresis of proteins.

Net cell surface charge can be assessed on the basis of "zeta potential," which is the electrical potential of the interfacial region between the cellular surface and the aqueous region. In other words, for a charged particle or cell, moving with respect to the solution phase, the potential at the shear surface, with respect to the bulk solution, is commonly referred to as the zeta potential (Miller et al., 1990). The zeta potential is measured because it is an indicator of changes of the cell wall components. Zeta potential can be estimated by measuring cellular velocity, or electrophoretic mobility, in an electric field. The velocity of particles or cells moving in an electric field can be directly measured by determining the frequency change of the laser light they scatter and is dependent on various factors such as temperature, ionic strength, pH of the medium, electric field strength, and the net surface charge of the particle (Wilson et al., 2001). Direction of the movement is affected by the charge of the particle, e.g., negatively charged particles are attracted to the positive electrode, while positively charged particles are attracted to the negative electrode. Measurements of zeta potential have been made in the past by several researchers, but on a relatively restricted range of samples. Collins and Stotzky (1992) made zeta potential measurements using a Zeta-Meter apparatus (Zeta-Meter, Inc., Long Island City, N.Y.). Ware and Flygare (1971, 1972), used a Coherent Radiation Model apparatus, an electrophoretic light scattering method to measure zeta potential.

The zeta potential of a single chitosan molecule in a solution has not yet been measured because of the relatively small size of the molecule. However, chitosan clusters in solutions have been evaluated and surface charge of 43 mV has been recorded (Calvo et al., 1997). This makes chitosan attractive as a potential antimicrobial agent, since most living cells possess net negative charges; therefore, chitosan would bind to the negatively charged microorganisms (Table 1).

Zeta potential is influenced by environmental factors such as pH, temperature, ionic strength, heavy metals and culture age. Environmental pH can permanently alter cell wall composition and microbial virulence (Montville, 1997). The net surface charge of some yeasts and bacteria can be altered due to the presence of heavy metals at elevated pH. The ability of a metal to cause charge alterations appears to be related to the speciation of the metal that occurs at various pH and to the ability of some speciation forms to be specifically adsorbed on the cell surface (Collins and Stotzky, 1992). Collins and Stotzky (1992) concluded that species differences played a role in electrophoretic mobility. However, the differences between the speciation forms of the metals were more noticeable than the type of cell or particle analyzed.

According to Lytle et al. (1999), ionic strength and pH impacts surface charge of microbes, and increasing ionic strength impacts the electrostatic properties of *E. coli* O157:H7 strains. This observation was explained by the electrostatic attraction of cations (Na^+) in the phosphate buffer to the anionic bacterial surface.

Primary existing methods (surface charge)

Various analytical methods have been used in the past to analyze electrostatic properties of microbial cell surfaces. Micro electrophoresis involves the placement of a cell suspension in an electrophoresis cell, applying voltage across the cell, microscopic observation of the microbial movement over a given distance, and velocity is used to calculate electrophoretic mobility (Moyer, 1936). Instruments that function using this technique (electrophoresis) include Zeta Meter (Zeta Meter, New York, NY, USA), FACE Zeta-Potential Meter ZPOM (Kyowa Interface Science, Tokyo, Japan) and Lazer Zee Meter 501 (PenKem, Bedford Hills, NY, USA). Electrostatic interaction chromatography (ESIC) is a less laborious method for characterization of cell surface charge. ESIC originally was employed as a method for isolation of microorganisms (Wood, 1980), but has also been used to study microbial physiology (Pederson, 1981). Electrophoretic light scattering (ELS) involves the velocity of particles moving in an electric field directly being measured by determining the frequency change of the laser light they scatter, yielding their electrophoretic mobility (Blake et al., 1994). The ELS method has shown to be of substantial value regarding a variety of physiological applications. ELS has proven to be a relatively easy and rapid method for estimating zeta potential.

Chitosan and mode(s) of action

Chitin, poly-β-(1→4)-N-acetyl-D-glucosamine, is a cellulose-like biopolymer distributed in marine invertebrates, insects, and fungal cell walls. It is commercially produced from shellfish wastes (Roller, Covill, 1999). Chitin and chitosan have attracted interest in scientific areas, such as the biomedical, food, and chemical industries (Peter, 1995). According to Cabib et al. (1988), chitin is the first structural polysaccharide of the fungal cell wall whose mechanism of synthesis was discovered, and it remains the most extensively studied. Chitosan, the deacylated derivative of chitin, is a naturally versatile biopolymer with numerous food applications (Shahidi et al., 1999). Chitosan acts as a metal chelator, binding metals such as lead, iron, copper, cadmium, and magnesium. Among the earliest applications of chitosan was to remove harmful metal ions from industrial waste waters and removing suspended solids from food processing wastes (Knorr, 1984). Chitosan possess numerous functional properties. It can be used as a thickening agent in beverages and semi solid foods, clarifying agent in wine and juice processing (Li et al., 1997), a mineral and lipid binder, as a flavor and color carrier, and for production of coatings and edible films (Ravi Kumar, 2000). It has been demonstrated that chitosan inhibits growth of foodborne fungi, yeasts and bacteria. It appears, however, that chitosan has stronger bactericidal effect against Gram-positive than against Gram-negative bacteria (No et al., 2002). This is probably due to the lipopolysaccharide layer (LPS) distinction amongst Gram-negative and Gram-positive organisms as well as differences in the net electronegativity of the cell wall between Gram-positive and Gram-negative bacteria, which ultimately affect the degree of binding of polycationic chitosan to bacterial surfaces. Since chitosans exhibit antimicrobial

properties, they have received attention for their potential as natural food preservatives (Gooday, 1994; Helander et al., 2001).

Attempts to explain the antimicrobial mode of action of chitosan have been focused in two directions. One hypothesis is that positively charged chitosan interacts with the negatively charged cell surface, interrupting cellular metabolic activity and eventually resulting in inhibition of cell growth and division, leading to cell death (Helander et al, 2001; Tsai and Su, 1999). However, Hadwiger et al. (1986) hypothesized that chitosan oligomers penetrate into the cell nucleus, interfering with DNA transcription, mRNA function, and protein synthesis. This mechanism is limited to eukaryotic cells and does not explain antibacterial effects. Although strong evidence of chitosan antimicrobial properties exists, both hypotheses lack direct confirmation and validation of a mechanism of chitosan activity. Chitosan currently is only approved by the Food and Drug Administration (FDA) for use in dietary supplements.

Yeast flocculation

Yeast flocculation has been defined as the phenomenon wherein yeast cells adhere in clumps and sediment rapidly from the medium in which they are suspended (Stewart et al., 1976). The mechanism of initiation of flocculation is not known. Initially, flocculation was reported to be a process predominately based on ionic interactions, with Ca^{2+} ions acting as bridges between yeast cells (Mill, 1964). A requirement for Ca^{2+} in flocculation of yeast cells is commonly cited, but magnesium and manganese ions may act as substitutes (Miki et al., 1982). Some researchers suggest that the yeast cell wall composition is a significant indicator of the rate and extent of cell wall flocculation

(Calleja, 1987). Sratford and Keenan (1987, 1988) showed evidence that agitation is required to initiate cell flocculation. This indicates that physiochemical cell surface interactions may be involved in flocculation. A correlation between flocculation and electrophoretic mobility of yeast cells under specific conditions was reported by Beavan and Belk (1979). Smit et al. (1992) reported a correlation between nutrient limitation, hydrophobicity, and flocculation for some *S. cerevisiae* strains. Their study demonstrated that magnesium-limited *S. cerevisiae* cells are strongly affected in surface hydrophobicity and their ability to flocculate. A proteinaceous cell surface factor(s) was identified as a flocculin. This component appears to be involved in both cell surface hydrophobicity and flocculation capability of yeast cells. Therefore, nutrient limitation ultimately appeared to trigger an increase in cell hydrophobicity and flocculation. Wilcock and Smart (1995) state that yeast cell surface influences flocculation, and it is strain dependant. It is also suggested that surface charge and the non-separation of progeny from mother cells rather than hydrophobicity influences flocculation of yeasts. The basis of flocculation is still insufficiently understood, although researchers realize the significance of this process in industrial processes.

The surface charge (zeta potential) and flocculation patterns of yeasts were observed in this study because the yeast cell wall is an important indicator of the rate and extent of cell flocculation; therefore, surface charge influences flocculation (Calleja, 1987).

Research objectives

The overall goal of this research was to determine correlations between microbial surface charge and susceptibility of microorganisms to the antimicrobial biopolymer chitosan. Specific objectives of this study were: to develop methodology using bovine serum albumin; to determine the surface charge of *S. cerevisiae* cells as affected by environmental pH and culture age; to determine the surface charge of three yeast species; and to measure the susceptibility of these yeasts to chitosan. One of the possible mechanisms of chitosan antimicrobial activity is its interaction with the yeast cell wall, causing disruption of normal transfer of nutrients and flocculation of the cells. Therefore, we investigated flocculation kinetics of *S.cerevisiae, C. krusei,* and *Z. bailii* as influenced by medium molecular weight chitosan at concentrations ranging from 0.00001 to 0.001%, and conducted plate counts to determine cell viability after exposure to chitosan.

