IDENTIFICATION OF BACTERIA IN A BACTERIAL MIXTURE USING RAMAN SPECTROSCOPY

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ABSTRACT

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The need for a quick, accurate and reliable method for detection of pathogenic bacteria, especially in mixture, is increasing day by day. A lot of detection methods have been developed since the discovery of the first microorganism, most of which are based on biochemical and immunological properties. However, their long sample preparation and procedure times, expensive and heavy instrumentation and need for both well- educated and trained staff are some of the disadvantages of these methods.

Raman spectroscopy is a promising technique for fast detection and identification of bacterial cells. It can be applied directly on the sample and there is no need for pre- test procedures. Besides, Raman spectra is not affected by water, which is a natural component of the cells or growth media and measuring and processing times are very short.

Considering the facts about Raman spectrometry which is a fast, reliable and feasible method for identification of bacterial cells in a mixture was the goal of this study. To achieve this goal five different bacteria (BFK13, BHK7 and DH5α varieties of *Escherichia coli*, *Proteus vulgaris* and *Shigella sonnei*) were used together as a model. First, the binary and ternary mixtures of bacteria were prepared. Then, Raman spectra were obtained from particular microorganism and their binary and ternary mixtures. When collected data were processed and statistically analyzed using SPSS software they were applied to plot 2D charts of Euclidean distance. As a result, it was shown that Raman spectra of each bacterium and their mixtures are very similar. However, each spot representing spectra of species fall at different coordinate on 2D charts of Euclidean distance.

ÖZET

RAMAN TAYFÖLÇÜMÜNÜ KULLANARAK BAKTERİ KARIŞIMINDAN BAKTERİ TESPİTİ

Özellikle karışım halinde bulunan patojen bakterilerin hızlı, doğru ve güvenilir tespit yöntemine olan ihtiyaç her geçen gün artmaktadır. İlk mikroorganizmanın keşfinden bu yana, büyük kısmı onların biyokimyasal ve immünolojik özelliklerine dayanan birçok tespit yöntemi geliştirilmiştir. Buna karşın uzun test öncesi ve test süresi, pahalı ve ağır cihazlar, iyi eğitmli ve tecrübeli personel bulundurma zorunluluğu bu yöntemlerin dezavantajlarından bazısıdır.

Raman tayfölçümü bakteri hücrelerinin hızlı tespit ve teşhisi için gelecek vadeden bir tekniktir. Bu yöntem, test öncesi işlemlerine gerek duymadan doğrudan örnek üzerine uygulanabilir. Bununla beraber, büyüme ortamının ya da hücrelerin yapısında doğal olarak bulunan su Raman tayfını etkilememektedir ayrıca ölçüm ve işlem süreleri çok kısadır.

Bu çalışmanın amacı, Raman tayfölçümünün, karışım halinde bulunan bakteri hücrelerinin teşhisi için hızlı, güvenilir ve uygulanabilir bir yöntem olduğunu göstermektir. Bu amaca ulaşmak için model olarak beş farklı bakteri türü kullanıldı (*Escherichia coli'* nin BFK13, BHK7 and DH5α alt türleri, *Proteus vulgaris* ve *Shigella sonnei*). İlk olarak bu bakterilerin ikili ve üçlü karışımları hazırlandı, daha sonra her bir mikroorganizmadan ve onların ikili ve üçlü karışımlarından Raman tayfı elde edildi. Toplanan veriler işlendikten ve istatistiksel olarak SPSS yazılımında çözümlendikten sonra iki boyutlu Öklid uzaklığı haritalarını çizmek için uygulandı. Sonuç olarak her bakterinin ve onların karışımlarının Raman tayfının çok benzer olduğunu, bunun yanında türlerin tayfını temsil eden her noktanın iki boyutlu Öklid uzaklığı haritalarının farklı koordinatlarına denk geldiğini gösterdik.

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LIST OF SYMBOLS / ABBREVIATIONS

DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *E.coli*
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic *E.coli*
EIEC	Entreoinvasive *E.coli*
EMB	Eosin methylene blue agar
EPEC	Enteropathogenic *E.coli*
ETEC	Enterotoxigenic *E.coli*
IR	Infrared
KCN	Potassium cyanide
MHA	Mueller- Hinton agar
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction
P. vulgaris	*Proteus vulgaris*
S. sonnei	*Shigella sonnei*

1. INTRODUCTION

Microorganisms are the most primitive and oldest form of life on our planet. Mankind has always been in contact with this tiny life form. They play a crucial role in our biosphere. For example, the cycle of all major chemical, biochemical elements and molecules are based on the activities of microorganisms and their ability to biodegrade. The breakdown and production of many biochemical components is mainly done by them. Bacteria are vital in recycling nutrients, with many steps in the nutrient cycle depending on these organisms. They also consume dead and decaying matter and preserve the ecosystem. Saprotrophic microorganisms attack and decompose organic matter. Some microorganisms, for example *Pseudomonas putida* has been created by using genetic engineering techniques and new abilities have been given to them. This bacteria can break down some dangerous chemicals [1]. The ability of bacteria to degrade a number of organic compounds is notable and has been used in waste processing. For example, some species are used to decompose sewage waste in big cities. Another group, capable of digesting the hydrocarbons in petroleum is often used to clean up oil spills [2]. Bacteria are also used for the processing and detoxification of industrial toxic waste [3].

Microbes play an major and unique role in the cycle of fundamental organic elements like nitrogen, sulfur and carbon. Nitrogen fixing bacteria living in root nodules of plants helps in the fixation of nitrogen from the air [4]. This is used for the synthesis of proteins and nucleic acids necessary for plants living at nitrogen- poor soil. Cyanobacteria (blue-green algae) can reduce air nitrogen to nitrate which is used by plants [5]. Another group of microorganisms called "methanogenic bacteria" can release methane gas [6].

Chemical industries utilize microorganisms in different manufacturing processes. They are commercially used to produce various pure chemicals in huge amounts for use as pharmaceuticals or agrochemicals [7]. In metal mining the bacteria and other microorganisms are cultured in containers and then used to extract metals like copper and gold in pure forms [8]. *Clostridium butyclicum* is used in fiber retting to separate fibers of plants. In this process the plant tissues are immersed in water and when they swell, water inoculated with bacteria hydrolyzes pectin substance of the cell walls and separates the

fibers. These fibers are used to make ropes and sacks [9]. *Corynebacterium ammoniagenes* is used for commercial preparation of riboflavin (vitamin B2) [10]. Many microorganisms can also be used in biological pest control. Commonly used toxins from the bacteria *Bacillus thuringiensis* are Lepidopteran-specific insecticides [11]. These pesticides are considered to be friendly to environment, they possess little or no effect on humans and wildlife, and does not kill other beneficial insects.

Mankind has been getting benefits from microorganisms since ancient times. Some species of bacteria such as *Lactobacillus* in combination with yeasts and molds have been used for thousands of years in the preparation of fermented foods such as cheese, wine, and yogurt.

Biotechnology is one of the most important developments in the history of mankind. The abilities of synthesis and biodegradation of microorganisms, especially bacteria, are widely used in biotechnological applications. Developments in genetic engineering have helped us to transform bacterial DNA and forced them to produce biologically important molecules for us. Some common examples are the production of human insulin, the human growth hormone and antibodies [12, 13]. Genetically transformed bacterial cells are used in the production of commercially important products. Pharmaceuticals, such as antibiotics, vaccines and steroids are all produced by them. *Streptomyces spp*. produce streptomycin, actinomycetes produce actinomycin [14].

Microorganisms can be beneficial for animals and plants, too. Some bacterial strains living in the stomach of herbivores produce an enzyme called cellulase which makes the digestion of the cellulose possible. Cellulose is the main source of energy for these animals. Pathogenic bacteria, on the other hand, cause severe and sometimes fatal diseases in humans, animals, and plants. The first bacterial disease ever discovered was anthrax (caused by *Bacillus anthracis*) of cattle and sheep in 1876 [15]. Plant pathogenic microorganisms cause a lot of different kinds of symptoms that include galls, leaf spots and over- growths.

Bacteria benefit humans in a number of different ways. The adult intestine contains a large number of bacteria. This load is ten times greater than the number of cells within our

body [16]. *Escherichia coli* that live in the human large intestine work for us and synthesizes beneficial products such as vitamins and releases it for human use. Most of the microorganisms in our body work to support us, as they break down foods and other larger molecules into smaller, more bio-available constituents that our cells can use. They also help us to eliminate waste and other toxins. The greatest health benefits come from the bacterial colonies that are housed in mucosal membranes such as vagina. They keep mucosal enviroment at a constant pH that is not suitable for the other types of bacteria. This prevents colonisation of the non- beneficial microorganisms. One of these benefits is that they help to strengthen the intestinal wall, preventing unwanted molecules from infiltrating through the intestinal wall into the bloodstream.

Not all kind of microbes are beneficial to humans. A large number of them cause infections and other important pathologic conditions in the human body. In fact, bacteria have been the cause of some of the most deadly diseases and widespread epidemics to the human civilization. Diseases such as typhus, tuberculosis, plague, diphtheria, typhoid, cholera, dysentery and pneumonia have taken a heavy toll on humanity. At the beginning of the twentieth century, pneumonia, tuberculosis and diarrhea were the three leading causes of death. The percentages are shown on Figure 1.1 [17].

