ANTAGONISTIC EFFECT OF BACTERIAL STRAINS ISOLATED FROM PRESERVED SEA FOOD

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Abstract

Seafood is a nutrient-rich part of a healthful diet, and seafood consumption is associated with potential health benefits. Seafood is responsible for an important proportion of food-borne illness and outbreaks worldwide. *Vibrio sp* and *Clostridium botulinum* have the major role of the seafood contaminations. There have been relatively less efforts to search antagonistic bacteria from preserved seafood harboring microbial diversity. Hear, is to evaluate the antimicrobial activity against human pathogenic bacteria. A total of two unknown strains (S1, L1) were isolated from prangipettai, Tamil Nadu, India. The two unknown (S1, L1) strains were under the biochemical and staining techniques. The two unknown strains (S1, L1) were standardized under the condition of EUBAC and 16S rRNA gene. The PCR results that the strain S1 is *Bacillus cereus* and the strain L1 is *Bacillus anthracis.* The standard antibody was tested against the (S1, L1) sample. The antimicrobial result is that *Bacillus anthracis* against the *Klebsiella pneumonia.* The results from the present study will provide a basis for developing possibly involving the conservation and management of the species.

Key words: antimicrobial activity, *Bacillus cereus*, *Bacillus anthracis*, *Klebsiella pneumonia*.

INTRODUCTION

Seafood includes mollusks (e.g., oysters, clams, and mussels), finfish (e.g., salmon and tuna), marine mammals (e.g., seal and whale), fish eggs (roe) and crustaceans (e.g., shrimp, crab, and lobster). Some seafood commodities are inherently more risky than others owing to many factors, including the nature of the environment from which they come, their mode of feeding, the season during which they are harvested, and how they are prepared and served. Fish, mollusks, and crustaceans can acquire pathogens from various sources.

All seafood can be susceptible to surface or tissue contamination originating from the marine environment. Bivalve mollusks feed by filtering large volumes of seawater. During this process, they can accumulate and concentrate pathogenic microorganisms that are naturally present in harvest waters. Contamination of seafood by pathogens with a human reservoir can occur when growing areas are contaminated with human sewage. Outbreaks of seafood-associated illness linked to polluted waters have been caused by calici virus, hepatitis A virus, and *Salmonella enterica* serotype Typhi (Desenclos, *et al* 1991). Identified sources of seafood contamination have included overboard sewage discharge into harvest areas, illegal harvesting from sewage-contaminated waters and sewage runoff from points inland after heavy rains or flooding. Additionally, seafood may become contaminated during handling, processing, or preparation. Contributing factors may include storage and transportation at inappropriate temperatures, contamination by an infected food handler, or cross-contamination through contact with contaminated seafood or seawater. Adequate cooking kills most pathogens; however, unlike other foods, such as meat and poultry, that are usually fully cooked, seafood is often consumed raw or prepared in ways that do not kill organisms.

A number of bacterial illnesses may arise from the consumption of seafood that has either been contaminated at source or during the processing and retail chain. Such illnesses may arise from infection with the bacteria themselves or by the ingestion of toxins formed in the foodstuff prior to consumption. This division is actually too simplistic: for example, the toxins of *Clostridium botulinum* and *Staphylococcus aureus* are preformed in food during bacterial growth, the toxin of *Clostridium perfringens* is usually only formed when the bacteria sporulate in the intestinal tract while the toxin of *Vibrio cholerae* O1 (and O139) is produced when the bacteria multiplies in the intestinal tract. One of the most common illnesses associated with seafood is scombrotoxin, due to the production of histamine and possibly related compounds by certain types of Gram-negative bacteria growing on histidine rich fish tissue. There is increasing interest in the vibrios as agents of bacterial illness associated with the consumption of bivalve molluscan shellfish and other types of seafood and particular attention will be paid to this group of bacteria.

Seafood-borne illnesses may be caused by biological, chemical or physical agents. Biological pathogens are represented by a vast array of bacteria, viruses and parasites. According to HUSS et al., (Huss, 2000) pathogenic bacteria found in seafood and related products may be divided into three groups:

1) Bacteria that may be naturally present in the habitat of the consumed species, such as *Vibrio spp. (V. parahaemolyticus, V. cholerae, V. vulnificus), non-proteolytic Clostridium botulinum type B, E and T, Plesiomonas shigelloides and Aeromonas spp;*

2) Bacteria present in the environment in general (Listeria monocytogenes, proteolytic *Clostridium botulinum* type A and B, *Clostridium perfringens and Bacillus spp;*

3) Bacteria which have their usual habitat in human or animals (*Salmonella spp., Shigella spp., Escherichia coli, Campylobacter jejuni and Staphylococcus aureus).*

The presence of Huss Group 1 and 2 bacteria in live fish or fresh raw fish is rarely a safety concern because tissue concentrations are too low to cause disease. However, the accumulation of large numbers of pathogens (*Vibrio spp*.) in filter-feeding shellfish represents a risk, especially since shellfish are often consumed raw. Pre-harvesting contamination with pathogens from the animal-human reservoir (Huss Group 3) may pose a risk since in some cases, where a very low infective dose is enough to cause illness (*Shigella and Salmonella serotypes*). Normal cooking procedures will eliminate the risk of contamination. Safety concerns are therefore primarily related to the consumption of raw shellfish and raw fish dishes like ceviche or sushi (Huss 2000).

