PRESERVING BIOLOGICAL MATERIALS IN PROTECTIVE POLYMERS

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PRESERVING BIOLOGICAL MATERIALS IN PROTECTIVE POLYMERS

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PRESERVING BIOLOGICAL MATERIALS IN PROTECTIVE POLYMERS

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April Anne Krumnow, daughter of James Stickler and Regina Heisler was born April 11, 1974, in Salem, Indiana. She graduated from Columbia High School in Columbia, Pennsylvania in 1992. She graduated summa cum laude from Jefferson Community College in Watertown, NY in May, 2000 earning an Associate in Science. She then, transferred to Clarkson University in Potsdam, NY and graduated summa cum laude with a Bachelor of Science degree in Bio-Molecular Science in December, 2001. She entered Graduate School at Auburn University in May 2002. She married Kevin Lynn Krumnow, son of Kenneth Krumnow and Donna Kelly on January 18, 1997 in Waimea, HI. She gave birth to Amanda Irene Krumnow on March 4, 1993.

DISSERTATION ABSTRACT

PRESERVING BIOLOGICAL MATERIALS IN PROTECTIVE POLYMERS

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Acacia gum (AG) is a water soluble natural polymer that has many desirable properties for a vast range of applications including those which are used in pharmaceutical, textile and food industries. In this study, a novel hypothesis utilizing AG as a protectant for biological materials during desiccation and storage was tested. Preserving biological materials in AG may be applied to large scale applications, for example, preservation of bacteria for crop protection and bioremediation. In addition, forensic and pathological samples of unknown content may be preserved in AG. The goal of this project was to develop a simple process and formulas for preserving biological materials in AG without applying specialized equipment and refrigeration. Biologicals tested were *Escherichia coli*, *Bacillus subtilis*, single-stranded DNA and double stranded dsDNA.

There were two types of experiments performed for all biologicals: (1) storage at various temperatures and low humidity and (2) storage at various humidity and ambient temperature. The level of protective effect on preserving biological materials in AG was object-specific. *E. coli* dried in AG was less sensitive to degradation compared to controls under all storage conditions. *E. coli* maintain viability up to day 128 when stored at 5 and 15°C and up to day 64 when stored at 25 and 40°C. For humidity experiments, *E. coli* remained viable up to day 32 for humidity levels of 46, 76 and 86%. For *B. subtilis* cultures, the viability remained constant for all storage conditions up to the maximum time tested for both experiments: 615 days for temperature experiments and 128 days for humidity experiments. It was determined that AG provides an adequate environment for *B. subtilis* spore formation. Electron micrographs of *E. coli* cells in AG had distorted morphologies and invaginations in cell walls and membranes compared to control. Electron micrographs of *B. subtilis* illustrated spore formation in AG samples but not in control samples. ssDNA was protected in AG when stored at low temperatures and humidity. On day 118 of the long-term temperature experiment, PCR were performed on ssDNA samples preserved in AG which were stored at all experimental temperatures. The PCR products obtained from one set of primers were of the same size as ssDNA control stored at -20°C. The DNA sequences of experimental PCR products were identical to control. dsDNA samples were successfully protected in AG stored at 5, 15, 25 and 40° C up to 64 days. For humidity experiments, more dsDNA was recovered from AG samples stored at low humidity (46 and 53%) up to 64 days. Examples of potential applications for preserving biologicals in AG include pesticides, bacterial preparations for bioremediation, oral vaccines, and probiotics.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Preservation of biological samples gathered from various environments is of great importance in many fields of science. For example, such samples might be needed in effort to fight bioterrism, and can include, among other biological materials, different types of microorganisms collected by air filtration at a crime scene. Current methods for preserving forensic or pathological samples have limitations such as requirements for immediate refrigeration or complete drying. The scale and numerous applications that involve dried bacterial and DNA preparations in industry, agriculture and medicine determine the significance of this dissertation.

This work concentrates on designing and developing a method for preserving biological materials while protecting structural integrity and vitality during desiccation and storage under various environmental conditions using acacia gum (AG) polymer. This method has potential for protecting many genera of bacteria and forms of DNA from adverse environmental conditions for an extended period of time without refrigeration. The preservation design involves a single compound and a simple procedure. The developed process of immobilization in the protective polymer does not include any

additives, accelerators, or plastifiers, nor does it involve elevated temperatures or radiation to promote polymerization. The polymerization and replacement of water occur spontaneously in a single process. The developed method is reversible, and the polymer can be safely removed from bacteria and DNA.

Properties of the proposed system include: (1) compatibility with many biological molecules, including structural, enzymatic, and immunological proteins, various types of DNA, and microorganisms, (2) long-term preservation, and (3) recovery of the preserved materials via solubilization in water. The process is a simple, rapid, one step procedure. Hardening of the polymer is performed under simple conditions (no temperature above 40°C, no chemicals or radiation). The polymer is non-toxic and environmentally friendly, mechanically sound, and economically sound. The polymer can appear in a desirable form and shape including liquids, films, tablets, granules, powder, etc.

The main goal of this work was to develop a simple process using acacia gum (AG) polymer as a protective agent for biologicals during desiccation and storage. The two main objectives are described as follows:

I. Develop a simple process for preserving biological materials (*Escherichia coli*, *Bacillus subtilis,* ssDNA, and dsDNA) in AG without using specialized equipment or complex formulations.
    1) Determine optimal conditions for long-term storage.
    2) Test viability and integrity of biologicals during the course of long-term preservation at various temperatures and at various humidity.

II. Evaluate biologicals stored in AG for alterations in structures.

1) For bacterial samples, the ultrastructure and cell membrane intactness was evaluated using electron microscopy (EM) and fluorescent microscopy (FM).
2) For DNA samples, DNA integrity and the ability to amplify and sequence the DNA was evaluated via quantitative gel electrophoresis, PCR, and DNA sequencing.

In the first chapter of this dissertation, current methods of preservation and protective agents for preserving biologicals are reviewed. The first chapter also describes characteristics and applications of AG (experimental) and pullulan (control) polymers. Additionally, this chapter briefly discusses the biologicals used in the study.

In the second and third chapters of this dissertation, *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*) preservation experiments are described, respectively. These experiments include optimizing AG concentration, determining optimal container for samples, and drying method. Additionally, experiments testing viability of bacteria after long-term storage in AG under various temperatures and humidity are described.

The forth chapter of this dissertation describes experiments performed to evaluate the ultrastructure and cell membrane intactness of bacterial cells preserved in AG. Structural characteristics in bacterial cells are determined before and after drying and during storage in AG using electron and fluorescent microscopy.

The fifth chapter of this dissertation describes experiments to determine the optimal AG concentration and method of drying for preserving ssDNA and dsDNA. This chapter also includes results from long-term preservation of both types of DNA stored at various temperatures and humidity. The conclusions are presented in Chapter 6.

1.2 Literature review

1.2.1 Polymers

1.2.1.1 Acacia gum polymer

Acacia gum (AG, also named gum arabic) is an exudate collected from *Acacia senegal* stems and branches (Joseleau and Ullmann, 1990; Baldwin et al., 1999a). Most of the AG (90 %) collected for commercial use comes from Sudan, Africa (Joseleau and Ullmann, 1990). This natural heterogeneous hydrocolloid is a mixture of different molecular weight polysaccharides including arabino-galactan protein (2.3 x 106 g mol-1) and arabino-galactan (2.7 x 105 g mol-1) (Baldwin et al., 1999a; Sanchez et al., 2002a). These polysaccharides are composed of a galactose backbone with arabinose, ramnose and glucuronic acid side chains (Sanchez et al., 2002a). The percentages of these components are as follows: D-galactose (~40%), L-arabinose (~24%) L-ramnose (~13%), D-glucuronic acid (~21%), 4-O-methyl-D-glucuronic acid (~2%), and polypeptide (~2%) (Michel et al., 1998; Mocak et al., 1998). AG also consists of a small percentage of glycoproteins. AG readily dissolves in cold and hot water in concentrations up to 50%

(JECFA, 1998). The solutions are characterized by a low viscosity and pH range 4.5-5.5. This polymer is indigestible to both humans and animals, not degraded in the intestine, but fermented in the colon by microflora (Michel et al., 1998). AG polymer is biodegradable and has no ecological impairments.

AG has been used for thousands of years. In Ancient Egypt, it was used as a pigment binder and adhesive in paints for making hieroglyphs, binder in cosmetics and inks, and for adhering flaxen wrappings for embalming mummies (Sanchez et al., 2002b). In current times, AG is used extensively in food industry as an emulsifier, flavoring agent/adjuvant, formulation aid, stabilizer/thickener, humectant, and surface-finishing agent (Baldwin et al., 1999b). In the pharmaceutical industry, AG functions as a suspending agent, emulsifier, adhesive, and binder in tabletting and in demulcent syrups (Sanchez et al., 2002b). In cosmetics, AG acts as a stabilizer in lotions and protective creams, adhesive agent in blusher; and foam stabilizer in liquid soaps (Sanchez et al., 2002b). AG is utilized in lithography, textiles and painting as dispersant in paints and insecticidal emulsions (Joseleau and Ullmann, 1990; Baldwin et al., 1999a; Sanchez et al., 2002a; Sanchez et al., 2002b).

Another application involves delivering microencapsulated probiotic bacteria to the gastro-intestinal tract. It has been reported that AG exhibits prebiotic effects when added to human intestinal microflora, *in vitro*; whereby, it decreased the levels of toxin forming bacterium *Clostridium* sp., and promoted probiotic bacteria *Lactobacillus* sp. (Michel et al., 1998). Thus, AG could have a dual role in delivering and maintaining normal intestinal microflora. Another application for encapsulation of bacteria in AG could be adapted for delivering effective doses of attenuated live microbial vaccinations

for mucosal and systemic immunity (Aldwell et al., 2003). Additionally, the presence of the acacia gum in vaccine preparations could work as an adjuvant because of the repeated polysaccharide moieties and protein content (Baldwin et al., 1999a). A study using an oral vaccine of polyacryl starch microparticles conjugated to diphtheria toxin reports significant mucosal and systemic immunity (Rydell and Sjoholm, 2004).

AG preservation is an attractive technique for industrial applications. For example, AG preservation process could improve current methods for storage, transportation, and application of bacterial pesticides. In addition, the process to produce such large volumes of bacterial pesticide and transportation are costly (Navon, 2000). Bacterial pesticides generally require water during application in order to adhere to plants. On the other hand, bacteria preserved in AG may not require water upon application, could adhere to desired plant with minimal moisture provided by the plant, and the cost for preparing, transporting, and applying bacteria pesticides could be reduced. This preservation method could also be applied to bioremediation of heavy metals, various hydrocarbons, and radionuclide contaminants, etc. Because AG polymer is biodegradable and ecologically safe, it can be applied to delivering vast amounts of fastidious microbes to areas of contamination.

AG preservation could be used for preserving lactic acid bacteria (LAB) (Desmond et al., 2002) for food production. Preserved LAB under current methods are not directly inoculated into cheese vats due to decrease cell viability and function after preservation (Broadbent and Lin, 1999). Preserving LAB in AG could prove to be beneficial in obtaining concentrated viable and active bacteria for direct inoculation in food production. One more application to consider is collecting samples in the field. It

has been shown in our preliminary studies that AG is capable of preserving bacteria in stringent conditions such as high temperature and humidity without refrigeration. Thus, collecting biological samples (i.e. forensic, pathological specimens) at remote locations using AG may be applied to preserving bacteria for further testing in a laboratory.

1.2.1.2 Pullulan polymer

Pullulan was used as a control in AG preservation experiments because it is a natural water soluble polymer with similar uses in industry to that of AG. Pullulan is an extracellular polymer synthesized from starch by *Aureobasidium pullulans* (fungus). It is a linear homo-polysaccharide of glucose composed of alpha-(1-6) linked polymer of maltotriose subunits. Pullulan is edible, biodegradable, water-soluble, non-toxic, non-mutagenic, odorless, and tasteless. This polymer forms solutions of low viscosity. Pullulan has numerous uses in foods, pharmaceuticals, manufacturing, electronics, etc.

1.2.2 Biological materials

To study AG 's capacity for protecting biologicals during desiccation and storage, *E. coli*, *B. subtilis*, single-stranded DNA (ssDNA), and double-stranded DNA (dsDNA) were used. The integrity of these biologicals was tested before and after drying and during storage via tittering and EM and FM for bacteria, and quantitative gel electrophoresis and PCR for DNA. The following section describes the characteristics of these biologicals.

1.2.2.1 Bacteria

The bacteria selected to represent gram-negative non-spore forming bacteria and gram-positive spore forming bacteria were *Escherichia coli* (ATCC 11775) and *Bacillus subtilis* (ATCC 6051), respectively. These strains of bacteria were chosen for this study because they are well characterized, non-pathogenic, economical and widely used (Jinks et al., 1985; Cohen et al., 1996; Roberts et al., 1996; Jeffreys et al., 1998; Atrih et al., 1999; Ivanova et al., 1999; Giraffa et al., 2000; Staudinger et al., 2002; Wang et al., 2002; Belanger et al., 2003; Fite et al., 2004; Langsrud et al., 2004; Waller et al., 2004b).

The general structure of bacterial cells consists of a cell wall, cell membrne and the cytoplasm which contains the nucleoid, ribosomes, plasmids and vacuoles. In general, bacteria are categorized by their cell wall composition as either gram-negative or gram-positive. The cell walls of gram-negative bacteria consist of an outer membrane containing lipopolysaccharide (endotoxin), a thin layer of peptidoglycan, polysaccharides, lipids and proteins. Gram-negative bacteria have a distinguishable gap between the cell wall and cell membrane which is called the periplasmic space. This space contains proteins such as digestive enzymes which eliminate harmful substances and transport proteins which transfer metabolites into the bacterial cytoplasm.
The periplasm consists of peptidoglycan, proteins, metabolites and sugars such as glucose to maintain osmotic pressure. Conversely, the cell walls of gram-positive bacteria lack an outer membrane, lipopolysaccharide, and many of the proteins and lipids present in gram-negative bacterium. The composition of gram-positive cell walls differs from gram-negative bacteria in that it contains a thick layer of peptidoglycan and teichoic or

teichuronic and lipoteichoic acids. These bacteria also lack a periplasmic space between the cell wall and cell membrane. Nonetheless, the cell walls of both types of bacteria function to maintain characteristic shape, regulate osmosis and serve as a course sieve.

The cell membranes of both types of bacteria are very similar. The main structure of the cell membrane consists of phospholipids which are made up of a hydrophilic head and hydrophobic tails. The tails composing of fatty acids hydrocarbons extend inward of the phospholipid bilayer creating a hydrophobic barrier. Whereas, the heads are composed of negatively charged phosphate make up the outer edges of the membrane which interact with the cytoplasm and the cell's microenvironment. The main function of the cell membrane is to transport materials such as proteins in and out of the cell. The cell membrane also plays a role in cellular respiration, DNA replication, cell wall synthesis, attachment of flagella or cilia and other processes needed for cell survival.

The main internal components which are contained by the cell membrane include the cytoplasm, nucleoid, ribosomes, plasmids, vacuoles and granules. The cytoplasm consists of mainly water with dissolved and suspended materials such as amino acids, proteins, sugars, lipids and minerals. The nucleoid or nuclear region is generally located in the center of the cell, and it contains DNA arranged in one circular chromosome, RNA and proteins. Ribosomes are the protein synthesizing structures composed of protein and RNA. Plasmids are small circular DNA molecules found in some bacteria that provide additional genetic material for the cell. Vacuoles are membrane enclosed structures that contain a variety of substances such as water or gases. Granules are dense bodies made up of a specific component such as glycogen or polyphosphate. These cell components are characteristic of vegetative cells that metabolize nutrients and proliferate.

B. subtilis cells are more robust than *E. coli* because of their efficient defense mechanisms against stress. This is greatly due to the presence of stress responding transcription factor σ^B. Stress induces activation of this protein which regulates at least 22 operons that are involved with the protection against adverse conditions (Scott et al., 2000). One such mechanism is the formation of spores under various environmental conditions such as desiccation and lack of nutrients (Stragier and Losick, 1996). *B. subtilis* spores are structurally different than bacterial cells because they are designed to withstand adverse conditions. The major layers of the spores are the core, the cortex and the coat. The core is the inner most part of the spore and it contains the DNA. The cortex is the next layer, and it contains peptidoglycan structure. The outer layer is the coat which is composed of highly crosslinked proteins (reviewed in (Jedrzejas, 2002; Driks, 2003). *E. coli,* on the other hand, is sensitive to desiccation and requires more protection such as the production of trehalose (Tunnacliffe et al., 2001).

1.2.2.2 ssDNA and dsDNA

There were two types of DNA used in this study, ssDNA and dsDNA, which were isolated from fd-tet bacteriophage. This phage is a filamentous virus that contains a single-stranded DNA genome. This type of phage infects K91 BKan *E. coli* via attachment to bacterial pilus. Once phage ssDNA is inside of the bacterial cell, it replicates the viral genome via rolling circle method using bacterial enzymes. During this process, the ssDNA (phage genome) is complimentarily base paired and becomes double stranded. The phage dsDNA within a bacteria cell is the replicative form (RF) by

which copies of the phage genome are produced and are packaged into major coat proteins. This type of RF DNA is covalently closed circular dsDNA. The ssDNA is isolated from phage itself, and the dsDNA is isolated from bacterial cells infected with phage.

1.2.3 Preservation methods

The most popular preservation techniques applied to biological materials include lyophilization, cryopreservation, and spray drying. These methods have serious drawbacks and limitations which include complex preservation formulas, specialized equipment, and equipment maintenance.

1.2.3.1 Cryopreservation

Freezing preserves biological materials by suspending the material in freezing media and storing samples at temperatures of -20°C or below. Freezing affects gene expression and regulation and causes DNA damage (Swartz, 1971; Mackenzie, 1973; Oliver et al., 2001). Protectants commonly used in cryopreservation include glycerol, DMSO and osmotic sugars such as trehalose, lactose, and sucrose (Malik, 1999; Panoff et al., 2000b). DMSO protects intracellularly by preventing ice formation at freezing temperatures (Panoff et al., 2000b). Sugars protect bacteria by inducing an adaptive response which leads to the upregulation of osmoticum-resposive gene (Schwartz et al., 1998). Sugars may cause cells to dehydrate prior to freezing in order to decrease

intracellular ice formation (Panoff et al., 2000b). Cryoprotectants such as glycine, carnitine and lactose broth have been found to induce cold tolerance in some bacterial cultures, but viability depends on strain (Smith, 1996; Lorca and de Valdez, 1998). High molecular weight molecules such as polyvinylpyrrolidone (PVP) and dextran have been used as protectants for many types of cells including bacteria and rabbit erythrocytes, yet levels of success depend on cooling rates and thawing (Ashwood-Smith and Warby, 1971). Cryopreservation is applicable for preserving most bacteria (Yamasato et al., 1973), but it is not practical for large scale industrial use.

1.2.3.2 Lyophilization

Freeze-drying is a process that first freezes the biological material, and then, the material is warmed under pressure to dehydrate the samples via sublimation. Freeze-drying which became popular in the 1950's induces mutation and low survival rates (Malik, 1988b). Parameters involved in reducing bacterial damage include storage atmosphere, exposure to air, storage temperature and biological water content (Wang et al., 2004). Another aspect for preservation is the physiologlogical state of the bacteria. It is reported that stationary phase bacteria are less sensitive to freeze-drying than other growth phases (Palmfeldt et al., 2003). Other factors to consider when lyophilizing microorganisms involve initial freezing temperatures, rates of freezing and sublimation, and protective media (Harris, 1951; Benedict et al., 1958; Barbaree, 1976). A study that exposed freeze-dried cultures to various environments before and during re-wetting of samples, indicate that exposure to oxygen was the main contributing factor to loss of cell

viability (Benedict et al., 1958). In addition, exposure to the environment may be due to the oxygen permeability of rubber stoppers used to cap the vials containing lyophilized material (Barbaree et al., 1985).

Protectants used in lyophilization include peptone, soluble starch (Annear, 1954), skim milk (Joubert and Britz, 1987; Moreira et al., 1995; Champagne et al., 1996; Safronova and Novikova, 1996), DMSO, glycerol, casein hydrolysates, egg white, yeast extract, acetamide and malic acid (de Valdez et al., 1983). In addition, high-molecular weight polymers such as bovine serum albumin, glycogen, dextran, PEG 1000 are only capable of protecting the cell wall since they do not permeate the cell membranes (de Valdez et al., 1983); (Champagne et al., 1996). Some types of bacterial cultures preserved by freeze-drying in solutes such as sucrose or glycerol and a high molecular weight component such as dextran or ficoll have shown to preserve better together than separately (MacKenzie and Orndorff, 1972). Polyols used to protect bacteria during lyophilization include glycerol, β-glycerophosphate and amino acids such as glutamate, asparagine and cysteine (de Valdez et al., 1983). Protectants such as raffinose and skim milk have been used for preserving some sensitive bacteria, but results are inconsistent and vary depending on bacterial strain (Malik, 1988a). Trehalose is widely used as a protectant for many types of bacteria during freezing drying (Israeli et al., 1993; Welsh and Herbert, 1999; Benaroudj et al., 2001). Freeze-drying is an intricate process that involves numerous formulations that vary depending on the type of organism preserved (Barbaree, 1976). Since freeze-drying is so complex, this gives rise to designing a method much simpler for use in developing countries (Siberry et al., 2001) or for large scale production of bacteria for crop protection.

1.2.3.3 Spray drying

Spray drying is a process which biological material is mixed with coating materials and protectants, then rapidly spray-dried at high temperatures which is capable of damaging the microbes beyond repair (O'Riordan et al., 2001; Lian et al., 2002; Corcoran et al., 2004). Generally, the viability of spray-dried bacteria is typically less than freeze-dried bacteria (Wang et al., 2004), but the viability varies depending on many factors. These factors include bacterial strain, drying temperatures, storage conditions, and protectants. Some bacterial strains are more adaptive to heat stress caused by drying temperatures and are more viable after spray-drying (Desmond et al., 2002). The loss of bound water from structural components of the cell may lead to an accumulation of unstable Mg^{+2} which degrades ribosomes (Desmond et al., 2001). Most bacterial spray-drying involves the use of skim milk as coating material and protectant (In't Veld, 1998), but some experiments have used fermented soymilk as the protectant and wall material (Wang et al., 2004). Some spray-drying techniques involve a two phase system where the product is coated two times with oppositely charged polymers (Millqvist-Fureby et al., 2000). Spray-drying is an area of preservation that is becoming more popular because it is more cost-effective and less time-consuming than freeze-drying (Costa et al., 2002).

1.2.3.4 Other drying methods

Methods described in this section are mainly used in the event when freezing or freeze-drying are not applicable. Liquid drying is a technique which utilizes vacuum drying at non-freezing temperatures and can be applied to microorganisms sensitive to freezing and freeze-drying (Malik, 1990a; Malik, 1999). Anaerobic liquid drying has been found to be an effective technique when preserving sensitive bacteria such as *Campylobacteraceae*, yet this method may not be applicable for large scale preservation (Malik and Lang, 1996). Liquid drying *Archaebacteria* that are sensitive to freezing and freeze-drying can be preserved using this method in combination with complex formulations which include a variety of protectants such monosodium glutamate, adonitol, and sorbitol (Sakane et al., 1992). Protectants used in liquid drying sensitive bacteria include skim milk, active charcoal, glutamate, mesoinositol, raffinose, trehalose or honey (Malik, 1992). Some bacteria have been found to increase their desiccation tolerance when vacuum dried in the presence of trehalose (Garcia De Castro et al., 2000).

The high cost of freezing and freeze-drying bacterial samples preserved for serotyping and further study in developing countries has lead to the use of sand desiccation preservation. This method is cheap and possible to maintain, yet the viability of bacterial cultures may not be preserved for long-term period of time (Brahmadathan et al., 1995). Some cultures have been protected using glycerol-chocolate broths, rabbit blood, sheep blood, and skimmed milk using sand desiccation (Siberry et al., 2001). Other cultures have been preserved for short-term via sand desiccation in sheep blood and stored under vacuum and refrigeration (Brahmadathan et al., 1995). This method

employs the use of silica desiccant and vacuum to keep the chamber free form moisture and oxygen. This method is inexpensive and applicable for storing small volumes of bacteria; however, viability of most cultures declines steadily at higher storage temperatures (Koshi et al., 1977).

1.2.4 Preservation protectants

Many of the techniques as aforementioned have employed the use of protectants such as honey, polyvinyl pyrollidone, colloids, glutamate, meso-inositol, raffinose, glucose, serum albumin, trehalose, DMSO, glycerol, gelatin, amino acids, lactose, sucrose, and skim milk (Malik, 1988b; Broadbent and Lin, 1999; Conrad et al., 2000; Panoff et al., 2000a; Carvalho et al., 2004). Protective agents prevent (1) intracellular ice crystal formation, (2) total desiccation which damages DNA and kills cells, and (3) neutralizes the electrolytic effects and free radicals (Malik, 1988b). Many of these protectants are thought to work by protecting cytoplasmic membranes from dehydration damage and prevent the loss of intracellular components such as RNA and proteins (Malik and Lang, 1996). Sugars such as trehalose are reported to protect intracellular proteins and membranes by forming hydrogen bonds where structural water is removed during dehydration (Wolkers et al., 2002). In addition, sugars are critical components for the formation of a glass matrix in the cytoplasm which immobilizes activity and protects the cell (Wolkers et al., 2002).

1.2.5 Mechanisms of degradation

The most popular preservation techniques include freeze-drying, freezing, and spray-drying all of which cause various degrees of structural damages to the cell wall, cell membrane and DNA (Malik, 1988c; Broadbent and Lin, 1999; Beal et al., 2001; Gouesbet et al., 2001; Carvalho et al., 2004). Consequences of freeze-drying and freezing include protein denaturation that leads to a decrease in cell viability (Leslie et al., 1995a; Panoff et al., 2000b; Carvalho et al., 2004). Subzero temperatures cause damage to bacterial cells via ice-crystal formation, osmotic stress, desaturation of fatty acids and unwinding RNA (Panoff et al., 2000b). The removal of water may cause irreversible changes to structure and function of proteins and lipids (Ananta et al., 2005). Freeze-drying and desiccation cause a variety of mutations in bacterial cells such as DNA single strand breaks which may be caused by free radicals (Ashwood-Smith, 1980a). On the other hand, freeze-drying may induce the activation of different types of bacterial prophage which may cause the DNA breaks (Ashwood-Smith, 1980b). Although mutation does occur during freeze-drying, some bacteria are capable of repairing DNA damage (Heckly and Quay, 1981). Lyophilizing may not interfere with cell viability but plasmid DNA may degrade, so genetically engineered bacteria are more sensitive to freezing and desiccation than wild-type (Lang and Malik, 1996).

Additional reasons for microorganism damage during freeze-drying include large cell size, complex enzyme systems, presence of plasmids, poly-β-hydroxy butyrate, absence of spores, cysts, and sensitivity to desiccation (Malik, 1988b). Bacterial degradation during desiccation may be caused by reactive oxygen species and

environmental stress such as drought and cool temperatures (Oliver et al., 2001). Freeze-drying also increases the formation of radicals that attack phospholipids, DNA and proteins (Oliver et al., 2001).

CHAPTER 2

PRESERVATION OF GRAM-NEGATIVE BACTERIA IN PROTECTIVE POLYMERS

2.1 Introduction

Preservation of vegetative cells of non-spore forming bacteria is of great importance in many fields that utilize microbiology and biotechnology (Malik, 1988b). Because bacteria undergo spontaneous genetic mutations at a rate of 10^{-3}-10^{-9} per cell division when maintained in growth cultures (Black, 1999), immobilizing bacteria and inhibiting growth through desiccation for long-term preservation sustains genetic consistency. This is crucial for bacteria used in agriculture, food, health, energy and bioremediation industries which depend on the authenticity and viability of particular strains (Don and Pemberton, 1981; Malik and Claus, 1987). Traditional methods for immobilizing and preserving vegetative cells for long-term storage include lyophilization, cryopreservation, liquid-drying and spray-drying. Lyophilization and cryopreservation are generally effective in preserving many types of bacteria. However, these methods can initiate structural damages to the cell wall, cell membrane and DNA which are undesirable when recovery of viable non-mutant bacteria is essential (Malik, 1988b; Malik, 1988c; El-Kest and Marth, 1992; Miyamoto-Shinohara et al., 2000).

In addition, desiccated bacteria may lose viability due to rehydration which may alter protein structures (Peccia et al., 2001). It is detrimental that bacteria revive, maintain cellular functions and propagate after dehydration, storage, and rehydration in order to be useful for industrial applications (Malik, 1988c; Potts, 2001).

Preservation methods generally involve complex formulations, specialized equipment and skilled personnel, and storage at low temperatures (Malik and Lang, 1996). Numerous protective agents are used to create formulations for preservation of bacteria. Protective agents include glutamate (Miyamoto-Shinohara et al., 2000), adonitol, sorbitol, thioglycollate (Sakane et al., 1992), trehalose, (Israeli et al., 1993; Malik and Lang, 1996; Panoff et al., 2000b; Palmfeldt et al., 2003), gelatin, collagen, raffinose, meso-inositol, honey (Malik, 1988b; Malik, 1990a; Malik and Lang, 1996), skim milk (Barbaree et al., 1982; Joubert and Britz, 1987; Malik, 1988b; Miyamoto-Shinohara et al., 2000; Palmfeldt et al., 2003), glycerol (Beyersdorf-Radeck et al., 1993a; Panoff et al., 2000b), cellulose, mono-sodium glutamate (Sakane et al., 1992) methylcellulose (Diez et al., 1994), polyvinylpyrrolidone (Barbaree et al., 1982), dextran (Antheunisse et al., 1981), polyvinylethanol (Beyersdorf-Radeck et al., 1993a), DMSO (Panoff et al., 2000b) and more. The specialized equipment for lyophilization requires a freeze-dryer and electrical power, involves low temperatures and complex formulations (Champagne et al., 1996; Palmfeldt et al., 2003). Cryopreservation requires a constant cooling system such as a freezer or liquid nitrogen, and many cryoprotectants such as DMSO or polyvinylethanol would need to be removed from the storage medium before using the microorganisms (Beyersdorf-Radeck et al., 1993a; Panoff et al., 2000b). Liquid drying has been a successful method of preservation for various species of

Helicobacter, *Campylobacter* and other bacteria which are sensitive to freezing and freeze-drying; however, this method usually requires refrigeration after drying for long-term storage (Iijima and Sakane, 1973; Malik, 1990a; Sakane et al., 1992; Malik and Lang, 1996). In spray-drying, the wall material is the protectant formulation that encapsulates and immobilizes bacteria. This is added to a bacterial suspension and sprayed in a hot chamber and dried. The problem with spray-drying is that it, in vast majority of cases, requires high temperatures to produce a dry product that will not cake; these high temperatures damage or kill most bacteria (Kailasapathy, 2002). These disadvantages provide cause for designing a simpler method for preserving vegetative bacterial cells for industrial applications.

In this chapter, a method for preserving gram-negative vegetative *E. coli* cells using acacia gum (AG) as the protective medium during desiccation is described. Since no refrigeration is required to dry or store bacterial samples for short-term, the method using AG as the protectant could be used in cases when refrigeration and specialized equipment such as freeze- dryers are not practical. Preserving bacteria in AG may be applied to a variety of microorganisms (Desmond et al., 2002; Boza et al., 2003) and can be dispersed into the environment for bioremediation or plant protection without hazardous consequences (Anderson, 1986). The use of AG for preserving microorganisms is attractive because this formulation is non-toxic (DeCloux et al., 1996), non-mutagenic (Anderson, 1986), inert (Mahler et al., 2000), ingestible (Al-Mosawi, 2002), organic (Rehman et al., 2003), inexpensive, simple (Vodyanoy et al., 2003) and biodegradable. This process could be an alternative method for preserving microorganisms for

immediate short-term preservation when refrigeration is not available and for large batches of bacterial cultures used for bioremediation or plant protection (Israeli et al., 1993; Khan et al., 1996; Fujii et al., 2003). AG could be used for preserving bacteria inoculums used in the food industry (van de Guchte et al., 2002). Since AG is a traditional food additive (confectionaries, ice cream, soda pop, TUMS®) known to be safe and non-toxic (Minhas et al., 2002; Gamal el-din et al., 2003), there is no need to separate AG from preserved bacteria for starter cultures in fermented foods like yogurts and cheeses. Some protective agents such as DMSO would not be favorable for preserving starter inoculums.

The work described here focuses on developing a simple process for preserving *E. coli* in protective polymers without using specialized equipment or complex formulations. This method involves bacteria suspensions mixed with AG polymer and air dried at 40°C. The samples were stored at various temperatures and humidity, and tested for viability before and after drying and during storage. The controls in this study were a physiological buffer (PBS) and a natural polysaccharide (pullulan) produced by a fungus, *Aureobasidium pullulan*. These control solutions were mixed with bacterial suspensions in the same ratio as AG and dried at 40°C. Successful preservation of bacteria was indicated by viability testing via colony plate count method.

2.2. Materials and methods

2.2.1 General material and methods

In this section, general information regarding preparation of polymers and storage conditions for long-term preservation experiments is presented. Specific methods concerning sample preparation and analysis for individual biologicals are presented in the chapters devoted to each biological. In this chapter, *E. coli,* a gram-negative bacterium, is tested. In chapter 3, *B. subtilis,* a gram-positive bacterium, is the biological studied. ssDNA and dsDNA are studied in Chapter 5 and 6, respectively.

2.2.1.1 Materials

Acacia gum (AG) and pullulan polymer powders were purchased from Frontier™ Natural Products Co-Op (Norway, IA), and Sigma-Aldrich (St. Louis, MO, cat # P-4516), respectively. Salts, listed in Table 1, were purchased from Fisher Scientific (Hampton, NH): potassium carbonate (cat # P179-500), ammonium chloride (cat # A687-500), potassium nitrate (cat # P263-500), calcium nitrate (cat # C109-500), and ammonium sulfate (cat # A938-500).

2.2.1.2 Polymer preparation

Polymer solutions were prepared by mixing polymer powders with sterile water in concentrations of 10, 15, 20 or 25% w/v and stirred for 2-3 h, or until powder was completely dissolved. AG colloidal solutions were filtered using two Brew Rite® coffee filters (Rockline Industries, Sheybogan, WI) in a Büchner funnel with vacuum filtration. Filters were changed after every 10-20ml, and the solutions were filtered twice and autoclaved at 120°C for 15 min, cooled and stored at 4°C. The pullulan polymer solutions were sterilized by filtration using 0.22µm Millipore Stericup™ (Fisher Scientific, cat # SCGV-U01-RE). The Stericups containing pullulan were capped, covered with aluminum foil, and stored at 4°C.

2.2.1.3 Long-term sample storage conditions

There were two types of long-term preservation experiments conducted for all biologicals: (1) storage at various temperatures and constant humidity and (2) storage at various humidity and constant temperature. Usually, samples were tested on days 2, 4, 8, 16, 32, 64, and 128 of experiments. For temperature experiments, samples were sealed after drying and placed in holding containers as shown in Fig. 1A-C. Samples were stored in incubators set at 5, 15, 25, and 40ºC and constant humidity (~33%) for long-term storage.

Table 2.1

Saturated salt solutions at 25° C.

Saturated salt solutions were prepared and placed in RH chambers. To insure complete saturation, additional salt crystals were added to the saturated solutions in the RH chambers.

Humidity, %	Salt	Salt, g	H_2O, ml
46	$K_2CO_3 \cdot 1.5H_2O$	200	100
53	$Ca(NO_3)_2 \cdot 6H_2O$	470	100
76	$KNO_3 + NH_4Cl$	50 + 50	100
86	$(NH_4)_2 SO_4$	100	100

For humidity experiments, saturated salt solutions were used to maintain 46, 53, 76, and 86% relative humidity (RH) at 25ºC (Table 1). Each salt, if mixed to the saturation point in water, maintains an associated characteristic RH at a given temperature in the microenvironment in the RH chambers. For example, if air of low relative humidity is introduced into the chamber, water molecules will evaporate from the saturated solution until the characteristic RH is achieved. Conversely, if air of high relative humidity is introduced, the solution will absorb water molecules from the air until that RH is reached. Samples were placed uncapped in RH chambers containing saturated salt solutions (Fig. 1D-F). RH chambers with samples were sealed and put in a large incubator set at 25°C for long-term storage.

2.2.2 E. coli cultivation and preparation of concentrated cell suspension

Escherichia coli (ATCC 11775) was purchased from American Type Culture Collection in Manassas, VA. Fresh *E. coli* cultures were grown in Difco nutrient broth (Fisher cat # DF0003) for 18 h in a shaker incubator set at 200 rmp and 37°C. Bacterial cells were harvested by centrifuging for 10 min at 5,000 rpm; then the supernatant was discarded. The concentrated cell suspensions were prepared by adding 1 ml of PBS to each pellet formed from 50ml of original culture.

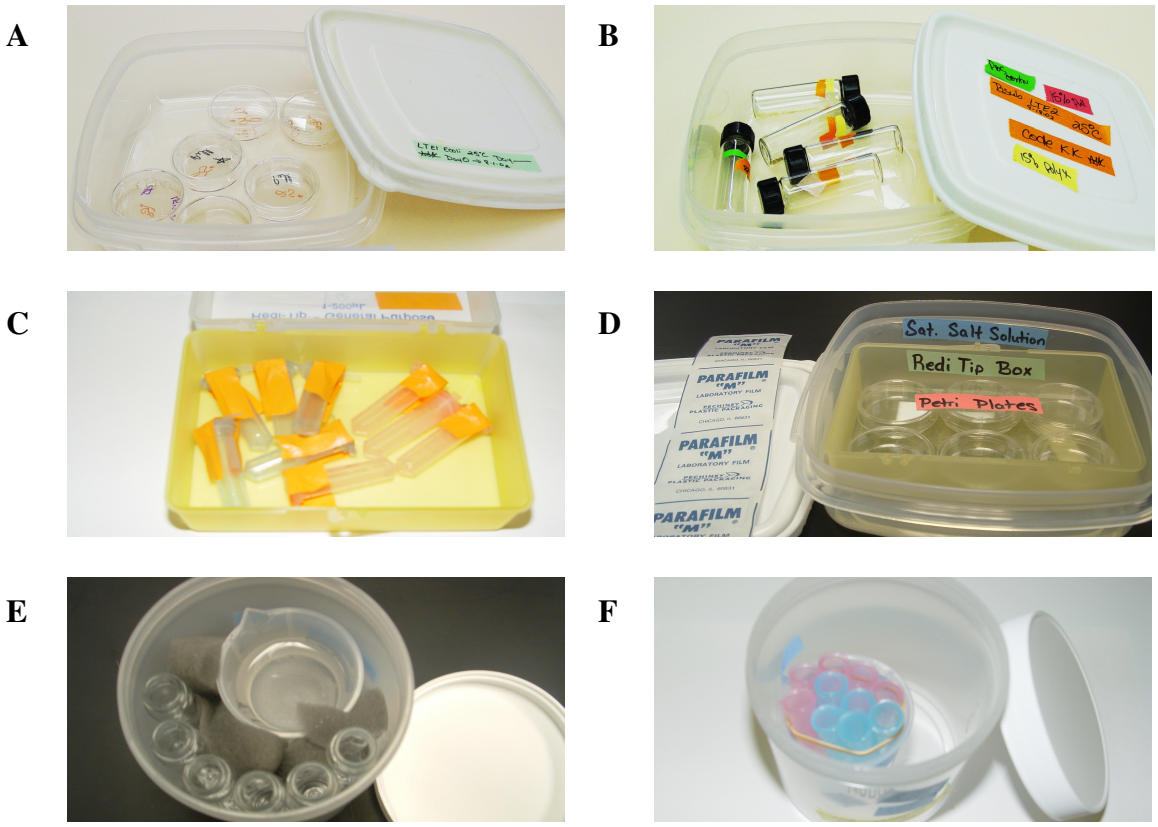

Fig. 2.1. Storage containers for temperature (A-C) and humidity (D-F) experiments. (A) *E. coli* samples in Petri plates, (B) *B. subtilis* samples in glass vials, and (C) DNA samples in microcentrifuge tubes were sealed and stored at various temperatures for long-term storage. (D) *E. coli* samples in Petri plates, (E) *B. subtilis* samples in glass vials, and (F) DNA samples in microcentrifuge tubes were placed in RH chambers designed to maintain a particular humidity for long-term storage.

2.2.3 Estimation of viability of bacterial samples

Bacterial titers were determined before drying, after drying and after storage on days specific for type of experiment. For re-hydration of samples, sterile de-ionized water was added in the amount of water loss and incubated at ambient temperature for 30-45 min. Bacterial viability was estimated by preparing serial dilutions and plating 10µl of each dilution on Difco nutrient agar obtained from Fisher Scientific (cat # DF0001-17-0). The bacteria were grown under aerobic conditions in a 37°C incubator for 18-24 h. The dilutions that contained 30-300 colonies were counted. Data were expressed as colony forming units per ml (CFU ml^{-1}). The bacterial colonies were observed for any morphological or pigmentation changes.

