

**BACTERIAL ADHESION TO SOLID SURFACES AND ITS
PREVENTION BY THE APPLICATION OF SILVER**

by

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To My Father,

Mother,

and Brothers

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BACTERIAL ADHESION TO SOLID SURFACES AND ITS PREVENTION BY THE APPLICATION OF SILVER

ABSTRACT

Silver, under different forms, has been proved effective for the elimination of bacteria and other microorganisms from liquid mediums, solutions and solid materials used in industrial, sanitary, and biomedical fields, such as reactors, pipes, filters, medical devices, biomaterials, etc. The effectiveness of different forms of silver antimicrobial application depends on the nature of these forms, the methods of application, and the field of application.

The main objective of the thesis is to present a theoretical and experimental studies involving bacterial growth in liquid suspensions and solid surfaces and its prevention (disinfection) by different application of different forms of antibacterial silver: electro-colloidal silver, silver nitrate, silver sulfadiazine, and antimicrobial ceramics impregnated with silver ions. Bacterial growth is detected by viable bacterial agar plate counting and by observation of turbidity in suspensions. The experimental results showed superiority of the antimicrobial effect electro-colloidal silver over other used forms of silver used to disinfect liquid suspension. It was also shown that the antibacterial properties of the hygienic ceramics impregnated with silver ions were strong and durable.

Keywords: Bacterial adhesion, bacterial counting, disinfection, colloidal silver, hygienic ceramic.

BAKTERİLERİN KATI YÜZEYLERE TUTUNMASI VE GÜMÜŞ UYGULANMASI İLE ÖNLENMESİ

ÖZET

Gümüşün, farklı biçimler altında, reaktörler, borular, filtreler, tıbbi cihazlar, biyomedikal malzemeler, endüstriyel, sıhhi tesisat ve biyomedikal alanlarda kullanılan akışkan ortam, karışım ve katı malzemelerden bakteri ve diğer mikroorganizmalar artılmasında etkin bir rol oynadığı ispatlamıştır. Gümüş farklı biçimlerinin antimikrobik uygulamalarının etkinliği, bu biçimlerin doğasına, bu uygulama yöntemlerine ve uygulama alanına bağlıdır.

Bu tezin başlıca amacı, elektro-kolloidal gümüş, gümüş nitrat, gümüş sulfadiyazin, ve gümüş iyonlarıyla doyurulmuş antimikrobik seramik gibi değişik biçimlerdeki antibakteriyel gümüş vasıtasıyla, katı yüzey ve akışkan asılılarda bakteriyel büyümeyi önleyen uygulamaları içerir deneysel çalışma sunmaktır. Bakteriyel büyüme, yaşayabilen bakteriyel agar levhası, sayımı ve asılılardaki bulanıklığın gözlemelenmesiyle algılanır. Deneysel sonuçlar, akışkan asılıların dezenfektaninde elektro-kolloidal antimikrobik etkisinin kullanılmış diğer gümüş türlerine nazaran üstünlüğünü göstermiştir. Bu çalışmada, gümüş iyonlarıyla doyurulmuş hijyenik seramiklerin antibakteriyel özelliklerinin kuvvetli ve kalıcı olduğu da gösterilmiştir.

Anahtar kelimeler: Bakteriyel yapışma, bakteriyel sayma, dezenfeksiyon, kolloida gümüş, hijyenik seramik.

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LIST OF ABBREVIATION

<i>A</i>	absorbance
ASS	atomic absorption spectroscopy
ATP	Adenosine Triphosphate
Avg	average
<i>c</i>	velocity of light
CFU	colony forming unit
CS	colloidal silver
DNA	Deoxyribonucleic Acid
<i>E</i>	energy
<i>h</i>	Plank's constant
HA	Hydroxylapatite
λ	wavelength
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
MKC	minimum killing concentration
ν	frequency
ppb	part per billion
ppm	part per million
Std Dev	standard deviation
UV	Ultra-violet

1. INTRODUCTION

Bacterial adhesion to biomaterial surfaces is the major barrier to the extensive use of prosthetics, since it causes biomaterial-centered infection and the lack of successful tissue integrity or compatibility with biomaterial surface [1]. This complication may lead to complete failure of the implant, possible amputation, prolonged hospitalization, and even death [2]. In addition, industrial and sanitary application of materials is also faced by the problem of adhesion of microorganisms on solid material surfaces, which is sometimes called biofouling. Therefore, antimicrobial substances are being developed for application with materials in the medical and industrial fields.

Antimicrobial agents used on biomaterials are classified into two groups: the synthetic or natural inorganic groups, and the synthetic or natural organic groups. Inorganic or metallic antimicrobials, which depend on the metal ions, have defects related to toxicity and coloring. Organic antimicrobials also have defects related such as high volatility, low heat resistance, coloring and high solubility for water and organic solvents [3]. There is a new tendency merging towards the use of metallic antimicrobials in different methods for different biomedical and industrial applications. These new methods try to avoid the known disadvantages of the use of metallic antimicrobials.

The antimicrobial effects of metallic ions such as Ag, Au, Pt, Pd, Ir (i.e. noble metals), and Cu, Sn, Sb, Zn is known [4]. The metallic salts of the above mentioned metals, whether organic or inorganic, have been known to have especially superior disinfectant property for bacteria such as *E. coli*, *S. aureus*, *Bacilli*, *Vibrio*, *salmonella*, and other microorganisms such as molds or round worms [5].

It is considered that the antimicrobial metals exhibit antimicrobial activity because when metal ions are absorbed into microbial cells, they inhibit respiration and basal metabolism of electron transfer and the transport of substrate through cell membrane [6]. Also, silver ions are able to complex with cellular DNA. This complex

formation interrupts the normal role of DNA and thus prevents the multiplication of bacterial cells [7].

Of the metallic ions with antimicrobial properties, silver is perhaps the best known due its unusually good bioactivity at low concentration. This phenomenon is termed as oligodynamic action [4]. Silver in its metallic state is safe because it is little absorbed in the human body [7]. When the National Aeronautic and Space Administration (NASA) was searching for a mean to purify the recycled water for the Apollo astronauts, they selected silver because of its high potency disinfecting capabilities.

Silver that is used for antimicrobial function is of different types:

Electro-colloidal silver (electrically sintered into pure water).

Mild silver proteins (MSP): This process chemically infuses or binds the silver to a protein. The part per million vary from 20 PPM to 200 PPM and concentrated as high as 5000 PPM.

Powdered silver: A very high voltage is used to disintegrate the silver, also known as water-soluble silver.

Ground silver: silver is pulverized and ground into a fine powder.

Silver oxide: Once used for chores and epilepsy

Silver salts: These silver compounds cannot be truly considered as colloidal silver. They are produced chemically or electrochemically. They may possess a positive charge but are usually considered as silver compounds. Below are examples of these silver compounds and their uses.

Silver sulfadiazine: Antibacterial and antifungal use in burn cases.

Silver nitrate: Disinfectant uses. Prodiatry, prevent blindness in infant's eye (used at birth), ulcer treatment.

Silver iodide: Disinfectant qualities.

Silver chloride: Disinfectant qualities.

Silver lactate: Astringent and antiseptic.

Silver picrate: Used for monilliasis and trichomoniasis [8].

1.1 Objective

The objective of this thesis is to study the antimicrobial efficacy of different forms of silver: electro-colloidal silver, silver nitrate, and silver sulfadiazine. These compounds are used with bacterial suspensions of *S. epidermidis*. In addition, these compound are to be applied on solid surfaces of ceramics and their antimicrobial effects are compared to that of a manufactured antimicrobial ceramic where silver ions were impregnated.

1.2 Outline

In this first section, an introduction to the thesis was given. In the second section, a study about bacterial structure and growth is presented. In the third section, a literature study about bacterial adhesion to solid surfaces and the factor governing it is presented. The fourth section includes a review about physical and chemical control of the growth of microorganisms. The fifth section includes a literature survey about the biomedical and industrial application of silver and silver compounds as antimicrobial agent. The sixth section includes a study of experimental techniques for producing electro-colloidal silver solutions and their analysis for silver content and the results of the application of these techniques. It also includes an introductory study of bacterial culturing, microscopic observation, and enumeration. Afterwards, a description of an experimental work involving culturing the bacterium *Staphylococcus epidermidis*, its enumeration, and bacterial application of silver under different forms against this bacterium in suspension and solid surfaces. Section 7 includes a conclusion for the thesis.

2. BACTERIAL STRUCTURE AND GROWTH

All bacteria (both archaeobacteria and eubacteria) and only bacteria have prokaryotic cells. Prokaryotic cells have an undifferentiated, grainy interior with a few organelles, and a nuclear region or nucleoid (a mass of DNA) rather than a membrane bound nucleus. Prokaryotic cells are usually much smaller than eukaryotic cells, which allows prokaryotes to grow and multiply much faster than eukaryotes [9].

2.1 Overview of Bacterial Cell Structure

The prokaryotic cell has two basic parts: the envelop (the outside of the cell) and the cytoplasm (which fills the interior and houses the nucleoides and other structures). Some bacterial species have appendages (structures attached to the envelope and extending beyond the cell). (Figures 2.1 and 2.2) [9].

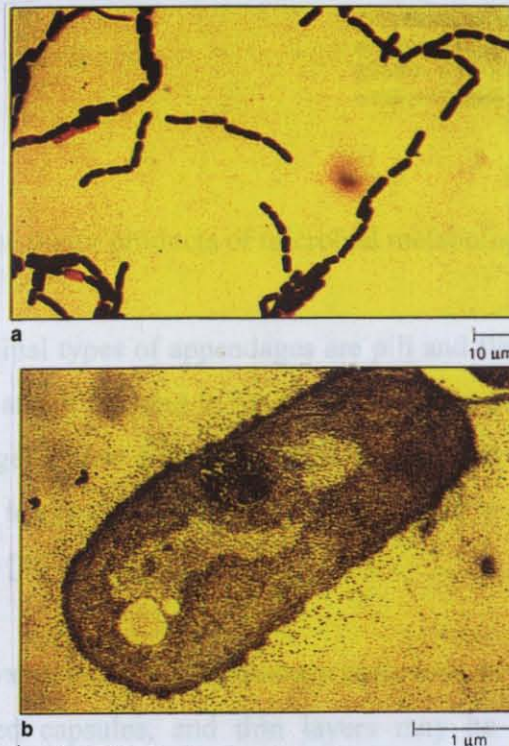


Figure 2.1 Prokaryotic cell. This is the bacterium *Bacillus megatrium* under (a) the light microscope and (b) the transmission electron microscope. In both, it looks like a typical prokaryote-colorless, grainy and undifferentiated [9].

and on the outer surface, lipopolysaccharide (LPS). LPS is not found in any other living thing. Only Gram-negative bacteria has an outer membrane (some literature consider it a part of the cell wall). The periplasm, only found in Gram-negative bacteria, is a gelatinous organelle containing enzymes and proteins [9].

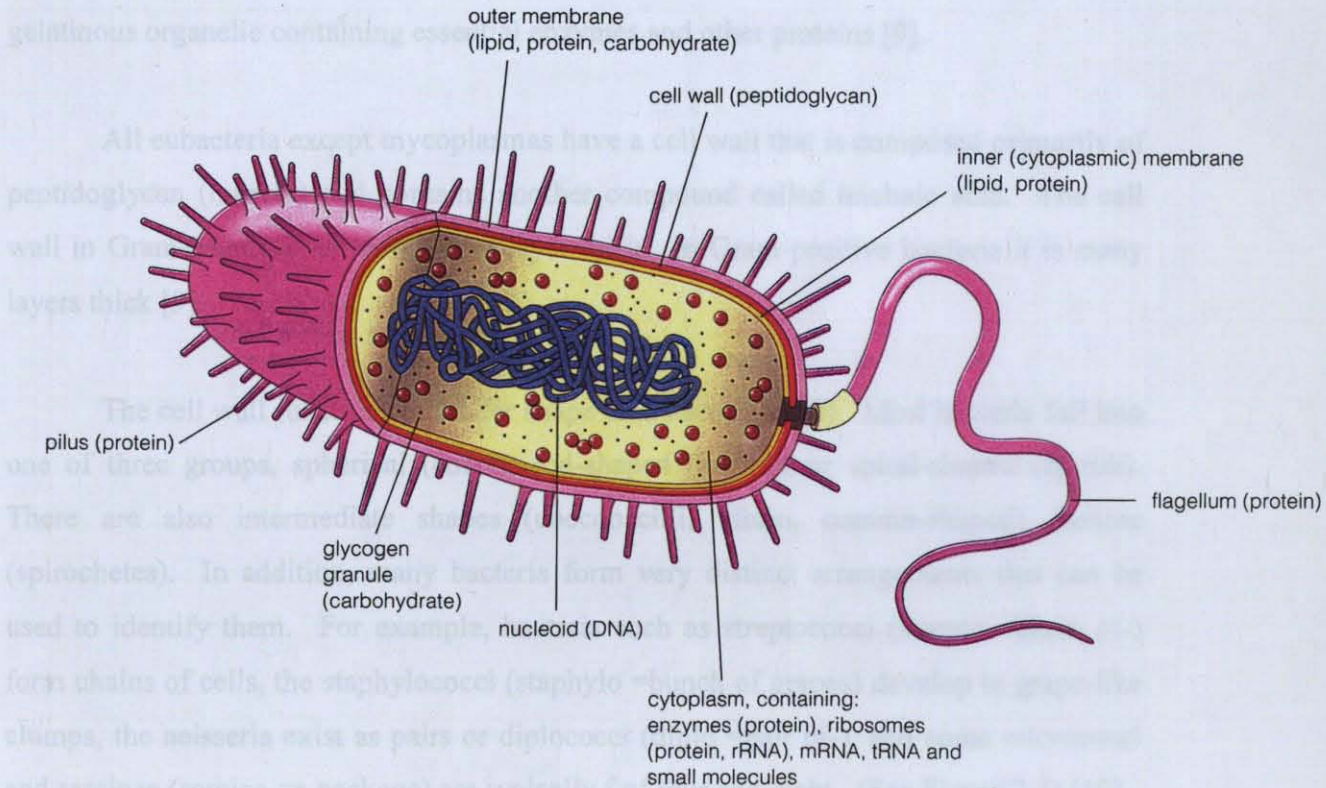


Figure 2.2 The major products of microbial metabolism in a new cell [9].

The two principal types of appendages are pili and flagella. Pili are straight hair-like appendages that attach bacteria to other cells. The sex pilus attaches mating bacteria. Flagella have a complex three-part structure that allows them to rotate and propel the cell toward favorable environment and away from harmful ones (a behavior called taxis) [9].

The glycocalyx is the slimy or gummy outermost layer of the envelope. Thick layers are also called capsules, and thin layers may be called slime layers. The glycocalyx offers protection from phagocytosis and helps the cell adhere to surfaces. The outer surface is a bilayer composed of phospholipids on the inner surface

and on the outer surface, lipopolysaccharide (LPS). LPS is not found in any other living thing. Only Gram-negative bacteria has an outer membrane (some literature consider it a part of the cell wall). The periplasm, only found in Gram-negative bacteria, is a gelatinous organelle containing essential enzymes and other proteins [9].

All eubacteria except mycoplasmas have a cell wall that is composed primarily of peptidoglycan (murein) and contains another compound called teichoic acid. The cell wall in Gram-negative bacteria is one layer thick. In Gram-positive bacteria it is many layers thick [9].

The cell wall functions to confer shape on a bacterium [9]. Most bacteria fall into one of three groups, spherical (cocci), rod-shaped (bacilli), or spiral-shaped (spirilli). There are also intermediate shapes (coccobacilli, vibrio, comma-shaped), helices (spirochetes). In addition, many bacteria form very distinct arrangements that can be used to identify them. For example, bacteria such as streptococci (strepto =chain of-) form chains of cells, the staphylococci (staphylo =bunch of grapes) develop in grape-like clumps, the neisseria exist as pairs or diplococci (diplo =pair of-), and some micrococci and sarcinae (sarcina =a package) are typically f of four and eight. (See Figure 2.3) [10].

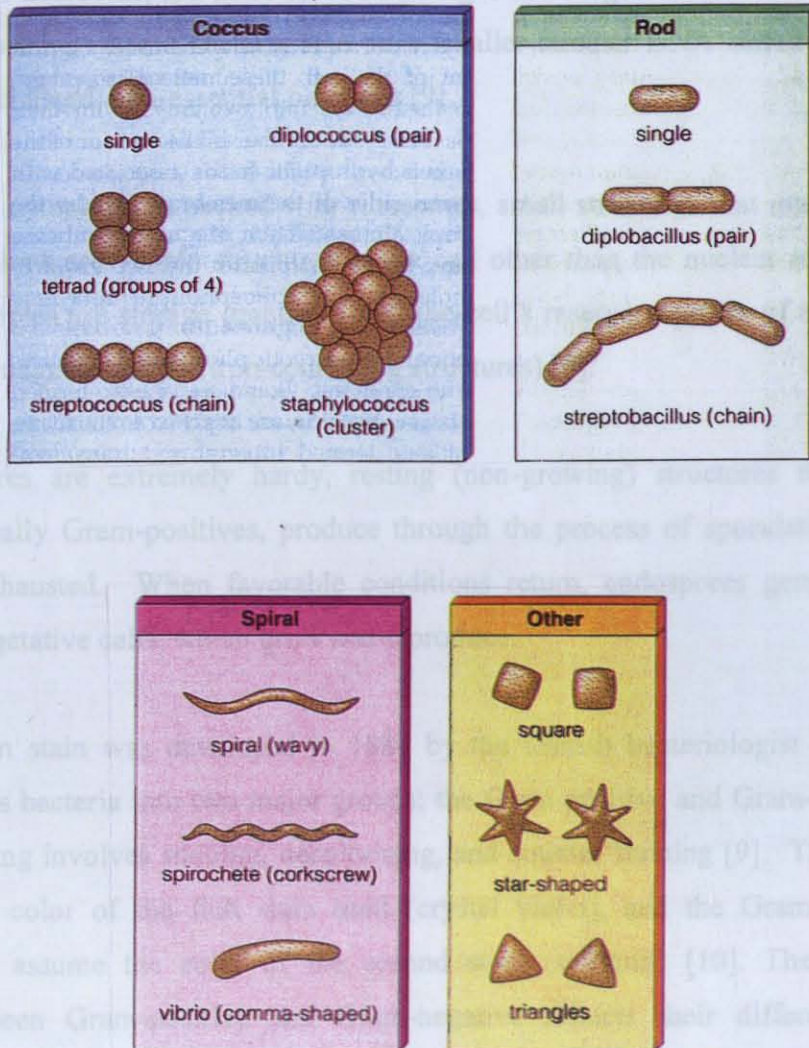


Figure 2.3 Shapes and Arrangements of Bacterial Cells [9].

The cell wall also contains a cell's turgor pressure, the internal pressure from its contents. A bacterial cell's turgor is extremely high because its contents are concentrated. The major functions of the plasma membrane are to contain the cytoplasm and to transport substances in and out the cell. The plasma membranes of bacteria are very similar to plasma membranes in eukaryotes [9].

The cytoplasm of all bacteria contains a nuclear region and ribosomes. The nuclear region, or nucleoid, is a mass of DNA arranged in a single circular molecule, the bacterial chromosome. Some bacteria also have smaller circular DNA called plasmids that encode specialized, non-essential functions [9].

Bacterial cytoplasm is packed with ribosomes, small structures that manufacture proteins. Inclusions are visible structures in the cell other than the nuclear region and ribosomes, examples are storage granules (hold the cell's reserve supplies of nutrients), gas vacuoles or magnetosomes (iron-containing structures) [9].

Endospores are extremely hardy, resting (non-growing) structures that some bacteria, principally Gram-positives, produce through the process of sporulation when nutrients are exhausted. When favorable conditions return, endospores germinate to produce new vegetative cells, which grow and reproduce.

The gram stain was developed in 1884 by the Danish bacteriologist Christian Gram. It divides bacteria into two major groups: the Gram-positive and Gram-negative. The Gram-staining involves staining, decolorizing, and counter staining [9]. The Gram-stain retain the color of the first stain used (crystal violet), and the Gram-negative bacteria is that assume the color of the second stain (safranin) [10]. The staining difference between Gram-positive and Gram-negative reflects their different outer surface. Gram-negative bacteria have a thin cell wall with a membrane outside it. Gram-positive bacteria have a thick cell wall with no membrane outside it [9].

2.2 Growth of Bacteria

Bacterial growth refers to growth of a population-an increase in the number of cells, not an increase in the size of an individual cell [9].

Time		Cells/milliliter		
Min.	Hours	Numbers	Scientific Notation	Logarithm
0	0	1000	10^3	3.0
20	0.33	2000	2×10^3	3.301
40	0.66	4000	4×10^3	3.602
60	1.00	8000	8×10^3	3.903
80	1.33	16,000	1.6×10^4	4.204
100	1.66	32,000	3.2×10^4	4.505
120	2.00	64,000	6.4×10^4	4.806
140	2.33	128,000	1.28×10^5	5.107
160	2.66	256,000	2.56×10^5	5.408
180	3.00	512,000	5.12×10^5	5.709
200	3.33	1,024,000	1.02×10^6	6.010

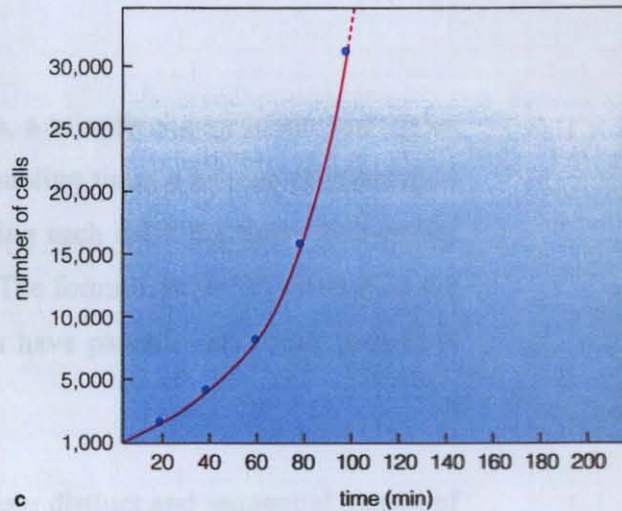
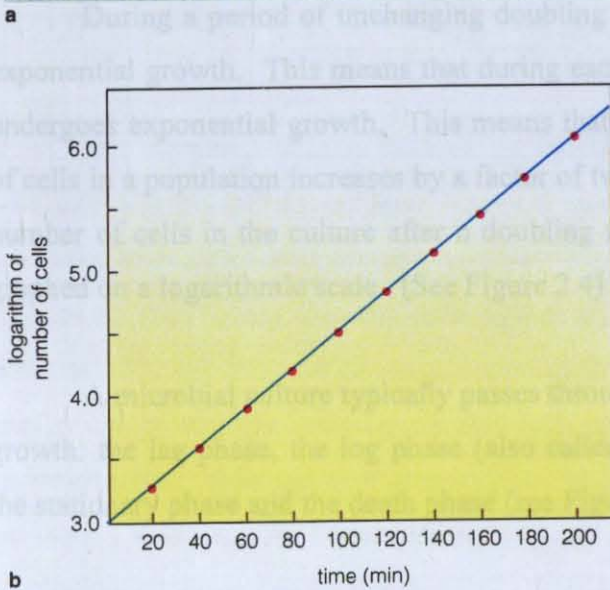


Figure 2.4 Exponential growth of a bacterial culture. (a) The number of cells per milliliter in the exponentially growing culture is measured every 20 minutes and recorded as numbers, by scientific designation, and by logarithm of the number. (b) Plotting the logarithm of the numbers against time gives a straight line. (c) Plotting the actual number against time gives a curve with sharply increasing slope. Because the number of cells is doubling every 20 minutes, the doubling time is 20 minutes and the growth rate is 3.0 (60/20) doubling per hour. A culture growing at this rate increases from 1000 to more than 1 million cells per milliliter in 200 minutes [9].

Doubling time (formerly referred to as generation time) is the period required for cell in a microbial population to grow, divide, and produce two new cells for each one that existed before. Growth rate is usually measured as doubling time per hour. The doubling time figure tells us how fast the population is growing, but the relationship is opposite. That is, population with low value of doubling time is growing rapidly, while population with higher value of doubling time is growing slowly. Doubling time vary depending on the species of microorganisms and on the growth conditions of the population [9].

2.2.1 Exponential Growth

During a period of unchanging doubling time, a microbial population undergoes exponential growth. This means that during each doubling time, a microbial population undergoes exponential growth. This means that during each doubling time, the number of cells in a population increases by a factor of two. The formula is $N = 2^n$ where N is the number of cells in the culture after n doubling times have passed. Microbial growth is graphed on a logarithmic scale. (See Figure 2.4) [9].

A microbial culture typically passes through four distinct and sequential phases of growth: the lag phase, the log phase (also called the logarithmic or exponential phase), the stationary phase and the death phase (see Figure 2.5) [9].

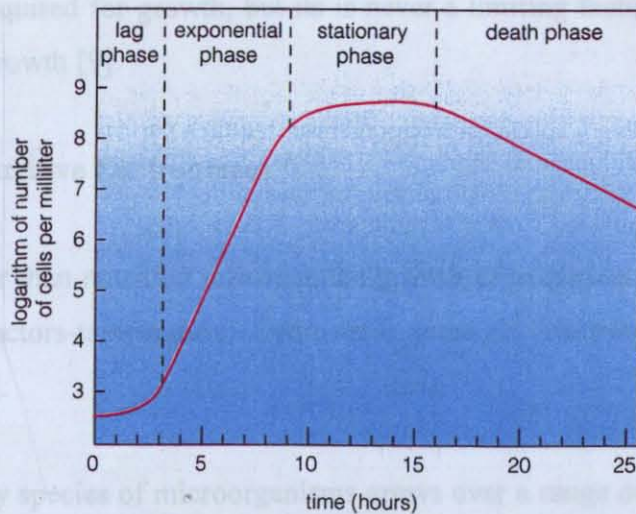


Figure 2.5 Phase of growth in bacterial culture. (This graph is based on the culture described in Figure 2.4). The death phase is exponential decline, like the exponential phase, and is, therefore, a straight line but it occurs at a much slower rate [9].

The lag phase usually follows the inoculation of stationary phase or death phase cells into a fresh culture medium. It is a no-growth period, but there is considerable metabolic activity as cells prepare to grow. The log phase does not continue indefinitely because of intervening factors, such as depleted nutrients or an accumulation of toxic products. No net increase of mass occurs during the stationary phase, but cells become smaller and synthesize components that help them survive non-growth periods. During the death phase, cells die exponentially but at a much lower rate than they grow during the log. Death usually occurs because a cell has depleted its intracellular reserve of Adenosine Triphosphate (ATP) to repair cellular components [9].

2.2.2 What Microorganisms Need to Grow

2.2.2.1 Nutrients

Nutrients that various microorganisms need to grow include a source of carbon, oxygen, nitrogen, phosphorous, sulfur, trace elements, and organic growth factors. Cells use these nutrients to generate driving forces (e.g. ATP) or precursor metabolites.

Hydrogen is also required for growth, but its is never a limiting factor in a medium that otherwise support growth [9].

2.2.2.2 The Non-nutritive Environment

Factors other than nutrition influence the growth of microbial populations. They include physical factors-temperature, hydrostatic pressure, chemical factors-pH, and osmotic strength [9].