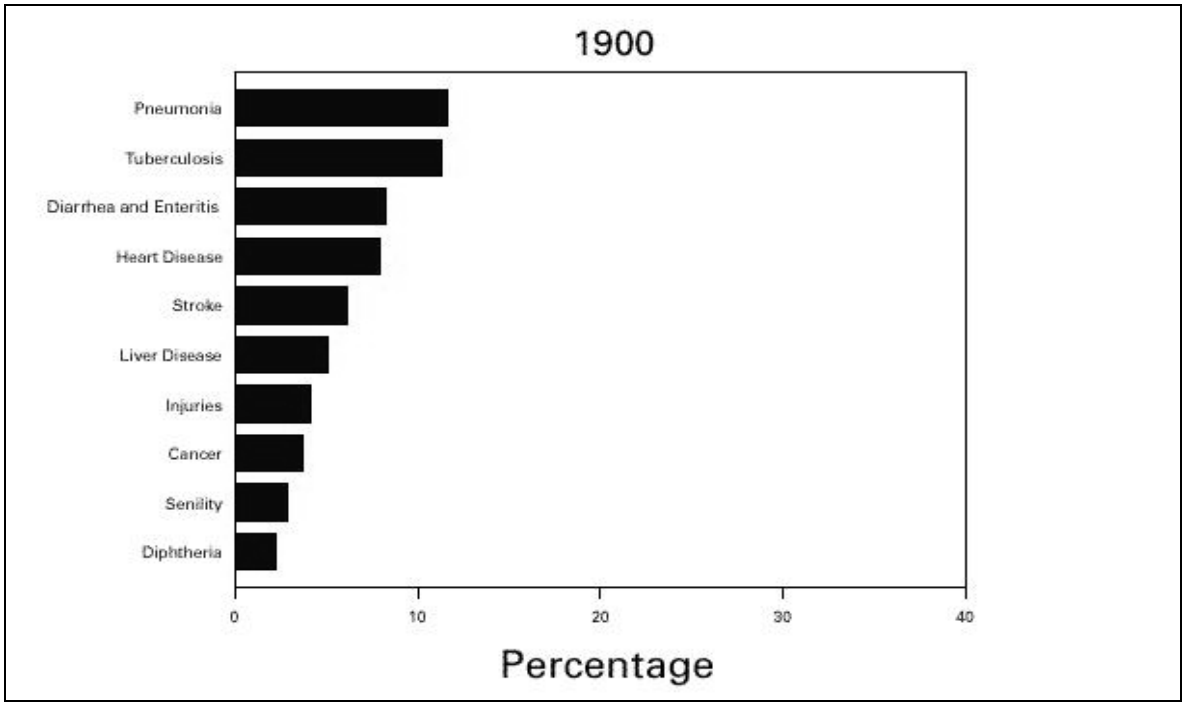

Figure 1.1. The 10 leading causes of death as a percentages of all deaths in USA for the year 1900 [17]

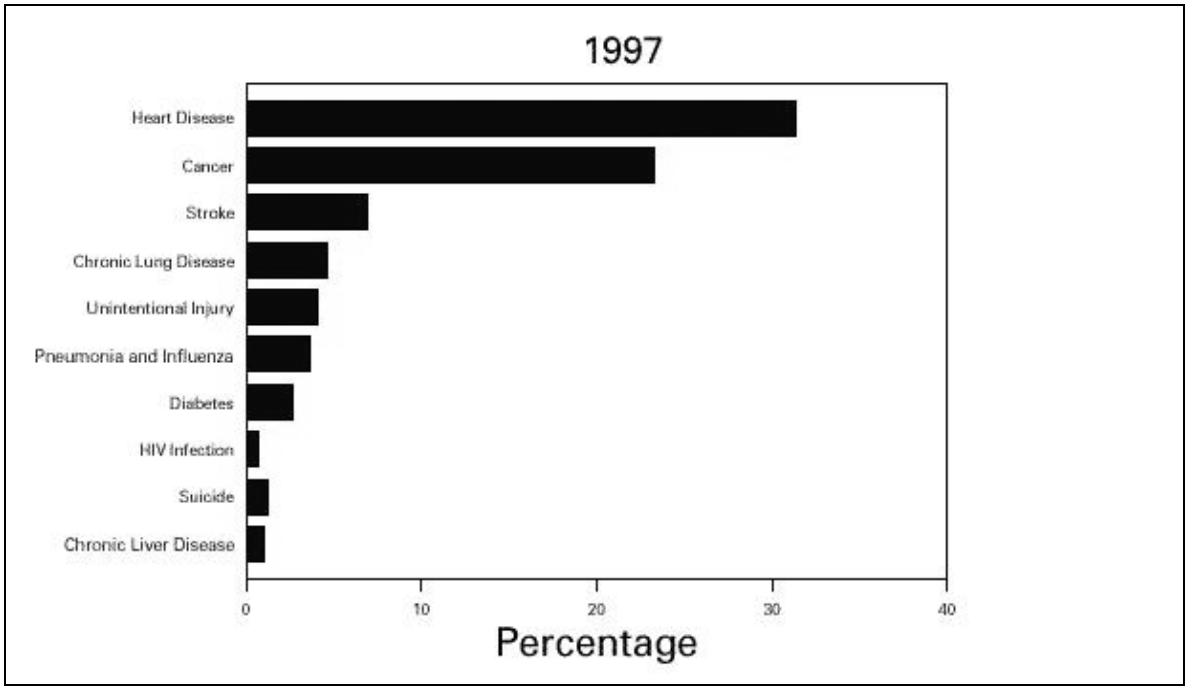

Figure 1.2. The 10 leading causes of death as a percentages of all deaths in USA for the year 1997 [17]

Some bacterial diseases have been conquered after the discovery of penicilin and other antimicrobial agents and this has changed the course of death rates, as it is shown on Figure 1.2. However, many new pathogens are recognized every day and many "old" pathogens, such as *Staphylococcus aureus* and *Mycobacterium tuberculosis* have emerged with new forms and patterns of resistance to antibiotics [17].

Table 1.1. Important bacteria that are pathogens of humans and diseases related with them [17]

BACTERIAL PATHOGEN	**DISEASE(S)**
Gram negative bacteria	
Escherichia coli	Gastroenteritis, urinary tract infections, diarrhea, hemolytic uremic syndrome
Salmonella enterica	Gastroenteritis
Salmonella typhi	Typhoid fever
Shigella dysenteriae	Bacillary dysentery
Pseudomonas aeruginosa	Opportunistic infections, cellulitis, pneumonia
Vibrio cholerae	Cholera
Haemophilus influenzae	Meningitis, pneumonia, sinusitis
Neisseria gonorrhoeae	Gonorrhea
Neisseria meningitidis	Meningococcemia, meningitis
Gram positive bacteria	
Staphylococcus aureus	Food poisoning, wound infections, toxic shock syndrome,
Streptococcus pneumoniae	Pneumonia, otitis media, meningitis,
Bacillus anthracis	Anthrax
Clostridium tetani	Tetanus
Clostridium difficile	Antibiotic-associated diarrhea, colitis
Corynebacterium diphteriae	Diphteria
Listeria monocytogenes	Listerosis

The major groups of bacterial pathogens are spirochetes, spirilla and other curved bacteria, vibrios, the Gram-negative aerobic rods and cocci, enteric bacteria, pyogenic cocci, endospore-forming bacteria, actinomycetes and related bacteria, rickettsias, chlamydiae and mycoplasmas [18]. Some bacterial patogens and diseases related with them are shown in Table 1.1.

No single group of microorganisms have received greater attention from the medical and scientific communities than the family *Enterobaceriaceae,* which are a large group of Gram-negative rods whose natural habitat is the intestinal tract of human and animals. This family includes aerobic and facultatively anaerobic Gram-negative bacilli. They are oxidase- negative, usually catalase- positive and also ferment glucose to acid. Family members generally are motile by peritrichous flagella, however some bacteria are nonmotile. Common genera of the family *Enterobaceriaceae* include *Citrobacter, Enterobacter, Erwinia, Escherichia, Hafnia, Klebsiella, Proteus, Providencia, Salmonella, Serratia, Shigella* and *Yersinia*. The *Enterobaceriaceae* are widely regarded as being mesophilic [19].

It is fact that enterobaceria are colonised in the gastrointestinal tract of humans. Over the years, it has been shown that enterobacteria also play notable roles in extraintestinal diseases like blood- borne infections, respiratory and urinary tract infections, infectious processes of wounds and illness involving the nose, mouth, throat, eye and ear.

The emergence of enterobacteria as major players in serious life- threatening diseases can be based on to several interrelated factors. Statistics indicate that life expectancy continues to rise. In the beginning of twentieth century there were only 15 million people in the world at age 65 and above. By now, this figure had risen to more than 350 million and is projected to reach 2 billion by 2050 [20]. Along with improved life expectancy come a number of indirect consequences. For example, a higher number of immunocompromised people now survive life- threatening conditions, such as cancer or liver disease. Such people are at increased risc of developing serious infections resulting from surgery, chemotherapy or other medical procedures. Another indirect consequence of this increasing “old” population is that many people will require comprehensive, long-term care due to complications with cardiovascular system or chronic diseases like diabetes

or pulmonary disease. Finally, a number of genera and species in the family *Enterobaceriaceae* have developed important detoxification mechanisms that make some members of this family resistant to many antimicrobial agents [21].

In our study we used three different genera of *Enteobacteriaceae*: *Escherichia, Proteus* and *Shigella. Escherichia coli* is a part of the normal intestinal flora and cause disease accidentally, while the *Proteus* and *Shigellae* are always patogenic for humans. They are generally identified and classified by their biochemical specifications. *Escherichia coli* typically produces positive tests for indole and produces gas from glucose. An isolate from urine can be quickly identified as *E.coli* by its hemolysis on blood agar and with an iridescent "sheen" in differential media such as Eosin Methylene Blue agar. This bacterium is the leading cause of urinary tract infections. Enteropathogenic *E.coli* (EPEC), enterotoxigenic *E.coli* (ETEC), enterohemorrhagic *E.coli* (EHEC), entreoinvasive *E.coli* (EIEC) and enteroaggregative *E.coli* (EAEC) are the major cause of diarrhea diseases. Sepsis and meningitis are the other examples for diseases related to these microorganisms.

Proteus is a member of *Proteus- Morganella- Providencia* group [22]. The members of this group are motile, can grow on potassium cyanide medium (KCN) and ferment xylose. *Proteus* species are urease- positive and move very actively because of their peritrichous flagella. Genera members lead to infections in humans only when the bacteria leave the intestinal tract. They cause urinary tract infections and produce bacteremia, pneumonia and lesions in patients with low-movement ability. *Proteus* species produce urease, an enzyme hydrolysing urea to ammonia. As a result, the urine becomes alkaline and this promotes the formation of kidney stones [18].

Shigella are narrow, nonmotile Gram-negative rods. All shigellae ferment glucose but do not ferment milk sugar (lactose), except *S. sonnei*. They are facultative anaerobes but grow very well under aerobic conditions. Genera include four *Shigella* species which are immunologically closely related to *E.coli*. *Shigella* species share many antigens with one another and with other enterobacteria. They live naturally in intestinal tracts of humans and other primates, where under certain conditions they can lead to bacillary dysentery.

The importance of early and rapid diagnosis comes forth, considering the potential of these bacteria to lead to many illnesses. A lot of methods have been developed over the years to identify particular microorganisms. These range from conventional methods utilizing immunological and biochemical approaches and PCR- based assays to optical spectroscopy techniques, but there are some disadvantages. Most of them are slow, cannot be operated under field conditions, usually require medical staff trained in bacteriology, or a combination of all these. The fact is that the search still goes on for general, rapid, automated, easily portable new methods that will not require specific, time-consuming, analyte-dependent sample preparation.

Since its discovery, a lot of PCR-based techniques have been developed for identification of pathogens [23]. However, these assays typically require species or strain specific probes that may not be allowed for a particular bacteria. Mass spectrometry is another promising method for rapid bacterial diagnostics. Nevertheless, mass spectrometry can be applied only if target pathogens are well known. The most important fact is that neither PCR nor mass spectrometric approaches can be applied to live bacteria. Optical spectroscopies such as fluorescence and photoluminescence are good candidates for rapid detection; however, fluorescence spectroscopic techniques lack specific chemical information because fluorescence spectra of different biological species are quite similar due to the presence of some common compounds [24].

