CHAPTER 4

4. MORPHOLOGICAL CHARACTERIZATION, BIOCHEMICAL CHARACTERIZATION AND MOLECULAR IDENTIFICATION OF PROBIOTIC BACTERIA

4.1. Introduction

Classical microbiological tools are founded in observational techniques for bacteria, some of which originated with the invention of the microscope by Leeuwenhoek in 1674. Notably, less than 5% and perhaps as little as 1% of all bacteria can be cultured in the laboratory. If one includes all viruses infecting all species on earth, the number becomes lower by several orders of magnitude. Hence, one caveat of classical microbial methods is that the tools used to describe microbial development and provide systematic organization are based on a statistically minor portion of all bacteria (DeLong and Pace 2001).

This is acceptable for medically important species since classical microbiological techniques, especially those adapted to the clinical laboratory, are based for analysis of human pathogens. When microbial analysis techniques require description and typing of environmental organisms rather than microbial pathogens, many laboratory methods especially clinical techniques may be inappropriate or give false information. This is especially true for automated laboratory tools which require matching to known signatures in the database.
Environmental microbes, since they do not produce disease and therefore are not isolated from patient samples, are not frequently represented in clinical databases.

Classical microbiology uses both gross and microscopic morphology to identify microbes. Gross morphology includes colony shape, size, and surface features. For example, *Bacillus atropheus* strain *globigii* produces an orange-pigmented colony on tryptic soy agar but produces small white colonies on other media. The structures assigned to bacteria cocci (round), bacilli (rods), or spirochetes (corkscrew) can be readily seen via light microscopy with no sample preparation. Generally, bacterial isolates are further differentiated at the time of microscopic examination by staining. The gram stain is one of the most useful and commonly used tools to differentiate bacteria beyond the genus level. This staining procedure named for its inventor, Hans Christian Gram, supplies both biochemical information about the composition of bacteria and special information regarding the distribution of chemicals within the cell. Gram-negative bacteria are unique in that they contain lipopolysaccharide (LPS), a polymeric structure found between the cell wall and the cellular membrane (found in all bacteria). LPS lies internal to the cell wall and helps regulate the permeability of the cell among other functions (Murch, 2003).

The first stain is crystal violet which stains all bacteria blue-purple followed by an iodine fixative. The critical step is then the decolorizer which is methanol. Methanol fixes the cells causing the pores in the walls of gram-positive cells to collapse and become sealed thus retaining the blue dye. The methanol also dissolves the lipid portion of the cell wall of gram-negative cells causing
them to become more porous and allowing the dye to leak out. At this point, gram negative cells can be distinguished from gram positive cells because they are clear. A red counterstain of fuchsin or safranin is used, giving the gram-negative cells a pink appearance to make their shape and size easier to see microscopically (Fletcher et al., 2006).

The patterning of staining can be important in categorizing bacteria to the species level. For example, the staining pattern of the bio threat agent, *Yersinia pestis* is described as “safety pin” since the gram-negative rod-shaped bacteria stains pink with the exception of blue staining at the ends of the rods indicating the specific exclusion of LPS from apical ends. Viruses, in contrast, have many more shapes by which they are categorized but can only be viewed using electron microscopy. Categorization by shape is still useful in this molecular age and is still the gold standard by which new viruses are identified. The newly emergent coronavirus, the causative agent of severe acute respiratory syndrome (SARS), was identified first by electron microscopic examination of patient samples followed by the use of molecular tools for genetic and immunological identification (Ksiazek et al., 2003).

Therefore, a toolbox for forensic analysis should include a combination of methods based on both classical methods and new molecular tools. High confidence can be assumed when these tests agree especially since the databases and signature libraries in general were built from and validated by organisms identified by classical methods (Scherer et al., 2003). Thus, there is an internal dependence of the new tools on the results predicted by the older classical
Casano et al., 1987 reported that a great deal of forensic information can be obtained from analysis of the composition of microbes beyond genetic information. Since microbes and microbial communities are dependent upon other community members in biofilms or microbial mats or on their hosts, significant amounts of information about microbial composition may not be directly determined from genome analysis. Growth conditions and growth media can play a significant role in the process of attribution.