Temperature: Every species of microorganisms grows over a range of temperature, from the minimum temperature of growth, the lowest that will support growth, to the maximum temperature of growth, the highest that will support growth. A species' optimum temperature is that at which it grows most rapidly. Themophiles grow best at temperatures above 50°C. Mesophiles grow best near 37°C, the temperature of the human body. Psychrophiles grow best at temperatures below 5°C, refrigerator temperature. The heat stability of a microorganism's protein determines the maximum temperature at which it can grow. Minimum temperature is determined by weakening of hydrophobic interactions of proteins [9].

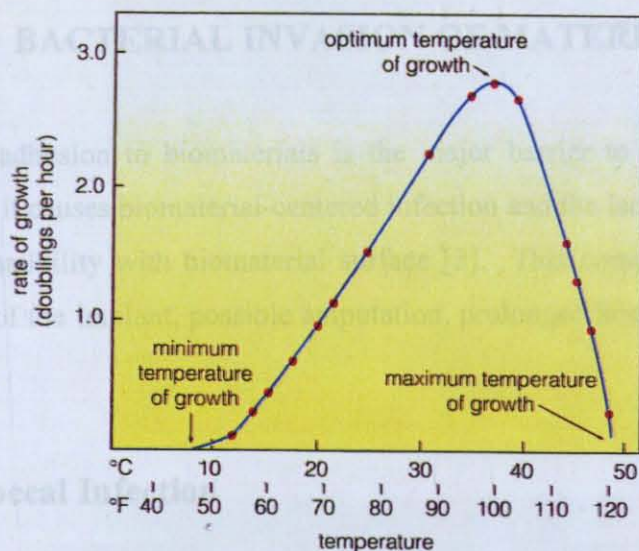


Figure 2.6 Rate of growth of *Escherichia coli* in a rich medium at various temperatures. *E. coli* grows from 8° to 48°C. The rate of growth increases rapidly above 8°C. At 37°C, *E. coli*'s optimum growth temperature, growth rate is 2.8 doubling per hour, a doubling time of about 21.4 minutes. At higher temperatures growth rate slows, and growth stops at 48°C, its maximum temperature of growth [9].

Hydrostatic pressure: Hydrostatic pressure is pressure applied to a liquid. Most microorganisms can tolerate high hydrostatic pressures. Barophiles grow better (facultative) or only (obligatory) at pressures greater than one atmosphere [9].

Turgor pressure: Bacteria maintain a negative turgor pressure: their internal cell osmotic strength is greater than the osmotic strength of their external environment. Certain bacterial species, the halophiles, require high external osmotic strength. Plasmolysis occurs when an increase in the osmotic strength draws water out of the cell, shrinking the cytoplasm [9].

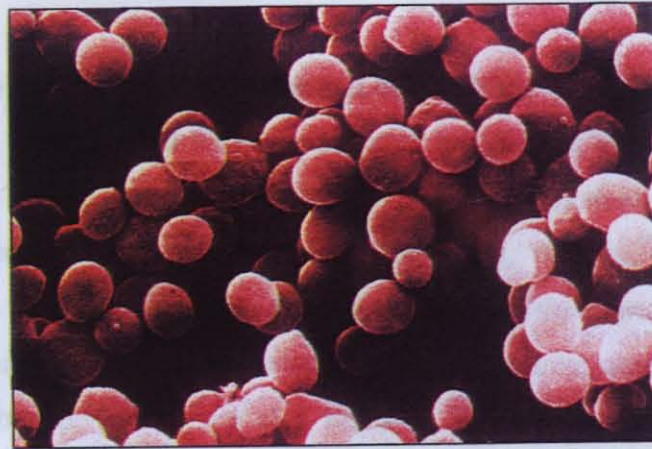
pH: In general, bacteria grow best at a slightly alkaline pH. Some bacteria called acidophiles and alkaliphiles thrive in low-pH environment or high-pH environment, respectively [9].

3. BACTERIAL INVASION OF MATERIALS

Bacterial adhesion to biomaterials is the major barrier to the extensive use of prosthetics, since it causes biomaterial-centered infection and the lack of successful tissue integrity or compatibility with biomaterial surface [3]. This complication may lead to complete failure of the implant, possible amputation, prolonged hospitalization, and even death [4].

3.1 Staphylococcal Infection

The genus *Staphylococcus*, and especially the species *Staphylococcus epidermidis*, is a major infecting agent that invades biomedical material and device surfaces. Treatment. That is why the genus *Staphylococcus* is presented as the model in this discussion of bacterial adhesion to material surface, and studied in terms of its growth, adhesive and infective properties, and its susceptibility to antimicrobials. The genus *Staphylococcus* is divided into two broad groups. The virulent *Staphylococcus aureus* strains produce the enzyme coagulase and hemolysin, ferment mannitol, and grow as yellowish colonies. The non-virulent or weakly virulent *Staphylococcus epidermidis* strains do not produce coagulase or hemolysine, nor does it ferment mannitol, and grow as whitish colonies (see Figure 3.1). Staphylococcal cells, which are Gram positive bacteria, form grape like clusters. They are facultative anaerobes. In the absence of oxygen, Staphylococci ferment sugars, producing lactic acid [9].



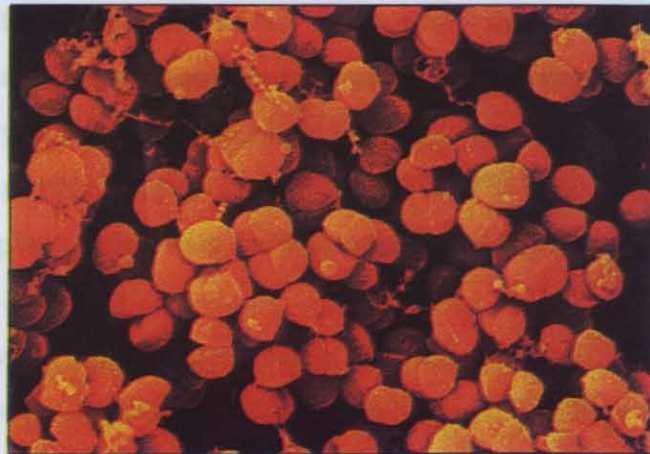
a

1 μm



b

1 μm



c

0.5 μm

Figure 3.1 The Gram-positive cocci are grouped by morphology. Some genera are identified by the way cells remain attached after division. (a) *Staphylococcus* cells form grape like clusters. (b) *Streptococcus* forms long chains. (c) *Sarcina* forms cubical packets [1].

Staphylococcal species are part of the normal human body's normal flora, where they live as commensals, meaning that they cause neither benefit nor harm to the human body. The sites of the human normal flora are the skin, conjunctivae (external eye mucous membrane), nasal cavity, mouth, intestinal tract, vagina, and urethra. *Staphylococcus aureus* is found on the intact skin of 5 to 10% of the population, usually around the anus. *S. epidermidis* are found also in the nasal cavity, eye conjunctivae, mouth, vagina, and urethra. *S. aureus* are also found in these sites but is much less common in the population [9].

Staphylococcal species, especially *S. epidermidis*, normally inhabit the skin, and until fairly recently were considered as harmless commensals. Clinical scientists now realize, however, that each Staphylococcal species has a disease causing potential but severity varies. *S. aureus* is well known of their capacity to invade and infect healthy tissues, and it is responsible for a variety of serious infections. Examples are wound infections, skin infections such as impetigo, boils, abscesses, and cellulitis. Strains of *S. aureus* producing a potent toxin called toxic shock syndrome toxin (TSST) can cause toxic shock syndrome if it passes through an abrasion in the vagina, for example, and infect different parts of the body. *S. aureus* causes more than half of the cases of bacterial endocarditis. Staphylococcal species can cause bacterial pericarditis, spreading from blood stream. They are also responsible for many opportunistic infections in susceptible people. For example, Staphylococcal species are the most common cause of infection in artificially implanted materials, such as catheters and artificial joints [9, 11].

Specifically, *S. aureus* is a frequent cause of orthopedic infections in humans. The bacterium expresses several adhesins that facilitate bacterial adhesion to bone matrix and to bone implant biomaterials coated with host plasma constituents [12].

Bone is a mineralized tissue which can be thought of as a composite material made up of collagen fiber matrix stiffened by hydroxylapatite (HA) whose formula is

$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ crystals [13]. Synthetic hydroxylapatite is a ceramic with a good biocompatibility, osteoconductive properties, and the ability to bond with bone [14].

HA has been tested many times as an artificial bone replacement, especially as augmentation material in oral and plastic surgery, or as coating material on bioinert implant material and has excellent biocompatibility and bonding characteristics [15]. A major application of HA in orthopedics is as plasma-sprayed coating coatings on total joint prosthesis to provide an early fixation to bone. Deep bacterial infection is the most serious complication of total joint replacement and a reason for revision surgery. Infection of HA coating with *S. aureus* or *S. epidermidis* could result in substantial damage to it, manifested with loss of integrity and loosening of HA-coated implants [14].

In fact, the coagulase-negative Staphylococci (CoagNS), particularly *Staphylococcus epidermidis*, cause more infections of medical devices than any other category of microorganisms. This capacity to cause disease is surprising because outside the setting of an infected medical device, these organisms rarely cause infection [11].

3.2 Role of Biofilms and Slimes in Bacterial Adhesion to Biomaterials

In general, the process of bacterial adhesion to biomaterial surface includes physicochemical interaction (phase I), and a late cellular and molecular (phase II) [16].

Phase I is correlated to the different components of surface free energy of biomaterials: dispersion, polarity, hydrogen bonding, van der Waals forces, and electrostatic and hydrophobic interactions [17, 18]. It seems that bacterial adhesion to a surface depends on the hydrophobicity of both bacteria and synthetic surface or host tissue [19, 20]. This observation is supported by the fact that grafting hydrophilic passivating groups uniformly on synthetic surfaces prevent bacterial adhesion to those surfaces [21]. Also, relatively hydrophilic strains have less affinity to hydrophobic substratum surfaces than the hydrophobic strains [22], and cell hydrophobicity was markedly reduced in parallel to the inhibition of adherence [23].

Phase II is a complicated process affected by many factors, including the characteristics of bacterial themselves (i.e. the presence of specific receptors at the coating), the proteins present at the target surfaces [16, 17]. In fact, exposure of any biomaterial to blood results in the immediate formation of a conditioning layer of blood proteins and cells on its surface. These protein layers may influence the presence of the process of staphylococcal attachment by two different ways. The first one is masking non-specifically any direct interaction between bacterial adhesive surface components and artificial surfaces. The second one is increasing more specifically the attractive properties of biomaterials for staphylococci. The blood components and tissue proteins containing specific binding sites for staphylococci are evidently ideal candidates for such a phenomenon, if they can be adsorbed in adequate amounts on the surface of biomaterials [11]. For example, *S. aureus* has been found to bind vitronectin, fibrinogen, fibronectin, thrombospondin, and other proteins [2].

It is proposed that the unusual capacity of pathogenic CoagNS strains to attach to and colonize medical devices be partially due to their ability to produce tenacious bacterial biofilms known as slimes (see FIGURE 3.2) [11]. Slimes are adherent multilayered biofilms embedded into glycocalyx composed of exopolysaccharides on implanted devices and are believed to be essential for the pathogenesis of *S. epidermidis* [24]. The slime film protects *S. epidermidis* from antimicrobial drugs and may be an inhibitor of phagocytosis by neutrophils [25].

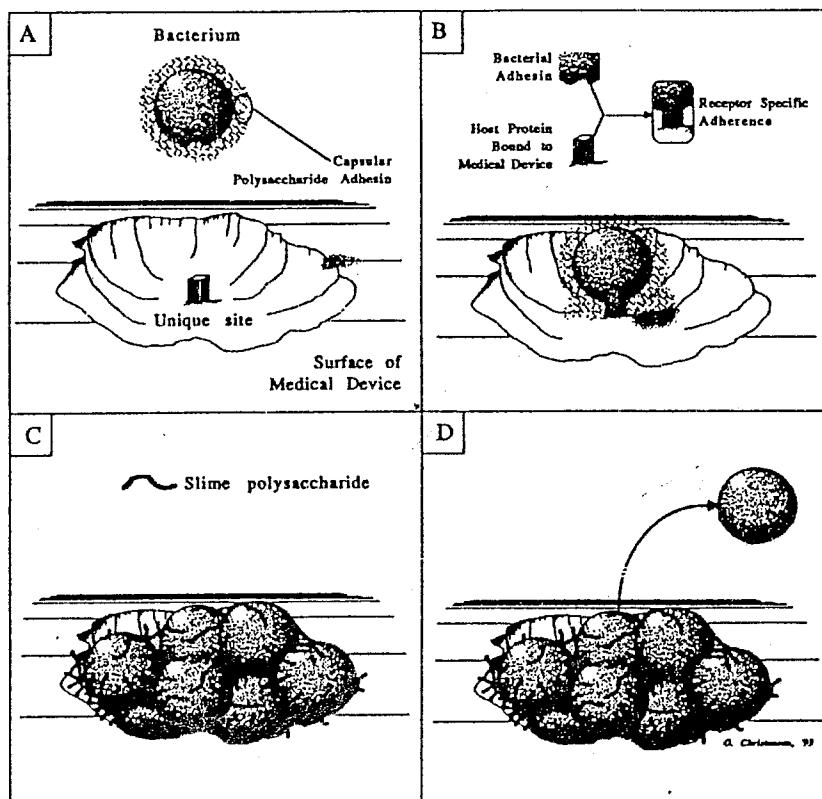


Figure 3.2 Bacterial colonization over a surface. (A) Bacterial colonization begins with exposure of the device to bacteria. By virtue of mass effect, a certain proportion of the bacteria in the bathing solution will associate with the surface of the device. The association will always take place, even with surfaces that are designed to repel bacterial attachment. (B) Resulting bacteria attachment to the surface. This initial attachment is promoted by the unavoidable presence of “unique sites” on the surface of the device.

These unique sites include local variations in surface hydrophobicity, surface irregularities (demonstrated in the three panels as surface crater), and the presence of host proteins adsorbed onto the surface of the device. (C) The rare *S. epidermidis* cells that attach to the surface at unique sites can proceed to successfully colonize the surface by producing slime. The slime stabilizes the cell-to-cell and the cell to surface associations, allowing the bacteria to accumulate on the medical device. (D) Final stage in surface colonization. By virtue of phase variation or phenotypic modulation, daughter cells that are not bound to the colony by slime emerge from the microcolony. These cells are then free to drift to new sites to repeat the colonization process [11].

Microbial cells (predominantly bacteria), and their extracellular polymers associated with a substratum are called biofilms. Microbial biofilms constitute a major reason for infections to occur and persist at various sites in the human body, especially associated with medical devices, the microorganism have host proteins and other substances coating them. Once adherent, the bacteria multiply and anchor themselves in quite intricate structures that appear to allow for communication and information transfer between organisms. For the treating physician, many antibiotics are unable to eradicate dense biofilms [26].

Bacteria in biofilms bind together in a sticky web of tangled polysaccharide fibers, which connect cells like strands of spaghetti and anchor them to a surface and to each other. Within this microcosm, anaerobic and aerobic bacteria can thrive along side each other, sharing water passageways and a complex structure. Water flows in connective patterns through the channels that deliver nutrients and remove waste like a circulatory system. Bacterial attachment and biofilm formation and persistence are governed by a complex set of physical, chemical, and biological processes acting simultaneously [27]. Biofilm formation may be separated into primary attachment of bacteria to native or modified polymer surfaces followed by proliferation of attached bacterial cells leading to accumulation of multilayered cell clusters and glycocalyx formation [24].

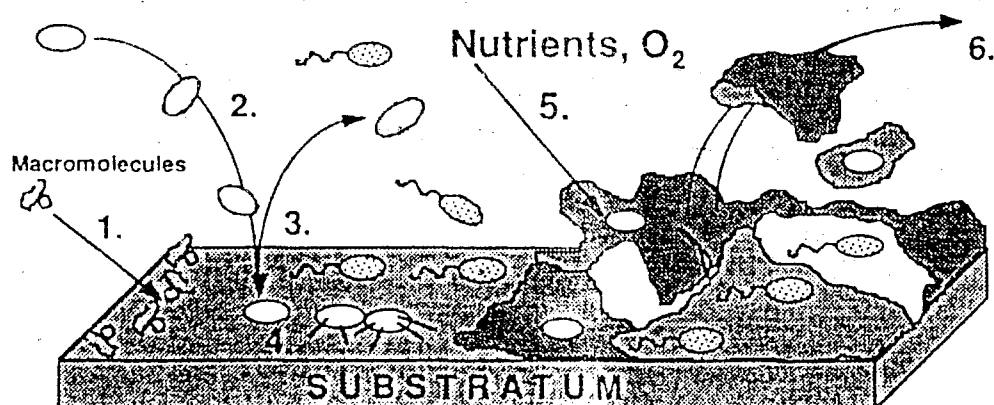


Figure 3.3 Process governing biofilm formation. Numbered processes are defined in the text [28].

As shown in Figure 3.3, development of a bacterial biofilm comprises the following fundamental processes: (1) Substratum pre-conditioning by adsorption of fluid phase organic molecules; bacterial cells deposition to the conditioned substratum; (2) cells transport to the surface; (3) cell adsorption; (4) cell adhesion to substratum; (5) Cell metabolism (cell substrate conversion, cell growth and replication, extracellular exopolymer production, cell starvation, lysis); and (6) biofilm removal (cell and biofilm detachment, biofilm sloughing). Naturally, the relative influences of each process is dependent on the specific system, the prevailing environmental conditions, and slow biological changes throughout the lifetime of the biofilm [28].

The prevention of colonization by an eradication of biofilm associated microorganisms is an important, and often difficult to solve problem in medicine. Unlike planktonic organisms, which are relatively susceptible to biocides, e.g., antibiotics, the structural matrix established during biofilm formation can make the colonizing cells able to withstand normal treatment dose of biocides, i.e. planktonic biocidal concentration

(PBC). It is known that when organisms are isolated from biofilms then grown in planktonic culture, they lose many of the characteristics associated with the progenitor cells, in particular, the ability to produce glycocalyx. In the biofilm, the glycocalyx matrix appears to serve as a barrier which protects and isolates the microorganisms from host defensive mechanisms, such as antibodies and phagocytes, as well as from antimicrobial agents such as surfactants, biocides and antibiotics. In one study, biofilm associated bacteria were able to survive a concentration of antibiotics 20 times the concentration effective to eliminate the same species of bacteria grown in planktonic culture [20].

Biofilm infection can occur in a variety of disease conditions. In some tissues, such as prostatic, the infective bacterium is capable of growing in the infected tissue in both biofilm (sessile) and circulating (planktonic) forms. Although growth of the planktonic cells can be controlled by antibiotic treatment, the biofilm itself may be refractory to antibiotic treatment providing, in effect, a reservoir of infection which can lead to recurrence of the infection after antibiotic treatment. Biofilm formation can also be a serious application in bioimplants, such as bone prosthesis, heart valves, pace maker, and the like. Biofilm formation on exposed surfaces of bioimplants can degrade the function of the implant, as in the case of bone prosthesis and all cases provide a source of difficult-to-treat septic infections [20].

4. CONTROL OF BACTERIAL GROWTH

4.1 Bacterial Death

A microbial cell is considered dead when it fails to divide to form new cells and consequently a colony on a solid medium or turbidity, sediments, or pellicles in a liquid medium. The rate of microbial death for any particular treatment can be predicted knowing the decimal reduction time (D-value). D-value is the time in minutes it takes 90% of the cells in a population. This value is constant for each reduction but could be affected by several factors: temperature, type of microorganisms, their physiological state, and the presence of other substances. Temperature could increase the rate of death reaction. Different types of microorganisms vary considerably in their resistance to killing. Log-phase cells are in a physiological state that makes them more susceptible to treatment from cells in their stationary state. The presence of certain substances, especially protein, in a culture protects microorganisms from many lethal treatments [9]. In addition, the amount of water present affects the time it takes to sterilize or disinfect a microbial population. The drier the environment the longer it takes to contaminate microorganisms [10].

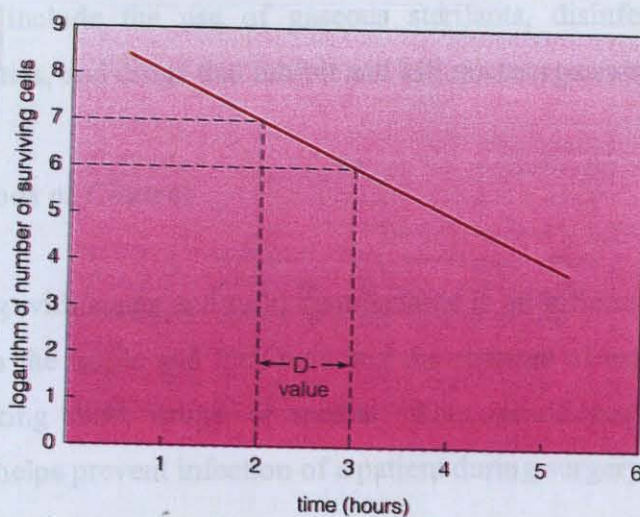


Figure 4.1 Microbial Death. A graph of microbial death, like a graph of microbial growth, is a straight line when plotted on a logarithmic scale. When exposed to lethal conditions, the number of surviving live cells in a microbial population declines 90 % (ten-fold or one logarithmic value) in the same period of time, called decimal time, or D-value. In this plot, the D-value is one hour [1].

4.2. Control of Microorganisms

Various characteristics of the disinfecting or sterilizing agents determine their effectiveness. The higher the concentration of an antimicrobial or the higher the temperature, the more rapidly a population is killed. In addition, Control may mean sterilization, i.e. treatment to destroy all microbial life. Disinfection (or sanitation) to reduce the number of pathogens to a level at which they pose no danger of disease. Decontamination is to render an instrument or surface that has been heavily exposed to microorganism safe to handle. Antisepsis is to kill microorganisms on skin or other living tissue [9].

Methods of control are often divide into physical methods of control and chemical methods of control. Physical methods of control include hand washing and the use of surgical masks, sterile clothing, and gloves to restrict the transfer of microorganisms. Heat and ultraviolet light are important physical agents to kill microorganisms. Chemical

methods of control include the use of gaseous sterilants, disinfectants, antiseptics, preservatives, antibiotics, and drugs that inhibit and kill microorganism's [10].

4.2.1 Physical Methods of Control

Hand washing with soaps and mild disinfectants is an efficient way of removing microorganisms from the hands and thus reducing the chances of infecting animals and plants or contaminating food, drugs, or media. The use of surgical masks, sterile clothing, and gloves helps prevent infection of a patient during surgery [10].

Heating

Heating is the most efficient and cost-effective way of killing organisms and is used to sterilize glassware, heat-stable media, and equipment. Heat sterilization could be dry or moist. All heat treatment kill by denaturing proteins. However, dry heat treatment kills largely by speeding oxidation, which inactivate macromolecule. Boiling or autoclaving (Fig. 15.1) is a moist heat treatment of heat-stable materials. In general, hot moist air kills more effectively than dry air at the same temperature [9, 10].

Cold

Cooling is familiar physical method of control that temporarily inhibits the growth of organisms. Cooling and freezing often does not kill the organisms, and consequently cannot be used when sterility is required [9, 10].

Filtration

Filtering is an important method for eliminating microorganisms, other than viruses, from heat-labile liquid media, vitamin solution, or heat-sensitive antibiotics (figure 4.2) and from air in surgical room and cell culture room [9].

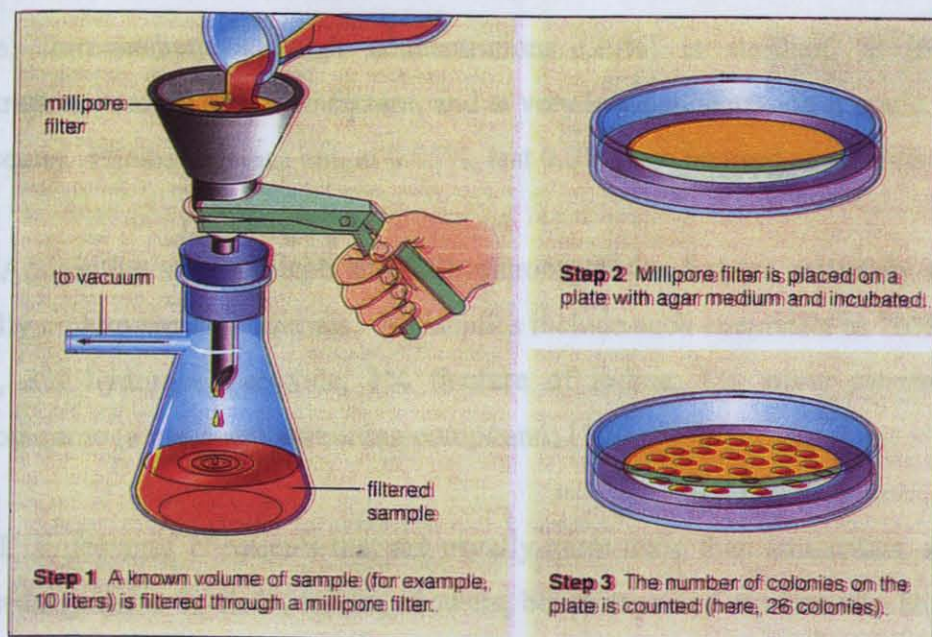


Figure 4.2 Filtration of bacteria [9].

Radiation

Because UV light is a potent mutagen, it is often used to kill microorganisms in the air and on the surface of working areas. Many cell culture rooms have UV light installed in their ceilings. These UV lights are turned on during the night when no one is working in the rooms. X-ray and gamma radiation is sometimes used to kill microorganisms in heat-labile medical supplies and some food [9, 10].

In addition, drying through vaporization or sublimation, and increasing osmotic strength by high concentration of sugars or salt could be used to control microbial growth especially in food industry [9].

4.2.2 Chemical Methods of Control

Antimicrobial agents, or germicides, are somewhat artificially divided into the following categories: antiseptics, disinfectants, sterilants, chemotherapeutic agents, pesticides, and preservatives. The distinction is sometimes blurred because some of the chemicals can fall into a number of categories depending on their concentrations. For

instance, formaldehyde at high concentrations (20%) is sterilant, at intermediate concentration, it could be a disinfectant, and at very low concentration it could be used as an antiseptic. Phedisinfectant but, at 0.05%, is at 0.05% can be used as an antiseptic [10].

Antiseptics are chemicals that kill microorganisms but are relatively safe to use externally on humans and animals. Antiseptics include such chemicals as 70% isopropyl alcohol, 3% hydrogen peroxide, 1% tincture of iodine, 1% silver nitrate, and 1% mercurochrome (a mercury-containing compound) [10].

Disinfectants, chemicals that are usually more toxic than antiseptics, are used to kill microorganisms on floor, toilets, showers, bench tops and equipment. Disinfectants include chemicals such as 0.5% chlorine bleach (chlorox), 3% phenol (carbolic acid), 5% quaternary ammonium compounds (Roccal a% ethyl alcohol and 80% isopropyl alcohol. Alcohols are considered as disinfectants rather than antiseptics because at these concentrations they are not safe to use on mucous membranes. Many disinfectant are composed of a mixture of chemicals: Lysol disinfectant consists of 0.1% o-phenylphenol and 79% ethyl alcohol while Lysol cleaner is made up of 2.7% alkyldimethylbenzyl ammonium chloride, 0.13% tetrasodium ethyldiamine tetraacetate, and 0.34% ethyl alcohol [10].