2.2.4 Optimization experiments

Short-term experiments were performed to determine the optimal conditions for long-term storage of *E. coli* samples. These conditions included AG concentration, drying temperatures, sample volume and container.

2.2.4.1 Polymer concentration determination procedure

Small polystyrene Petri plates purchased from Fisher (cat # 08772-30) were labeled. One part concentrated *E. coli* suspension was mixed with four parts of 10, 15, 20 or 25% AG. Five hundred microliters of these *E. coli* suspensions in AG were aliquoted

into these dishes. These samples were dried uncovered in 40°C incubator (Lab Line Imperial III Model 302, Fisher, cat # 11-702-10) containing silica desiccant beads for ~20 h. Dried samples were covered and stored at 25°C. Bacterial viability was estimated before and after drying and after storage for 2 and 6 days as described in section 2.2.3.

2.2.4.2 Container, sample volume and drying temperature determination procedure

E. coli bacterial suspensions in 15% AG and PBS were prepared as described in section 2.4.1 and aliquoted into containers as outlined in Table 2. The following containers used in optimization experiments were purchased from Fisher: 2.0ml microcentrifuge tubes (cat # 05-408-138), 6 well plates (cat # 08-772-49), (3.5 cm) small Petri plates, and 12.5cm^2 cell culture flasks (cat # 08-772-1F). Table 2 summarizes the parameters of sample volume, drying temperature, type of drying and time to dry. Bacterial viability was estimated via the plate count method before and after drying.

2.2.5 Long-term storage experiments for E. coli samples

Based on optimization procedures, the following protocol was used for long-term preservation of *E. coli* cells. Bacterial suspensions in 15% AG, 15% pullulan, and PBS were prepared as described in section 2.4.1. An aliquot of 500µl of sample was placed into a labeled small Petri plates. The plates were placed in an incubator containing silica desiccant and were dried at 40°C for 22-24 h.

For temperature experiments, dried samples were capped and placed into 470ml square containers (Fig. 1A) which were sealed with parafilm. These containers were stored at 5, 15, 25, and 40°C. Bacterial viability and stability were tested as described in section 2.4, before and after drying and after storage on days 2, 4, 8, 16, 32, 64, and 128. For humidity experiments, samples dried in small Petri plates were placed in redi-tip boxes, which were placed in 470ml square Rubbermaid® container holding saturated salt solutions as shown in Fig. 1D. The containers were sealed tightly with lids and reinforced with parafilm to prevent water evaporation during long-term storage. The RH chambers were sealed and stored at 25°C. Bacterial viability and stability were tested as described in 2.4, before and after drying and after storage on days 2, 4, 8, 16, 32, 64, and 128.

2.2.6 Calculations

Statistical analyses (t- Test and ANOVA) were performed using Microcal™ Origin® version 6.0 (Northhampton, MA). The data are presented as means of at least two independent analysis (titer of bacterial samples in each independent experiment was determined twice) ± standard deviation

Temperature-dependent degradation model is comprised of two components: (1) first-order or zero-order reaction kinetics for the degradation process; (2) Arrhenius equation for the first-order reaction kinetics on media temperature.

Table 2.2

Summary of parameters for optimizing the drying method for bacteria in AG.

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Container	Sample volume, µl	Drying temp,°C	Method of drying	Time to dry, hr
2.0 ml micro-centrifuge tubes	50, 100	Ambient	Vacuum centrifuge	5
	50, 100	Ambient	High vacuum	.75
6 well plates	500	Ambient	Circulating air under laminar hood	4
	500	40	Static air incubator	5
	500	37	Static air incubator	5
Small Petri plates (3.5cm)	50	Ambient	Vacuum desiccator	3.5
	50	40	Static air incubator	2
	500	Ambient	Vacuum desiccator	6
	500	40	Static air incubator	20
Culture flasks ($12.5cm^2$)	500	25	Static air incubator	72
	500	40	Static air incubator	48
Glass vials (7.4 ml)	500	40	Static air incubator	48

(1) The first-order degradation kinetics may be expressed as

$$\frac{dN}{dt} = -kN \qquad (1)$$

where N is the concentration of a degraded bacteria or phage; k is the first-order degradation rate constant; and t is time. The solution of the equation 1 is expressed as

$$N = N_0 e^{-kt} \qquad (2)$$

where N_0 is the initial concentration of cells.

The equation (2) can be written as follows:

$$\log(N / N_0) = -kt \qquad (3)$$

If time is expressed in days, k is the first-order degradation rate constant in $(day)^{-1}$.

The zero-order degradation kinetics may be expressed as a percent of residual product or activity

$$P = P_0 + k_0 t \qquad (4)$$

where P is a percent of residual product or activity after degradation during the time t; P_0 is the regression intercept, and k_0 is the zero-order degradation rate constant in % per day.

(2) Temperature dependence of the reaction kinetics

Arrhenius equation is used to represent the kinetics dependence on temperature:

$$\log k = -\frac{\Delta H}{2.303R} \times \frac{1}{T} \qquad (5)$$

where ΔH is the heat of activation of the reacting molecules; R is the universal gas constant; and T is absolute temperature.

2.3. Results and discussion

3.1 Optimization experiments for long-term preservation of E. coli

Numerous dehydration and short-term storage experiments were performed to optimize the conditions for sample preservation and analysis; among them, determination of AG concentration, storage containers, drying temperature, method of drying and duration of drying, and storage temperature. In order to compare AG to a substance with similar applications, natural pullulan polymer was used as a control polymer.

To find the optimal AG concentration for preservation of *E. coli*, the viability of bacteria was tested when dried and stored for six days in various polymer concentrations ranging from 10-25%. Degradation curves for *E. coli* in all concentrations of AG are shown in Fig. 2.2. The percent of viable cells remaining after drying of *E. coli* samples in 10, 15, 20 and 25% was 48.5, 40.4, 30.7 and 14.9 %, respectively. More viable cells were recovered from bacterial samples dried in 10 and 15% than those samples dried in 20 and 25% AG. For cell viability, samples were tested on day 6. The percent of viable cells remaining after storage in 10, 15, 20 and 25% were 17.3, 17.6, 7.6, and 7.1, respectively. Cell viability for each parameter was tested in triplicate ($a = 0.05$). The greatest amount of viable *E. coli* cells were recovered from samples dried and stored in 15% AG.

In addition to determining the optimal concentration of AG, several variables were tested in order to find the most favorable container and method of drying *E. coli* (for experimental design see Table 2.2). Desirable qualities of the drying method include

quick and simple drying procedure, ability to dry many samples simultaneously and maximum recovery of viable cells. The results from several optimization experiments are illustrated in Fig. 2.3. Since the drying time for each parameter varied, the relative numbers of viable cells recovered after drying were compared. Results from samples dried in 12.5cm^2 culture flasks and glass vials were not presented in Fig. 2.3 because time to dry exceeded 24 hours, and this was undesirable for our design. Bacterial samples in AG dried under high vacuum at ambient temperature took less than 1 h to dry, but recovered cells were less than 1% of original titer, and there were limitations to the capacity of the static high vacuum machine. Samples that were dried in vacuum centrifuge and desiccator were favorable because the drying time was ~ 6 h. However, bacterial titers measured after drying were between 1-3% of original titer. Vegetative cells recovered after drying in 6 well plates at 40°C in a static incubator were less than 0.5% of the original titer. The best and most consistent results were obtained when *E. coli* samples in AG were dried in small Petri plates at 40°C in a static air incubator containing silica desiccant.

Vegetative cells recovered from 50 and 500µl of dried samples in Petri plates were 4.0% and 9.0% of the original titer, respectively. The difference in titers of the varying volumes was likely due to the rate of drying. Evaluation of bacterial degradation due to desiccation is discussed in Chapter 4 of this dissertation.

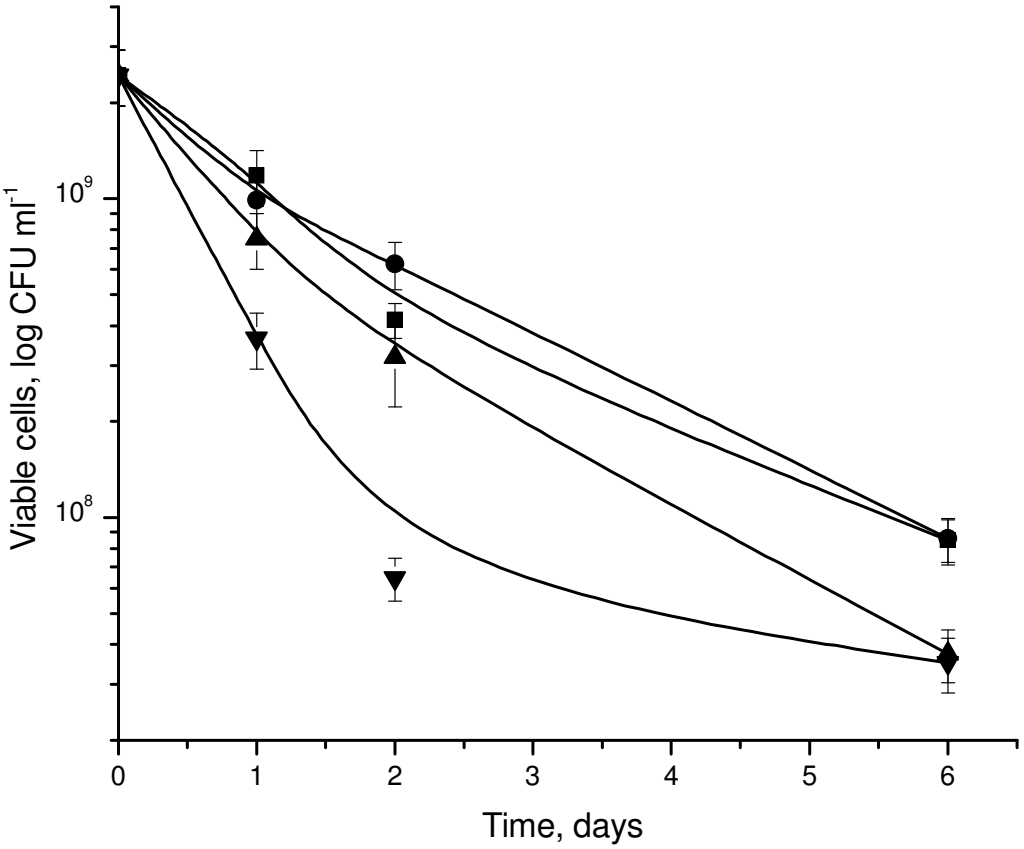


Fig. 2.2. Viability of *E. coli* cells preserved at various AG concentrations. AG was prepared in concentrations of 10 (■), 15 (●), 20 (▲), and 25% (▼). Bacterial suspensions were mixed with the various AG concentrations, aliquoted into small Petri plates, dried at 40°C overnight and stored at 25°C. Errors bars are indicative of standard deviation calculated using results from three independent analyses.

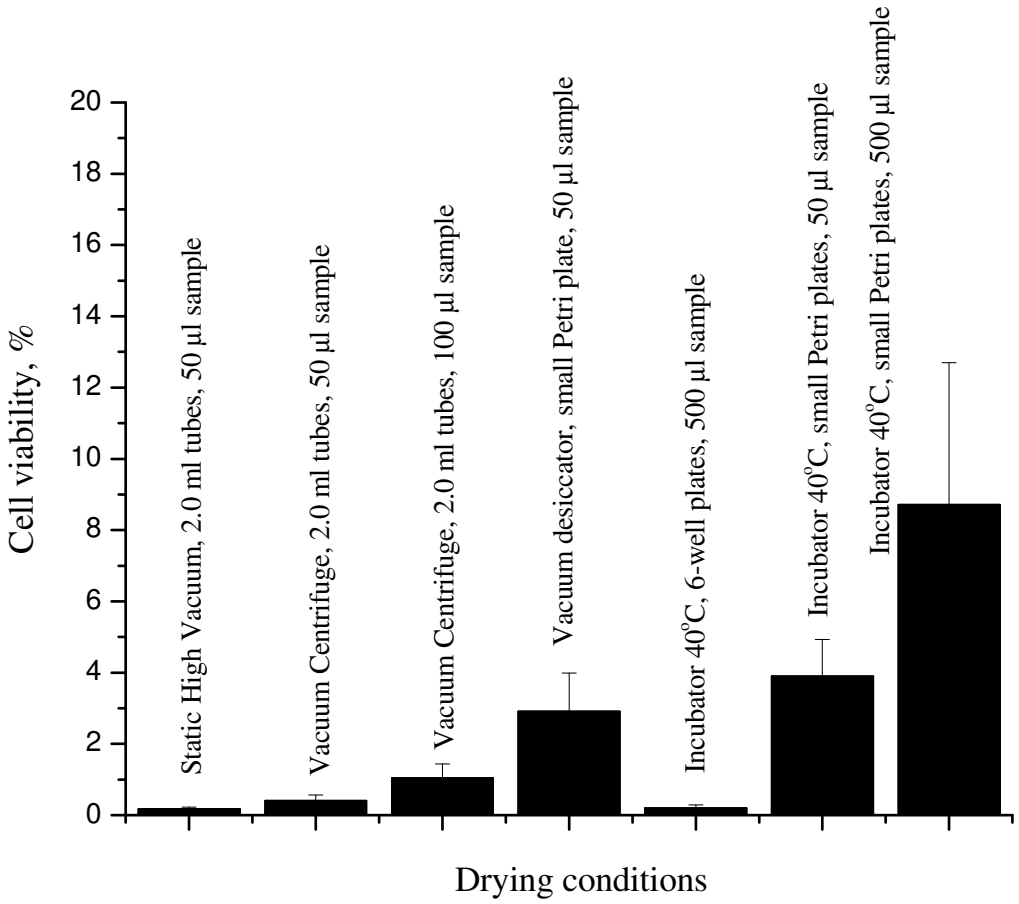


Fig. 2.3. Cell viability of *E. coli* samples dried in 15% AG at various conditions (method of drying and container type). The x-axis shows various methods of drying, containers, and sample volume. The y-axis represents % of viable cells recovered after drying. A summary of drying parameters are listed in Table 2.2.

2.3.2 Long-term preservation of E. coli cells

To determine survival of bacterial cells after preserving in protective polymers for long-term storage under various conditions, *E. coli* samples in 15% AG, 15% pullulan (control polymer), and PBS (control buffer) were dried at 40°C. After drying, two types of long-term preservation experiments were performed: (1) storage at various temperatures and constant humidity and (2) storage at various humidity and constant temperature. These are referred to from this point forward as temperature and humidity experiments.

For temperature experiments, samples were dried, stored at 5, 15, 25, or 40°C, at constant humidity (~33%) and tested for cell viability on days 2, 4, 8, 16, 32, 64 and 128 of storage. The titers for AG, pullulan and PBS samples after drying were 9.0, 1.0 and 0.03% of original titer, respectively. Bacterial degradation plots for all preserving media and temperatures are shown in Fig. 2.4 A-D. All *E. coli* samples exhibited an initial sharp decrease in viability followed by a slower linear decline. The duration of the initial drop appeared to be during drying and the stabilization period that took up to 8 days. Long-term preservation of bacterial cells is described by comparing the loss of viability in the various preserving media and storage temperatures. Bacterial titers for AG, pullulan and PBS were 1, 2 and 3 logs less than original titer (OT) on day 4 when stored at 5°C, respectively. On day 8, bacterial titers obtained from AG, pullulan and PBS samples were 1, 2, and 4 logs less than OT, respectively, when stored at 5°C. Bacterial titers measured from AG samples on subsequent days 16, 32 and 64 appeared to stabilize

until the last day for testing. On day 128 when stored at 5°C, *E. coli* samples preserved in pullulan and PBS recovered viable cells 4 logs less than OT, and bacterial titers obtained from testing AG samples were 3 logs less than OT. Similar trends were present when storing samples at 15°C; however, the rate of bacterial degradation appeared to increase. Bacteria recovered from AG and pullulan samples stored at elevated temperatures 25 and 40°C remained viable up to day 64. *E. coli* in AG stored at 25 and 40°C were 270 and 63 $CFUml^{-1}$, respectively. Viable cells in pullulan stored at 25 and 40°C were 1800 and 6.0 $CFUml^{-1}$, respectively. Whereas, bacteria in PBS rapidly declined to approximately 230 $CFUml^{-1}$ by day 64 at 25°C and few viable cells were detected by day 8 at 40°C. The low number of viable cells confirms that this strain of bacteria is sensitive to desiccation. The results indicate that AG provides adequate protection for *E. coli* cells during desiccation and storage at low temperatures and humidity.

E. coli samples stored at various temperatures declined in viability with increasing storage temperature. This linear relationship can be used to estimate the shelf-life of bacteria stored at a particular temperature. Various mathematical models have been employed to extrapolate cell viability of stored bacteria by applying the Arrhenius equation to determine thermal degradation (Greiff and Rightsel, 1965; Barbaree et al., 1982). Arrhenius temperature degradation plot for *E. coli* cells preserved in AG with relation to storage temperature is shown in Fig. 2.5. The Arrhenius plot indicated that

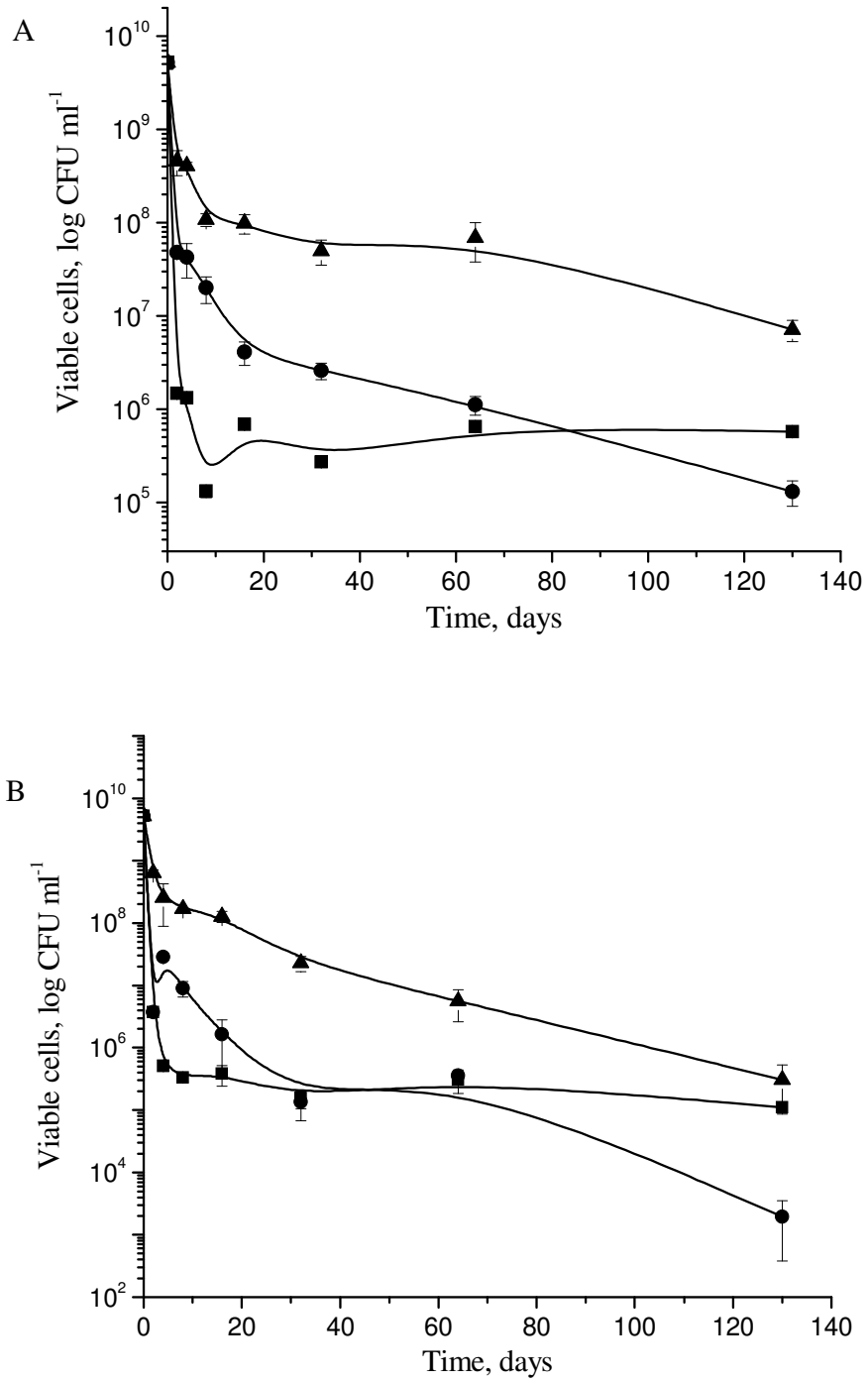


Fig. 2.4. *E. coli* degradation curves for long-term storage at various temperatures. AG (▲), pullulan (●), and PBS (■) samples were stored at 5 (A), 15 (B), 25 (C), and 40°C (D).

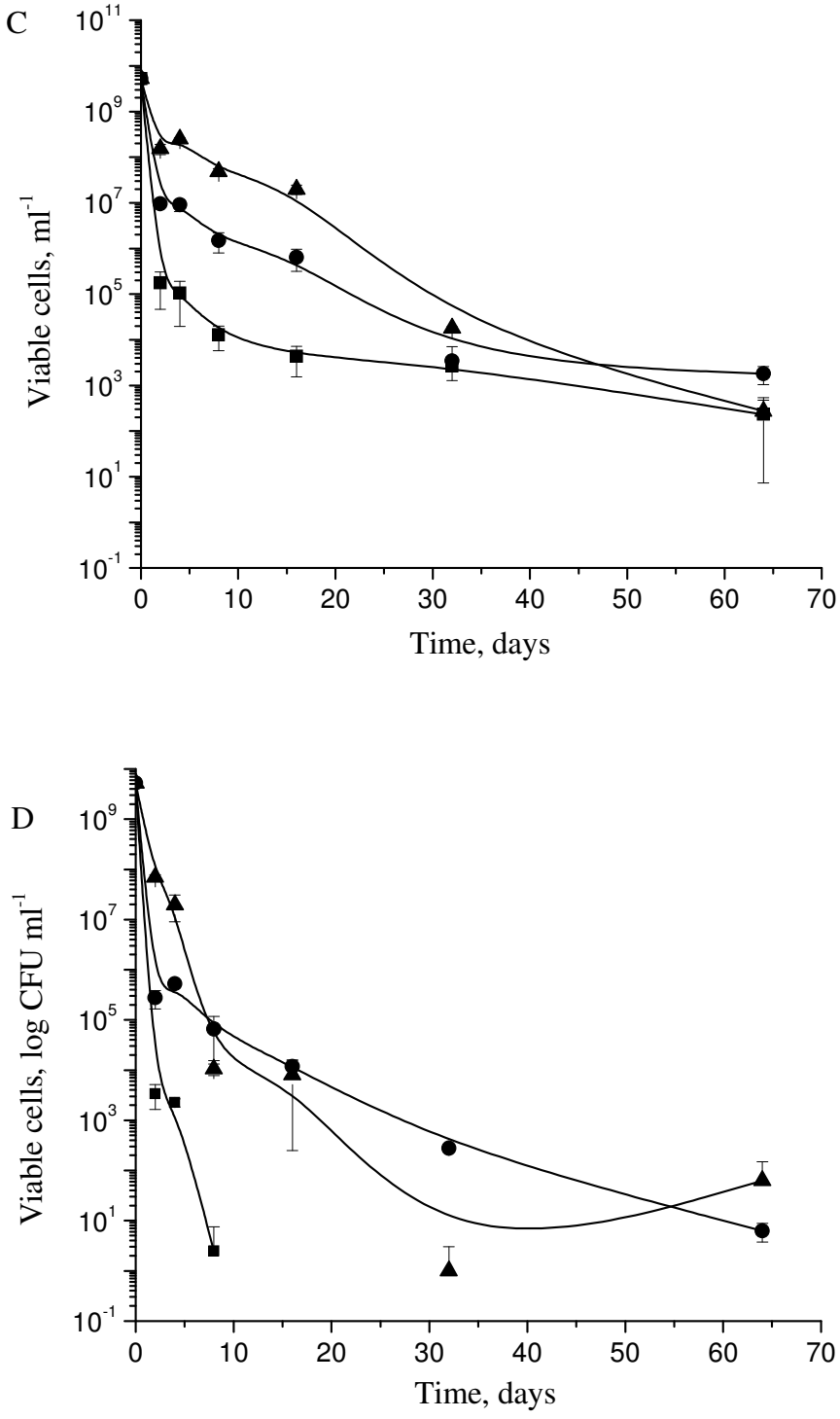


Fig. 2.4. continued.

E. coli preserved in AG follows this temperature related degradation model for 128 days. The activation energy for degradation was derived using the following formula (Segal, 1976)

$$k = Ae^{(-Ea/RT)}$$

where the degradation constant is k, the constant for a particular reaction is A, the activation energy for the reaction to proceed is $E_{a,}$, the universal gas constant is R, and absolute temperature is T.

To determine the activation energy of the biological degradation of *E. coli*, a derivation of the above equation gives

$$\text{Slope} = -Ea/2.3R.$$

The slope of this equation is obtained from the slope of the Arrhenius plot (log k vs 1/T) derived using our experimental data. The Arrhenius plot is shown in Fig 2.5. The slope was calculated to be -3.537×10^3. Using this slope in the above equation results in and activation energy of 16,107 cal/mole.

Humidity experiments are summarized in degradation curves shown in Fig. 2.6. Viability of *E. coli* preserved in AG and control samples declines steadily for all humidity levels. More viable cells were recovered from AG samples stored in 46% humidity than any other humidity. *E. coli* remained viable up to 32 days in AG when stored at 46, 76, and 86% humidity. The titers for AG samples stored at 46% for 32 days declined by 7 logs from original titer, and no viable bacteria were present in the PBS and pullulan control samples. AG and PBS samples contained no viable bacteria on day 32 when stored at 53% humidity; whereas, viable bacteria were present in pullulan which was 7 logs less than original titer. The titers for AG and pullulan samples declined 8 and

7 logs on day 32 when stored at 76% humidity, respectively. There were no viable bacteria present in PBS samples. AG samples stored at 86% for 32 days recovered viable bacteria 8 logs less than original titer; whereas, there were no viable bacteria present in pullulan and PBS samples. All samples stored at 76 and 86% humidity were no longer dry because they gained moisture from within their relative humidity chambers by day 32 of testing. There were no viable bacteria recovered from all samples tested on day 64 of storage.

2.3.3 Discussion

In this study, a non-pathogenic strain of *E. coli* was successfully preserved in AG for 64 days when stored at 25 and 40°C and 128 days when stored at 5 and 15°C. Because viability was not tested past 128 days of storage, we estimated the times for *E. coli* cells to degrade to 100 CFU which were 660, 240, 65, and 20 days for storage temperatures of 5°, 15°, 25°, and 40°C at low humidity, respectively. These results could be improved by making a few adjustments to the current protocol that would decrease the exposure to oxygen. It is well documented that desiccated bacterial samples exposed to oxygen in the air during and after lyophilization are prone to damage (Cox et al., 1971; Ashwood-Smith and Grant, 1976). Damage to freeze-dried bacteria exposed to oxygen involves the bacterial membrane and DNA initiation complex (Israeli et al., 1975).

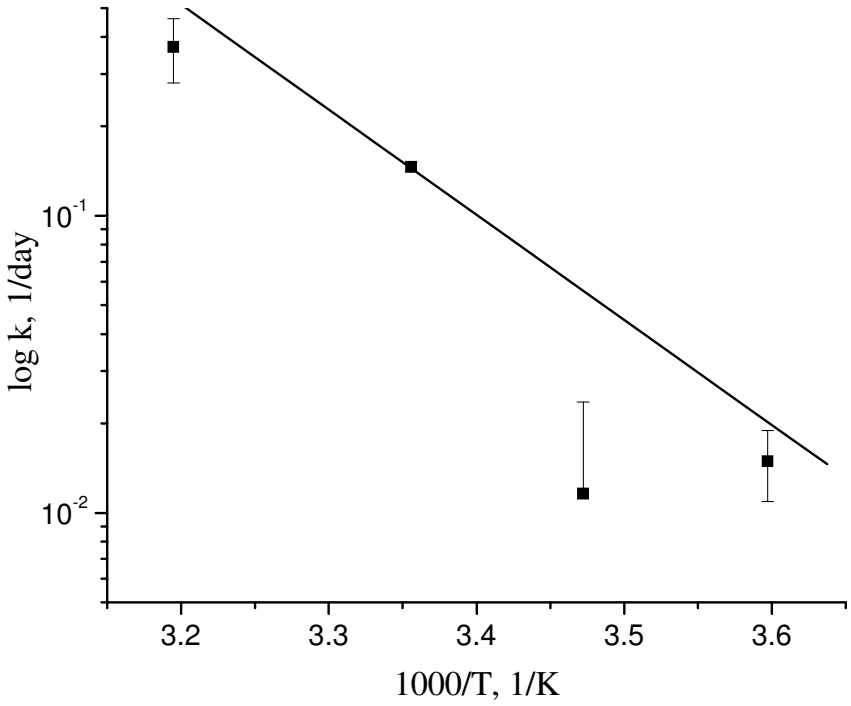


Fig. 2.5. Arrhenius temperature degradation plot for *E. coli* in AG. The linear plot was derived from experimental values of the rate constant for thermal degradation (k) at various temperatures. The equation for this line is y = -3.54x + 11.03 (P=0.023).

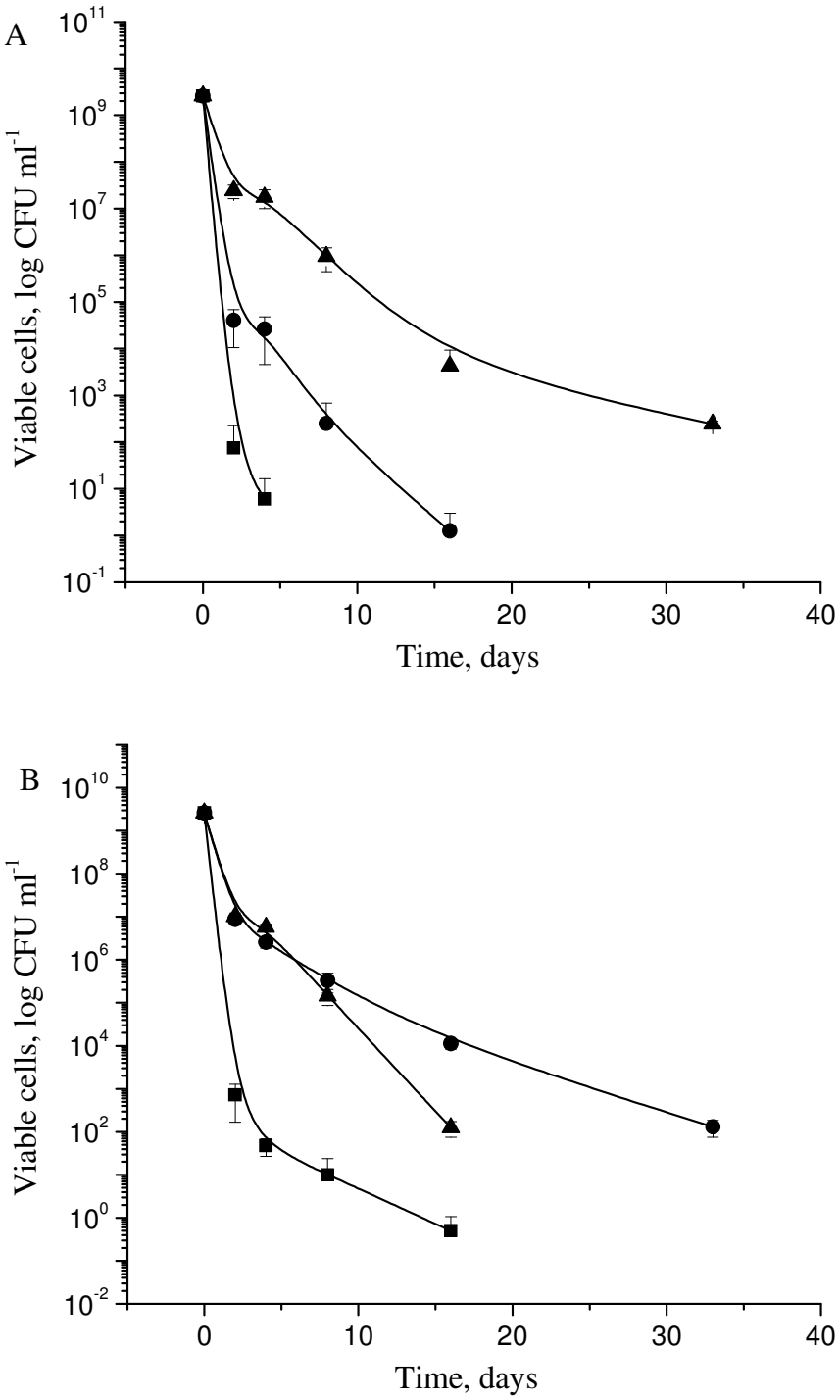
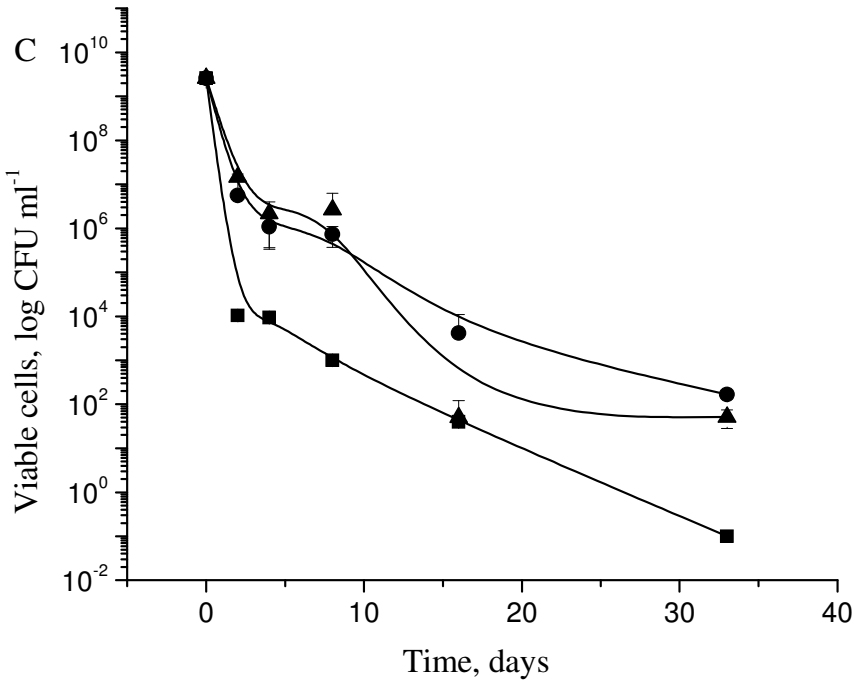


Fig. 2.6. *E. coli* degradation curves for long-term storage at various humidity. AG (▲), pullulan (●), and PBS (■) samples were stored at 46 (A), 53 (B), 76 (C), and 86% (D).



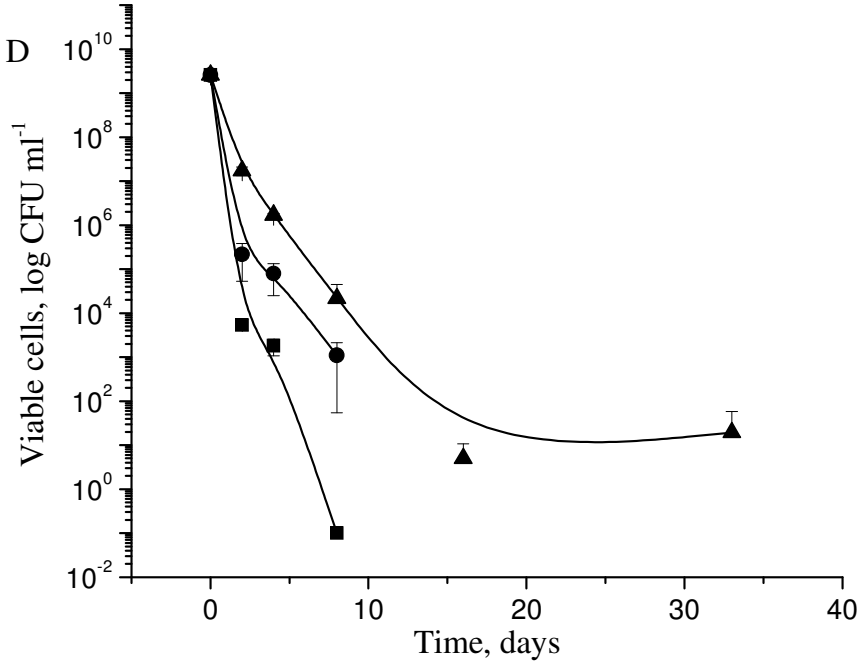


Fig. 2.6. continued.

Membranal site for DNA synthesis becomes damaged when bacteria are freeze-dried and exposed to light and air (Israeli et al., 1993). This site is sensitive to air and oxygen because of membranal phase transitions (Israeli et al., 1993). It is reported that desiccated *E. coli* release nucleases which may be the cause of DNA single strand breaks and induction of mutation (Ashwood-Smith, 1980a). Cells may be able to repair DNA damage caused by desiccation. However, bacteria with active DNA repair mechanisms were found to contain more mutations than bacteria preserved via desiccation (Heckly, 1980). The samples in our study were exposed to air oxygen before and during drying. This could contribute to the increase in cell death compared to other preservation methods such as lyophilization where exposure to oxygen is minimal (Malik, 1988b). Preservation of bacteria in AG could be improved by drying under low-vacuum to eliminate exposure to oxygen in the air (Iijima and Sakane, 1973; Israeli et al., 1975). In addition, removal of oxygen from AG (degassing AG) before mixing with bacteria could also contribute to increase cell viability by decreasing the exposure to oxygen. Another aspect that should be considered, is the addition of substances that are good scavengers of toxic oxygen such as activated charcoal (Malik, 1990b; Malik, 1992). Although our optimization experiments showed that drying under vacuum centrifuge was not favorable for this strain, this could be a result of other factors such as drying rate and sample volume. Another factor that may cause loss of viable cells after freeze-drying is rehydration (MacKenzie and Orndorff, 1972; Heckly, 1980). This factor appears to be negligible since the re-entry of water to bacterial samples preserved in AG was relatively slow (~ 30 min with little agitation). An additional factor that may result in loss of

bacterial function is the time between reconstitution and testing. This was relevant in the study where *Yersinia pestis* increased in virulence the longer it remained in spent media due to repairing injuries caused by lyophilization (Heckly and Quay, 1981). There are inexhaustible possibilities for improving the survival rate of *E. coli* preserved in AG which have not been fully explored and are currently under investigation. Our current results indicate that *E. coli* could be dried and preserved in acacia gum for short-term at elevated temperatures up to 40°C.

AG preservation process could be considered for other strains of gram-negative bacteria. The bacterium tested in this study was confirmed to be desiccate sensitive strain of *E. coli* during our own testing when cells were dried and stored in physiological buffers without protectants. The *E. coli* strain used in our study was also successfully preserved by others in a skim milk complex stored under "cooled conditions" up to 3 years using a freeze-drying method (Joubert and Britz, 1987). However, the complexity of the protocol in this study appeared to be time consuming and involved special equipment and extensive preparation (Joubert and Britz, 1987). Our method for preserving bacteria with AG is most similar to liquid drying except our method did not utilize a vacuum. It has been reported that the liquid drying method recovers more viable and functional gram-negative bacteria when compared to freeze-drying (Lang and Malik, 1996). Viability of bacteria after drying in vacuo (similar to liquid drying) varies depending on the strain. For example, 42% of viable *Aerobacter aerogenes* cells were recovered after 6 months of storage at 37°C; whereas, only 3% of *E. coli* cells were recovered using the same method (Iijima and Sakane, 1973). Freeze-drying is the most popular method for preserving bacteria because the viability remains high after

lyophilization and long-term storage; for example, 50% of gram-negative bacteria were viable after lyophilizing in skim milk and sodium glutamate and storing at 5°C for up to 15 years (Miyamoto-Shinohara et al., 2000). A method for freeze-drying desiccant sensitive *E. coli* was successful when using trehalose and exposing the product to various environmental conditions (Israeli et al., 1993). However, trehalose is relatively expensive compared to AG, and the method involves freeze-drying and special formulations. The variations in recovered cell viabilities are in part a result of the varying species, so strains less sensitive to desiccation may be preserved much longer than the strain of *E. coli* used in our study.