Unlike mammals and higher eukaryotes, growth medium may significantly affect the molecular and structural composition of bacteria as well as the isotopic profile of its components. For example, the placement of freshly isolated samples of bacteria on laboratory media alters the fatty acid composition of the bacteria (Scherer et al., 2003; Kankaanpää et al., 2004). Fatty acid composition can be an important tool for microbial identification and strain distinction. It is unclear from these studies whether laboratory media are in themselves selective for new variants in the isolated sample with altered fatty acid profiles or whether the isolate itself changes to meet its changing nutrient availability (Scherer et al., 2003). Important to address is the issue of clonality in cultured materials. When isolating bacteria on plated media, most procedures are initiated from a single isolated colony on a plate. The incorrect assumption is that each colony arises from a single bacterial cell; however, in reality it can only be stated that a colony arose from at least one bacterial cell.

Many organisms do not grow as isolated individual strains. The bacilli and cocci shaped microbes discussed above do not always grow as individual
cells but may grow as chains or clusters, respectively. The average length of the chains or numbers in each cluster is a growth characteristic which is useful in distinguishing bacteria to the genus or species level. Both chain length and cluster size are influenced by culture media effects (Rhee and Pack 1980; Murdoch and Greenlees, 2004).

Microscopic examination of cultures of *Staphylococcus aureus* show “grape-like” clusters of cocci shaped bacteria the smallest of which is generally made of diplococcic (two cells) and the largest is 12 or more cells without robust efforts to disassociate these clusters prior to plating, it is unlikely that a single colony of *Staphylococcus* has descended from a single cell. Hence, enumeration of bacteria by colony count (serial dilution followed by plating on growth media) is really only an estimate and frequently an underestimate of the total number of viable bacteria present (Colwell, 2000). Depending on the viability of the sample, it may or may not reflect the number of target bacterial genomes present since polymerase chain reaction (PCR) based technologies do not differentiate between viable and dead cells. Better agreement is expected using direct counting via a Petroff Hauser chamber. The Petroff Hauser chamber is a low-tech solution in that it merely provides a calibrated area in which individual objects are counted microscopically. It does not discriminate between live or dead (Xu HS *et al.*, 1982).

Use of the Petroff Hauser chamber and colony counts can provide a good estimate of the health, i.e., relative viability of the sample. Even simple direct microscopic examination of raw samples combined with microbial culture can
provide the investigator with a better estimate of the initial concentration of microbial targets being analyzed in the original sample. Such examination can provide estimates of sampling variability based on the distribution of material in the sample. Unlike chemicals, bacteria and viruses are discrete units which sometimes adhere to each other or other materials present within a sample creating an uneven distribution and distortion of estimates of microbial targets when using indirect methods to evaluate concentration such as culture. It is important to note that culture, long considered the gold standard of microbiology, is being replaced by direct methods of microbial counting and detection with the development of culture independent methods for microbial evaluation of samples, it is possible to evaluate unculturable organisms in a sample. The ability to identify unculturable microbes further enhances the forensic analysis of a sample (Oliver, 2005).

4.2. Review of Literature

Oliver, 2005 reported that unculturable organisms may not be the target of concern for bio crimes since one of the underlying requirements for nearly all types of infectious agents are that they are easily cultured; however, contaminating unculturable microbes can provide information regarding comparative temporal and geolocation typing of microbial communities. At the species level and, in particular, at the strain level, the overall makeup of microbial communities can be extremely specific. Some microbes will persist in environments with few alterations for years, while other organisms will persist seasonally or be otherwise chronologically limited. In this case, both the
dynamics and stability of the population contain useful information for comparative purposes. It is important to stress comparison when considering characterization of unculturable microbes since the application space afforded by characterizing microbial communities and its variations can be infinite. Culture-independent tools include the use of PCR, microarrays, and mass spectrometry (described below) to identify the presence of bacterial species (Foschino et al., 2004).