In order to control these bacteria, from contaminating seafood and further causing illness, extensive knowledge is required regarding their origin, biology, physiology, ecology, survival, growth and prevalence in seafood and related products, along with the epidemiology and symptomatology of the diseases with which they are associated.

Clostridium botulinum:

Clostridium botulinum is a strictly anaerobic, Gram-positive, peritrichous, rod-shaped, spore and gas forming bacterium ubiquitous in soils and aquatic sediments. The organism is classified into types according to serological specificity. Each type secretes a different toxin, referred to as A, B, C, D, E, F and G. When found in humans, the E type is usually associated with the consumption of seafood and related products (Frazier, *et al* 1988). According to ketcham & gomez (Ketcham, 2008), spores of the E type can germinate at temperatures below 3˚C and are often found in association with cold-stored seafood.

Botulinum toxin is an active exotoxin (even more potent than tetanus toxin) with neurotropic action (affecting the nervous system). It is the only bacterial toxin which can be fatal upon ingestion and may be regarded as a biological poison. It is lethal at very small doses (1/100-1/120 ng). Unlike the spores, the toxin is thermo liable and is destroyed if exposed to 65-80 ºC for 30 minutes, or to 100 ºC for five minutes (Elliot,1995), whereas according to Ketcham & Gomez (2008), spores in contaminated food may be destroyed if exposed to 120˚C for 30 minutes. In packaged and sealed foodstuffs, spores germinate under anaerobic conditions provided the pH value is above 4.5 and there is enough water activity. Thus, once the spores in packaged foods have germinated, vegetative cells will produce *botulinum* toxin during storage (Scarcelli, 2008).

Non-proteolytic *Clostridium botulinum* type B, E and F primarily inhabit temperate and arctic aquatic environments, whereas type E multiplies in putrefying aquatic organisms, usually at low densities (< 0.1 spores/g fish, though exceptionally up to 5.3 spores/g fish) (Huss.2000)

Vibrio spp:

The genus *Vibrio* consists of Gram-negative, curved rod-shaped facultative anaerobes endowed with a single polar flagellum. The genus includes at least 12 species pathogenic to human, 10 of which may be food borne. Most *Vibrio*-related food-borne illnesses are caused by *V. parahaemolyticus, V. cholerae and V. vulnificus* (Dalsgaard *et al* 1996) *V. parahaemolyticus and V. cholerae* have been isolated in cases of gastroenteritis caused by contaminated food (both species) and contaminated water (the latter*). V. vulnificus* is mainly observed in extraintestinal infections (septicemia, wounds etc.). Primary septicemia caused by *V. vulnificus* is usually associated with the consumption of seafood, especially raw bivalves.

Pathogenic Vibrios, especially *V. cholerae*, also occur in fresh water and in estuaries (Desmarehelier 1997) to which it may be introduced by way of fecal contamination. Unlike most other Vibrios, *V. cholerae* can survive infresh water. The mechanisms of epidemic pathogenicity of *V. cholerae* and *V. parahaemolyticus* have been extensively investigated and are well known. Environmental strains of these bacteria may be virulent or not, depending on their ability to produce virulence factors. An important pathogen, *V. vulnificus* is associated with high levels of fatality, but fortunately infections are rare and tend to be limited to individuals with chronic disease or immunodeficiency.

Characteristics shared by most *Vibrio* species include sensitivity to low pH values, infrequent association with highly acidic foods, and inhibition of virulence by adequate cooking. However, the three most pathogenic vibrio species differ in a number of important aspects.

Vibrio parahaemolyticus:

This mobile, Gram-negative and rod shaped microorganism is distributed worldwide in marine environments, but it is most abundant in warmer regions. It is often isolated from seafood from marine and estuarine origin, especially bivalves (Su, 2007). It is found on aquatic animals, especially crustaceans and mollusks, at temperatures above 8˚C and thrives in alkaline media containing 2-4% NaCl at 37˚C. Due to its high multiplication rate (5-10 min) under appropriate conditions and its ability to compete with other microorganisms, a small number of infecting cells in seafood at room temperature rapidly becomes a threat to seafood consumers.

V. parahaemolyticus can cause diarrhea, cramping abdominal pain, vomiting, fever and headaches at a concentration of 10^6 - 10^9 CFU/g. As a mesophilous organism, *V. parahaemolyticus* is easily eliminated from seafood by exposure to heat, but when seafood is served raw (such as oysters, mussels, sushi, sashimi and carpaccio), consumers are at risk of infection (ICMSF 1978). According to Brazilian regulations (RDC 12), the maximum concentration of *V. parahaemolyticus* allowed in foodstuffs is 10^3 CFU/g.