Compared to fluorescence and luminescence methods, vibrational spectroscopy provides specific information about biological analytes and it is a promising detection technique for rapid microorganism identification [25, 26]. Raman spectroscopy appears to be better suited for the identification of bacteria, carrying advantages both of electronic (based on transitions between molecular electronic levels, absorption and emission in the visible and the ultraviolet spectrum) and magnetic (such as nuclear magnetic resonance) spectroscopies. Raman spectroscopy has a much higher and sharper spectral information content compared to electronic spectroscopy, and it does not require labeling, as in the case of fluorescence spectroscopic approaches. Also, the equipment required is simpler, more mobile and cheaper than that typically used in NMR. Another important technique, Infrared absorption spectroscopy (IR), seems to be more useful compared to vibrational spectroscopy technique, mainly due to its higher sensitivity and cheaper equipment

compared to Raman spectroscopy. However, IR spectroscopy can not be used in biological systems because the IR light is strongly absorbed by water that is present in biological systems. All these facts leave Raman spectroscopy as the most suitable vibrational spectroscopic technique for biological samples. Raman spectroscopy has the big advantage of being blind to aqueous environments. The Raman intensity of water is very small. Furthermore, recent progress in development of Raman instrumentation has increased the sensitivity and decreased the cost of Raman spectrometers. All of these specifications and developments make Raman spectroscopy the natural choice for investigating bacteria and microorganisms [27].

2. THEORETICAL BACKGROUND

General information about Raman spectroscopy and structure and molecular composition of a bacteria is provided in this part.

2.1. RAMAN SPECTROSCOPY

The most important technique used today to investigate molecular transitions, which can be rotational or vibrational is a Raman spectroscopy [28]. It is based on inelastic scattering of monochromatic light, generally produced from a laser in the visible or other frequency range. Inelastic scattering means that the frequency of photons in monochromatic light changes upon interaction with a molecule. In spite of this, elastic scattering which occurs with no change in photon frequency is called Rayleigh scattering. In Raman spectroscopy, Raman scattering is attended by the change in photon frequency due to excitement or inactivation of vibrations at the molecular level. Photons of the laser light are absorbed by the sample and then re-emitted. The frequency of the re-emitted photons is increased or decreased in contrast to the original frequency, which is called the Raman effect. Raman scattering is a good example of inelastic scattering because there happens to be an energy exchange between the laser photons and the investigated molecules.

In this process, a laser beam is used to enlight a part of the investigated sample. Reflected light from the enlighted spot is collected via a lens and is sent through an interference filter to a spectrophotometer to obtain the spectrum of a sample. Photons of reflected light with wavelengths close to the laser's original wavelenght are filtered out while the rest of the collected light is sent to a detector. The laser light changes the vibrational states of molecules in the sample. Interaction with these molecules changes the photon energy of the laser light and the change of energy provides information about the vibrational conditions in the investigated sample [28].

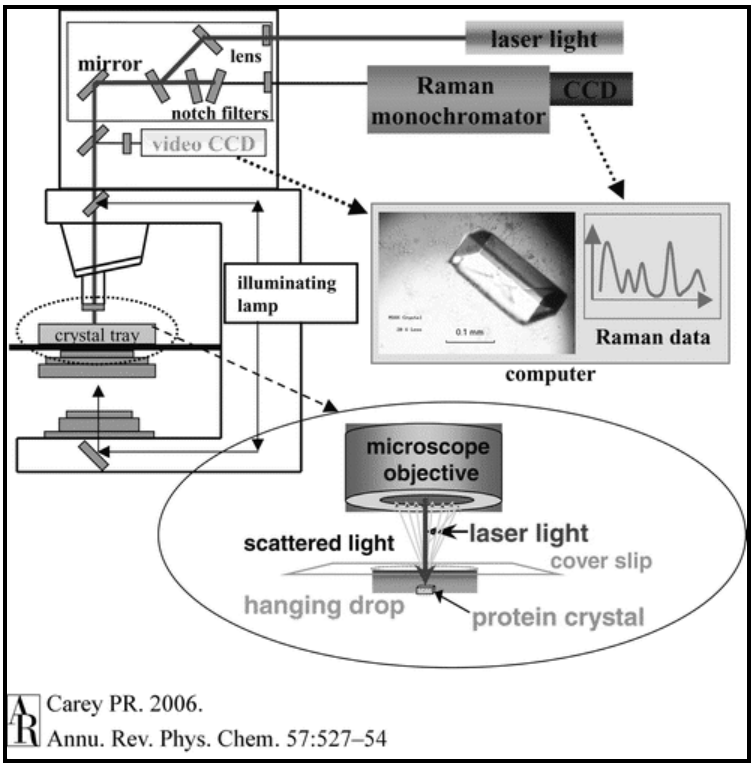

Figure 2.1. Schematic representation of Raman spectrometer [29]

2.1.1. Basic theory

The Raman effect occurs when light encroaches upon a molecule and interacts with the electron cloud and the bonds of that molecule. In the common Raman effect, a photon from a laser beam excites the target molecule from the basic state to a virtual energy state. After that, when the molecule goes back to its “zero” state it looses emited energy as a photon and returns to a different rotational or vibrational state. The difference in energy between the basic state and this new state leads to a shift in the emitted photon's frequency. The Raman effect is different from absorption where the molecule is excited to a distinct, not virtual ener

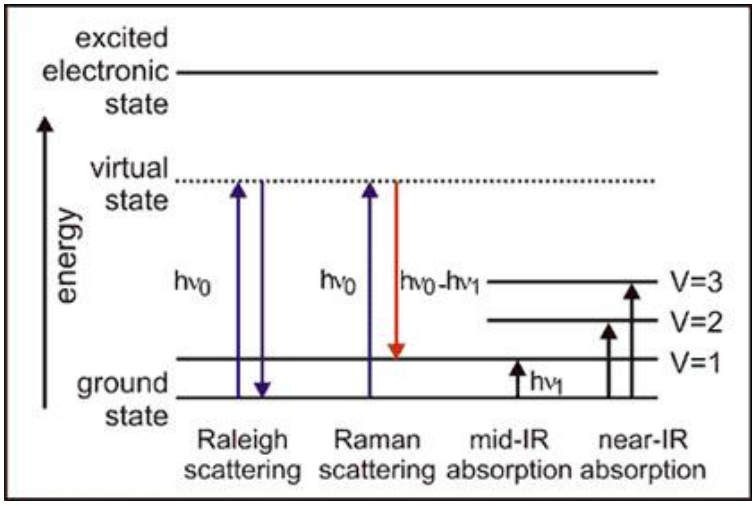

Figure 2.2. Schematic representation of the difference between Raleigh and Raman scattering and absorption [30]

The electron envelope of a molecule must be distorted to reveal a Raman effect. When the amount of distortion is measured the difference will determine the Raman scattering intensity. Raman spectra are typically shown in wavenumbers and most commonly, the unit chosen to indicate wavenumber in Raman spectra is inverse centimeters (cm^{-1}) [28].

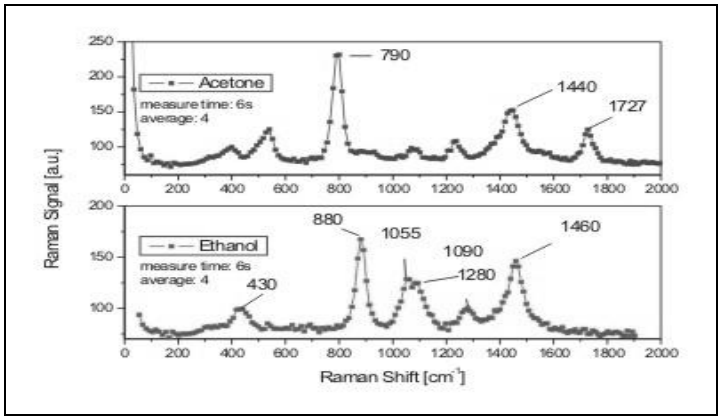

Figure 2.3. Examples of Raman scattering spectra [31]

As for it as a scattering technique, Raman spectroscopy has a lot of advantages for microscopic analysis. One of them is that specimens do not need to be fixed with chemicals or sectioned. Also, Raman data can be gained from a very small area or volume

and this makes possible the identification of species present in that volume. The spectral fingerprint of water is very weak and it has no effect on the Raman spectra of specimens. All of the specifications stated above make Raman spectroscopy one of the most suitable techniques for the microscopic examination of cells and proteins.

2.2. STRUCTURE AND MOLECULAR COMPOSITION OF A BACTERIA

Bacteria are prokaryotic, single celled microscopic organisms. Prokaryotic means that their cells have neither a membrane-separated nucleus nor other membrane- enclosed organelles like mitochondria and chloroplasts. The relatively small size, usually on the order of micrometers in diameter is the primary differentiative characteristic of the prokaryotes.

Bacterial cells have different morphologies. Generally they are rod or sphere shape but some species looks like a comma or a spiral. They can live in different enviroments. Some are living in great depths in oceans under enormous pressure of water, others can be seen in acidic conditions, hot springs, in high altitudes of atmosphere or in our planet's crust. Live or dead plant and animal bodies are also invaded by microorganisms.

Bacteria were first observed by Dutch scientist Antonie van Leeuwenhoek in 1676, using a microscope of his own design [32]. The observed microorganisms previously were called "animacules" but in 1828 German scientist Christian Gottfried Ehrenberg introduced the term "bacterium" [33].

The DNA of almost all bacteria is circular with an overall length of about 1 mm. The nuclear material is folded more than a thousand times to fit within the prokaryotic cell membrane. The specialised region of a prokaryotic cell containing DNA is termed "the nucleoid" and can be visualised by electron microscopy. Some prokaryotes have subcellular structures like the chromatophores of photosynthetic bacteria, surrounded by a membrane. Thus, it would be a mistake to conclude that subcellular differentiation is lacking in all prokaryotes.

2.2.1. Prokaryotic cell structure

The prokaryotic cell is simpler than the eukaryotic cell but an important exception is that the cell envelope is more complex.

The prokaryotic nucleoid is the equivalent of the eukaryotic nucleus. It can be seen with a light microscope if it is stained. The absence of a nuclear membrane, mitotic apparatus and other subcellular structures can be seen clearly in electron micrographs. The nuclear region is filled with DNA fibrils. The nucleoid of bacterial cells has long been considered to consist of a single circular molecule but this view has recently been revised [34, 35]. Studies have revealed that some prokaryotes like *Borellia burgdorferi* have a linear chromosome [36].