With the revolution in molecular biology, the characterization and typing of microbes has become a matter of some debate. The source of much discussion among systematic biologists is the ordering of bacteria into specific classes. Much of this reassignment is due to the use of genetic methods to identify bacteria based on their DNA profiles rather than their morphology. Like the science of forensics, which is a continuum of methods from exclusion to attribution (Murch 2003), genetic profiling of microbes is a continuum from which it is sometimes difficult to make clear distinctions. Instead of cut offs or limits, it is better to compare based on a degree of “sameness” when comparing two strains of bacteria. PCR is a process by which small amounts of nucleic acid is synthesized in vitro to make large amounts of an exact or nearly exact copy of nucleic acid. As a result, PCR has enabled countless new applications in human forensics, medicine, and agriculture by providing enough material for more robust analyses without the need for a living organism. PCR has been the subject of many excellent recent forensic reviews and so will be discussed here relative to microbial applications (Budowle et al., 2005; Gill, 2002). Briefly, PCR works by amplifying DNA or, in the case of reverse transcriptase PCR (RT-PCR),
amplifying RNA. This is possible due to the discovery of thermo stable polymerases which retain their ability to extend short oligomers of nucleotides known as primers (Saiki, 1988).

Lindstedt, 2005 reported that all DNA polymerases, a template is required in order for the polymerase to extend the new complementary DNA strand. Therefore, extension of the primer only occurs when the primer is bound to its DNA target. The number of DNA targets is limiting in the reaction initially. So to increase the number of DNA templates after the first extension is complete, the two DNA strands are dissociated by raising the temperature to the point where the DNA melts. When the temperature is lowered, the next primer which is in high concentration in the reaction binds to the DNA template, and the process begins again. Using one primer, the process is rather slow and the increase in the DNA is linear. To make the process of amplification logarithmic, a second primer is added which binds downstream of the first primer on the complementary DNA template and any amplified DNA which have the DNA template sequence. Therefore, after the first few cycles of primer binding (annealing), primer extension by the polymerase, and DNA melting, amplification of the original DNA template is rare in comparison with amplifications of primers binding and re amplifying newly synthesized DNA (Denoeud and Vergnaud, 2004).

The product of this reaction is a short segment of tens to thousands of base pairs of double-stranded DNA and, in acknowledgement of its synthetic origin, is known as an amplicon. In their most primitive form, amplicons are visualized by gel electrophoresis and staining to determine if the reaction
succeeded in producing product and if the product’s size is consistent with predicted results. A whole variety of tools have been developed to make the detection of the amplified products more efficient such as (1) the addition of fluorophores to the end of the primer for direct fluorescent detection of amplicons without staining, (2) the use of internal probes whose exact binding to sequences within the amplicon produces a light-emitting reaction known as Fluorescent Resonance Energy Transfer (FRET) (FRET emission can only occur if the two internal primers are within one base pair of each other and if the amplicon sequence matches perfectly, thus providing sequence information about the amplified product as well as its size), and the addition of intercalating DNA dyes into the reaction. The intercalating dye fluorescence is enhanced only when the dye is able to bind double-stranded DNA. Generally, the template DNA is present in too small a quantity to contribute to the reaction. Amplicons can then be used in other assays, sequenced directly, or inserted into other genes or organisms. By carefully designing the complementary primer sets used for amplification, it is possible to amplify any sequence of DNA uniquely. Because of this, PCR based methods have caused concern in the law enforcement and scientific community, as it has been demonstrated that whole microorganisms can be regenerated by this means (Atlas et al., 2003).

Nevertheless, PCR has provided investigators with a molecular tool needed to examine small amounts of materials for low abundance microbial targets. PCR has been used to detect pathogens directly by a number of methods. Most common is to target a gene which is important to the function of a pathogen. Panels of primer sets have been published for nearly every pathogen
including bioterrorism agents. Most of the gene targets for these panels use virulence factors as a condition of pathogenicity. For example, initial screening of forensic samples to find the source of a suspected *Bacillus anthracis* attack would likely include amplification and detection of genes for protective antigen (pag), lethal factor (lef) or edema factor (ef), and capsule (cap). All of these genes must be present for virulence (Koehler, 2002).

Rapid testing of these targets and others such as BA 813, a chromosomal marker specific for the *Bacillus cereus* subspecies, can be accomplished on suspected spore preparations, i.e., white powders, without extensive sample cleanup. Amplification of BA 813 is evident when whole spores or vegetative cells are added directly to the PCR reaction without extraction or extensive methods to destroy the cell structure autoclaved spores which have been thermally disrupted and intact spores added to a PCR reaction amplify similarly. This method has been used for the toxin genes named above with similar results and sensitivities (Jackman, personal communication). This method works well because DNA is found on the external surface of spores (Thibodeaux *et al.*, 2007).