Numerous applications can be explored for using AG as a desiccated bacterial protectant. AG could be used in junction or replacement of trehalose which is used in lyophilizing modified *Janthinobacterium lividum* bacteria for bioluminescent assays (Cho et al., 2004). Microorganisms mixed with AG could possibly be dried on microbial biosensers for use in detection of harmful chemicals or biologicals (Beyersdorf-Radeck et al., 1993b; Zourob et al., 2004; LaGier et al., 2005; Peltola et al., 2005). For instance, *Alcaligenes eutrophus* are well known for degradation of xenobiotics (Don and Pemberton, 1981; Don and Pemberton, 1985). These bacteria have been successfully cryopreserved in polyvinylethanol and glycerol using liquid nitrogen; however, the bacteria must be isolated from protective agents before it can be used as a sensing component of a biosensor (Beyersdorf-Radeck et al., 1993b; Gu et al., 2001). Using AG as a protectant may provide means that would not require complete removal of AG from the bacteria in order for it to regain physiological activity. Acacia gum has been used in enrichment media to cultivate recovered bacteria from freeze drying. The recovery of

freeze-dried *Streptococcus thermophilus* was increased when plating media was supplemented with AG (Morichi and Irie, 1973). This indicates that the presence of AG in the growth media had a positive effect on cellular function upon recovery from desiccation. AG is a well established substance with many applications and to this list we add preservation of bacterial vegetative cells. The method developed in our study provide an alternative for economically preparing industrial volumes of bacterial product and for preserving bacteria when traditional methods are impractical such as collecting complex biological samples in the field at ambient temperatures.

2.4. Conclusions

E. coli viability varies significantly depending on storage conditions. *E. coli* vegetative cells were best preserved when diluted in 15% AG and dried in small Petri plates in a 40° C incubator containing silica desiccant for ~20 hours. Long-term storage was optimal when samples were maintained at 5° C at low humidity. The time to degrade *E. coli* to 100 CFU when stored at 5, 15, 25, and 40° C are 660, 240, 65, and 20 days, respectively. *E. coli* cells preserved in AG were less sensitive to desiccation than control and retain their viability under cool and dry storage conditions. For extended period of time, *E. coli* cells retain their viability in AG at temperatures and humidity levels of 5-15ºC and 30-46%, respectively.

CHAPTER 3

PRESERVATION OF GRAM-POSITIVE SPORE-FORMING BACTERIA IN PROTECTIVE POLYMERS

3.1. Introduction

In general, gram-positive bacteria are robust, so they are capable of withstanding adverse conditions during preservation processes. Contributing factors to their resistance include the bacterial cell wall composition and production of endospores by many gram-positive bacteria. The most important aspect for preserving bacteria is maintaining viability and genetic consistency. Factors that play a role in successful preservation are mode of drying, drying and storage temperature, protectants, and osmotic stress (de Valdez et al., 1983; Desmond et al., 2001; Desmond et al., 2002). In addition, the preservation process and protectants used must be appropriate for specific applications. For example, protectants should be categorized as GRAS (generally recognized as safe) when preserving bacteria for probiotics or food starters (Holzapfel et al., 1995; Schillinger et al., 1996; Plessas et al., 2005). When preparing large volumes of preserved bacteria, protectants should be inexpensive, non-toxic and biodegradable for applications such as bioremediation (Khan et al., 1996) or crop protection (Collins and Jacobsen, 2003).

Current methods for preserving gram-positive bacterial cultures for industrial use predominately include spray-drying and freeze-drying (Tsvetkov et al., 1980; Desmond et al., 2002; Leverrier et al., 2005). Spray-drying generally involves high temperatures that are harmful to some gram-positive bacteria (Ananta et al., 2005), which contributes to the loss of viability (Desmond et al., 2001). These high temperatures may cause denaturation of structural and functional proteins that are detrimental for cell survival (Hutter et al., 1995). The complexity of the spray-drying process may involve multiple encapsulation stages (Millqvist-Fureby et al., 2000). Lyophilization can be challenging because success depends on numerous parameters such as chemical characteristics of protective media, freezing and drying time, and bacterial strain (Barbaree, 1976). In addition, many freeze-driers may be problematic due to aseptic transferal of bacterial product (Joubert and Britz, 1987). Another method for preserving bacteria uses a fluidized bed dryer where viability depends on the rehydration temperature of the dried bacterial samples (Mille et al., 2004). In summary, current techniques for preserving gram-positive bacteria have several drawbacks. Such techniques cause damage to cell wall, cytoplasmic membrane and DNA during desiccation or freezing

There are many types of protective agents used in all methods of preservation. Most methods employ the use of skim milk as the major protective agent in freeze-drying and spray-drying (Tsvetkov et al., 1980; de Valdez et al., 1983; Moreira et al., 1995; Desmond et al., 2002; Leverrier et al., 2005). In addition, milk proteins such as casein have been successfully used to preserve lactic acid bacteria (Mille et al., 2004). Other protective agents include polymers such as sodium alginate, xanthum (Leverrier et al., 2005), bovine albumin and dextran, polyols such as glycerol, amino acids such as

glutamate and cysteine (de Valdez et al., 1983). Most of these agents have not been proven to be effective protectants for thermophilic lactic acid bacteria during freeze-drying (de Valdez et al., 1983). Whey protein is a natural polymer used along with milk fat to immobilize *Bifidobaterium* for probiotic supplementation in yogurt (Picot and Lacroix, 2004). There are numerous methods and protectants for preserving gram-positive bacteria. The development of a process that is applicable for many types of bacteria and utilizes inexpensive materials (Yamasato et al., 1973) would be attractive for industrial production bacteria used in probiotic, food starters, bioremediation and crop protection.

Acacia gum is a natural polymer that may be a useful protectant for many types of bacteria. AG has been shown to improve survival of probiotic *Lactobacillus* during spray-drying, storage and gastric transit (Desmond et al., 2002). AG could be used to microencapsulate and protect probiotic bacteria during transit to the intestines (Desmond et al., 2002) such as *Bifidobacteria longum* and *Bifidobacterium breve* which are sensitive to gastric acids and bile (Picot and Lacroix, 2004). The benefits for using the AG preservation process for immobilizing bacteria are numerous. AG is non-toxic (DeCloux et al., 1996; Gamal el-din et al., 2003), non-mutagenic (Anderson, 1986), inert (Mahler et al., 2000), ingestible (Al-Mosawi, 2002), organic (Rehman et al., 2003), inexpensive, simple (Vodyanoy et al., 2003) and biodegradable. Because of these attributes, AG can be used to preserve bacteria for release into the environment without hazardous effects (Anderson, 1986).

In addition, AG is GRAS (Anderson et al., 1982); thus an edible polymer (Gamal el-din et al., 2003) which can be used for preserving bacteria intended for food starters or probiotics.

The main focus of this work was to design and develop a process for long-term preservation of *B. subtilis* at ambient temperature. *B. subtilis* vegetative cells were subjected to a preservation process that promotes spore formation during desiccation. This method involves bacteria suspensions mixed with AG polymer and air dried at 40°C for ~48 h. The samples were stored at various temperatures and humidity, and tested for viability before and after drying and during storage.

3.2 Materials and methods

3.2.1 B. subtilis cultivation, preparation of concentrated cell suspension, and estimating cell viability

Bacillus subtilis ATCC 6051 was purchased from ATCC. *B. subtilis* cultures were grown in Difco nutrient broth for 18 hours (beginning of stationary phase) at 200 rmp and 37° C. Bacterial cells were harvested by centrifuging for 7 min at 4,500 rpm and discarding the supernatant. The concentrated cell suspension was prepared by adding 1 ml of PBS to each pellet formed from 50 ml of original culture. Viability of cultures before and after drying and during storage was tested by diluting samples and plating them on nutrient agar. The detailed protocol for estimating cell viability is described in section 2.2.3

3.2.2 Spore staining

To determine the presence of spores, *B. subtilis* suspension before and after drying were smeared onto glass slides and heat fixed. The stains used for this procedure were 5% malachite green oxalate (cat # M-290) and safranin-S (cat # 212534) purchased from Fisher Scientific. The smears were stained as described by Pierce and Leboffe (Pierce, 1999). These slides were observed using a bright-field Nikon Eclipse E800M microscope. Pictures were obtained digitally using SPOT V.4.0.4 camera model 2.3.0 V1.0 from Diagnostic Instruments, Inc., Sterling Heights, MI. The percent of spores produced were determined by counting spores and comparing these numbers to the vegetative cells present in the pictures.

3.2.3 Optimization experiments

Short-term experiments were performed to determine the optimal conditions for long-term storage of *B. subtilis* samples. The parameters that were optimized included AG concentration, drying temperatures and time, sample volume and containers.

3.2.3.1 Polymer concentration determination procedure

One part concentrated *B. subtilis* suspension was mixed with four parts of 10, 15, 20 or 25% AG. Five hundred microliters of these *B. subtilis* bacterial suspensions in AG were aliquoted into glass vials purchased from Fisher (cat # 03-338C). The samples were

dried uncovered in 40°C incubator (Lab Line Imperial III Model 302, Fisher, cat # 11-702-10) containing silica desiccant beads for approximately 48 h. Dried samples were covered and stored at 25°C. Bacterial viability was tested as described in section 2.2.3 before and after drying and storage on day 11.

3.2.3.2 Container, sample volume and drying temperature determination procedure

B. subtilis bacterial suspensions in 15% AG and PBS were prepared as described in section 3.2.3.1. Container, sample volume, and drying temperature experiments were carried out as described in section 2.4.2 using *B. subtilis* bacterial suspensions. Bacterial viability was tested as described in Chapter 2 section 2.3, before and after drying.

3.2.4 Long-term storage experiments for B. subtilis samples

Based on optimization procedures, the following protocol was used for long-term preservation of *B. subtilis* cells. Bacterial suspensions in 15% AG, 15% pullulan, and PBS were prepared as described in section 3.2.3.1. Bacterial suspensions in 15% pullulan and PBS were used as controls. Glass vials (7.4 ml) were labeled and 500µl of diluted bacterial suspensions were aliquoted into these vials. The samples were dried uncovered on their sides at 40° C in a Lab Line Imperial III incubator (Model # 302) containing silica desiccant beads for approximately 48 h.

For temperature experiments, dried samples were sealed and placed into 470ml square containers as shown in Fig. 2.1 B. Then, the containers were sealed with parafilm

and placed in incubators set at 5, 15, 25, and 40°C. Bacterial viability was tested as described in Chapter II section 2.3, before and after drying and after storage on days 2, 4, 8, 16, 32, 64, and 128.

For humidity experiments, un-capped vials containing dried *B. subtilis* samples were placed in RH chambers as shown in Fig. 2.1 E. RH chambers contained dried bacterial samples in glass vials and small beakers filled with saturated salt solutions (Table 2.1) to maintain relative humidity. The RH chambers were sealed and stored at 25°C. Bacterial viability was tested as described in section 2.2.3, before and after drying and after storage on days 2, 4, 8, 16, 32, 64, and 128.

3.3 Results and discussion

3.3.1 Optimization experiments for long-term preservation of B. subtilis

Numerous drying and short-term storage experiments were conducted to determine the optimal conditions for preserving *B. subtilis* in AG. The parameters that were optimized included concentration of AG, storage containers, drying temperature, method and duration of drying, and storage temperature. A detailed summary of these parameters are shown in Table 2.2. Similar to *E. coli* preservation, controls for *B. subtilis* experiments were PBS (buffer control) and pullulan (polymer control).

Determining the optimal concentration of AG for preserving *B. subtilis* was accomplished by testing bacterial viability after samples were dried and stored for 11 days in various concentrations of polymer ranging from 10 to 25%. Viability curves for

B. subtilis in all concentrations of AG are shown in Fig 3.1. After drying in glass vials, the percent of viable cells of *B. subtilis* samples remaining in 10, 15, 20, 25% AG was 49.0, 57.8, 25.8, and 2.3%, respectively. By day 11 of storage at 25°C, the percent of viable cells preserved in 10, 15, 20, 25% AG was 59.7, 61.4, 25.9, 2.4%, respectively. Similar to *E. coli*, more viable cells were recovered from *B. subtilis* samples dried in 15% AG.

Several parameters were tested to determine the optimal container and drying method for preserving *B. subtilis*. Desirable qualities of the drying method include simple drying procedure that would maximize the number of viable cells recovered. The results from these experiments are shown in Fig. 3.2. Bacterial samples dried under static high vacuum, vacuum centrifuge, low vacuum desiccator and incubator in 2.0 ml tubes were less than 3.0% viable. In addition, bacterial cultures dried in small Petri plates and 6-well plates at 40°C recovered 5.5 and 7.8% of viable cells, respectively. The viability of bacterial samples in AG and dried in glass vials for 48 h at 40°C was ~100%. This was indicated by colonies formed from these samples after drying and reconstitution in water. The optimal parameters for recovering *B. subtilis* from 15% AG were determined to be drying cell suspensions in glass vials for 48 hr in 40°C incubator containing silica desiccant. The high viability of *B. subtilis* cultures after drying for 48 h implied that spore formation was probable. Spore staining was performed before and after drying in AG. Vegetative cells produced 99% of free spores after 48 hr of drying (Fig 3.3 B). Drying *B. subtilis* vegetative cells in glass vials for 48 hr at 40°C produced viable spores.

3.3.2 Long-term preservation of B. subtilis

B. subtilis samples were dried in 15% AG, 15% pullulan (control polymer) and PBS (control buffer) at 40°C for 48 hr to determine the viability of cells during long-term preservation under various conditions. There were two types of experiments performed: (1) storage at various temperatures and constant humidity and (2) storage at various humidity and constant temperature. From this point forward, these experiments are referred to as temperature and humidity experiments. For both types of experiments, *B. subtilis* vegetative cells were mixed with 15% AG, placed into glass vials and dried for 48 h at 40°C. There was no significant loss of bacterial viability of cells dried in AG (~100%); whereas, bacterial viability of *B. subtilis* cells dried in pullulan and PBS were two logs less than original titer (OT).

For temperature experiments, vials containing dried samples were sealed and stored at various temperatures (5, 15, 25 and 40 °C) and constant humidity (~33 %). Viability curves for *B. subtilis* stored at various temperatures are shown in Fig. 3.4. After drying for 48 hr, spores were stabilized, and there was no significant decline in bacterial titer up to 128 days of storage for all samples. The experiment was extended to 615 days where the viability of *B. subtilis* spores remained consistent with the titer obtained after drying. The percent of viable *B. subtilis* cells recovered from AG on day 615 when stored at 5, 15, 25, 40°C was 55.9, 106.3, 69.5, and 86.8, respectively.

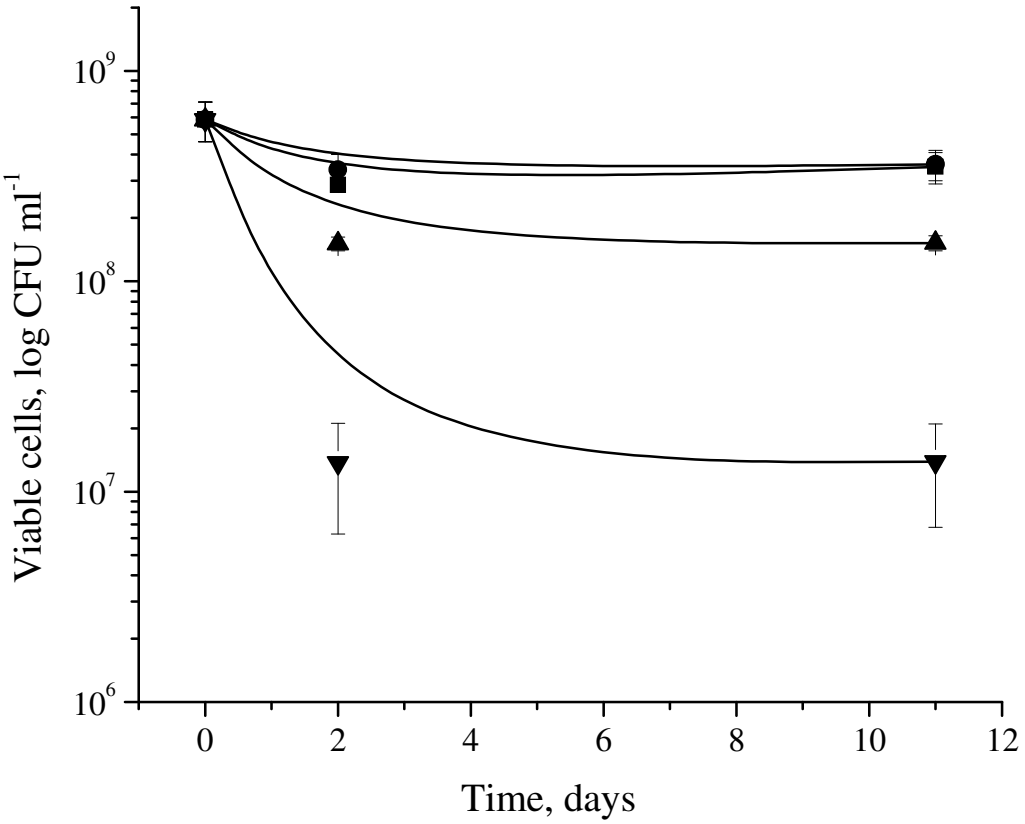


Fig. 3.1. Optimizing AG concentrations for preserving *B. subtilis* for long-term storage. AG was mixed with water in concentrations of 10% (■), 15% (●), 20% (▲), and 25% (▼). Bacterial suspensions were mixed with the various AG concentrations, aliquoted into glass vials and dried at 40 °C for approximately 48 hours. Samples were stored at 25°C. Errors bars were indicative of standard deviation calculated from 3 samples.

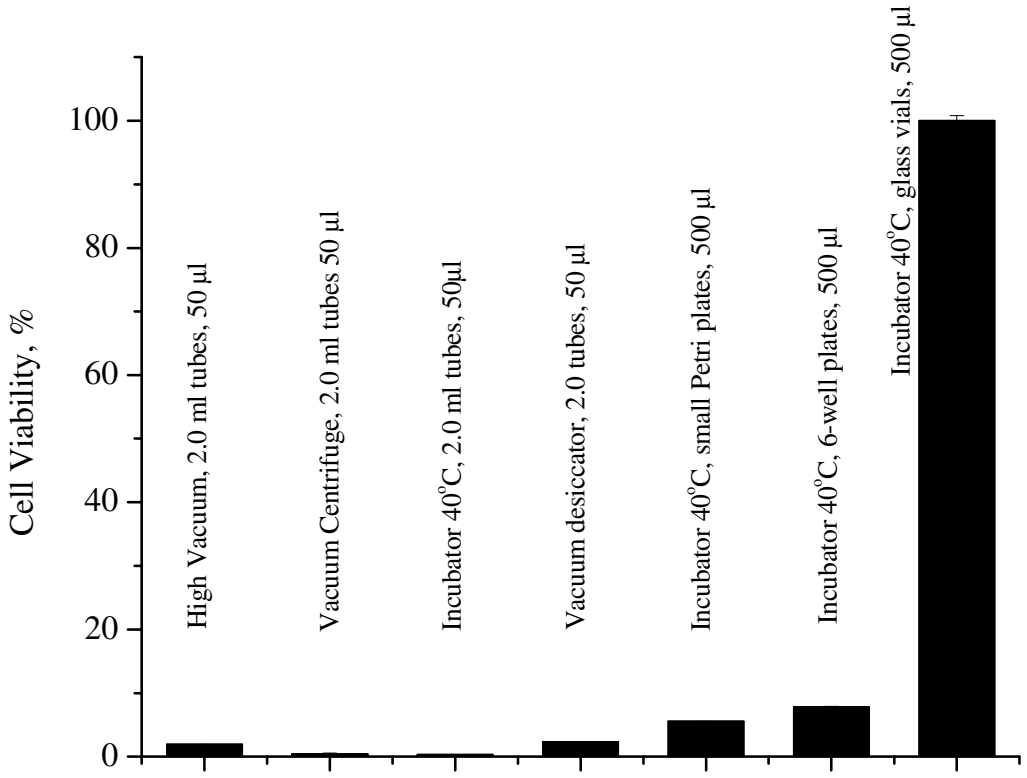


Fig. 3.2. Determination of an optimal method for preserving *B. subtilis* in 15% AG. The x-axis depicts various methods of drying, containers, and sample volume. The y-axis represents relative % of viable cells recovered after drying (CFU ml^{-1}). A summary of all parameters tested are listed in Table 2.2.

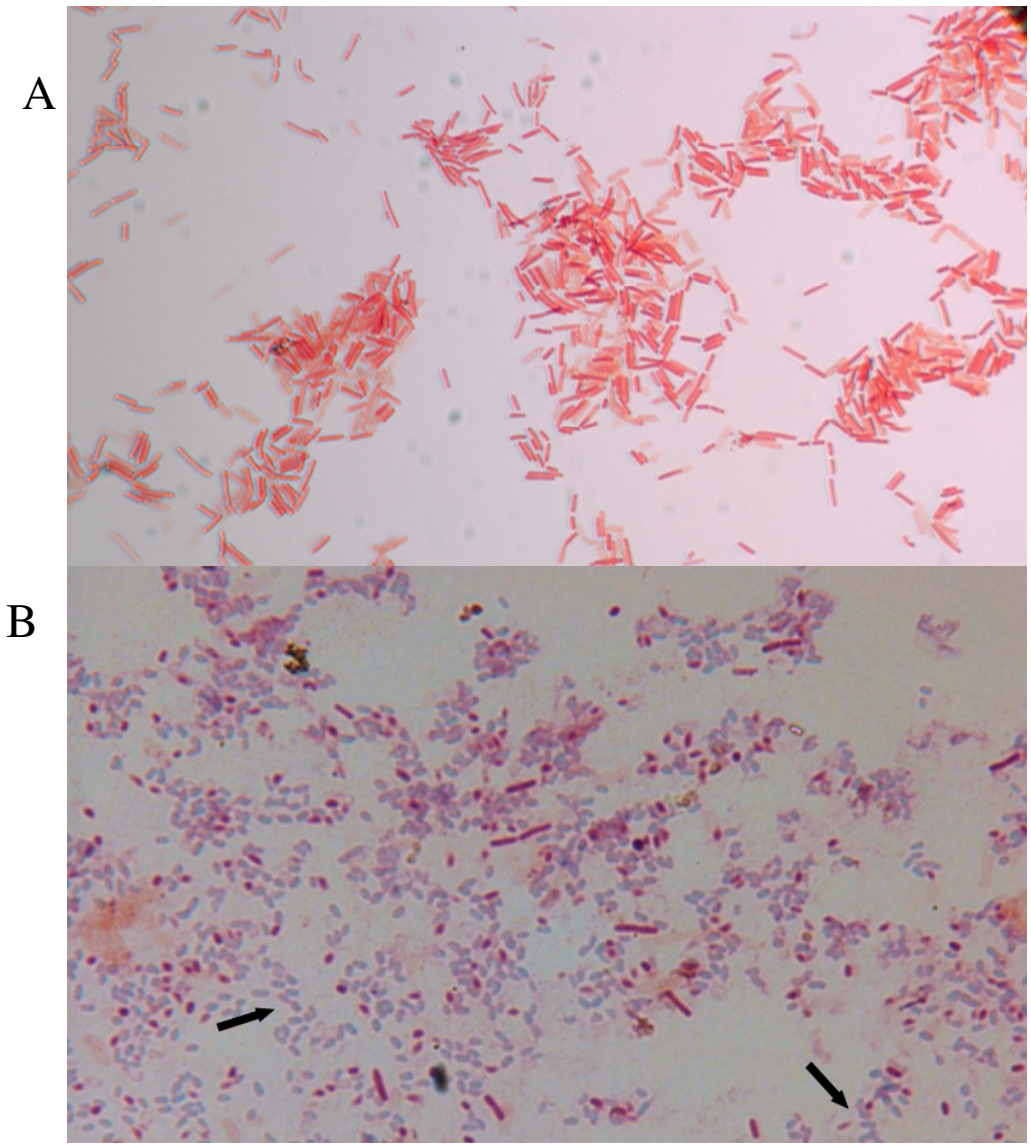


Fig. 3.3. Bright-field microscopy revealing formation of spores during desiccation in AG. (A) *B. subtilis* culture before desiccation in 15% AG. Vegetative cells when stained are fuchsia. (B) *B. subtilis* culture after drying for 48 hr. Spores when stained are green (arrows are pointing to spores). Magnification 1000 x.

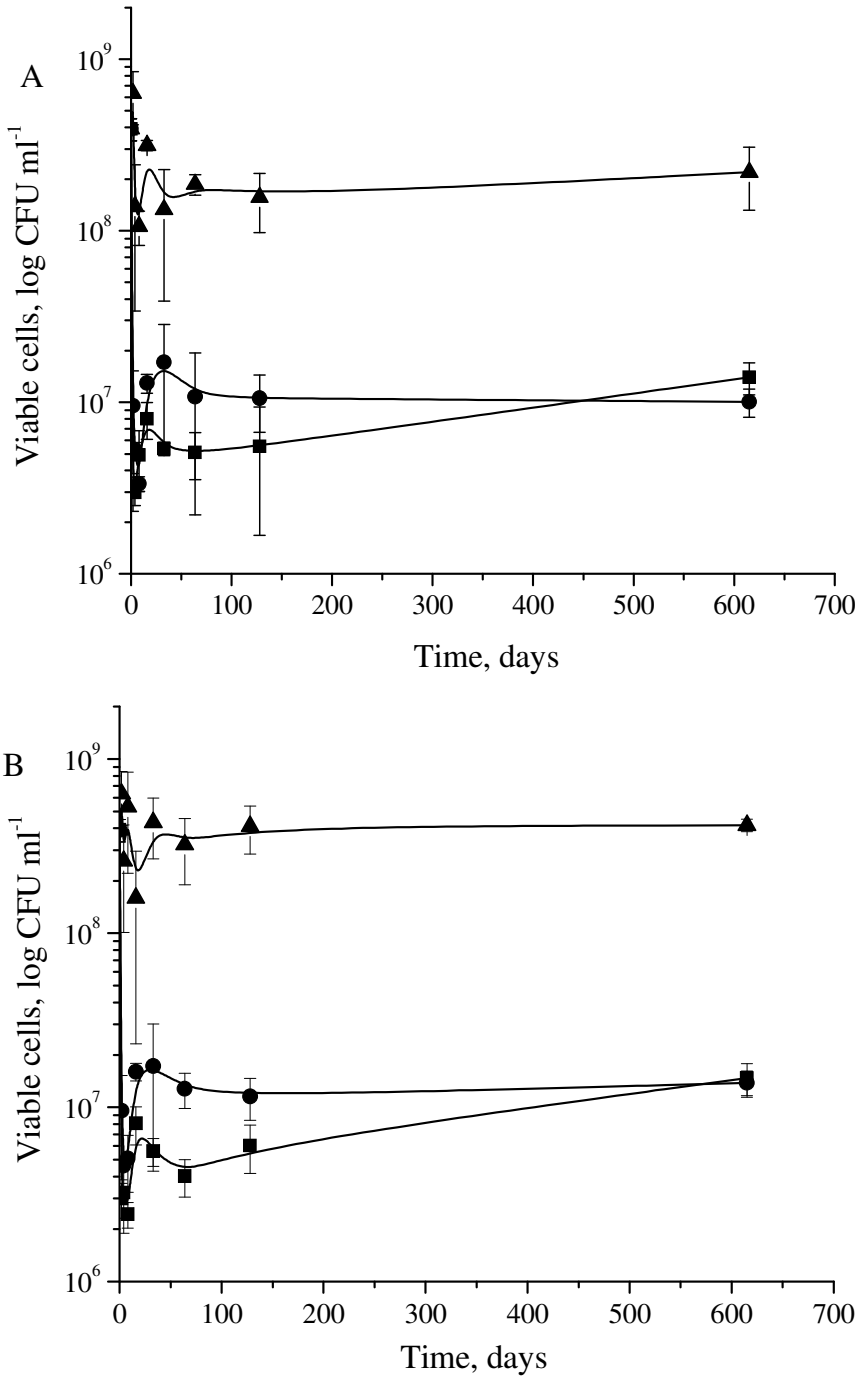


Fig. 3.4. *B. subtilis* degradation curves for long-term storage at various temperatures. AG (▲), pullulan (●), and PBS (■) samples were stored at 5°C (A), 15°C (B), 25°C (C), and 40°C (D).

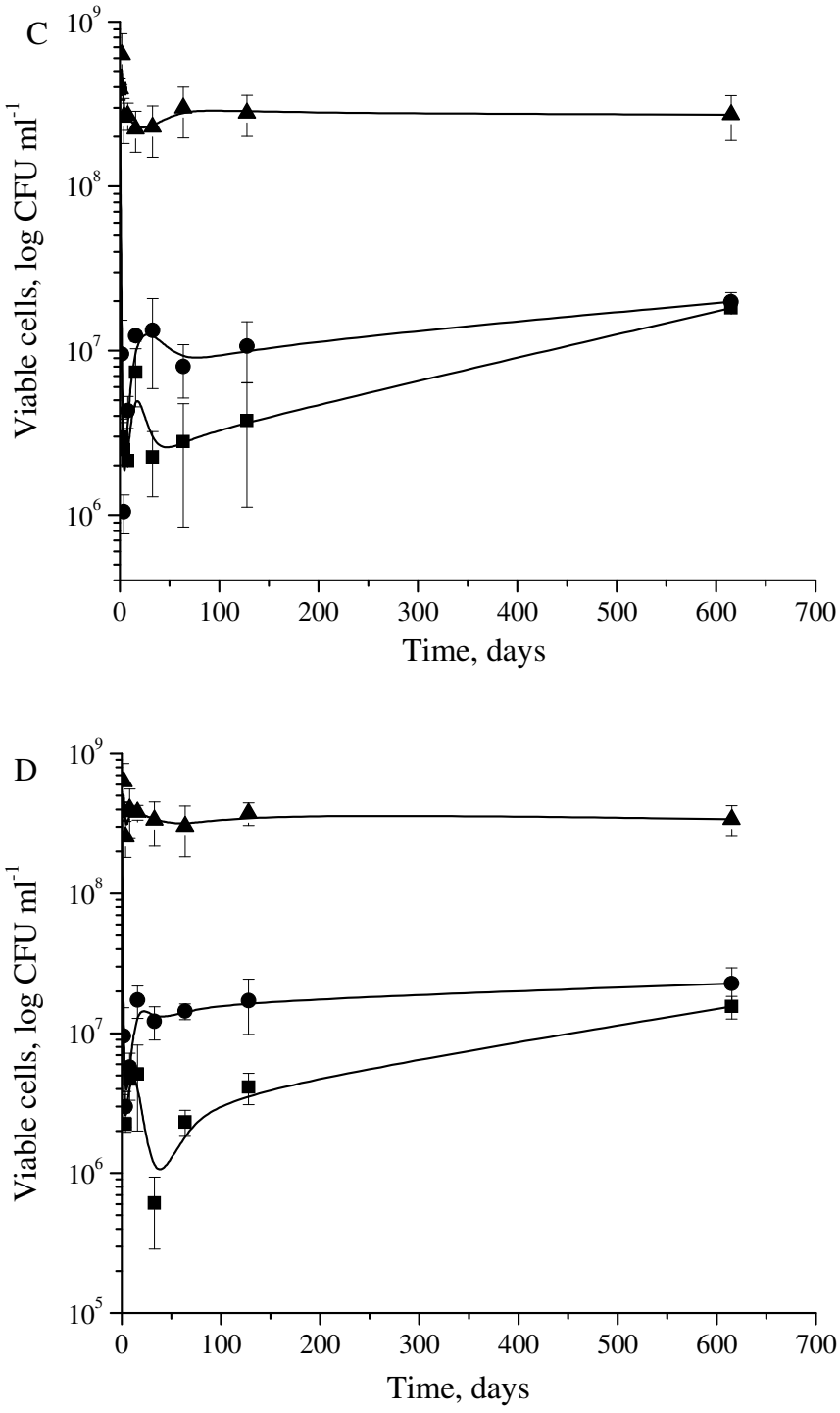


Fig. 3.4. Continued.

B. subtilis samples were subjected to 46, 53, 76 and 86% humidity at constant temperature (25°C) for 128 days of storage. Humidity experiments are summarized in viability curves shown in Fig. 3.5. Initially, more viable cells were recovered from AG samples than pullulan and PBS after drying; however, the viability of *B. subtilis* cells remained steady for all humidity levels up to 128 days.

3.3.3 Discussion

Preservation of spores is generally unproblematic because they are highly resistant to adverse conditions such as freezing, dehydration and high temperatures up to 100°C (Ishihara et al., 1994; Stragier and Losick, 1996). The obstacles faced with preserving spores for numerous applications involve the processes of growing and harvesting spores. Another concern is the aggregation or clumping of spores during removal from solid media (Jinks et al., 1985). For example, spore suspensions used in vaccine studies are prepared by growing *B. subtilis* on Difco-sporolation media (Duc et al., 2003). Then, the cells are treated with lysozyme and washed several times (Duc et al., 2003). Another example, a method for preparing and preserving spores for large scale laboratory use involves growing cells on agar, harvesting, homogenizing and then freezing in glycerol (Stastna and Janda, 1983). The aforementioned procedures for spore preparations involve transferring spores and several steps which increase the chances of contamination. The method for preparation and preservation of spores used in this study does not require harvesting from sporulation medium because spores are formed in synchronization with the preservation process.

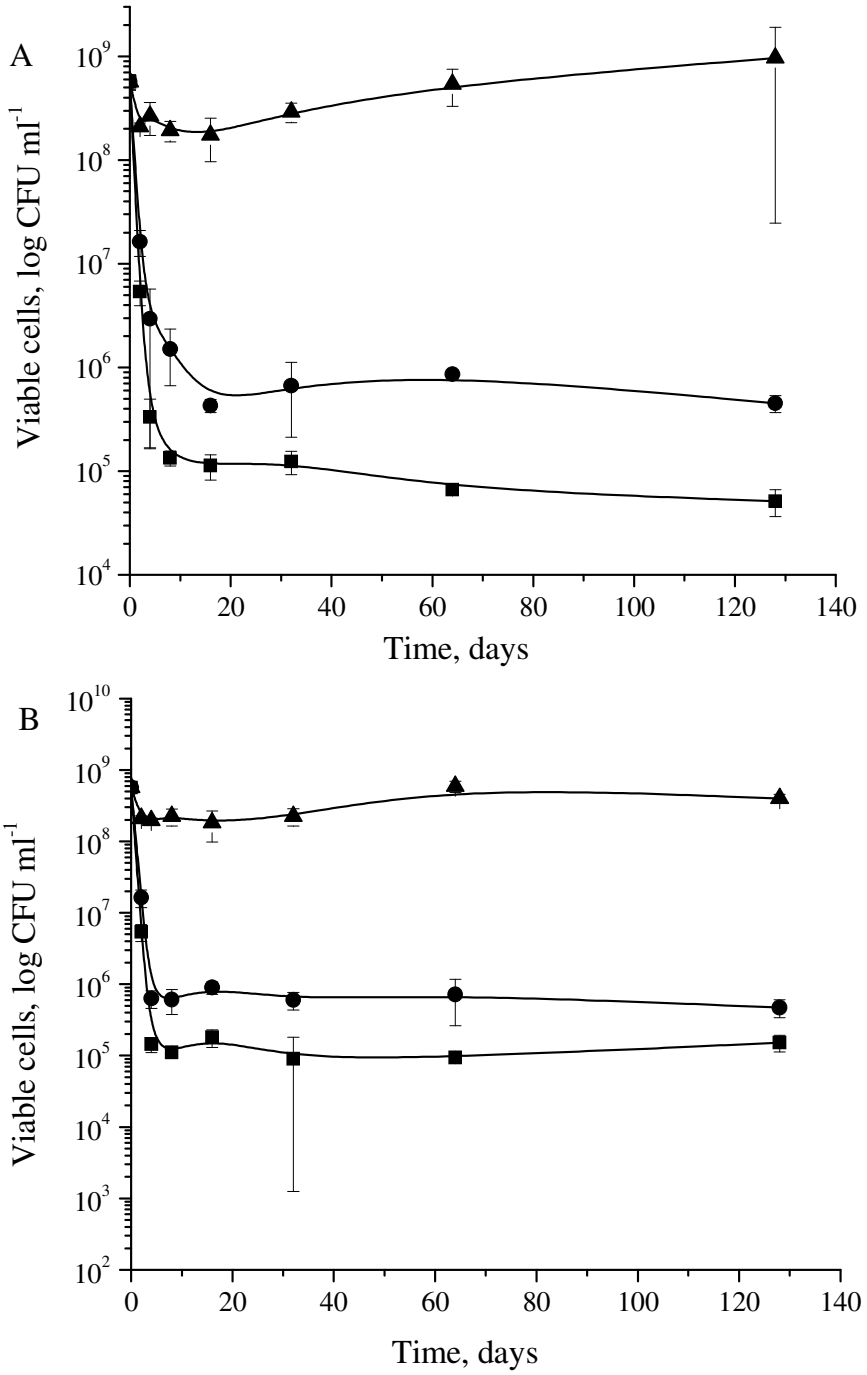


Fig. 3.5. *B. subtilis* viability curves for long-term storage at various humidity. AG (▲), pullulan (●), and PBS (■) samples were stored at 46% (A), 53% (B), 76% (C), and 86% (D).

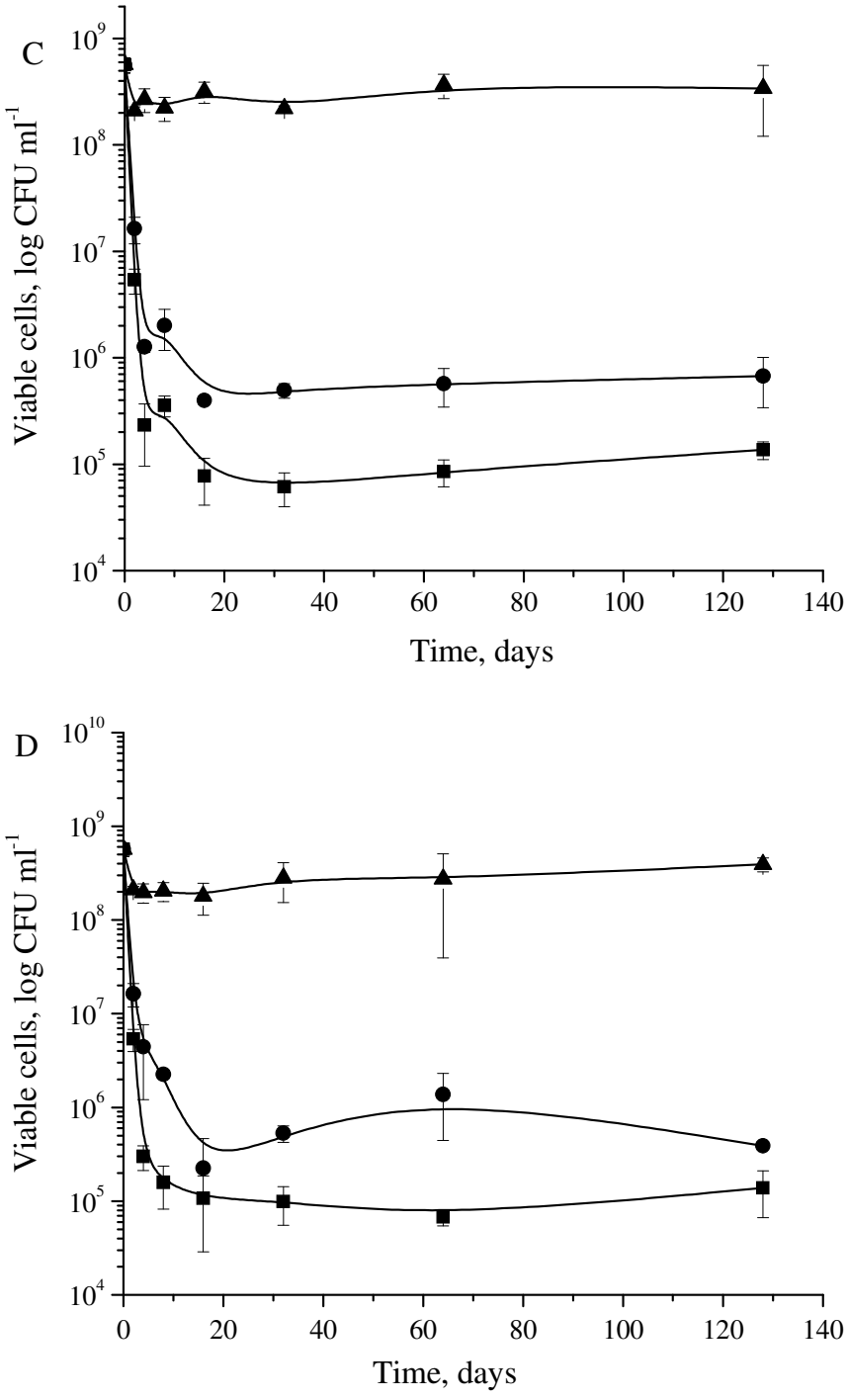


Fig. 3.5. Continued.

In this study, the formation of mature *B. subtilis* spores was found to be critical for long-term preservation at all tested conditions. This was noticed in the course of short-term optimization experiments. Those samples which dried rapidly and did not have time to form spores had less numbers of viable cells compared to the samples that were dried for longer period of time and formed approximately 100% spores. *B. subtilis* cells dried in 20 and 25% AG did not recover as many viable cells as those samples dried in 10 and 15% AG. Samples in higher concentration of AG take less time to dry, so the shorter drying time seemed to have an effect on spore maturation and viability. When *B. subtilis* vegetative cells were dried for 24 hr, survival was less that 8% for all containers, sample volumes and drying methods tested. In agreement with Magyarosy *et al*.(Magyarosy et al., 2002), our results confirmed that drying time needed to be longer for complete endospore formation and maturation.