In 1965, an analysis of strains of *V. parahaemolyticus* from gastroenteritis patients at a hospital in Yokohama, Japan, revealed a hemolytic enzyme - thermostable direct hemolysin (TDH) - not observed in isolates from seafood and the environment. The presence of TDH, as detected by the Kanagawa test, is associated with enteropathogenicity (Wong, 1999). Strains of *V. parahaemolyticus* can cause serious infectious outbreaks if they carry the genes *tdh* and/or *trh* (Kaper 1995) or if they are capable of hydrolyzing urea and inducing beta-hemolysis in blood agar (Okuda 1998).

The risks associated with the consumption of seafood contaminated with *V. parahaemolyticus* were evaluated in a recent study published jointly by the World Health Organization and the Food and Agriculture Organization of the United Nations.

Vibrio cholerae:

V. cholerae occurs naturally in fresh and brackish water in tropical, subtropical and temperate regions. Strains of serotype O1 and O139 usually carry the gene ctx and produce cholera toxin. These toxigenic strains are responsible for cholera epidemics around the world. The disease is exclusively human and human feces are the primary source of infection. Cholera epidemics are mostly restricted to developing countries at warmer latitudes (Wachamuth 1994). The contamination of environments involved in food production (including aquaculture ponds) with feces from infected individuals may indirectly introduce toxigenic *V. cholerae* strains into foodstuffs. The concentration of free toxigenic *V. cholerae* in the natural environment is low, although the species is known to be capable of attaching to and multiplying on zooplankton especially copepods (Hug 1983).

Strains of *V. cholerae* O1 may be classified into two biotypes, Classic and El Tor, based on phenotypic traits (Kaper 1995). For unclear reasons, infection tends to be more severe when caused by the Classic biotype than by El Tor. Approximately 20% of infected individuals develop acute aqueous diarrhea, which in 10-20% of cases evolve into severe aqueous diarrhea with vomiting. Without immediate and proper treatment, the infection can lead to intense dehydration and death in a matter of hours, with a fatality rate up to 30-50% among family members. However, when treatment is timely and adequate, fatality rates are reduced to less than

1% O serotypes other than O1/O139, referred to as non-O1/O139 strains, can induce food-related diarrhea which is less severe than illness associated with cholera.

Vibrio vulnificus: Phenotypically, V. vulnificus is highly homologous with *V. parahaemolyticus*, but differs by its ability to ferment lactose, justifying its early classification as "lactose-positive vibrio". According to (elliot et al.1995), strains of *V. parahaemolyticus and V. vulnificus* may be differentiated by several biochemical tests, such as the b-galactosidase assay. The name *V. vulnificus* was formally adopted in 1979 (Horre 1996).

Clinical and epidemiological investigations have shown that *V. vulnificus* can cause septicemia and death in human through contaminated seafood (by penetrating the blood stream from the gastrointestinal tract) or through wounds exposed to contaminated marine environments (Almeida, 2004).

According to (huss et al 2008), *V. vulnificus* produces extracellular cytotoxins and hydrolytic enzymes capable of rapid muscle tissue degradation during infection. The presence of capsular polysaccharide is essential to trigger the infectious process. Three biotypes of *V. vulnificus* have been identified; approximately 85% of strains isolated from clinical samples belonging to biotype 1, while biotype 2 is known to cause infection in eels. Biotype 3 was first described recently in association with seafood related bacteremia.

Antibiotics are substances used to treat infection caused by microbes. Antibiotics acts by inhibiting or destroying bacterial cells (Demain, 1998) that cause certain disease. In nature, antibiotics are secondary metabolites produced by bacteria (Teasdale *et al.,* 2004) in order to maintain their niche and territory. Only limited groups of microorganisms are responsible for the sources of clinically usable antibiotics. As stated by Cooke and Gibson (1983), only those have an effect on bacterial cells but not the host cells like human categorized as useful antibiotics. Besides, there are only limited numbers of antibacterial actibiotics that can be used to treat bacterial infections (Motta *et al* 2004). Antibiotics can be found easily but only few are useful. Hence, concerted efforts have been carried out by many scientists in order to screen for novel antibiotic producing microbes. Through their efforts, many antibiotics have been successfully discovered in order to combat pathogenic bacteria that cause disease. However, the emergence of new diseases and reemergence of multiple-antibiotic resistant pathogens have rendered the existence used antibiotics ineffectively.

Among the diverse sources of antibiotics, canned sea food is the most important target for scientists in the discovery of novel antibiotics. According to Dulmage and Rivas (1978), canned sea food microorganisms have continually been screened for their useful biological active metabolites such as antibiotics since long ago.

Based on this concept the present study has been carried out to find the alternate method for the inhibition of pathogenic bacteria.

AIM AND OBJECTIVES

1. To isolate bacteria from preserved sea food.
2. To identify the isolated bacteria by biochemical and molecular techniques.
3. To standardize DNA isolation.
4. To optimize PCR amplification of 16S rRNA gene.
5. To evaluate antimicrobial activity against human pathogenic bacteria.