Prokaryotic cells lack autonomous plastids such as chloroplasts and mitochondria. In bacterial cell the electron transport enzymes are localised on a cytoplasmic membrane. In some photosynthetic bacteria the photosynthetic pigments are localised in spherical vesicles underlying the cell membrane. Microtubular structures, which are characteristics of eukaryotic cells are generally absent in prokaryotes. But in a few instances, electron microscope studies have revealed bacterial structures that resemble microtubules [37]. The general structure of a bacterial cell can be seen in Figure 2.4.

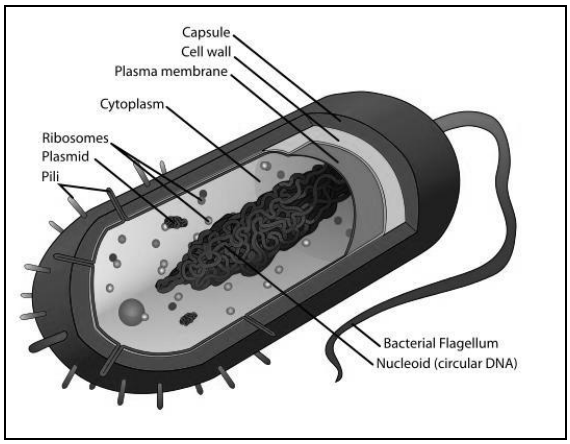

Figure 2.4. Schematic representation of a bacterial cross- section [38]

2.2.2. The cell envelope

The cell envelope that surround the prokaryotic cell is a multilayered structure. The cell envelope consists of the cell wall and the cytoplasmic membrane. The stucture and organisation of a layered cell wall differ in Gram-positive and Gram-negative bacteria and this difference defines these two major bacterial classes.

Many Gram-positive and Gram-negative bacteria possess a two-dimentional lattice of protein or glycoprotein molecules. This layer is the outermost component of the cell envelope and it is generally composed of a single molecular species. The function of this glycoprotein layer is uncertain, however it has been shown to protect the cell from wall-degrading enzymes and bacteriophages, play a role in the maintenance of cell shape and in cell-adhesion to host epidermal surfaces [39].

2.2.2.1. Gram-positive Cell Wall

The cell wall of Gram-positive cells is relatively simple, consisting of two or three layers: the cytoplasmic membrane, a thick peptidoglycan layer and in some bacteria an outer layer called the capsule, as seen in Figure 2.5.

2.2.2.2. Gram-negative Cell Wall

Gram-negative cell walls are highly complex, multilayered structures. The cytoplasmic membrane, also called the inner membrane in Gram-negative bacteria, is surronded by a single two- dimensional sheet of peptidoglycan to which is anchored a complex layer called the outer membrane. An outermost capsule may be also present. The space between the inner and outer membrane is called the periplasmic space. The schematic structure of the Gram-negative cell wall is represented on Figure 2.5.

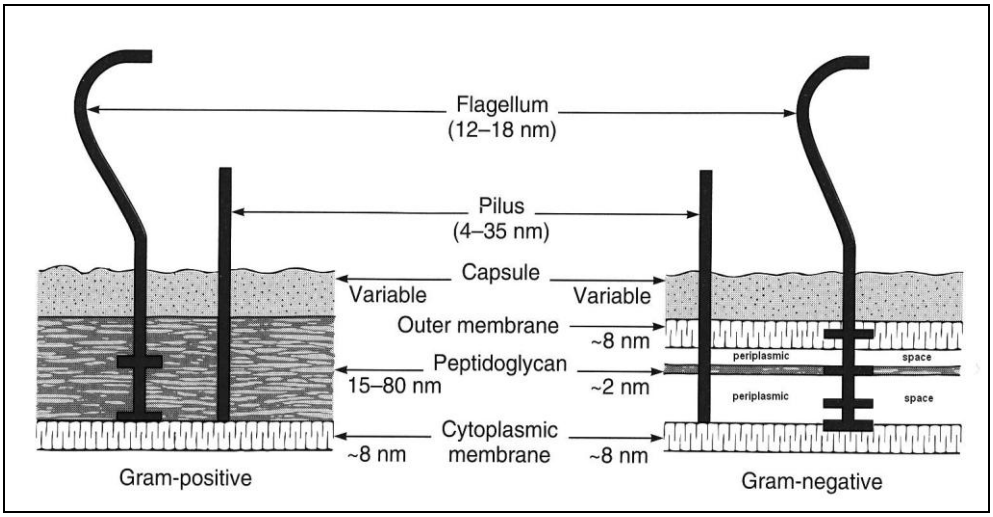

Figure 2.5. Comparison of the structure of Gram-positive and Gram-negative cell envelopes [18]

2.2.3. The cytoplasmic membrane

The bacterial cytoplasmic membrane, also called the cell membrane, is a typical "unit membrane" composed of phospholipids and proteins, as it is shown on Figure 2.6. The membrane of prokaryotes are distinguished from those of eukaryotic cells by the absence of sterols such as cholesterol [40]. Selective permeability, active transport of solutes, electron transport and oxidative phosphorylation, excretion of enzymes, bearing the enzymes, receptors, other proteins and carrier molecules that have functions in the biosynthesis of different molecules, are some of the major functions of the cytoplasmic membrane.

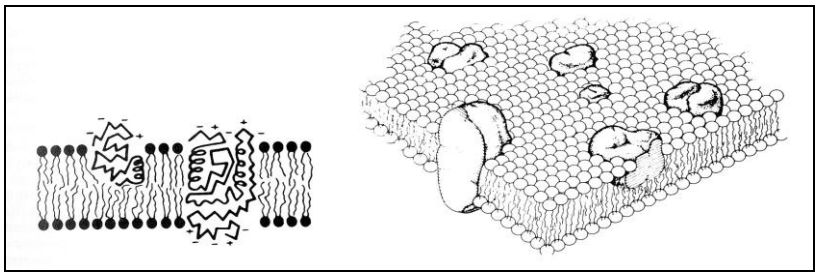

Figure 2.6. A model of a membrane structure. Folded polypeptide molecules are visualised as embedded in a phospholipid bilayer [18]

2.2.4. The cell wall

In bacterial cells the periplasmic space, peptidoglycan layer and outer membrane are collectively called as the “cell wall”. Peptidoglycan and teichoic acid are the main cell wall components of Gram-positive bacteria while in Gram-negative bacteria the cell wall is mainly composed of peptidoglycan, outer membrane and lipopolysaccharide layers. Peptidoglycan (murein, mucopeptide) layer gives stability and strength to the cell wall.

Bacteria are classified as Gram-negative or Gram-positive according to their response to the Gram staining procedure. This procedure is named for the Danish bacteriologist Hans Christian Joachim Gram (1853-1938). In Gram staining the cells are first stained with crystal violet for one minute, washed, stained with iodine and then washed with acetone or ethyl alcohol- acetone mixture. This step decolorises Gram-negative bacteria but not Gram-positive bacteria. Dilute carbol fuchsin or safranin is added for 30 seconds. Washing with water is the last step of procedure. The difference between Gram-negative and Gram-positive bacteria resides in the bacterial cell wall composition: Gram-positive cells can be decolorised with acetone or alcohol but Gram-negative can not be. However, the chemical process of staining is still unclear. The cell wall is, in general, nonselectively permeable; however, one layer of the Gram-negative wall- the outer membrane- blocks the passage of relatively large molecules.

Peptidoglycan is a complex polymer. Repeating N- acetylglucosamine and N-acetylmuramic acid molecules connected to each other by a set of identical peptide cross-bridges builds the backbone of the polymer. A set of tetrapeptide side chains are attached to N-acetylmuramic acid. All structure can be seen in Figure 2.7.

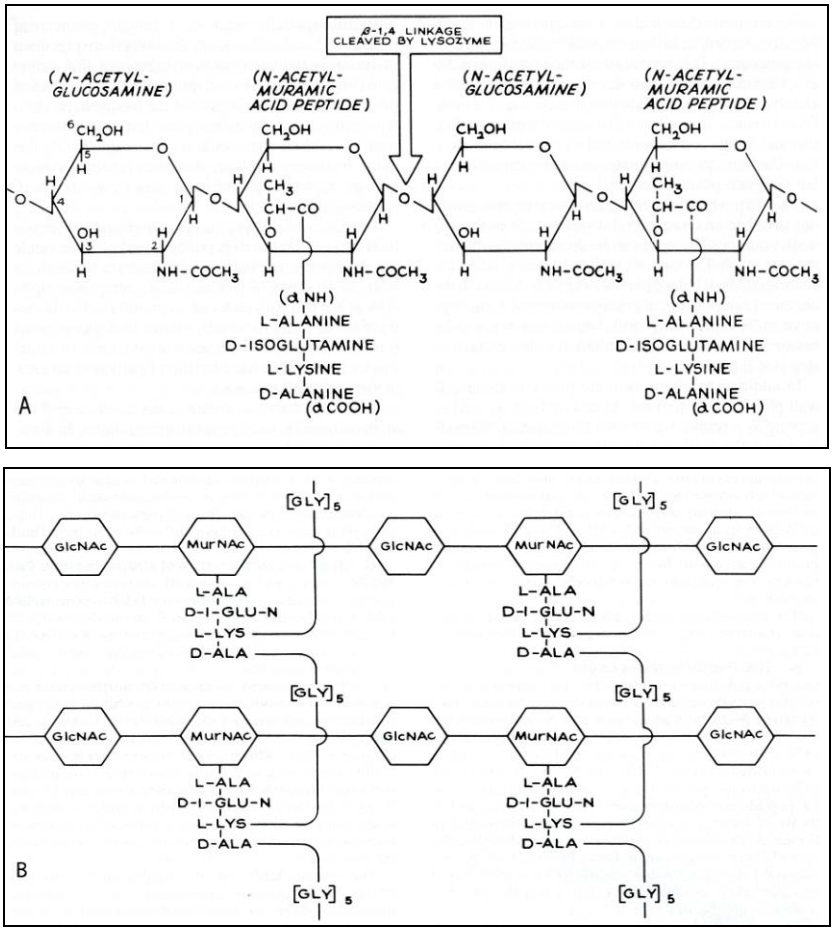

Figure 2.7. A segment of the peptidoglycan of *Staphylococcus aureus*(A); Schematic representation of the peptidoglycan lattice(B) [18]

The peptidoglycan backbone is the same in all bacterial species. The difference is made by tetrapeptide side chains and peptide cross- bridges. Cross-linking makes the peptidoglycan layer one single giant molecule. In Gram-positive bacteria there are a lot of sheets of peptidoglycan which makes the major part of the cell wall material. In Gram-negative bacteria there are a few sheets, comprising five or ten percent of the wall material.