Chemical sterilants are compounds that are very toxic to life and must be used with great caution. Chemical sterilants such as the gases ethylene oxide (12%) and propiolactone are used to kill microorganisms in heat labile materials such as petri dishes plastic pipettes, plastic filtering systems, etc., and surgical implants. Liquid glutaraldehyde (20%) has been used to sterilize anesthesia tubing and surgical instruments since it rinses off easily with water [10].

Pesticides are chemicals that are used against insects, arachnids, nematodes, algae, fungi, and bacteria. For example, solutions of copper sulfate (Bordeaux mixtures) kill algae and fungi, 1-% copper-8-hydroxyquinolate kills fungi and bacteria, and

400g/ml-ethylene bromide kill insects and arachnids in grains and on fruits as well as nematodes in soils [10].

Food preservatives are chemicals that are added to food in order to inhibit the growth of microorganisms. Some of the preservatives are natural compounds such as vitamin C and propionic acid and probably not harmful to the consumer at the added concentration. Many of the preservatives such as sodium nitrite, ethyl formate and polymyxin B can be dangerous if consumed in large counts. Common food preservatives include sodium chloride, sugars, and acetic (vinegar), ascorbic (vitamin C), benzoic, lactic, propionic, sorbic, and sulfurous acids. Fungi are inhibited by preservatives like benzoic acid, sorbic acid, propionic acid, sulfur dioxide, sodium diacetate, and ethyl formate, while bacteria are commonly inhibited by vitamin C, sodium nitrite, sodium chloride, sugars, and polymyxin B [10].

Chemotherapeutic agents are chemical substances that have a high degree of antimicrobial activity and can be used internally through ingestion or intravenous injection. Chemotherapeutic agents that are produced by microorganisms are called antibiotics. The best known antibiotics are penicillin (produced by a fungus) and chloramphenicol (produced by a bacterium). The chemotherapeutic agents that are produced by scientists are called synthetic drugs [10].

Factors that influence the effectiveness of chemical control agents include: The size of microbial population; the organisms' susceptibility to the antimicrobial; the volume of material to be sterilized; the length of time the antimicrobial is used on the microorganisms; the concentration of the antimicrobial; the temperature; the water activity of the substance to be sterilized; and the amount of organic matter [10].

The microbiocidal efficiency of a chemical is often determined with respect to phenol, a commonly used disinfectant. The efficiency of a chemical with respect to phenol is called phenol coefficient (PC). The phenol coefficient is calculated by dividing the highest dilution of the antimicrobial of interest that kills microorganism after

incubation for 10 minutes but not after incubation for 5 minutes by the highest dilution of phenol that has the same characteristics. Chemicals that have phenol coefficients greater than 1 are more effective than phenol [10].

The best chemotherapeutic agents are those that kill microorganisms rather than inhibit their growth, but lysis or cell rupture do not occur. Agents that kill are called microbiocidal, while those that inhibit are called microbiostatic. An antimicrobial agent may be microbiostatic at low concentration and microbiocidal at high concentration [10]. Microbiolytic agents are those agents that kill by cell lysis, which is observed as a decrease in cell number or in turbidity after the agent is added [29]. A chemotherapeutic agent is supposed to kill the microorganisms at risk, with little or no effect on host. This is called selective toxicity [10].

Some antimicrobials do not kill organisms but merely inhibit their growth and reproduction. Other antimicrobials are inhibitory if used at low concentrations but kill if used in high concentration. Because of this, it is important to know the minimum inhibitory concentration (MIC) and the minimum killing concentration (MKC) for an antimicrobial with organism that may be present [10].

Some antimicrobials affect a large number of different organisms and are known as broad-spectrum antimicrobials. Others affective against a small group of organisms and consequently they are known as narrow spectrum antimicrobials. The best antimicrobials are those that kills undesirable microorganisms at low concentrations but have no toxic effect on the host (plant, animal and human) [10].

Here, some types of germicides and the way they kill are briefly described.

Phenol and Phenolics: Phenol consists of a hydroxyl group attached to benzene ring. Phenolics have this structure and additional components. Both act by denaturing proteins. Phenolics also act on lipids. Hexachlorophene is a phenolic once used as antiseptic but now replaced by chlorhexidine, which is less toxic to humans [9].

Alcohols: Alcohols are compounds with a hydroxyl group. They kill microorganisms by denaturing protein and disrupting lipids in the plasma membrane. They do not kill endospores. Ethanol and isopropanol are commonly used clinical disinfectants [9].

Halogens and hydrogen peroxide: Halogens are oxidizing agents. They inactivate enzymes by oxidizing certain functional groups. Iodine is antiseptic and chloride is a disinfectant. Hydrogen peroxide is not a halogen but acts in the same way. It is used as a weak antiseptic for cleaning wounds and disinfecting fragile medical instrument and contact lenses [9].

Surfactants: Surfactants are compounds with hydrophilic and hydrophobic parts that penetrate oily substances in water and form an emulsion. Soaps and detergents are surfactants. They do not kill microorganisms but control them by washing them away. Quaternary ammonium salts are powerful surfactant germicides that are cationic. They kill all classes of microorganisms and viruses that have membranes, though higher concentrations are required for Gram negative bacteria. Anionic surfactants are mild disinfectants that act like quaternary ammonium salts but less effectively. They are used to disinfect skin and other surfaces, such as bench top in microbiological laboratories [9].

Alkylating Agents: Alkylating agents attach short chains of carbon atoms to enzymes, which inactivate them and kills the cell. Formaldehyde, formaline, glutaraldehyde ethylene oxide are examples. Ethylene oxide is a gas and a sterilizing agent used for heat sensitive materials and unwieldy objects. However, it is very toxic to humans [9].

Heavy Metals: Salts of heavy metals react with sulfhydryl (-SH) groups of proteins, “poison” enzymes and thereby kill microbial cells. Salts of mercury and silver have been used in medicine for years. The mercury salt mercury chloride have been used was once widely used as an antiseptic but is no more used today because it is highly toxic. Instead, organic compounds that contain mercury, including Merthiolate and Mercurochrome,

which are less toxic are used. Both are basic home medicine chest and first aid kit supply for disinfecting skin and mucous membranes [9].

Silver salts and colloidal silver (finely dispersed silver particles) were once widely used antiseptics. State law uniformly required that a solution of silver nitrate be applied to the eyes of newborns to avoid ophthalmic gonorrhea and the blindness that results. Today, however, the trend is away from using silver nitrate and toward using antibiotics. In general, silver salts have limited use today because they can irritate skin; though they continue to be helpful in preventing infections of burned skin [9]. Silver antimicrobials are discussed in more details in coming sections.

4.2.3 Aseptic Techniques

Aseptic techniques are the procedure used to prevent contamination of previously uncontaminated and to obtain and perpetuate pure cultures of microorganisms. A surgical team, performing even the most insignificant of procedures, employs the aseptic technique in order to prevent the contamination of surgical incisions or internal organs with microorganisms. Rudimentary aseptic techniques would include personnel washing their hands with antiseptic and mild disinfectants, treating bench tops with disinfectants, sterilizing all materials and equipment used in operation (including instruments, gloves, surgical drapes, masks and gowns), and maintain their sterility by careful wrapping and handling [9].

5. ANTIMICROBIAL SILVER MATERIALS

As mentioned before, antimicrobial agents used on biomaterials are classified into two groups: the synthetic or natural inorganic groups, and the synthetic or natural organic groups. There is a new tendency merging towards the use of metallic antimicrobials in different methods for different biomedical and industrial applications. These new methods avoid the known disadvantages of the use of the metallic antimicrobials [3].

5.1 Methods of Antimicrobial Application of Silver

In modern medical practice, both inorganic and organic soluble silver salts are used to prevent and treat microbial infection. While these compounds are effective as soluble salts, they do not provide prolonged protection due to loss through removal or complexation of free silver ions. They must be reapplied at frequent intervals to overcome this problem. Reapplication is not always practical, especially, where an indwelling or implanted medical device is involved. Attempts were made to slow the release of silver ions during treatment by creating silver-containing complexes that lower the level of solubility-like colloidal silver proteins. Such compounds are formulated as creams. [30]. The following is a presentation of new method for biomedical and hygienic application of antimicrobial silver in different forms.

5.1.1 Antimicrobial Silver with Surgical Bioinert Implants

Surgical implants, especially endoprosthetic orthopedic implants and sutures, are rendered antimicrobial by the presence of a bioerodible metallic silver component. This component, especially as a surface coating, provides in vivo a sustained release of silver ions in a concentration sufficient to provide a localized antimicrobial effect insufficient to cause harm to tissue [31].

In early years of implant surgery, silver was employed in the manufacture of the endoprosthetic implants. However, the use of silver and silver plated implants has

generally ceased by about 1935 in the ever continuing search for greater bioinertness for implant materials. In the particular case of orthopedic implants, silver is generally considered unacceptable as an implant material because of poor mechanical properties, connective tissue reaction and excessive subperiosteal bone growth [31].

It has been proposed that inorganic silver compounds should be incorporated in bone cement to reduce the risk of postoperative infection following insertion of endoprosthetic orthopedic implant. In particular, it was proposed that low concentrations of inorganic silver compounds should be incorporated in polymethyl methacrylate bone cement for this purpose. The compounds that they evaluated for this purpose were silver chloride (AgCl), silver oxide (Ag_2O), silver sulfate (Ag_2SO_4), silver phosphate (Ag_3PO_4), and chloridal silver (Ag-AgCl). The least effective of the compounds evaluated was chloridal silver. It has also been reported that the electrically generated silver ions constitute a potent broad-spectrum antibacterial agent of use as adjunctive treatment of osteomyelitis [31].

The clinical use of metallic silver is limited and, in particular, that diffusion of silver ions from a metallic surface such as silver foil is negligible. The use of metallic silver in endoprosthetic implant is counter-indicated. The failure of metallic silver coating to generate the required antimicrobial efficacy is due to the fact that silver metal surface does not release antimicrobial amount of silver ion [30]. Further, in the field of orthopedic surgery the usage of silver salts and electrically generated silver ions for the prophylactic or adjunctive treatment of postoperative infection following implant surgery is recommended [31].

Metallic silver should be activated in the sense that it erodes in vivo to provide a sustained release of silver ion at a concentration sufficient to produce a localized antimicrobial effect but insufficient to cause significant damage to connective tissues. The other criterion is that the structural material of the implant should be a substantial bioinert material so that the mechanical integrity of the implant is retained despite the erosion of the metallic silver [31].

A method was designed to render antimicrobial an endoprosthetic implant, comprising a permanent implant structure formed of a substantially bioinert structural material providing permanent implant structure having permanent mechanical integrity. Also, the implant is treated so that the antimicrobial component latently bioerodible to provide in vivo a sustained release of silver ions in a concentration sufficient to provide a localized antimicrobial effect but insufficient to provide alcoholized antimicrobial effect but insufficient to cause significant damage to connective tissues. This method could be used for prophylactic treatment of postoperative infection following endoprosthetic surgery [31].

The implant can be made of any structural material which is substantially bioinert but usually will be made of titanium alloy, or cobalt-chrome-molybdenum alloy, or ceramic material, or any combination of these materials [31].

The metallic silver component can be made of commercially pure (i.e. 99.99% pure) silver metal or of a silver alloy, for example dental amalgam or silver solder. In order to promote galvanic action producing silver ions, there can be used an alloy of silver with a nobler metal such as gold or platinum. The silver component will be deposited in or on the permanent implant structure [31].

The quantity of silver in the composite implant and the rate of erosion in vivo are such as to provide a sustained release of silver ions in sufficient concentration to produce a localized antimicrobial effect but insufficient to damage connective tissues. The required concentration balance readily can be achieved because antimicrobial activity is provided at a concentration of the order of nanograms/ml, whereas connective tissue damage results of six order higher concentrations, i.e. mg/ml [31].

5.1.2 Antimicrobial Silver with Hydroxyapatite

A method for making antimicrobial hydroxyapatite powders consists of including hydroxyapatite which has good affinity for living body with antimicrobial ions such as silver, copper or zinc, or organic antimicrobials such as honokitiol, tannin, lysozyme, protamine, or sorbic acid. These powders widely used in the medical dental and hygienic field such as artificial bones, artificial root of a tooth, dentifrice, dental cement, and supplement for bone for bone deficit and cosmetic materials [3].

As mentioned before, hydroxyapatite has a good affinity for living tissues such as bones and teeth, and hence a good affinity for biomolecules and microorganisms. Therefore bacteria are absorbed and breed in hydroxyapatite [3]. It is even used as a substance to concentrate bacteria from foods and other stuffs [32]. Accordingly, when hydroxyapatite is used as a living body material, it is necessary to consider its contamination with bacteria. To solve this problem, hydroxyapatite is to be used with antimicrobials [3].

Antimicrobial hydroxyapatite containing antimicrobial metal ions could be producing by having antimicrobial metal salts present with hydroxyapatite when being manufactured, or by reacting hydroxyapatite with these salts. For example, a calcium chloride solution can be dripped into or otherwise added to a solution containing disodium monohydrogen phosphate and antimicrobial metal salt selected from silver, copper, and zinc salts. The HA is produced from the solution, then filtered, washed with distilled water, dried and crushed. Alternatively, hydroxyapatite produced by the usual method can be suspended in water, and water-soluble antimicrobial salts added to the suspension. Then the precipitates are washed with distilled water, dried and crushed. The acid radicals, metal salts and calcium salts produced by changing the calcium ions of hydroxylapatite to one of the three metal ions, coexist with the antimicrobial hydroxyapatites. Thus, it is necessary to remove these contaminants by washing fully antimicrobial hydroxyapatite with water. The antimicrobial hydroxyapatites produced retain their antimicrobial activity for a long period, and the amounts of metal soluble in

water are ppm or less. Thus, the product can be used safely and their antimicrobial properties taken advantage of by adding the hydroxyapatite in amount of 10% or less preferably 0.5-5% to other materials [3].

The antimicrobial hydroxyapatite prepared following this method are useful because they are safe and have good affinity for a living body in the fields of foods, living body materials, cosmetics, fibers, cellulose, plastics, filters, and water absorption, polymers where antimicrobial properties are needed. When used in a dentifrice, an anticaries effect is obtained by the protein removal action and by the fine filling effect of hydroxyapatite. Pharmaceutical agents stay on the surface of a tooth with hydroxyapatite for a long period and the highly preventive agents of dental carries are developed by this method [3].

5.1.3 Antimicrobial Silver with Other Ceramics

An light antimicrobial ceramic contains an antibacterial material is produced by loading an antibacterial metal such as silver on a calcium ceramic carrier, an aggregate such as cordite and has a bulk density of 0.6-1.2 g/ml [33].

There has been conventionally been antibacterial metal such as silver, copper, and zinc loaded on a ceramic. Such antibacterial ceramics are used as soil conditioners due to their antibacterial property. An antibacterial ceramic having the shape of a pellet with enlarged specific surface area or the shape of Rasching ring has been used for treating water to improve its quality. Incidentally, the aforementioned antibacterial ceramic is formed to be relatively porous to enhance its antimicrobial property. However, many of such conventional antimicrobial ceramics are dense and heavy [33].

Firing a mixture of an antimicrobial metal and a specific material, and controlling the bulk density and porosity of the sintered body leads to the completion of this invention [33].

The method for producing a light weight antimicrobial ceramic of the present invention include steps of: preparing an antimicrobial ceramic material by loading an antimicrobial metal or metallic ion on a carrier; mixing the obtained antimicrobial ceramic material of 0.01-20 (%wt) with an oxide inorganic material and /or non-oxide material of 80-99.99 (%wt) to obtain a first mixture; mixing a combustible of 5-50 weight parts to obtain with the mixture of 100 parts weight with the mixture of 100 weight part to obtain a second mixture; and firing the second mixture with at a temperature 500-1300°C [33].

The antimicrobial metal or metallic ion could be silver, copper, zinc, platinum, chromium, tin, arsenic, lead, cadmium, or many mixtures of these components. The aforementioned carrier may be phosphate silica, zeolite, or many mixtures of these components. Phosphate for the carrier may be calcium phosphate, zinc phosphate zirconium phosphate, aluminum phosphate, or any mixture of these components. Calcium phosphate for the carrier may be tricalcium phosphate, calcium phosphate, calcium metaphosphate, hydroxyapatite, apatite halides or any mixture of these components [33].

The methods for loading the antimicrobial metals or metallic ions on the aforementioned carriers are not limited. Suitable methods include, for example, ion exchange, or firing at about 800°C or higher after ion exchange, or firing at about 80°C or higher an aqueous solution containing a water-soluble salt having antimicrobial component and then collecting the precipitate. Among these methods, the preferred is a one in which a water-soluble salt having an antimicrobial component is used because this method prevents elusion of the antibacterial metallic component and reduces the production cost [33].

Another type of disinfectant bioceramic material which can be easily manufactured, has a stable disinfectant property, catches heavy metals which are harmful to human body, and radiates far infra red rays with a positive influence on living things. Examples of the base materials use are compounds containing alkalineaining alkaline

earth metals and phosphorous such as granulated bone, keel (fossil of vertebra), artificial apatite, phosphate calcium ore, tricalcium phosphate, $Mg_3(PO_4)_2 \cdot 5H_2O$, $MgO(SiO)_3 \cdot xH_2O$ (x is a coefficient indicating the number of water molecules) in which compounds containing calcium and phosphorous are not preferable, with these base materials, by reacting metals with disinfectant and harmless property such as silver, copper, or zinc, a disinfectant bioceramic material is obtained. A polymer or wallpaper is manufactured and a proper amount of the above bioceramic composition is added [5].

The above bioceramic composition with its useful properties, could be made from the burning of the bone of vertebrate animals such as cows, pigs, etc. It could be also made of a compound containing phosphorous and alkaline earth metals such as artificial apatite, as a base material with metals of disinfectant and harmless properties such as silver, copper or zinc. Compounds containing calcium and phosphorous are most preferred in that they have disinfectant properties against harmful bacteria by themselves. Moreover, these compounds activate beneficial bacteria for the human body. Using the base materials manufactured as above by reacting various kinds of metals with disinfectant and harm free properties such as, silver, copper or zinc, a disinfectant ceramic material of the present invention could be obtained. Here, the ionic tendencies of the above-mentioned metals (silver, copper, and zinc) are lower than those of alkaline earth metals, and hence a substitution reaction is expected. In the case where disinfectant metals have a substituting reaction with the base materials in accordance with the above-mentioned ratio, the ceramic materials thus made are capable of having a disinfectant property due to the disinfectant metals substituted. At the same time, alkaline earth metals remaining non-substituted can catch harmful ions of heavy metals such as Hg^{2+} , Pb^{2+} , and Cd^{2+} by substitution and further get rid of harmful anions such as Cl^- , and F^- . The examples of the base materials which are most preferable for using as containers or wrappers for food are compounds containing calcium and phosphorous such as TCP, artificial hydroxylapatite, or $Ca_{10}(PO_4)_6(OH)_2$ (which is a primary composition of granulated bone of vertebrate [5]).

However, the artificial apatite is too expensive, and TCP has an inferior affinity with the human body compared to the granulated bone. For base materials other than the food storing container or other kitchen goods, TCP or phosphate calcium ore can be used as base materials [5].

When bone of a vertebrate animal is burnt at a temperature of 1000° C, most of the organic and inorganic substances evaporate, and the remains after the evaporation become porous materials which are able to absorb impurities and heavy metals with ease. Thus, they can promote the effect of catching heavy metals. When granulated into very fine particles having an average particle diameter less than 0.2 μm , the porous materials comes to have a specific surface area of roughly 200 m^2/g . Since, these granulated particles have a property of radiating far infra-red rays, they can also be used as the materials for making a far infra-rg ceramic material [5].

Also, in the case of using the base materials as an artificial bone or tooth for man, by adding a proper amount of the above disinfectant metals, the occurrence of inflammation or carious tooth after an operation can be remarkably reduced. Additionally, a container for food made of the base materials containing calcium salt, such as the above granulated bone, may help water or wine to become more tasteful by eluting small quantities of calcium, which may supply a human body with calcium ions, which are likely to be in short supply [5].

5.1.4 Silver with Biomedical Polymer-Based Antimicrobial Devices

5.1.4.1 Antimicrobial Silver Complex Salt with Silica Gel

A new antimicrobial composition of silver thiosulfate complex salt is carried on silica gel particles having an equilibrium water content of 10% or smaller. At least a part of the surface of the silica gel particles carrying silver thiosulfate complex salt is covered with a coating layer composed of silicon dioxide formed by means of hydrolysis of alkoxy silane [34].

The silver salt antimicrobial agents have a disadvantage that silver ions of the agent would be allowed to react with chloride ions in tap water to form insoluble silver chloride, resulting in a loss of their antimicrobial effect. Further, the silver salts, since they have a high photochemical reactivity, suffer another disadvantage that they become black and deteriorate their antimicrobial performance by being transferred into metallic silver or silver oxide, when exposed to light. In addition, the silver salt antimicrobial agent have a further disadvantage that in case of incorporating them into the resin by kneading or compounding and the resin is molded at a temperature of 200°C or above, it is difficult to obtain a white or transparent resin molded product because the antimicrobial agent may sometimes discolor or make a dull color at the heating temperature during the heating process [34].

An antimicrobial composition that avoid the above disadvantages and deficiencies inherent to the prior art silver salt antimicrobial agents and composition. The presented antibacterial composition in accordance with the present invention hardly produces white turbidity when contacted with tap water, showing no formation of AgCl. Thus a stable antimicrobial effect. Also, this composition will not discolor at the molding temperature of resin and can give a white or transparent resin molded product [34].

This new composition consists of a silver thiosulfate complex salt and silica gel particles having an equilibrium water content of 10% per weight or smaller at room temperature and ordinary humidity (at 25°C, relative humidity is 70%), and carrying said silver thiosulfate complex salt [34].

Silica gel particles as the carrier having two hydroxyl groups (-OH) or less for nm^2 of their surface are particularly preferred in point of view of the thermal stability of the carried silver thiosulfate complex salt. In the above mentioned antibacterial composition of this method, at least part of the surface of the silica gel particulate carrier is preferably covered with a coating layer. In the compliance with a covering area, thickness, porosity, and the like of the layer of the coating material, the degree and the

rate of releasing of the silver thiosulfate complex salt carried on the carrier are properly controlled [34].

The present composition comprises potassium silver thiosulfate salt carried on silica gel particles. Although the silver thiosulfate complex salt is difficult to obtain as a single substance in a solid state, it is possible to immobilize the salt in a state of being carried on a carrier, by first preparing as a water soluble compound, then impregnating a silica gel carrier with an aqueous solution of the compound, and drying the impregnated carrier [34].

The silver thiosulfate complex salt can be obtained by adding at least one salt selected from the group consisting of a sulfite salt and a hydrogen sulfite salt to a water soluble silver salt, and then adding a thiosulfate salt. Each of said sulfite salt, hydrogen sulfite salt and thiosulfate salt is preferably a sodium salt, and more preferably a potassium salt [34].

This method further provides an antibacterial composition comprising a potassium salt of silver thiosulfate complex carried on silica gel particles [34].

The silver thiosulfate complex salts used in the present invention form radicals near them by an action of oxygen in the air, and the radicals exert an influence on the surface of a membrane of the microorganism which accesses to the radicals and thus the silver salts demonstrate the antibacterial activities. Such an influence is called oligodynamic reaction. That is, it is presumed that the silver thiosulfate complex salts convert the oxygen near the salts into active oxygen by their catalytic action. The active oxygen gives a damage on a cell membrane covering the surface of the microorganism and demonstrates their antibacterial actions on the microorganisms by giving a defect on a genetic information, thereby suppressing the growth of microorganisms [34].

5.1.4.2 Antimicrobial Composition of Silver Nitrate and Gentian Violet

Indwelling devices such as catheters are made silastic and polymer-based with improved antimicrobial properties. They would include low amount of gentian violet or a combination of gentian violet and silver nitrate. These devices are made of polyurethane, silicon, or other similarly absorptive materials [35].

Catheters for patient care that are treated to include metal antimicrobial compounds, such as silver and silver oxide, gold thioglycolate and copper oxide. Silver nitrate pretreatment of silastic catheters (using a silver nitrate in alcohol technique) has previously been reported by the present inventor to impart *E. coli* resistance to a treated surface. Silver nitrate suspended in alcohol has also been observed to provide a more effective and rapid impregnation of silastic rather than silver nitrate in water. Silver sulfadiazine with chlorhexidine and other sulfa drugs have also been used with commercial catheters to enhance infection resistance [35].

This method demonstrates that low amounts of gentian violet, or a combination of gentian violet and silver nitrate, provides for an improved and microbially resistant device using anti-microbial agents that do not give rise to antibiotic resistant microbial strains. The present invention, in a general and overall sense, concerns medical devices of silicone or polyurethane, and like materials, that includes relatively low concentrations of gentian violet or mixtures of gentian violet and silver nitrate. These devices are found by the present inventors to have extraordinarily effective antibacterial and antifungal activity. These elegantly simple devices provide amazingly effective antimicrobial activity at concentrations several folds lower than gentian violet concentrations previously contemplated in the literature. Potential undesirable characteristics associated with “tattooing” or discoloration of tissues are thus reduced, without loss of bacteriostatic activity. Other advantages of the invention include improved, longer-term protection against infection of both bacterial and fungal origin. Such is accomplished without antibiotics, thus reducing the incidence of resistant bacterial infection [35].

5.1.4.3 Antimicrobial Synergistic Composition of a Silver Salt and Chlorhexidine

A method of preparing an infection-resistant medical device comprising one or more matrix-forming polymers selected from the group consisting of biomedical polyurethane, biomedical silicones and biodegradable polymers, and antimicrobial agents, especially a synergistic combination of a silver salt and chlorhexidine (or its salts); also disclosed are medical devices having the synergistic composition therein or compositions thereon [36].

The charge that the polyurethane polymer carries influences the affinity for the antimicrobial agent. When the antimicrobial is silver chlorhexidine acetate (CHA), the polymer of choice would be a polyether polyurethane, specifically Pellathane [36].

The biomedical Silicones that can be used in this composition include the silicon rubbers or elastomers. The selection of specific polymeric coating agent to form a coating agent to form a coating matrix will depend upon the nature of the surface to which the coating will be applied. It is preferred that biomedical polyurethane be applied to polyurethane surface to assure good adherence. A biomedical silicone such as a mixture of Silastic type A Medical Adhesin and MDX4-4159, is suitable to coat a device that is fabricated of silicone, polyurethane or of latex [36].

It has further been found that use of a biodegradable polymer in the coating composition of this invention, either alone or in combination with one or more of the other biomedical polymers, enhances the character of the polymer matrix [36].

The use of degradable polymer modulates the release of antimicrobial agents. The initial burst of agent which during the first few days after implantation is mere of less eliminated since the agent is bound in the biodegradable polymer and will be released only when degradation of the polymer occurs. Inclusion of a biodegradable polymer such as polylactic acid (PLA), in the matrix gives prolonged biocidal activity [36].

An additional advantage of using a biodegradable polymer such as PLA in a polyurethane matrix is to allow improved tissue ingrowth simultaneously with a prolonged antimicrobial effect as the biodegradable polymer degrades. Thus, this embodiment of the invention is particularly important in orthopedic applications as well as in such devices as arterial grafts where there is a need for formation of the pseudo intima or the growth of tissue into the interstices of orthopedic implants and arterial grafts, as well as cuffs which anchor catheters in place [36].