REVIEW OF LITERATURE

Conventional histological staining methods, including stain selection for specific situations, have long been established. Descriptions of the most frequently used staining methods should be sufficient for day-to-day practice. Longer fixation in formaldehyde or in higher concentrations of formaldehyde can lead to sediments of formalin pigment. If the assessment of tissue sections will be affected by such sediments, pretreatment should be considered (Kardasewitsch reaction; Kardasewitsch, *1952*). Depending on which tissue is to be investigated, the fixation technique can influence the microscopic image. Thus, for example, the influence of fixation on the development of pulmonary alveoli has been investigated (*Hausmann et al*. 2004). In some cases, alternative fixing solutions are used: Bouin's solution, Zamboni solution, "NoTox" (*Meyer et al.* 1996), pure alcohol, etc. In cases where an electron microscopic investigation is needed, glutaraldehyde is typically chosen as a fixative (3% solution for 24 h at 4°C, followed by phosphate buffer solution; additional fixation in 1% osmium acid, embedded in Epon). It should be noted that fixative selection and duration can have a direct bearing on potential molecular gene tic investigations (Kuhn and Krugmann 1995). Such investigations can be difficult or even impossible and special pretreatment methods are sometimes suggested (*Ananian et al.* 2010). Immunohistochemical evidence can be found in formalin-fixed tissue, depending on the antigen, as is the case for viral antigens (*Lozinski et al* 1994), but also in other molecular genetics investigations (*Miething et al.* 2006). Antigen-conserving methods are also discussed in order to overcome antigen loss or difficult detectability due to autolysis (*Pelstring et al.* 1991). Microwave pretreatment can accelerate fixation with formaldehyde (*Login et al*. 1987). In addition to conventional histology, which has long been common practice, immunohistochemical techniques have also found their way into forensic diagnostics (Bratzke and Schröter 1995).

DNA isolation is aprocess of purification of DNA from sample using a combination of physical and chemical methods. The first isolation of DNA was done in 1869 by Friedrich Miescher (Dahm, 2008). Currently it is a routine procedure in molecular biology or forensic analyses. For the chemical method, there are many different kits are used for extraction, and selecting the correct one will save time on kit optimization and extraction procedures. PCR sensitivity detection considered to show the variation between the commercial kits (Yoshikawa, 2011).

First study about chemical composition of cell was done by Friedrich Miescher. In 1869, he used leukocytes that he collected from the samples on fresh surgical bandages and conducted experiments to purify and classify proteins contained in these cells. During his experiments he identified a novel substance in the nuclei, which he called "nuclein" (Dahm .2005). He then developed two protocols to separate cells' nuclei from cytoplasm and to isolate this novel compound, nowadays known as DNA, which differed from proteins and other cellular substances. (Holmes, 2001). This scientific finding, along with the isolation protocols used, was published in 1871 in collaboration with his mentor, Felix Hoppe-Seyler (Dahm, 2005). However, it was only in 1958 that Meselson and Stahl (*Meselson et al*, 1958.), developed a routine laboratory procedure for DNA extraction. They performed DNA extraction from bacterial samples of *Escherichia coli* using a salt density gradient centrifugation protocol. Since then, DNA extraction techniques have been adapted to perform extractions on many different types of biological sources (*Meselson et al*, 1958.).

DNA extraction methods follow some common procedures aimed to achieve effective disruption of cells, denaturation of nucleoprotein complexes, inactivation of nucleases and other enzymes, removal of biological and chemical contaminants, and finally DNA precipitation.[5] Most of them follow similar basic steps and include the use of organic and nonorganic reagents and centrifugation methods. Finally, they have developed into a variety of automated procedures and commercially available kits (*Carpi et al* 2007*)*

DNA precipitation is achieved by adding high concentrations of salt to DNA-containing solutions, as cations from salts such as ammonium acetate counteract repulsion caused by the negative charge of the phosphate backbone. A mixture of DNA and salts in the presence of solvents like ethanol (final concentrations of 70%–80%) or isopropanol (final concentrations of 40%–50%) causes nucleic acids to precipitate. Some protocols include washing steps with 70% ethanol to remove excess salt from DNA. Finally, nucleic acids are resuspended in water or TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid [EDTA]) (Price *et al* 2009*)* TE buffer is commonly used for long-term DNA storage because it prevents it from being damaged by nucleases, inadequate pH, heavy metals, and oxidation by free radicals. Tris provides a safe pH of 7–8, and EDTA chelates divalent ions used in nuclease activity and counteracts oxidative damage from heavy metals (Herzer 2001).

Members of the *vibrio* genus are gram negative, halophilic bacteria indigenous to coastal marine systems (*Thompso*n *et al*., 2003). While these common bacteria persist as a natural component of the marine microbial flora, a small percentage of environmental isolates carry the genetic determinants for human pathogenesis (*Rivera et al.*, 2001).