Chapter II

Materials and Methods

Bovine serum albumin solution

Protein bovine serum albumin (BSA) was utilized in this study as a model to develop an accurate methodology procedure. Few if any scientific studies have been published regarding consistent zeta potential measurements of yeast cells. However, an abundance of articles exist concerning BSA and zeta potential measurements. Therefore, zeta potential readings were first performed using BSA, ultimately to optimize conditions of the analysis. To determine the effects of particle concentration, sample conductance, and pH of the solvent on zeta potential of measured particles, several preliminary experiments were conducted with BSA.

The protein concentration of BSA ranged from 1 to 5%, and conductance was altered with 1 and 10 mM potassium chloride (KCl). Optimum conditions were found to be 5% BSA in 1 mM KCl, and this BSA solution was utilized because it gave less standard deviation within zeta potential measurements. The solution pH was adjusted using 0.01 N HCl and 0.1 N NaOH. To validate the conditions, we measured the BSA surface charge at pH ranging from 2 to 11, using a zeta potential analyzer (Zeta PALS), calibrated with Zeta PLUS software (Brookhaven Instruments Corporation, Holtsville, New York). Ten measurements were taken per sample.

BSA solution consisted of 5g BSA crystals (Sigma Aldrich co., St. Louis, Missouri) and 100 ml of 1 mM KCl (Fisher Scientific co., Fair Lawn, New Jersey). Five grams of BSA were weighed in a 100 ml volumetric flask and brought to volume with 100 mM KCl. This solution was mixed for one hour. Potassium chloride was prepared using HPLC grade water (18.0 F.W., Fisher Scientific, Fair Lawn, New Jersey).

Yeast strains and cultivation

Saccharomyces cerevisiae (KE 162), *Zygosaccharomyces bailii* (NRRL 7256) and *Candida krusei* (NRRL 7179) held in the University of Tennessee, Knoxville food microbiology laboratory culture collections were utilized in this study.

Inoculation of media: One loopful of yeast cells were inoculated into 40 ml sterilize yeast and mold (YM) broth (Difco Becton Dickinson Microbiology Systems, Sparks, MD) and incubated for 48 hr at 25ºC under continuous orbital shaking (100 rpm Controlled Environmental Incubator Shaker, New Brunswick Scientific Co., Inc., Edison, NJ). After 48 hr, 0.1 ml of yeast suspension was transferred to 350 ml of sterile YM broth and incubated under the same conditions for 24 hr (early stationary phase). Cells were harvested by centrifugation for 10 minutes at 8000 rpm at 4ºC (Biofuge 17R, Baxter Scientific Products, McGaw Park, IL) and washed with deionized water, mixed, and centrifuged again. The cells were consequently washed and centrifuged two times in 1 mM KCl, mixed, and centrifuged. Surface charge of the yeast cells was determined in a suspension of cells in 1 mM KCl with absorbance values (A_{600nm}) of 0.1 to 0.2, zero set against KCl buffer. Absorbance was measured using a UV Scanning Spectrophotometer (UV-2101PC) (Shimadzu Scientific Instruments, Inc., Columbia, MD**).**

Determination of effects of species on zeta potential of yeast cells

The cell wall composition of microorganisms varies depending on components within the cell membrane; different species contain different cell wall materials, which influence zeta potential readings. Gram-positive bacteria posses a thick peptidoglycan layer while, Gram-negative bacteria do not, and this difference affects the charge of these organisms. Aware of cell composition differences, three different yeast species were utilized in this study to observe differences in zeta potential measurements among different yeast species.

S. cerevisiae, Z. bailii and *C. krusei* were utilized in this study to detect zeta potential differences influenced by species. One loopful of cells were inoculated into 40 ml sterilized YM broth and incubated for 48 hrs. at 25ºC under orbital shaking. After 48 hrs., 0.1 ml of yeast suspension was transferred to 350 ml of sterile YM broth and incubated under the same conditions for 24 hrs. (early stationary phase). Cells were harvested by centrifugation for 10 minutes at 8000 rpm at 4ºC and washed with deionized water, mixed, and centrifuged again. The cells were consequently washed and centrifuged two times in 1 mM KCl, mixed, and centrifuged. Surface charge of yeast cells was determined in a suspension of cells in 1 mM KCl with absorbance (A_{600nm}) of 0.1 to 0.2.

Determination of effects of culture age on zeta potential of yeast cells

The cell wall composition of microorganisms changes during various stages of growth. For instance, phospholipid or protein contents may increase or decrease due to age of the organism. According to Jay (2000), some cells become smaller as they age.

This suggests that a change in composition of cell wall material may occur during the various growth stages due to cell shrinkage. For this reason, zeta potential was measured at various growth stages using *S. cerevisiae* to determine if cell culture age impacts zeta potential.

In order to determine the growth cycle of *S. cerevisiae*, a growth curve was constructed using YM broth and plate counts were determined on YM agar after 48 hr incubation (25ºC). Zeta potential of yeast cells at various stages of growth was measured. Yeast cells were cultured as indicated above, except, once cells were transferred to 500 ml flask containing 350 ml of sterile YM broth, samples were collected over time, adjusted to appropriate absorbance ($A_{600nm,}$,0.1-0.2), and zeta potential was measured using the Zeta PALS instrument. Measurements of *S. cerevisiae* (KE 162) were taken at 12, 18, 24, 36, 48 and 72 hour culture ages; zeta potential, therefore, was determined in the log phase, and at the beginning and during the stationary phase.

Effect of pH on surface charge of *Saccharomyces cerevisiae*

Cell wall composition changes differently depending on a range of environmental circumstances. Organisms come into contact with unfavorable living conditions frequently, and they must adapt to these stressful environments. Such changes may be made possible due to the complexity of the cell structure. The exchange of nutrients and ions from the inside of the cell to the exterior all impact the overall composition of the cell. The environment plays a major role in microbial survival and the behavior of the organism upon exposure to antimicrobial agents such as chitosan. Yeasts are capable of developing acid resistance under acidic growth conditions, but how this affects the

surface charge of the cell is not known. To determine this, *S. cerevisiae* was briefly exposed to various pH, and zeta potential was measured.

To evaluate short term effects of environmental pH on yeast surface charge, pH of 1 mM KCl solution was adjusted with 0.1 N NaOH or 0.1 N HCl to obtain solutions with pH ranging from 3 to 11. Cells were grown and harvested as described previously, and 1 mM KCl of the appropriate pH was used for washing the cells and as a medium for cell suspension. This was done to determine whether cell charge would be affected by short term exposure to suspensions with different pH. Zeta potential was determined in cell suspensions adjusted to the appropriate absorbance (A_{600nm}, value 0.1-0.2).

Effect of pH on surface charge of pH adapted *Saccharomyces cerevisiae*

Cell wall composition changes differently depending on various environmental factors. For instance, the microbial adaptation process occurs over time by allowing the organism to develop key defense mechanisms. Organisms are able to adapt to stressful environments by initiating specific genes to activate specific resistance responses, such as defense responses to adverse pH and heat. These changes may be made possible due to the alteration of the components within the cell wall.

To evaluate prolonged effects of environmental pH on yeast surface charge, cells were adapted to various pH environments using 1 N NaOH and 1 N HCl (adapted to pH 3, 4, 8). To determine whether yeast surface charge is altered by extended culturing at non-optimal pH, cultures were adapted to pH after being inoculated into 100 ml of pH adjusted YM broth and transferred to sterile YM broth (with appropriate pH) every 3-5 days; pH adapted *S. cerevisiae* cells were maintained on pH adjusted YM agar slants at

4ºC. Cells were grown and harvested by centrifugation, as described, and 1 mM KCl of the appropriate pH was used for washing the cells and as a medium for cell suspension. Zeta potential was determined in cell suspension with appropriate absorbance.

Yeast cell flocculation and plate counts

Since flocculation of cells is known to be influenced by charge due to the components within the cell wall, flocculation was observed in this study. During the early stationary phase, cells were centrifuged (8000 rpm, 10 minutes, 4ºC), washed twice in 0.1 M sodium chloride solution (NaCl) (Fisher Scientific co., Fair Lawn, New Jersey), and resuspended in 0.1 M acetate buffer (pH 4.0) (Anhydrous sodium acetate, Sigma-Aldrich co., St. Louis, MO). A target absorbance of 3.0, corresponding to about 8 log cfu/ml was desired (A_{600nm}). Next, 9 ml of the yeast suspension, including cells and acetate buffer solution, was added to 1 ml of chitosan suspension (see below). The mixture was gently shaken for 10 minutes, and absorbance and plate count measurements were determined (0-8, 24 hrs). Flocculation of yeast cells during 24 hr incubation at 25ºC was measured as a decrease in absorbance at 600 nm using a UV-VIS spectrophotometer. Along with absorbance readings, YM agar plate counts (incubated at 25ºC) were performed to determine yeast viability and how they were impacted by various chitosan concentrations as compared to the control. Specifically, three yeast species (*S. cerevisiae*, *C. krusei*, and *Z. bailii*) were incubated in YM broth at 25ºC overnight with orbital agitation until the early stationary phase was reached. The cultures were subsequently serially diluted (with buffer as the blank) to about 4 log cfu/ml, and chitosan was added to the cell suspension to obtain various concentrations of chitosan.