Antimicrobial agents useful according to this first embodiment of the invention include the biguanides, especially chlorhexidine and its salts, including chlorhexidine acetate, chlorhexidine gluconate, chlorhexidine hydrochloride, and chlorhexidine sulfate, silver and its salts, including silver acetate, silver benzoate, silver carbonate, silver iodate, silver iodide, silver lactate, silver laureate, silver nitrate, silver oxide, silver palmitate, silver protein, and silver sulfadiazine, polymyxin, tetracycline, aminoglycosides, such as tobramycin and gentamicin, rifampicin, bacitracin, neomycin, chloramphenicol, miconazole, quinolones such as oxolinic acid, norfloxacin, nalidixic acid, pefloxacin, enoxacin and ciprofloxacin, penicillins such as oxacillin and piperacil, nonoxynol 9, fusidic acid, cephalosporins, ephalosporins, and combinations thereof

From the above list, unexpectedly, some special combinations have been found. The combination of the biguanides, especially chlorhexidine and its salts with silver salts cause a special synergistic sustaining of antimicrobial action, as described in the second embodiment of the invention below. The biguanides are also synergistically effective with nalidixic acid and its derivatives. Another effective combination is chlorhexidine is chlorhexidine acetate and where the antimicrobial agent used is insoluble in the coating vehicle, as is the case with most of the silver salts and the water insoluble chlorhexidine, it is preferred that the agent be very finely subdivided, as by grinding with a mortar and pestle. A preferred product is micronized, e.g., a product wherein all particles are of a size of $5\mu\text{m}$ or less. In the case of the preferred silver preferred silver sulfadiazine, a micronized product may be used. The only way to use chlorhexidine internally is in the

time release matrix system described above that allows for a dose that is non-toxic to the patient but effective against microorganisms [36].

5.1.4.4 Silver with heart prosthetics

Another form of silver application includes an antimicrobial metallic coating on portions of the prosthesis, usually fabric, to enhance the overall acceptability of the implantable device. The preferred metal is silver, which is applied to surfaces that are exposed to the heart tissue. Mechanical and bioprosthetic heart valves benefit from this coating and it may also be applied to annuloplasty rings, composite valved grafts, sutures, pledges, a heart girdle or other implantable devices. The silver-treated portions of these devices inhibit or greatly reduce colonization of endocarditis-causing bacteria without affecting the overall biocompatibility of the device [37].

There are several ways to provide the silver coating to the devices. First, the fabric used in the construction of the devices may be coated after the fabric is formed. Second, the yarn or fiber that makes up the fabric can be coated before the fabric is formed. Third, after the fabric portion is constructed, the fabric portion itself may be coated with the silver. In addition, the silver coating may be applied directly to a device. In one embodiment, the amount of silver required is quite small, generally in the range of 1-100 mg of silver per gram of fabric, preferably 20-50 mg of silver per gram of fabric. Coating the fiber may be advantageous because this technique may produce a more optimal distribution of the silver in the completed product. For all the coating methods, the porosity of the textile must be preserved so that the tissue ingrowth properties are adequate [37].

For stented bioprosthetic heart valves, the fabric used for the sewing cuff could be coated in the same manner as described above. It is also contemplated that the stent for these valves may be coated with silver to further enhance the infection resistance of the device. In stentless bioprosthetic heart valves, the outer fabric wraps may also be treated with the silver coating [37].

The thin film silver coating may be deposited or carried in the fabric member, fibers, or sewing cuff using methods known to the art. Through action the term “coating” or “coated” may be used to mean that the silver may be coated on the surface of the device or component or may be implanted within or into the device or component as well as on the surface of the device or component, depending on how the silver was applied. For instance, the silver coatings may be applied by vapor-deposition or by sputtering. Although any of a number of techniques can be used to create the silver coating, it is important that the coating be extremely adherent to the fabric or other materials to prevent excessive circulation of cytotoxic silver materials throughout the body, while retaining porosity necessary for tissue ingrowth [37].

As is known in this art, other metals may be adhered to the fiber before the coating of silver is applied. Similar methods may be employed for coating the fabric or the assembled sewing cuff by utilizing fixtures, such as mandrel, to rotate or position the fabric or cuff to provide a uniform coating of silver [37].

To achieve uniform and tightly adherent silver coating, vapor deposition techniques, such as ion beam implantation and ion beam-assisted deposition (IBAD) are contemplated within this disclosure [37].

5.1.4.5 Silver with Antimicrobial Resin for Ophthalmic Solution

A method for improving the antibacterial efficacy of an ophthalmic solution, particularly a solution for the care of contact lenses, comprising providing an article molded from plastic resin including an inorganic carrier retaining antibacterial metal ions, and placing the ophthalmic solution in contact with the plastic resin [38].

It is understood that the term “improve the antibacterial efficacy” includes imparting antibacterial properties to a solution, such as solutions which contain no preservatives or other antibacterial agents, as well as enhancing antibacterial properties of a solution that already contains an antibacterial component [38].

The antibacterial efficacy of the solution is improved by contacting the solution with the article composed of a plastic resin having incorporated therein antibacterial metal ions retained in an inorganic carrier [38].

The method is particularly useful for contact lens cases since the prevention of bacterial contamination is more dependent on the user following care regimen instructions. For example, a user may fail to properly clean the lens case between uses, and if the case becomes contaminated, a biofilm can then form on the surfaces of the case from colonization of the contaminating microorganisms. Such a biofilm provides means for bacteria to survive even when later subjected to a preserved solution since the biofilm “protects” microorganisms from contacting the solution. The invention provides a method of inhibiting the formation of such a biofilm on the surfaces of the contact lens case to further enhance the efficacy of an ophthalmic solution when placed in contact therewith [38].

The plastic resin is preferably a thermoplastic or thermosetting resin suitable for molding, such as injection molding or blow molding. Additionally, the resin is selected such that it is compatible with the inorganic carrier retaining the antibacterial metal ions. One class of resins are thermoplastic polyolefin resins, including high-density polyethylene, low density polyethylene and polypropylene. Other resins include polycarbonates, polyvinyl chlorides, polystyrene, as well as other known plastic resins [38].

The inorganic carrier must be one that retains the antibacterial metal ions and which is suitable for molding processes when combined with the plastic resin. Particularly, it is desirable that when the ophthalmic solution is in contact with the plastic resin, only the metal ions leach in the solution while the inorganic carrier remains in the resin [38].

A preferred inorganic carrier is a zeolite. Zeolites may be defined as a silicate of aluminum and a metal oxide having a three-dimensional skeletal structure, and represented by the formula $xM_{1/2}O \cdot Al_2O_3 \cdot ySiO_2 \cdot zH_2O$, wherein M is a univalent or bivalent metal ion such as sodium or calcium, n is the valence of this metal ion and x is a coefficient of this metal ion, y is a coefficient of the silica, and z is the number of water crystallized. For the zeolite employed in the method of this invention, the metal ion M is exchangeable with the antibacterial metal ions, such as silver ions, copper ions or zinc ions, whereby the antibacterial metal ions are retained in the zeolite. Silver metal ions may be incorporated in the zeolite at 0.001 to 10% by weight (based on weight of anhydrous zeolite), more preferably 0.001 to 5%; zinc or copper ions may be incorporated at 0.01 to 25% by weight, more preferably 0.01 to 15% by weight. Another inorganic carrier that can retain antibacterial metal ions is an amorphous aluminosilicate. Such materials have an amorphous three-dimensional skeleton and may be represented by the formula $xM_{1/2}O \cdot Al_2O_3 \cdot ySiO_2$ wherein the variables are defined as above for the zeolite. Silver metal ions may be incorporated in the carrier at 0.001 to 20% by weight (based on a dry weight of the carrier), more preferably 0.001 to 12%; zinc or copper ions are incorporated at 0.01 to 15% by weight, more preferably 0.01 to 10% by weight [38].

5.1.4.6 A Crystalline Silver Antimicrobial Composition with Polymers

The method below relates to a novel antimicrobial polymer composition comprising a novel crystalline antimicrobial composition. More particularly, the present invention relates to an antimicrobial polymer composition comprising a crystalline antimicrobial composition and a polymer, wherein said crystalline antimicrobial composition comprises a crystalline silicon dioxide containing silver ions and one or two optional metal ions selected from the group consisting of zinc and copper [39].

This method provides an antimicrobial polymer composition comprising a crystalline antimicrobial composition and a polymer, wherein said crystalline antimicrobial composition comprises a crystalline silicon dioxide containing silver ions and one or two optional metal ions selected from the group consisting of zinc and copper.

The present invention also provides a process for preparing an antimicrobial composition of said antimicrobial polymer composition comprising steps of 1) preheating an antimicrobial composition having an antimicrobial coating of an aluminosilicate provided on the surface of silica gel, wherein said aluminosilicate contains silver ions and one or two optional metal ions selected from the group consisting of zinc and copper, to a temperature between 250° and 500° C. to substantially remove water, 2) sintering at a temperature from 800° to 1300° C., and 3) mixing the obtained crystalline antimicrobial composition with a polymer [39].

Both halogenated and non-halogenated organic polymers may be used in preparing the antimicrobial polymer composition of the present invention. Non-halogenated organic polymers may be synthetic or semi-synthetic and include the following: thermoplastic synthetic polymers such as polyethylene, polypropylene, polystyrene, polyamide, polyesters, polyvinyl alcohol, polycarbonates, polyacetals, ABS resins, acrylic resins, fluorine resins, polyurethane elastomers and polyester elastomers; thermosetting synthetic polymers such as phenolic resins, urea resins, melamine resins, unsaturated polyester resins, epoxy resins and urethane resins; and regenerated or semi-synthetic polymers such as rayon, cuprammonium rayon, cellulose monoacetate, cellulose diacetate and cellulose triacetate. If a strong antimicrobial and/or microbicidal effect is necessary, the polymer composition is preferably foamed or otherwise shaped into a net, a fiber, etc. Preferred from this viewpoint are organic or fiber-forming polymers such as synthetic polymers exemplified by nylon 6, nylon 66, polyvinyl alcohol, polyethylene terephthalate, polybutylene terephthalate, polyacrylonitrile, polyethylene, polypropylene and copolymers thereof, and regenerated or semi-synthetic polymers exemplified by rayon, cuprammonium rayon, cellulose monoacetate, cellulose diacetate and cellulose triacetate. Halogenated organic polymers that can be used in the present invention are also not limited to any particular kinds and may be exemplified by polyvinyl chloride and polyvinylidene chloride [39].

5.1.5 Some Other Applications of Antimicrobial Silver

A method and a portable apparatus is provided which can be transported to the area of treatment, generate silver ions or ions of other selected elements such as copper, uniformly distribute the ions over both confined and extended areas of water and land, and destroy infectious bacteria and/or other undesirable microorganisms in such areas. Water under pressure is passed through a manifold containing silver electrodes (or other desired metal electrodes) which are energized to produce ions, (primarily silver and secondarily copper). Being the reference base for the International Ampere and Faraday's Law, it was well known to be readily ionized. Accordingly, N.A.S.A. developed a simple electrolytic system to generate silver ions to be circulated in the water to be treated. A control unit generates a direct current which is applied across electrodes of specially formulated silver alloy immersed in the water being treated. The dose rate at which these ions are generated and liberated into the water is set and maintained by solid-state microprocessor circuitry in the control unit. This same system, with the addition of copper to the silver and with other minor adaptations, is now being used in the treatment of water in many commercial and industrial applications such as swimming pools, spas, air conditioning cooling towers, food processing, etc. At the same time, several patents have been issued in recent years which cover unique ways of using silver in water treatment and disinfecting [40].

A method was provided for preventing a microorganism from growing on surfaces such as the skin, walls, floors, countertops, and food preparation surfaces as well as in absorbent materials such as diapers, clothing, bedding, bedpads surgical apparel, surgical masks and the like. This method includes incorporating an effective amount of zeolite onto the surface or into the absorbent material to inhibit such microorganisms from growing [41].

According to the present invention, zeolites prevent microbial growth thereby inhibiting urea from converting to ammonia and carbon dioxide. Therefore, zeolites have two beneficial effects: inhibition of bacterial growth and inhibition of ammonia and

carbon dioxide production. Zeolites can be chemically synthesized and occur naturally in volcanic rocks, altered basalts, ores and clay deposits. Zeolites include crystalline, hydrated alkali-aluminum silicates of the general formula: $M_{2/n} O \cdot (Al_2O_3) \cdot [y(SiO_2)] \cdot wH_2O$ wherein M is a cation of valence n, w is the number of water molecules, and y is 2 or more. The cation is mobile and can undergo ion exchange [41].

5.1.6 Depositing Antimicrobial Silver on Surfaces

Metallic compounds may be applied to a surface by metal surface deposition, electroless plating, chemical derivatization, and coating [42].

Metallic silver is deposited upon the surface of a non-conductive substrate using a multi-step-wet deposition. The surface is cleaned, and then activated in an aqueous solution containing stannous tin. The silver is deposited as a colloidal material from a solution containing a silver salt and a reducing agent that reduces the salt to form metallic silver by nucleating throughout the solution. After the substrate is coated, the coating is stabilized in an aqueous solution of a salt of a noble metal like gold or platinum, dissolved in a dilute, hydrophobic acid. This process is particularly effective for depositing uniform film of 2 to 2000 Angstrom thickness, which strongly adhere to the substrate. A thin film can reduce the incidence of infection caused by a device introduced into the human body [43].

In one method, metallic silver coating is accomplished by contacting the surface with a carbonyl compound, e.g. an aldehyde such as glutaraldehyde, a sugar such as glucose, or an aldehyde functionally generating compound. Then, the substrate is dried and contacted with a metal salt, e.g. silver nitrate, or metal carboxylate solution, e.g. a dichloride solution to deposit the metal on the surface. In another embodiment, the substrate is contacted with an activator, e.g. a dichloride solution, dried, and then contacted with a metal salt or metal carboxylate salt solution, either with or without an amine containing compound solution, so as to deposit the metal on the surface of the substrate [44].

Some antimicrobial materials are molecularly designed to enable a matrix bound biocide retain high activity without elution of any compound into contacting solutions, carriers or other materials. Their activity stems from the cooperative biocidal action of its components. Selective transfer of one component from the matrix directly to the microorganism upon contacting is achieved via a “hand-off” mechanism upon engagement and penetration of the microorganism’s cell membrane [44].

The antimicrobial material of this type comprises a combination of an organic material which forms a matrix, and a broad spectrum biocide intercalated in the matrix that interact sufficiently strongly with the organic material that the biocide does not leach from the matrix. The organic material must possess two important properties: it must be able to bind or complex with the biocide, and must be capable of dissolving into or adhering to the cell membrane of the organism. The organic material preferably is capable of being immobilized on a surface and which binds the biocide in such a manner as to permit release of the biocide into the organism but not the surrounding environment. The biocide preferably is a low-molecular-weight metallic material that is toxic to the microorganism and is capable of complexing or reversibly binding to the organic matrix material, but which preferentially binds to cellular proteins of the microorganism [44].

5.2 Colloidal Silver

5.2.1 Structure, Concentration, and Antimicrobial Activity of Colloidal Silver

At least four different products in the market are being called colloidal silver. The first one is the original kind called also electro-colloidal silver. The second is mild silver protein, which may appear as transparent clear or amber. The third is the ground or powder silver, with a silver content of 100-500 ppm. It is made when a silver wire is rapidly disintegrated by a high voltage electric discharge. The microscopic dust is collected and then dissolved in water or added to salves or cream for topical use. The fourth one is a group of different ionic silver salt solutions [45].

Colloidal silver is pure elemental silver that is evenly distributed into a second substance, i.e. water. The silver is not dissolved but suspended evenly throughout the solution as a result of the positive electric charge that is attached to each particle of silver. The charged silver particle, i.e. silver ions, form into aggregate of atom microcluster of silver that interact with each other and create a synergistic effect, each electrically charged particle of the microcluster has a magnetic field. Together the particles create a phenomenon called Brownian movement, which is a sort of random oscillating motion. In other words, the microclusters also have a positive charge and repel each other, that is how they stay suspended and evenly distributed in the water. This colloidal condition is most easily detected by what is called Tyndal effect where a narrow beam of light is shined through the liquid to produce a cone shaped dispersion of light. The particles so illuminated exhibit a random, zigzag motion called Brownian motion when observed under a microscope. When something is completely dissolved, both the Brownian and Tyndal effect disappear [8, 45].

Electro-colloidal silver is both ionic and colloidal. It is considered colloidal because of the particle size and ionic because of the particle size. In fact, most of the biological studies suggest that the ionic characteristics of colloidal silver are what make such a good germicide [45].

In order to produce electro-colloidal silver, at least .999% pure silver is placed in water, an electrical charge is induced into the silver, and as a result particles of silver are electrically sintered off of the metallic silver rods or wire into water. In the water, the silver forms submicroscopic clusters (atomic microclusters) optimally 10 to 100 Angstrom. Parts per million is a way to express the concentration of colloidal silver [8]. It is nearly impossible to standardize Electro colloidal silver. Even when the voltage, the water and the water temperature are identical, different batches will proceed at different rates in different days. The rate of the reaction can vary by over 100% depending on the day [45].

The total allowable impurities in .999 pure silver are 1000 ppm. These impurities and their maximums are copper (800 ppm), lead (250 ppm), iron (200 ppm), and bismuth (10 ppm). When this product is used to make electro-colloidal silver at a concentration of 5 ppm, the total impurity concentration drops to 4-ppb (parts per billion) copper, 1.25 ppb lead, 1 ppb iron, 0.05 ppb bismuth. In fact when analyzing samples of colloidal silver, the concentration of impurities is much more greater than the above figures and different elements such as Ca, Mg, K, and Mn are detected, showing that most of the impurities usually come from the used water [45].

In a previous study, various forms of silver were tested for their antimicrobial activities. Electro-colloidal silver out performed silver nitrate, silver chloride and silver sulfadiazine as a broad-spectrum germicide. For all classes of bacteria, fungi, and molds, colloidal silver performed better and at lower concentration. A conclusion was drawn that any additives reduce the effectiveness of silver ion, as silver salts being 100 times less effective than colloidal silver [45].

The positive effects of colloidal silver are not yet fully understood; therefore, the following explanation is hypothetical. The observed antimicrobial activity of colloidal silver appears to be the result of silver microcluster's frequency, motion, and magnetic field that the particles generate [8].

The Brownian movement and collective charge of each colloidal silver microclusters have several effects. These microclusters of silver have a frequency that is apparently disruptive to pathogens the oscillating particles seemingly gyrate in the fluids of the body interacting with cells including pathogens. Then acting as catalysts, the silver disables the oxygen-metabolizing enzyme, destroying these pathogenic particles, not by attack, but by creating an environment hostile to its existence [8].

The colloidal silver microclusters frequency appears to be disruptive to the pathogenic cells' ability to manufacture the enzyme used to process oxygen. Aerobic pathogenic cells need to quickly replace their spent oxygen used to survive, without the required enzyme pathogenic cells need to metabolize oxygen they are in effect suffocated within 6 minutes of initial contact with the silver [8].

Furthermore, researchers have also noticed that the Hexhermer effect, also referred to as a healing crisis or healing response caused by a reaction in the body to the toxins by the dead pathogenic cells as they "die off" and decompose, is lessened or not as apparent with the use of colloidal silver [8].

Some researches indicate that colloidal silver appears to concentrate in the Kupffer cells of the liver. Colloidal silver localized in the Kupffer cells of the liver may be allowing the body to grab and filter out of the pathogen with the help of silver that is localized in the liver Kupffer cells and sweep them before the pathogen is lysed (decomposed/dissolved). What appears to happen is that the dead pathogens are immediately swept from the human body before there is a substantial toxic build-up [8].

5.2.2 Some Applications of Colloidal Silver

Colloidal silver could be used as a disinfectant for a hemodialysis system. Colloidal silver is introduced to the blood and dialysate sides of a hemodialysis session and is not flushed from the system until another dialysis is to be performed. In the past, formaldehyde and renaline (a hydrogen peroxide-peracetic acid based product) were

used. Being extremely toxic if introduced in a patient, formaldehyde was carefully applied in a way to avoid its presence in the blood, tubing, and blood side of dialyzer before reuse. Therefore, there was a need for a disinfectant that is chemically pure, not toxic, and easily removable from the dialyzer. The need can be filled by properly using colloidal silver as disinfectant, preferably at a concentration of 3 ppm. This disinfectant is circulated to fill the dialyzer and associated set of dialysate and blood tubing and related apparatus after they have been rinsed followtreatment. The preferred concentration of the used colloidal silver is 3 ppm ($\pm 10\%$). Greater concentrations could be used but there is no significant increase in disinfecting effectiveness, and there is the disadvantage of being more costly. In fact, a concentration above 5 ppm does not practically give as good results as a concentration about 3 ppm [46].

A colloidal silver solution can be produced in a batch procedure by suspending two substantially pure silver electrodes, as anode and cathode, in distilled water and subjecting the electrodes to a relatively high alternating current voltage as, for example, 440 V. This procedure is continued until sufficient silver particle has sloughed into then water to achieve the desired concentration of 3 ppm. These silver particles are very minute, typically in the range of about 0.005 to 0.015 μm and normally have the same polarity. Hence, these particles remain suspended in the water in a colloidal state [46].

A composition of matter comprising a therapeutic active component with antiseptic and osmotic characteristics for treatment of burns and open wounds experienced by animals and man. The compound is in solution form and composed of colloidal silver, *Helichrysum angustifolium* or *Helichrysum italicum* oil and raw honey emulsified with water lecithin by agitation [47].

Silver complex compounds and colloidal silver are known are known in the prior art for use as therapeutic and antimicrobial treatments of wound and thermal injuries experienced by humans [47]. Also, colloidal silver has been used topically for conjunctivitis, urethritis, and vaginitis [7]. Colloidal silver, earlier used as an antibacterial therapy, has declined in use as a result of the development of modern

antibiotics, the cost of the colloidal silver production (which has become much lower nowadays) and the possible adverse reaction of skin discoloration. The present compound is of low viscosity colloidal silver based compound which has eliminated the risk of skin discoloration and which is produced at little cost. Helichrysum is distilled oil product, flowering heads of *Helichrysum angustifolium* or *italicum*. Raw honey provides antiseptic qualities and retards the loss of fluids from the trauma site. Water-soluble lecithin is used as an emulsifier. Colloidal silver effectively eliminates bacteria, virus, and fungi precluding mutation and thus resistance to future uses [47].

6. EXPERIMENTAL STUDIES

6.1 Preparation of Colloidal Silver

The silver that is prepared and used in the following experimental studies is low-voltage Electro-colloidal silver. The instrument used to produce colloidal silver is connected to the general AC power supply by an AC-DC adapter that transforms the 220-V AC current to 12-V DC current (See Figure 6.1).



Figure 6.1 Instrument for Producing Electro-colloidal Silver.

The process involves the electrolysis of two plates of 99.999% pure silver in steam distilled water to produce a suspension of clusters of silver ions. Steam distilled water is recommended because a pure silver suspension, with minimum content of other elements or chemicals, is desired. Besides, the presence of an excess of mineral salts can cause production of large silver clusters that will precipitate instead of staying suspended.

Distilled water was heated before usage. Heating increases the rate of reaction. The usage of pure steam distilled water allows the generation of small particles of silver due to its high resistance. This process, that may take 45 to 75 minutes, allows the very finest silver particles to slowly sinter off the larger (gross) piece of silver. At the end of the process the solution will appear almost clear or may slightly gold, depending on the particle size of colloidal silver (10-to 100-Angstrom sized particle gives light gold color). At a volume of 100 ml, the expected silver concentration will be 20 to 26 ppm. After being prepared, the solution should be kept away from light, strong magnetic fields and extreme temperature because these factors cause deionization and hence the precipitation of silver particles. An aluminum sheet was used to cover the bottle where the silver solution was stored.

6.2 Analysis of Colloidal Silver by Atomic Absorption Spectroscopy



Figure 6.2 Perkin-Elmer Flame Atomic Absorption Spectrometer.

6.2.1 Introduction

Our obtained electro-colloidal silver solutions were of 3.32 and 12.72 ppm. These concentrations were determined by Atomic Absorption Spectroscopy (AAS) (see figure 6.2). This technique involves valence electron transition yielding radiation with wavelength in the ultraviolet-visible region of the spectrum [48].

Atomic Spectra

Electron orbits in an atom are characterized by the major (n) and azimuthal (l) quantum numbers. When an electron undergoes a transition from the high energy level (E_{nl}) to a lower energy level ($E_{n_1l_1}$), light of frequency

$$\nu = (E_{nl} - E_{n_1l_1})/h = \Delta E/h \quad (6.1)$$

is given off. In terms of wavelength

$$\lambda = c/\nu = hc/\Delta E. \quad (6.2)$$

The constant h and c in these equations are Planck's constant and the velocity of light, respectively. The parameters ΔE , ν and λ are unique for a given electronic transition. An element can undergo many electronic transitions. This results in a series of sharp lines-a spectrum-which is uniquely characteristic of each element [48].

Emission and absorption can be related as shown in figure 6.3. An electron of an atom in its lowest energy state, the ground state, can absorb a quantum of energy ($+\Delta E$) and undergo transition to a low-lying excited state. Emission occurs when this quantum of energy is released ($-\Delta E$) and the electron returns to the ground state. A transition to and from the ground state, is called a resonance transition. Resonance lines are the most useful analytical lines for atomic absorption spectroscopy [48].

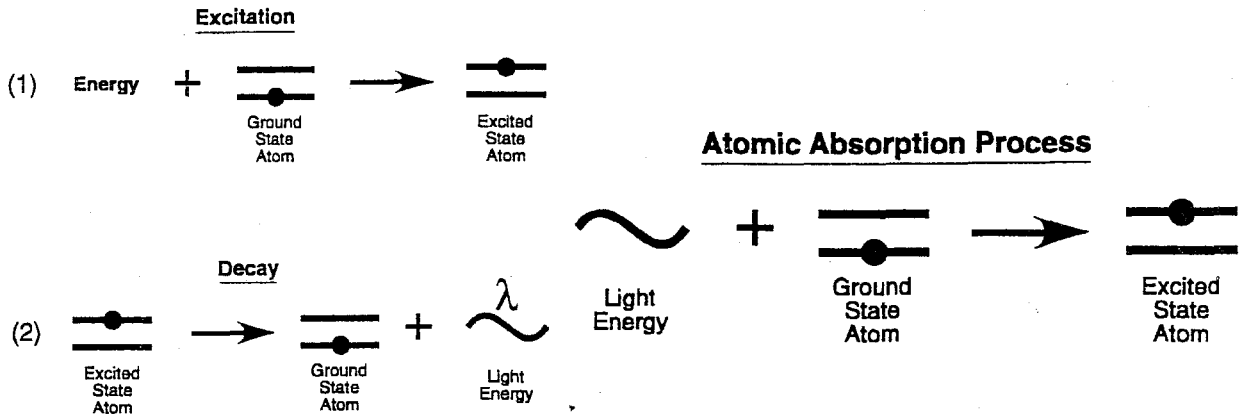


Figure 6.3 Excitation, Decay, and Atomic Absorption Process [48].

Absorption Expression

Absorption of monochromatic radiation is governed by Lambert and Beer's laws. Lambert's law states that light absorbed in a transparent absorption cell is independent of incident light intensity. An equal fraction of the light is absorbed by each successive layer of absorbing medium. Beer's law states that absorption of light is likewise exponentially proportional to the number of absorbing species in the path of the light beam [48].