The massive uses of antibiotics in human therapy, bacteria have developed several resistance mechanisms including the efflux of antibiotics (*yala et al.*, 2001).

Agarose gel is a three-dimensional matrix formed of helical agarose molecules in supercoiled bundles that are aggregated into three-dimensional structures with channels and pores through which biomolecules can pass (*Joseph Sambrook et al*).The 3-D structure is held together with hydrogen bonds and can therefore be disrupted by heating back to a liquid state. The melting temperature is different from the gelling temperature, depending on the sources; agarose gel has a gelling temperature of 3542 °C and a melting temperature of 85-95 °C. Low-melting and low-gelling agaroses made through chemical modifications are also available.

Agarose gel has large pore size and good gel strength, making it suitable as an anticonvection medium for the electrophoresis of DNA and large protein molecules. The pore size of a 1% gel has been estimated from 100 nm to 200-500 nm, and its gel strength allows gels as dilute as 0.15% to form a slab for gel electrophoresis (Philip Serwer 1983). Low-concentration gels (0.1 - 0.2%) however are fragile and therefore hard to handle. Agarose gel has lower resolving power than polyacrylamide gel for DNA but has a greater range of separation, and is therefore used for DNA fragments of usually 50-20,000 bp in size. The limit of resolution for standard agarose gel electrophoresis is around 750 kb, but resolution of over 6 Mb is possible with pulsed field gel electrophoresis (*Joseph Sambrook et al*). It can also be used to separate large proteins, and it is the preferred matrix for the gel electrophoresis of particles with effective radii larger than 5-10 nm. A 0.9% agarose gel has pores large enough for the entry of bacteriophage T4 (Philip Serwer 1983).

The agarose polymer contains charged groups, in particular pyruvate and sulphate . These negatively charged groups create a flow of water in the opposite direction to the movement of DNA in a process called electroendosmosis (EEO), and can therefore retard the movement of DNA and cause blurring of bands. Higher concentration gel would have higher electroosmotic flow. Low EEO agarose is therefore generally preferred for use in agarose gel electrophoresis of nucleic acids, but high EEO agarose may be used for other purposes. The lower sulphate content of low EEO agarose, particularly low-melting point (LMP) agarose, is also beneficial in cases where the DNA extracted from gel is to be used for further manipulation as the presence of contaminating sulphates may affect some subsequent procedures, such as ligation and PCR. Zero EEO agaroses however are undesirable for some applications as they may be made by adding positively charged groups and such groups

can affect subsequent enzyme reactions(Joseph Sambrook *et al*). Electroendosmosis is a reason agarose is used in preference to agar as the agaropectin component in agar contains a significant amount of negatively charged sulphate and carboxyl groups. The removal of agaropectin in agarose substantially reduce the EEO, as well as reducing the non-specific adsorption of biomolecules to the gel matrix. However, for some applications such as the electrophoresis of serum proteins, a high EEO may be desirable, and agaropeptin may be added in the gel used (David 2003).

Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. It is an easy and cheap tool to amplify a focused segment of DNA, useful for such purposes as the diagnosis and monitoring of genetic diseases, identification of criminals (in the field of forensics), and studying the function of a targeted segment of DNA.

Developed in 1983 by Kary Mullis,(*Bartlett et al* 2003) PCR is now a common and often indispensable technique used in clinical laboratories and research laboratories for a variety of applications (*Saiki et al* 1985). These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and DNA paternity testing); and the detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR.

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase, which the method is named after, are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations. PCR is not generally considered to be a recombinant DNA method, as it does not involve cutting and pasting DNA, only amplification of existing sequences.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase (an enzyme originally isolated from the bacterium *Thermus aquaticus*). This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample through a defined series of temperature steps.

In the first step, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting. In the second step, the temperature is lowered and the two DNA strands become templates for DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

Klebsiella pneumonia over 100 years ago as a cause of community acquired *pneumonia* and is the opportunistic pathogen that can cause *pneumonia*, urinary tract infections and bacteremia (*Wu et al.*, 2012).

MATERIAL AND METHODS

SAMPLE COLLECTION

1. The sample was isolated from preserved sea food.
2. The sample was homogenized.

MOTILITY TEST

HANGING DROP METHOD

1. A drop of the bacterial culture (optimally from a young broth culture) was placed in the middle of a cover slip.
2. A thin line of petroleum jelly was placed around the edge of the cover slide.
3. The depression slide was turned to upside-down (depressed area facing down) and gently touches the cover slide. (The jelly holds the cover slip to the slide and also keeps the suspension from drying out).
4. Then the entire microscope slide/cover slip combination was turned over and observed under micorscope

BIOCHEMICAL TESTS

INDOLE TEST

1. Indole medium (tryptophan broth) was inoculated with test bacterium and incubated at optimum temperature for 48 hours.
2. After incubation, 4 to 5 drops of Indole Reagent (Kovacs) was added to down the inner wall of the tube.