Yeasts and buffer solution without chitosan served as the control. Samples were plated onto YM agar over time. Plates were incubated at 25ºC for 48 hrs., and plate counts were determined.

Chitosan stock solution

One percent medium molecular weight chitosan (Sigma-Aldrich, St. Louis, MO) in 1 % acetic acid was added to cell suspensions to achieve concentrations of 0.1, 1.0, and 10 ppm (0.00001, 0.0001, and 0.001 %) and volume was adjusted with acid solution. Cell suspensions with addition of the same volume of acetic acid, with no chitosan, served as the control.

Phase contrast microscopy

Phase contrast microscopy was utilized to observe changes in cell appearance and morphology of yeasts adapted to various pH environments and exposed to chitosan. *S. cerevisiae* cells were revived from YM agar slants, inoculated into 40 ml of sterile YM broth, incubated at 25ºC under agitation for 48 hr, and then transferred to YM broth for 24 hr and observed under phase contrast microscopy (Olympus Optical Co., New York, NY). *S. cerevisiae* pH adapted cells were adjusted to pH as described previously and observed under phase contrast at each pH and after chitosan was added.

Cell harvesting was done as previously mentioned, after 24 hr incubation and yeast cells were adjusted to an absorbance of 3.0 (A_{600nm}). Yeast and chitosan suspension was prepared as follows: 9 ml of yeast cells and 0.1 M acetate buffer solution were added

to 1 ml chitosan solution (0.001 %), and held for four hours. Next, 10 µl werecarefully removed from the sedimentation, placed into 1 mL of HPLC grade water, and held for 2 minutes. A loopful from the bottom area of the tube was applied to a microscope slide and observed under 100x magnification using phase contrast.

Data analysis

The statistical model consisted of a repeated measures and a completely randomized block design (CRD). Statistical analysis was conducted using the mixed model procedure (PROC MIXED) of SAS® 8.2 (Statistical Analysis Systems Institute, Cary, North Carolina) and significance factors set at $P>0.05$. Analysis of variance (ANOVA) was used to determine statistical differences in zeta potential as influenced by treatment conditions (species, age, and pH).

Chapter III

Results and Discussion

Surface charge of bovine serum albumin

BSA was utilized in this study as a standard. Few, if any, studies have been published regarding measuring zeta potential of yeast cells. However, several studies have demonstrated measurement of the surface charge of BSA. Therefore, this protein was used to ensure accurate zeta potential measurements before measuring surface charge of yeast cells. The BSA results from this study indicated that BSA has a negative charge at neutral pH, and a more negative charge at basic pH. In other words, the higher the pH, BSA is more negatively charged (Figure 1; all figures are displayed in the appendix). These results are confirmed by Vilker et al. (1980); in their study, as pH increased, the BSA surface charge became more negative. The isoelectric point (pI) is the pH at which the net surface charge or zeta potential is 0. The pI of BSA was determined in this study to be at pH 4.6 - 4.8, corresponding to pI data for BSA.

According to Kitano et al. (1998), results of their study indicated that the protein phase had an important role in the determination of the physiological zeta potential. We also believe that the constituents present in the yeast cell wall (i.e. protein and polysaccharides) will affect zeta potential measurements. We have developed a sufficient method for measuring the zeta potential of proteins, and the results are more consistent

and accurate than the results yielded by the more commonly used method involving the formation of a protein emulsion.

Yeast species zeta potential comparison

Yeast species play a significant role in cell wall properties such as surface charge and flocculation rates, even within species, there may be differences in the components in the cell that affect the properties mentioned. Therefore, yeast strain is important when investigating cellular properties and components that affect zeta potential or electrophoretic mobility of cells. Table 1 (see appendix) demonstrates how different genera and species posses different electrophoretic mobility measurements. For these reasons, three yeast species were utilized to determine zeta potential differences.

Zeta potential measurements were performed using *S. cerevisiae*, *C. krusei* and *Z. bailii* cells. Statistical analysis indicates that there are significant differences in zeta potential values depending on the yeast species utilized (4 replications). Results obtained reveal that the average zeta potential of *S. cerevisiae* cells was -19.6 mV, -12.07 mV for *C. krusei*, and -25.82 mV for *Z. bailii*. *C. krusei,* with the greatest (least negative) surface charge, was the least affected by the antimicrobial effects of chitosan. *Z. bailii* had the most negative charge, which may be the reason chitosan was effective against *Z. bailii*. This study demonstrates the possibility that chitosan acts more effectively as an antimicrobial agent against microbial cells with a more negative charge, since chitosan is a positively charged macromolecule (Table 2).

Effect of culture age

According to Jay (2000), older yeasts are typically smaller than young cells. This indicates that yeast physiological properties may be affected by culture age. As a consequence of yeast cell aging, they undergo constant modifications in morphology, gene expression, and physiology. The rate at which cells flocculate or sediment may vary depending on their age, and it has been shown that flocculation usually develops during the stationary growth phase (Powell et al., 2003).

Results obtained in this study demonstrate that *S. cerevisiae* cultured in YM broth at 25ºC reached the late log phase in 18 hr and the early stationary phase at 24 hr. The cell surface charge became slightly less negative as cultures aged. The difference was significant after 48 hr, as the cultures entered the later stationary phase (Figure 2). Data analysis revealed that there are significant differences in yeast surface charge due to culture age. Therefore, the effectiveness of antimicrobial agents such as chitosan, may be impacted by the phase of microbial growth.

Environmental conditions

Saccharomyces cerevisiae not adapted to adverse pH environments

Results indicate that *S. cerevisiae* surface charge was affected by pH (Figure 3). When microorganisms are placed in acidic environments, the cells must either keep hydrogen ions (H+) from entering or release H+ ions rapidly as they enter. This is important because cellular components such as ATP and DNA require neutrality (Jay, 2000).

This study demonstrates that there were differences in charge in pH adjusted *S. cerevisiae* cells. Below pH 6, significant differences in surface charge were observed at different pH. However, at pH 6 and above, no significant differences were observed.

Saccharomyces cerevisiae pH adapted cells

Surface charge of *S. cerevisiae* cells adapted to pH 3, 4, and 8 was measured (3 replications). For the first replication, results indicate a surface charge trend (more negative trend). However, during the first replication, cells were adapted to pH during a longer period than the last two replications; the cells harvested during the first replication were transferred (every 4 days) over a month-long period before the adapted cells were measured for zeta potential. For the second and third replications, pH adapted cells were transferred only twice over a week-long period and measurements were taken. This difference in adaptation time may account for the surface charge pattern difference observed between replications (Figure 4). Adaptation time thus affected surface charge of yeast cells. However, a trend is more prominent when cells are adapted to pH for longer exposure periods.

Transfer times affect pH adaptability, which was shown in this study. The longer the cells were allowed to adapt to their environment, they likely became more pH resistant due to the longer exposure. Adaptation of yeast cells may result in cell alterations in cell membrane composition (e.g., phospholipids), which could affect the surface charge due to the adaptability of cells and their ability to adapt to the pH of the environment. Factors such as transfer times, acidulant utilized, and temperature all impact the adaptation process of microorganisms and determine their successfulness at surviving stressful environments (Hohmann & Mager 2003).

Results indicate that pH adapted *S. cerevisiae* cells show differences in zeta potential values among the pH levels evaluated. However, pH 3 and 4 adapted cells showed no differences in surface charge, while the zeta potential for cells adapted to pH 8 was significantly different from the other adapted cells (Figure 4).

Yeast flocculation

Yeast flocculation provides a natural mechanism for yeast removal in fermentation processes. Flocculation is mediated by the properties of the cell wall, which ultimately are genetically determined and influenced by environmental factors (Eddy, 1955; Miki et al., 1982; Powell et al., 2003). Recording flocculation rates of yeast cells allows the ability to determine if chitosan induces the rate and magnitude of flocculation. If so, chitosan may possess binding and inhibitory capabilities.

Flocculation kinetics of *S. cerevisiae*, *C. krusei*, and *Z. bailii* in medium molecular weight chitosan solutions were performed to determine flocculation patterns of the yeasts and to assist in determining the binding and clarifying properties of chitosan. Chitosan was observed at low concentrations due to the increase in viscosity when higher concentrations were used which caused a delay in flocculation.