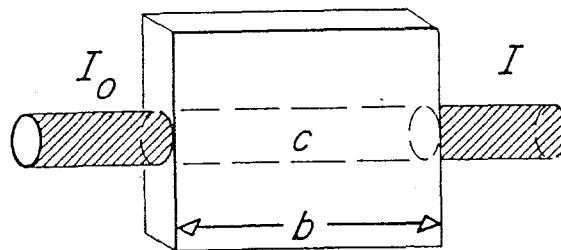


Figure 6.4 Atomic Absorption of Length b [48].

Referring to Figure 6.4, the incident beam of monochromatic radiation I_0 falls on an absorption cell of length b . The transmittance is given by

$$T = e^{-abc} \quad (6.3)$$

Then, since

$$\log_{10}(1/T) = \log_{10}(I_0/I) = abc$$

and

$$\log_{10}(I_0/I) = A,$$

where A is the absorbance, then

$$A = abc, \quad (6.4)$$

where a is a constant for a given system and c is the concentration of the analyte atoms in the flame. This expression, known as the Beer-Lambert law, predicts a linear relationship between absorbance and concentration, as long as a and b remain constant [48].

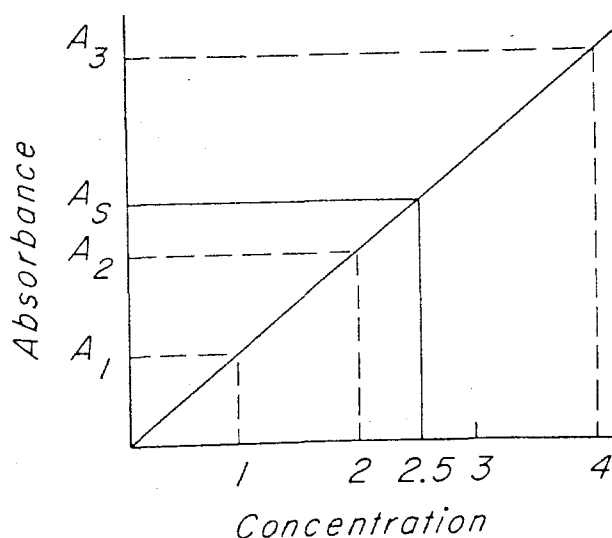


Figure 6.5 Linear Calibration Graph [48].

Figure 6.5 shows a linear calibration graph. Three solutions of known concentration 1, 2, and 4 ppb of an element is nebulized into an atomic absorption flame and the absorbances are found to be A_1 , A_2 , and A_3 respectively. As can be seen, this yields a straight line as predicted by the Beer-Lambert law. A sample to be analyzed is

then nebulized. The absorbance obtained is A_s . By interpolation on the calibration graph for the sample [48].

The concentration working range for an element is determined by atomic absorption spectroscopy is generally 4 to 5 orders of magnitude. At the upper end of the concentration range, it is common to have the graph bend toward the concentration axis (Figure 6.6, Curve B). Less frequently, the calibration curves throughout the whole working range (curve D) [48].

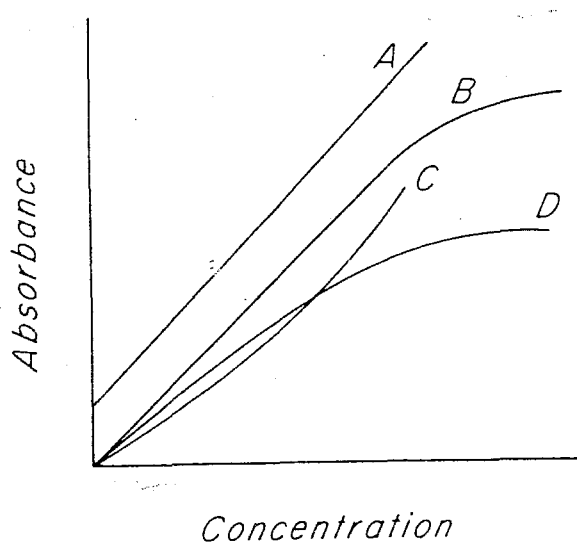


Figure 6.6 Calibration graphs showing departures from ideality [48].

Atomic Absorption Spectrophotometer

The essential components of an atomic absorption spectrometer are diagrammed in Figure 6.7. In most commercial instrumentation, A is a hollow cathode lamp, B a flame or electrothermal device, C a grating monochromator, and D a photomultiplier. The principle of AAS could be explained as follows [48].

The hollow-cathode lamp emits radiation characteristic of the cathode material, usually a single element (analyte). This beam consisting largely of resonance radiation is electronically or mechanically pulsed. Analyte atoms are produced thermally in the atom reservoir. Ground-state atoms, which predominate under the experimental conditions, absorb resonance radiation from the lamp, reducing the intensity of the incident beam. The monochromator isolates the desired resonance line and allows this radiation to fall on the photomultiplier. An electrical signal is generated. The electronics of the unit are designed to respond selectively to the pulsed radiation emanating from the radiation source. Signal processing occurs, which results in electronic output proportional to the absorption by analyte atoms [48].

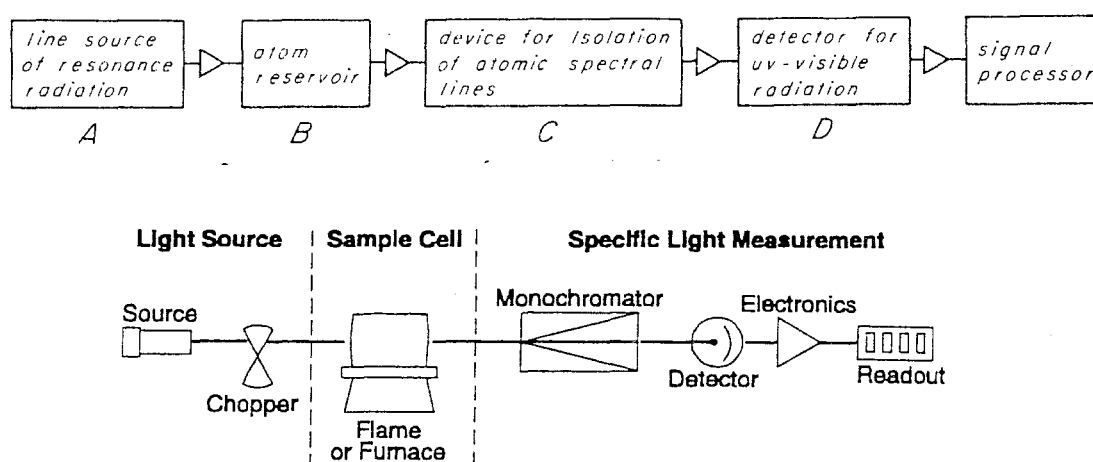


Figure 6.7 Atomic Absorption spectrometry. (a) A block diagram of an atomic absorption spectrometer. (b) A single-beam atomic absorption spectrometer [48].

Absorption spectra are relatively simple, consisting predominantly of resonance transitions. The monochromator need not be capable of especially high resolution, its function being to isolate the resonance line of interest and to diminish the light flux from non-absorbing lines in the source and emission from the flame does not produce noise. The very small fluorescence signal from analyte atoms is emitted isotropically and will be insignificant component of the signal falling on the photo-multiplier [48].

Instrument offerings to date are for single- or two-element analysis. Development of simultaneous multi-element atomic absorption equipment has been hindered by problems with optical alignment of components, need for different operating conditions for many elements, and the relatively short linear range of calibration curves [48].

6.2.2 Calibration Procedure

There are two commonly employed approaches to calibration in atomic absorption spectroscopy. These are the methods of direct comparison and standard addition. These are the methods of direct comparison and standard additions [48].

Direct comparison

This is the simplest approach, and when applicable can be used with many instruments to give a direct readout of the concentration of an element in an unknown sample [48].

A number of standards (usually three to five) in increasing concentrations, as well as a blank, are prepared to cover the concentration range. These solutions are run in absorbance to check for linearity of the calibration curve. If curvature is present, the fact is noted and, with most modern instruments, correction can be made to allow direct readout [48].

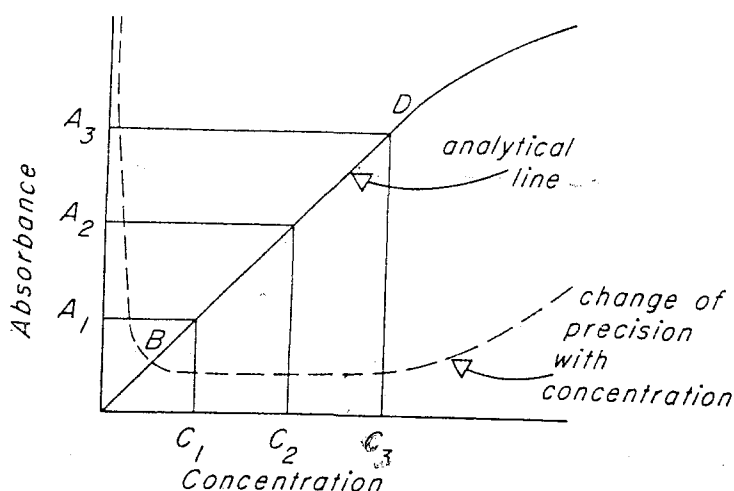


Figure 6.8 Calibration graph for method of direct comparison [48].

When absorbances have been obtained, these values (A_1 , A_2 , A_3 in figure 6.8) can be plotted against concentration (C_1 , C_2 , C_3) for a standard and best fit line drawn. The portion of the analytical line BD gives the best results, as shown by the dashed line. In many cases, the line thus produced will be straight. When curvature occurs, usually at higher concentrations, a French curve or other manual curve-fitting devices are used to draw the line. Much poorer results are obtained in the curved region of the calibration graph, and it is advisable, where possible, not to use the curved portion of the graph. Likewise, at low values of the absorbance, where the change of precision with concentration is high decision and hence poorer results are obtained. If the calibration graphs pass through zero absorbance and concentration, as in fig., then all is well. However, if the line intercepts on the absorbance axis after subtraction of the blank, an error due to non-specific absorption may be present [48].

Most new equipment has the capability for direct readout in concentration. It is very important to check calibration linearity. Any non-linearity is readily detectable and methods for curve correction can be devised. At least three standards should be used. The actual technique employed for direct readout and curve corrections depend on the particular equipment. Instruments with microprocessors can curve-correct readily [48].

It is not enough to read the standards only once during a run. For best accuracy, one of the standards is run at least three times over a set of 25 sample. This will allows a statistical analysis to be done. Means of standard readings are used and linear regression analysis is possible [48].

In both the direct readout and plotting methods, the importance of using blank cannot be over emphasized. In work at low concentration, the level of analyte in reagents is often an important consideration [48].

Output can be fed into a desktop or large-scale computers. This can be done through manual methods or with proper interfacing devices, automatically [48].

When an instrument is provided with a microprocessor, the calibration procedure is simplifies. Experimental parameters are entered by keyboard. Any integration time or scale expansion factors between fixed upper and lower limits can be chosen. Values for the standards are entered and the standards are subsequently nebulized. Linear regressions and fitting-curve programs are carried out automatically. Samples are then nebulized and direct readout of concentration is obtained. Some recent instruments do other statistical calculations on the data as well. Better precision and accuracy are obtained in the curved region compared to the manual methods. However, for better best precision and accuracy, the linear portion should be used [48].

Methods of Additions

When samples contain a low concentration of analyte in large amounts of varyoing matrix constituents, it is often difficult to prepare useful standard solutions. In tyhis case it is possible to add small amounts of conventional standard solutions, in increasing amounbt to aliquots of each sample. Graph such as that shown in Figure 6.9 can the be constructed. The method of stqandard addition is often used in work with electrothwermal atomizer. For this method to work the added analyte must behave

during the atomization process in an identical way to the analyte that was originally present in the sample, particularly using electrothermal techniques. The calibration curve must be linear within the concentration range used. Such condition commonly exists with flame techniques. However, it is well known that working curves for electrothermal methods, particularly in heavy matrix solutions, are often characterized by relatively short linear regions. The standard additions approach in this case can lead to serious errors [48].

The usual method for standard addition involves using three aliquots of the sample. The first aliquot is diluted to volume while the second and third are spiked with increasing amount of standard and then diluted to a same volume. It is best to spike samples such that the second volume has a concentration of approximately twice of the unknown and the third three times the unknown. The absorbances are obtained, A_1 , A_2 , A_3 , respectively, and the graph constructed as shown in figure 6.9. The line is extrapolated to intersect the x-axis. While it may be possible with some equipment to obtain a direct readout in concentration, the procedure is time consuming and subject to limitations of the instrument in generating negative numbers. If no-specific absorption is a problem background correction must be employed [48].

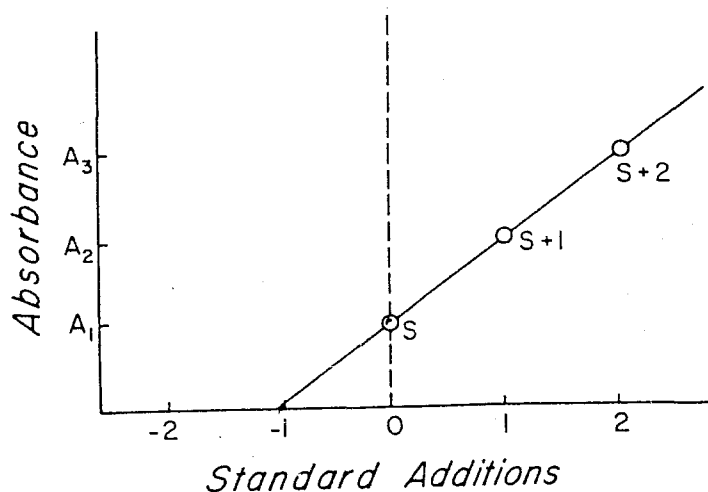


Figure 6.9 Calibration graph for method of standard additions [48].

6.3 Microscopic Observation of Bacteria

6.3.1 Microscopy

The main tool used for microorganism observation is a bright-field light microscope with digital camera attachment (Figure 6.10).



Figure 6.10 Optical, bright field microscope with digital camera attachment

A bright-field light microscope is a one in which the light source shines directly into the objective lens. Consequently, the field of view is generally bright [9, 10].

6.3.1.1 Bacterial Staining

Wet mounts are the most common preparation used to view living organisms. Placing a small drop of an aqueous suspension of the specimen on a clean slide and covering it with a clean cover slip makes wet mounts. Although wet mounts can be made rapidly, they dry up after five or ten minutes and so are not very useful if long-term

observations are required. A ring of Vaseline or petrol jelly around the edge of the cover slip keeps the hanging drop from drying out (see figure 6.11) [9, 10].

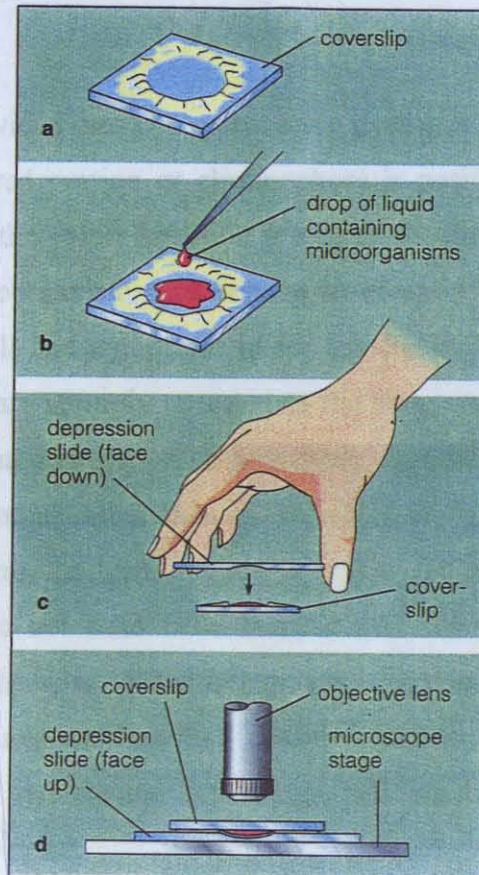


Figure 6.11 Hanging drop wet mount [9].

6.3.2 Bacterial Staining

Many microorganisms are difficult to see with the light microscope because they do not absorb, reflect, refract, or diffract much light. Because of this, stains, alcoholic or aqueous solutions of colored compounds called dyes, are often used to color microorganisms and/or their background. The use of stains often make it possible to see

structural components such as endospores and flagella as well as inclusions such as starch and phosphate granules. Stains that can be used to detect the presence or absence of specialized parts of a cell are known as structural stains. Differential stains, on the other hand, are procedures that enable one to distinguish between cell types. The Gram stain and the acid-fast stain are differential staining techniques that allow bacteria to be divided into major groups [10].

Dyes are often divided into two types: basic dyes and acidic dyes. Basic dyes are those in which the colored portion or chromophore is positively charged while acidic dyes are those in which the colored portion is negatively charged. Basic dyes are used most often because microorganisms tend to have an excess of negatively charged groups associated with their wall and cytoplasm under the conditions used for staining. The positively charged dyes are strongly attracted to the many negatively charged groups in cells, resulting in efficient staining. The negatively charged acidic dyes frequently are used to stain the background rather than the microorganisms themselves. Acidic dyes frequently are used to stain the background rather than the microorganisms themselves. Acidic dyes generally do not stain cells because they are repelled by the many negative charges associated with the cells. Nevertheless, acidic dyes occasionally are used to stain positively charged structures within cells. It is important to recognize that changing the pH of the environment can change the charge and binding properties of dyes and structure [10].

Whenever a staining technique results in the staining of the objects, the technique is described as a positive stain whether or not a basic (positive charge) or acidic (negative charge) has been used. Similarly, whenever the background is stained to create contrast with the unstained object, the stain technique is called a negative stain [10].

6.3.2.1 Positive and Negative Staining

A simple stain is a procedure in which only one stain is used to create a contrast between the specimen and its background. Generally, simple stains involve the use of

basic dyes such as crystal violet, methylene blue, basic carbofuschin (red), safarine (red), or malachite green. Infrequently, simple stain involve the use of acidic dyes such as nigrosine and Congo red [10].

Since simple staining procedure are rapid and easy to carry out, they are often used when information about cell shape, size, and arrangement is described (Figure 6.12). A simple stain helps microscopists determine the dimension and shape of microorganisms [10].

Usually, a simple stain involves the staining of dried preparation of cells on a glass slide (Figure 6.12). The dried preparation of cells is known as a smear. Smears can be prepared from cells in a liquid culture or from growth on an agar plate or slant. When using a liquid suspension, one to several loopfuls is smeared onto a glass slide and then allowed to air dry. The cells in the dry smear are attached or fixed to the slide by briefly heating the slide over a gas burner flame. This procedure is known as heat fixation. When using colonies or growth from a semisolid medium, a loopful of water is placed on the slide and a very small amount of material is mixed with the water to separate and suspend the cells. The suspension is then spread out, air-dried, and heat-fixed. In a good smear, individual organisms are visible microscopically and organisms are not piled on top of each other. In order to insure well-dispersed microorganisms, the drop used to make the smear should be only slightly turbid (cloudy) [10].



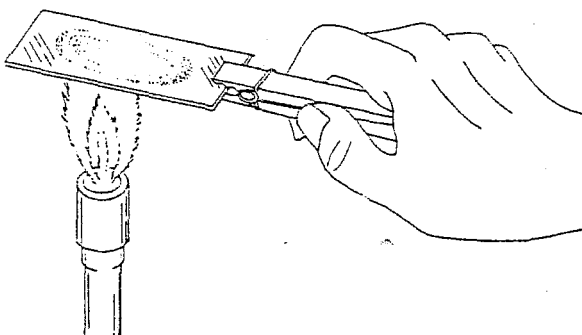
1. Place several large loopfuls of a liquid culture on a clean slide. If the culture is on an agar plate or slant, place several loopfuls of water on the slide and then mix in a very small amount of the solid growth (e.g. part of a colony).



2. Spread the liquid culture or mixture over the slide so as to create a thin film.



3. Air dry the smear. Use a warming plate if possible to speed up the drying, but do not use a gas burner.



4. Heat fix the dried smear by placing the bottom of the slide over the gas burner flame for three seconds. An alternate method is to pass the slide through the flame three times.

Figure 6.12 Procedure for positive staining [10].

6.3.2.2 Differential (Gram) Staining

Differential stains are very useful in microbiology because they can be used to distinguish between groups of bacteria. An important differential stain is the Gram stain. The gram stain is one of the most important steps in the characterization and identification of bacteria [10].

The Gram stain separates bacteria into two groups: the Gram-positive bacteria that retain the color of the first stain used (crystal violet), and the Gram-negative bacteria that assume the color of a second stain (safranin). Bacteria stain differentially because of the differences in the structure and chemical composition of their cell wall. The Gram-positive bacteria have a thick cell wall that consists primarily of peptidoglycan (figure 6.13). Many Gram-positive bacteria, however, have in their cell wall a polymer called teichoic acid in their cell walls which may account for as much as 50% of the wall's weight. The peptidoglycan layer of the cell wall consists of polysaccharides, made up of alternating N-acetylglucosamine and N-acetylmuramic acid units, cross-linked by short peptides, while teichoic acid are long polymers of alternating phosphate and carbohydrates (e.g., ribitol or glycerol). The cell wall of Gram-positive bacteria is generally between 20 and 80 nm [10].

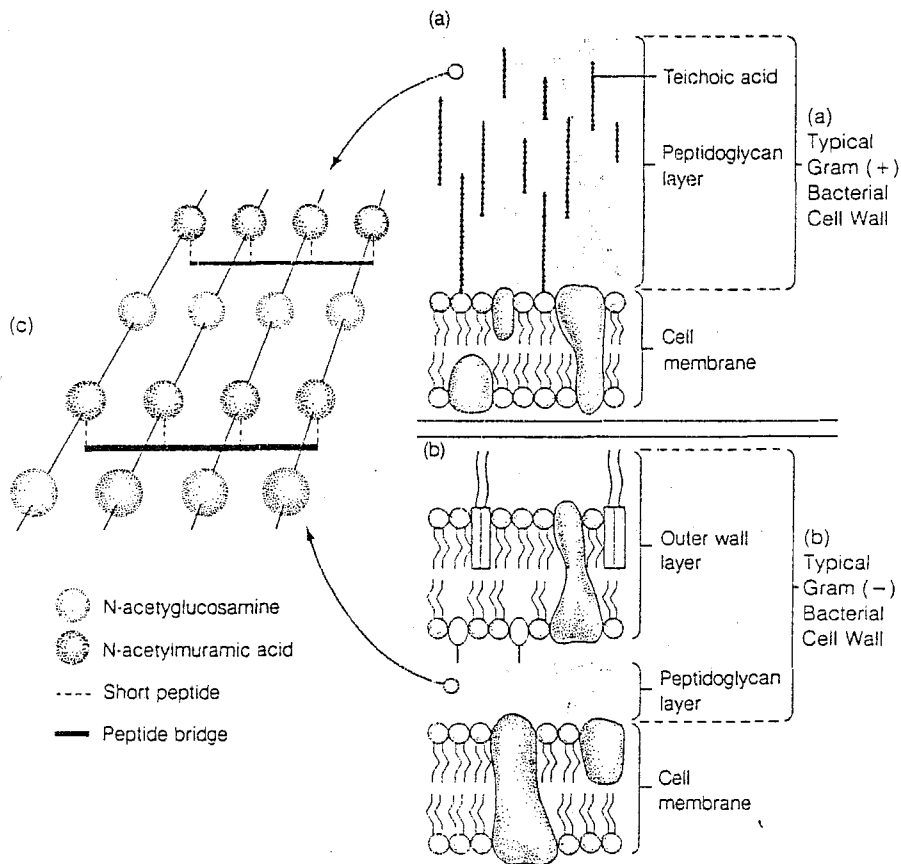


Figure 6.13 Cell wall of (a) Gram-positive and (b) Gram-negative bacteria. (c) Peptidoglycan structure [10].

The walls of Gram-negative have much less peptidoglycan than what those of Gram-positive bacteria have. The peptidoglycan layer, usually about 2 nm thick, is surrounded by a complex lipid bilayer called the outer membrane. Some microbiologists regard this outer membrane as part of cell wall while other scientists consider the outer membrane to be a separated and distinct envelope. No teichoic acids are associated with the cell wall of Gram-negative bacteria. The Gram stain procedure is illustrated in Figure 6.14 [10].

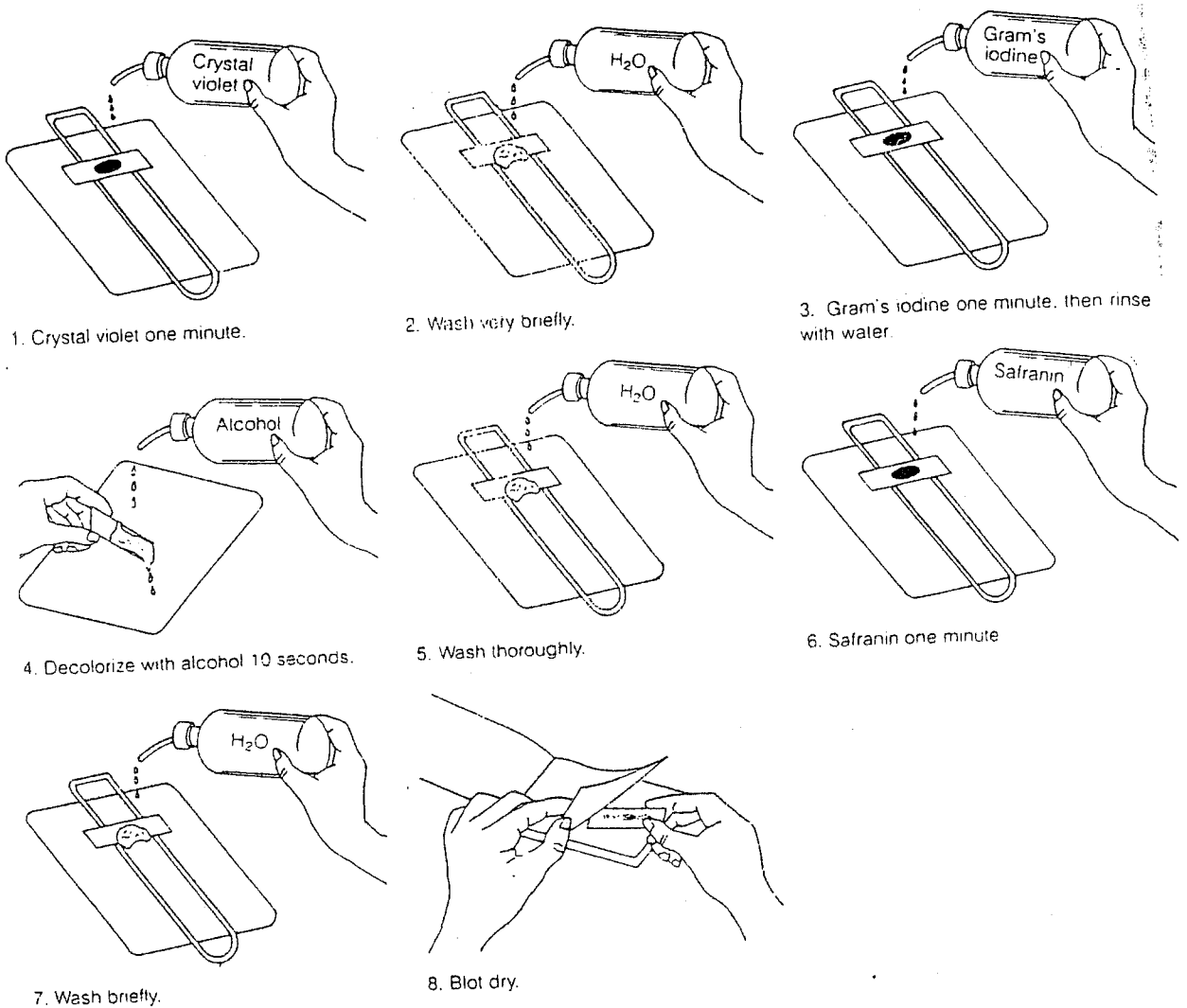


Figure 6.14 Gram Stain Procedure [10].