METHYL RED (MR) TEST

MR/VP broth was inoculated with a pure culture of test organism and incubated at optimum temperature for 48 to 72 hours (depends on the growth rate of the bacterium). After incubation, 5 drops of methyl red reagent was added directly to the broth.

VOGES-PROSKAUER TEST

1. MR/VP broth was inoculated with a pure culture of test organism and incubated at optimum temperature for 48 to 72 hours (depends on the growth rate of the bacterium).

2. 6 drops (0.6 ml) of 5% α-naphthol, followed by 2 drops (0.2 ml) of 40% KOH was added. (KOH must be added last. If KOH is added first a false positive may be obtained).

3. The tube was vigorously shaken to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 5 to 15 min. The tube can be rested at an angle to increase the surface area of the media (greater exposure to atmospheric oxygen).

CITRATE UTILIZATION TEST

Simmons citrate agar slant was inoculated with a pure culture of the test organism and incubated at optimum temperature for 48 hours.

DNA ISOLATION

STOCK SOLUTIONS

0.5M Tris HCL (pH-8.0)

Tris base - 3.028g

Distilled water - 40mL

The pH adjusted to 8.0 using HCL and the volume was made to 50mL, then autoclaved and stored at 4˚c.

0.5M EDTA (pH-8.0)

EDTA - 9.31g

Distilled water - 40mL

The pH adjusted to 8.0 using NaOH was made up the volume to 50mL, then autoclaved and stored at 4˚c.

10mM Tris HCL (pH-7.5)

Tris base - 0.03g

Distilled water -20mL

The pH adjusted to 7.5 using HCL was made up the volume to 25mL, then autoclaved and stored at 4˚c.

TAE Buffer (50X-1 litre)

Tris base - 242g

Glacial acetic acid - 57.1mL

EDTA - 100mL (0.5M, pH 8)

242g of Tris base, 57.1mL of glacial acetic acid and 100 mL of 0.5M EDTA, (pH8.0) were added and made up to 1 litre with distilled water.

WORKING SOLUTIONS

SOLUTION 1:

Tris HCL - 50mM (pH 8.0)

EDTA -20mM (pH 8.0)

SOLUTION 2:

Saturated NaCl solution - 6M

PROTEINASE K (20mg/mL)

Proteinase k - 10mg

Autoclaved distilled water -500µl

Proteinase K dissolved in distilled water and stored at -20˚c.

TE BUFFER

Stock 0.5M Tris HCL (pH 8) - 2.0mL (10mM)

Stock 0.5M EDTA HCL (pH 8) - 0.2mL (1mM)

Made up the solution to 100mL with distilled water, then autoclaved and stored at 4˚c.

PROCEDURE

1. The cells were grown overnight in nutrient rich broth (Nutrient broth).
2. 1.5ml of culture was transferred to a tube and centrifuged at 10,000 rpm for 2 minutes.
3. The pellets were collected and repeated the centrifugation with another 1.5ml of culture containing cells.
4. Drained the tubes on a paper towel briefly.
5. 400µl of solution 1 and 100µl of solution 2 and 10µl of 10% SDS were added.
6. 5µl of proteinase K (20mg/ml) was added.
7. Incubate at 55˚c for 2 hours.
8. After incubation it was chilled on ice for 10 minutes.
9. 250µl of 6M Nacl was added.
10. Again it was kept on freezer for 5 minutes.
11. After freezing the sample was spinned at 8000 rpm for 15minutes.
12. 500µl of supernatant was taken and transferred into a new 1.5ml tube.
13. 1ml of 100% ice cold ethanol was added and inverted several times.
14. Again the sample was spinned at 10,000 rpm for 15 minutes.
15. The supernatant was removed and rinse with 500µl of 70% ethanol.
16. The sample was spinned at 10,000rpm for 5 minutes.
17. The supernatant was removed and dry the pellet at room temperature.
18. 100µl of 1X TE buffer was added to the pellet.

19. 5µl of DNA sample was added to the 0.8% agarose gel.
20. Visualized under the UV Transilluminator.

AGAROSE GEL ELECTROPHORESIS

1. 0.24g of agarose in 30ml of TAE buffer was mixed.
2. The agarose solution was boiled till get a clear solution.
3. 1.5µl of EtBr was added the solution gets completely cooled..
4. The clear solution was poured in a gel casting plate with already adjusted gel comb.
5. The casting tray was cooled at room temperature for 30 minutes for solidification.
6. After solidified, 5µl of DNA sample with 2µl of loading buffer were mixed and load in the well.
7. Run the gel 50V for about 20 minutes.
8. Observed the bands in UV light.

POLYMERASE CHAIN REACTION

PRIMER MIX

1. Eubac 5'-AGAGTTTGATCCTGGCTC-3'
2. 1492RA 5'-GGTTACCTTGTTACGACTT-3'

MASTER MIX COMPONENTS

1.	Distelled water	-	16µl
2.	10X Assay buffer	-	2.5µl
3.	Primer mix	-	0.5µl
4.	dNTPs mix	-	2µl
5.	Mgcl (30mM)	-	3.0µl
6.	Taq polymerase	-	0.5µl
7.	Template DNA	-	1µl

PCR PROGRAMME FOR 16S rRNA

Polymerase chain reactions for EUBAC gene can be performed by following the temperature and timing condition programmed in a thermal cycler.