Several prokaryotic groups lack a peptidoglycan layer. They are collectively called the archaeobacteria. In some species within this group a similar polymer exists but in other archaeobacteria, a protein layer is present instead of peptidoglycan.

Most Gram-positive cell walls contain considerable amounts of teichoic and teichuronic acids, which may account for up to fifty percent of the dry weight of the wall

and ten percent of the dry weight of the total cell [41]. In addition, some Gram-positive walls may contain polysaccharide molecules.

On the other hand Gram-negative cell walls contain three components that are attached to the outer side of the peptidoglycan layer: lipoprotein, outer membrane and lipopolysaccharide. The lipopolysaccharide layer is attached to the outer membrane by hydrophobic bonds and is the most important part of a Gram-negative bacteria' s cell wall. It is extremely toxic to animals, and sometimes has been called an endotoxin of Gram-negative bacteria because it is firmly bound to the cell surface and is released only when the cells are lysed.

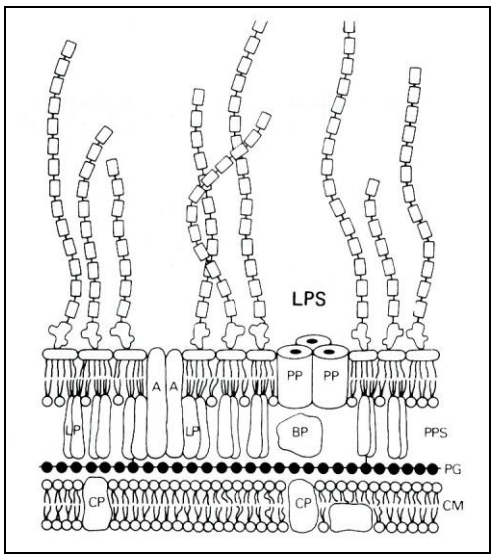

Figure 2.8. Molecular structure of the outer membrane of Gram-negative bacteria. (LPS, lipopolysaccharide; A, OmpA protein; PP, pore protein [matrix porin]; LP, lipoprotein; BP, nutrient- binding protein; PPS, periplasmic space; PG, peptidoglycan; CP, carrier protein; CM, cytoplasmic membrane [18].

3. MATERIALS

Ready-to-use Mueller- Hinton solid culture was purchased from Salubris A.S (İstanbul, Turkey). CaF_2 slides was obtained from Yeditepe University' s Nanobiotechnology laboratory.

4. METHOD

4.1. PREPARATION OF THE SAMPLES FOR RAMAN MEASUREMENTS

Shigella sonnei (Ss), *Proteus vulgaris* (Pv) and three *Escherichia coli* (Ec) strains (BFK13, BHK7, DH5α) were obtained from our microorganism collection (Yeditepe University, Genetics and Bioengineering Department). The identity of all strains of bacterial species in the present study was verified by the Microbial Identification System version 4.5 (MIDI) before use.

First, the bacteria in −80 ^{0}C stock were dissolved and cultivated on Mueller- Hinton Agar (MHA) solid culture for 24 hours. After the incubation the bacterial samples were collected with the sterile loops, suspended in 1 ml deionized water, vortexed, and centrifuged for 5 min at 7500 rpm. After centrifugation, the supernatant was removed with a help of a pipette. The washing procedure was repeated three times. The binary mixtures of Ss/Pv, BFK13/BHK7, BFK13/DH5α, and BHK7/DH5α as well as the ternary mixture of BFK13/BHK7/DH5α were prepared and used to test the feasibility of the approach in the study. A 5-µl aliquot of each bacteria sample (Ss, Pv, BFK13, BHK7, DH5α) their binary (Ss/Pv, BFK13/BHK7, BFK13/DH5α, and BHK7/DH5α) as well as the ternary mixtures of BFK13/BHK7/DH5α species was immediately dropped onto a clean CaF_2 slide and dried at room temperature before Raman analysis. The Bacterial biochemical metabolism shows variations depending on growth conditions and culture media used.

4.2. RAMAN MEASUREMENT

All Raman measurements were performed using a Renishaw inVia Reflex Raman Microscopy System (Renishaw Plc., New Mills, Wotton-under-Edge, Gloucestershire, UK) equipped with an 830- nm diode and a 514- nm argon ion laser. The instrument was automatically calibrated using an internal silicon wafer with the band centered at 520 cm^{-1}. The data were collected by WIRE 2.0 software. A laser power of 1.5–9 mW was selected; the exposure time was 10 seconds and light was focused onto the sample at the microscope

stage through a 50x objective. We used a double accumulation for all samples. A total of 10 spectra were taken from each sample.

4.3. STATISTICAL ANALYSIS

The statistical analyses were performed by using the SPSS software (version 11.5.0, SPSS Inc, Chicago, IL, USA). After the raw data of the Raman spectra were normalized, multidimensional scaling was performed in SPSS. Euclidean distance was selected as a scaling model when 2D charts were plotted.

5. RESULTS AND DISCUSSION

One of the challenges in bacterial identification is long procedures in a clinical setting. Most of the time, a clinical sample is recultured for the identification of the bacteria in the sample. During the reculturing process, each colony on the growth media is recultured, making sure that the isolated bacteria is a pure colony. These operations increase the time of identification, which may delay or hinder the starting of the real treatment. Therfore, there is a need for a technique to decrease the bacterial identification time. The main objective of this study is to test the feasibility of Raman spectropscopy for the identification of a bacterium in a bacterial mixture. When a sample is cultured, the colonies can be tested for their bacterial content.

Model bacteria at species and strain level used in this study are binary and ternary mixtures of *E. coli* strains and other bacteria; *E. coli*, *Shigella*, and *Proteus* are prepared. Figure 5.1, 5.2 and 5.3 show reproducibility of ten Raman spectra for *Proteus vulgaris*, *E. coli* BFK13 strain and *E.coli* mixture, respectively for each bacteria. The percent coefficient of variation was found to be 10.3, 11.3 and 11.6, respestively. As seen in Figures 5.1, 5.2 and 5.3 the reproducibility of each bacteria or mixture Raman spectra is very high.