In our experiments, the AG polymer was tested for its qualities as a protectant for long-term spore preservation and was compared to controls. In both controls, 15% pullulan and PBS samples, viability declined by 2 logs after drying. There was no significant decline in titer for 15% AG samples dried in glass vials for 48 hr at 40°C. Subsequent days tested for all samples showed no decline in titer up to 128 days of storage. This indicated that once spores were formed, they were stable throughout all conditions tested. Our results suggest that AG provides a suitable environment for a high degree of sporulation. In addition, the process for spore formation and preservation occurs simultaneously so the dried product can be used immediately or stored for long-term.

Traditional preservation techniques for gram-positive bacteria have several drawbacks such as utilization of complex equipment, involvement of multiple steps and cell damage. These techniques include lyophilization, freezing, spray-drying and vacuum desiccation. Microionization of freeze-dried probiotic bacteria was performed in an effort to protect bacteria from oxygen, acid, freezing and gastro-intestinal fluids (Picot and Lacroix, 2003). However, the bacterial viability declined even further after microionization with decreasing particle size (smallest particle size was one micron). This method appeared to damage bacteria by breaking cells and by increasing exposure to oxygen (Picot and Lacroix, 2003; Picot and Lacroix, 2004). Freezing and most spray-drying techniques involve extreme low and high temperatures to preserve gram-positive bacteria, respectively (Ananta et al., 2005). Our method is more favorable than traditional preservation techniques because it is simple and does not decrease viability of bacterial preparations. Since AG is non-toxic, biodegradable and GRAS, removal of AG from bacterial preparations can be eliminated when used for probiotics or food starters.

Ultimately the preservation of bacteria depends on the strain and its survival mechanisms such as spore formation. Some gram-positive bacterial strains are more sensitive to freeze-drying and spray drying than others such as lactobacilli are more delicate to desiccation than streptococci (Wang et al., 2004). These finding are indicative of the genomic variation (van Schaik and Abee, 2005), so each bacterium must be tested in order to determine if AG is an appropriate method for preserving many strains of bacteria.

3.4. Conclusions

B. subtilis is optimally recovered when suspended in AG and dried for 2 days in glass at 40° C. Bacterial titer in PBS and pullulan controls after drying are 100 fold less than bacterial titer in AG. *B. subtilis* vegetative cells form spores during the 2 day drying process. For both temperature and humidity experiments, *B. subtilis* viability remains consistent after spores are formed. AG provides better environment for spore formation than pullulan and PBS. *B. subtilis* cultures were successfully preserved in AG polymer up to 615 days.

CHAPTER 4

ULTRASTRUCTURAL ALTERATIONS IN BACTERIAL CELLS IN THE COURSE OF PRESERVATION IN ACACIA GUM

4.1. Introduction

When preserving bacterial cells via freeze-drying, spray-drying or air-drying, removal of water is necessary for bacteria to enter a quiescent state. This induction of water loss jeopardizes the structural integrity of DNA, proteins, carbohydrates and lipids within bacterial cells (Potts, 1994). In addition, it is well documented that the aforementioned preservation techniques induce structural damages to the cell wall and cell membrane (Broadbent and Lin, 1999; Beal et al., 2001; Gouesbet et al., 2001; Carvalho et al., 2004). Consequences of freezing and freeze-drying include protein denaturation that leads to the decrease in cell viability (Leslie et al., 1995a; Carvalho et al., 2004). Spray-drying is performed at elevated temperatures (O'Riordan et al., 2001; Lian et al., 2002; Corcoran et al., 2004) which damages DNA, cell membrane lipids and ribosomes (Teixeira et al., 1997). If cellular components such as DNA are damaged during the preservation process, some bacteria are capable of repair via nucleotide excision repair mechanisms. However, others may undergo irreversible damage or lack the ability to repair.

In general, gram-positive bacteria are more resistant to desiccation than gram-negative bacteria (Janning and in't Veld, 1994; Miyamoto-Shinohara et al., 2000). This resistance is partially due to the composition of the cell wall, cytoplasmic membrane and the defense mechanisms such as those that prepare bacteria for changes in osmolarity (Janning and in't Veld, 1994; Heermann and Jung, 2004). The cell walls of gram-negative bacteria consist of an outer membrane containing lipopolysaccharide, a thin layer of peptidoglycan, polysaccharides, lipids and proteins. Gram-negative bacteria have a distinguishable gap between the cell wall and cell membrane which is called the periplasmic space. The cell walls of gram-positive bacteria lack an outer membrane, lipopolysaccharide, and many of the proteins and lipids present in gram-negative bacteria. The composition of gram-positive cell walls differs in that it contains a thick layer of peptidoglycan and teichoic or teichuronic and lipoteichoic acids. These bacteria also lack a periplasmic space between the cell wall and cell membrane.

The central defense mechanism which involves over 125 genes for many gram-positive bacteria is the ability to form endospores (Stragier and Losick, 1996). During desiccation, sporulation is induced when the transcriptional protein Spo0A is activated (Roberson and Firestone, 1992; Stragier and Losick, 1996). This activation may be a result of signals from stress such as reduction of nutrients caused by decrease in water potential (Roberson and Firestone, 1992). Spores are highly resistant to adverse environmental conditions such as desiccation, exposure to oxygen, osmolarity, high levels of humidity, and high temperatures, which are general concerns when preserving vegetative bacterial cells (Tunnacliffe et al., 2001). The spores are resistant to

desiccation mainly because the outer core protects against dehydration that would compromise sensitive structures such as DNA (Ishihara et al., 1994).

Other mechanisms bacteria use for resisting desiccation include accumulation of protectant molecules. Trehalose and sucrose have been reported to increase desiccation tolerance by protecting proteins (Carpenter et al., 1987; Crowe et al., 1987) and replacing water that is removed from the cell membranes during desiccation (Crowe et al., 1988; Leslie et al., 1995b). Some bacteria are capable of producing molecules that protect against water loss. For example, when *Pseudomonas* sp. underwent desiccation, there was an accumulation of exopolysaccharide (EPS) which has a capacity for retaining water (Roberson and Firestone, 1992; Roberson et al., 1993). Although most bacteria are capable of withstanding some level of stress, the use of additional protectants during desiccation or freezing has been successful in preserving more viable and unaltered bacteria.

In our work (Chapter 2 and 3), acacia gum (AG) has been used as a protectant during desiccation of *E. coli* and *B. subtilis*. *E. coli* cells preserved in AG were less sensitive to desiccation than controls and retained their viability under cool and dry storage conditions. *B. subtilis* preserved in AG were more robust than control cells and retained their viability under all tested storage conditions. Here, we concentrate on structural alterations in bacterial cells that occurred after drying and storing in AG. This was accomplished by characterizing the ultrastructure of the cell wall, cell membrane and cytoplasm of bacteria in the presence of AG using electron microscopy. In addition, fluorescent microscopy was used to determine cell membrane intactness before and after drying in AG.

4.2. Material and methods

4.2.1 Preparation of bacterial samples

E. coli ATCC 1175 and *B. subtilis* ATCC 6051 were grown in Difco nutrient broth for 18 h in a shaker incubator set at 200 rmp and 37°C. Bacterial cells were harvested by centrifuging *E. coli* cultures for 10 min at 5,000 rpm and *B. subtilis* cultures for 7 min at 4,500 rpm. The supernatant were discarded. The concentrated cell suspensions were prepared by adding 1 ml of PBS to each pellet formed from 50ml of original culture. Bacterial samples in AG and PBS were prepared and dried as described in section 2.2.4.1 for *E. coli* samples and section 3.2.3.1 for *B. subtilis* samples. The dried samples were stored at 5 and 25°C until testing days.

4.2.2 Electron microscopy

Bacterial samples before and after drying, and after storage in 15 % AG and PBS were reconstituted with 500 µl of water and fixed over night in 1 ml of EM fixative (1% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer, pH 7.4) at 4°C. The bacterial cells were agar-embedded in 1% SeaPlaque purchased from FMC Marine Colloids (Rockland, ME). The preparations were post-fixed for 2 h at room temperature in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4. Then, samples underwent dehydration in ascending concentrations of ethanol. The samples were treated twice with transitional solvent propylene oxide each time for 10 min at room temperature.

Then, the samples were infiltrated into spur resin and the blocks were cured. Ultra-thin sections (70 nm) were stained with uranyl acetate and lead citrate. These sections were observed with Philips 301 TEM operated at 60kV.

4.2.3 Fluorescent microscopy

Dried bacterial samples were reconstituted with 500 µl of water and exposed to 0.75 ul of each fluorescent dye SYTO 9 and propidium iodide (Live/Daed® Baclight™ Bacterial viability kit from Molecular probes (Eugene, OR, USA) for 15 min in the dark. After incubation, 5 µl of each sample was placed on microscope slide with cover slip. These samples were observed using a Nikon Eclipse E800M microscope with a dual cube filter, 51006 FITC/Texas Red® from Chroma Technology Corp. (Rockingham, VT). Pictures were obtained digitally using SPOT V.4.0.4 camera model 2.3.0 V1.0 from Diagnostic Instruments, Inc. (Sterling Heights, MI).

4.3. Results and discussion

4.3.1 Evaluation of structural alterations of E. coli cells dried in AG

Experiments were performed to detect alterations in the ultrastructure of *E. coli* cells during preservation. Using electron microscopy, *E. coli* samples in AG and PBS were examined before and after drying. Subsequently, the samples were stored at 5 and 25º C and tested in the course of preservation on day 14 and 139. Additionally, AG and

PBS samples stored at 5°C were plated on nutrient agar for 24 h to characterize the progeny of cells after preservation.

An electron micrograph of AG with no cells present is shown in Fig. 4.1. The polymer did not interfere with the EM processing, since AG particles could be differentiated from bacterial cells. *E. coli* cells in growth media (Fig 4.2) were used as a reference for detecting irregularities in ultrastructure of preserved cells. This micrograph shows that cells had smooth edges around the cell wall and cell membranes. The cells in nutrient broth had dense cytoplasms that maintained a normal matrix. There were vacuoles in some cells, but no invaginations in the cell walls or cell membranes. Fig. 4.3 A and B show freshly cultured *E. coli* cells that were suspended in AG and PBS, respectively. There were no apparent differences between cells suspended in nutrient broth, AG and PBS before drying. Fig. 4.3 C depicts *E. coli* cells dried in AG and reconstituted in water. Cells dried in PBS and reconstituted in water are shown in Fig. 4.3 D. Cells dried in AG (Fig 4.3 C) are more electron dense in the cytoplasm than cells in Fig. 4.2 and Fig. 4.3 A-B and D. The cells dried in AG appear to have invaginations in the cell wall and cell membrane which may imply dehydration.

Micrographs of samples tested on day 14 are shown in Fig. 4.4. *E. coli* cultures preserved in AG and stored at 5°C are shown in Fig 4.4 A. These cells had more invaginations and irregularities than those stored in PBS for 14 days (Fig. 4.4 B). *E. coli* cells in AG stored at 25°C were noticeably different than those in PBS stored at 25°C (Fig. 4.4 D). Regardless of storage temperature, there were no dissimilarities in ultrastructures of cells preserved in AG for 14 day.

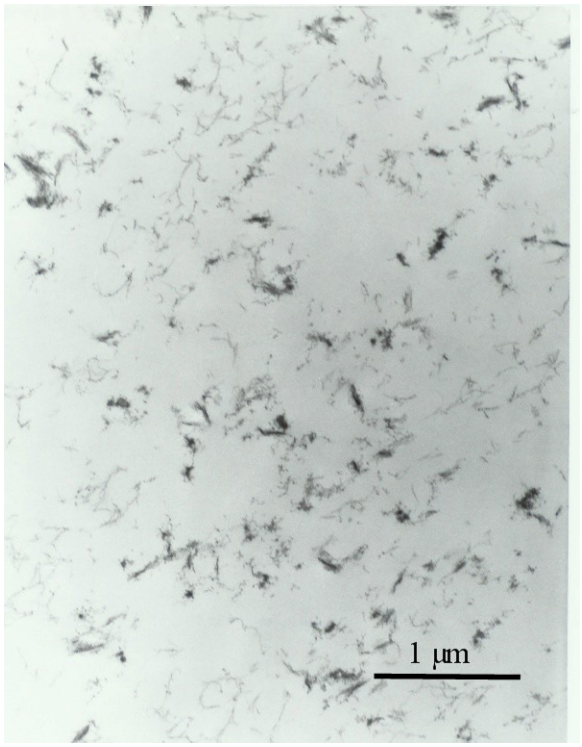


Fig. 4.1 Electron micrograph of AG with no bacterial cells present.

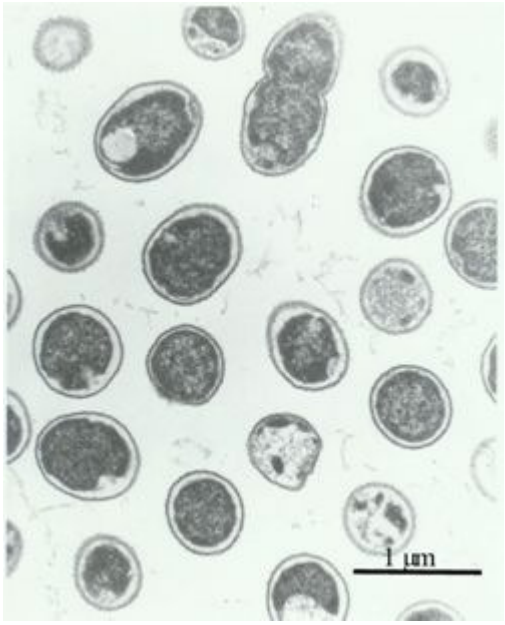


Fig. 4.2 Electron micrographs of fresh *E. coli* cultures in growth medium that was used as a reference.

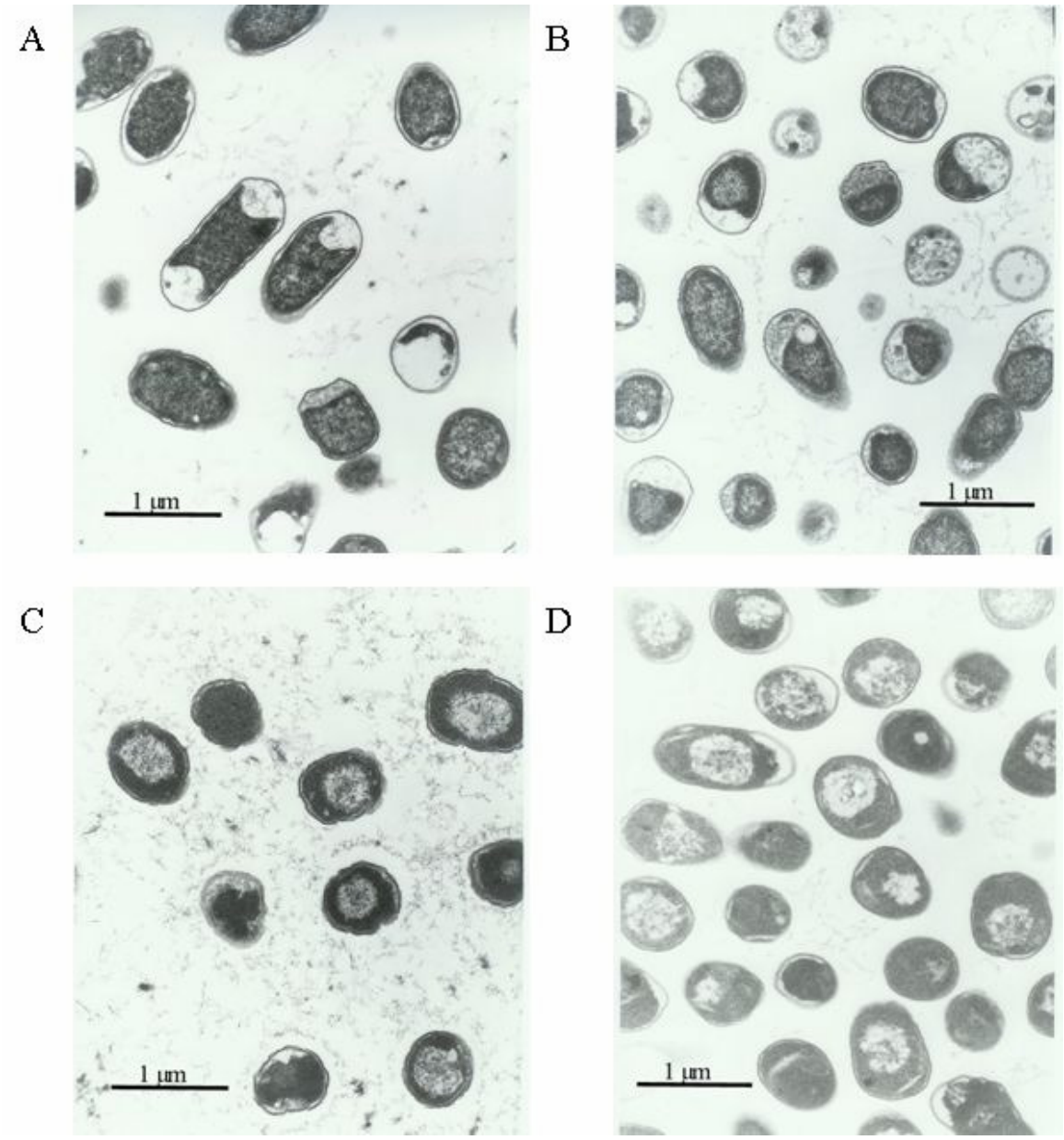


Fig 4.3. Electron micrographs of *E. coli* cultures. A. *E. coli* cells in 15% AG before drying. B. *E. coli* cells in PBS before drying. C. *E. coli* cells dried in AG for 24 h. D. *E. coli* cells dried in PBS for 24 h.

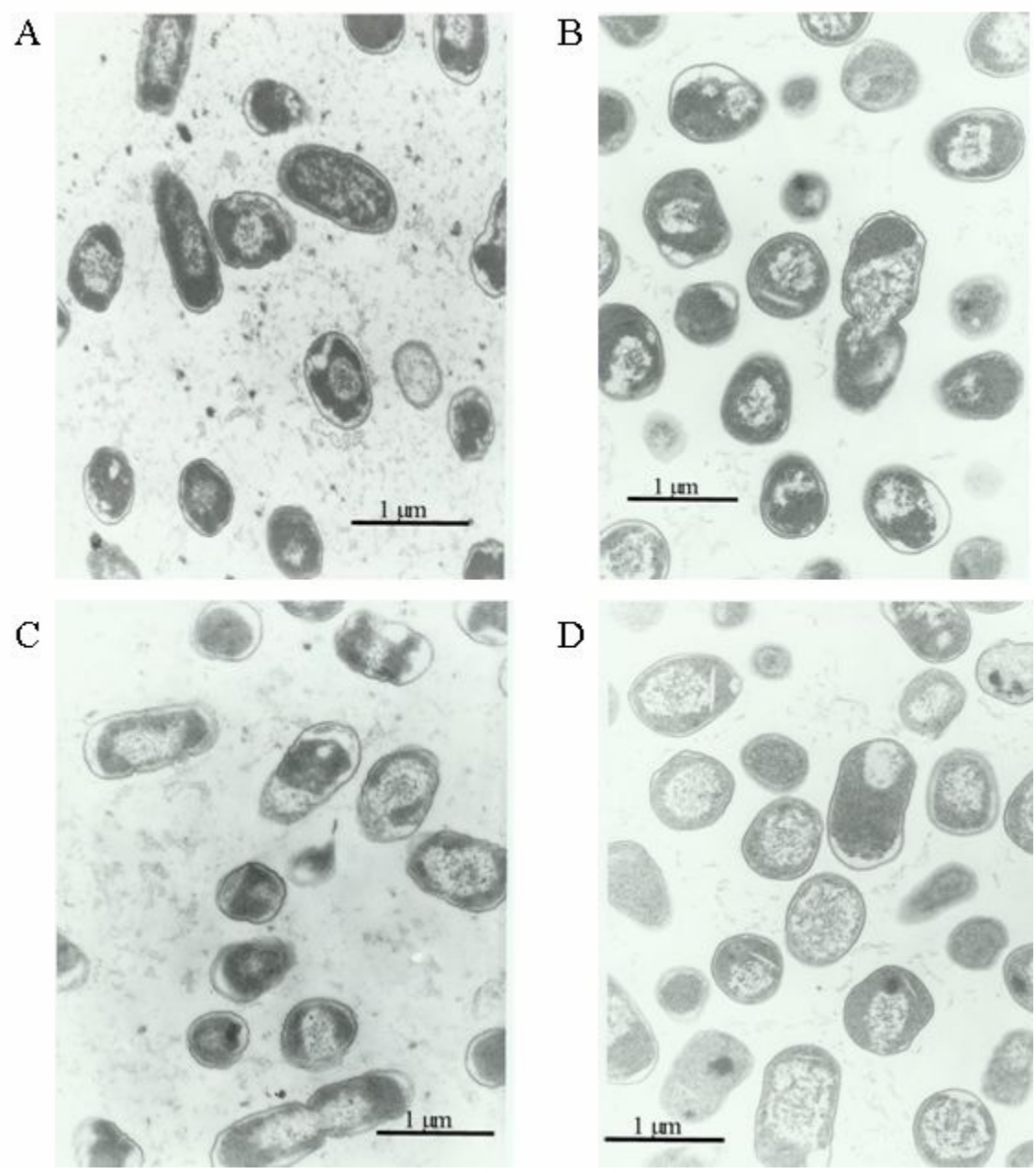


Fig. 4.4. Electron micrographs of *E. coli* cultures stored for 14 days. A. *E. coli* cells dried in AG and stored at 5°C. B. *E. coli* cells dried in PBS and stored at 5°C. C. *E. coli* cells dried in AG and stored at 25°C. D. *E. coli* cells dried in PBS and stored at 25°C.

To characterize progeny obtained from *E. coli* cells preserved in AG and PBS for 14 days, the samples were reconstituted and plated on nutrient agar. After 18 h, cells were removed from agar-grown colonies and examined by EM. Cells from AG and PBS samples were found to show no apparent irregularities, Fig 4.5 A and B, respectively. Although cells preserved in AG look abnormal directly after reconstitution (Fig. 4.4 A and C), their progeny appeared to maintain normal morphology after propagation for 18 h on growth medium.

Fig 4.6 (A-D) illustrates the ultrastructures of cells preserved in AG and PBS stored at 5 and 25°C for 139 days. Bacteria stored in AG at 5°C (Fig 4.6 A) had invaginations in cell walls; whereas, cells in PBS samples (Fig 4.6 B) maintained smooth edges along the cell walls and were similar in morphology to cells recovered from PBS directly after drying. In spite of changes observed, the titers of viable cells in AG samples were significantly higher than those in PBS samples (see Chapter 2). Cells in AG stored at 5°C for 139 days had similar characteristics compared to cells recovered from AG on days 1 and 14. In contrast, bacteria stored in AG at 25°C (Fig 4.6 C) appeared emaciated and were very small. These kinds of changes were indicative of cells that lost viability. The electron micrographs of bacteria in PBS stored at 25°C (Fig 4.6 D) illustrate remnants of bacterial cells and hollow bacteria.

To study cell membrane intactness of *E. coli* cells in AG before and after drying, the cells were stained with two fluorescent dyes and viewed using a fluorescent microscope (Fig 4.7). The SYTO 9 nucleic acid dye is capable of penetrating the cell membrane, so cells that appear green have intact cell membranes. The propidium iodide nucleic acid dye is unable to penetrate intact cell membranes, so cells stained red indicate

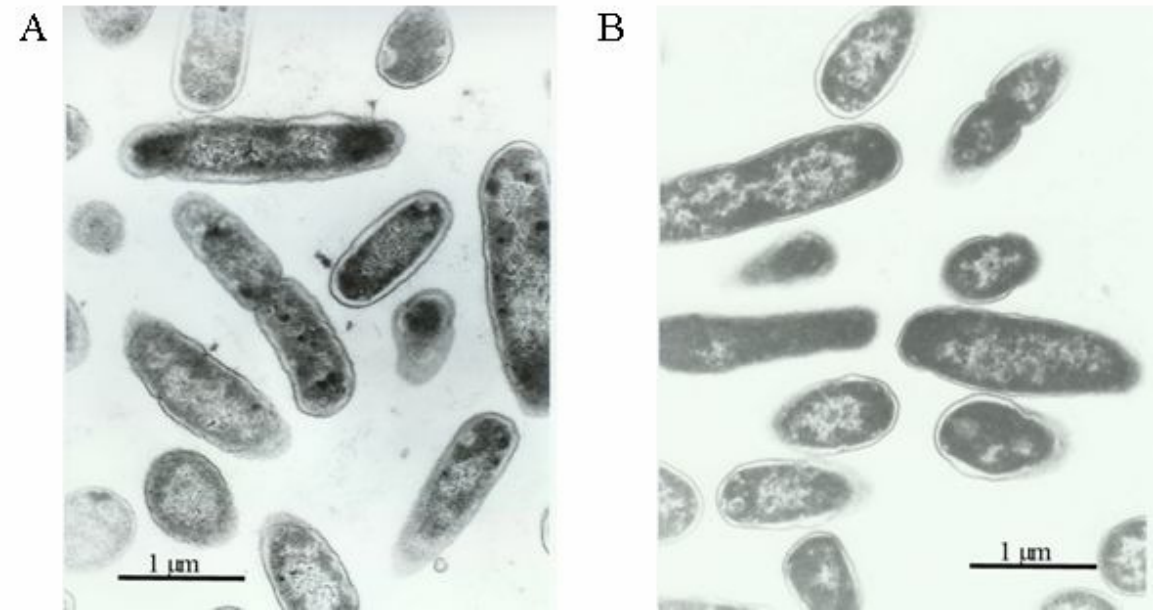


Fig. 4.5. Electron micrographs of *E. coli* cells obtained from colonies grown on nutrient agar for 18 h from preserved cultures stored for 14 days. A. Recovered *E. coli* cells dried in AG and stored at 5°C. B. Recovered *E. coli* cells dried in PBS and stored at 5°C.

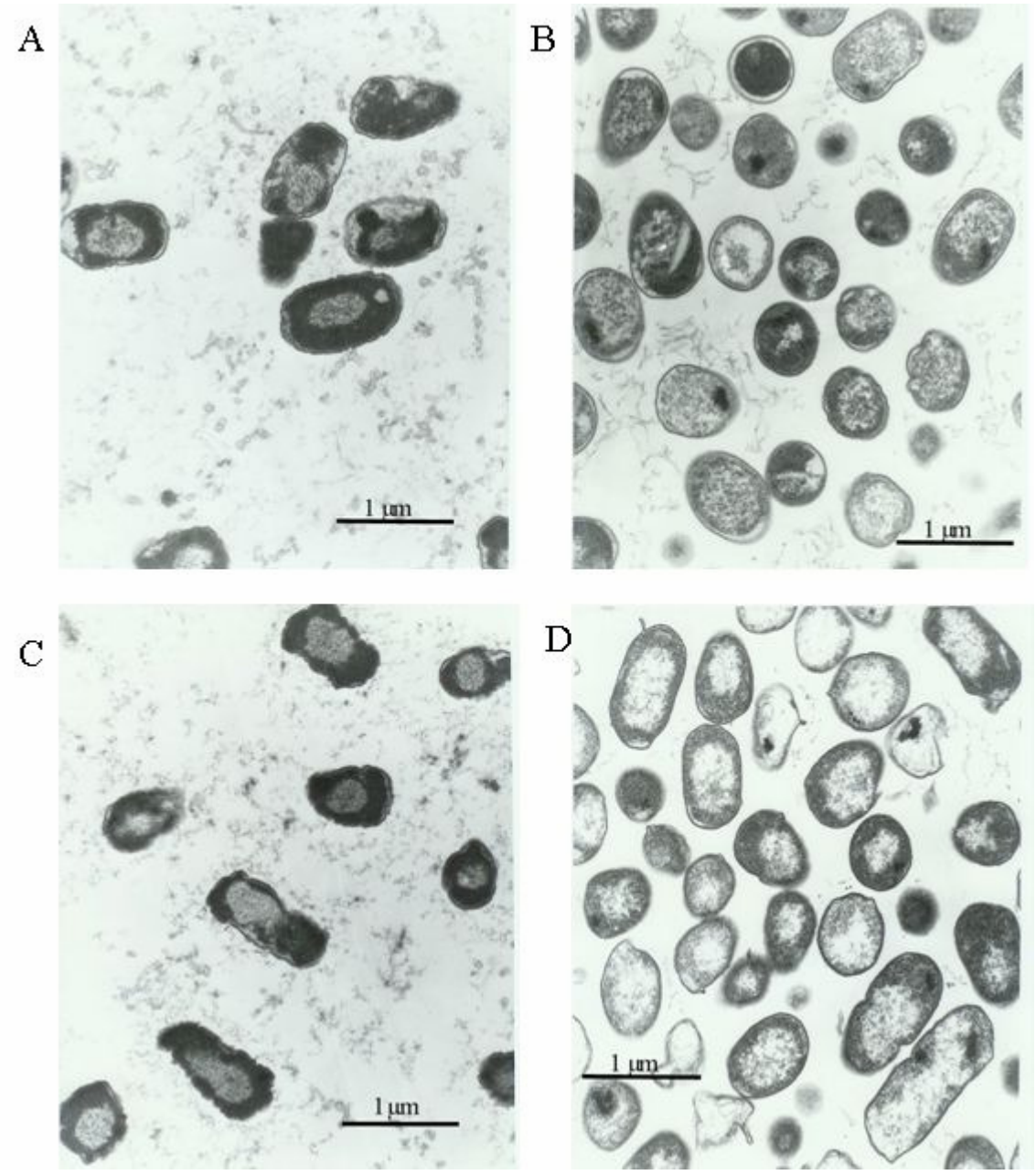


Fig. 4.6. Electron micrographs of *E. coli* cultures stored for 139 days. A. *E. coli* cells dried in AG and stored at 5°C. B. *E. coli* cells dried in PBS and stored at 5°C. C. *E. coli* cells dried in AG and stored at 25°C. D. *E. coli* cells dried in PBS and stored at 25°C.

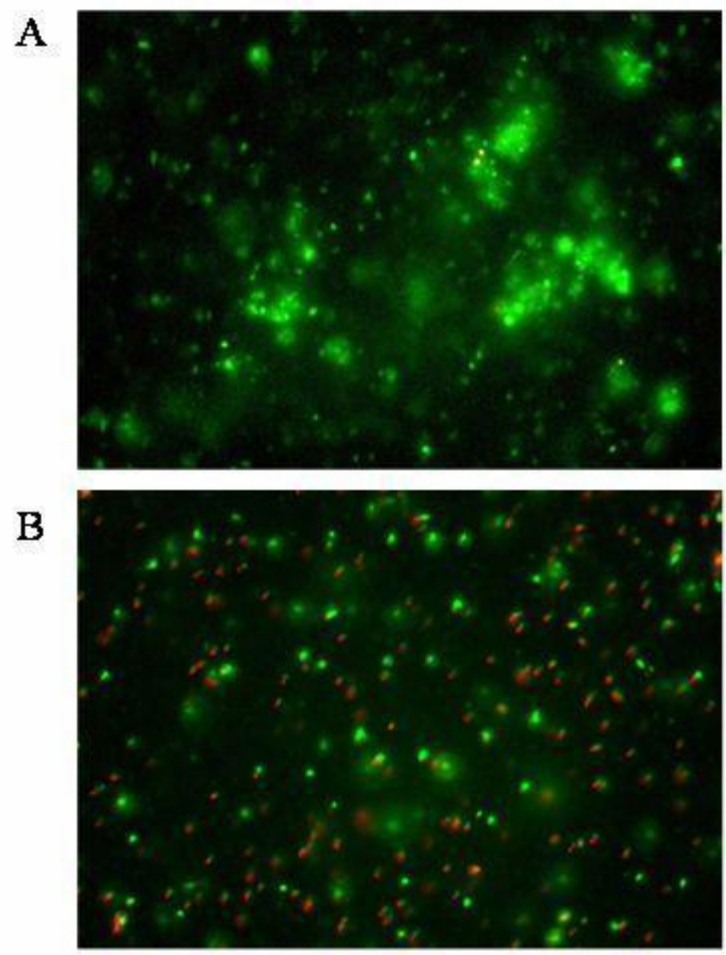


Fig. 4.7. Fluorescent Microscopy of *E. coli* samples tested for cell membrane intactness. These cells were stained with SYTO 9 (green) and propidium iodide (red). Bacterial cells with intact cell membranes fluoresce green, and those with damaged cell membranes fluoresce red. A. Fresh *E. coli* cells in 15% AG before drying. B. Recovered *E. coli* cells recovered from the samples dried in 15% AG for 24 h.

compromised, ruptured or leaky cell membranes. *E. coli* cells in AG before drying were stained green which indicated cell membrane intactness (Fig 4.7 A). Fig 4.7 B illustrates stained *E. coli* cells that were dried in AG and reconstituted. These samples had both types of cells, with intact cell membranes (green cells) and damaged cell membranes (red cells), indicating that loss of cell membrane intactness could be a possible cause of decreased viability.

4.3.2 Evaluation of structural alterations of B. subtilis cells dried in AG

Experiments were conducted to detect alterations in the ultrastructure of *B. subtilis* while drying in AG and PBS. In addition, these experiments were carried out to confirm formation of *B. subtilis* spores in the presence of AG during drying. Using EM, *B. subtilis* samples in AG and PBS were examined before drying, in partially dried samples at 24 h and in completely dried samples at 48 h. The samples were stored at 5 and 25º C and tested on day 13 of storage.

B. subtilis spores in water and vegetative cells in growth media (nutrient broth) are shown in Fig. 4.8. These micrographs were used as a reference for detecting spore formation and irregularities in ultrastructure of *B. subtilis* samples. The micrograph in Fig. 4.8 A shows structures that are characteristic of *B. subtilis* spores. Vegetative *B. subtilis* cells shown in Fig. 4.8 B are dense throughout the ultrastructure of the cytoplasm with smooth edges around the cell wall. *B. subtilis* cells that were suspended in AG and PBS before drying are shown in Fig. 4.9 A and B, respectively. There were no apparent differences between cells suspended in nutrient broth, AG and PBS before drying.

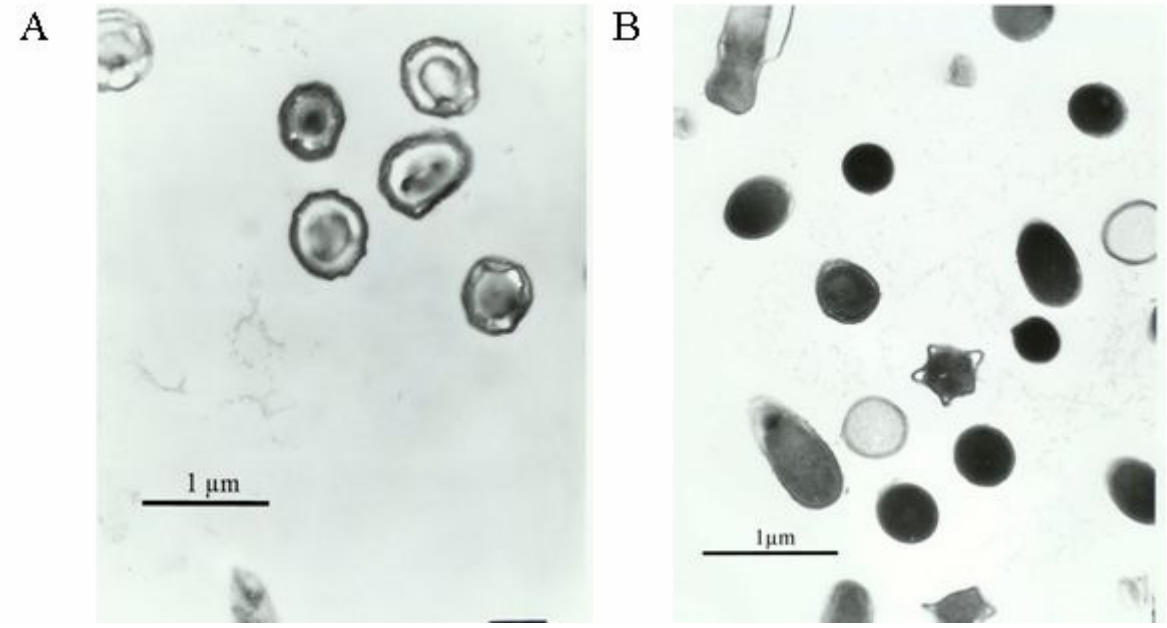


Fig. 4.8. Electron micrographs of *B. subtilis*. A. Freshly harvested *B. subtilis spores* in water. B. Fresh *B. subtilis* vegetative cells in growth medium.

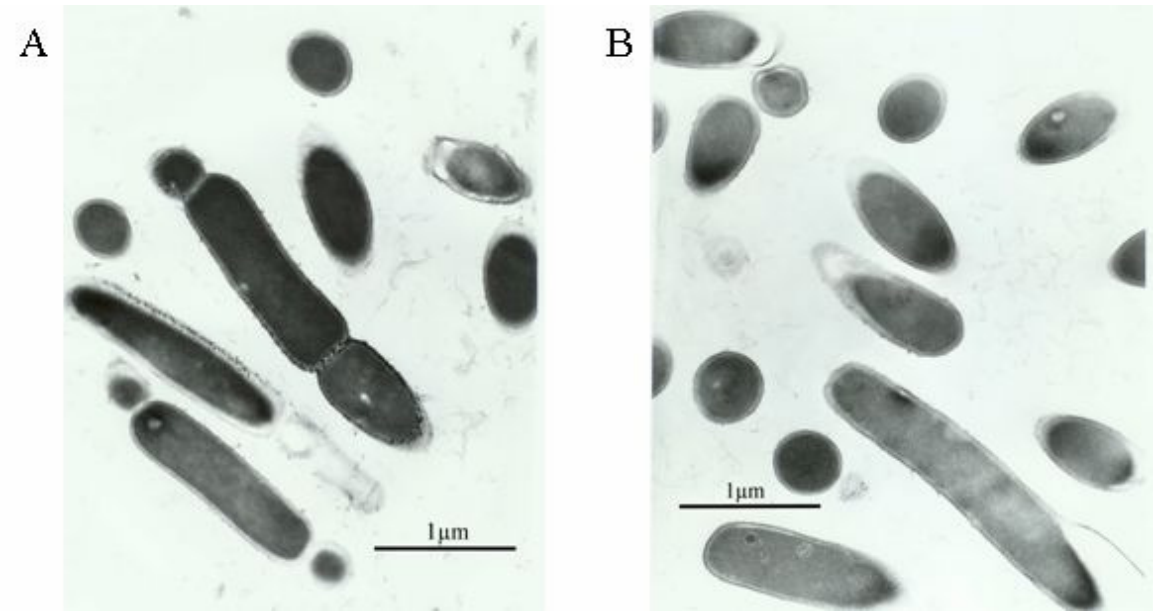


Fig. 4.9. Electron micrographs of *B. subtilis* cultures. A. *B. subtilis* cells in 15% AG before drying. B. *B. subtilis* cells in PBS before drying. C. *B. subtilis* cells in AG after 24 h of drying. D. *B. subtilis* cells in PBS during 24 h of drying. E. *B. subtilis* cells in AG after drying for 48 h. F. *B. subtilis* cells in PBS after drying for 48 h.