In order to obtain a reliable Gram stain, it is necessary to use a young culture of organisms, no older than 24 to 48 hours. If older cultures are used, there is the possibility of ambiguous results. As culture age, progressively more cells sustain damage to their cell walls. This damage apparently allows dyes to be more easily leached out of the cell by decolorizing agents. Thus, gram-positive cells with damaged cell walls tend to lose their ability to retain crystal violet-Gram's iodine complex and consequently stain as if they were Gram-negative. Damaged cells in a pure culture of Gram-positive bacteria are sometimes the reason a mixture of blue-violet is seen in Gram stained smear of pure microorganisms [10].

6.4 Culturing Bacteria

6.4.1 Introduction

The cultivation of microorganisms in the laboratory requires that the needed nutrients and suitable environmental conditions be provided. In the laboratory, the nutrients are supplied to microorganisms in culture media. The culture medium may be in a liquid or in a solid gel form. Solid media are usually made by the addition of a polysaccharide called agar that is derived from some red algae [9, 10].

When the culture medium has been prepared, it is not ready for use, since microorganisms commonly associated with the nutrients, water, glassware, media maker, and air are likely to have contaminated it. It is necessary to sterilize the medium before it is used. Laboratory sterilization procedures generally are carried out using autoclaves (figure 6.13). Autoclaves sterilize and are carried out for 15 to 20° C at a pressure of 15 pounds per inch² above normal atmospheric pressure, which allows the steam temperature to reach 121° C. Heat-sensitive solutions are commonly sterilized using special filters that have pores with diameters of 0.45 micrometer. These filters prevent microorganisms from passing into the collecting chamber [10].

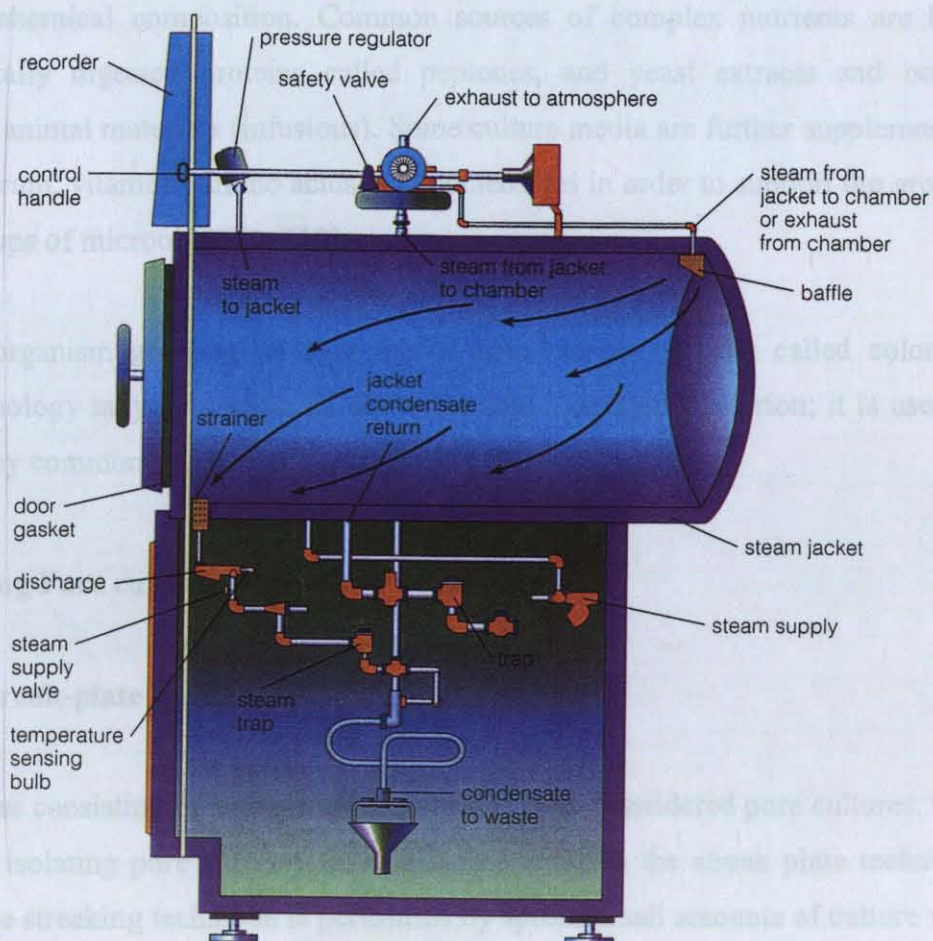


Figure 6.15 Autoclave [9].

Two categories of culture media based upon their formulation are routinely used in the laboratory. These categories are chemically defined and chemically complex (undefined) media. A chemically defined medium is formulated so that the concentration and chemical nature of each added compound is known. The ingredients used to prepare chemically defined media are highly purified (reagent grade) inorganic salts and simple organic compounds. Chemically defined media are often necessary in the study of the

physiology and genetics of microorganisms. However, if a defined medium calls for many ingredients, it can be tedious to prepare. Moreover, reagent grade chemicals are often expensive. Chemically complex culture media are prepared using natural products of unknown chemical composition. Common sources of complex nutrients are beef extracts, partially digested proteins called peptones, and yeast extracts and boiled vegetable and animal materials (infusions). Some culture media are further supplemented with blood, serum, vitamins, amino acids, and nucleosides in order to support the growth of special groups of microorganism [10].

Microorganism growing on agar media from masses of cells called colonies. Colony morphology may be used as an aid to the sole identifying criterion; it is used to recognize many common types of microorganism [10].

6.4.2 Obtaining Pure culture

6.4.2.1 The streak-plate method

Cultures consisting of a single species (or stain) are considered pure cultures. One technique for isolating pure cultures from mixtures involves the streak plate technique (fig. 6.16). The streaking technique is performed by spread small amounts of culture with an inoculating loop over an agar surface. As the microorganisms are spread over the surface, the concentration of microorganisms on the inoculating loop decreases. Eventually, a single microbial cell will be deposited on an area of the agar medium, multiply, and form a colony. This colony, derived from a single cell, can then be picked with a bacteriological loop (or a needle) and a subculture onto a fresh culture medium. In this way, a pure culture of the isolated colony can be obtained and maintained [9].

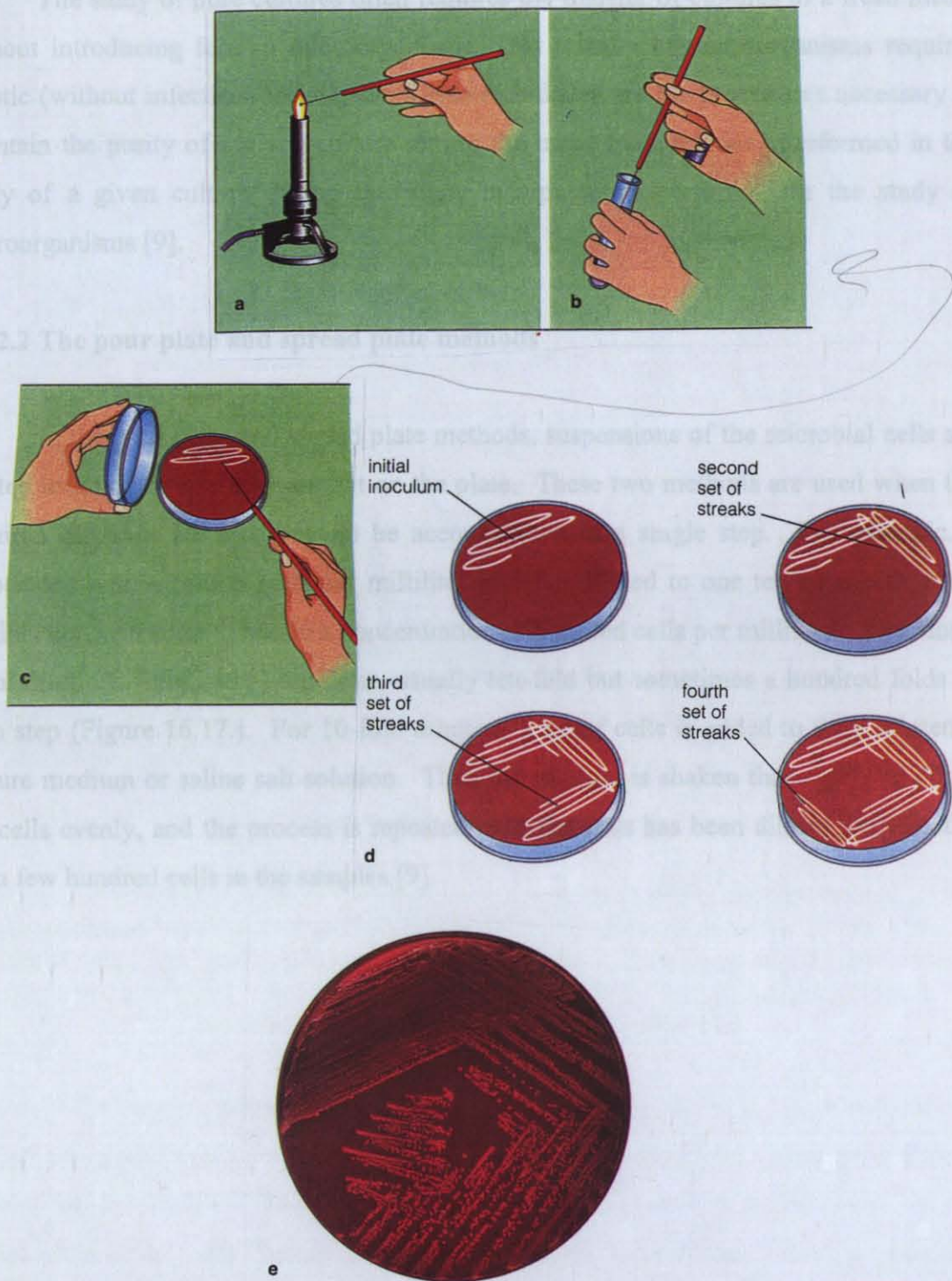


Figure 6.16 Streak plate method. (a) Sterilization of inoculating loop. (b) Inoculation of bacteria from suspension. (c) Streaking bacteria on agar. (d) Multiple streaking. (e) After incubation [10].

The study of pure cultures often requires the transfer of cultures to a fresh media without introducing foreign microorganisms. This transfer of microorganisms requires aseptic (without infection) techniques. These techniques are the procedures necessary to maintain the purity of a given culture during the many manipulations performed in the study of a given culture during the many manipulations performed in the study of microorganisms [9].

6.4.2.2 The pour plate and spread plate methods

In the pour plate and spread plate methods, suspensions of the microbial cells are diluted in liquid before they are put on the plate. These two methods are used when the required dilutions are too great to be accomplished in a single step. For example, a suspension with a billion cells per milliliter must be diluted to one ten-millionth of its original concentration to obtain a concentration of hundred cells per milliliter. Therefore, serial dilutions (multi-step) are done, usually ten-fold but sometimes a hundred folds in each step (Figure 16.17.). For 10-fold dilution, 1 ml of cells is added to 9 ml of sterile culture medium or saline salt solution. Then the mixture is shaken thoroughly to dilute the cells evenly, and the process is repeated until the cells has been diluted sufficiently, i.e. a few hundred cells in the samples [9].

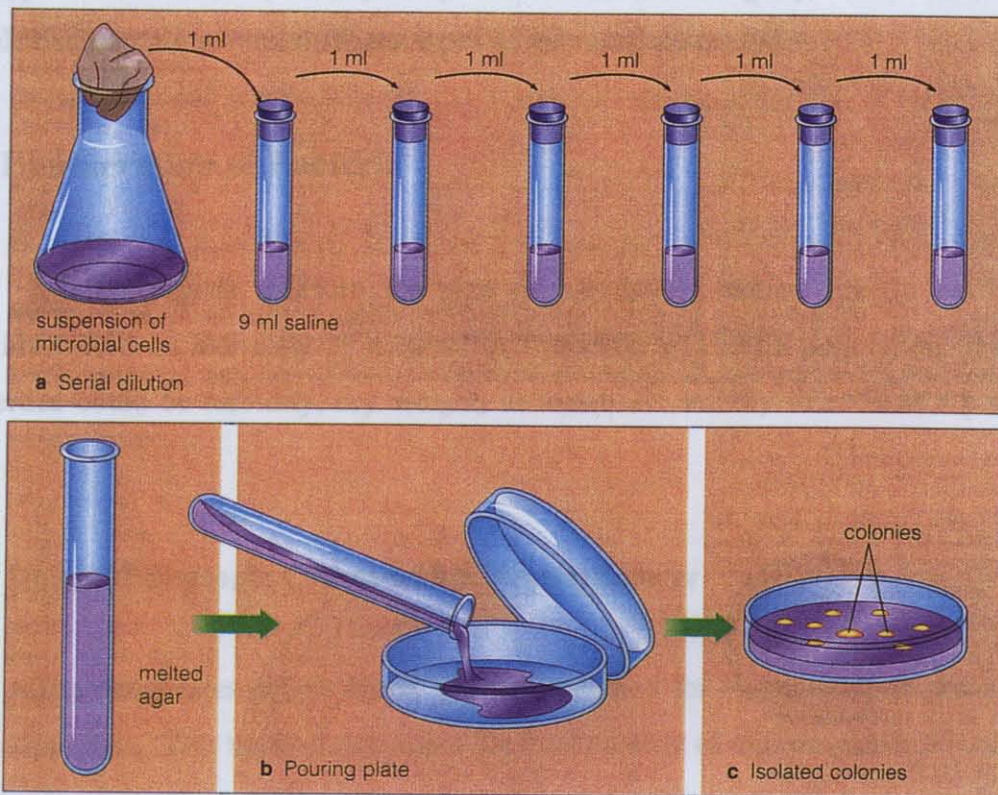


Figure 6.17 Pour plate method. To obtain a pure culture by the pour plate method, (a) a suspension of bacterial cells is serially diluted to get a sample containing only a few hundred cells. (b) The sample is poured into a tube of melted agar, mixed, and poured into a petri dish. (c) After incubation, isolated colonies develop in the agar (small colonies) and on its surface (large colonies) [9].

In the pour plate method, the diluted sample is added to melted agar, mixed, and poured into a petri dish. Some colonies that develop are embedded on the agar and some develop on the surface. The surface colonies spread and appear to be larger. In the spread plate method, the diluted sample is poured onto the surface of an agar plate and spread evenly with a sterile glass rod. The liquid and dissolved materials are absorbed into the agar, leaving microbial cells in the surface. In either method the plates are incubated until individual colonies appear. As with the streak plate method, there is no assurance that the colonies that develop on spread plates or within the solidified agar of poured plates are pure cultures until the process is repeated [9].

The pour plate and spread plate methods produce more isolated colonies than the streak plate method does, so they are preferred when isolating a particular strain from a sample that contain several different types of microorganisms [9].

6.5. Enumeration of Bacteria

Microbiologists measure the size of populations either directly by counting individual cells or indirectly by measuring a property of certain population. Measured properties could be turbidity, dry weight, or metabolic activity of cells in a population [9].

6.5.1 Direct Microscopic Counts with a Hemocytometer

The direct microscopic count is a rapid method for enumerating microorganisms in a suspension. This method determine the total number of microorganisms (both living and dead) of microorganisms in a suspension by counting a number of organisms in a known volume of fluid contained inside a counting chamber such as the hemocytometer or the Petroff-Hauser chamber [9, 10].

The hemocytometer (Fig. 6.18) is a slide with a counting chamber 0.1-mm deep. On the bottom of the counting chamber there is an etched square divided into 9 square each 1 mm^2 . The central square is divide into 25 smaller square and each of these in turns is divided into 16 square (Figure 14.b). Therefore, within the central square millimeter there are 400 small squares ($25 \times 16 = 400$). The method for calculating the number of cells/ml of suspension is as follows:

The number of cells in five of the $(1/25)\text{-mm}^2$ squares (the central square and the 4 corner squares) in the central area of the hemocytometer is counted. The number of cells counted in the 5 square is multiplied by 5 to estimate the number of cells/ mm^2 of the surface. If the cells were diluted, multiply by the inverse of the dilution. The number of

cells/mm² is multiplied by 10 to obtain the number of cells /mm³. The number of cells/mm³ is multiplied by 1,000 to obtain the number of cells/ml in the suspension. For diluted cultures the estimate number of cells inn the central area (25 of the (1/25)mm² squares) is multiplied by 10,000 (this number is derived from the logic described above) and express the results in cells/ml of suspension [10].

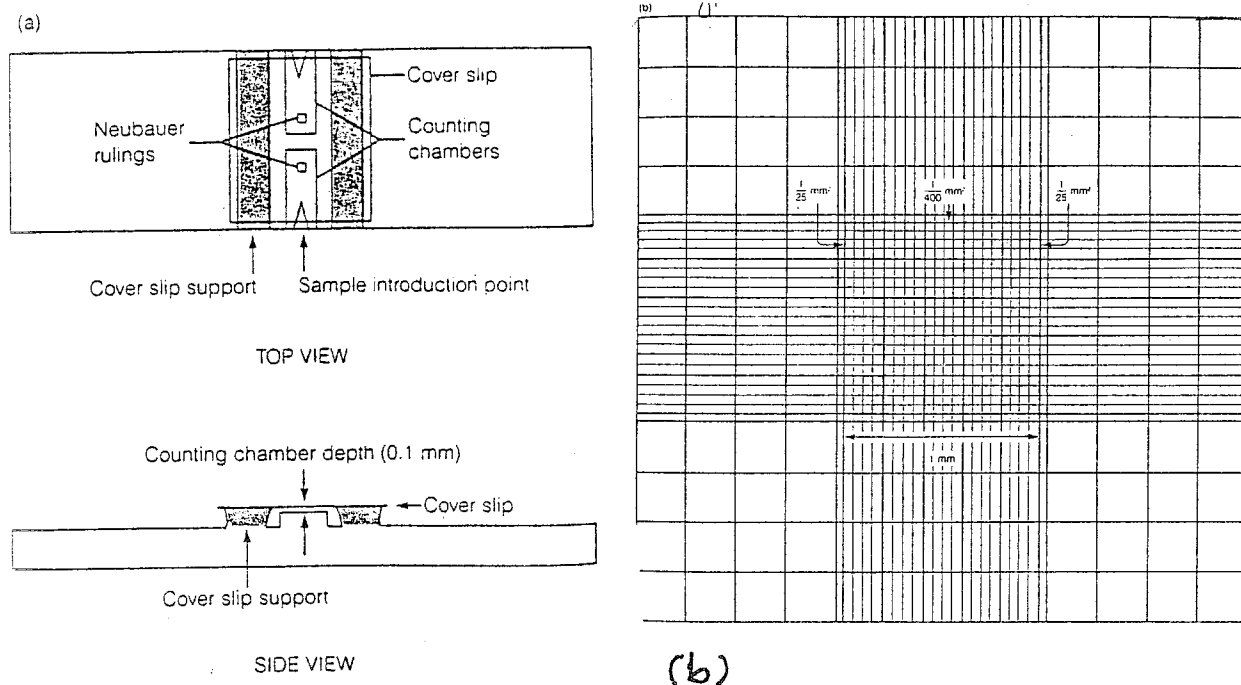


Figure 6.18 The Hemocytometer and Its Use. (a) Top and side views of a cytometer illustrating the construction of a hemocytometer. (b) Enlarged view of Neubauer ruling of the hemocytometer. The central area, which is a large square measuring 1 mm² and delimited by double lines, consists of 25 squares, each measuring 1/25 mm². Each of these squares, in turn, are subdivided into 16 squares, each measuring 1/400 mm² [10].

Microscopic counting is necessary to discriminate bacteria if the sample contain foreign particles, such as blood cells or soil particles [9].

Direct count can also be done electronically by using an instrument called Coulter counter. Electronic counting is possible because microbial cells do not conduct

electricity as well as the medium in which they are suspended. A measured volume of the culture to be counted is placed in one chamber of the counter and passed through a small pore (about 15 μm in diameter) into another chamber by a vacuum pump. The pore is part of an electric circuit, so each time a cell pass through, the conductivity of the circuit decrease and the presence of the cell is tallied electronically on the counter [9].

6.5.2 Plate or Viable Count

The enumeration of microorganisms by viable count is based on the assumption that each viable microorganism in a suspension will rise to a single colony after incubation in suitable media under favorable conditions. After incubation, the number of colony formed is counted to arrive to an estimation of the number of microorganism in the original suspension [10].

The viable count is considered to be a minimum count because the number of colonies on the plate represents only those microorganisms that can multiply under the condition that have been established. For example, a soil sample can contain both aerobic and anaerobic bacteria, but if the plates are incubated in the presence of air, then only aerobic and facultative anaerobic will grow. Similarly, if a sample is plated on a culture system devoid of growth factors like biotin, only microorganisms that do not require biotin in their growth will form colonies. In fact, the cultural and/or nutritional conditions can be manipulated so that only certain types of microorganisms can grow. This method sometimes is used to determine the presence of pathogens or spoilage microorganisms in water, foods, and dairy products [10].

Not all cells give rise to a colony because certain microorganisms have a tendency to clump or aggregate. When plated onto suitable culture media, a clump will give rise to only one colony, regardless of how many cells exist in the clump. For this reason, microbiologists express the results obtained by viable plate counts as colony forming units (CFU)/ml rather than cells/ml. Ideally, only plates containing 30 to 300 colonies are counted because this will enhance the accuracy of the count. If the sample contains

too many microorganisms (many more than 300 per plate) the microorganisms will be too crowded and will cover the entire plate. For this reason, samples are sometimes diluted. However, if the sample is too dilute, the results obtained are statistically invalid [10].

Viable count has numerical applications in microbiology. When samples are tested for disease causing or spoilage microorganisms (not dead ones) can cause infectious diseases or spoil foodstuffs [10].

The technique for performing a plate count is the same as the spread and the pour plate methods of culturing microorganisms described above. A sample of the culture is serially diluted, usually tenfold at each dilution, and then a small amount of each dilution is spread on a plate or mixed with melted medium and incubated under appropriate growth conditions. After visible colonies have formed, usually a day or so later, plates with clearly separated colonies are selected and counted. Choosing plates with between 30 and 300 colonies offers a good compromise between speed and accuracy. The number of colonies on a plate, along with dilution of the sample that was spread on that plate, allows calculating the concentration of cells that are present in the original sample (Figure 16.19). Accuracy increases with the increase of the number of colonies counted because sampling error is decreased. Sampling error is inevitable inaccuracy that results, no matter how carefully dilutions and plating are done, because all samples are not completely representative of the total population [9].

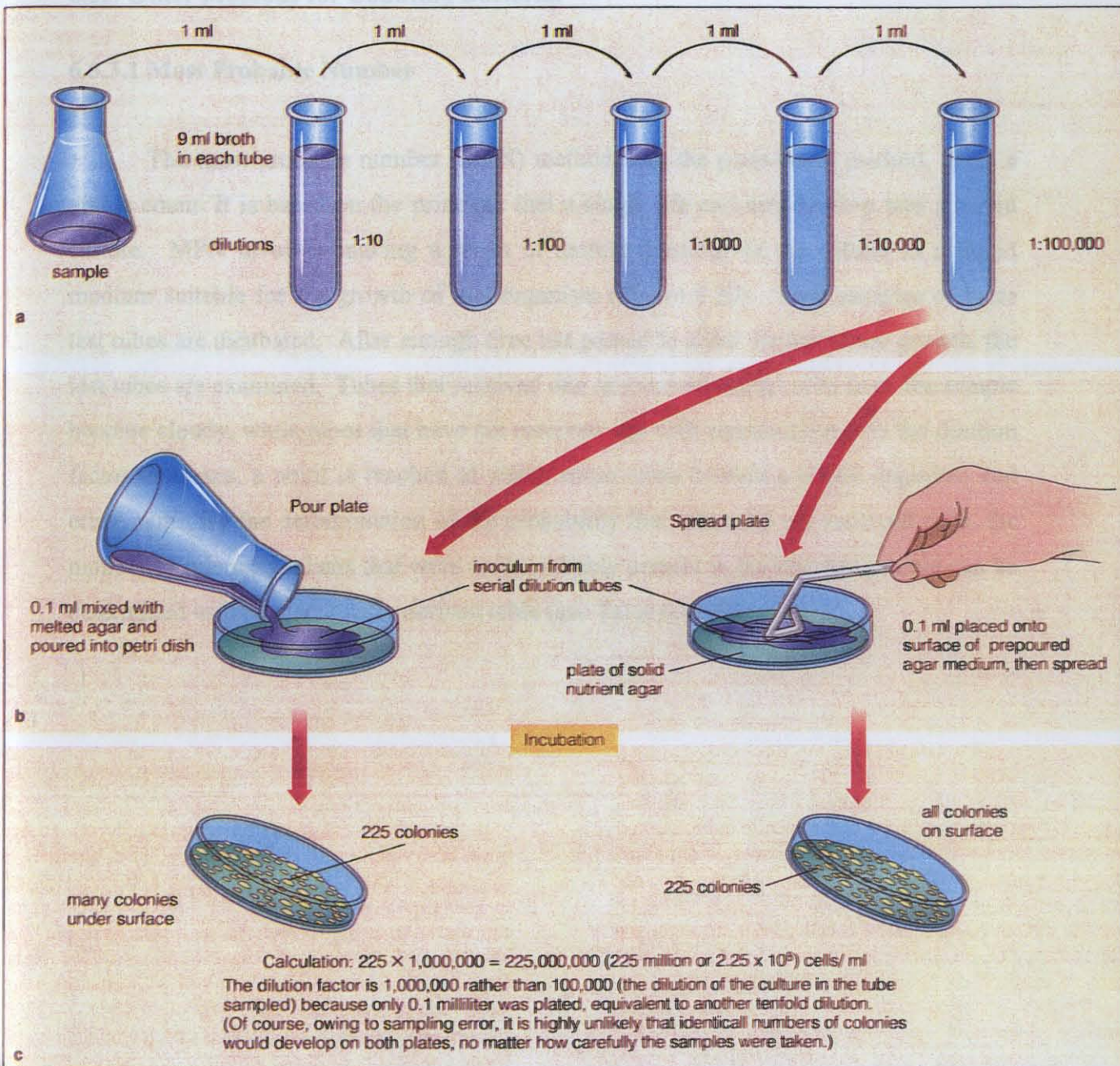


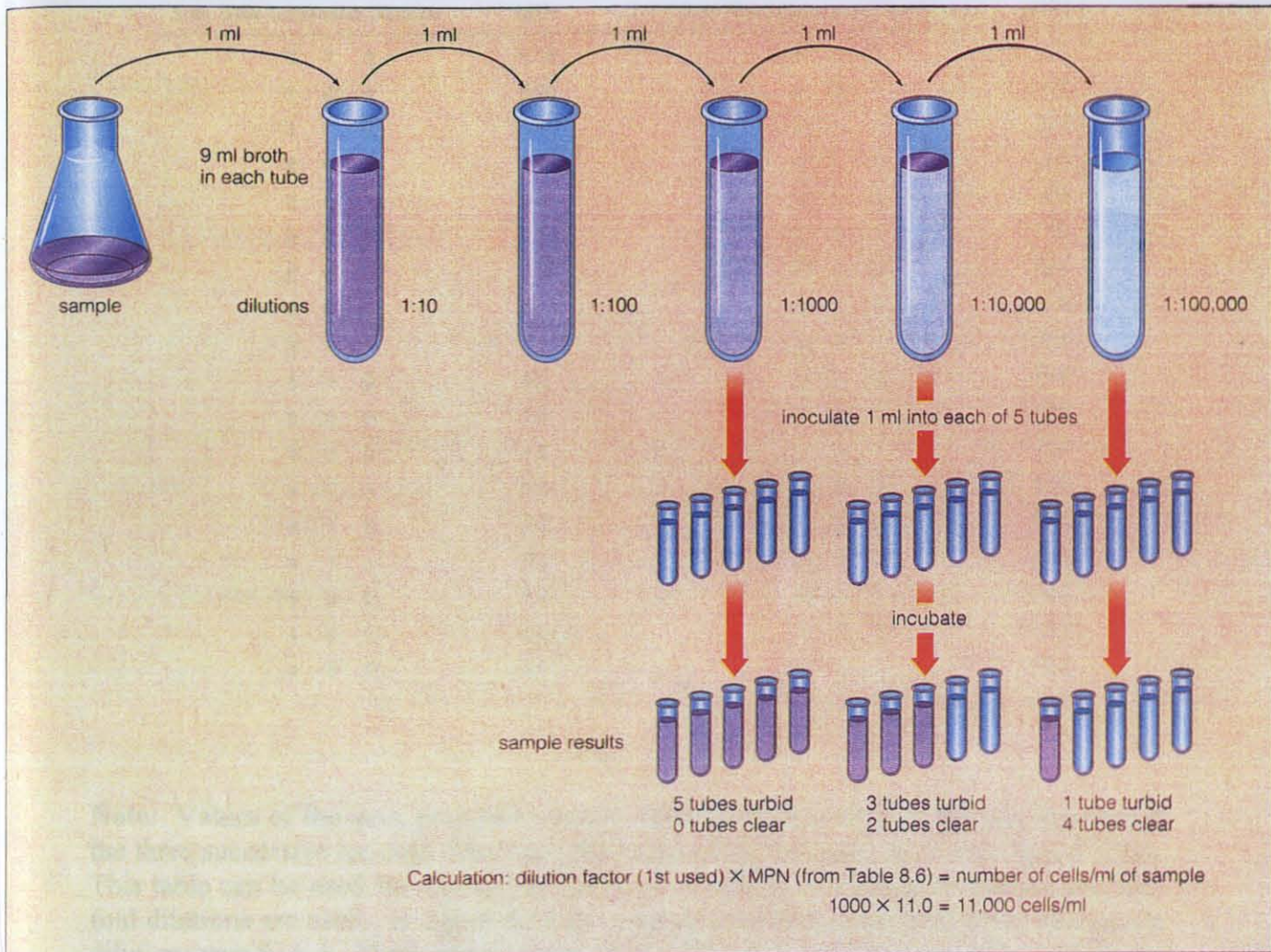
Figure 16.19 A plate count is a direct measurement and gives a viable count. First the sample is serially diluted by transferring 1 ml of the sample to 9 ml of sterile medium and mixing thoroughly. This process is repeated until an appropriate dilution is obtained (in this example 1:100,000). The 0.1 ml is added to a plate of nutrient agar either by spreading it on the surface (spread plate method) or by pouring it with the medium. The plates are incubated. The colonies that develop are counted. For statistical reasons, plates should contain between 30 and 300 colonies. In this example, the plates has 225 colonies [1].