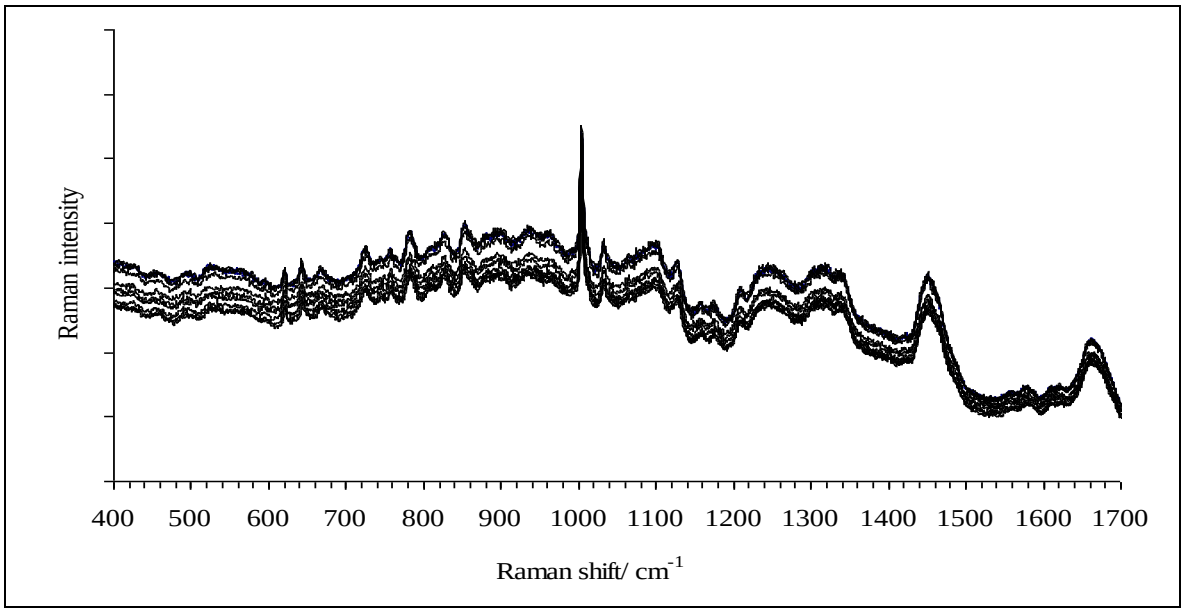

Figure 5.1. Reproducibility of Raman spectra of *Proteus vulgaris*

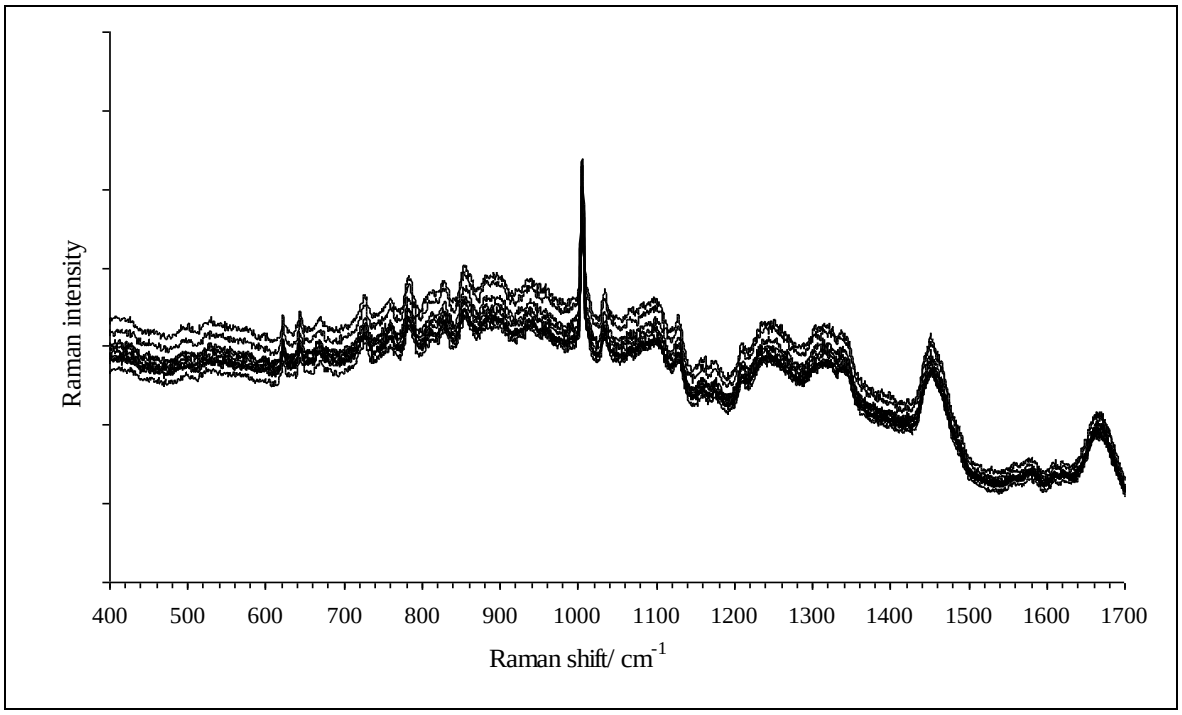

Figure 5.2. Reproducibility of Raman spectra of *E. coli* BFK13 strain

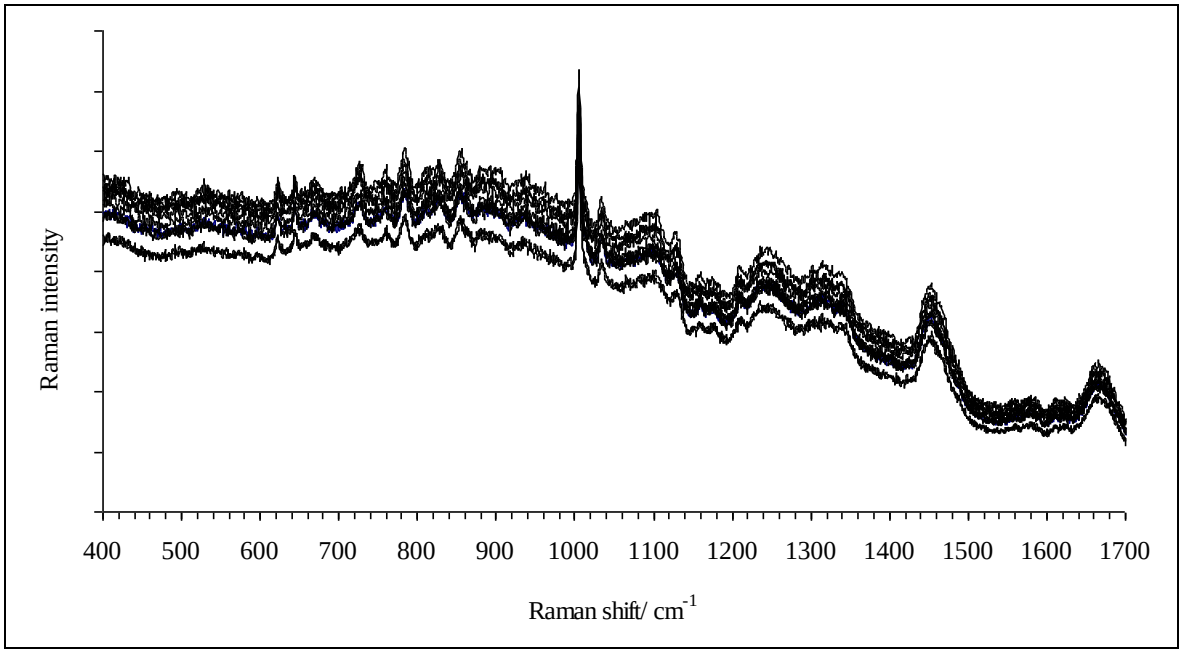

Figure 5.3. Reproducibility of Raman spectra of *E.coli* mixture

The next step was to compare the spectra of bacteria at species and strain level. Figures 5.4, 5.5 and 5.6 show the Raman spectra of *E. Coli' s* BFK13, BHK7 and DH5α strains compared to their binary mixture. The differences between spectra cannot be seen with unaided eye, unless processed mathematically.

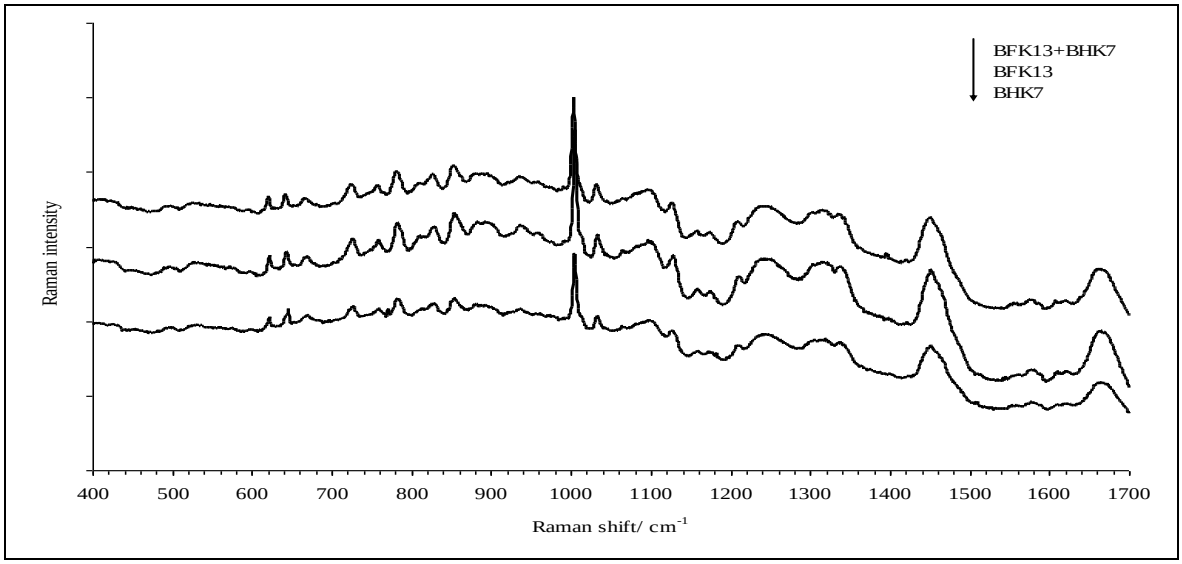

Figure 5.4. Comparative Raman spectra of *E. coli* BFK13, BHK7 strains and their mixture.

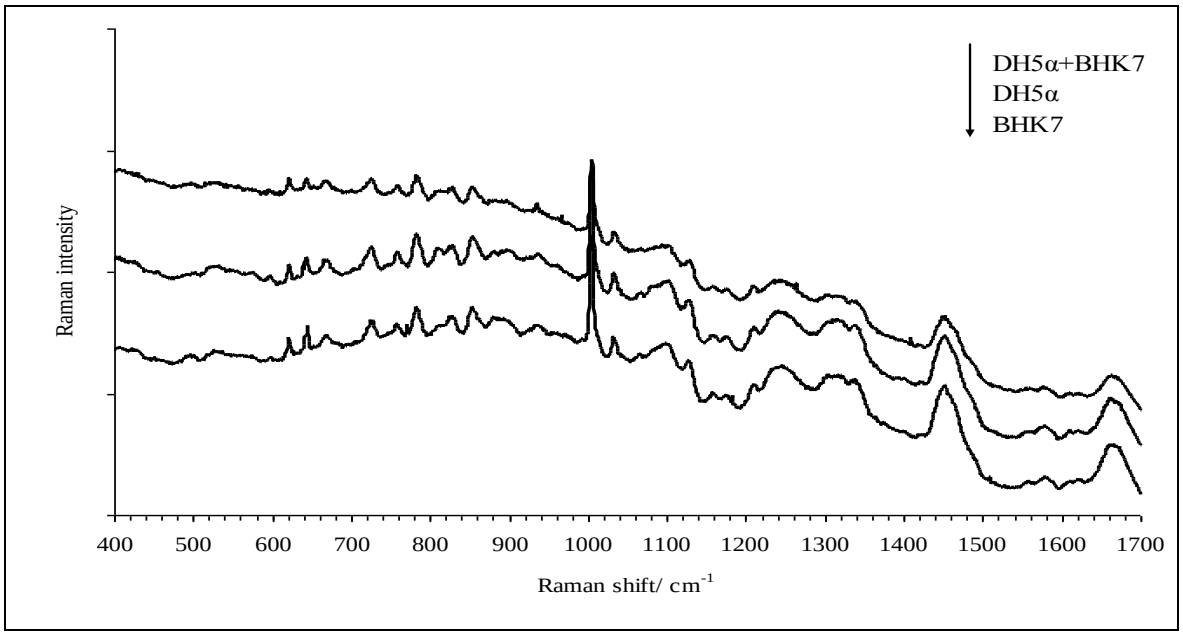

Figure 5.5. Comparative Raman spectra of *E. coli* DH5α, BHK7 strains and their mixture.

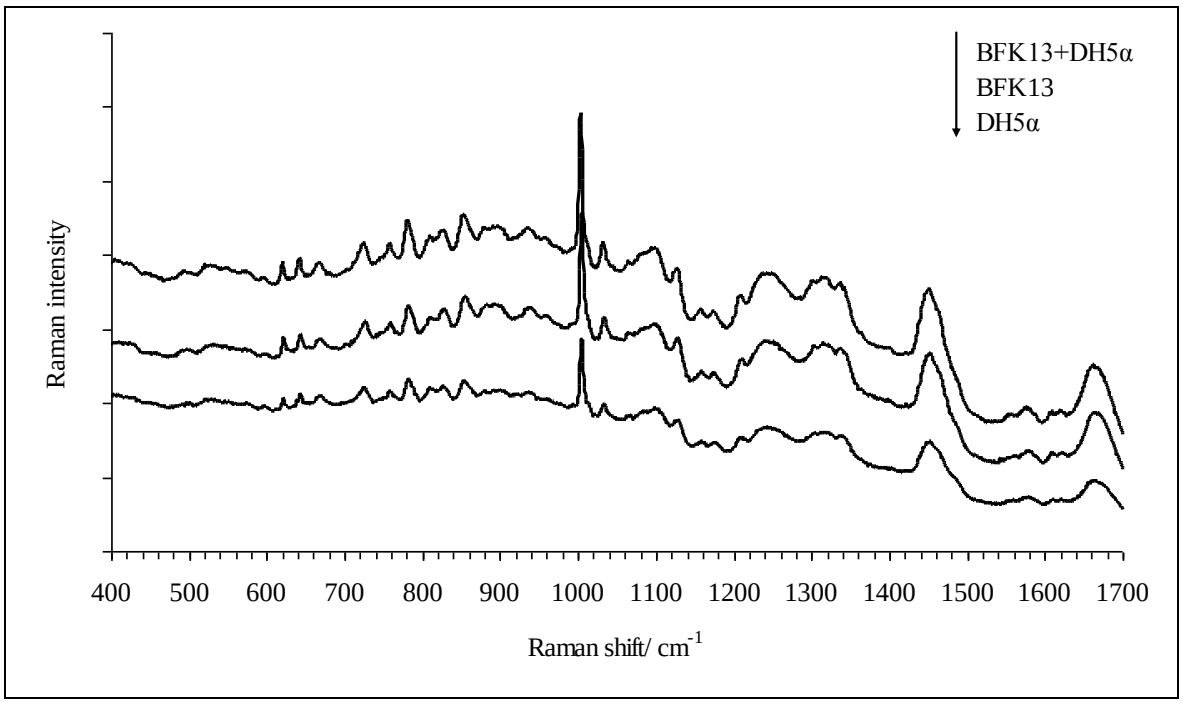

Figure 5.6. Comparative Raman spectra of *E. coli* BFK13, DH5α strains and their mixture.