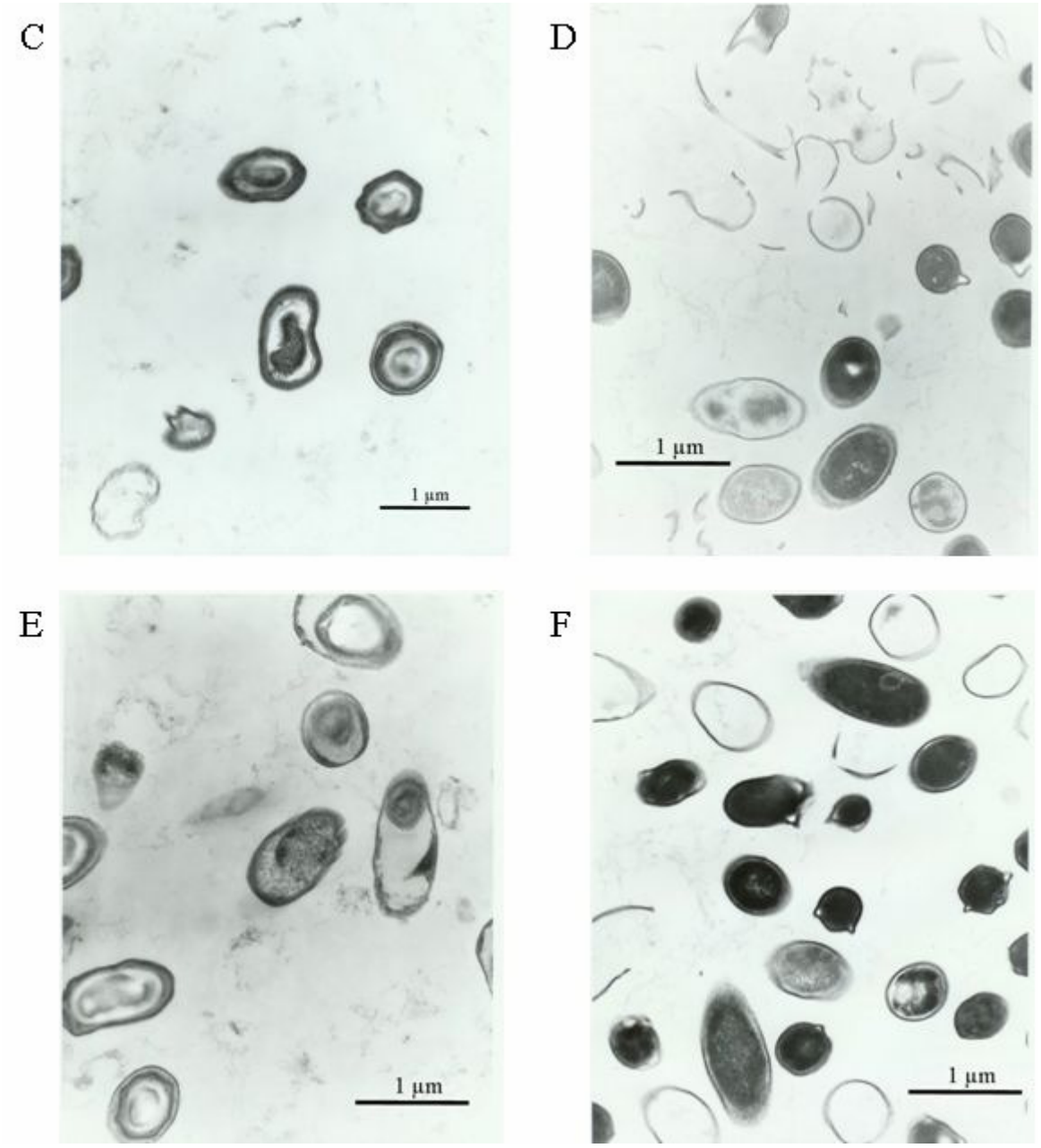


Fig. 4.9. continued.

To study spore formation in the course of drying, AG and PBS samples were tested at 24 h before complete drying (Fig. 4.9 C-D). Cells in AG have formed spores. Remnants of cells in AG samples suggested lysing of spores from mother cells. In Fig. 4.9 D, the cells in PBS showed hollow cell bodies, some cells looked damaged and emaciated. A few cells in PBS were comparable to vegetative cell reference with no apparent spore structures present.

Micrographs of *B. subtilis* in AG and PBS were tested at 48 h after complete drying (Fig. 4.9 E-F). AG samples shown in Fig. 4.8 E contained structures comparable to spores (4.8 A) with some structures that were indicative of cells containing endospores. In Fig 4.9 F, cells in PBS samples were comparable to vegetative cell reference. In addition, the PBS samples included hollow bodies, cellular debris and no apparent spores were present.

To characterize alterations in structures of *B. subtilis* samples after storage in AG and PBS for 13 days, electron microscopy was performed (Fig. 4.10). *B. subtilis* cultures preserved in AG and stored at 5°C are shown in Fig. 4.10 A. These micrographs displayed cells containing endospores and spores; whereas, micrographs of PBS samples stored at 5°C revealed hollow bodies and few intact cells (Fig. 4.10 B). There was no substantial evidence of spore formation. Samples preserved in AG and stored at 25°C (Fig. 4.10 C) were structurally similar to cells stored at 5°C (Fig 4.10 A). Once spores were formed, the ultrastructure remained consistent for all storage temperatures tested.

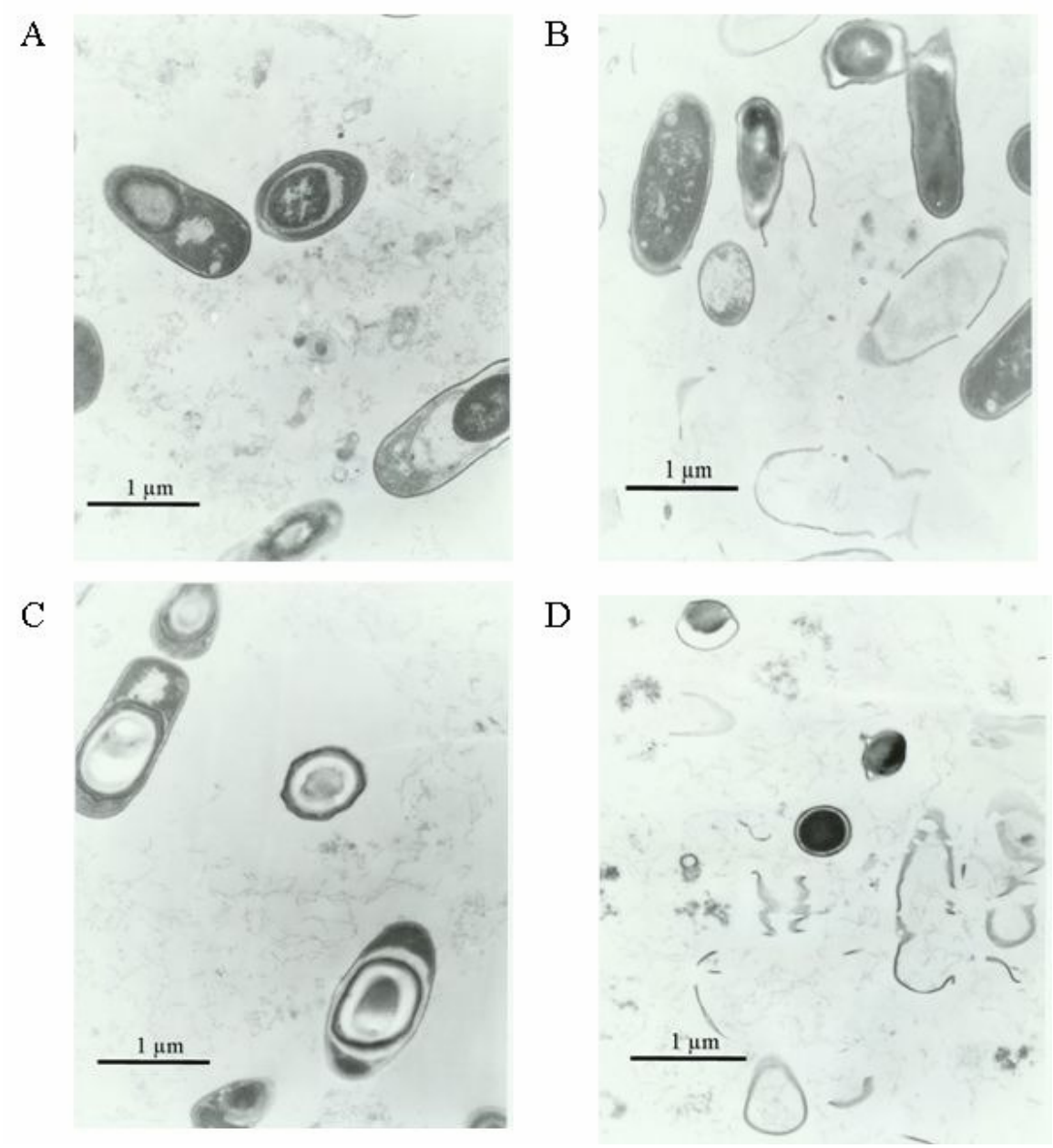


Fig. 4.10. Electron micrographs of *B. subtilis* cultures stored for 13 days. A. *B. subtilis* cells dried in AG and stored at 5°C. B. *B. subtilis* cells dried in PBS and stored at 5°C. C. *B. subtilis* cells dried in AG and stored at 25°C. D. *B. subtilis* cells dried in PBS and stored at 25°C.

PBS samples stored at 25°C are shown in Fig 4.10 D. These samples were similar to those stored at 5°C with a few intact vegetative cells and cellular debris which represented the major portion of the sample. In addition, spores were not present in these samples (Fig. 4.10).

To determine cell membrane intactness of *B. subtilis* cells in AG after drying, samples were stained as aforementioned in section 4.3.1 for *E. coli*. *B. subtilis* samples before and after drying are shown Fig. 4.11. Bacterial cells before drying were fluorescent green indicating cell membrane intactness (Fig 4.11 A). Fig. 4.11 B illustrates *B. subtilis* cells that were dried in AG, reconstituted, and stained with fluorescent dyes. These samples contained cells with intact cell membranes (green cells) and compromised cell membranes (red cells). It was previously determined that spore formation occurred in AG samples; therefore, red cells may be the result of spores lysing from cells instead of representing dead cells.

4.3.3 Discussion

Preservation of bacteria in AG has shown promising results in our viability studies. However, cell viability varied significantly depending on the type of bacteria and preservation conditions. Viability of *E. coli* preserved in AG was significantly greater than controls; nonetheless, viability of these samples was compromised after drying and decreased at a faster rate when stored at elevated temperatures and humidity. The viability of *B. subtilis* cultures preserved in AG was 100% after drying and maintained viability throughout storage regardless of storage temperature and humidity.

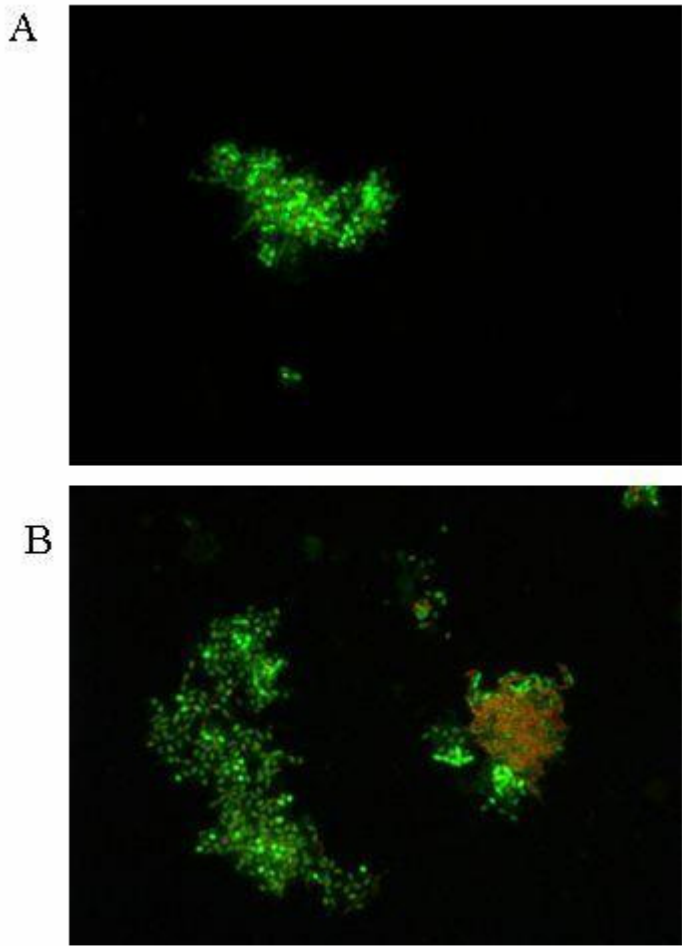


Fig. 4.11. Fluorescent microscopy of *B. subtilis* samples tested for cell membrane intactness. These cells were stained with SYTO 9 (green) and propidium iodide (red). Bacterial cells with intact cell membranes fluoresce green, and those with damaged cell membranes fluoresce red. A. Fresh *B. subtilis* cells in 15% AG before drying B. Recovered *B. subtilis* cells recovered from the samples dried in 15% AG for 48 h.

These results along with evidence obtained from spore staining assays indicated that *B. subtilis* cells formed spores. Presently, the mechanism for degradation is unknown in the course of preserving bacteria in AG. The numbers of viable and functioning bacteria could be improved significantly if more is known about structural irregularities in cells after drying and storing in AG. To ascertain the mechanism of cellular degradation, ultrastructural alterations of bacteria preserved in AG were studied using electron and fluorescent microscopy (EM and FM).

In micrographs of *E. coli* samples, alterations in cells preserved in AG included invaginations in the cell wall and decreased periplasmic space after desiccation and rehydration. The cells in AG looked emaciated which was due to the invaginations in the cell walls causing the morphology to be deformed. AG samples did not appear to contain many hollow bodies, so it was suggestive that AG protects bacterial cells by coating the cell wall to provide structural support when water is lost and to prevent complete dehydration. The micrographs were also suggestive in that the increase in density around cell wall maybe due to the accumulation of AG. Since AG is a mixture of high molecular weight molecules (put references for size of AG) it is unlikely that internal structures were protected. Thus, *E. coli* cells preserved in AG would have to use their own defense mechanisms for internal protection during desiccation and storage.

Irregularities in bacterial ultrastructure could be caused by osmotic stress during desiccation and/or shock due to dehydration (Malik, 1988c), high oxygen levels and acidic environments (Picot and Lacroix, 2003). Causes for *E. coli* degradation may include lack of intracellular protection from desiccation. As water leaves the cell, intracellular proteins compensate by interacting with each other, and upon rehydration,

desiccation-sensitive bacteria are unable to retain their native protein structures (Wolkers et al., 2002). In some bacteria, molecules such as exopolysaccharide (Roberson and Firestone, 1992), sucrose or trehalose (Leslie et al., 1995b) accumulate. In general, sugars such as threhalose are able to protect proteins during the course of drying and preservation by inhibiting conformational changes in bacterial molecules and chemical degradation (French et al., 2004). In addition, trehalose protects bacteria cell membranes from leakage upon rehydration by replacing water between lipid headgroups (Leslie et al., 1995b). Samples drying too fast may not allow for these molecules to accumulate. Therefore, adding an intracellular protectant for desiccation sensitive bacteria may improve survival and prevent subsequent degradation.

EM studies of *B. subtilis* samples in AG were accomplished to determine alterations in cell ultrastructure and formation of bacterial spores. It was first discovered in viability experiments (Chapter 3) that spores were formed during drying. This was confirmed in EM experiments. *B. subtilis* spores maintained a characteristic shape with three main layers of their outer coat as described by Waller, Fox *et al*. (Waller et al., 2004a). The outer most layer is known as the glycoprotein nap; the next layer in is the outer core; and the next layer in is the inner core. These structures are visible in electron micrographs of spores in water (Fig. 4.8 A) and in AG samples drying for at least 24 h (Fig. 4.9 C and E, Fig. 4.10 A and C).

To determine cell membrane intactness of both types of bacterial cells after drying, fluorescent microscopy was performed. It appeared that approximately one-half of the *E. coli* and *B. subtilis* cultures dried in AG and reconstituted had leaky or damaged cell membranes. Viability using this assay was unattainable because some cells can

repair this damage, and some cells may have formed spores. Cell membrane damage for *E. coli* cells is mainly due to dehydration, so upon rehydration the structural integrity of cell membranes become compromised and are leaky. Another cause for damage may include enzyme degradation of lipid head groups into free fatty acids (Crowe et al., 2002). This results in damage to large sections cell membranes and repair is unlikely after rehydration. Those cell membranes that do remain intact rely on defense mechanisms as aforementioned such as the accumulation of sugars that replace water during dehydration. Other mechanisms include DNA repair such as nucleotide excision repair or recombination repair (reference). The formation of new proteins will restore cell membrane intactness and eventually normal cellular functions as long as transcription can occur (Potts, 1994).

4.4. Conclusions

E. coli cells dried and stored in AG exhibited irregularities in bacteria cells which included increase in electron density of cell walls and invaginations in cell walls and cell membranes which distort the normal morphology of the cells. These alterations were detected in AG samples up to day 139 for both storage temperatures. The ultrastructure of *E. coli* progeny recovered from cultures preserved in AG for 14 days appeared normal. *B. subtilis* cells dried in AG formed spores and spore morphology remained consistent up to 13 days of storage. AG provided adequate environment for complete spore formation

during the drying process. About one-half of both cell types dried in AG appeared to have compromised cell membranes. Nonetheless, *E. coli* cells may be able to repair this damage. In addition, *B. subtilis* cultures may appear to be injured, but this could be a result of spores lysing from cells.

CHAPTER 5

PRESERVATION OF DNA IN PROTECTIVE POLYMERS

5.1 Introduction

Preservation of DNA is of great importance in many fields such as biotechnology (Harry et al., 2000), biosensor technologies (Piunno, 1994), drug discovery (Williams et al., 2002), research and diagonostic work (Paoli, 2005). The conventional method for preserving DNA is mixing DNA in TE buffer then refrigerating at 4° C for short-term or freezing at -20° C for long-term storage. Although this method is suitable for laboratory use, alternative methods for preserving DNA at ambient temperatures are desirable, for instance, when transporting DNA samples from the field to the laboratory. Several methods tested that do not utilize refrigeration include preserving DNA on diamond chips (Takahashi et al., 2003), in bacterial samples dried in horse blood agar (Moore et al., 2005), on biosensors (Piunno et al., 1994), and on FTA paper (Seah and Burgoyne, 2001; Tack et al., 2005). However, some of these methods involve expensive materials or require specialized training. For example, DNA samples stored on FTA paper or diamond chips would have to be removed prior to biological assays such as PCR.

In previous chapters of this dissertation, AG and pullulan polymers were tested for their capacity to protect two types of bacteria during desiccation and storage under various environmental conditions. Particularly, the use of AG was our main interest because of its low cost. AG is capable of immobilizing many different types of biologicals (Vodyanoy et al., 2003). Thus, it is possible that AG could protect DNA from degradation in samples of unknown contents.

In this chapter, a method for preserving ssDNA and dsDNA using AG is described. This method could be adopted for collecting and transporting samples containing DNA without refrigeration. Although preservation of DNA in the laboratory is well maintained via freezing, the development of a method for immobilizing and protecting DNA from environmental factors such as humidity and temperatures is desirable. This method should be inexpensive and simple so specialized equipment or training is not required.

The DNA used in our experiments was isolated from fd-tet bacteriophage. This phage is a filamentous virus that contains a single-stranded DNA genome. This type of phage infects K91 BKan *Escherichia coli* via attachment to bacterial pilus. Once phage ssDNA is inside of the bacterial cell, it replicates the viral genome via rolling circle method using bacterial enzymes. During this process, the ssDNA (phage genome) is complimentarily base paired and becomes double stranded. The phage dsDNA within a bacteria cell is the replicative form (RF) by which copies of the phage genome are produced in order for phage to proliferate. This type of RF DNA is covalently closed circular dsDNA. Thus, the ssDNA is isolated from phage itself, and the dsDNA is isolated from bacterial cells infected with phage.

The main focus of this work was to design and develop a process for long-term preservation of DNA at ambient temperatures. Two types of DNA were subjected to a drying process in AG and pullulan in order to determine the protective capacity of these polymers. This method involves DNA in TE buffer mixed with AG polymer and dried under vacuum for 48 h at room temperature. The samples were stored at various temperatures and humidity, and tested for viability before and after drying and during storage.

5.2 Materials and methods

5.2.1 Isolation of ssDNA

ssDNA was isolated from cultured fd-tet phage (generous gift from Dr. Petrenko, Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL) using a DNA purification method described in *Molecular Cloning: a laboratory manual* (Sambrook et al., 1989). Five milliliters of cultured phage were placed into a 15 ml polypropolene tube with 5 ml of phenol. This was mixed for a 5 min and the phenol layer was discarded. Another 5 ml of phenol was added for 5 minutes and discarded. Then, 5 ml of phenol-chloroform was added for 5 min and discarded. Then, 5 ml of chloroform was added and discarded. The water layer was transferred into two 50 ml centrifuge tube and the volume level was adjusted to 5 ml with TE buffer.
Then, 0.5 ml of NaOAc and 12.5 ml of 100% ethanol was added to each tube. This mixture results in precipitation of ssDNA. This pellet underwent a series of rising steps

with ethanol and a final drying step overnight in a sterile environment. The dried ssDNA was reconstituted in TE buffer which results in a purified ssDNA in TE buffer.
The concentration of ssDNA was measured using Shimadzu UV160U spectrophotometer (Shimadzu Corporation, Japan) at λ 260 nm. Once concentration was known, ssDNA were diluted with TE buffer to 40 ng/µl.

5.2.2 Isolation of dsDNA

Fd-tet phage dsDNA was isolated from infected *Escherichia coli* K91 BKan culture according to protocol 15-8 in *Phage Display* (Barbas, 2001) with the following modification. In step 4, 4 ml of freshly prepared solution of lysozyme (10 mg/ml in 10mM Tris HCL [pH 8.0]) was added after the buffered glucose; this modification was derived from *Molecular Cloning: a laboratory manual* lysis by alkali plasmid vector protocol (Sambrook et al., 1989). After extraction, the dsDNA pellet was reconstituted in TE buffer. The concentration of dsDNA was measured using Shimadzu UV160U spectrophotometer, and the dsDNA samples was diluted with TE buffer to make a final concentration of 30 ng/µl.

5.2.3 Electrophoresis of DNA in AG samples

ssDNA in the amounts of 200 and 400 ng was measured into 10 µl of 10% AG or TE buffer. Each of these mixtures was combined with 2 µl of 70/75 bromophenol blue

(BPB) loading dye. One percent DNA grade agarose gels containing 20 wells were prepared as follows: 0.7 g of Agarose DNA grade (Fisher cat # BP164) was added to 70 ml 1x Tris-borate-EDTA buffer (TBE buffer) (TBE 5 x stock: 0.5M Tris, 0.5 M boric acid, 10mM EDTA), the mixture was heated in a microwave until agar dissolved, and the warm mixture was poured into a gel case with a 20 well comb. To these 1% gels, ssDNA samples containing BPB dye were loaded. Gels were run at 50 V for 2 h in TBE buffer. Gels were stained with SYBR® Green I nucleic acid gel stain (Cambrex Bio Science Rockland Inc., Rockland, MN, purchased from Fisher cat # BMA 50513) for 1 h. The gels were illuminated using a transilluminator DR-190M (Clare Chemical Research, Dolores, CO). Pictures of the stained gels were taken using Kodak EDAS 290 (Eastman Kodak Company, New Haven, CT).

5.2.4 Recovery of DNA from AG and pullulan polymers

Two kits were tested for DNA recovery from AG and pullulan: (1) GeneClean® Turbo for PCR kit (using the protocol for Rapid Isolation of 0.1-300kb DNA from solution purchased from Q-Biogene (Carlsbad, CA) and (2) QIAquick gel extraction kit from QIAGEN (Valencia, CA). Samples were prepared by mixing 200 ng of ssDNA in 50 µl of TE buffer, 10% AG or 10% pullulan. Then, these samples were purified one or the other kit. Since the volume of the recovered DNA samples was too much to load into the wells of the agarose gels, the purified samples were concentrated. The recovered samples were placed in vacuum centrifuge until dry (~3 h). The dried samples were reconstituted with 10 µl of sterile water for 10 minutes. Then, 2 µl of 70/75 bromophenol

blue (BPB) loading dye were added to each sample. The samples were loaded into agarose gels and electrophoresis was carried out as described in section 5.2.2.1.

5.2.5 Polymer concentration and drying temperature optimization

To determine the optimal concentration of AG, one part of ssDNA in TE buffer was mixed gently with four parts of 10, 15, 20 or 25% AG or TE buffer. Fifty microliters of prepared DNA mixtures were aliquoted into 2 ml polypropolene microcentrifuge tubes (Fisher cat # 05-408-141). These samples were dried uncovered in a vacuum desiccator containing silica gel desiccant at ambient temperature for ~ 2 days. The samples were reconstituted in 50 µl of sterile water for 10 minutes and purified using GeneClean® Turbo for PCR kit. The DNA samples recovered from AG were concentrated as described in section 5.2.4 and tested for integrity via electrophoresis as described in section 5.2.3.

To determine optimal drying temperature, ssDNA samples were prepared in 10% AG and TE buffer as abovementioned. These samples were dried in a vacuum desiccator at ambient temperature for 2 days or dried in a static incubator at 40°C for 24 h. After drying, the samples were reconstituted, purified, concentrated and tested for integrity via gel electrophoresis as described above.

5.2.6 Long-term storage experiments

Based on optimization experiments, the following protocol was used for long-term preservation of ssDNA and dsDNA. One part of ssDNA or dsDNA was prepared in four parts of 10% AG, 10% pullulan or TE buffer. Aliquots of 50 µl were measured into 2 ml centrifuge tubes. AG and pullulan samples were dried uncovered in a vacuum desiccator containing silica desiccant at ambient temperature for 2 days. As a reference, DNA samples in TE buffer were sealed and stored at -20° C.

For temperature experiments, DNA samples dried in AG and pullulan were sealed and placed into containers as shown in Fig. 2.1 C. These containers were placed in incubators set at 5, 15, 25, and 40°C. DNA integrity was tested after drying and after storage on days 2, 4, 8, 16, 32, 64 and 128. For humidity experiments, DNA samples dried AG and pullulan were placed into relative humidity (RH) chambers unsealed as shown in Fig. 2.1 F. In analogy to temperature experiments, DNA integrity was tested after drying and after storage on days 2, 4, 8, 16, 32, 64 and 128.

5.2.7 Testing DNA integrity using gel electrophoresis

On testing days, both ssDNA and dsDNA dried in AG and pullulan were reconstituted with 50 µl of sterile water for 10 min. Reference samples stored at -20°C were unthawed. All samples (including reference samples) were purified using GeneClean® Turbo for PCR kit. After purification, samples were dried in a vacuum

centrifuge for ~3 h or until dry. Then each sample was reconstituted in 10µl of TE buffer for 10 min and 2 µl of BPB loading dye were added.

For ssDNA samples, 1% agarose gels were prepared using 1x TBE. Samples were loaded into wells and gels were run at 50 V for 2 h in TBE buffer. For dsDNA samples, 0.8% agarose gels were prepared using 4x GBB buffer (GBB 40 x stock: 1.68 M Tris, 0.80 M sodium acetate, 72 mM EDTA). Lambda DNA-BstEII digest was used for the molecular weight standard (generous gift from Dr. Petrenko). Experimental samples and molecular weight standards were loaded into wells and gels were run at 50 V for 1.5 h.

All gels were stained with SYBR® Green I nucleic acid gel stain for 1hour. Gel pictures were taken using Kodak EDAS 290. The relative amount of DNA was determined using Kodak 1D version 3.6 software. DNA integrity was expressed as percent of intact DNA relative to control DNA stored at -20° C in TE buffer.

5.2.8 PCR Amplification of ssDNA recovered from AG and pullulan samples

PCR reaction was prepared using dNTP mixture (dNTP set purchased from Amersham Biosciences Piscataway, NJ cat. # 27-2035-01), F8 sense and F8 anti-sense primers (generous gift from Dr. Petrenko) and Taq polymerase (Promega Madison, WI Cat # M1661). The concentration of ssDNA from experimental samples was unknown. The concentration of the positive control was 40 ng/µl of ssDNA stored in TE buffer at -20°C. The thermocycler was programmed as follows: 94° C for 3 min followed by 35 cycles: 94°C for 10 s, 46°C for 20 s, and 72°C for 45 s; 72°C for 4 min.

The reaction mixtures was dropped to 4°C until samples were tested further. After the reaction was completed, the samples were loaded into gels as described in section 5.2.3.

PCR products were sequenced at the Auburn Genomics and Sequencing Laboratory (Auburn University, Auburn, AL) using S20 primer (generous gift from Dr. Petrenko). The sequences were analyzed using Chromas 1.45 (Technelysium Pty Ltd, Tewantin Qld 4565, Australia) and DNAstar (Madison, WI) programs.

5.3 Results and discussion

5.3.1 Optimization experiments for long-term preservation of ssDNA

ssDNA was used to determine optimal parameters for long-term preservation for both types of DNA. Because ssDNA is less stable and more susceptible to degradation than dsDNA, the optimal conditions for ssDNA would apply to dsDNA. Several experiments were conducted to determine the best method for DNA recovery from AG because DNA did not migrate under electrical current in the presence of AG as shown in Fig. 5.1. Two methods were tested to optimize recovery of DNA from AG. QIAquick gel extraction and GeneClean® Turbo kits were compared. DNA was successfully purified from AG samples using both methods; however, GeneClean® Turbo kit appeared to recover more DNA (Fig. 5.2), so this method was used from this point forward.

A number of experiments were performed to determine the optimal AG concentration and drying temperature for long-term preservation of DNA. ssDNA was

dried in 10, 15, 20 and 25% AG, reconstituted in water and tested via gel electrophoresis. ssDNA recovered from these samples are shown in Fig. 5.3. More ssDNA was recovered from decreasing concentrations of AG, so the most favorable AG concentration for ssDNA preservation was determined to be 10%. Drying temperatures tested were 25°C and 40°C. As shown in Fig. 5.4, the optimal drying temperature for DNA samples in AG was determined to be 25°C because more DNA was detected.

5.3.2 Long-term preservation of ssDNA

To determine integrity of ssDNA after preserving in protective polymers for long-term storage under various conditions, ssDNA samples in 10% AG and 10% pullulan (control polymer) were dried at 25°C. After drying, two types of long-term preservation experiments were performed: (1) storage at various temperatures and constant humidity and (2) storage at various humidity and constant temperature. These are referred to from this point forward as temperature and humidity experiments.

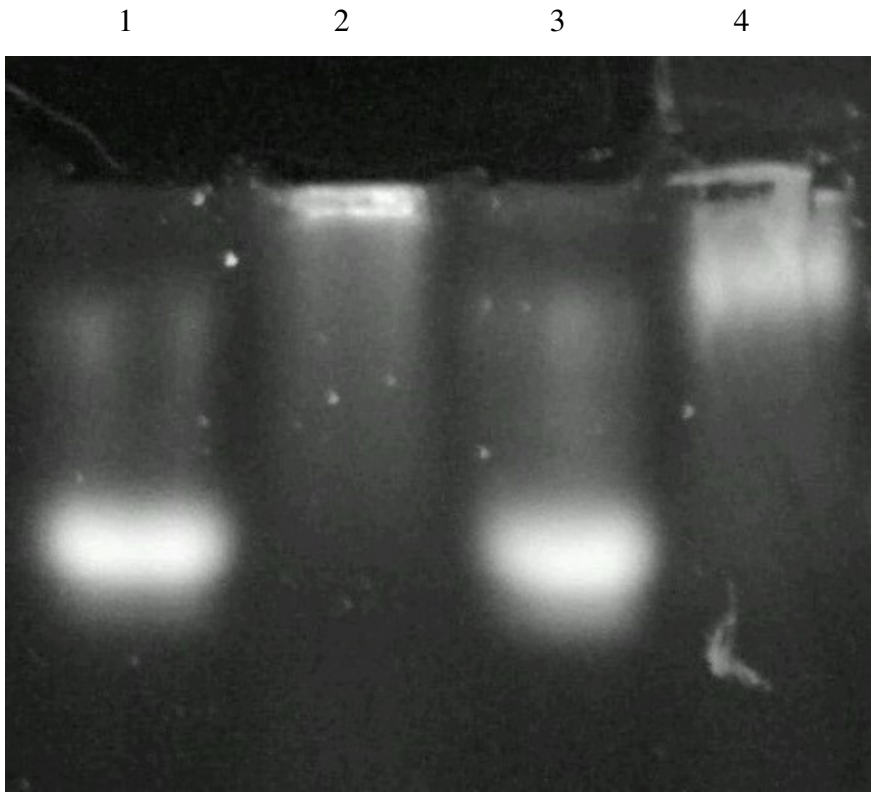


Fig. 5.1. ssDNA migration patterns in the presence and absence of AG. Lanes 1, 200 ng of ssDNA in TE buffer. Lane 2, 200 ng ssDNA in 10% AG. Lane 3, 400 ng of ssDNA in TE buffer. Lane 4, 400 ng of ssDNA in 10% AG.

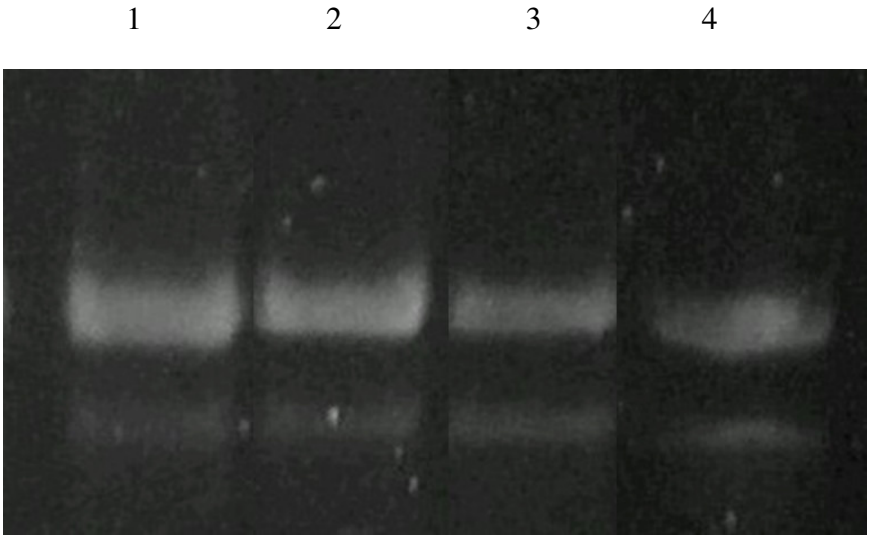


Fig. 5.2. Electrophoretic image of DNA samples purified with the use of different DNA purification kits. Lane 1, control ssDNA in TE buffer purified using GeneClean® turbo kit. Lane 2, ssDNA purified from AG using GeneClean® turbo kit. Lane 3, control ssDNA in TE buffer purified using QIAquick kit. Lane 4, ssDNA purified from AG using QIAquick kit.

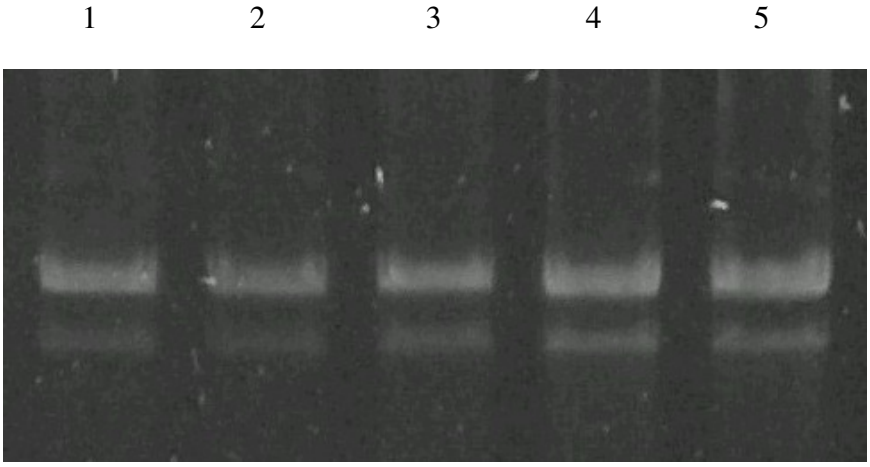


Fig. 5.3. Electrophoretic image of DNA samples prepared in different concentrations of AG. ssDNA were dried in various concentrations of AG and reconstituted with water. GeneClean® turbo kit was used to purify DNA from AG. Lane 1, ssDNA recovered from 25% AG. Lane 2, ssDNA recovered from 20% AG. Lane 3, ssDNA recovered from 15% AG. Lane 4, ssDNA recovered from 10% AG.

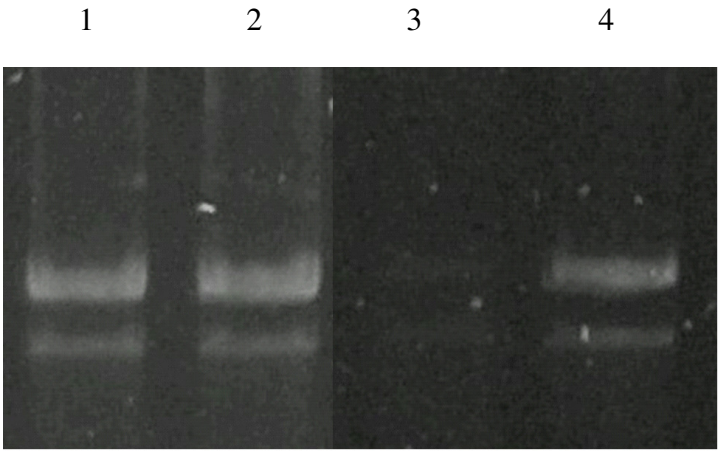


Fig. 5.4. Electrophoretic image of DNA samples dried at different temperatures. Lane 1, ssDNA in 10% AG dried at 25°C. Lane 2, ssDNA control in TE buffer dried at 25°C. Lane 3, ssDNA in 10% AG dried at 40°C. Lane 4, ssDNA control in TE buffer dried at 40° C.

For temperature experiments, samples were dried, stored at 5, 15, 25, or 40°C and constant humidity (~33%) and DNA integrity was tested on days 2, 4, 8, 16, 32, 64 and 128 of storage. The pictures of representative gels used to determine the relative amount of ssDNA remaining after storage at various temperatures and humidity are shown in Fig. 5.5 and Fig. 5.6, respectively (See Appendix for all experimental electrophoretic images). The relative amounts of ssDNA recovered from AG samples stored at 5, 15, 25, and 40°C and tested on day two of the experiment were 6.3, 6.3, 2.3, and 2.2%, respectively (Fig. 5.7). The relative amounts of ssDNA recovered from pullulan samples stored at 5, 15, 25, and 40° C and tested on day two of the experiment were 3.6, 2.0, 2.9, and 1.2%, respectively (Fig. 5.7). Intact ssDNA was detectable up to day 128 for AG samples stored at 5 and 15°C (Fig. 5.7 A-B). ssDNA dried in AG was undetectable on day 128 and day 8 when stored at 25 and 40°C, respectively (Fig. 5.7 C-D). For pullulan samples, ssDNA was detectable up to day 64, 32, 16, and 4 when stored at 5, 15, 25, and 40° C, respectively. In humidity experiments, ssDNA integrity diminished rapidly. For AG samples, ssDNA was visible up to day 32 when stored at 46 and 53 % humidity (Fig 5.8 A-B), and day 4 when stored at 76 and 86% humidity (Fig. 5.8 C-D). For pullulan samples, ssDNA was detected up to day 8 when stored at 46 and 53% humidity and up to day 4 for samples stored at 76 and 86% (Fig. 5.8).

5.3.3 DNA integrity tested by PCR amplification and sequencing of AG and pullulan samples

ssDNA samples stored at various temperatures (5, 15, 25, 40° C) were tested on day 118 to determine whether the remaining ssDNA were able to produce PCR products. In one set of experiments, AG and pullulan samples were purified from polymers using Gene Clean® Turbo Kit for PCR according to manufacturer's protocol. In addition, samples stored at 25°C for 118 days were tested to determine if samples could successfully undergo a PCR reaction without prior purification from AG and pullulan polymers.

In spite of the little amount or even absence of intact ssDNA present in AG samples stored at various temperatures detected via gel electrophoresis, the samples produced PCR products of correct size (Fig. 5.9 Lanes 2-4) and were successfully sequenced with no variation in ssDNA sequence compared to control (Fig. 5.10). Pullulan samples were barely visible in the electrophoretic image (Fig. 5.9 Lanes 6-8), but sequencing results indicate that there was no variation in DNA sequence compared to control. AG samples were also tested to determine if they could undergo PCR reaction in the presence of AG or pullulan. The entire pellet of dried AG or pullulan samples stored at 25°C for 118 days were placed into the PCR tube with reaction components.