1. Initial denaturation at 95˚c for 5 minutes.
2. Number of cycles 30.
3. Denaturation at 94˚c for 1 minute.
4. Annealing at 45˚c for 45 seconds.
5. Extension at 72˚c for 1 minute.
6. Final extension at 72˚c for 10 minutes.
7. Check the amplified products in 1.5% Agarose gel electrophoresis and the molecular weight was assessed using molecular weight marker (100bp ladder).

ANTIMICROBIAL ACTIVITY

1. The broth culture was prepared of test samples and human pathogens.
2. The broth was incubated at over night.
3. The nutrient agar plates were prepared and named properly.The standard antibiotic plates were prepared and named as duplicate plates.
4. The plated were allowed to solidify.
5. After solidification the human pathogens were inoculated by using cotton swab method.
6. Prepare well by using micropipette tips.
7. The test sample were added to the well as concentration about 20µl , 40µl, 60µl.
8. Place the plates in incubator for 24 hours.
9. Observed the plates and note the zone formation.

SEQUENCING EDITING

The obtained sequences were edited based on the electropherogram peak clarities. Sequences with noisy peaks were excluded from the analysis. The sequences were assessed to check the insertion or deletions and codons in MEGA 5.0 software.

SEQUENCING CHARACTERIZATION

Multiple sequence alignment and pairwise sequence alignment were performed using Clustal W program implemented in MEGA 5.0 in all the sequences. Nucleotide differences were carefully monitored and the differences were observed and edit manually. Sequences were translated into amino acid sequences using vertebrate mitochondrial codon pattern in the MEGA 5.0 for checking the pseudo-gene status. All the

sequences were correctly translated into amino acid sequences with their respective starting primers without any internal stop codon.

BLAST SEARCH

The amplified sequences of EUBAC were confirmed by similarity index built in the NCBI's BLAST program. Based on the percentage similarity and query coverage against the reference species, the species were confirmed.

RESULTS

	STAINING TECHNIQUES	FIGURE A *Bacillus cereus*	FIGURE B *Bacillus anthracis*
1.	Gram's staining	Positive	Negative
2.	Capsule staining	Negative	Positive
3.	Negative staining	Negative	Negative
4.	Simple staining	Negative	Negative
5.	Motility test	Positive	Positive

	BIOCHEMICAL TESTS	FIGURE A *Bacillus cereus*	FIGURE B *Bacillus anthracis*
1.	Indole test	Negative	Positive

2.	Methyl red test	Negative	Positive
3.	Vogesproskauer test	Negative	Positive
4.	Simmon citrate agar test	Negative	Positive

DNA ISOLATION

The DNA was isolated from two species of Bacteria by standardized procedure showed clear bands in the 0.8% agarose gel electrophoresis.

Genomic DNA isolated from two *Bacillus* species (lane 1 *Bacillus cereus* and lane 2 *Bacillus anthracis.*

PCR AMPLIFICATION OF 16S rRNA GENE

DNA region of 16S rRNA gene was successfully amplified using universal primers.

Agarose gel electrophoresis of amplified 16S rRNA gene (lane 1&2 amplified EUBAC gene)

ANTIMICROBIAL ACTIVITY

The antimicrobial result shown in the figure is *Bacillus anthracis* against the *klebsiella pneumonia.*

SEQUENCE OBTAINED AFTER SEQUENCING FOR 16S rRNA GENE

>Bacillus cereus (Present study)

TAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCC

CATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCA

TGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAG

CTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGAT

CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCT

TCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTC

GTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGT

ACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA

AGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTG

AAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAG

GAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGC

GAA

>Bacillus anthracis (Present study)

TAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCC

CATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCA

TGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAG

CTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGAT

CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCT

TCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTC

GTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGT

ACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA

AGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTG

AAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAG

GAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGC

GAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGG

ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA

DISCUSSION

In recent years, there has been increasing demand for safe and eco-friendly agricultural products (Huh and Kim, 2010). Accordingly, biological control practices are in much greater demand as alternatives to synthetic pesticides. Biological control practices are particularly important in organic crop production disease control (Cook et al., 2013).

Reports from several studies provide strong evidence that strains of *Bacillus* species, including *B. subtilis* (*Kim et al.,* 2012), *B. amyloliquefaciens* (*Kong et al.,* 2010a), *B. licheniformis* (*Govender et al., 2005*), *B. pumilus* (*Sari et al.,* 2007), *B. mycoides,* and *B. sphaericus,* significantly reduce disease severity on a variety of hosts in greenhouse or field conditions (*Kloepper et al*., 2004). *B. subtilis* and *B. amyloliquefaciens* have been used in commercial biological control products due to their excellent antagonistic effects and high stability in harsh environmental conditions (*Kwak et al.,* 2012).