Figure 5.7 shows *E. coli*' s single strains compared to its ternary mixture. There are subtle differences between BFK13 and BHK7 compared to DH5α and the *E. coli* mixture, particularly at the region between 1500 cm^{-1} and 1700 cm^{-1}

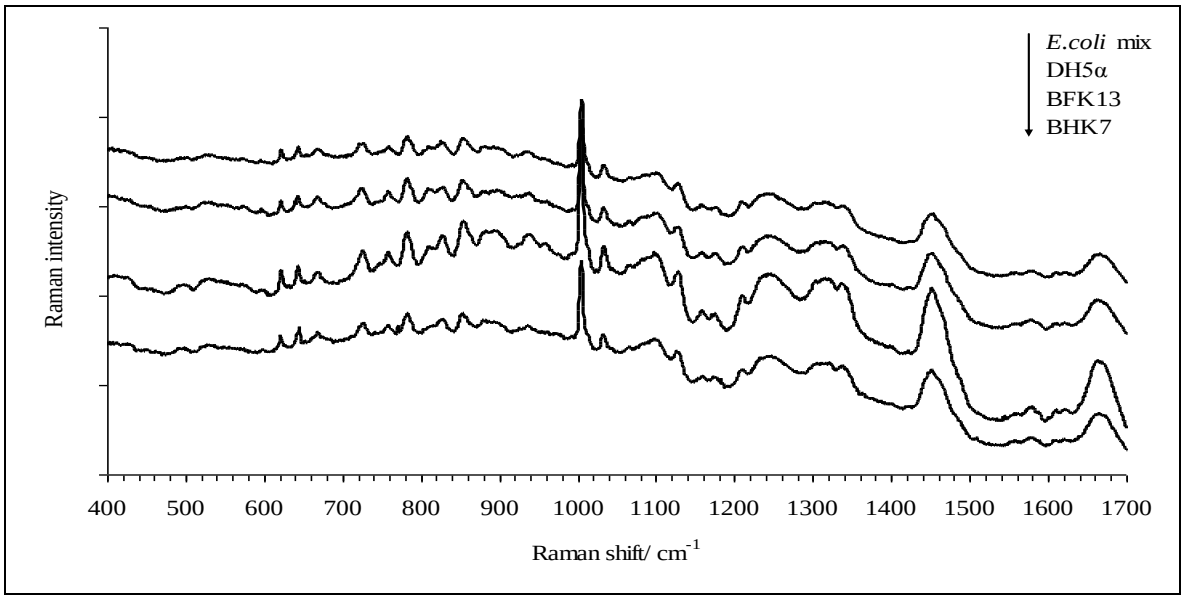

Figure 5.7. Comparative Raman spectra of *E. coli* mixture and three *E.coli* strains.

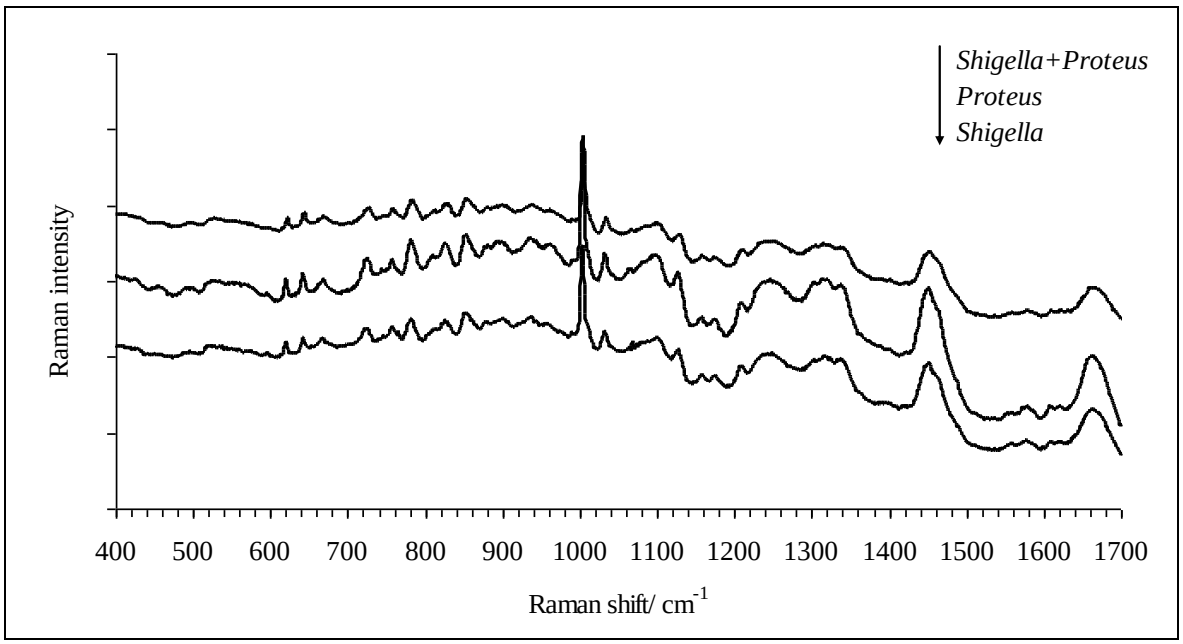

Figure 5.8. Comparative Raman spectra of *Shigella sonnei*, *Proteus vulgaris* and their mixture.

Comparative Raman spectra of *S.sonnei*, *P.vulgaris* and their binary mixture are shown at Figure 5.8. The peaks of *Proteus* and *Shigella* at 1500 cm^{-1} are very similar to each other, however Raman intensity of this peak becomes weaker in their mixture. Also peak intensity at region between 1100- 1200 cm^{-1} differs when measured in mixture.

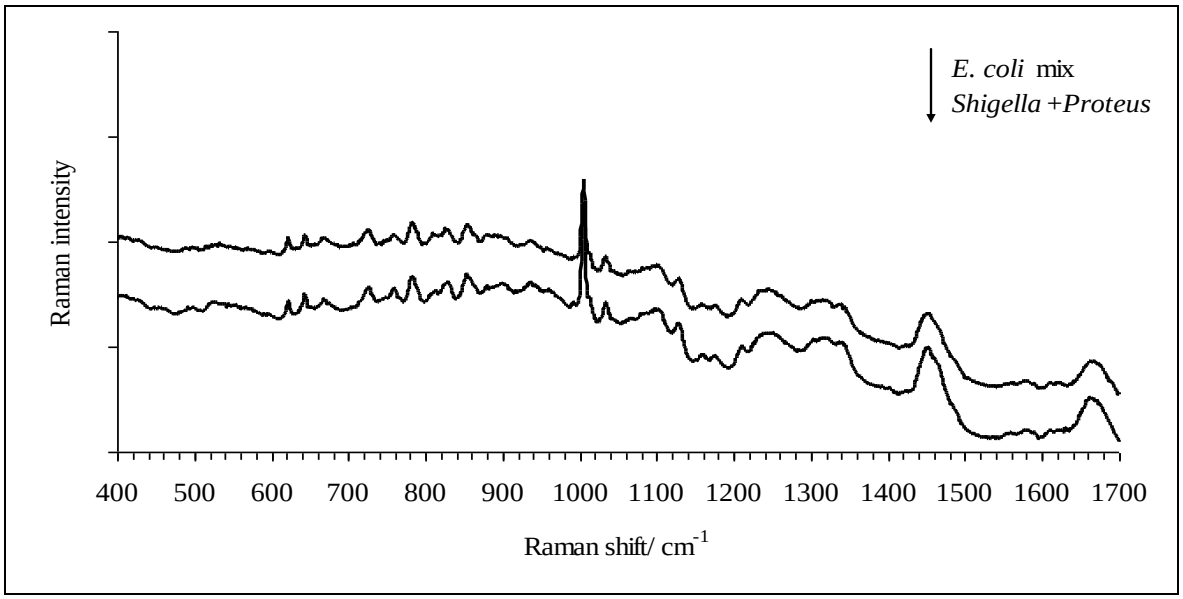

Figure 5.9. Comparative Raman spectra of *E. coli* mixture and *Shigella+Proteus* mixture.

The similarity of the Raman spectra of *E. coli* mixture and *Proteus*+ *Shigella* mixture can be seen clearly at Figure 5.9. All bacterial species belong to the same family, *Enterobacteriaceae*, thus their molecular and cellular compositions are closely related to each other and there are no major differences between them. When examined separately, each species reveal its specifications but when processed in a mixture the differences disappear and the Raman spectra of mixtures show great resemblance to each other.

The spectrum of each bacteria is reduced to a spot on a 2D Euclidean distance plot. Figures 5.10, 5.11 and 5.12 show the 2D Euclidean distance plots after processing. As seen in Figure 5.10, each bacterium is placed on a separate coordinate. This plot can be used to examine the relationship of spectra to each other. The spots representing the mixtures fall around the spot of the bacterium that makes the composition of the bacterial mixture. Interestingly, there is an accumulation of spots at upper right square of plot, when spectra obtained from mixtures containing *E. Coli* BFK13 strain is processed. The same effect can

be seen on raw Raman spectra of binary or ternary mixtures of that strain (graphic not shown). Intensity of peaks increases significantly and makes it appear like all signal come from BFK13 cells. According to this, when spectra are processed, spots representing binary or ternary mixtures of this bacterium groups get closer to each other, as seen in Figures 5.10 and 5.12.