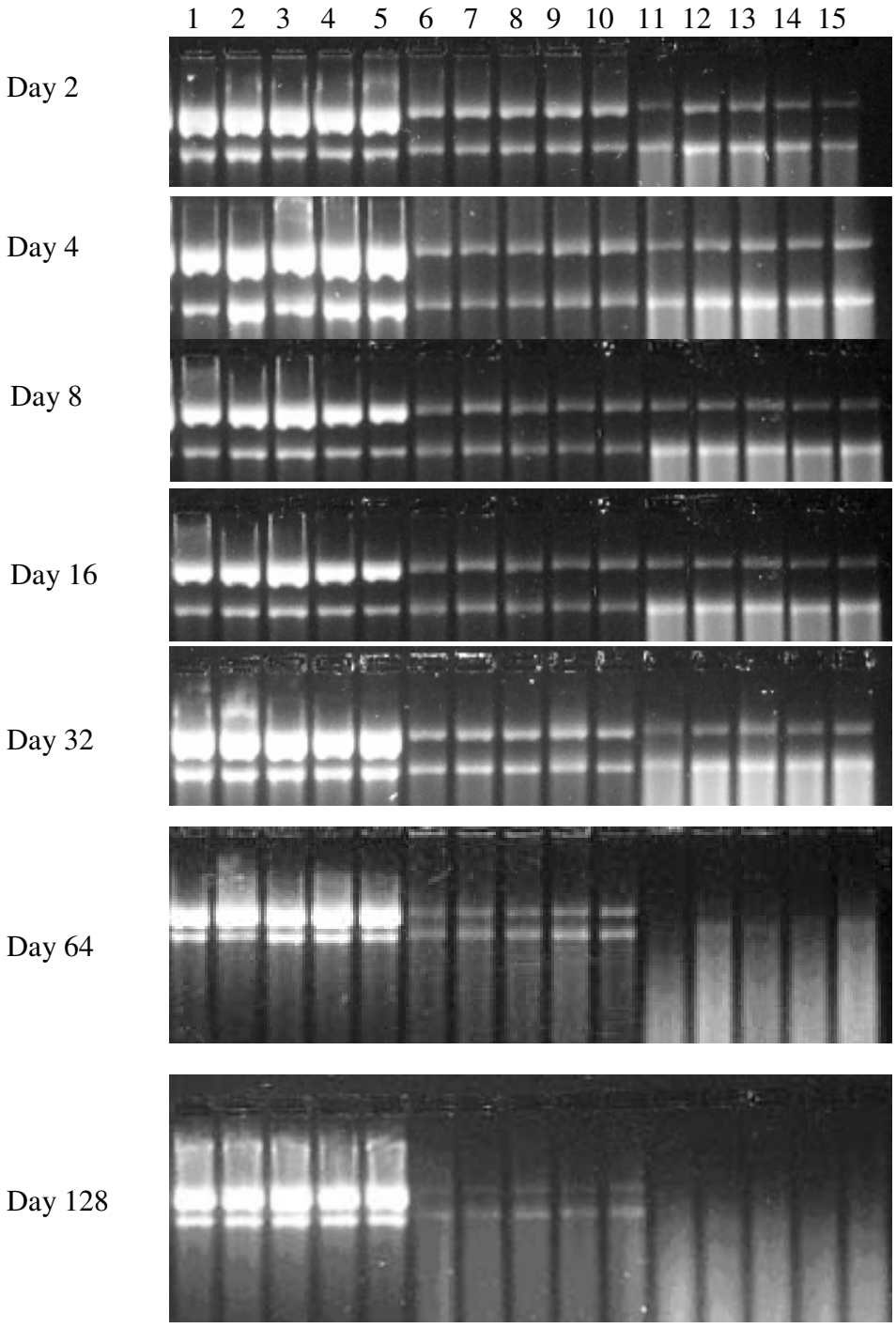


Fig. 5.5. Electrophoretic images of ssDNA samples stored at 15ºC. Lanes 1-5 contain 400 ng reference ssDNA stored at -20° C. Lanes 6-10 contain ssDNA recovered from 10%AG. Lanes 11-15 contain ssDNA recovered from 10% pullulan.

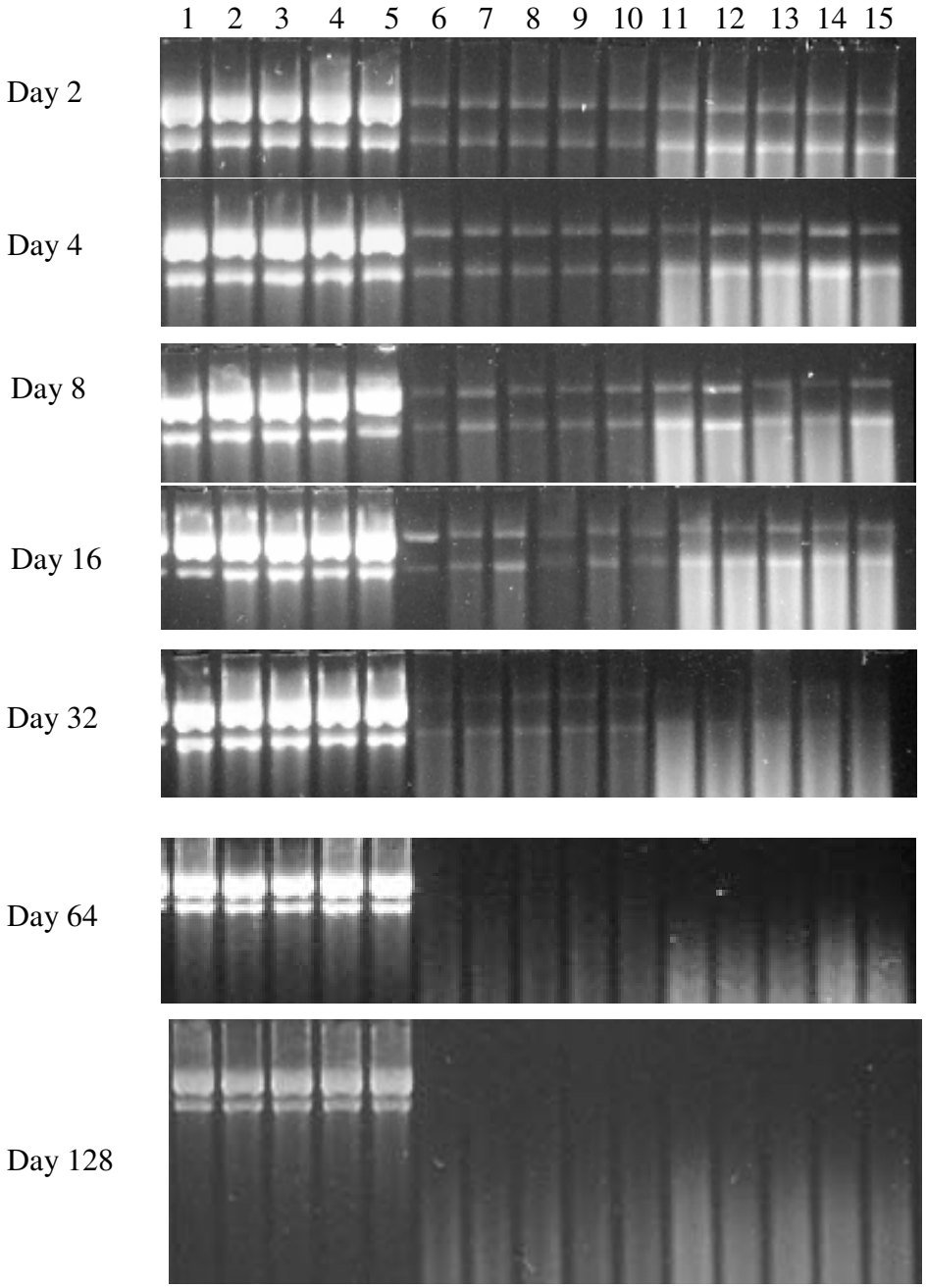


Fig. 5.6. Electrophoretic images of ssDNA samples stored at 46% humidity. Lanes 1-5 contain 400 ng reference ssDNA stored at -20° C. Lanes 6-10 contain ssDNA recovered from 10% AG. Lanes 11-15 contain ssDNA recovered from 10% pullulan.

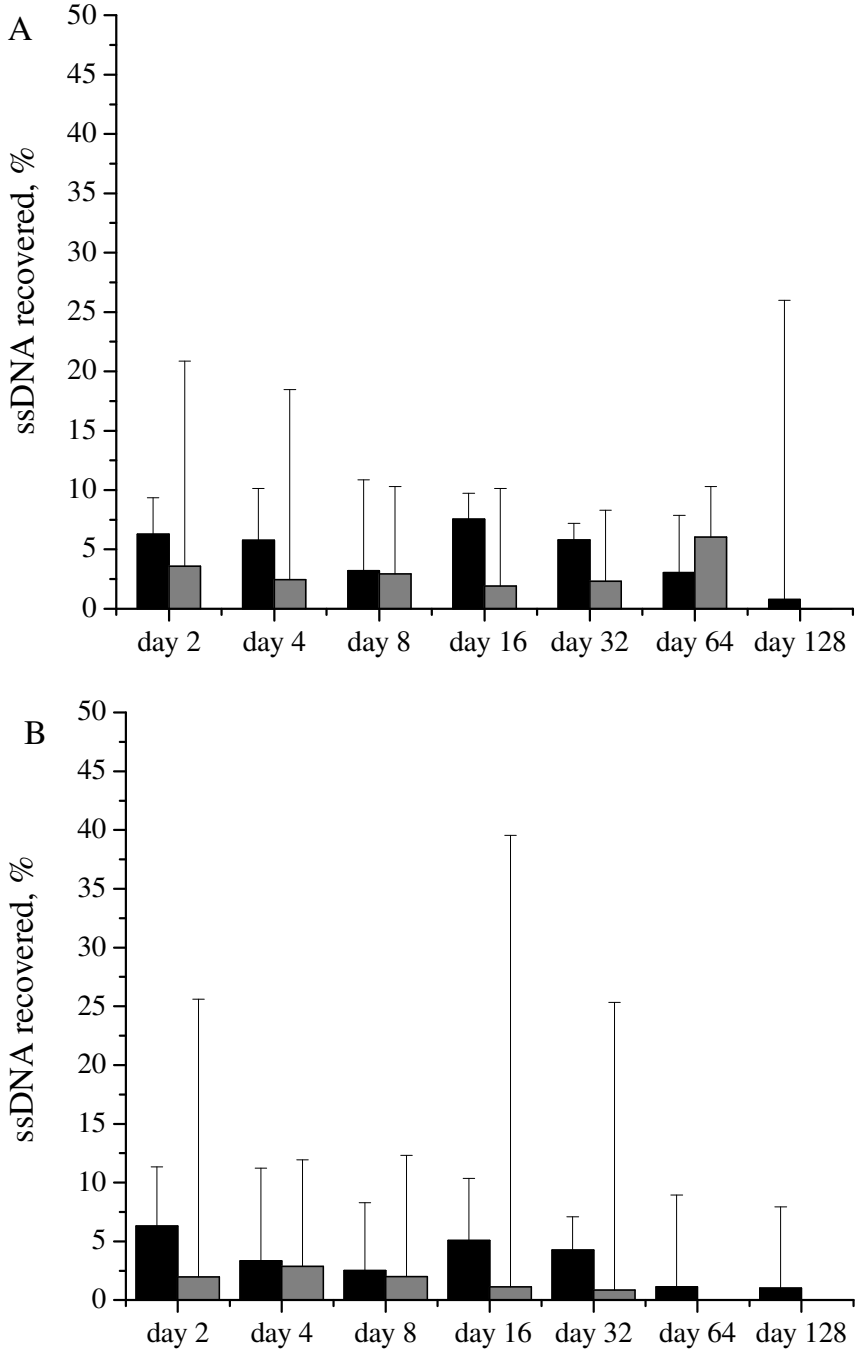


Fig. 5.7. Amounts of intact ssDNA recovered from AG (black bars) and pullulan (gray bars) that were stored at various temperatures. A. Storage at 5° C. B. Storage at 15° C. C. Storage at 25° C. D. Storage at 40° C.

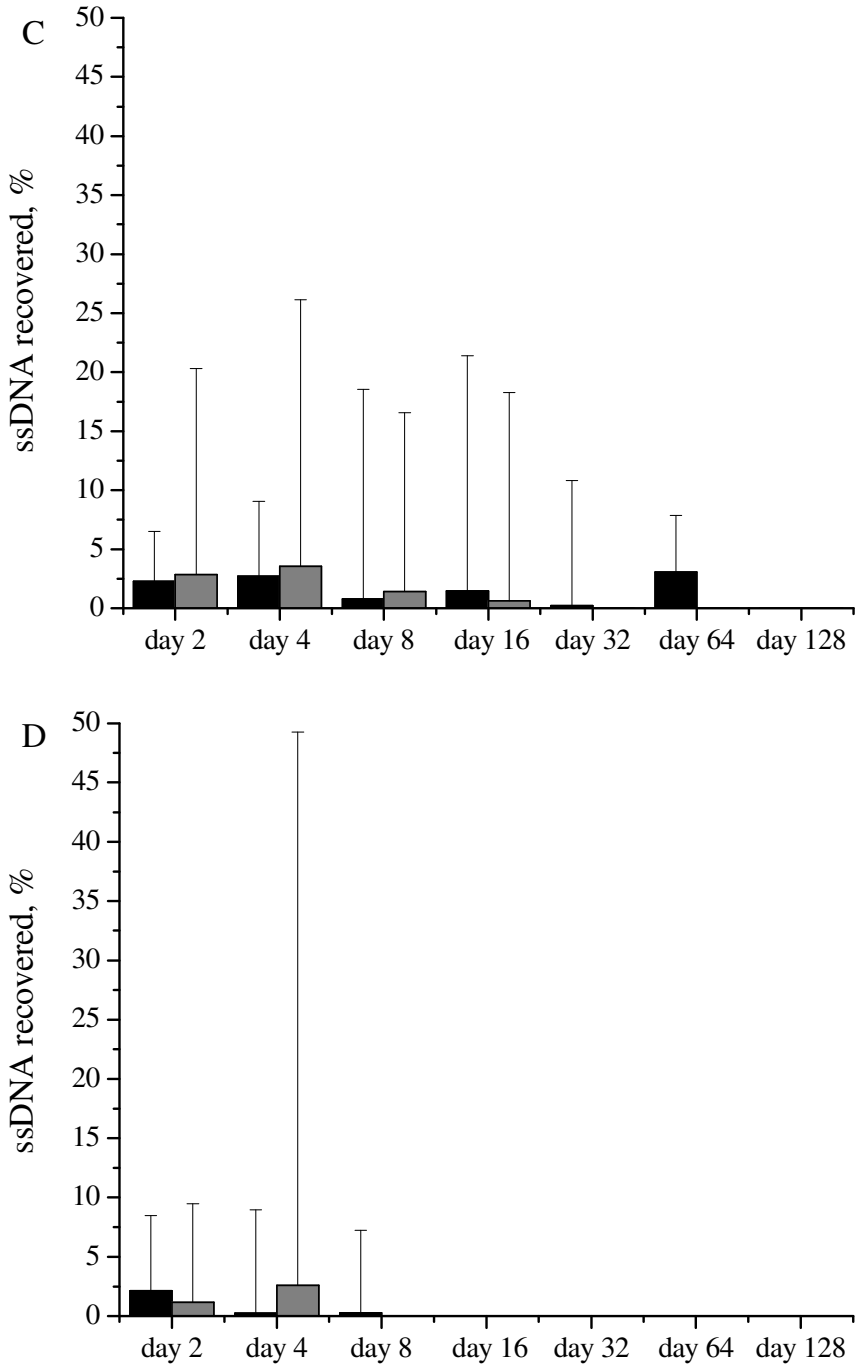


Fig. 5.7. continued.

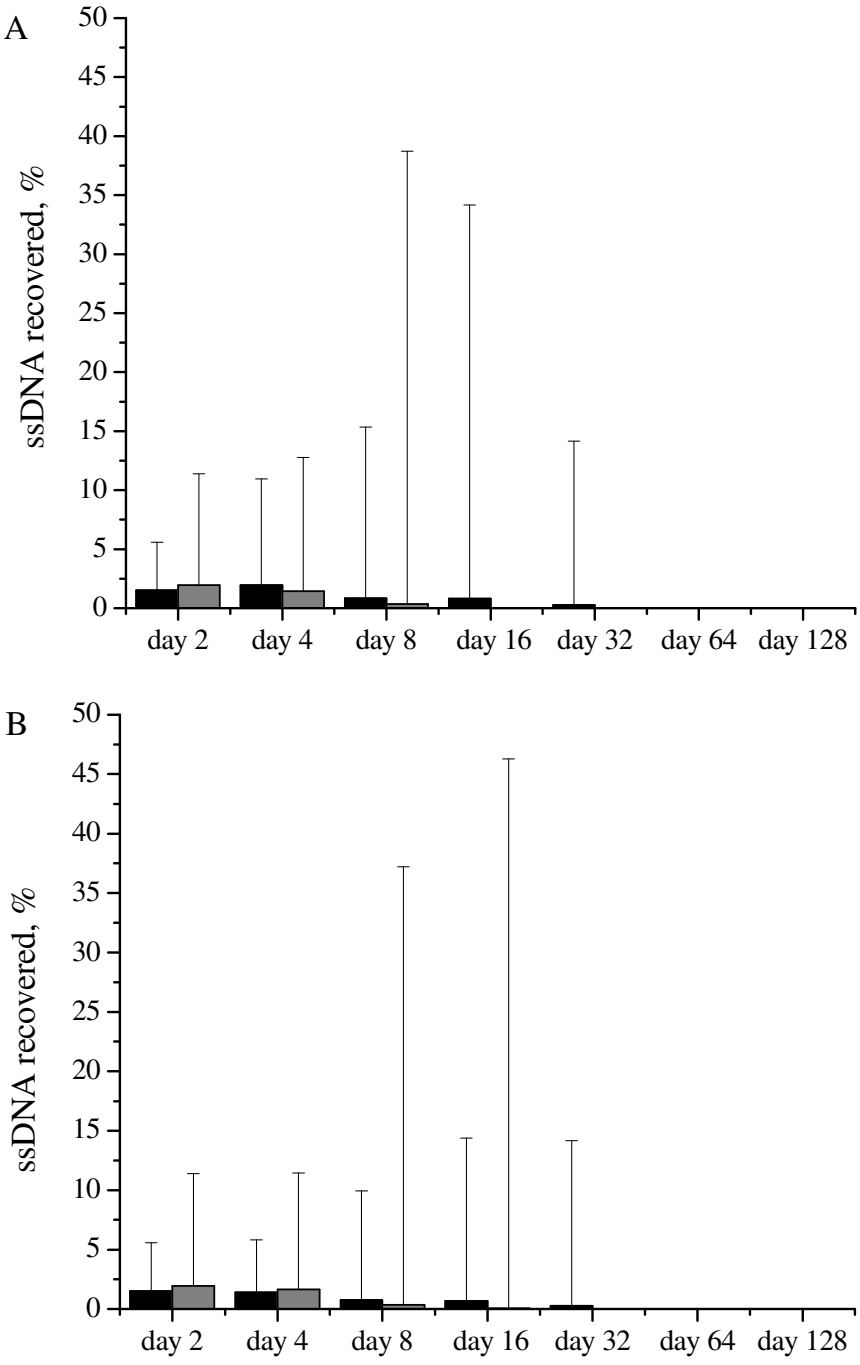


Fig. 5.8. Amounts of intact ssDNA recovered from AG (black bars) and pullulan (gray bars) that were stored at various humidity. A. Storage at 46% humidity. B. Storage at 53% humidity. C. Storage at 76% humidity. D. Storage at 86% humidity.

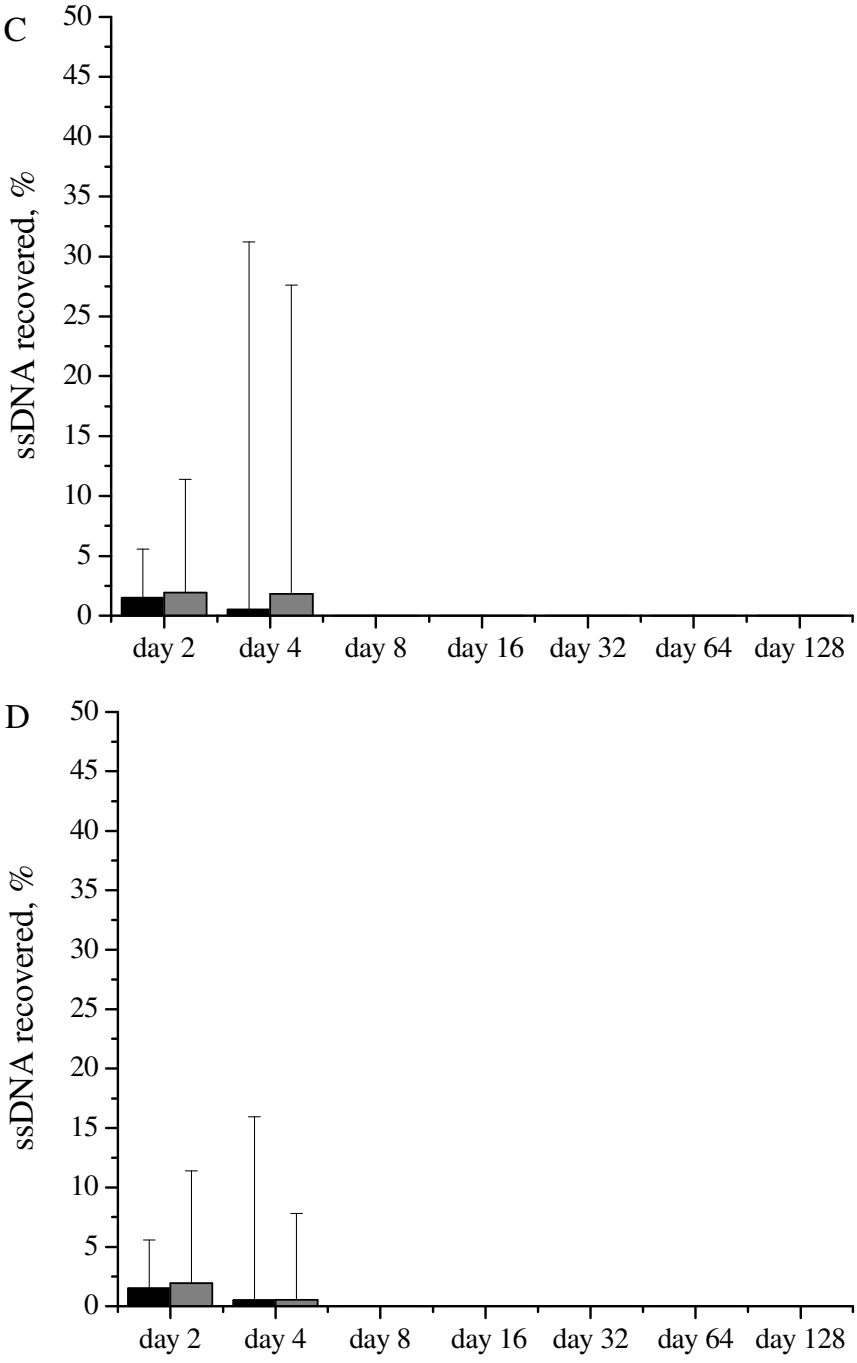


Fig. 5.8. continued.

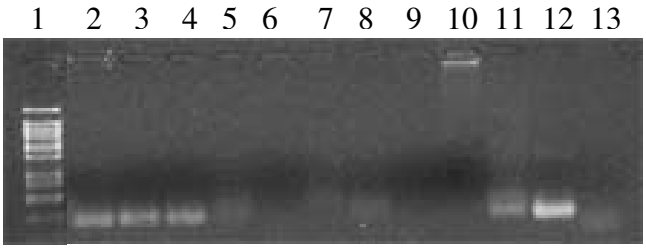


Fig. 5.9. Electrophoretic images of PCR products obtained from amplification of ssDNA samples on day 118 of storage. Lane 1, molecular weight marker (λ Bst EII digest). Lanes 2-5, ssDNA purified from in AG samples stored for 118 days at 5, 15, 25, and 40°C, respectively. Lanes 6-9, ssDNA purified from pullulan samples stored for 118 days at 5, 15, 25 and 40°C, respectively. Lane 10, ssDNA in ~16 % AG stored at 25°C. Lane 11, ssDNA in ~16% pullulan stored at 25°C. Lane 12, intact ssDNA stored at -20°C. Lane 13, negative PCR control (no DNA present).

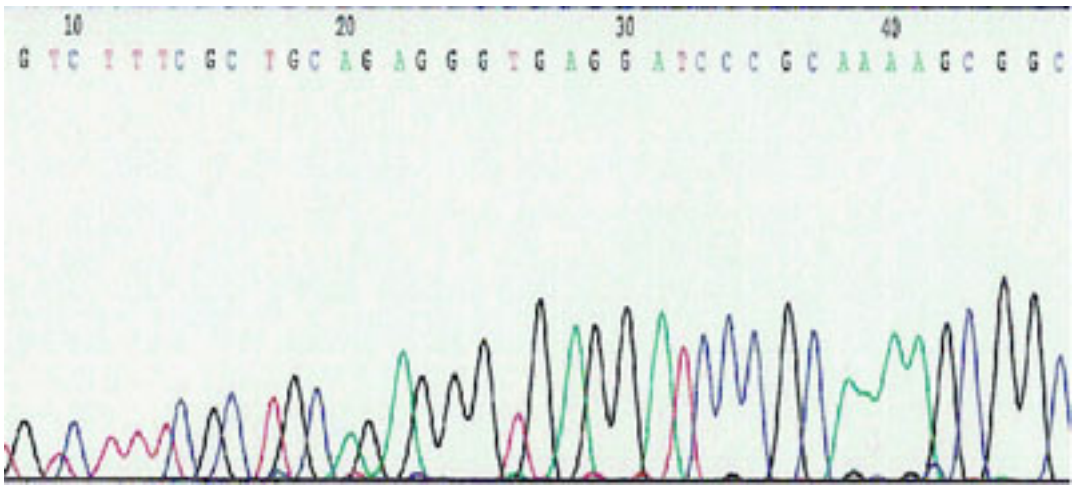


Fig. 5.10. Representative portion of DNA sequence of ssDNA preserved in AG for 118 days.

The PCR reactions were successful and DNA sequencing indicated that in the presence of ~ 16% AG and pullulan there was no variation in DNA sequence of the PCR products compared to control DNA samples stored at -20° C.

5.3.4 Long-term preservation of dsDNA

To determine integrity of dsDNA after preserving in protective polymers for long-term storage under various conditions, dsDNA samples in 10% AG or 10% pullulan (control polymer) were dried at 25°C. After drying, two types of long-term preservation experiments were performed: (1) storage at various temperatures and constant humidity and (2) storage at various humidity and constant temperature.

For dsDNA temperature experiments, samples were dried, stored at 5, 15, 25, or 40°C and constant humidity (~33%) and dsDNA integrity was tested on days 2, 4, 8, 16, 32, 64 and 128 of storage. Representative gels used for detection of intact dsDNA after storage at various temperature and humidity are shown in Fig 5.11 and Fig. 5.12, respectively. Samples were tested after drying for 2 days (Fig. 5.13). The relative amounts of intact dsDNA recovered from AG and pullulan samples after drying were 79.7 and 93.1%, respectively. The relative amounts of dsDNA recovered from AG stored at 5 and 15° C tested on day 4 were 77.0 and 73.6%, respectively (Fig. 5.14 A-B). After this initial period, there appeared to be no significant degradation of dsDNA up to day 64 for AG samples stored at 5 and 15° C. However, the relative amounts of dsDNA recovered from AG stored at 25 and 40° C significantly declined on day 16 and day 8, respectively (Fig. 5.14 C-D).

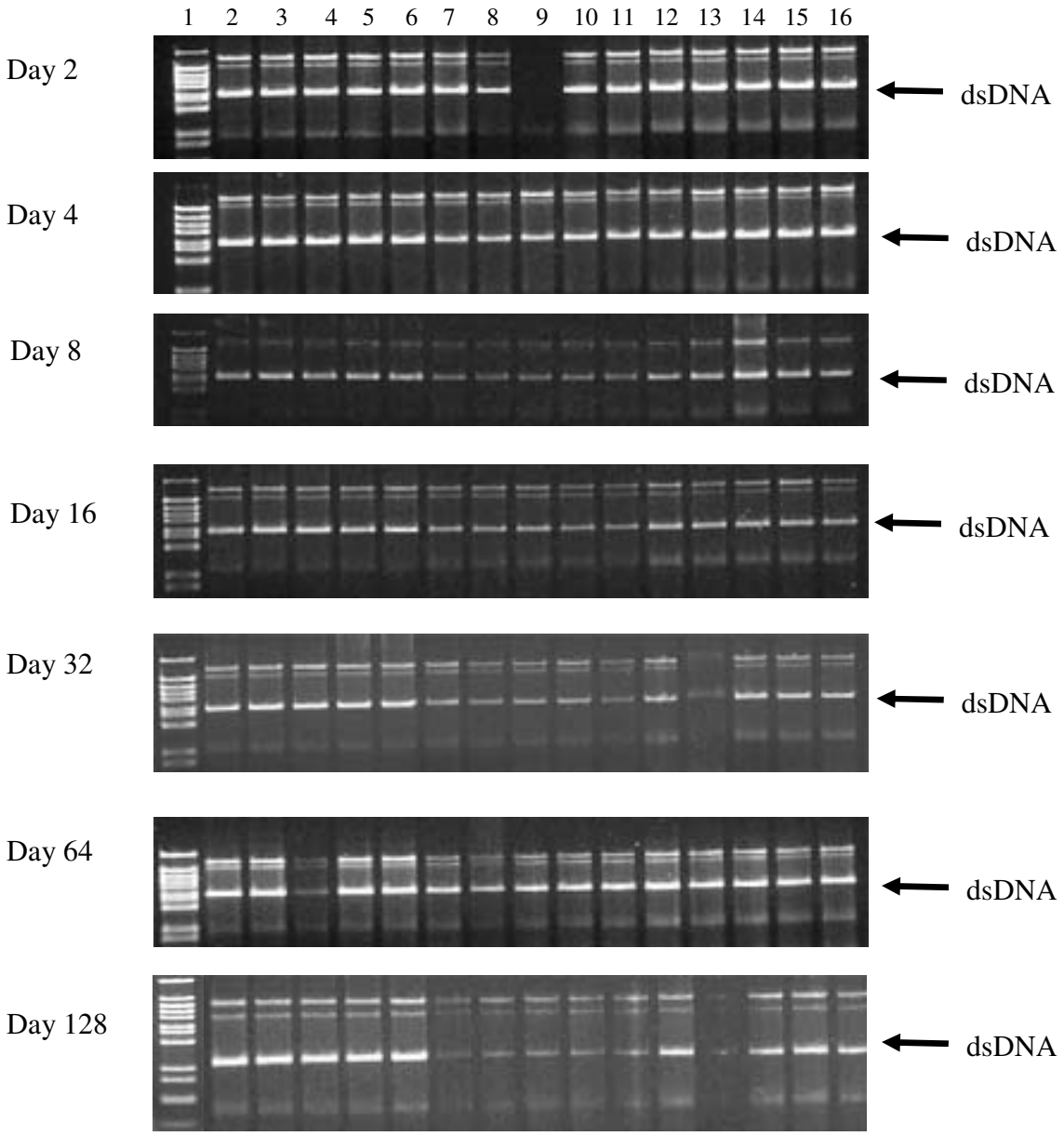


Fig. 5.11. Electrophoretic images of dsDNA stored at 40º C. Lane 1 is a molecular weight standard. Lanes 2-6 contain 400 ng of dsDNA reference stored at -20°. Lanes 7-11 contain dsDNA recovered from 10% AG. Lanes 12-16 contain dsDNA recovered from 10% pullulan.

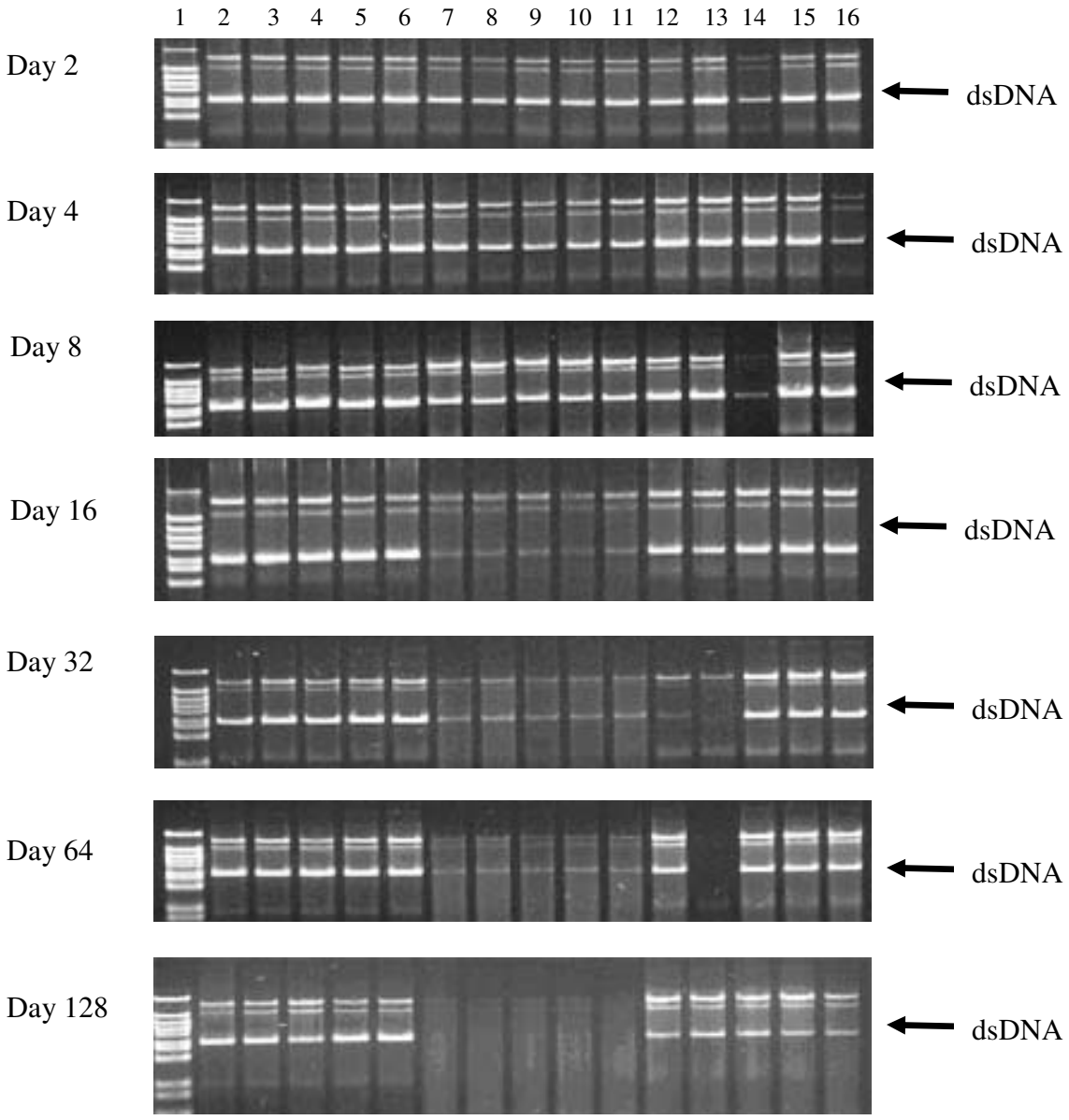


Fig. 5.12. Electrophoretic images of dsDNA stored at 53% humidity at 25°C. Lane 1 is a molecular weight standard. Lanes 2-6 contain 400 ng of dsDNA reference stored at -20°. Lanes 7-11 contain dsDNA recovered from 10% AG. Lanes 12-16 contain dsDNA recovered from 10% pullulan.

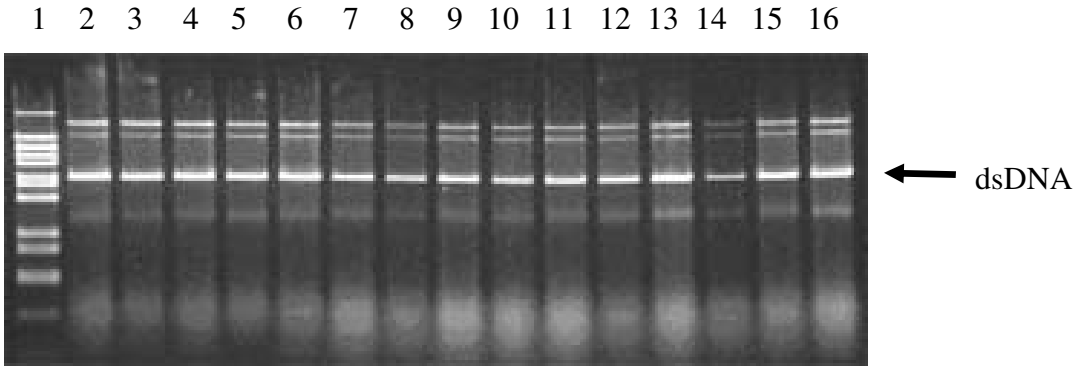


Fig. 5.13. Electrophoretic image of dsDNA after drying for 2 days at 25°C in 10% AG and 10% pullulan. Lane 1 is a molecular weight standard. Lanes 2-6 contain 400 ng of dsDNA reference stored at -20°. Lane 7-11 contain dsDNA recovered from 10% gum arabic. Lane 12-16 contains dsDNA recovered from 10% pullulan.

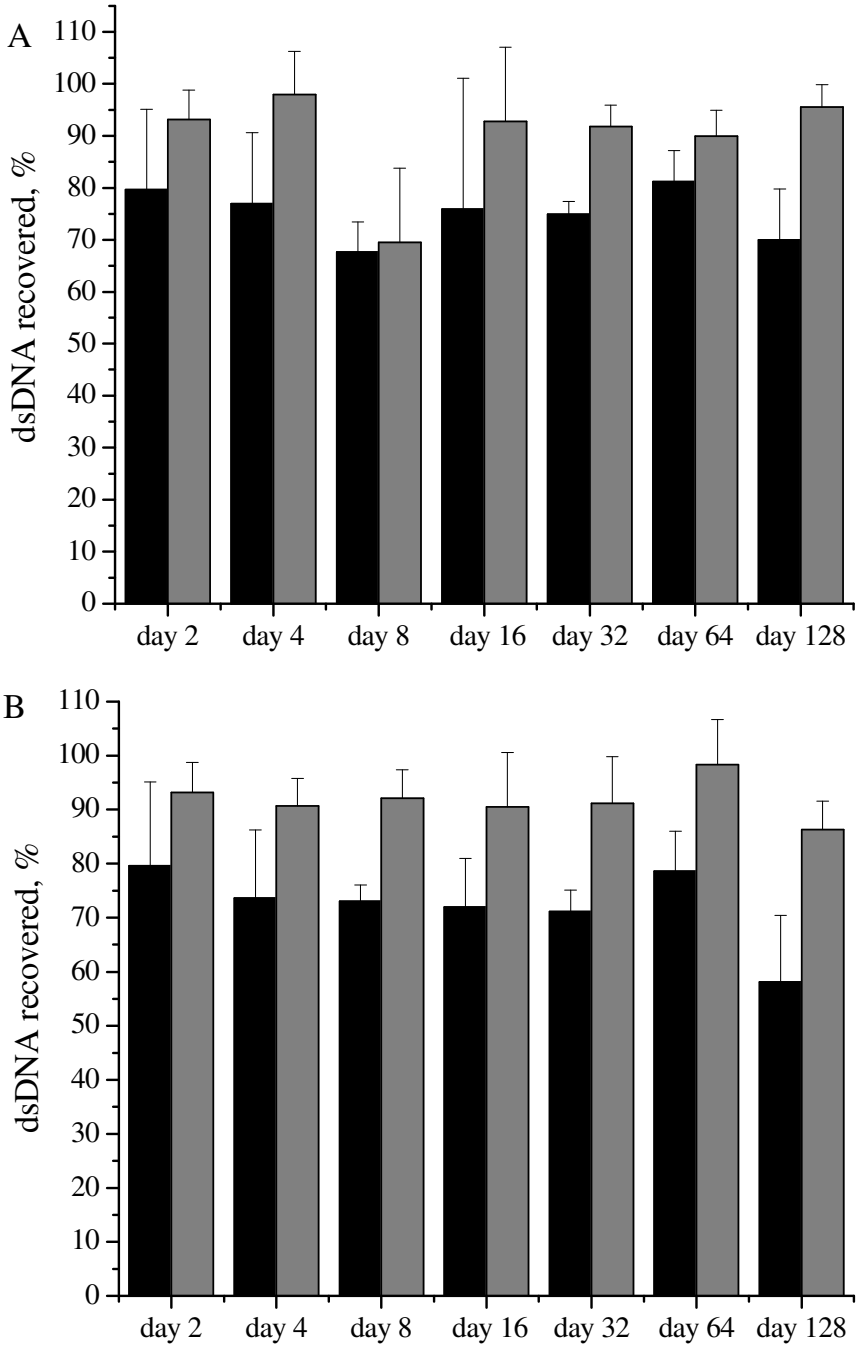


Fig. 5.14. Amounts of intact dsDNA recovered from AG (black bars) and pullulan (gray bars) that were stored at various temperatures. A. Storage at 5°C. B. Storage at 15°C. C. Storage at 25°C. D. Storage at 40°C.