6.5.3 Other Methods for Counting Bacterial

6.5.3.1 Most Probable Number

The most probable number (MPN) method, like the plate-count method, gives a viable count. It is based on the principle that a single life cell can develop into a turbid culture. MPN involves making a series of tenfold dilutions of the culture in a liquid medium suitable for the growth of that organism (Figure 6.20). Then samples of these test tubes are incubated. After enough time has passed to allow for microbial growth, the test tubes are examined. Tubes that received one or more microbial cells from the sample become cloudy, while tubes that have not received any cells remain clear. As the dilution factor increases, a point is reached at which some tubes contain a single organism and other, non. By the determination of the probability that tubes did not received cells, the number of microorganisms that were most probably present in the original sample can be determined using a statistically derived table (see Table 6.1) [9].

Table 6.1 Most Probable Number Table



sample (1 ml) from the previous dilution is added to 9 ml of broth in each tube.

6.5.3.2 Filtration

Figure 6.20 The most probable number (MPN) gives a viable count. As the dilution factor increases, a point is reached at which some tubes contain only a single organism and the others, none. From the pattern of the number of turbid tubes in three successive dilutions (here 5, 3, 1), the statistically most probable number of cells (11.0) in the first of the three dilutions can be determined by referring to a table of most probable numbers (Table 6.1). This value multiplied by the dilution factor gives the number of viable cells in the sample [9].

Table 6.1 Most Probable Number Table [9].

Table 8.6 Most Probable Number Table

Numbers of Turbid Tubes Inoculated from Three Successive Dilutions			MPN	Numbers of Turbid Tubes Inoculated from Three Successive Dilutions			MPN
0	1	0	0.18	5	0	0	2.3
1	0	0	0.20	5	0	1	3.1
1	1	0	0.40	5	1	0	3.3
2	0	0	0.45	5	1	1	4.6
2	0	1	0.68	5	2	0	4.9
2	1	0	0.68	5	2	1	7.0
2	2	0	0.93	5	2	2	9.5
3	0	0	0.78	5	3	0	7.9
3	0	1	1.1	5	3	1	11.0
3	1	0	1.1	5	3	2	14.0
3	2	0	1.4	5	4	0	13.0
4	0	0	1.3	5	4	1	17.0
4	0	1	1.7	5	4	2	22.0
4	1	0	1.7	5	4	3	28.0
4	1	1	2.1	5	5	0	24.0
4	2	0	2.2	5	5	1	35.0
4	2	1	2.6	5	5	2	54.0
4	3	0	2.7	5	5	3	92.0
				5	5	4	160.0

Note: Values of the most probable number (MPN) of viable cells in the first dilution of the three successive ten-fold dilutions used to inoculate five tubes (see also Figure 6.20). This table can be used for any unicellular microorganism as long as five tubes and ten-fold dilutions are used. In figure 6.20 the number of turbid tubes from three successive dilutions was 5, 3, 1. From this table we see that the most probable of viable cells in the sample (1 ml) from the first dilution (1:1000) added to each tube is 11.0. Therefore, the number of viable cells in the original sample is 11,000 per ml ($1000 \times 11.0 = 11,000$) [9].

6.5.3.2 Filtration

Filtration is done prior to measuring small numbers of microorganisms in large volume of liquid or gas. A known volume of liquid or gas is drawn through a membrane filter by vacuum. The pores in the filter are too small for microbial cells to pass through. Then the filter is placed on an appropriate solid medium and incubated. The number of

colonies that develop is the number of viable microbial cells in the volume of fluid that was filtered. Results are usually calculated as organisms per liter (See Figure 4.2) [9].

6.5.3.3 Dry Weight

Determining dry weight depends upon separating cells from their medium, drying them, and weighing them [9].

6.5.3.5 Metabolic Activity

There are several ways metabolic activity can be used to measure numbers: the rate of formation of metabolic products, such as gases or acids, that a culture produces; rate of utilization of a substrate, such as oxygen or glucose; rate of reduction of certain dyes [9].

6.5.3.6 Turbidity

Turbidity is measured with a spectrophotometer. The spectrophotometer measured how much light a liquid culture of microbial cells transmits. A standard curve is used to relate spectrophotometer readings to cell mass or numbers in a particular culture [9].

6.6 Laboratory Work Involving Culturing and Counting Bacteria

6.6.1 Materials and Methods

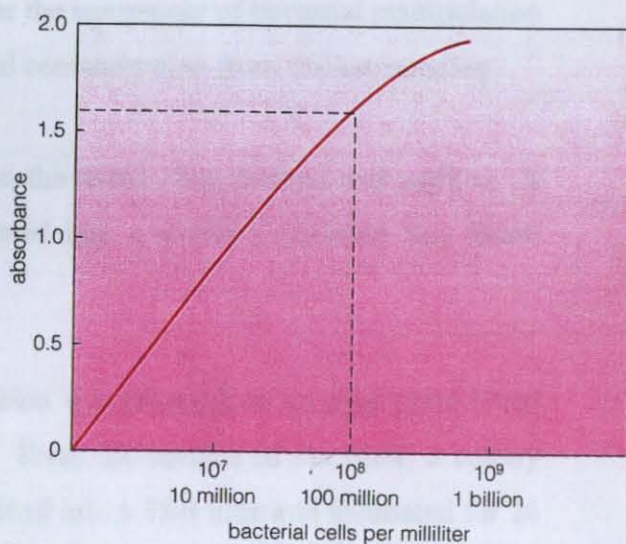
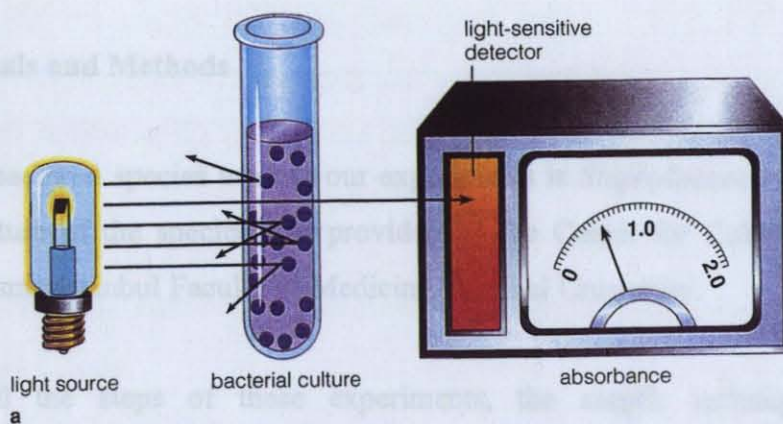


Figure 6.21 The spectrophotometer. (a) A spectrophotometer measures turbidity, the amount of light that a culture transmits in units of absorbance. The bacterial cells scatter light so it does not strike the light sensitive detector. (b) The tube on the left has no bacterial cells in it and is clear. The one on the right contains about a billion cells per ml and therefore appears turbid. (c) A standard curve relates absorbance to the number of particular kind of bacterial cells per ml. Once prepared, the curve can be used to convert any absorbance reading to cell number. For example, from this curve a reading of 1.6 absorbance units means the culture contains a hundred million cells per ml [9].

6.6 Laboratory Work Involving Culturing and Counting Bacteria

6.6.1 Materials and Methods

The bacterial species used in our experiments is *Staphylococcus epidermidis*. A bacterial culture of the species was provided by the Center for Culturing and Storing Microorganisms-Istanbul Faculty of Medicine, Istanbul University.

In all the steps of these experiments, the aseptic techniques like using disinfectants (70% ethyl alcohol) and detergents to clean the stuff (tubes, flasks, etc.) and the area of work. Also, UV light was turned on overnight to sterilize the experimental chamber. Further, a Bunsen flame was used near the equipment of bacterial manipulation and Petri plate preparation to minimize microbial contamination from the surrounding.

In order to obtain a pure bacterial culture, the streak plate method was applied. A loopful from the provided culture was inoculated into a sterile Trypticase Soy Broth (TSB) and incubated for 24 hours.

Then, a 0.1-ml aliquot from the suspension was pipetted on an agar plate (Petri dish) and spread, then incubated for 24 hours. From the surface of the plate, a colony (lenticular in form) was picked and again inoculated into a TSB tube and incubated for 24 hours. Afterwards the spread plate method was applied. The number of dilutions necessary to obtain a countable number of colonies on the Petri plate was estimated to be three hundred-fold dilutions, in each dilution a 0.1-ml of the initial suspension is diluted by TSB to obtain a 10-ml new suspension. Then, a 0.1-ml aliquot from the last suspension was pipetted onto a Petri dish and incubated for 24 hours. The above procedure was repeated three times.

6.6.2 Result

The average of the counted numbers of bacterial colonies in each plate was 155 colonies. The number of CFU/ml in the original suspension is $155 \times 100 \times 10^6 = 1.55 \times 10^{10}$ CFU/ml.

6.7 The Effects of Time and Concentration on the Antibacterial Efficacy of Colloidal Silver

6.7.1 Materials and Method

The bacterium used was *Staphylococcus epidermidis* ATCC 2311 in a 24-hour incubated suspension in Trypticase Soy Broth (TSB). Two solutions of colloidal silver (CS) of approximately 30 and 20 ppm CS (measured using a spectrophotometer of the TSE). Two 10-ml tubes the above solutions and seven tubes, each a range of dilutions were used to test for antimicrobial activity of CS. The diluted solutions were of 10, 7.5, 5, 2.5, 1, 0.5 and 0.1 ppm CS. The diluent was TSB. Each tube containing intact or diluted silver solution and additional control tube containing only TSB was inoculated by 0.1 ml from a two 100-fold diluted suspension of bacteria. An aliquot of 0.1 ml from each tube was pipetted onto a petri plate containing mannitol salt agar. This kind of agar does not allow the growth of any bacteria except Staphylococci, and hence contamination was reduced to the maximum. With the exception of the blank (control) tube, this procedure was repeated for each tube after 5, 10, 20, 40, 60, 80, and 120 minutes. The whole precedent procedure was repeated 10 times for statistical purposes.

6.7.2 Results

The results of the above experiment (the number of colonies in each tube) are presented in the following tables and graphs.

Table 6.2 Number of colonies resulting from Tube 1 (30 ppm).

Trial	1	2	3	4	5	6	7	8	9	10	Avg*	Std Dev**
0	111	106	98	104	102	103	100	108	113	94	104	5.8
5	54	42	47	44	49	38	42	45	41	51	45	4.9
10	7	4	11	8	7	10	6	90	5	8	8	2.2
20	0	0	1	0	0	5	3	0	0	0	1	1.8
40	0	0	2	0	4	0	0	1	0	0	1	1.3
60	0	0	1	1	0	0	3	0	1	0	0	1.0
80	0	0	0	0	1	0	0	0	2	0	0	0.7
120	0	0	2	0	0	0	0	0	0	2	0	0.8

*Avg= Average, **Std Dev= Standard Deviation, +mn= minute

The above table (of Tube 1) is represented graphically in Figure 6.22.

Colonies vs Time (1)

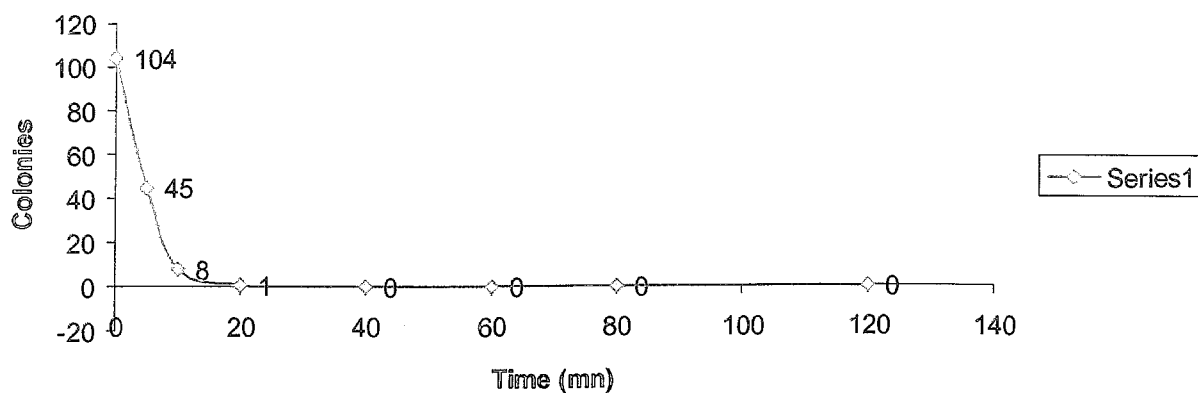


Figure 6.22 Average number of colonies versus time (Tube 1).

Table 6.3 Numbers of colonies resulting from Tube 2 (20 ppm).

Trial	1	2	3	4	5	6	7	8	9	100	Avg	Std Dev
Time (mn)												
0	103	109	112	105	97	107	101	104	96	106	104	4.9
5	63	57	60	64	61	59	69	62	65	67	63	3.7
10	24	28	21	19	25	31	23	27	25	30	25	3.8
20	8	5	9	7	3	7	5	6	2	4	6	2.2
40	0	0	0	3	0	4	2	0	0	0	1	1.5
60	0	0	0	0	1	0	0	0	2	0	0	0.7
80	0	5	0	0	0	3	0	0	0	0	1	1.8
120	0	0	0	0	0	0	3	0	0	0	0	1.0

The above table (Tube 2) is represented graphically in figure 6.23.

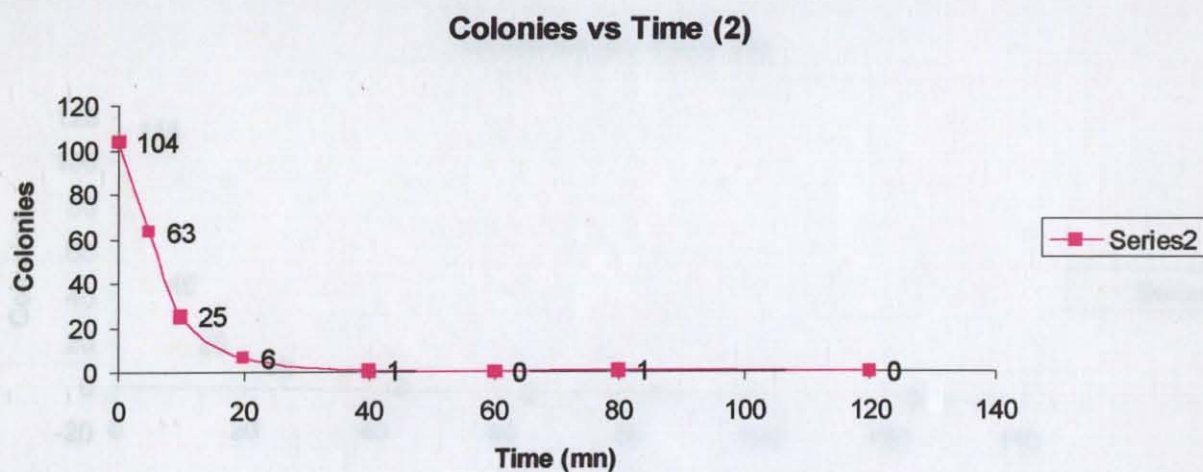


Figure 6.23 Average number of colonies versus time (Tube 2).

Table 6.4 Number of colonies resulting from Tube 3 (10 ppm).

Trial	1	2	3	4	5	6	7	8	9	10	Avg	Std Dev
0	113	107	107	111	118	115	119	114	118	105	114	5.0
5	52	47	43	46	47	54	41	51	53	39	46	5.2
10	16	21	14	20	25	23	19	26	17	21	20	3.8
20	12	7	15	10	13	5	6	11	7	9	10	3.3
40	0	0	3	0	7	2	0	0	1	2	2	2.2
60	0	0	0	0	0	1	0	0	0	0	0	0.3
80	0	0	0	4	0	2	0	0	0	0	1	1.4
120	0	0	0	0	0	2	0	0	0	0	0	0.6

Table 6.4 is represented graphically in figure 6.24.

Colonies vs Time (3)

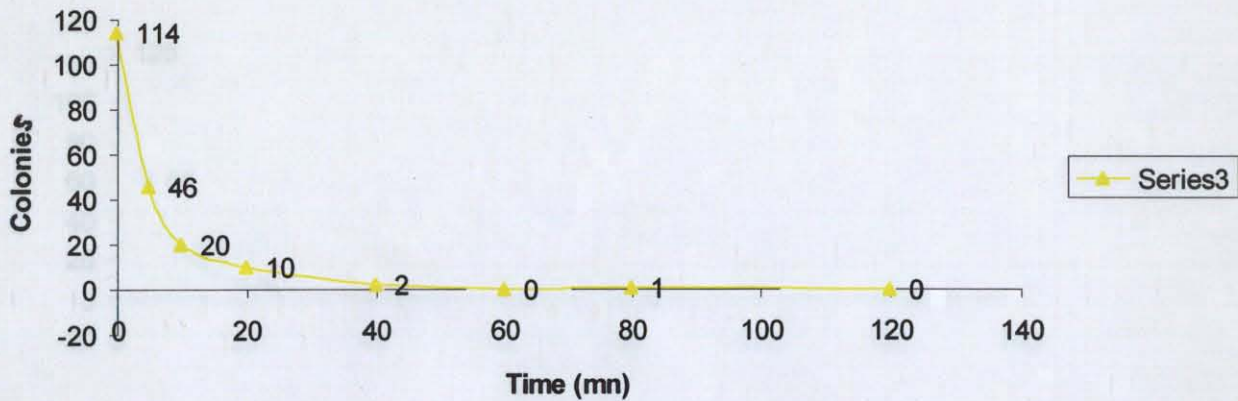


Figure 6.24 Average number of colonies versus time (Tube 3).

Table 6.5 Number of colonies resulting from Tube 4 (7.5 ppm).

Trial	1	2	3	4	5	6	7	8	9	10	Avg	Std Dev
0	122	127	118	131	125	129	117	132	126	121	125	5.2
5	67	54	58	63	63	55	62	69	55	57	60	5.3
10	26	18	23	22	23	31	24	29	30	21	25	3.7
20	7	5	13	10	15	4	8	10	6	6	8	3.6
40	0	0	0	0	2	0	0	1	0	4	1	1.3
60	0	0	1	0	0	0	0	0	3	0	0	1.0
80	0	0	0	0	0	1	0	0	0	0	0	0.3
120	0	0	0	0	0	0	0	0	0	0	0	0.0

Table 6.5 is represented graphically in Figure 6.25.

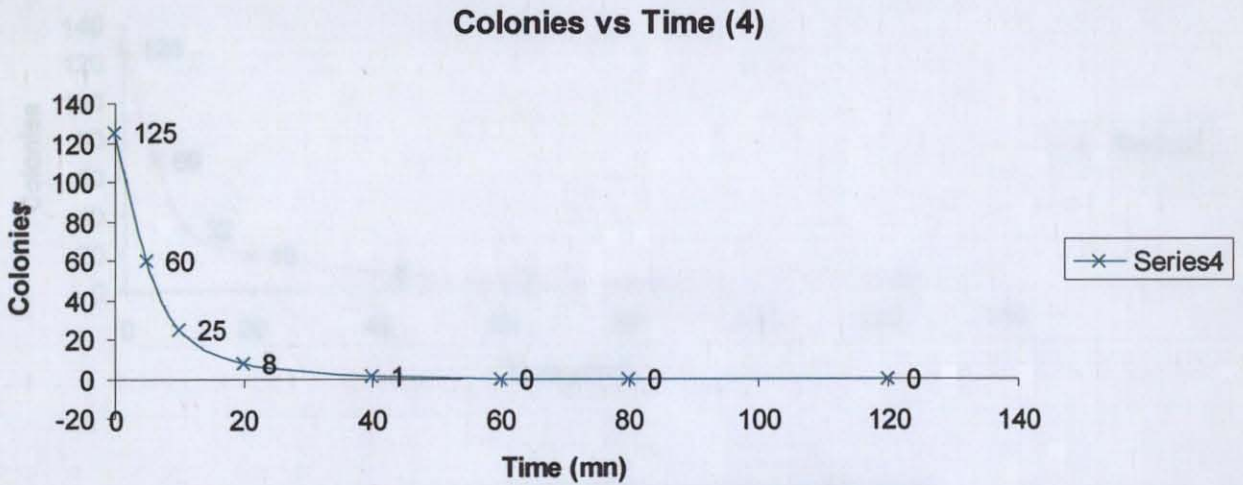


Figure 6.25 Average number of colonies versus time (Tube 4).

Table 6.6 Number of colonies from Tube 5 (5 ppm).

Trial	1	2	3	4	5	6	7	8	9	10	Avg	Std Dev
Time (mn)												
0	134	129	127	123	130	126	131	135	124	121	128	4.6
5	72	73	65	77	63	64	69	76	61	66	69	5.6
10	33	34	26	29	38	37	32	25	36	35	32	4.5
20	18	12	16	16	19	23	20	14	17	21	18	3.3
40	7	13	6	10	4	12	11	9	7	5	8	3.1
60	3	3	0	5	2	6	4	0	0	2	2	1.8
80	0	0	0	4	0	0	2	0	0	0	1	1.4
120	0	0	0	0	0	0	0	0	0	0	0	0.0

Table 6.6 is represented graphically in Figure 6.26.

Colonies vs Time (5)

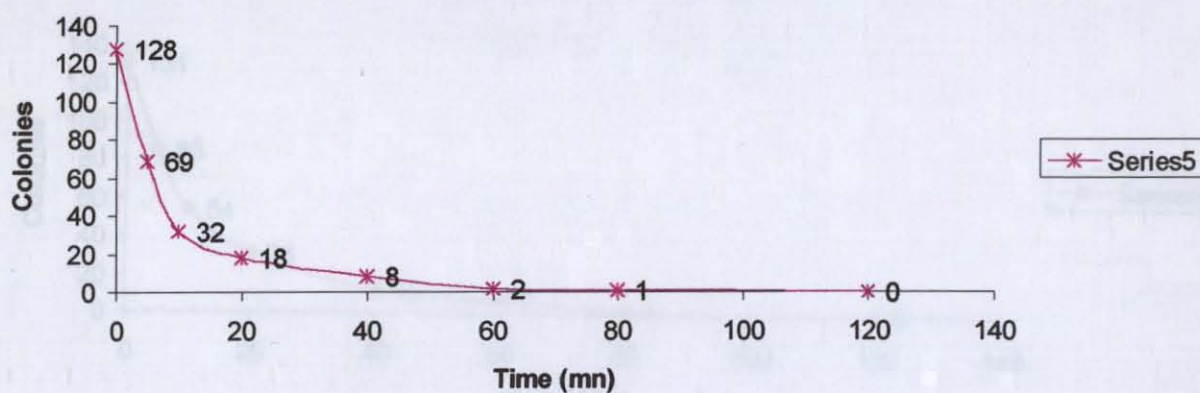


Figure 6.26 Average number of colonies versus time (Tube 5).

Table 6.7 Number of colonies resulting from Tube 6 (2.5 ppm).

Trial	1	2	3	4	5	6	7	8	9	10	Avg	Std Dev
0	134	129	133	124	127	125	138	136	134	128	131	4.8
5	88	91	82	81	86	87	89	79	85	84	85	3.8
10	52	53	59	56	48	47	55	54	61	60	54	4.7
20	35	36	32	28	26	31	34	21	32	37	31	5.4
40	10	16	13	9	11	8	17	12	12	14	12	2.9
60	4	7	3	6	8	5	10	7	6	11	7	2.5
80	0	2	2	0	0	5	1	3	0	0	1	1.7
120	0	0	0	0	0	0	0	0	0	0	0	0.0

Table 6.7 is graphically represented in Figure 6.27.