Flocculation can be easily quantified by observing the decrease in turbidity or optical density (A_{600}) of an undisturbed yeast cell suspension in acetate buffer overtime. According to the flocculation results (~8 log cfu/ml), the highest rate of flocculation of *S. cerevisiae* and *Z. bailii* cells was achieved with 0.001 % medium molecular weight chitosan (Figures 5, 7). However, this was not the case for *C. krusei* cells. This may be due to the morphology of *C. krusei,* which forms mycelia that could reduce flocculation.

After 2 hr incubation, absorbance (A_{600}) of *S. cerevisiae* suspensions decreased by 3, 6, 14, and 56% for control, 0.00001, 0.0001, and 0.001% chitosan, respectively. After 8 hr incubation, absorbance of *S. cerevisiae* suspensions decreased by 93, 92, 84, and 89% for control, 0.00001, 0.0001, and 0.001% chitosan, respectively (Figure 5).

Absorbance of *C. krusei* suspensions decreased by 0, 0, 1, and 1% for control, 0.00001, 0.0001, and 0.001% chitosan, respectively, after 2 hr incubation, and by 95, 95, 96, and 56% respectively, after 8 hr. Delay in flocculation with the highest tested concentration of chitosan and *C. krusei* might have been due to stability of the suspension due to the presence of mycelia (Figure 6).

After 2 hr incubation, absorbance of *Z. bailii* suspensions decreased by 23, 38, 25, 80% for control, 0.00001, 0.0001, and 0.001% chitosan, respectively, and absorbance decreased by 98, 96, 97, and 94% for control, 0.00001, 0.0001, and 0.001% chitosan, respectively, after 8 hr incubation (Figure 7). While there were flocculation pattern changes of the yeast cells when chitosan was applied at higher concentrations (0.001%), at lower concentrations of chitosan, absorbance did not change significantly.

The cationic nature of chitosan makes it significantly valuable for use as a flocculation agent of negatively charged particles, such as yeasts and other microorganisms. Adsorption and flocculation caused by cationic biopolymers have been intensely studied. However, there are a few theories regarding the mechanism of polymer induced aggregation or flocculation. One theory is by way of charge neutralization, another is flocculation caused predominately by bridging, a third suggested mechanism is patch flocculation due to attraction between oppositely charged particles covered with absorbed polymer, and the last theory suggests that flocculation is

highly dependent on the relative rates of polymer adsorption, polymer chain rearrangements, and particle collision, which are affected by mixing conditions and concentration of particles (Strand et al., 2001).

Plate counts

The first set of plate counts (YM agar, 25 ºC) were done using high inocula (~8 log cfu/ml) of *S. cerevisiae, C. krusei and Z. bailii* in chitosan suspensions (control, 0.00001, 0.0001, 0.001 %). Corresponding absorbance for the high inoculum was therefore determined to be 3.0 (A_{600}). Chitosan did not cause a reduction in cell numbers during incubation (Figure 8). These results are similar to those corresponding to Hoon et al. (2001) who reported that chitosan can be used as a food preservative to inhibit growth of spoilage organisms in mayonnaise.

Next, plate counts were performed using a low inoculum (~4 log cfu/ml) of yeast cells in chitosan suspensions (Figure 9). With low inocula, yeast counts decreased slightly over time, suggesting that inactivation may be influenced by the ability of chitosan to bind to cells. As such, high cell numbers may serve to "quench" chitosan at the concentrations tested.

Yeast cell observation

A possible mechanism of the antimicrobial activity of chitosan is its interaction with the microbial cell wall, causing disruption of normal nutrient transfer. For this reason, yeast cells were observed microscopically to identify microbial changes in cell

appearance and morphology when exposed to various environmental conditions such as pH change and chitosan.

Yeast cells were observed under phase contrast microscopy. *S. cerevisiae* pH adapted cells, in particular cells adapted to pH 3, manifested changes in morphology and physical appearance compared to control cells. For pH 3 adapted cells, the interior structures visible under phase contrast seemed deformed and less structured compared to control cells (Illustration 1, 2; all illustrations are in the appendix). The pH adapted cells changed their morphology, that is, they became more rounded and less elliptical. An interaction between hydrogen ions and enzymes in the cytoplasmic membrane occurs when microorganisms are adapted to adverse pH ranges. Therefore, the morphology of some organisms may be affected by pH (Jay, 2000).

Exposing *S. cerevisiae* to 0.001% chitosan suspension resulted in an in increase in cell aggregation or clumping (Illustration 5). Only the 0.001% chitosan suspension was evaluated since it was the most effective concentration, as determined from our studies. *S. cerevisiae* cells in chitosan suspension appeared less dense, as indicated by having a darker appearance (i.e., more light transmitted), and cells were more rounded than control cells. The clumping or clustering of cells caused by the application of chitosan may indicate a change in the surface charge of yeast cells.

Chapter IV

Conclusion

Food spoilage is an economic concern in the United States. Because of this, various additives (such as benzoic and sorbic acids) are used to prevent or control microbial growth in foods. Many additives have become less effective against some spoilage organisms that have adapted and developed a resistance to additives. Alternative yeast and bacterial inhibitors, such as the natural biopolymer, chitosan, should be investigated to compensate for these resistance issues.

Results of this study demonstrate that when microorganisms are adapted to non-optimal environmental pH, their surface charge can be affected. The length of the adaptation time used affected surface charge of yeast cells, with longer exposure to environmental pH, leading to greater changes in surface charge.

There are many factors that affect flocculation rates of microbial cells. These include species, strain, surface charge, pH, and nutrient limitation. Surface charge appears to be a major determinant in yeast flocculation. It has been suggested that age, environmental pH, species, yeast structure, and flocculation patterns all influence zeta potential of fungal cells. Our results confirm that yeasts exhibit negatively charged surfaces. This demonstrates that microbial surface charge is dependent on species and strain and environmental factors such as culture age and pH. Environmental conditions such as a

pH or the application of chitosan to yeast cell suspensions resulted in changes in yeast morphology and cell flocculation rates.

These changes are suggestive that physiological changes also occurred, thereby resulting in a change in surface charge. This study is important in order to determine favorable conditions for chitosan to be approved as a natural food antimicrobial or clarifying agent by the Food and Drug Administration.

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Appendix

Table 1. Electrophoretic mobilities of some yeasts, Gram-postive, and Gram-negative bacteria.

Microorganism	Electrophoretic Mobility (units)	Reference
Saccharomyces cerevisiae	-1.2 ($\mu m\ sec^{-1}v^{-1}cm^{-1}$)	Collins and Stotzky (1992)
C.andida albicans	-3.1 ($\mu m\ sec^{-1}v^{-1}cm^{-1}$)	Collins and Stotzky (1992)
Listeria innocua	-3×10^{-4} (cm^2/Vs)	Chang et al. (2002)
Escherichia coli O157:H7	-0.1 ($\mu m\ cm\ V^{-1}\ s^{-1}$)	Lytle et al. (1999)
Pseudomonas aeruginosa	-2.8 ($\mu m\ sec^{-1}v^{-1}cm^{-1}$)	Collins and Stotzky (1992)

Table 2. Variation in zeta potential among *Saccharomyces cerevisiae, Candida krusei* and *Zygosaccharomyces baili* (n=4).

Yeast Species	Mean	Standard Deviation	Standard Error
Saccharomyces cerevisiae	-19.57	3.11	0.31
Candida krusei	-12.07	5.58	0.62
Zygosaccharomyces bailii	-25.82	9.48	1.06

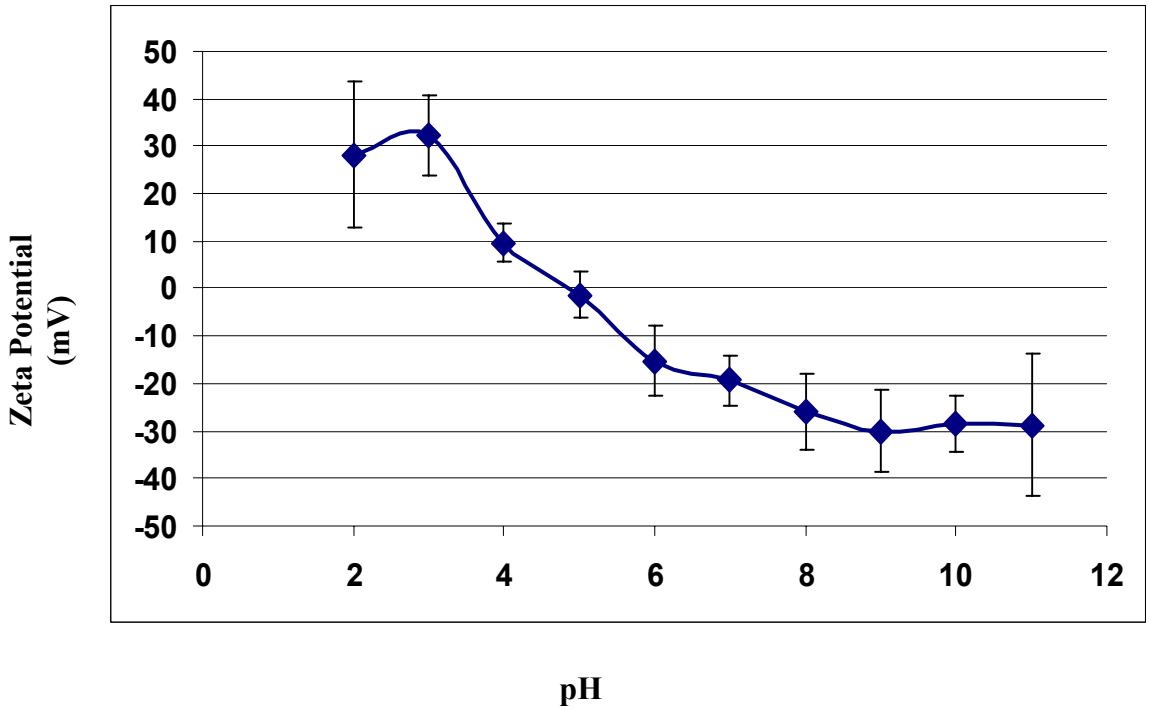


Figure 1. Zeta potential of 5% BSA solutions adjusted to pH 2-11.