The exosporal DNA is intrinsically associated with the spore surface. Copious washing does not remove the DNA even when other DNAs added to the spore preparation are easily removed, suggesting that this DNA is tightly retained by the endospore during the sporulation by an as yet unknown mechanism. Our estimates based on enumeration of spore preparations and quantification of DNA target copy number indicate that about 1–10% of total genomic DNA is retained.
on the spore surface (Drickamer and Taylor 1998). While the sensitivity of the test based on gene copy number is lower overall by about tenfold as compared to clean extracted DNA, the assumption for rapid screening for hazard assessment and sample collection involving white powders is that the number of spores is not limiting. Pure dry powdered *Bacillus anthracis* spore preparations contain between 10^{12} and 10^{14} spores per gram depending on the sample preparation methods and amount of contaminants remaining. When adding vegetative bacteria directly to the PCR reaction, amplification is likely enhanced by the release of internal DNA by the process of heating and cooling in a typical PCR cycle (Maidak *et al*., 1997).

Heat shock is used to kill off by lysis vegetative cells in microbial preparations where only the number of spores (typically heat resistant). This property of vegetative cells can be easily exploited by the PCR process to liberate intracellular DNA targets for amplification. Appropriate controls should be used always to verify that contaminants present in the sample do not inhibit the PCR reaction. Microbiologists have an extensive combination of tools available to them which include the old standards of culture, microscopy, and biochemistry upon which the field of microbiology was built as well as new standards which are heavily attentive to differences in DNA sequence (Drake *et al*., 1998).

While the genetic profiling methods will have a remarkable impact on the ability to rapidly identify an unknown pathogen, speciate microbes, and discriminate isolates, other techniques should be applied in order to capture information for attribution which is not located in the genome. In the post
genomic age, tools such as mass spectrometry hold promise for general application to the area of attribution by providing information regarding contaminants, co-associating viruses, media, or host residues and other hitchhiking factors. The highest confidence approaches will employ a variety of orthogonal and parallel processes in order to gain the greatest argument in support of an attribution source.

4.3. Materials and Methods

4.3.1. Morphological characterization

4.3.1.1. Gram’s staining

The Probiotic and amylase positive isolate AP3 was Gram stained according to the procedure (Cappuccino and Sherman, 2002). The isolate was smeared in the slide and heat fixed. The crystal violet dye was added, kept for 1 minute and washed in running water. Gram’s iodine was iodine was added, kept for 1 minute and washed in running water then the smear was decolorized with ethanol and finally the counter stain safranin was added after a minute washed in running water. It was observed under the binocular microscope (Olympus-mlx-B-Magnes) Gram-positive cells were purple in colors and Gram negative cells were red in colors.

4.3.1.2. Spore Staining

The Probiotic and amylase positive isolate AP3 was smeared in the slide and heat fixed. The malachite green dye was added and it was heat fixed, kept for 1 minute and washed in running water. It was decolorized with tap water and
counter stain safranin was added after a minute, washed in running water. The isolate was observed under the the binocular microscope (Olympus-mlx-B-Magnes). Spore formers were green in colors and nonspore formers were red in color.

4.3.1.3. Hanging drop method

Motility test was performed by hanging drop technique (Gunasekaran, 1995) cover slip was coated with Vaseline on it is edges. The isolate was transferred into center of the cover slip. The cavity slide was placed over the cover slip and turned over to prepare hanging drop the slide was viewed under the microscope. The motility was determined from the swarming movement of the microorganism.

4.3.2. Electron Microscopic analysis of bacterial structure

The scanning electron microscope (SEM) will scan the ultra image of the specimen. The size of the isolated bacterial strain was estimated with SEM. Sample photos were taken by a Quanta 200 scanning electron microscope made by FEI, at magnification of 5000 X and 10,000X at Alagapa Chettiyar Institute of Science and Technology, Chennai. The sample was dried with platinum coating under coating holder. This dried specimen was tested under high vacuum in natural state, without sputtering a platinum layer on the sample. We estimate the size of the specimen. The ultra structure of the specimen was recorded with a computer analyzer.
4.3.3. Biochemical characterization (Cappuccino and Sherman, 2002).