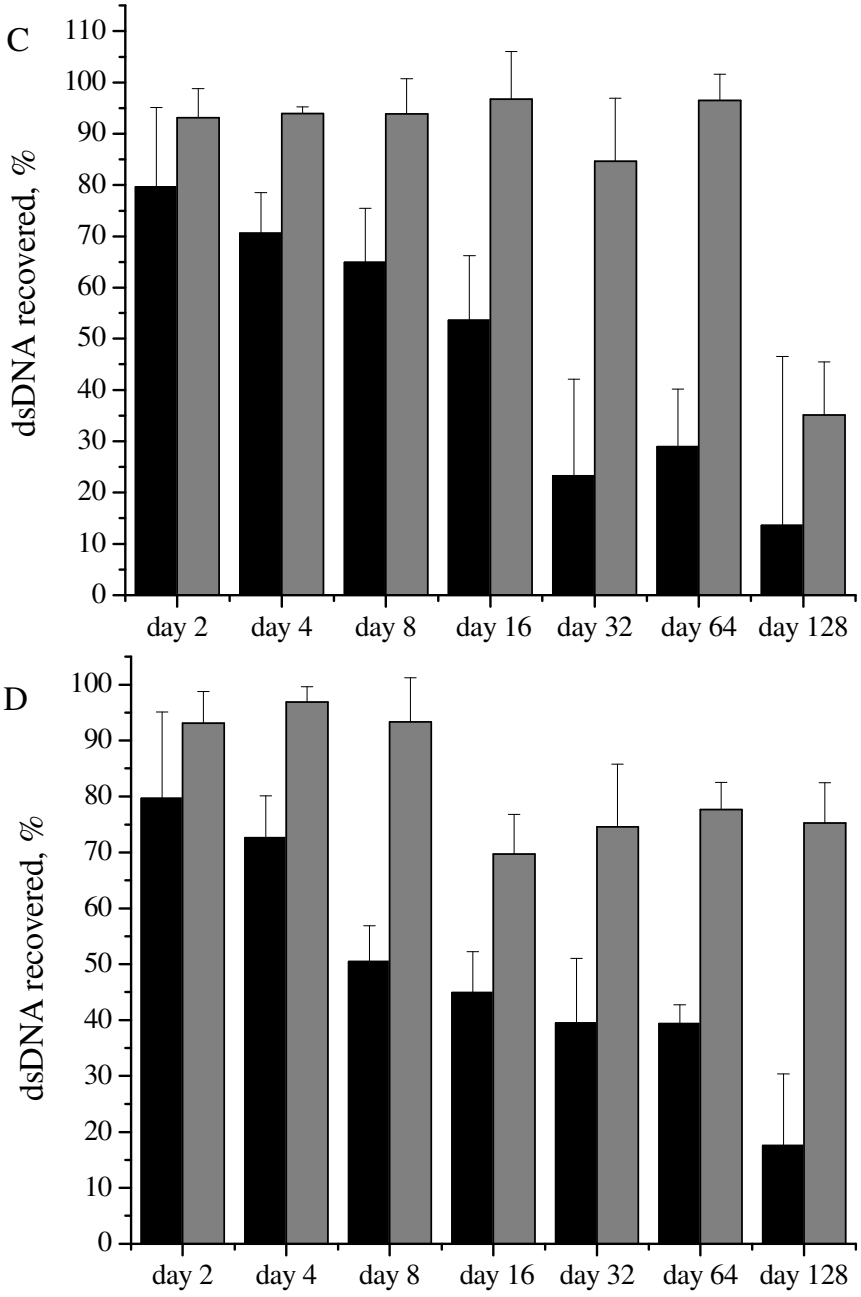


Fig. 5.14. continued.

For pullulan samples, no significant degradation occurred after the drying process. The relative amounts of intact dsDNA from pullulan samples stored at 5, 15 and 25° C up to day 64 were 90.0, 98.4 and 96.5%, respectively (Fig. 5.14). These samples were comparable to control stored at -20° C in TE buffer. Pullulan samples stored at 40°C were remarkably consistent and similar to controls until day 16 with a slight decline to 69.7 %, but less than that of AG samples (44.9%). It was evident that dsDNA were more stable in pullulan polymer through the drying and storing process than in AG polymer. Intact dsDNA were recovered from both formulations at all storage temperatures up to day 64.

Similar to dsDNA temperature experiment, in the humidity experiment the samples were dried for two days and tested. The relative amounts of intact dsDNA for AG and pullulan samples after drying were 78.3 and 89.8%, respectively. The relative amount of dsDNA recovered from AG samples stored at 46 and 53 % humidity began to decline on day 2 and became more noticeable on day 8 (Fig. 5.15). Intact dsDNA were recovered from storage at 46 and 53% humidity on day 64 but the amount was significantly less than control, 23.1 and 13.4%, respectively. The relative amounts of dsDNA recovered from AG samples stored at 76 and 86% humidity on day 8 were 20.6 and 35.5%, respectively (Fig. 5.15 C-D). There was a small amount of detectable dsDNA on day 16 for AG samples stored at 86%, but no intact dsDNA were detected for samples stored at 76% humidity on day 16. On day 32, there was not detectable dsDNA for either AG samples stored at 76 or 86% humidity.

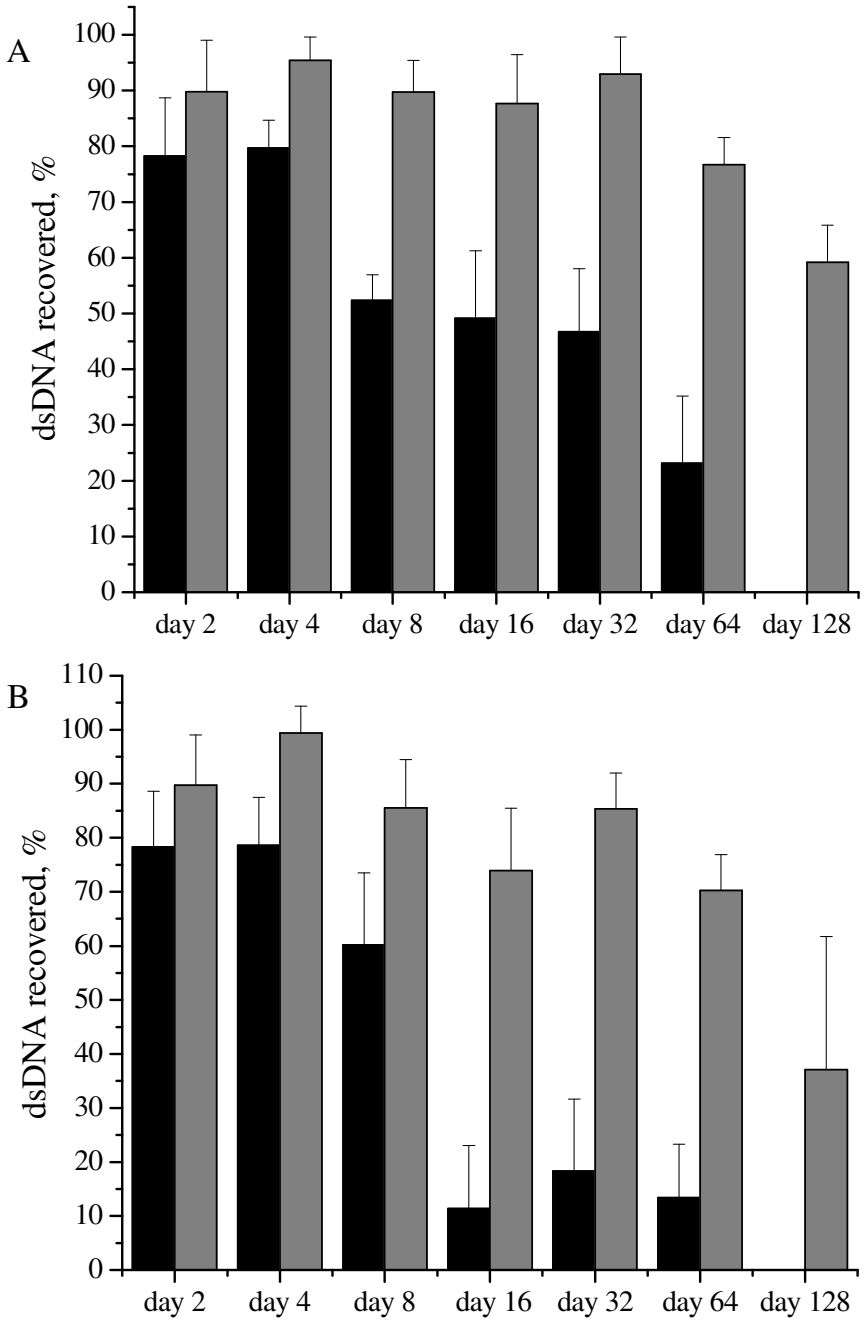


Fig. 5.15. Amounts of intact dsDNA recovered from AG (black bars) and pullulan (gray bars) that were stored at various humidity. A. Storage at 46% humidity. B. Storage at 53% humidity. C. Storage at 76% humidity. D. Storage at 86% humidity.

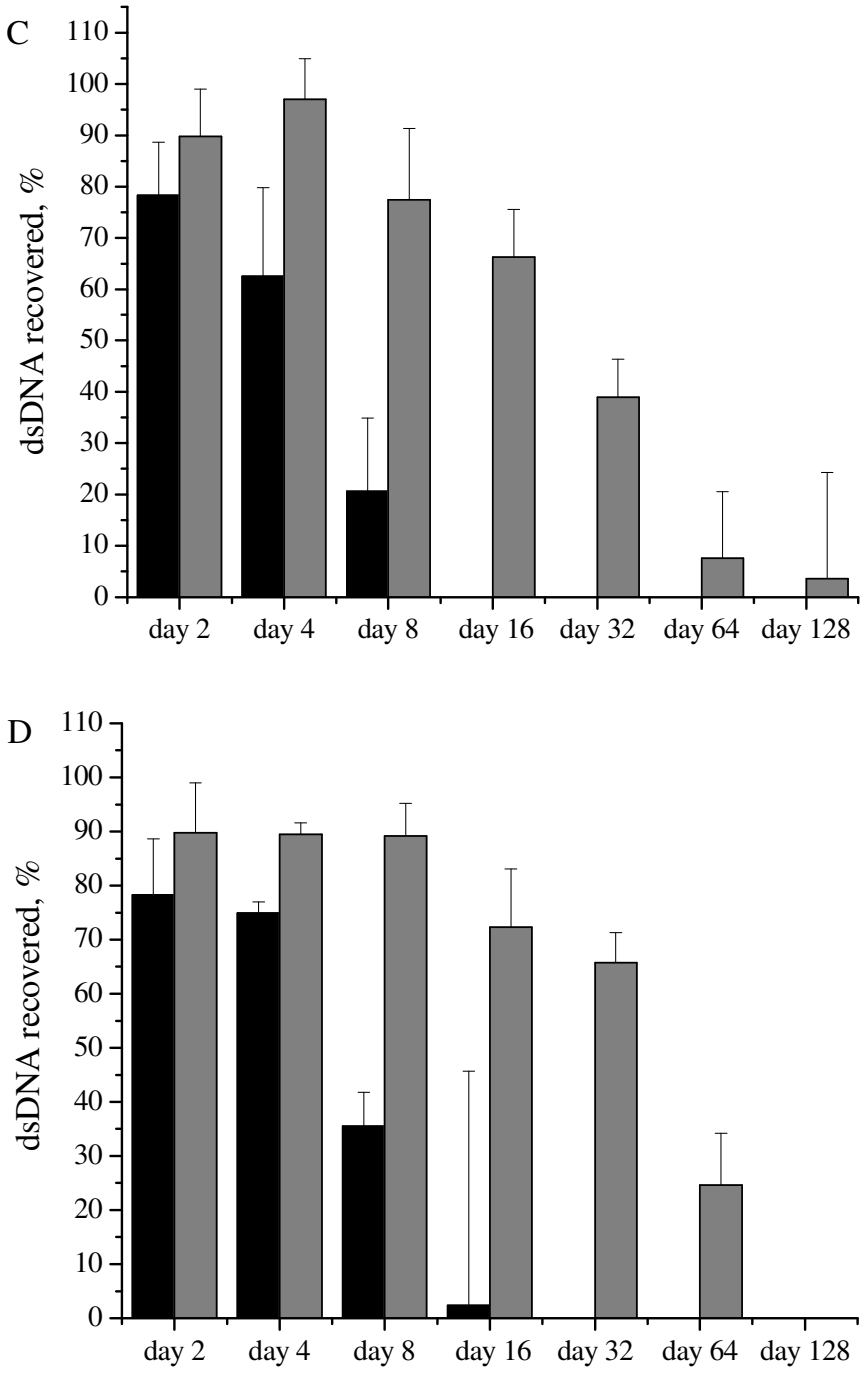


Fig. 5.15. continued.

dsDNA samples preserved in pullulan were more stable than dsDNA in AG. Samples stored at 46 and 53% humidity were similar to dsDNA pullulan samples stored at 25°C (Fig. 5.15 A-B). dsDNA pullulan samples stored at higher humidity recovered much less intact dsDNA with time. On day 64, the relative amounts of dsDNA recovered for 76 and 86% humidity were 7.6 and 24.6%, respectively (Fig. 5.15 C-D).

5.3.5 Discussion

The work described here was the first known study that utilizes acacia gum (AG) and pullulan polymers as a protectant for desiccated ssDNA and dsDNA. ssDNA was unstable and degraded fast during drying for 2 days at room temperature in both polymers. Once dried, more intact ssDNA was recovered from AG samples than pullulan for all conditions tested. There was no detectable ssDNA in AG and pullulan samples stored at elevated temperatures. On day 118 of preservation, successful PCR amplification for both types of ssDNA samples was accomplished. The sequence obtained from control ssDNA which was stored in TE buffer at -20°C was identical to experimental samples. dsDNA was very stable when preserved in both polymers and stored at cool temperatures up to 128 days. Degradation of dsDNA was faster for AG samples than pullulan samples when stored at elevated temperatures and all humidity levels tested. Nonetheless, intact dsDNA preserved in AG was detected up to day 128 day for samples stored at 33% humidity and 25 and 40°C and up to 64 days for samples at 46 and 53% humidity at 25°C.

The results obtained in these experiments are promising and show that AG and pullulan polymers protect DNA to some degree when dried and stored under various conditions using an inexpensive and simple process. DNA integrity could be prolonged when preserved in AG if more is known about the mechanism of DNA degradation during the drying process and storage in AG. For example, AG is a heterogeneous mixture of carbohydrates and proteins (Schmitt et al., 1998) and contains calcium, magnesium, potassium and sodium (Duke, 1983). It is possible that the AG used in this study contained divalent cations. The presence of divalent cations (Mg^{+2}, Ca^{2+} and Ba^{2+} and Mn^{2+}) are well known to effect oligonucleotide conformations (Patil, 2002), and binding of these cations to DNA has been shown to effect stability (Davey and Richmond, 2002). Other factors that may contribute to a decrease in DNA integrity could involve the loss of water (Wu et al., 2005) and the low pH of AG. In addition, enzymatic processes, temperature, humidity, and oxidation contribute to DNA degradtion. The most favorable conditions for preservation of DNA are reported to be low humidity and temperature and absence of microorganisms (Poinar and Stankiewicz, 1999).

This method is applicable for transporting DNA samples because no refrigeration is needed for recovery of adequate amounts of DNA for the use in laboratory setting. In our study, the PCR products amplified from ssDNA samples preserved in protective polymers were sequenced using specific primers. The sequences of all samples tested were identical to controlssDNA stored in TE buffer at -20°C. Degradation of ssDNA in AG and pullulan samples is fast and no ssDNA is detected when samples are stored at elevated temperatures on day 128. PCR products of the correct size were obtained from ssDNA stored in AG and pullulan at different conditions for 118 days. When the

amplified DNA were sequenced, there were no discrepancies between the DNA sequence of the experimental samples and control

5. 4. Conclusions

ssDNA is sensitive to temperature degradation even in the presence of protective polymers, AG and pullulan. The greatest amount of intact ssDNA was recovered from AG samples when stored at 5 and 15° C and low humidity (46 and 53%). In spite of fast degradation, PCR products of the correct size were obtained from ssDNA stored in AG and pullulan at different conditions for 118 days. These PCR products when sequenced were identical to control. dsDNA is resistant to degradation. For all storage conditions, intact dsDNA was recovered up to 64 days in pullulan. dsDNA was recovered from AG and pullulan up to 128 days when stored at cool temperatures. For all storage temperatures studied, more dsDNA was recovered from pullulan samples than AG.

CHAPTER 6

CONCLUSIONS

I. A simple process for preserving biological materials in acacia gum (AG) without using specialized equipment or complex formulations was developed. Biologicals used for testing the protective capacity of AG were *Escherichia coli*, *Bacillus subtilis* spores, ssDNA and dsDNA. The optimal drying and storage conditions for preserving each biological was determined as follows:

   1) *E. coli* vegetative cells were best preserved when diluted (1:4) in 15% AG and dried in small Petri plates in a 40° C incubator containing silica desiccant for ~20 hours. *E. coli* viability varied significantly depending on storage conditions. Long-term storage was optimal when samples were maintained at 5° C at low humidity. The time to degrade *E. coli* to 100 CFU when stored at 5, 15, 25, and 40° C are 660, 240, 65, and 20 days, respectively. *E. coli* cells preserved in AG were less sensitive to desiccation than control and retain their viability under cool and dry storage conditions. For extended period of time, *E. coli* cells retain their viability in AG at temperatures and humidity levels of 5-15ºC and 30-46%, respectively.

2) *B. subtilis* cells are optimally recovered when suspended in AG and dried for 2 days in glass at 40° C. Bacterial titer in PBS and pullulan controls after drying are 100 fold less than bacterial titer in AG. *B. subtilis* vegetative cells form spores during the 2 day drying process. For both temperature and humidity experiments, *B. subtilis* viability remains consistent after spores are formed. AG provides better environment for spore formation than pullulan and PBS. *B. subtilis* spores were successfully preserved in AG polymer up to 615 days.

3) ssDNA is sensitive to temperature degradation even in the presence of protective polymers, AG and pullulan. The greatest amount of intact ssDNA was recovered from AG samples when stored at 5 and 15° C and low humidity (46 and 53%).

4) dsDNA is resistant to degradation. For all storage conditions, intact dsDNA was recovered up to 64 days in pullulan. dsDNA was recovered from AG and pullulan up to 128 days when stored at cool temperatures. For all storage temperatures studied, more dsDNA was recovered from pullulan samples than AG.

II. Biological structures were evaluated for alterations after dehydration, storage and rehydration of samples in AG. The ultrastructure and cell membrane intactness of preserved bacterial cells in AG were evaluated for alterations using electron microscopy and fluorescent microscopy. The integrity of a specific sequence of ssDNA stored in AG was tested using PCR and DNA sequencing.

1) *E. coli* cells dried and stored in AG exhibited irregularities in bacteria cells which included increase in electron density of cell walls and invaginations in cell walls and cell membranes which distort the normal morphology of the cells. These alterations were detected in AG samples up to day 139 for both storage temperatures.
2) The ultrastructure of *E. coli* progeny recovered from cultures preserved in AG for 14 days appeared normal.
3) *B. subtilis* cells dried in AG formed spores and spore morphology remained consistent up to 13 days of storage. AG provided adequate environment for complete spore formation during the drying process.
4) About one-half of both cell types dried in AG appeared to have compromised cell membranes. In addition, *B. subtilis* cultures may appear to be injured, but this could be a result of spores lysing from mother cells.
5) Degradation of ssDNA in AG and pullulan samples is fast, and no ssDNA is detected when samples are stored at elevated temperatures on day 128. PCR products of the correct size were obtained from ssDNA stored in AG and pullulan at different conditions for 118 days. When the amplified DNA was sequenced, there were no discrepancies between the DNA sequence of the experimental samples and control.

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APPENDIX

The following electrophoretic images were used to determine the relative amount of intact DNA recovered from polymers remaining after long-term storage at various conditions. There were a few electrophoretic images included in Chapter 4, the following images complete the set of data obtained to produce bar graphs in Chapter 4.

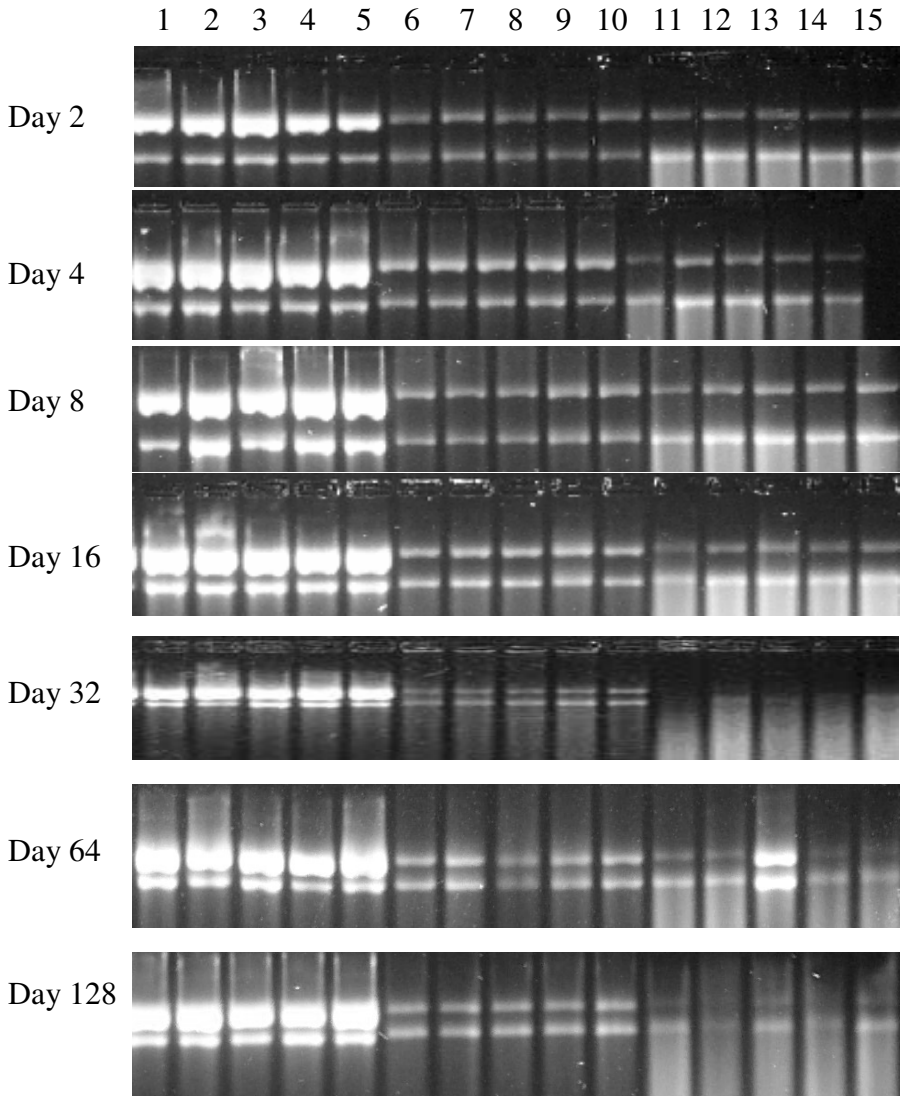

Fig. A.1 Electrophoretic images of ssDNA samples stored at 5ºC. Lanes 1-5 contain 400 ng reference ssDNA stored at -20° C. Lanes 6-10 contain ssDNA recovered from 10%AG. Lanes 11-15 contain ssDNA recovered from 10% pullulan.

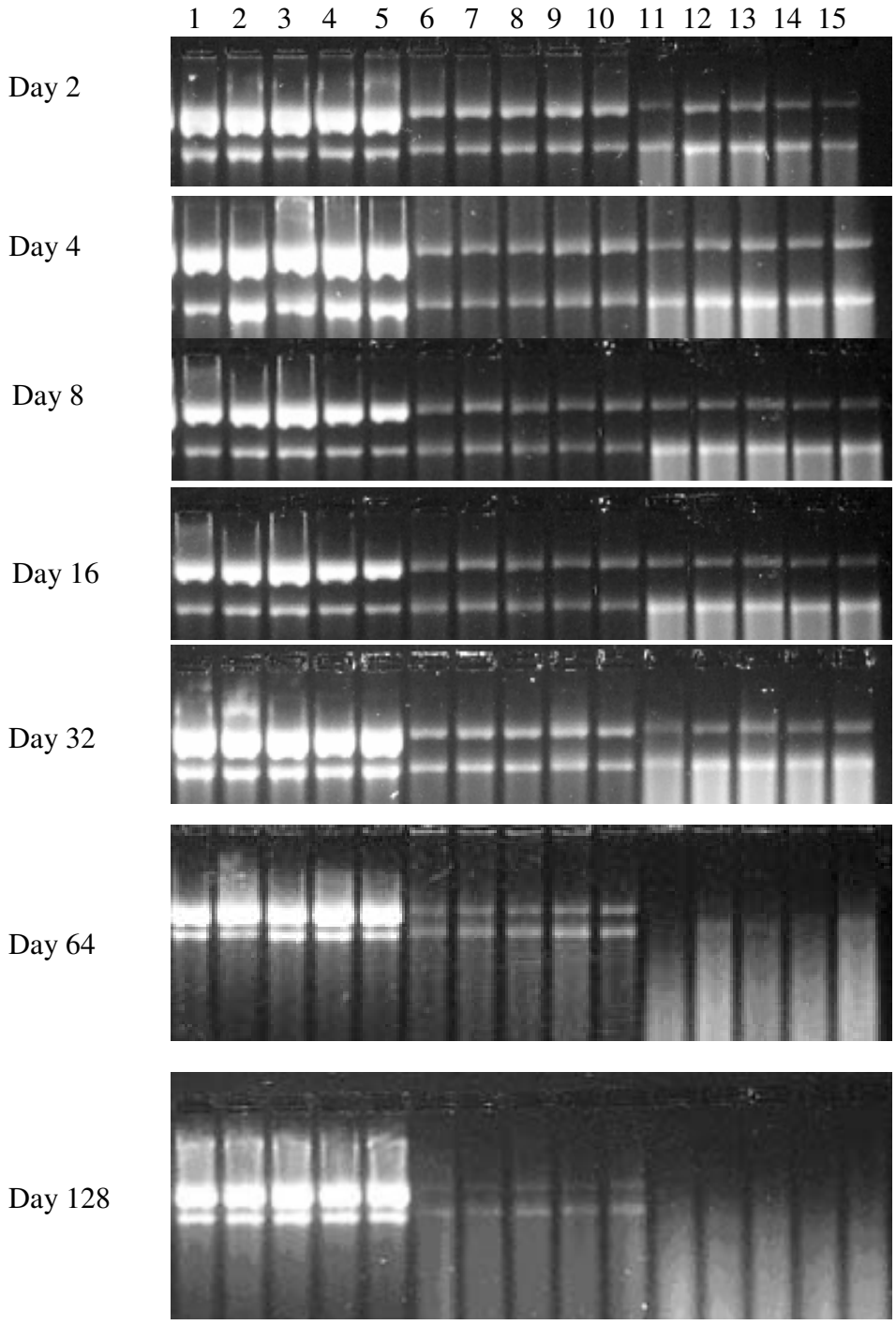

Fig. A.2 Electrophoretic images of ssDNA samples stored at 15ºC. Lanes 1-5 contain 400 ng reference ssDNA stored at -20° C. Lanes 6-10 contain ssDNA recovered from 10%AG. Lanes 11-15 contain ssDNA recovered from 10% pullulan.

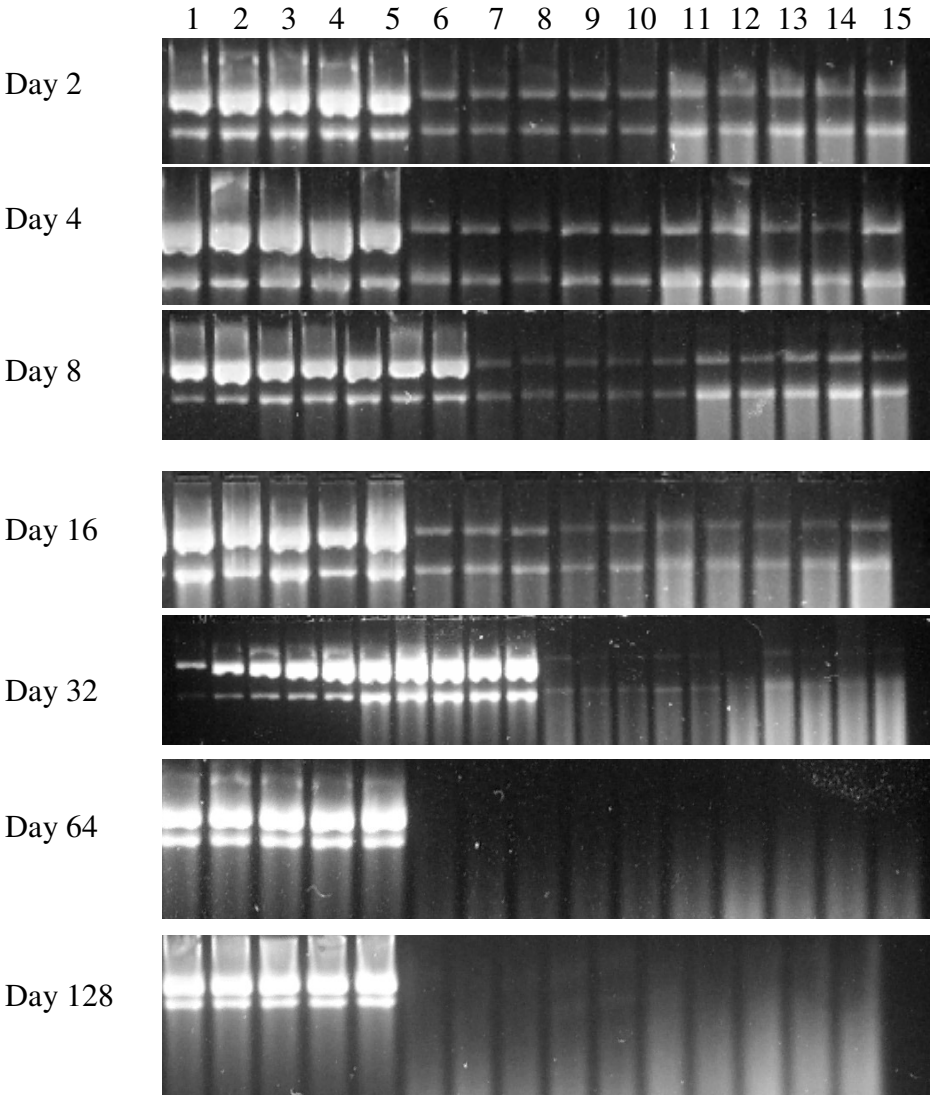

Fig. A.3 Electrophoretic images of ssDNA samples stored at 25ºC. Lanes 1-5 contain 400 ng reference ssDNA stored at -20° C. Lanes 6-10 contain ssDNA recovered from 10%AG. Lanes 11-15 contain ssDNA recovered from 10% pullulan.

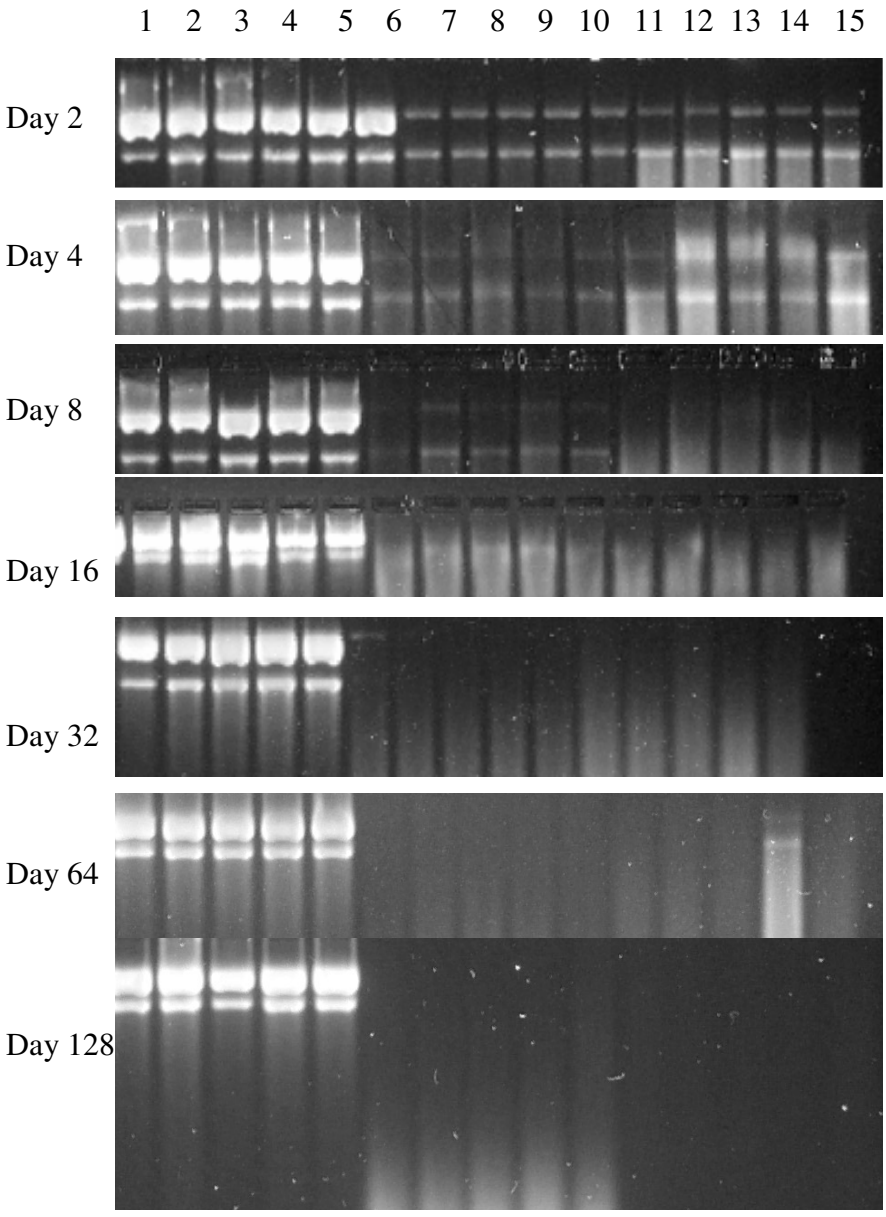

Fig. A.4 Electrophoretic images of ssDNA samples stored at 40ºC. Lanes 1-5 contain 400 ng reference ssDNA stored at -20° C. Lanes 6-10 contain ssDNA recovered from 10%AG. Lanes 11-15 contain ssDNA recovered from 10% pullulan.

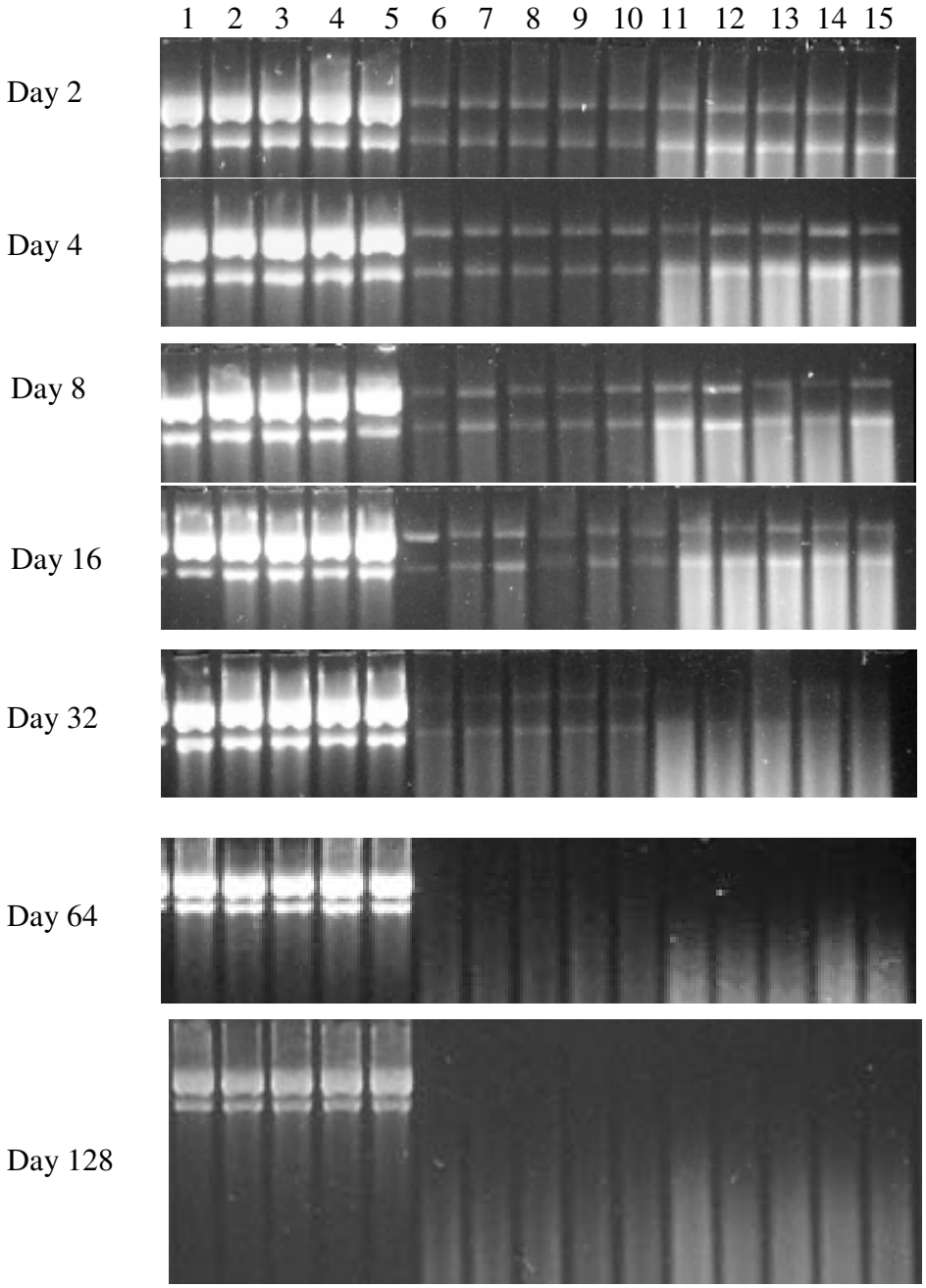

Fig. A.5 Electrophoretic images of ssDNA samples stored at 46% humidity. Lanes 1-5 contain 400 ng reference ssDNA stored at -20° C. Lanes 6-10 contain ssDNA recovered from 10% AG. Lanes 11-15 contain ssDNA recovered from 10% pullulan.

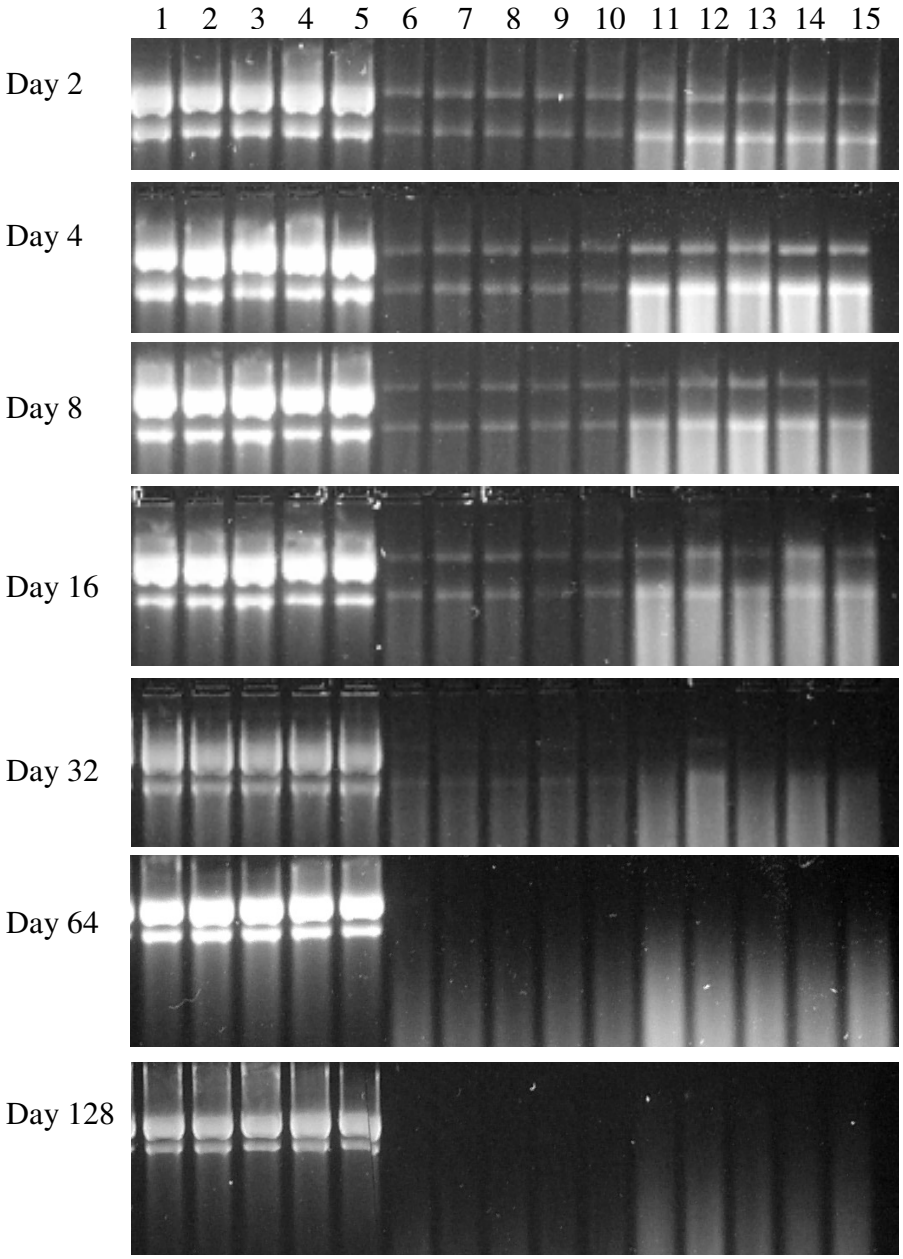

Fig. A.6 Electrophoretic images of ssDNA samples stored at 53% humidity. Lanes 1-5 contain 400 ng reference ssDNA stored at -20° C. Lanes 6-10 contain ssDNA recovered from 10% AG. Lanes 11-15 contain ssDNA recovered from 10% pullulan.