Isoelectric point was determined to be at pH 4.7.

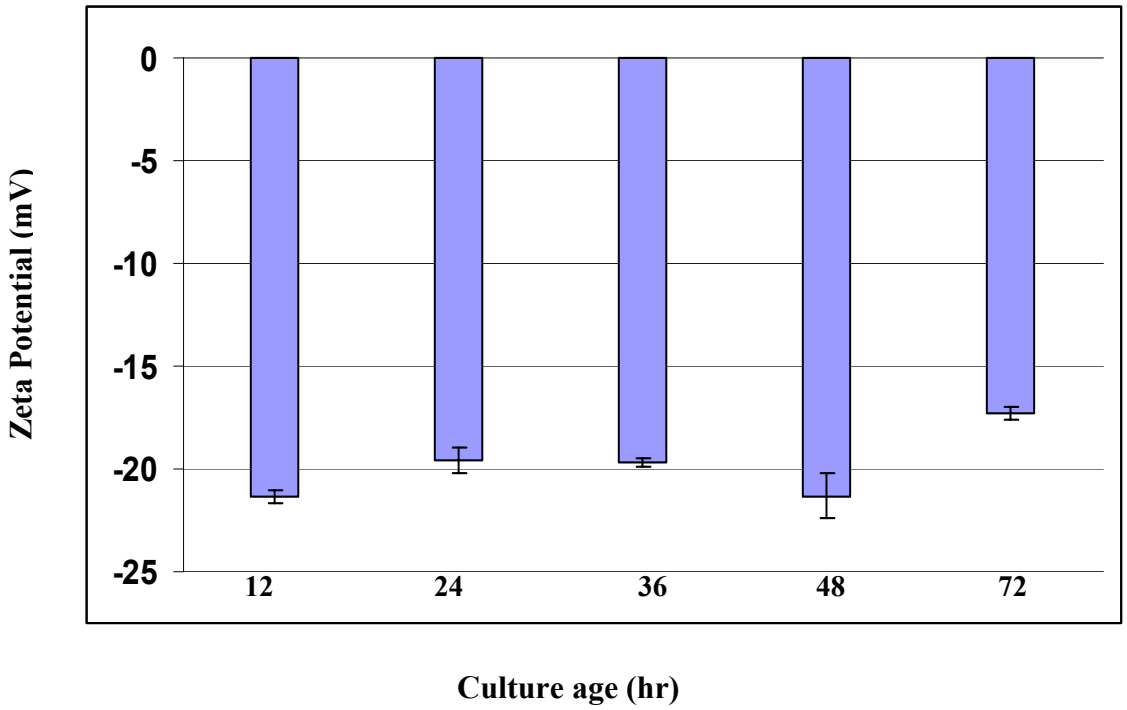


Figure 2. Effect of culture age on zeta potential of *S. cerevisiae* (n=5).

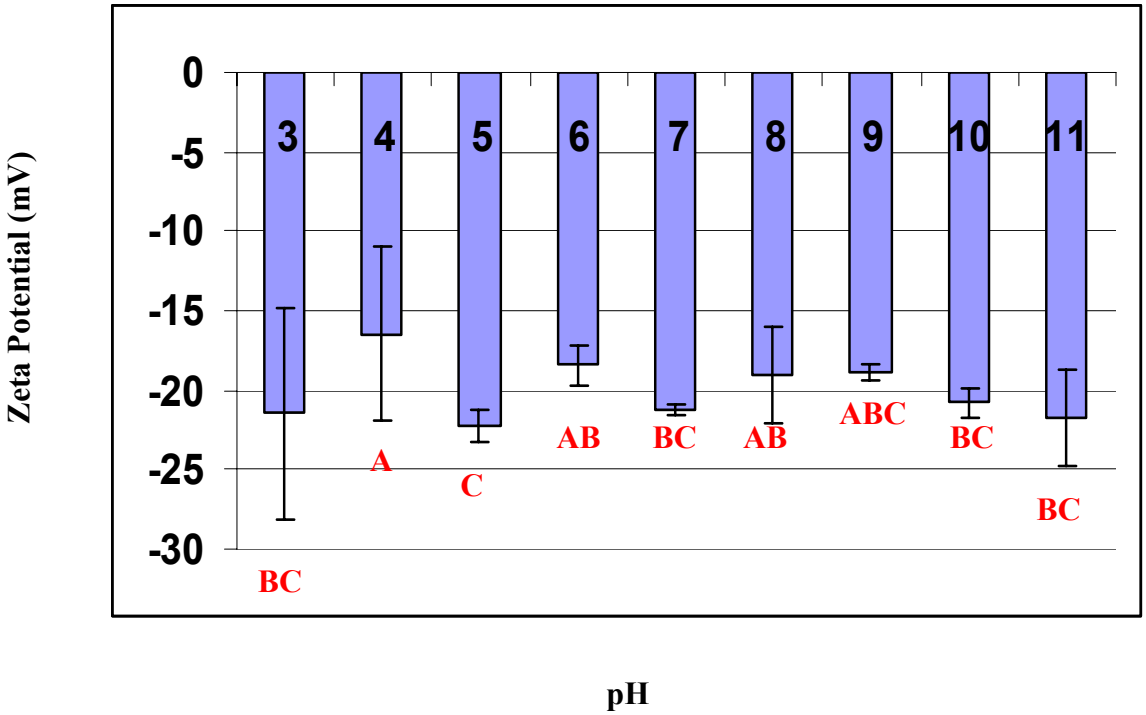


Figure 3. Effect of buffer pH on zeta potential of *S. cerevisiae*.

Significant differences represented by different letters ($p<.05$) ($n=5$).

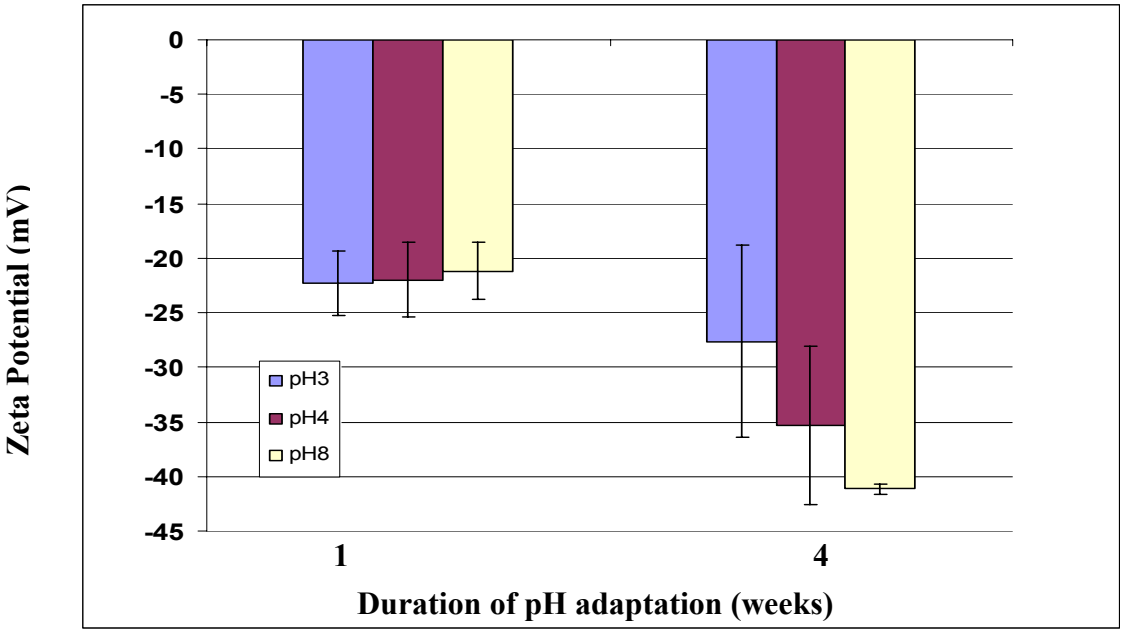


Figure 4. Effect of pH adaptation and duration of adaptation on the zeta potential of *S. cerevisiae.*