4.3.3.1. Indole Production Test

Indole production test was used to test whether the organism can oxidize tryptophan resulting in the formation of indole, pyruvic acid and ammonia. Tryptone broth was inoculated with the isolate and one tube was kept as an uninoculated comparative control incubate tubes for 24-48 hrs at 37ºC. The indole production during the reaction was detected by adding Kovac’s reagent (dimethyl aminobenzaldehyde) which produces a cherry-red layer in the top of the test tube was indicates positive. There was no development of a cherry (deep) red colour in the top layer of the tube indicates negative reaction.

4.3.3.2. Methyl Red test

The methyl red test was employed to detect the ability of microorganisms to oxidize glucose with the production of high concentration of Acid and acid products. MR-VP broth tubes were inoculated with the isolate. One tube was kept as uninoculated comparative control. Incubate tubes for 24-48 hrs at 37ºC. After 48 hrs of incubation, 5 drops of methyl red indicator was added, the colour of methyl red turned to red indicates positive test. The colour of methyl red turned to yellow indicates negative test.

4.3.3.3. Voges-Proskaur test

Voges-Proskaur test was used to differentiate the microorganisms to produce some non acidic or neutral end product such as acetyl methyl carbinol
and 2, 3 butanediol. The isolate was inoculated with test tubes containing MR-VP broth. One tube was kept as uninoculated comparative control. Incubate tubes for 24 hrs at 37°C. Development of deep red colour in the inoculated tubes 15 minutes after addition of Barrit's reagent was the indication of positive the absence of red colouration indicates negative.

4.3.3.4. Citrate utilization test

Citrate test was used to differentiate bacteria on the basis of their ability to utilize citrate as the sole carbon source. The utilization of citrate depends on the presence of an enzyme citrase produced by the organism that breaks down the citrate to oxaloacetic acid and acetic acid. Simmon’s Citrate agar slants were inoculated with the selected isolate. An uninoculated Simmon’s Citrate agar slant was kept as control. Then these tubes were incubated at 37°C for 24-48 hrs. Development of green to blue color and this constitutes a positive test. No change in the colour of the medium indicates negative.

4.3.3.5. Catalase tests

Trypticase soyagar slants were inoculated with the selected isolate. An uninoculated trypticase soyagar slant was kept as control. Then these tubes were incubated at 37°C for 24-48 hrs. While holding the inoculated tube at an angle, allow 3-4 drops of hydrogen peroxide to flow over the growth of each slant culture. Catalase positive microorganisms were indicated by production of bubbles of oxygen within one minute after addition of H₂O₂. Catalase negative microorganisms were indicated by no bubble of oxygen production.
4.3.3.6. Oxidase Test

Oxidase test to check the presence of the electron transport chain that was the final phase of aerobic respiration of microorganisms. Normally, oxygen was the final electron acceptor for this system. In the oxidase test, an artificial final electron acceptor (N,N,N',N'-tetramethyl phenylenediamine dihydrochloride) was used in the place of oxygen. This acceptor changes color to a dark blue or purple when it takes the electron from the last element cytochrome oxidase in the electron transport chain. With a sterile swab, obtain a small amount of isolated culture from an agar plate. Place one drop of oxidase test reagent onto the culture on the swab. Positive reactions turn the bacteria violet to purple immediately or within 10 to 30 seconds. Delayed reactions should be ignored.

4.3.3.7. Starch hydrolysis

The ability to degrade starch was used as a criterion for the determination of amylase production by a microbe. Starch hydrolysis test to determine the absence or presence of starch in the medium by using iodine solution as an indicator. Starch in the presence of iodine produces a dark blue colouration of the medium and a clear zone around a colony indicates amylolytic activity. The isolate was streaked on sterile starch agar plates and incubated at 37ºC for 48 hrs. The plates were flooded with 1 % of iodine. Amylase production was indicated by colourless clear zone surrounded by the microbial isolate and rest of the plate appeared dark blue colouration.
4.3.3.8. Carbohydrate fermentation (Glucose utilization test)

About 2 or 3 loopful of 18-24 hrs old broth culture of isolate was inoculated into the glucose or sucrose broth medium and incubated for 24 and 48 hrs at 30 ºC. The collection of gas inside the Durham’s tube was found positive for gas production, and the change in colour of the medium to yellow indicated acid production. No gas and colour change was indicates negative (Sharma, 2002).