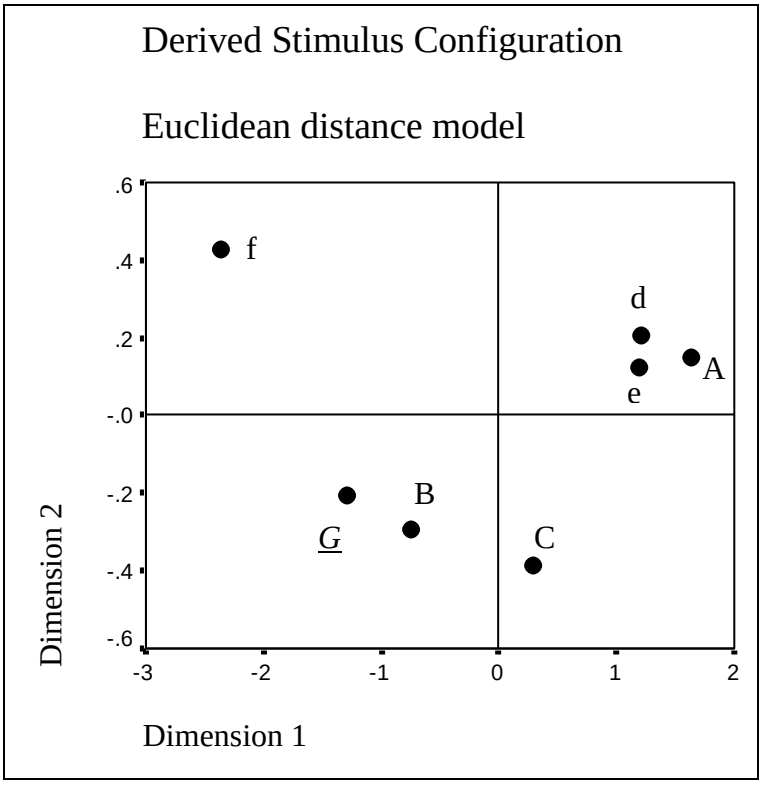

Figure 5.10. Euclidean distance plot for single *E.coli* strains (BFK13(A), DH5α(B) and BHK7(C); binary mixtures BFK13+ DH5α(d), BHK7+ BFK13(e) and BHK7+ DH5α(f); ternary mixture of *E.coli* strains (*G*)

Figure 5.10 shows Euclidean distance plot for single *E.coli* strains, their binary and ternary mixtures. It can be seen that the spot representing each bacterium falls at different coordinates on the the plot. Single strains, namely BFK13, DH5α and BHK7 are marked with capital A, B and C, respectively. All letters can be seen at different coordinate. Binary mixtures are marked with small letters and ternary mixture is marked as *G*. Binary mixture of strains BFK13+ DH5α marked as "d" is close to A- B line but fall close to spot representing BFK13 (A). The same thing happens to A- C line. The mixture of

BHK7+ BFK13 is marked as “e” and it is represented very close to A- C line. But, the “e” spot falls close to BFK13 (A), too. The main reason for this phenomenon is stated above.

The spot representing DH5α (B) +BHK7 (C) mixture is marked as “f”. This spot falls at the top left quarter of plot, not close to B- C line. Apparently, Raman spectra of mixture has different specifications than spectra of its components.

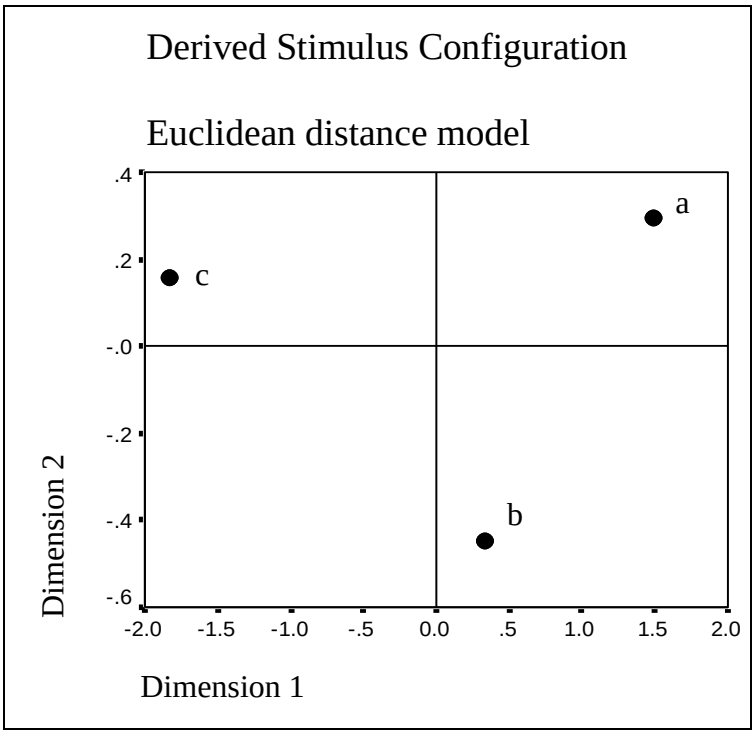

Figure 5.11. Euclidean distance plot for *Proteus vulgaris* (a), *Shigella sonnei* (b) and their mixture (c).

Euclidean distance plot for *Proteus vulgaris*, *Shigella sonnei* and their mixture is shown on Figure 5.11. The dissimilarity of their Raman spectra is shown at Figure 5.8. This difference is clearly represented on Euclidean plot, when spectra are processed statistically. All spots fall at separate quarters.

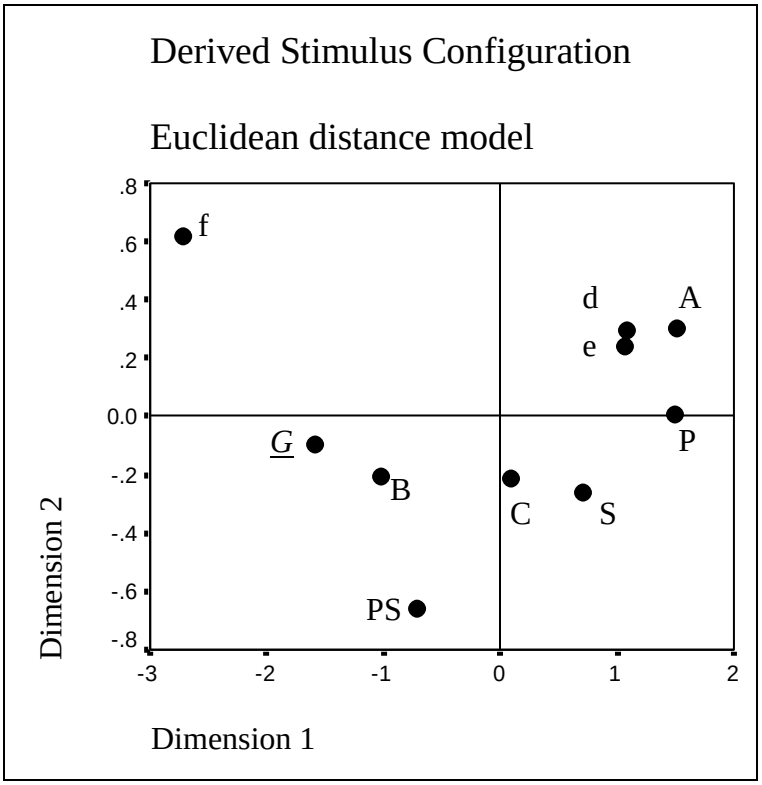

Figure 5.12. Euclidean distance plot for single *E.coli* strains, *Proteus vulgaris* and *Shigella sonnei* and their binary and ternary mixtures. BFK13(A), DH5α(B), BHK7(C), BFK13+ DH5α(d), BHK7+ BFK13(e), BHK7+ DH5α(f), ternary mixture of *E.coli* (*G*), *Proteus vulgaris*+*Shigella sonnei* mixture(PS), *Shigella sonnei*(S) and *Proteus vulgaris*(P)

Euclidean distance plot for single *E.coli* strains, *Proteus vulgaris* and *Shigella sonnei* and their binary and ternary mixtures are shown on Figure 5.12. This figure represents all used bacteria, with their binary and ternary mixtures shown on the same Euclidean plot. The coordinates for *E. coli* and mixtures of its strains are the same in Figure 5.10. However there is a little difference: The spot of *Proteus* +*Shigella* mixture (PS) lays on different coordinate, compared to Figure 5.11. Dimensions of the Euclidean plot changes when all data is shown on one plot, thus spots representing bacteria fall at different coordinate. To reveal the reasons of all phenomena, further investigations and deep analysis are needed.

6. CONCLUSION AND RECOMMENDATIONS

6.1. CONCLUSION

In this study, our main goal was to determine the feasibility and reliability of Raman spectroscopy as a fast and reliable method for identification and detection of different bacteria species and strains. Five types of bacteria, two at a species level (*Proteus vulgaris* and *Shigella sonnei*) and three at a strain level (*Escherichia coli*'s BFK13, BHK7 and DH5α strains) were used as a model. Binary and ternary mixtures of *E. coli*, its strains and mixtures of other bacteria were prepared and investigated.

We obtained Raman spectra of single types of bacteria and their binary and ternary mixtures. Then the produced data were normalised, processed and statistically analysed using SPSS software. Euclidean distance was selected as a scaling model when 2D charts were plotted.

In our study we used bulk Raman to investigate bacterial cells, and no additions of any chemicals or nanoparticles were made. Although there are subtle differences, the Raman spectra shows great similarity. Microorganisms choosen as a model are relatively close to each other, their cellular composition at the molecular level shows great resemblance but the structure of a cell wall is slightly different compared to each other. Small differences in the Raman spectra may be related to this specifications of the bacteria.

A clear difference can be seen on the Euclidean plots. There is a single point representing each individual strain, bacteria or their binary or ternary mixture falling at a different coordinate on the plot. Each point has its own coordinate and spots of binary or ternary mixtures are close to the line connecting spots representing components of mixture separately, except the spots of mixtures containing *E. coli*' s BFK13 strain.

In conclusion, it was shown that Raman spectroscopy can be used for identification of bacteria in a bacterial mixture.

6.2. RECOMMENDATIONS

There are lots of bacterial identification procedures used regularly in laboratories all around the world. Most of them are based on biochemical reactions occuring in microorganisms. There are also immunological, PCR- based and microscopical tests for determination of bacteria, but all of these procedures are very long, slow and need well educated and trained technical staff. One of the challenges in bacterial identification is decreasing the time used for tests, also reliable and feasible method for identification of a pathogen bacteria is needed. Raman spectroscopy has a great advantage because it does not need long and difficult sample preparation procedures. At the same time, Raman spectra is not affected by aqueous enviroment of media. The investigated bacterial cells can be directly collected from growth media and put on a substrate for analysis.

In our study we used five types of pathogenic bacteria, two at a species and three at a strain level as a model. In further studies, there can be used other pathogens and their coordinates should be specified. All pathogenic microorganisms will have an specific Euclidean coordinate, at last. By using the coordinates, it will be possible to create a “map” of disease causing microbes. In future, when the unknown bacteria will be processed we will find its coordinates and place it on the map. When the coordinate matches any microorganism of which coordinates had been found, then the reason for the illnesses will have been found. If the found point falls between any two points or among more, it will be understood that the factors of illness are more than one. And that will play an important role in determining and taking an appropriate treatment method for the illnesses fast. Besides all this, genetic and molecular relationships between different microorganisms can be detected. Future tests of huge matrices of different bacterial species and strains would be required and may be prohibitive.

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