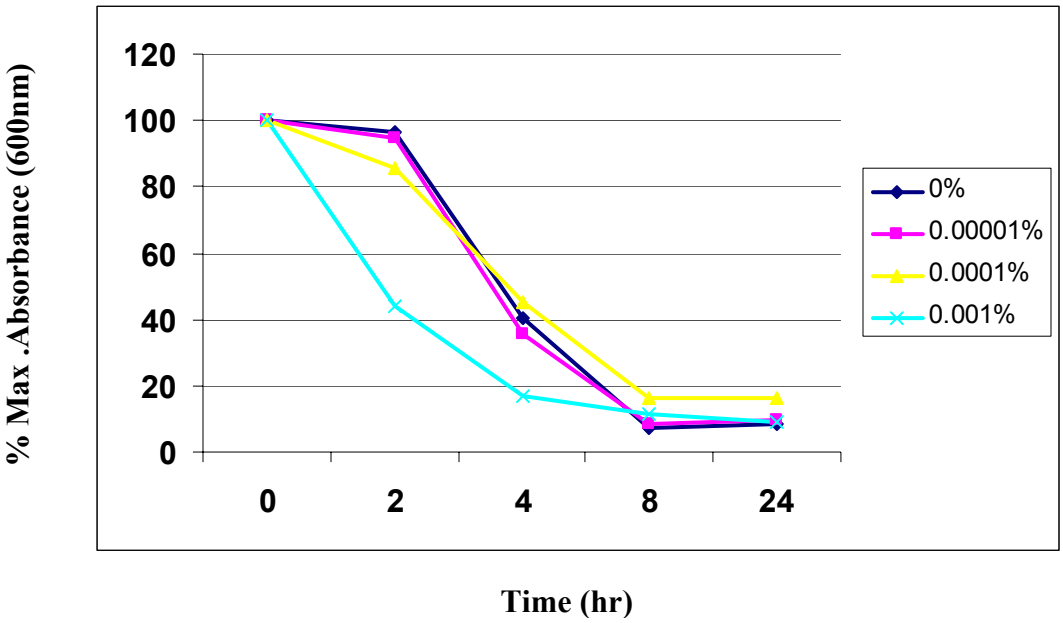


Figure 5. Effect of chitosan concentration in acetate buffer (pH 4) on flocculation of *S. cerevisiae.*

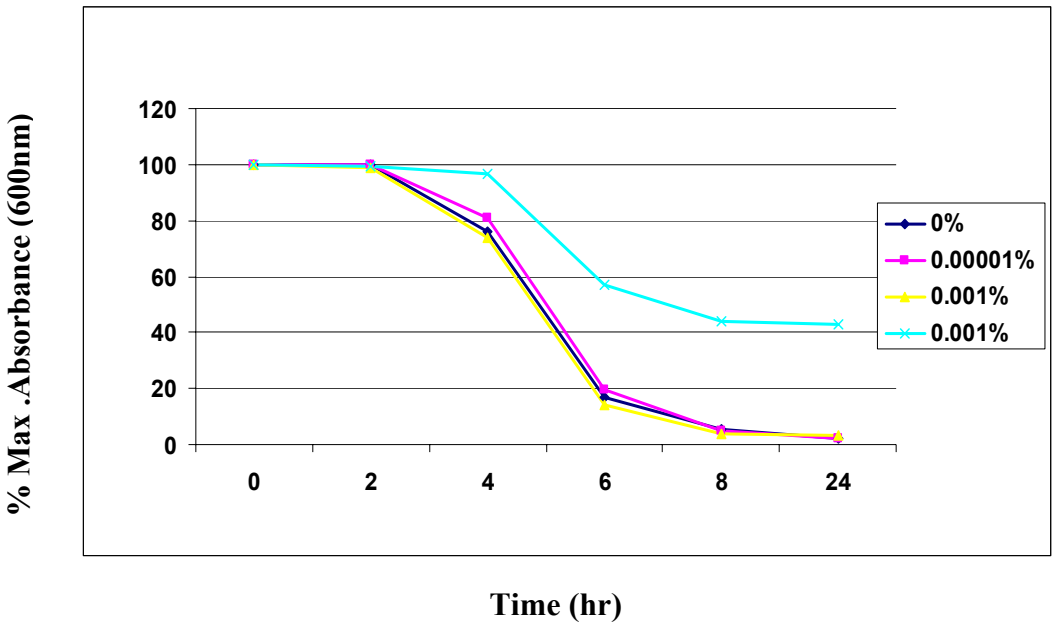


Figure 6. Effect of chitosan concentration in acetate buffer (pH 4) on flocculation of *Candida krusei*.

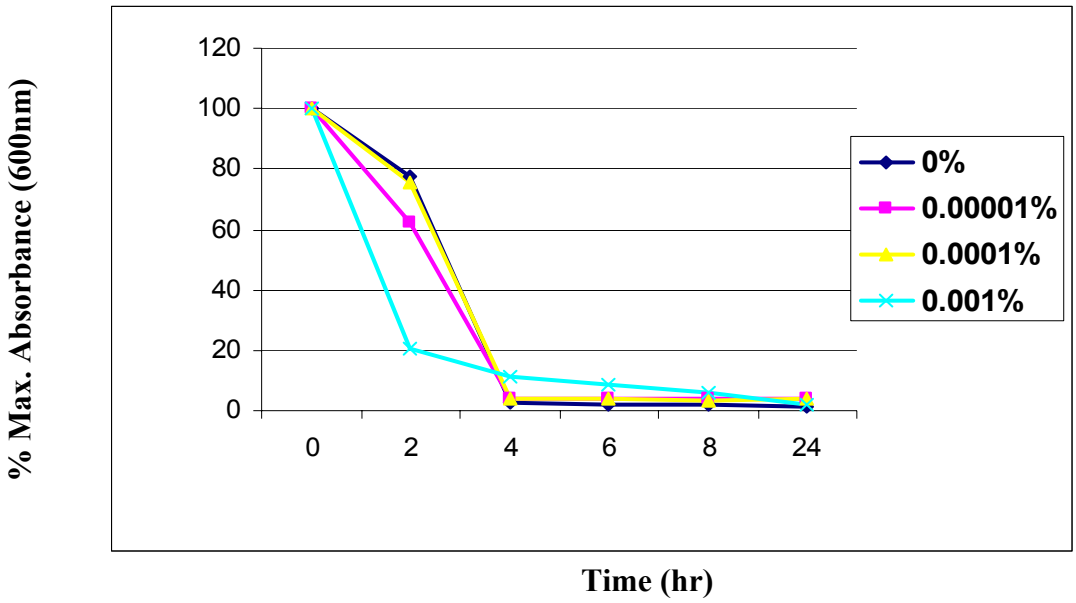
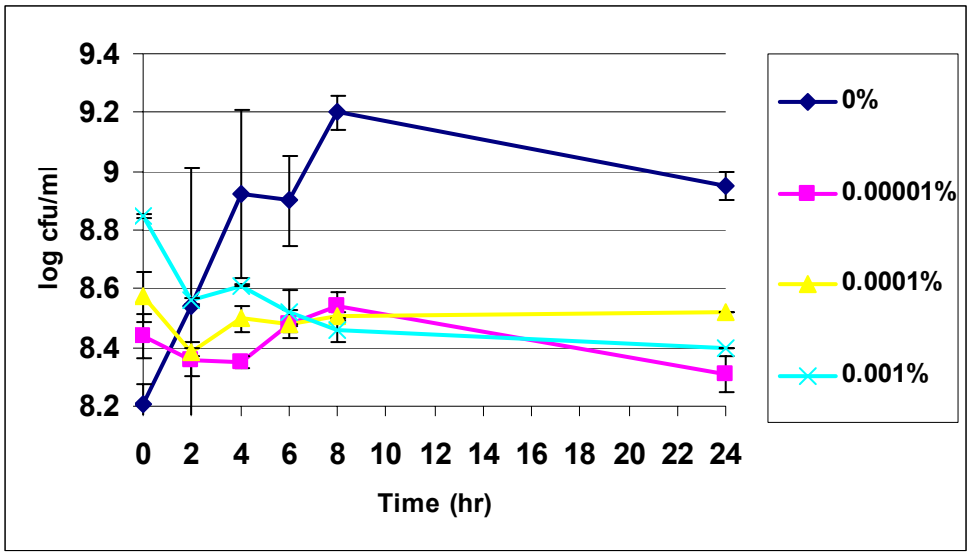
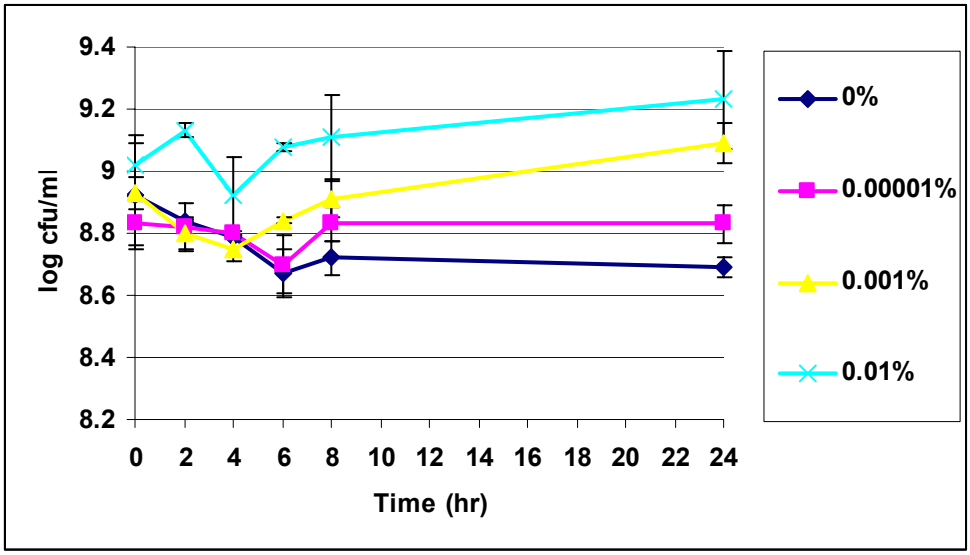