4.3.3.9. Nitrate Reduction

Nitrate broth was inoculated with a loopful of selected isolate and incubated at 28ºC for 7 days. Uninoculated nitrate broth was kept as control. Control was also run without inoculation. After incubation two drops of sulphanilic acid followed by two drops of α – naphthylamine solution were added. The presence of nitrate was indicated by a pink, red or orange colour and absence of colour change was considered as nitrite negative.

4.3.3.10. KBOO2 Hi Assorted™ biochemical Test (By Kit)

The biochemical test were carried out for citrate utilization, lysine decarboxylase, ornithine decarboxylase, urease, phenylalanine deamination, nitrate reduction, H₂S production, glucose, adonito, lactose utilization, arabinose utilization, sorbitol according to company instruction (Hi Media Mumbai) 50µl of the isolated organism was inoculated into each well in the test kit by surface inoculations method. The kit was incubated at 37°C for 24 hrs based on the principle of pH change after substrate utilization. On incubation, organisms
undergo metabolic changes which were indicated by color changed in the media that can be either interrupted visually or after the addition of reagent.

4.3.3.11. KBOO9 Hi Carbohydrate\textsuperscript{TM} utilization Test (By Kit)

The carbohydrate test based on the principle of pH change and substrate utilization. On incubation organism undergo metabolic changes which were indicate by a Spontaneous colors change in the media. The organisms were analysis for the utilization of Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Rabinose, Trehalose, Melibiose, Sucrose, L-Arabinose, Mannose, Inulin, Sodium Gluconate, Glycerol, Salicin, Glucosamine, Dulcitol, Inocitol, Sorbitol, Mannitol, Adonitol, α-Methyl-D-glucoside, Ribose, Rhamnose, Cellobiose, Melezitose, α-Methyl-D-mannoside, Xylitol, ONPG, Esculin, D-Arabinose, Citrate, Malonate, Sorbose 50\(\mu\)l of broth containing the sample was inoculated into the well provided in the kit and incubation was carried out at 37\(^\circ\)C for 24 hrs.

4.3.4. Molecular identification of bacteria

4.3.4.1. Genomic DNA isolation

The genomic DNA was isolated by the method of Veggin and Giovannoni (1999). A single colony was inoculated into 50 ml of LB medium and incubated at 37 \(^\circ\)C under agitation. The cells were collected by centrifugation at 10,000 rpm for 10 min. The pellet was resuspended in 10 ml Tris-buffer containing 10% glycerol (Tris 25 mM; EDTA 10 mM; pH 8.0). Lysozyme (10 mg) was added to the above contents and incubated overnight at 37 \(^\circ\)C. Proteinase-K was added to
the final concentration of 200 μg/ml and allowed to stand at 50 °C for 12 h. 1 ml of 10% SDS was added and incubated overnight further at 50 °C. The solution was cooled to room temperature and then extracted with phenol, phenol: chloroform and then chloroform. The aqueous phase was transferred and 0.2 Volume of 10 mM sodium acetate and 2.5 vol of ethanol was added and kept at -70 °C for an hour. DNA was recovered by centrifugation. The pellet was rinsed with 70% ethanol and air-dried. The pellet was suspended in TE (Tris-HCl 10 mM, EDTA 1 mM) buffer and the absorbance was measured at 260 and 280 nm spectrophotometer.

4.3.4.2. DNA Quantification

The DNA concentration and quality were calculated using the absorbance at A₂₆₀ and A₂₈₀ by the spectrophotometer measurement and adjusted to a final concentration of 0.5 μg/µl. $50 \times 200 \times OD_{260} = DNA \ Concentration \ (µg/ml)$

Where ‘50’ was the correction coefficient for the double stranded DNA, ‘200’ was the dilution factor (1:200).

4.3.4.3. DNA extraction from gel using gel extraction column

DNA from the 0.7% agarose gel was extracted using Prefect prep Gel extraction column (Eppendorf, Germany) according to manufacture instruction.