A previous study used tidal flat sediments and jeotgal, a salted-fermented fish product, to find antagonistic microorganisms in Korea. The antagonistic bacteria included *Paenibacillus macerans, B. atrophaeus,* and *B. pumilus* from todal flat sediments, and *B. atrophaeus, Paenibacillus sp. Virgibacillus pantothenticus* , *B. subtilis,* and other *Bacillus* sp. from jeotgal (*Kim et al.,* 2010).

Consistent with previous reports that *B. atrophaeus* suppreses fungal plant pathogens (*Kim et al.*, 2010), our study demonstrated that three *B. atrophaeus* strains, LB14, HM03, and HM17, were effective for not only inhibiting mycelial growth, but also inhibiting conidial germination of *C. acutatum* and *C. gloeosporioides*. Furthermore, we showed that chinase, protease, siderophore, and phosphate solubilization, associated with LB14, HM03, and HM17, ma y contribute to suppression of *C. acutatum* and *C. gloeosporioides.*

Fungal cell walls play important physiological roles, in addition to providing structural barriers, in regulating the passage of molecules into cells and their development and survival in response to ambient conditions. The fungal cell wall is a matrix composed of polysaccharides, small amounts of proteins, and other components. Many fungal pathogens in ascomycota and basidiomycota have a mixture of chitin, polymers of glucan, and mannoproteins in their cell walls (Vega and Kalkum, 2012). Many antagonistic bacteria secrete lytic enzymes capable of dissolving fungal cell walls, resultin g in exhibition of predatory activity of fungal pathogens (*Xu et al.,* 2014). Strong activity of chitinase and protease in the *B. atrophaeus* strains LB14, HM04, and HM17 are therefore consistent with the observed growth inhibition of *C. acutatum* and *C. gloeosporioides.*

Proteases, in particular, play a key role in the cell lysis process. Proteases bind to the outer mannoprotein layer of the cell wall, open the protein structure, and expose inner glucan layers and chitin microfibrils. Among the three B. atrophaeus strains, LB01 (*B. amyloliquefaciens*) exhibited strong protease activity. However, activity of chitinase and protease was almost absent in LB15 (*B. pumulis*). This difference ma y explain the reduced effectiveness of LB15 compared to the other *B. atrophaeus* and *B. amyloliquefaciens* strains in inhibiting mycelial growth and conidial germination of *C. acutatum* and *C. gloeosporioides*.

The three *B. atrophaeus* strains, together with *B. amyloliquefaciens* and *B . pumilus* strains isolated from tidal flat sediments, demonstrated a high capacity to solubilize an insoluble phosphate compound. This is an important trait of antagonistic bacteria in supplying phosphate, a major nutrient required for plant growth. Uptake of phosphate by plants is limited due to low solubility of phosphate compounds in soil. Many bacteria are known to increase solubilization of insoluble phosphate forms by releasing organic acids and phosphatase enzymes (*Halder et al.,* 1990). Therefore, phosphate solubilizing bacteria, especially those with antifungal activities, may promote crop productivity, by not only providing plant-absorbable forms of phosphate, but also by effectively protecting plants from fungal soil-borne diseases (*Dey et al.,* 2004).

In addition, production of siderophore by the three *B. atrophaeus* strains may be a key factor in promoting plant growth and protecting plants from human pathogens. Iron, an essential cofactor for cellular processes, is abundant in nature, but iron bioavailability is very limited in soils due to low solubility under aerobic conditions and in the presence of a neutral pH. A number of bacteria, including plant pathogens, produce iron-chelating siderophores. Siderophores produced by antagonistic microorganisms may inhibit the growth of plant pathogens, but may enhance plant growth by increasing iron in the root zone. Studies have demonstrated that beneficial bacteria-producing siderophores stimulate plant growth and inhibit germination of a soil-borne fungal pathogen (*Alexander et al., 1991*). Similarly, the variable efficiency in inhibiting conidial germination of *C. acutatum* and *C. gloeosporioides* may be correlated with siderophore production b y the *B. atrophaeus* and *B. pumilus* strains, although the effect of strong antifungal substances produced by *B. atrophaeus* strains should also be considered. Further evaluation of LB14 should be performed under different environmental conditions, cultural practices to obtain more knowledge of efficacy in the field. Knowledge of the mechanisms and performance of antagonistic microorganisms will be helpful in developing reliable biological systems for disease control.

SUMMARY AND CONCLUSION

Two *Bacillus* species, *Bacillus cererus, Bacillus anthracis* were collected by Parangipettai estuary. The morphological characters of these two species were compared and found they are distinct. DNA was isolated from fresh broth culture by standardized method and the purity was checked. The PCR conditions for the amplification of EUBAC and 16S rRNA gene were standardized. All the sequences were checked for species confirmation by BLAST in NCBI. Based on the similarity search the two species were identified as *Bacillus cererus*, *Bacillus anthracis*. This study serves as a basis for future studies possibly involving the conservation and management of the species.

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