Figure 7. Effect of chitosan concentration in acetate buffer (pH 4) on flocculation of *Zygosaccharomyces bailii.*

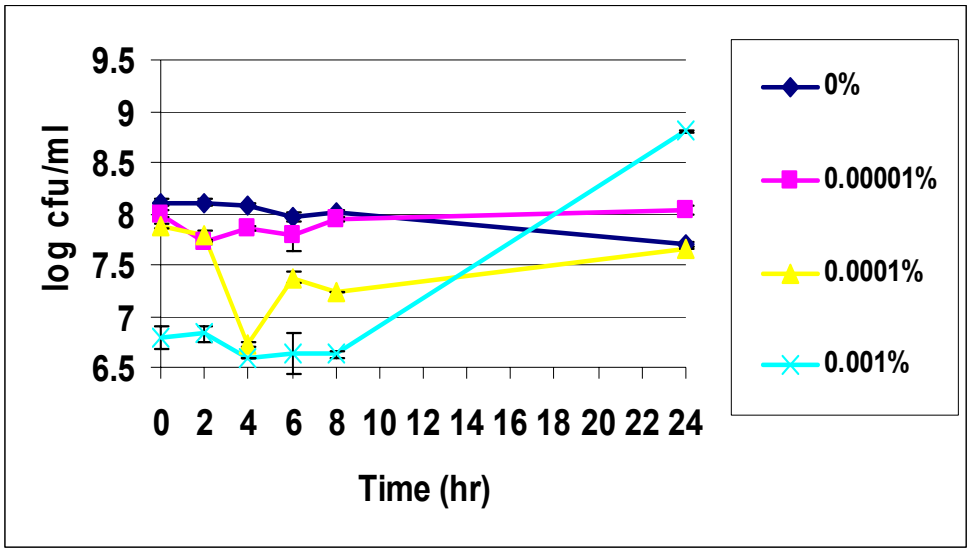


A.



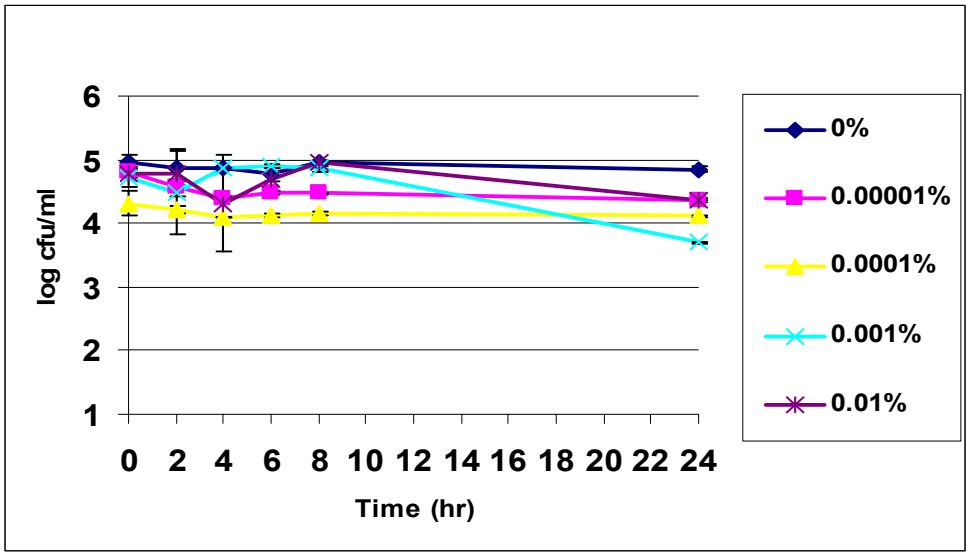
B.

Figure 8. Effect of chitosan concentration in acetate buffer (pH 4) on survival of *Saccharomyces cerevisiae* (A), *Candida krusei* (B), and *Zygosaccharomyces bailii* (C) at high inoculum populations.

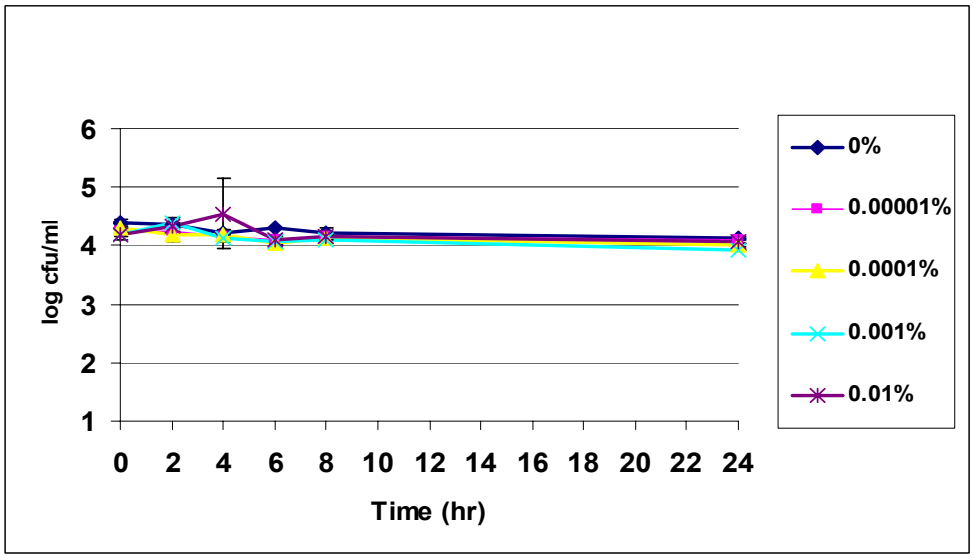


C.

Figure 8. Continued.

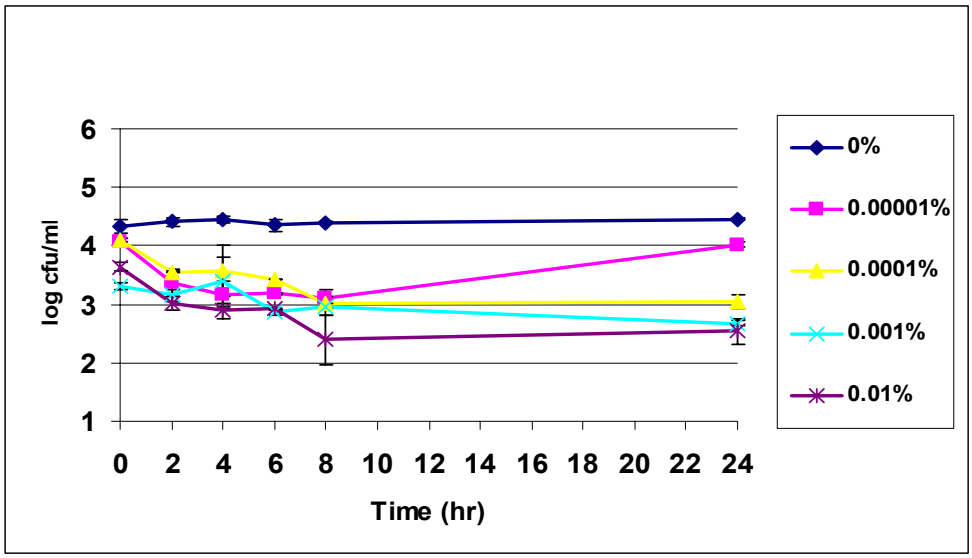


A.