4.3.4.4. Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with 0.5X TAE as described by Veggin and Giovannoni (1999). The
DNA samples were diluted with 1/6 volume of 6X loading buffer and deionized water. Samples were loaded on ethidium bromide containing (0.5 µg/ml) agarose gel along with a size standard. Electrophoresis was performed at 5 v/cm until the run was complete. After completion of electrophoresis, the gel was visualized with 254nm transilluminator and photographed with a gel documentation system.

4.3.4.5. 16S ribosomal RNA (rRNA) gene sequencing

PCR on the extracted DNA was performed in a 100-µl volume. Oligonucleotide primers with specificity for eubacterial 16S rRNA genes, primers 16S rDF CGCTGGCGGCAGGCTTAACA); 16S rDR (CCAGCCGCAGGTTCCC CT) were used to amplify the 16S rRNA gene fragments with template DNA originating from bacteria. The following conditions were used for DNA amplification: 35 cycles consisting of denaturation at 94ºC for 0.5 min, annealing at 55 º C for 1 min, and extension at 72 ºC for 1.5 min followed by a final extension at 72ºC for 3 min. Amplified PCR products of the proper size (about 1500 base pair fragment) were confirmed by electrophoresis of 10 µL sub samples through a 1% horizontal agarose gel containing 0.5 lg/mL ethidium bromide. Gels were examined under UV light and photographed. (Acinas et al., 2004).

PCR products were purified using QIA quick Spin columns (Qiagen Inc., Chatsworth, CA). A Perkin Elmer 377 DNA sequencer, in combination with Dye Deoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, CA) was used for sequencing the purified PCR products by the help of MWG bioinformatics centre. Nucleotide sequences were compared with sequences in the
National Centre for Biotechnology information (NCBI). GenBank database using the BLASTn program and Ribosomal Database Project (RDP) database using the sequence matching program.

4.3.4.6. Phylogenetic analysis

Once the sequencing was done, the resultant nucleotide sequence subjected to n-BLAST analysis (http://blast.ncbi.nlm.nih.gov) in National Center of Biotechnology Information (NCBI) and deposited in genbank database for universal accession. By using query and control sequence a phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis Tool (MEGA 5.1) (Felsenstein, 1985; Tamura, 2011).

4.4. Results and discussion

4.4.1. Morphological Characterization

The selected antagonistic isolate AP3 was Gram positive, rod shaped, spore forming motile bacteria (Table 4.1 and Plate 4.1). Gram-positive, rod-shaped bacteria that differentiate into heat-resistant endospores under aerobic conditions were placed in the genus Bacillus (Priest, 1993). Bacteria belonging to the genus Bacillus have a long and distinguished history in the realms of biotechnology. They were probably first used by the Japanese in the preparation of a traditional fermented food from rice straw and soybean, itohiki-natto. This derives from the action of “Bacillus natto” (a derivative of B. subtilis) on steamed soybean and results in a viscous, sticky polymer (primarily polyglutamic acid) that forms long, thin threads when touched. Natto has been prepared in
Japan for at least four hundred years, and currently consumption was about 108 kg per annum. Exploitation of *Bacillus* in the west is more recent. Manufacture of extracellular amylases and proteases for industrial applications began early this century, but significant production and usage was delayed until after the 1950s when the advantages of including the alkaline protease of *Bacillus licheniformis* in washing detergents was realized. This was followed by developments in the starch processing industry based on the a-amylase from *B. licheniformis*, particularly the conversion of starch to high-fructose corn syrups as sucrose replacements in foods and beverages.

4.4.2. Electron Microscopic analysis

Under SEM, the morphology of the organism all possessed appendages on their surface. In addition, there were no visible changes in the outer surface of the spores. Plate (4.2) shows an electron micrograph of ultra structure of AP3 revealed the rod shaped cylindrical molecule with a diameter of 50 nm. (Ragkousi and Setlow, 2004), suggesting that *B. subtilis* also cross linked in the exosporium. Thus, the lack of cross linking may affect the structural stability of the exosporium in the deficient spores.