Colonies vs Time (6)

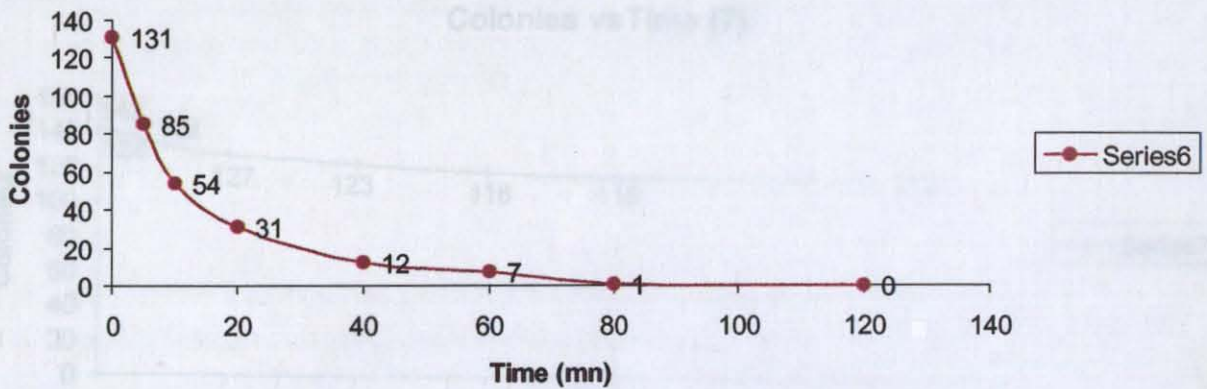


Figure 6.2 Average number of colonies versus time (Tube 6).

Figure 6.28 Average number of colonies versus time (Tube 8).

Tube 8 (0.5 ppm): The number of colonies is 120 and 140 and the antimicrobial effect.

Table 6.8 Numbers of colonies resulting from Tube 7 (1 ppm).

Trial	1	2	3	4	5	6	7	8	9	10	Avg	Std Dev
0	141	145	146	139	142	136	136	142	148	143	142	4.0
5	135	132	139	138	143	133	137	141	144	136	138	4.0
10	131	134	128	133	136	131	126	132	137	127	132	3.7
20	127	124	128	131	133	122	129	122	130	125	127	3.8
40	124	118	126	123	122	118	127	125	120	124	123	3.2
60	117	112	119	115	123	122	114	115	118	120	118	3.6
80	112	110	117	118	114	120	113	120	109	115	115	3.9
120	110	112	116	115	108	117	106	115	116	113	113	3.7

Table 6.8 is represented graphically in Figure 6.28.

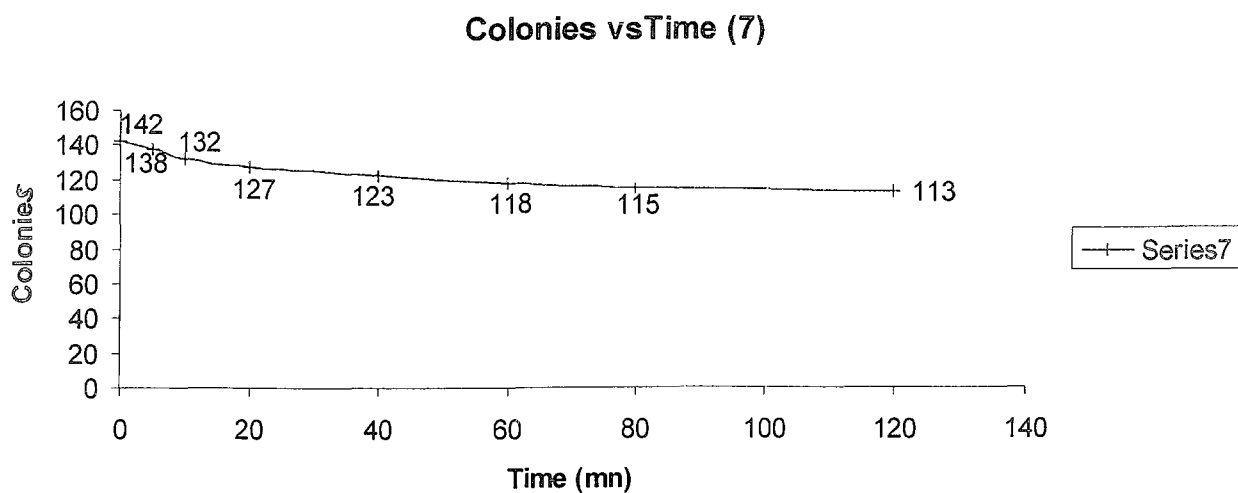


Figure 6.28 Average number of colonies versus time (Tube 7).

Tube 8 (0.5 ppm): The number of colonies is 120 and 140 and this result show little or no antimicrobial effect.

Table 6.9 Number of colonies resulting from Tube 9: blank.

Trial	1	2	3	4	5	6	7	8	9	10	Avg	Std Dev
Result	147	147	152	154	149	144	145	146	155	153	149.2	4.0

Colonies vs Time (1 to 7)

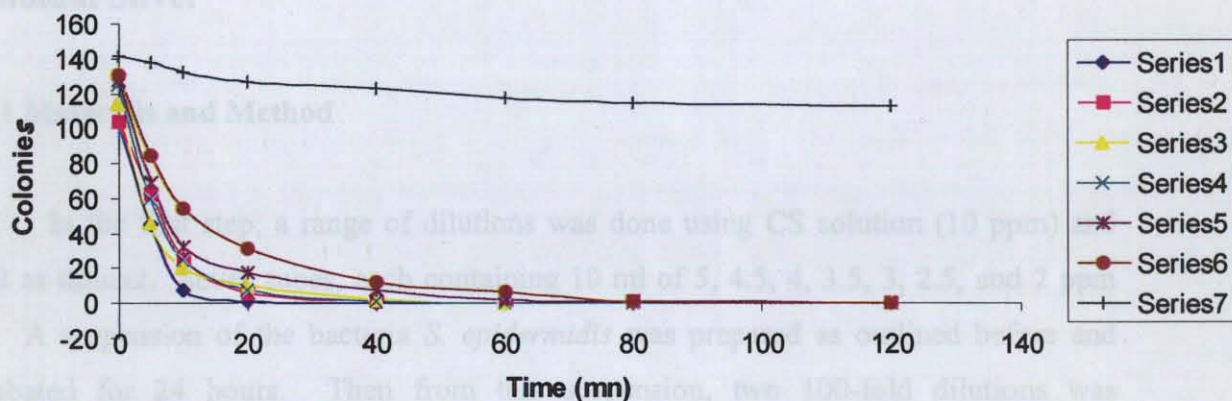


Figure 6.29 Average number of colonies versus time (Tube 1 to 7). In this graph, the curves corresponding to each table (drawn before separated) are placed together for comparison. The descending trends of curves are decreasing as the CS concentrations of their corresponding tubes are increasing.

6.7.3 Discussion

All tubes that contain CS of concentration more than 1 ppm show antimicrobial effects, as they caused noticeable decrease in the number of colonies. This antimicrobial effect reached the degree of total elimination after 120 minutes of exposition to CS as the number of bacterial colonies obtained was less than 10. Following the gradual decrease in the concentration of CS used in each tube, the decreasing trends of each of the corresponding graphs show differences, which proves the decrease of effect of CS as its concentration decrease. In addition, bacterial cells seemed to be affected by the metabolite released from dead cells in suspensions as time passed, which also account for the dramatically decreasing trends of the graphs. Also, the decrease in the number of

colonies on the table inoculated with suspension at the starting point (0 mm) as the concentration of CS increased showed that CS particles continued to slightly act after spreading on the plate surfaces (see Figure 6.29 and Tables 6.2 to 6.8). The above experiment indicated also the range of concentrations that should be used for the coming experiment aiming to determine the minimum inhibitory (bacteriostatic) and killing (bactericidal) concentration of colloidal silver. This range is between 5 and 2 ppm CS.

6.8 Minimum Bactericidal and Bacteriostatic Concentrations of Colloidal Silver

6.8.1 Materials and Method

In the first step, a range of dilutions was done using CS solution (10 ppm) and TSB as diluent. Seven tubes, each containing 10 ml of 5, 4.5, 4, 3.5, 3, 2.5, and 2 ppm CS. A suspension of the bacteria *S. epidermidis* was prepared as outlined before and incubated for 24 hours. Then from this suspension, two 100-fold dilutions was performed. Form the diluted suspension, an 0.1-ml aliquot was pipetted into each of the seven prepared tubes. Then all the tubes were incubated for 48 hours. In the second step, after incubation, A 0.1-ml aliquot from the tubes that shows no turbidity as a sign of bacterial growth was pipetted into a sterile 10-ml TSB and incubated for 48 hours. The above procedure was repeated ten times for accuracy.

6.8.2. Results

The concentrations that resulted in the inhibition of bacterial growth are in the interval between the minimum bactericidal (killing) concentration (MBC or MKC) and the minimum bacteriostatic (inhibitory) concentration (MIC). The lack of bacterial growth, as an indication of an effective concentration (between MIC and MKC) of the CS, is noted by obtaining a clear TSB solution. Whereas the bacterial growth is noted by the turbidity of the suspension, indicating an ineffective concentration, that is less than the MIC. These observations are tabulated in Table 6.10.

Table 6.10

Table 6.10 Bacterial growth in tubes containing 5 to 2.5 ppm CS, respectively (Step 1).

In each column, the presence or absence of bacterial growth is indicated for each tube corresponding to each used concentration of CS (first step).

Trial	1	2	3	4	5	6	7	8	9	10
5	-	-	-	-	-	-	-	-	-	-
4.5	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
3.5	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
2.5	-	-	-	-	-	-	-	-	-	-
2	+	+	+	+	+	+	+	+	+	+

- no growth, + growth

From Table 6.10, it is clear that a concentration less than 2.5 ppm CS has no antibacterial efficacy against *S. epidermidis*. Hence, the MIC is estimated to be approximately 2.5 ppm. On the contrary, all suspensions containing 2.5 ppm and more of CS shows no bacterial growth.

Table 6.11 Bacterial growth in the tubes of Step 2. In each column, the presence or absence of bacterial growth indicated for the tubes of sterile TSB that were inoculated from the previous tubes that showed no bacterial growth (second step). See Table 6.10.

Trial	1	2	3	4	5	6	7	8	9	10
5	-	-	-	-	-	-	-	-	-	-
4.5	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+
3.5	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+
2.5	+	+	+	+	+	+	+	+	+	+

From Table 6.11, CS concentrations that are below 5 ppm shows bacterial growth when the treated bacteria were inoculated in a TSB medium free of antimicrobials. Thus, these concentrations were effective only in inhibiting bacterial growth not for total elimination of bacteria.

From the above results, the MBC and MIC of CS estimated to be approximately 5 and 2.5 ppm, respectively.

6.8.3 Discussion

According to the literature, both the MIC and MBC of CS for *S. aureus* are found to be 5 ppm [49]. However, the time of incubation of the suspensions treated with CS was 24 hours, which seems not to be enough for showing the antibacterial effect of the CS of concentrations less than 5 ppm. As mentioned before (section 6.8.1), the incubation time of the first step of the experiment is 48 hours, as recommended in Ref. 10. This long period of incubation is necessary to show the inhibitory effect of CS on bacteria. On the contrary of the previous experiment that involved spreading bacteria onto plates, in which the time of exposure in suspensions did not exceed 120 mn, this experiment that showed the effect of antibacterial CS in suspension needed that long

period of incubation. The reason for this is that the presence of bacterial growth in suspensions, indicated by the presence of turbidity, cannot be seen after a short time of exposure. Whereas transferring bacteria on agar plates after these relatively short time of exposure is enough because the effect of the used antimicrobial is greatly reduced by spreading aliquots of bacteria over agar surface. This allowed the approximate determination of the time needed for the antimicrobial, depending on its concentration to exert its effect on bacteria.

6.9 Minimum Bactericidal and Bacteriostatic Concentration of Silver Nitrate

6.9.1 Materials and Methods

A 0.1 % w/w (i.e. 1000 ppm) solution of AgNO_3 was used as a stock solution, from which a range of dilutions was made for testing antimicrobial activity. Again, *S. epidermidis* was used as the testing organism. The concentrations of the diluted AgNO_3 (with TSB as diluent) are 500, 450, 400, 350, 300, 250, 200, 150, and 100, respectively. These solutions, together with the original stock solution were used to determine the MIC and MBC in the same way as outlined before for CS.

From this table (6.12), the MIC of the AgNO_3 is estimated to be approximately 150 ppm because it is the lowest concentration that allowed bacterial growth.

Table 6.13 Bacterial growth in the tubes of the second step. The presence or absence of bacterial growth in the sterile TSB tubes inoculated with aliquots from the tubes with no bacterial growth from the first step is indicated.

Trial	1	2	3	4	5	6	7	8	9	10
1000	-	-	-	-	-	-	-	-	-	-
500	-	-	-	-	-	-	-	-	-	-
450	-	-	-	-	-	-	-	-	-	-
400	+	+	+	+	+	+	+	+	+	+
350	+	+	+	+	+	+	+	+	+	+
300	+	+	+	+	+	+	+	+	+	+
250	+	+	+	+	+	+	+	+	+	+
200	+	+	+	+	+	+	+	+	+	+
150	+	+	+	+	+	+	+	+	+	+

Table 6.13 shows that the concentrations of AgNO_3 below 450 ppm allowed bacterial growth when an aliquot of the suspensions were transferred to sterile TSB mediums without antimicrobials. Thus, from the above tables, the MBC and MIC of AgNO_3 are 450 and 150 ppm, respectively, which are much larger than those of CS.

6.10 The Efficacy of Antimicrobial Ceramics

6.10.1 Materials and Method

As explained before ceramics are rendered antimicrobial by treatment with silver ions. Ceramics of this sort (Vitra from Eczacibasi) was used in this experiment. Discs

of diameter of 3 cm were placed in Petri plates. A mannitol salt agar was poured into each of the plates in which the antimicrobial ceramic discs were placed. Then a three 100-fold diluted bacterial suspension (*S. epidemidis*) were used as the source of 0.1-ml aliquots that were spread over the plates, one of which was a control plate without antimicrobial ceramics. The plates were incubated for 48 hours. The whole procedure was repeated many times for accuracy.

6.10.2 Results

The results (number of colonies in each plate) of the precedent experiment are in the table below.

Table 6.14 The number of colonies resulting on the plates with the antibacterial ceramic disks, after 48-hour incubation.

Trials	1	2	3	4	5	6	7	8	9	10
Plates										
1	4	0	0	2	0	0	1	2	0	7
2	0	0	0	0	5	0	0	0	0	3
3	1	0	0	0	1	0	4	0	0	0
4	0	2	5	6	0	0	6	0	4	0
5	0	7	5	0	3	0	0	0	0	2
6	0	0	0	4	0	0	0	3	5	0
7	3	2	3	4	0	2	0	0	3	0
8	5	0	1	0	0	0	0	0	0	2
Blank	147	142	151	150	144	148	140	145	148	152

6.10.3 Discussion

From the above table, it is concluded that the used antimicrobial ceramic is efficiently bactericidal, since the number of colonies on each of the plates that contains the ceramics did not exceed 10 colonies. Also, this antimicrobial activity is continuous

and durable. A continuous release of silver ions and their diffusion through agar during incubation caused the inhibition of bacterial growth and even the killing of a large portion of the bacterial population. In fact, this result conforms to the test done by TUBITAK that used microscopic direct bacterial count and showed no bacterial growth after incubation [50].

6.11 Antimicrobial Activity of Ceramics Covered with Colloidal Silver, Silver Nitrate, and Silver Sulfadiazine

6.11.1 Materials and Method

Normal ceramics under the form of discs 3-cm diameter were treated with a CS solution (30 ppm), silver nitrate (0.1%), and silver sulfadiazine in the form of a commercial cream used for treatment of burns (Silverdine). As for the precedent experiment with antimicrobial ceramics, the treated ceramic disks were placed in petri dishes and bacterial growth (*S. epidermidis*) at the upper surface of agar was tested. For each silver compound, seven plates were used, with an additional blank (control) plate. The whole procedure was repeated ten times for accuracy.

6.11.2 Results

The numbers of colonies obtained on the surface of the plates containing treated ceramics were not significantly different from the blank plate (in the range between 120 and 150 colony).

6.11.3 Discussion

The above result shows an obvious ineffectiveness of silver liquid agents and creams for continuous and durable antimicrobial treatment of solid surfaces if continuous reapplications or coating techniques, as discussed in previous sections, are not employed. Effective silver ions should have diffused or washed away during manipulation of the

treated ceramic plates, and hence no antimicrobial effect was manifested over the growing bacterial colonies on agar surfaces. In addition, a better method, especially for ceramics is the impregnation of silver ions during the manufacturing of antimicrobial ceramics.

7. CONCLUSION

The experimental work involving the use of silver against bacteria showed clearly the antibacterial efficacy of this element, in different forms and compounds, in disinfecting mediums and solid ceramic surfaces.

First, the application of colloidal silver to prevent bacterial growth and killing bacteria was tested by growing and incubating treated bacteria both in suspension and on agar plates. This antibacterial effect was dependent on concentration and the time of exposure, as was shown in the experiments that involved plating. The values of the MIC and MKC of CS were close to those found in the literature.

Second, from the estimation of the MIC and the MKC of AgNO_3 , which were much higher than those of CS, the superiority of CS over silver salts as antimicrobial was proved, which conformed to the literature.

Third, the durable effect of antimicrobial ceramics where silver ions are impregnated within the ceramics during manufacturing was reproved experimental using viable plate counts. This durable antimicrobial effect is not shown for silver when used in liquid (solutions or colloidal suspensions) or pharmaceutical creams.

In these in vitro experimental works, bacteria were used in the planktonic form. As mentioned before bacteria in sessile form found in biofilms are more resistant to antibacterial treatment. Therefore it is recommended to experiment different forms of silver against biofilms grown on solid surfaces. In addition, it is recommended to experiment the antimicrobial silver treatment of biomaterials such as ceramics, metal, polymer, or composite form in vivo to prove its effectiveness in producing biocompatible antimicrobial implants

REFERENCES

1. Gristina, A. G., G. Girhard, B. L. Gabriel, P. T. Naylor and Q. N. Myrvik, "Cell Biology and Molecular Mechanisms in Artificial Device Infection," *International Journal of Artificial Organs*, Vol. 16, No. 11, pp. 755-763, November 1993
2. An, Y. H. and R. J. Friedman, "Laboratory Methods for Studies of Bacterial Adhesion," *Journal of Microbiological Methods*, Vol. 30, No. 2, pp. 141-152, August 1997.
3. Sakuma, S., K. Atsumi and K. Fujita, "Antimicrobial Hydroxyapatite Powders and Methods for Preparing Them," *US Patent 5009898*, April 1991.
4. Burrell, R. E., L. R. Morris, P. S. Apte, S. B. Sant and K. S. Gill, "Antimicrobial Materials," *US Patent 6017553*, January 2000.
5. Kweon, Y. J., "Disinfectant Ceramic Composition," *US Patent 5187124*, February 1993.
6. Hoshimora, S. and H. Minami, "Antimicrobial Agent," *US Patent 5187124*, February 1993.
7. Steele, J. W., J. T. Birbara, R. W. Marsh and T. D. Scull, "Antimicrobial Hydrophilic Coating," *US Patent 5264250*, November 1993.
8. Goetsch, V., *Colloidal Silver Maker and Researcher Manual*, SynerGenesis TM, Hot Springs, AR, January 1998.
9. Ingraham J. L. and C. A. Ingraham, *Introduction to Microbiology*, Wadsworth publishing Company, California, 1995.

10. Kubinski, A.M., D. V. Grady, J. S. Colome and R. J. Cano, *Laboratory Exercise in Microbiology*, West Publishing Company, U.S.A., 1986.
11. Bisno, A. L. and F. A. Waldvogel, *Infections Associated with Indwelling Medical Devices*, Second Edition, American Society for Microbiology (ASM), Washington D.C., 1994.
12. Hudson, M. C., W. K. Ramp and K. P. Frankenburg, "Staphylococcus adhesion to bone matrix and bone-associated biomaterials," *FEMS Microbiology Letters*, Vol. 173 (2), pp. 279-284, April 1999.
13. Şendemir, A. and S. Altıntaş, "Production of hydroxylapatite-reinforced polymer composites for biomedical applications," *Proceedings of the 1998-2nd International conference (Biomedical Engineering Days, Istanbul Turkey, 20-22 May 1998)*, pp. 114-117, 1998.
14. Vereheyen, C. P.M., W. J. A. Dhetr, P. L. C. Petit, P. M. Rozing and K. de Groot, "In vitro study of the integrity of a hydroxylapatite coating when challenged with staphylococci," *Journal of Biomedical Material Research*, Vol. 27, pp. 775-781, 1993.
15. Bagambisa, F. B., U. Joos, and W. Scilli, "Mechanisms of structure of bone between bone and hydroxylapatite ceramics," *Journal of Biomedical Material Research*, Vol. 27, pp. 1047-1055, 1993.
16. An, Y. H. and R. J. Friedman, "Concise review of mechanism of bacterial adhesion to biomaterial surfaces," *Journal of Biomedical Material Research*, Vol. 43, pp. 338-348, 1992.

17. Kitano, T., Y. Yutani, A. Shimazu, , I. Yano, H. Ohashi and Y. Yamano, "The role of physicochemical properties of biomaterials and bacterial cell adhesion in vitro," *International Journal of Artificial Organs* Vol. 19 No. 6, pp. 353-358, June 1996.
18. Dickinson, R. B., J. A. Nagel, R. A. Proctor, and S. L. Cooper, "Quantitative analysis of bacterial adhesion to biomaterial surfaces under well-defined flow conditions," *Materials in Clinical Applications*, Vincenzini, P., (editor), Techna Srl, 1995.
19. Galliani, S., A. Cremieux, van der P. Auwera and M. Viot, "Influence of strain, biomaterial, proteins, and oncostatic chemotherapy on *Staphylococcus epidermidis* adhesion to intravascular catheters in vitro," *Journal of Laboratory Clinical Medicine*, Vol. 127, pp. 71-80, 1996.
20. Woodson, L. P., "Biofilm reduction sterilizer," *US Patent* 6004438, December 21, 1999.
21. Cunliffe, D., C. A. Smart, C. Alexander and E. N. Vulfson, "Bacterial adhesion at synthetic surfaces," *Applied and Environmental Microbiology*, Vol. 65, No. 11, pp. 4995-5002, November 1999.
22. Van der Mei, H., B. van der Belt-Gritter, G. Reid, K. Bialkowska and H. J. Busscher, "Adhesion of coagulase-negative staphylococci grouped according to physico-chemical surface properties," *Microbiology*, Vol. 143, pp. 3861-3870, 1997
23. Kadry, A. A., A. F. Tawfik, A. Abu EL-Asrar, and A. M. Shibl, "Reduction of mucoid *Staphylococcus epidermidis* adherence to Intraocular Lenses by Selected antimicrobial agents," *Chemotherapy*, Vol. 45, pp. 56-60, 1999.
24. Mack, D., "Molecular mechanisms of *Staphylococcus epidermidis* biofilm formation," *Journal of Hospital Infection*, Vol. 43, pp. S113-S125 (Suppl.) December 1999.

25. Konig, D. P., F. Perdreau-Remington, J. Rutt, P. Stoberger, R. D. Hilger and G. Plum, "Slime production of *Staphylococcus epidermidis*," *Acta Orthopédica Scandinavica*, Vol. 69, No. 5, pp. 523-526, 1998
26. Reid, G., "Biofilm in infectious disease and medical devices," *International Journal of Antimicrobial Agents*, Vol. 11, No. 3, pp. 223-226, May 1999.
27. Potera, C., "Biofilms invade microbiology," *Science*, Vol. 273, pp. 1795-1797, 27 September 1996.
28. Bryers, J. D., and S. Hendricks, "Bacterial infection of biomaterials, experimental protocols for in vitro adhesion studies," *Annals New York Academy of Science*, Vol. 831, pp. 127-137, 31 December 1997.
29. Brock, T. D., W. S. Davis and T. M. Michael, *Biology of Microorganisms*, Englewood Cliffs, N. J., Prentice-hall, 1984.
30. Burrell, R. E. and L. R. Morris, "Antimicrobial coating for medical devices," *US Patent 5770255*, June 23, 1998.
31. Scales, J. T., and M. J. Wilkinson, "Antimicrobial surgical implants," *US Patent 4615705*, October 7, 1986.
32. Berry, E. D., and G. R. Siragusa, "Hydroxyapatite adherence as a mean to concentrate bacteria," *Applied and Environmental Microbiology*, Vol. 63, No. 10, pp. 4069-4074, October 1997.
33. Shikarawa, H., O. Ymakawa, H. Nihonmatsu and K. Atsumi, "Light-weight antibacterial ceramic and antibacterial filter," *US Patent 5618762*, April 8, 1997.

34. Tomioka, T., K. Tomita, H. Oka, K. Hoshino, A. Nishino, "Silver thiosulfate silica gel antibacterial composition," *US Patent 5651978*, July 29, 1997.
35. Illiner, H., "Silastic and polymer-based catheters with improved antibacterial/antifungal properties," *US Patent 5709672*, January 20, 1998.
36. Fox, C. L. and S. M. Modak, "Infection-resistant compositions, medical devices, and surfaces and methods for preparing and using them," *US Patent 5019096*, May 28, 1991.
37. Tweden, K. S., W. R. Holmberg, D. G. Bergman and T. L. Shepherd, "Coated prosthetic cardiac device," *US patent 5895419*, April 20, 1999.
38. Raheja, M. K. and S. J. Wroble, "Method for improving antibacterial properties of ophthalmic solutions," *US Patent 5320843*, January 14, 1994.
39. Hagiwara, Z., K. Kishimoto and H. Yamazaki, "Antimicrobial polymer composition," *US Patent 5827524*, October 27, 1998.
40. Woodside, A. G., "Method and apparatus for killing infections bacteria and undesirable microorganisms in large land and water areas," *US Patent 5281312*, January 25, 1994.
41. Engler, P. V., "Antimicrobial Compositions," *US Patent 5900258*, May 4, 1999.
42. Sawan, S. P., S. Subramanyam and A. Yurkovetski, "Non-leaching antimicrobial films," *US Patent 6030632*, February 29, 2000.
43. Sodervall, B. V. and T. Lundberg, "Deposition of silver layer on non-conducting substrate," *US Patent 5695204*, October 12, 1999.

44. Sawan, S. P., T. Shalon, S. Subramanyam, and A. Yurkovetski, "Contact-killing antimicrobial devices," *US Patent 5817325*, October 6, 1998.
45. www.elixa.com/silver/lindmn.htm.
46. Johnson, L. D., "Disinfecting system for hemodialysis apparatus," *US Patent 6027469*, Feb. 22, 2000.
47. Tyler, K. A., "Disinfecting system for hemodialysis apparatus," *US Patent 5785972*, February 22, 2000.
48. Van Loon, J. C., *Analytical Atomic Absorption Spectroscopy, Selected Methods*, New York Academic Press, 1980.
49. www.natural-acne-remedy.com/1d_antibiotic_comparison.htm.
50. www.vitra-artema.eczacibasi.com.tr.