B.

Figure 9. Effect of chitosan concentration in acetate buffer (pH 4) on survival of *Saccharomyces cerevisiae* (A), *Candida krusei* (B), and *Zygosaccharomyces bailii* (C) at low inoculum populations.



C.

Figure 9. Continued.

Illustrations

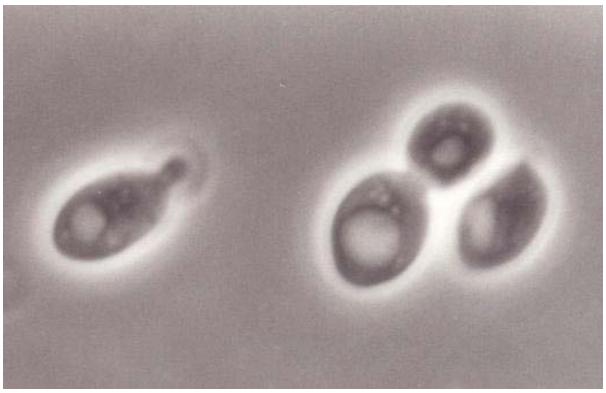


Illustration 1. Phase contrast micrograph (100x) of *Saccharomyces cerevisiae* control cells grown in YM broth (pH 6.8) and harvested during the stationary growth phase.

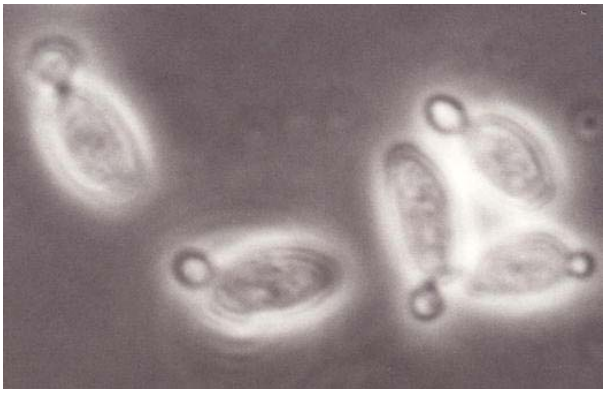


Illustration 2. Phase contrast micrograph (100x) of *Saccharomyces cerevisiae* adapted to pH 3 in YM broth and harvested during the stationary growth phase.

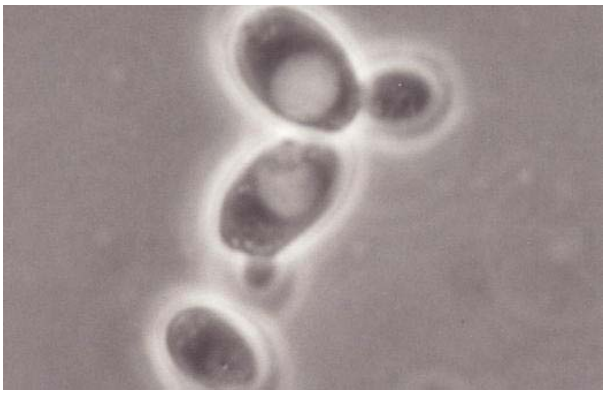


Illustration 3. Phase contrast micrograph (100x) of *Saccharomyces cerevisiae* adapted to pH 4 in YM broth and harvested during the stationary growth phase.

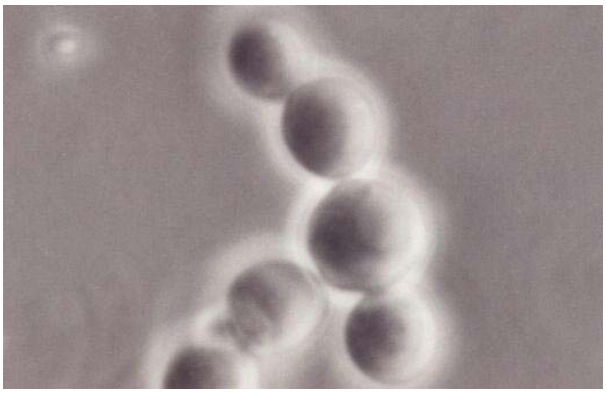


Illustration 4. Phase contrast micrograph (100x) of *Saccharomyces cerevisiae* adapted to pH 8 in YM broth and harvested during the stationary growth phase.

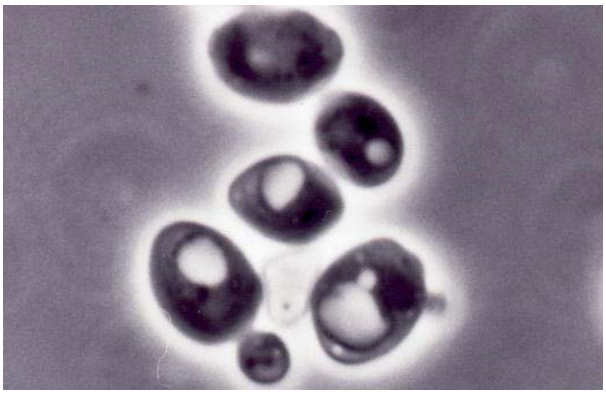


Illustration 5. Phase contrast micrograph (100x) of *Saccharomyces cerevisiae* control cells grown in YM broth (pH 6.8), harvested during stationary phase, and then exposed to 0.001% chitosan for 10 minutes.

SAS Programming: Impact of yeast species on zeta potential

```
proc mixed data=species;
class species measure;
model surf_chrg=species|measure/ outp=rrr ddfm=kr;
repeated measure;
lsmeans species/pdiff;
title 'Repeated Measures ANOVA: species and surface charge';
run;


proc univariate data=rrr normal;
var resid;
title2 'Test of residual normality';
run;


proc means mean std stderr maxdec=2 data=species;
class species;
var surf_chrg;
types species;
title 'Descriptive Stats';
run;
```

SAS Programming: Impact of culture age on the zeta potential

```
proc mixed data=age;
class hour measure;
model surf_chrg=hour|measure/ outp=rrr ddfm=kr;
repeated measure;
lsmeans hour/pdiff;
title 'Repeated Measures ANOVA: culture age and surface
charge';
run;


proc univariate data=rrr normal;
var resid;
title2 'Test of residual normality';
run;
```

```
proc means mean std stderr maxdec=2 data=age;
class hour measure;
var surf_chrg;
types hour measure;
title 'Descriptive Stats';
run;
```

SAS Programming: Impact of adjusted pH on the zeta potential

```
proc mixed data=adjusted_ph;
class ph measure;
model surf_chrg=ph |measure/ outp=rrr ddfm=kr;
repeated measure;
lsmeans ph/pdiff;
title 'Repeated Measures ANOVA: adjusted ph  and surface
charge';
run;


proc univariate data=rrr normal;
var resid;
title2 'Test of residual normality';
run;


proc means mean std stderr maxdec=2 data=adjusted_ph;
class ph;
var surf_chrg;
types ph;
title 'Descriptive Stats';
run;
```

SAS Programming: Impact of adapted pH on the zeta potential

```
proc mixed data=adapted_ph;
class tperiod ph measure rep;
model surf_chrg=tperiod|ph|measure/ outp=rrr;
random rep(ph tperiod measure);
lsmeans tperiod*ph/pdiff;
title 'Repeated Measures ANOVA: adapted ph and surface
charge';
run;
```

```
proc univariate data=rrr normal;
var resid;
title2 'Test of residual normality';
run;


proc means mean std stderr maxdec=2 data=adapted_ph;
class tperiod ph;
var surf_chrg;
types tperiod ph tperiod*ph;
title 'Descriptive Stats';
run;
```

SAS Programming: Impact of chitosan on cell viability

```
proc mixed data=use;
where rep=2;
class species_num concent;* rep;
model platecntlog_=species_num|concent/outp=rrr;
*repeated time;
lsmeans species_num*concent/pdiff;
title 'Repeated Measures ANOVA: platecntlog';
run;


proc univariate data=rrr normal;
var resid;
run;


proc means data=use mean std stderr maxdec=2;
class species concent;
var platecntlog_;
types species concent species*concent;
run;


proc means data=use mean std stderr maxdec=2;
class species concent time;
var abs;
types species*concent*time;
run;
```

VITA

LaQuita M. Michael was born in Knoxville, Tennessee on August 26, 1980 to parents Joseph and Betty Michael Jr. LaQuita was raised in Knoxville with her older sibling, Charles Michael, and graduated from Fulton High School in 1998. She then attended The University of Tennessee, were she received the Bachelor of Science degree in Agriculture in 2003. Ms. Michael majored in Food Science and Technology and minored in Business. In August 2003, she began the M.S. program in Food Science and Technology, specializing in Food Microbiology, and graduated from this program in August 2005.

During her college years, LaQuita was actively involved in the Food Science Club, Minorities in Agriculture and Natural Resources and Related Sciences (MANRRS), and The Ronald McNair Post Baccalaureate Program at the University of Tennessee.