4.4.3. Biochemical Characterization

The isolate AP3 exhibited positive to voges proskauer, citrate utilization, oxidase, nitrate reduction, gelatin liquefaction, starch hydrolysis, arbinose, lycine decarboxylase and Ornithine decarboxylase. Further this isolate showed negative for the following reactions, such as indole, methyl red, catalase, hydrogen
sulphide, urease, glucose utilization, and Adonitol. These results suggested that all the isolate belong to the genus *Bacillus* sp. (Table 4.2 and Plate 4.3).

4.4.4. Analysis for carbon utility test

Among the thirty five carbon sources, this strain used and ferment the xylose dextrose, galactose, lactose, melibiose, L-arabinose, mannose, glucosamine, sorbitol, mannitol, D- arabinose and citrate as a sole source of carbon for their growth. However, they could not hydrolyze maltose, fructose, rabinose, trehalose, sucrose, inocitol, sorbitol, adonitol, α-methyl-D-glucoside, ribose, rhamnose, cellobiose, melezitose, α-methyl-D-mannoside, xylitol, ONPG and esculin. Based on the characteristic features exhibited by the isolate. Isolates was confirmed as *Bacillus* sp. (Table 4.3 and Plate 4.4).

4.4.5. Molecular identification

The 16S rRNA gene of the AP3 strain was amplified by PCR techniques using the 16S rDNA universal primers and both the strands were sequenced (Plate 4.5). The resultant nucleotide sequences subjected to n-BLAST analysis (http://blast.ncbi.nlm.nih.gov) in National Center of Biotechnology Information (NCBI) and deposited in genbank database for universal accession. Using query and control sequence a phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis Tool (MEGA 5.1) (Plate 4.6 and 4.7). Sequence analysis had revealed that the strains were phylogenetically closely related to the genus *Bacillus* sp. BLAST analysis of the 16S rRNA sequence of AP3 isolate revealed that it was more similarity score bit with *Bacillus subtilis*. 

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4.4.6. Tree inference

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates were taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1491 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

16S rRNA sequencing revealed that *B. subtilis* was the predominant species followed by *B. cereus*. *B. pumilus* belongs to *Bacillus* group of aerobic spore-forming organisms, which has lately evoked considerable research interest due its involvement in cases of extra cellular enzyme synthesis (Fritze, 2002; Acinas *et al*., 2004). A study by Banerjee *et al*., 2007 reported experimental induction of *Bacillus* controlling pathogenic bacteria from shrimp and Artemia while some compounds produced by *B. subtilis* were reportedly toxic to fish pathogens (Priest, 1993).

With the introduction of modern taxonomic techniques such as numerical phenetics, DNA base composition determinations and DNA reassociation experiments which allow DNA sequence homology between strains to be
estimated, it became apparent that the bacilli were more heterogeneous than hitherto suspected. The range of DNA base composition among strains was a good indicator of genetic diversity indeed it was generally agreed that species in a genus should vary by no more than 10–12 mol% G+C (Brosius et al., 1978; Joung and Cote, 2002). In the case of *Bacillus*, the range is about 33 to 65% although strains of most species cluster between 40 and 50% (Priest, 1993). This indicates considerable genetic diversity among species and suggests that the genus should perhaps be split into several, more homogeneous taxa.

Numerical classification has also helped clarify relationships between bacilli at the species level, although in most cases this was better done by DNA reassociation studies. It was reassuring that, in general, numerical classification and DNA homology has given concordant results. In many areas, for example *Bacillus circulans*, *B. megaterium*, *B. sphaericus*, *B. Stearothermophilus* and *B. subtilis*, examination of strains by these techniques has revealed that Chun and Bae, 2000 “lumped” strains into species rather too enthusiastically and that each of these species probably represents several taxa (Saitou and Nei, 1987). *B. subtilis sensu lato*, for example, was now known to include *B. amyloliquefaciens* and *B. atropheus* as well as *B. subtilis* itself and *B. circulans sensu lato* encompasses numerous species including *B. alginolyticus*, *B. amylolyticus*, *B. chondroitinus*, *B. glucanolyticus*, *B. lautus*, *B. pabuli* and *B. validus* as well as some unnamed DNA homology groups. Roberts *et al.*, 1994 reported that these revisions of several taxa, together with the isolation and naming of new strains, has led to the expansion of *Bacillus* and the genus now includes at least 67 validly described species.