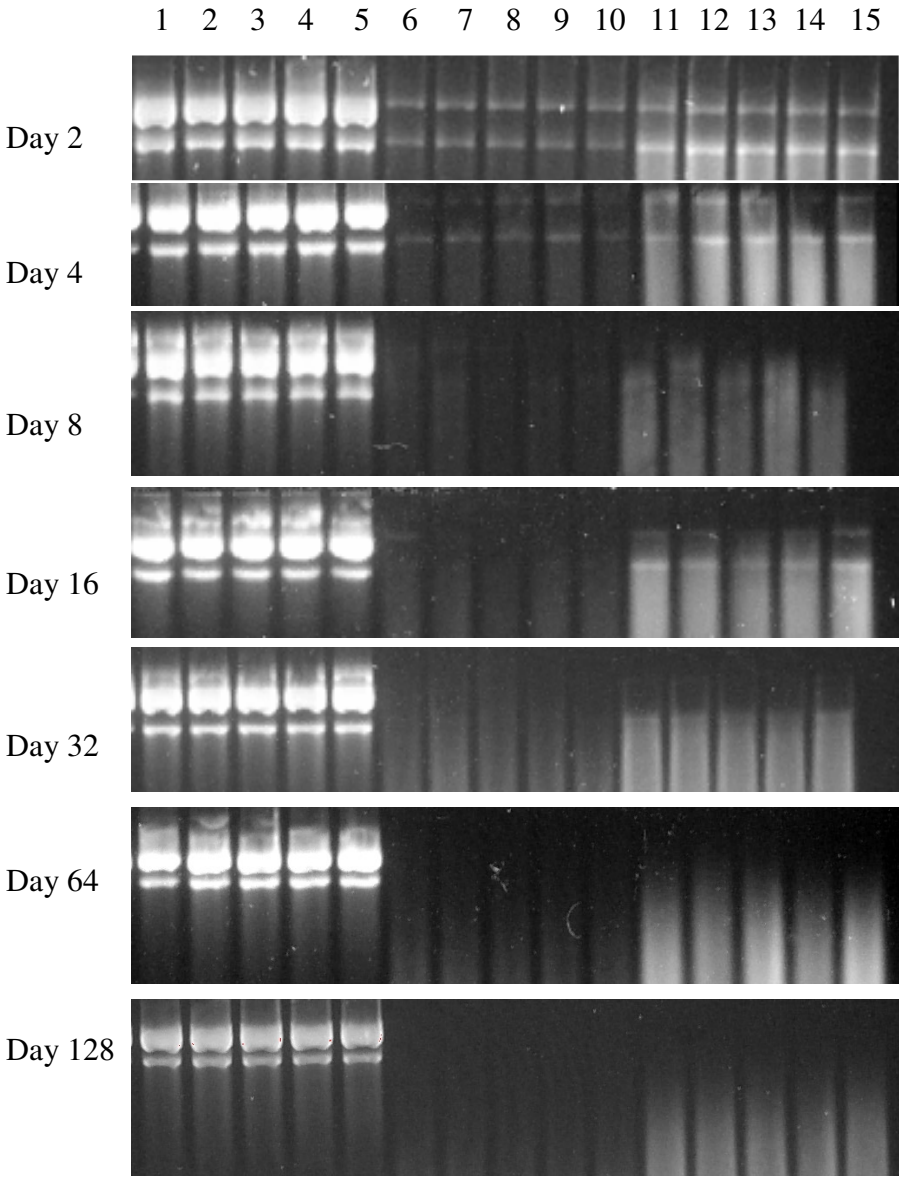

Fig. A.7 Electrophoretic images of ssDNA samples stored at 76% humidity. Lanes 1-5 contain 400 ng reference ssDNA stored at -20° C. Lanes 6-10 contain ssDNA recovered from 10% AG. Lanes 11-15 contain ssDNA recovered from 10% pullulan.

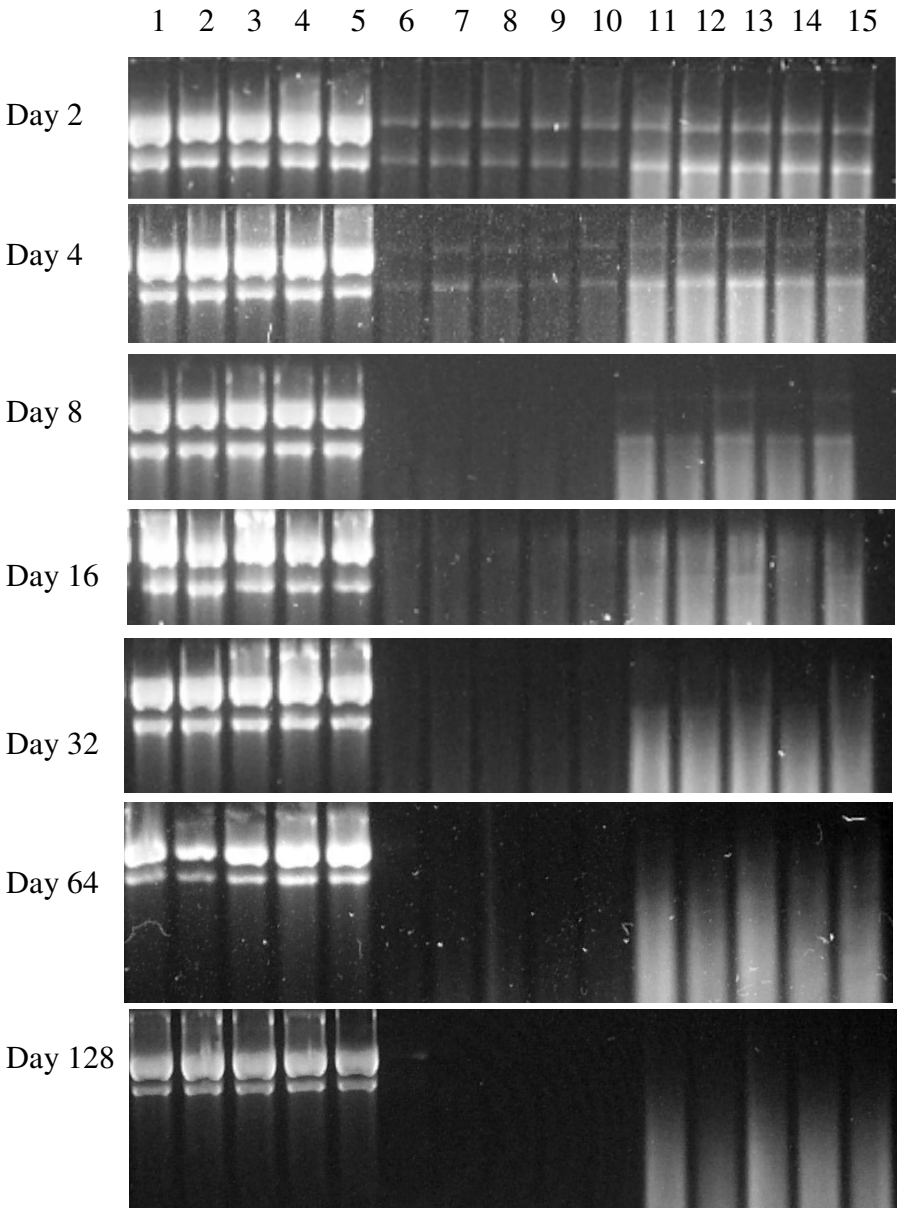

Fig. A.8 Electrophoretic images of ssDNA samples stored at 86% humidity. Lanes 1-5 contain 400 ng reference ssDNA stored at -20° C. Lanes 6-10 contain ssDNA recovered from 10% AG. Lanes 11-15 contain ssDNA recovered from 10% pullulan.

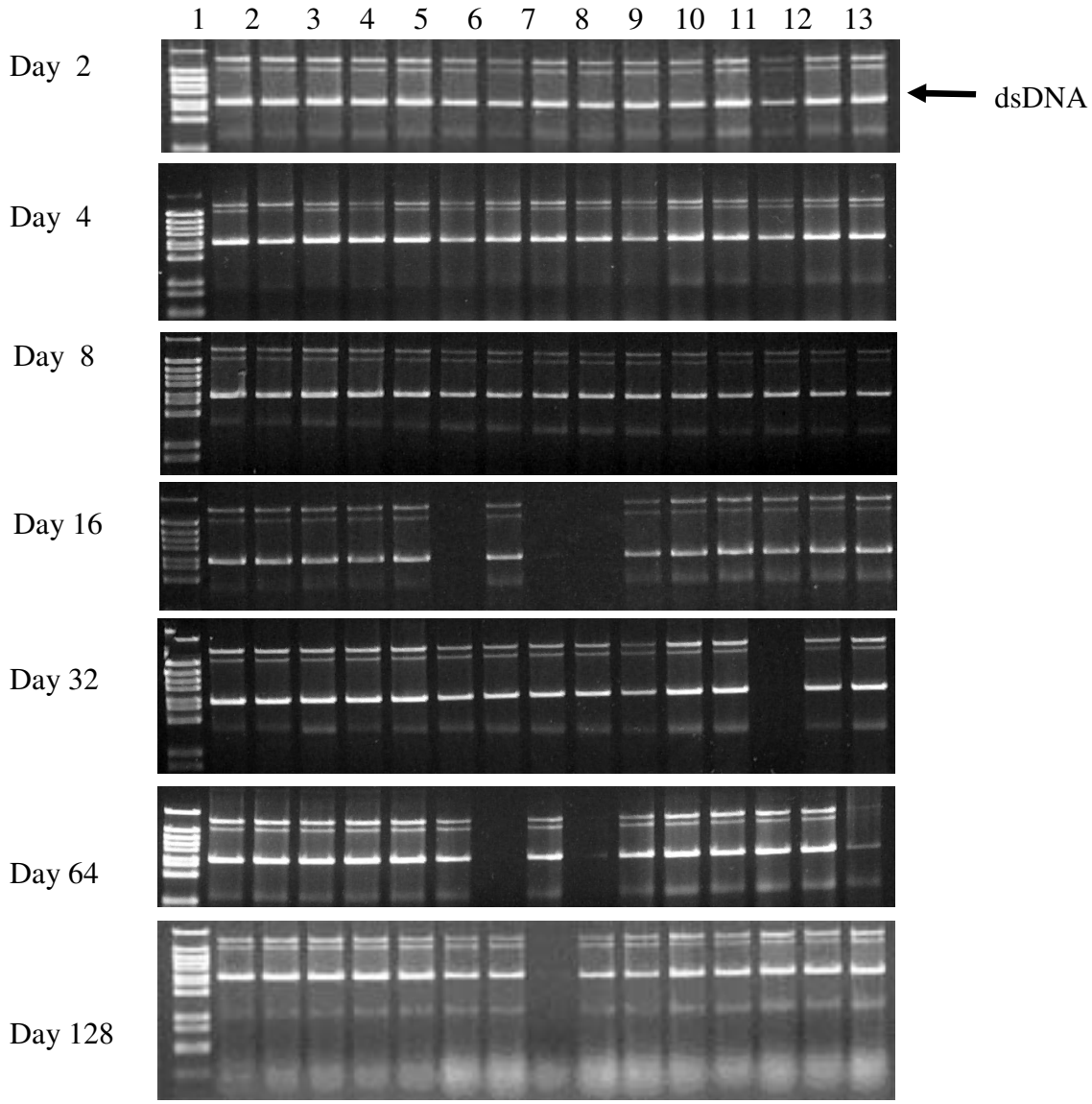

Fig. A.9 Electrophoretic images of dsDNA stored at 5º C. Lane 1 is a molecular weight standard. Lanes 2-6 contain 400 ng of dsDNA reference stored at -20°. Lanes 7-11 contain dsDNA recovered from 10% AG. Lanes 12-16 contain dsDNA recovered from 10% pullulan.

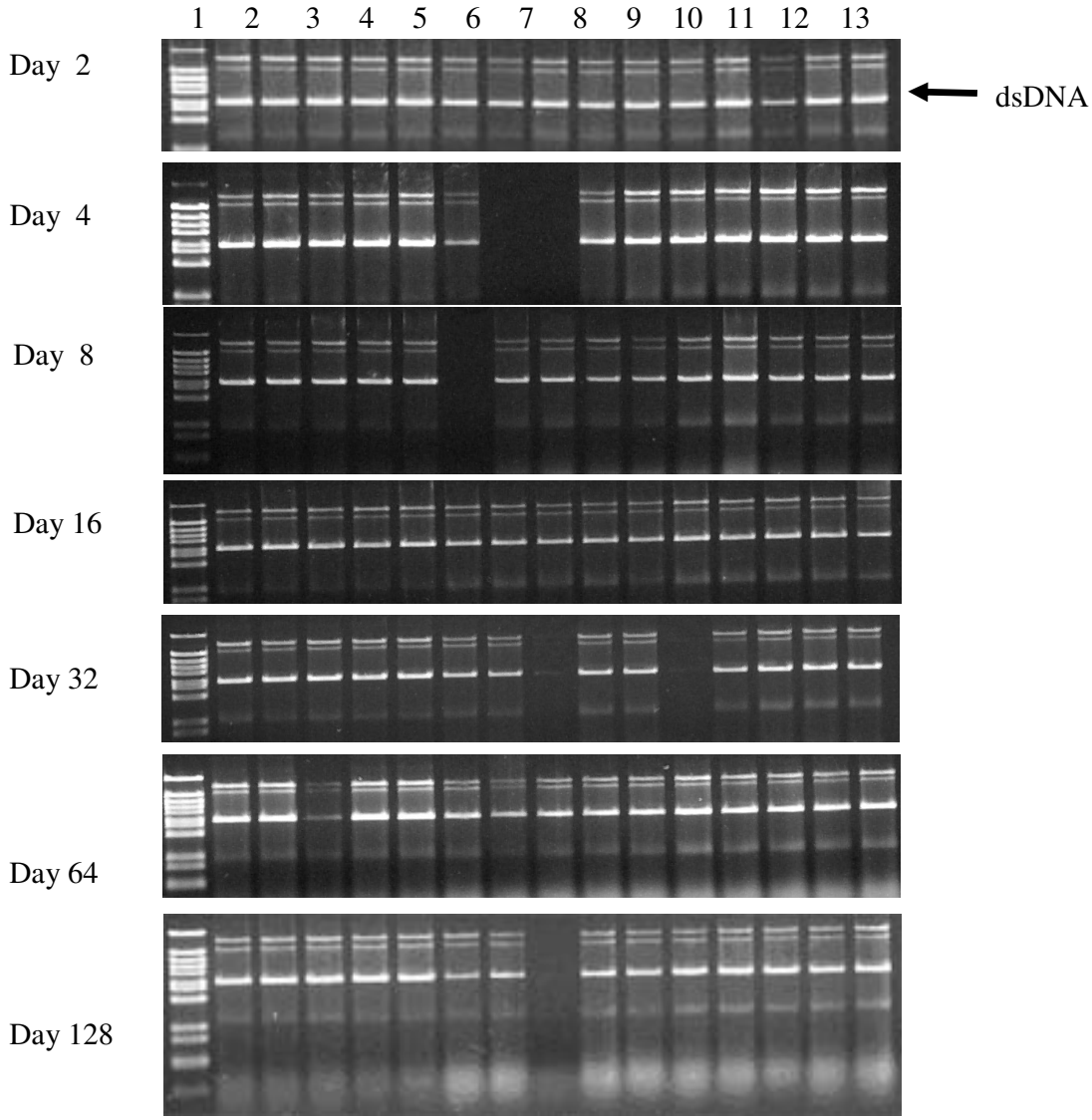

Fig. A.10 Electrophoretic images of dsDNA stored at 15º C. Lane 1 is a molecular weight standard. Lanes 2-6 contain 400 ng of dsDNA reference stored at -20°. Lanes 7-11 contain dsDNA recovered from 10% AG. Lanes 12-16 contain dsDNA recovered from 10% pullulan.

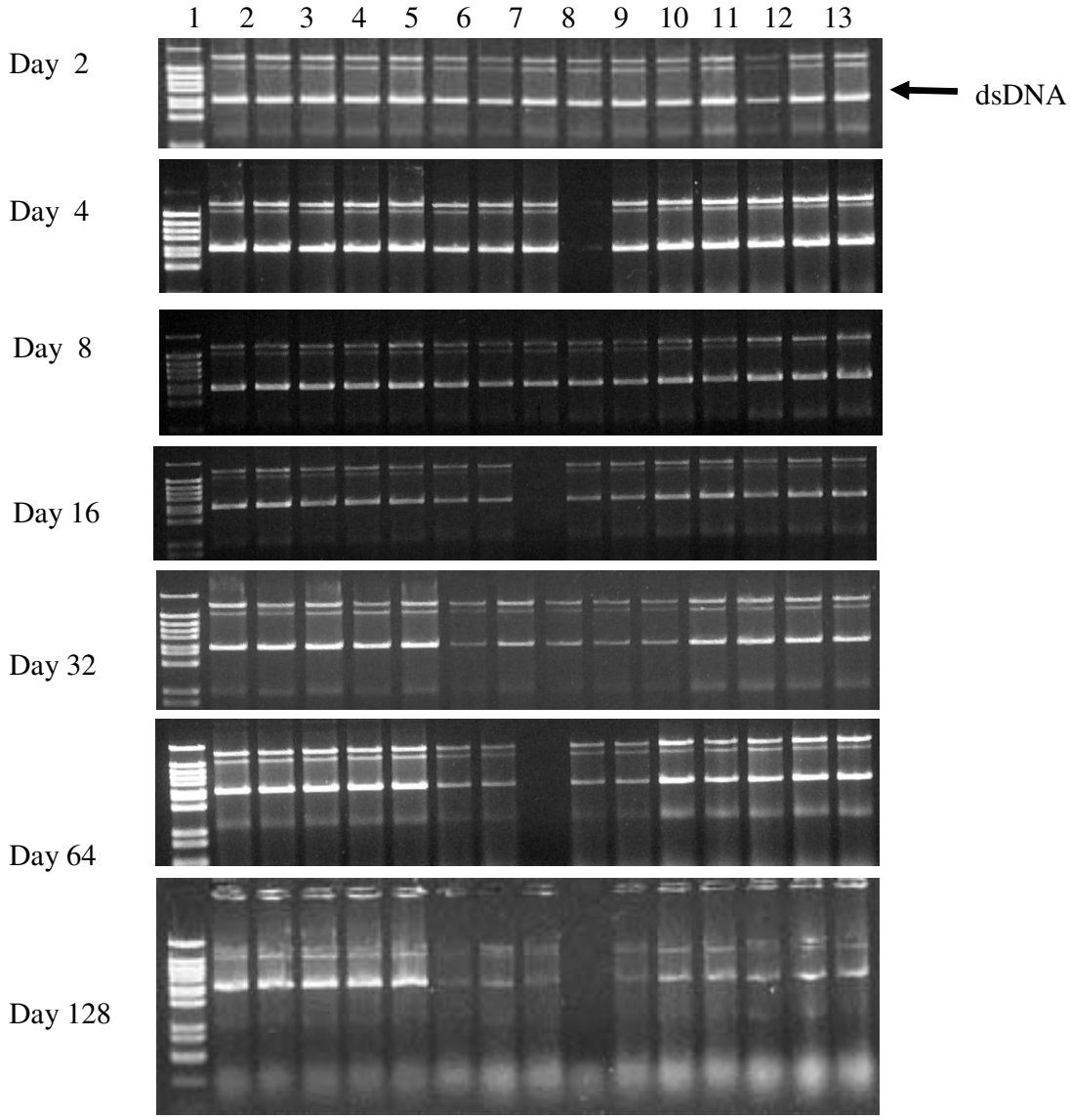

Fig. A.11 Electrophoretic images of dsDNA stored at 25º C. Lane 1 is a molecular weight standard. Lanes 2-6 contain 400 ng of dsDNA reference stored at -20°. Lanes 7-11 contain dsDNA recovered from 10% AG. Lanes 12-16 contain dsDNA recovered from 10% pullulan.

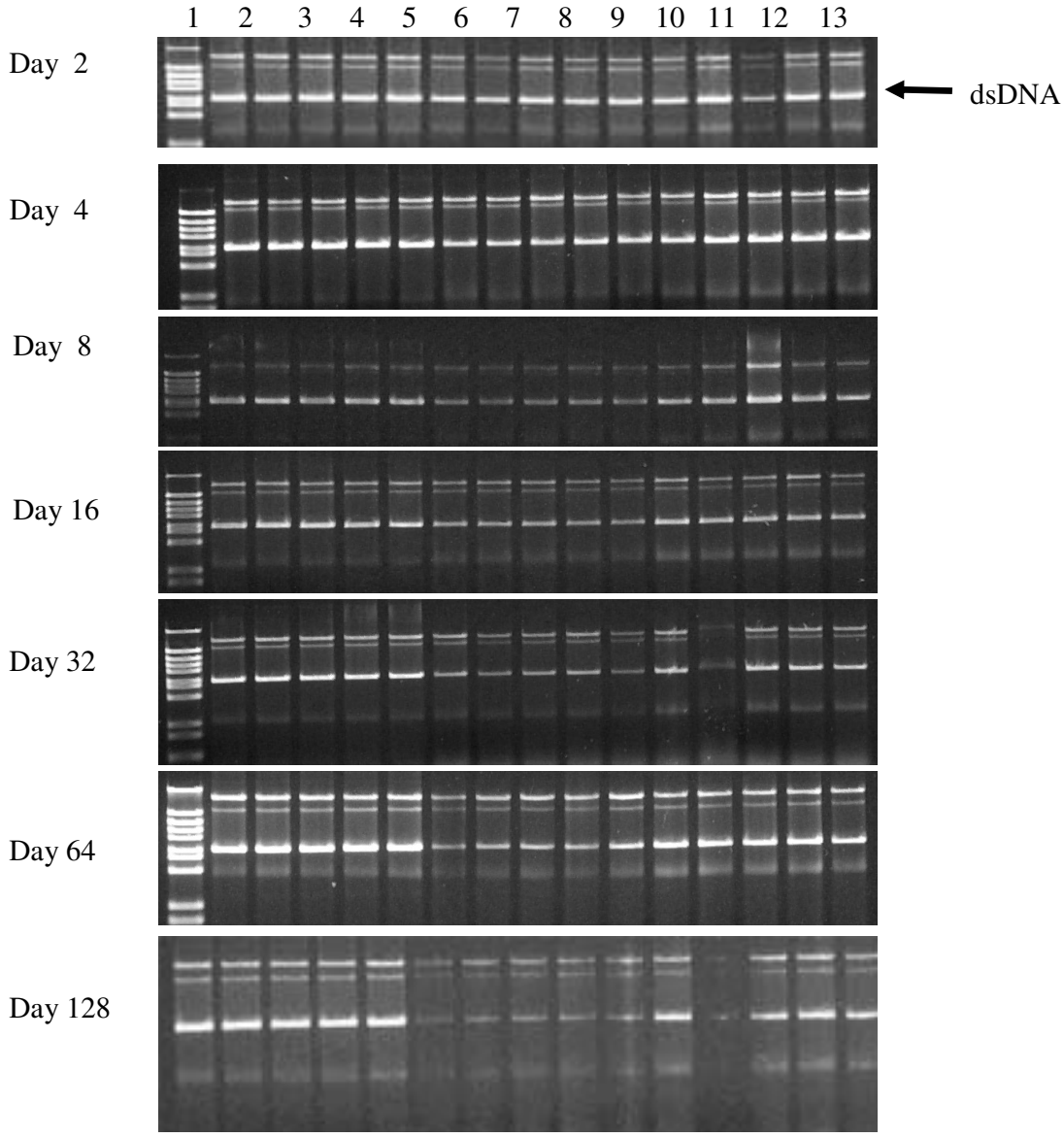

Fig. A.12 Electrophoretic images of dsDNA stored at 40º C. Lane 1 is a molecular weight standard. Lanes 2-6 contain 400 ng of dsDNA reference stored at -20°. Lanes 7-11 contain dsDNA recovered from 10% AG. Lanes 12-16 contain dsDNA recovered from 10% pullulan.

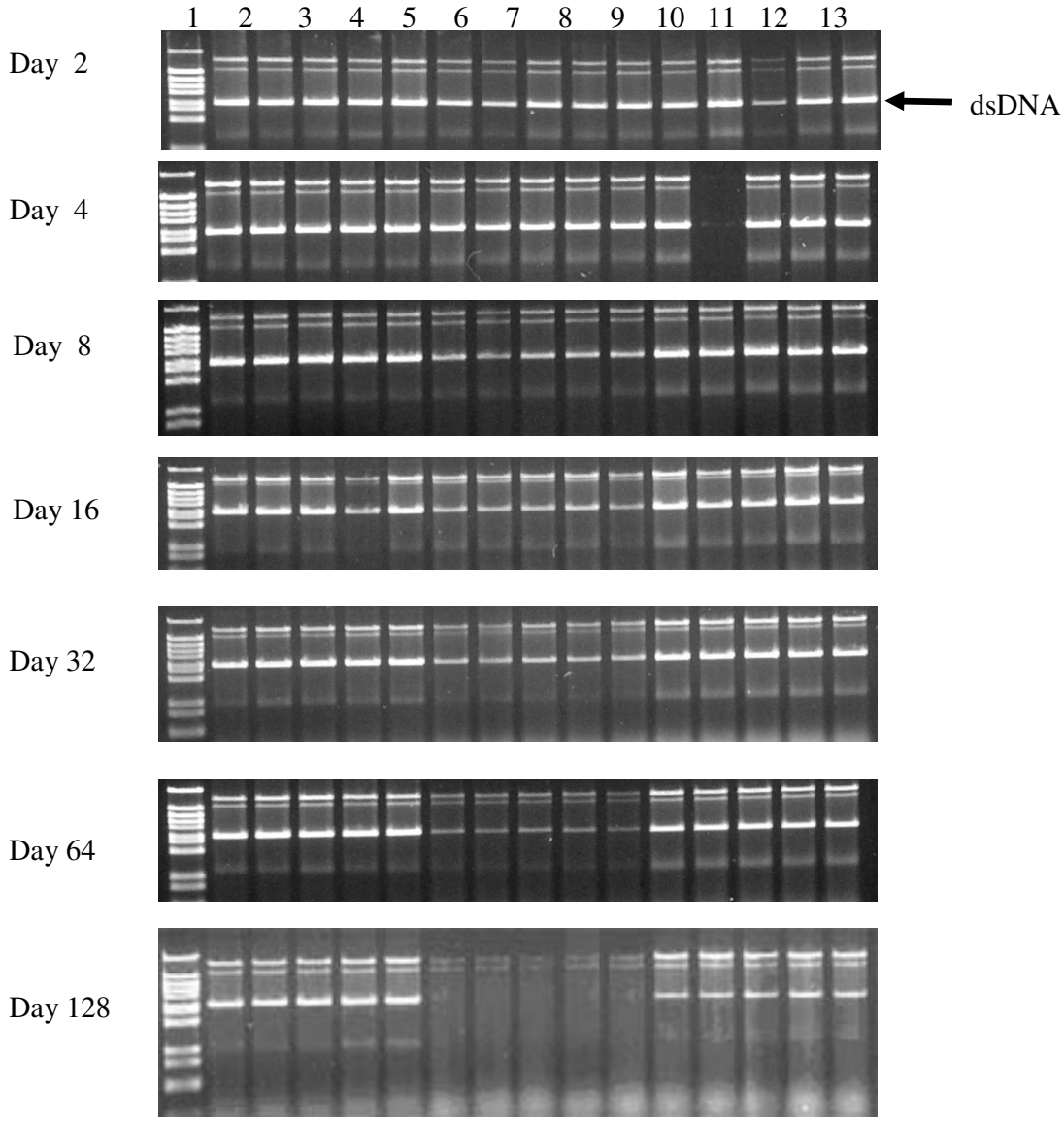

Fig. A.13 Electrophoretic images of dsDNA stored at 46% humidity at 25°C. Lane 1 is a molecular weight standard. Lanes 2-6 contain 400 ng of dsDNA reference stored at -20°. Lanes 7-11 contain dsDNA recovered from 10% AG. Lanes 12-16 contain dsDNA recovered from 10% pullulan.

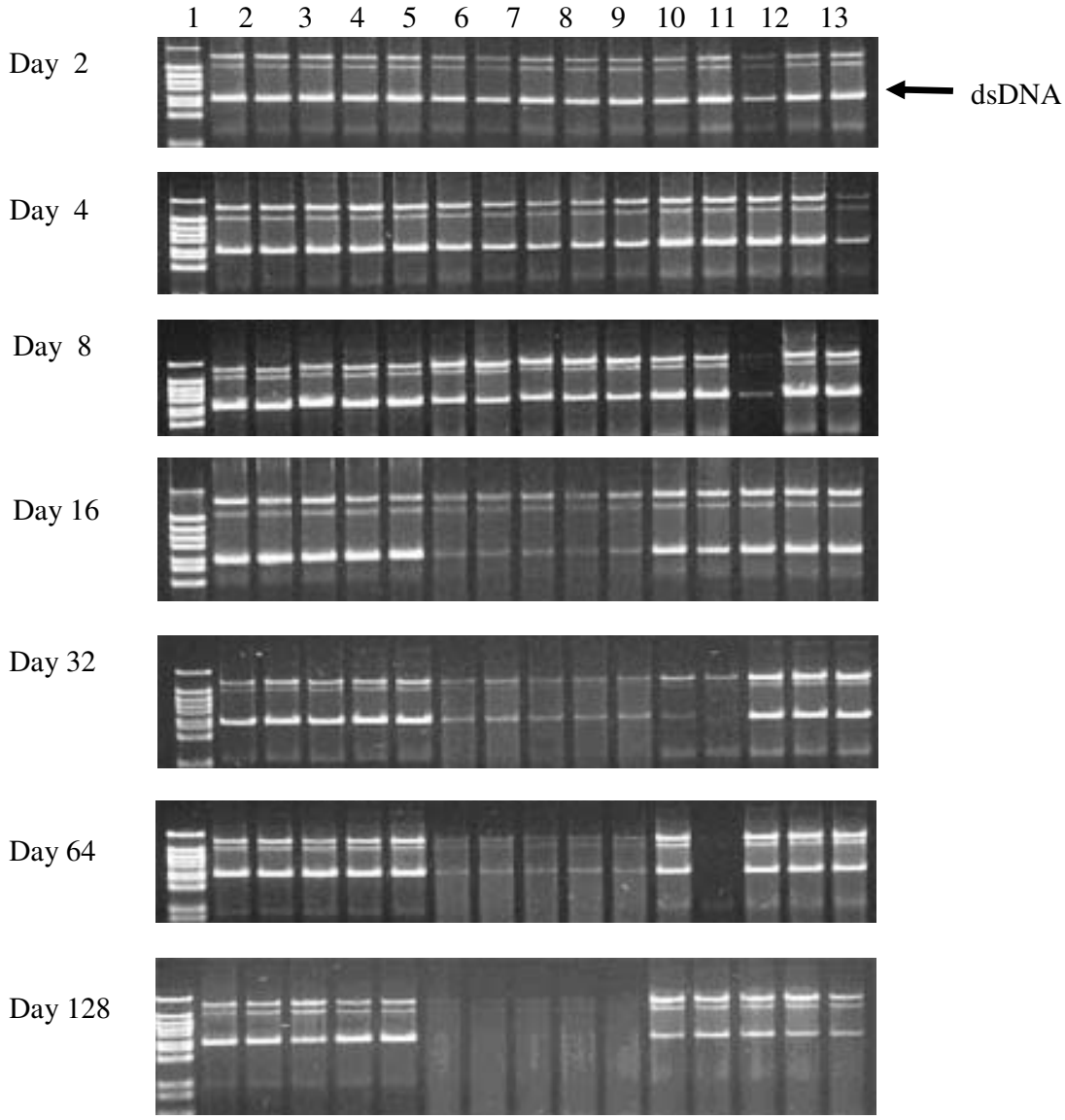

Fig. A.14. Electrophoretic images of dsDNA stored at 53% humidity at 25°C. Lane 1 is a molecular weight standard. Lanes 2-6 contain 400 ng of dsDNA reference stored at -20°. Lanes 7-11 contain dsDNA recovered from 10% AG. Lanes 12-16 contain dsDNA recovered from 10% pullulan.

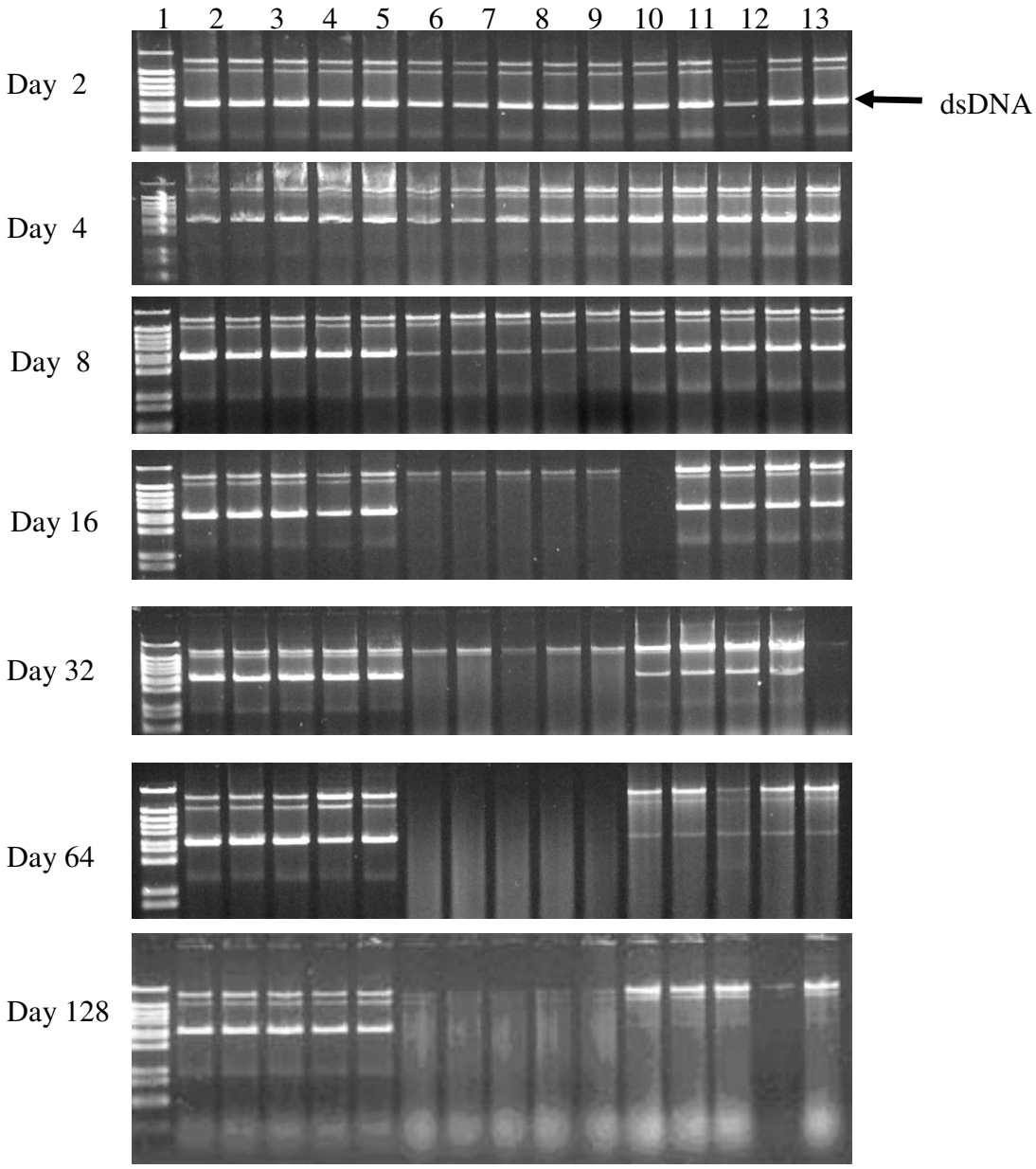

Fig. A.15. Electrophoretic images of dsDNA stored at 76% humidity at 25°C. Lane 1 is a molecular weight standard. Lanes 2-6 contain 400 ng of dsDNA reference stored at -20°. Lanes 7-11 contain dsDNA recovered from 10% AG. Lanes 12-16 contain dsDNA recovered from 10% pullulan.

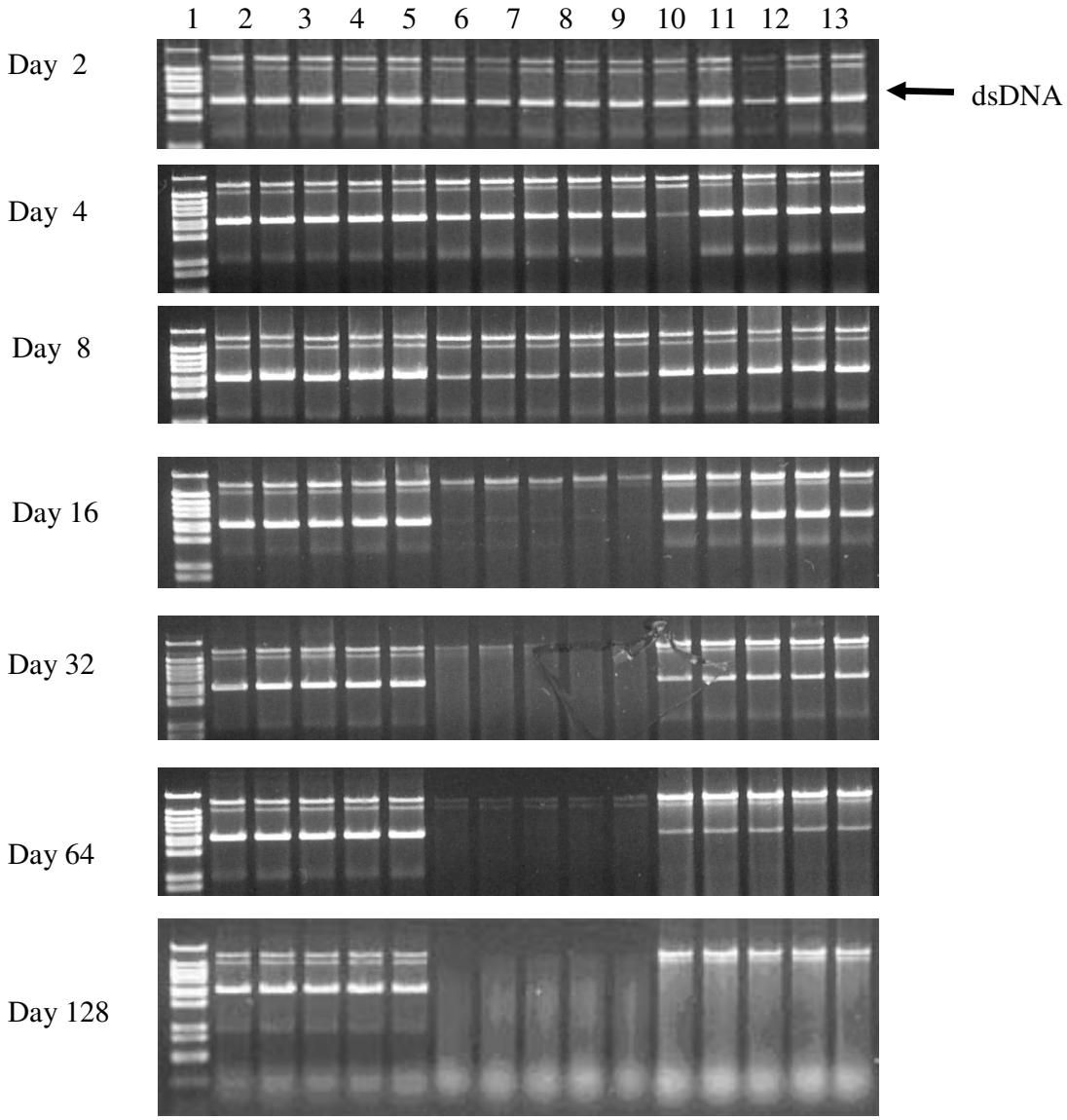

Fig. A.16. Electrophoretic images of dsDNA stored at 86% humidity at 25°C. Lane 1 is a molecular weight standard. Lanes 2-6 contain 400 ng of dsDNA reference stored at -20°. Lanes 7-11 contain dsDNA recovered from 10% AG. Lanes 12-16 contain dsDNA recovered from 10% pullulan.