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Antibacterial Activity of Chitosan on Faecal Indicator Bacteria Isolated from Sewage Outfall Nearby Kanyakumari Coast

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Abstract

Untreated wastewater discharges into nearby water bodies can pose a significant health risk as it serves as a reservoir for pathogens. Therefore, treating wastewater is necessary to reduce the risk of transmitted diseases and environmental pollution. This study used chitosan to eliminate the faecal indicator and pathogenic bacteria isolated from the sewage outfall of Kanyakumari Coast, where untreated wastewater is dumped directly onto the coast. The control sample had a bacterial count of 6.42×10^8CFU/100ml, but the sample treated with chitosan did not have any bacterial growth recorded. The antibacterial effect of chitosan was reported in this study by inhibiting the growth of faecal indicator and pathogenic bacteria.

Keywords: Sewage, Chitosan, Faecal indicator bacteria, Antibacterial effect

1.Introduction

Globally, sewage is a major component of marine pollution from land-based activities, which account for roughly three-fourths of all pollutants entering the world's oceans (Rapaport and Dave, 1996). Land-based sources of marine pollution are contributing to an alarming decline in the health of the world's marine ecosystems and their ability to provide for human needs.

Wear and Vega Thurber, 2015 reported that sewage pollution is not a single, simple stressor; rather, it is complex and can introduce diverse pollutants, including nutrients, microbial pathogens, and chemical contaminants which can have detrimental effects on coastal ecosystems. Pollution from microbial contaminants (Despland *et al.*, 2012; Wang *et al.*, 2014) found in sewage threatens coastal water quality and the health of humans and ecosystems. Water quality in developing nations and in rural areas is particularly challenged because adequate sewage treatment infrastructure is often lacking (Wiegner *et al.*, 2016).

Human exposure to sewage can increase the risk of diseases and infections most commonly skin and urinary tract infections, hepatitis and gastroenteritis (Betancourt *et al.*, 2014; Cheung *et al.*, 2015). Shuval, 2003 reported that annually, there are over 120 million gastroenteritis cases worldwide associated with sewage contaminated waters. Efforts to address coastal water-quality problems over the past 20 years have not solved all our problems, yet progress has been made. While the problems of the future are complex, there is now a greatly improved scientific understanding of physical and ecological processes and improved techniques for managing coastal resources.

Removal of pathogenic microorganism from sewage is a matter of concern in last few decades. Untreated waste water discharged from increased numbers of hotels and restaurants, holiday spots, amusement parks, hospitals and the coastal settlements goes through different discharge points terminating into the coast, produces health hazards to the living organisms. Various methods have been employed, to eradicate microorganisms in waste water before discharge. They are expensive and having demerits too.

The self-purification processes using biomaterial is the best known method, which effectively removes the bacteria and improves water quality. Among the biomaterials, chitosan is a versatile biomaterial, has recently emerged as a useful raw material with positive environmental, economic and technical added value whose main application have attracted considerably due to their antimicrobial and antifungal activities (I. Junceda-Mena *et al.*, 2023; Jung *et al*., 1999). Chitosan is a wealth from waste. Chitosan is the de-acetylated derivative of chitin.

Chitin is a biological material that is one of the most ubiquitous biopolymer present in the exoskeleton of crustaceans which can be obtained from the shell waste of the crab, and shrimp. It is also found in a wide range of natural sources, such as fungi, yeast and insects (Kumar, 2000). It is said to be the second most abundant natural biopolymer on earth next to cellulose (Yadav *et al.*, 2015; Usui *et al.*, 2004; Hudson and Smith, 1998).

Chitin can be quickly processed into chitosan, which is a fiber-like polysaccharide, hence chitosan is a derivative compound of chitin that can be obtained by partial de-acetylation (Kurita, 2006). Chitosan is composed of a copolymer of D-glucosamine and N-acetyl-D-glucosamine. The number of D-glucosamine and N-acetyl-D-glucosamine residues in the co-polymer varies depending on the varying degree of de-acetylation (Islam *et al*., 2017).

Due to its several unique properties, including low cost, renewable, biodegradability, biocompatibility, and low toxicity, chitosan has been extensively investigated for applications in many fields. For example, chitosan has been used as a flocking agent in water treatment (Nasrollahzadeh *et al.*, 2021; Liaw *et al.*, 2020; Morsi *et al.*, 2017; Picos-Corrales *et al.*, 2020) an elicitor to activate plant defenses (Vanda *et al*., 2019; Varlamov *et al.*, 2018; Xing *et al*., 2015) and a supplement during food preservation and in food additives (Dutta *et al*., 2012; Morin-Crini *et al*., 2019; Phillibert *et al*., 2017). Chitosan has distinctive biological properties such as antimicrobial, and is now widely applied in functional food, environmental protection, and biotechnology (Jeon *et al.*, 2001; No *et al*., 2002 and Assis and Britto, 2008). However, the effectiveness of the antimicrobial activity of chitosan is highly dependent on the type of target microorganism (Li *et al*., 2013; Rabea *et al.*, 2003; Varlamov and Mysyakina, 2018; Kong *et al*., 2010). Chitosan and its derivatives can be used to remove various pollutants from the environment (Takeshita *et al*., 2021; Bakshi, *et al*., 2020; Vidal and Moraes, (2019).

Chitosan has been proven to suppress the growth of bacteria, filamentous fungi, and yeast strains. Chitosan has also been discovered as an antibacterial agent (Zheng *et al.*, 2000), though its capacity to do so is uncertain due to the fact that its character has been attributed to several unique mechanisms.

Chitosan has a broad spectrum of action and a high mortality rate against gram-positive and gram-negative bacteria (Chung *et al*., 2004).

As coastal populations grow, there is a greater need for remedies to find solutions for sewage pollution using low-cost, non-toxic biodegradable material, and infrastructure development, including wastewater treatment plants and sewer systems. Planning and managing this development sustainably are essential to protect coastal ecosystems. In this study, mechanically stable chitosan beads were prepared (without blending with other material or polymer), and used to eliminate faecal indicator and pathogenic bacteria isolated from wastewater from the sewage outfall of Kanyakumari Coast where untreated sewage is dumped directly into the coast.

2. Materials and methods

2.1 Sample Collection

Samples were collected from sewage outfall off the coast of Kanyakumari, a scenic coastal town located in the southern tip of India (8.08^0 N, 77.55^0 E). 1000 ml sterile bottles were used to collect sewage waters, 0–20 cm below the surface and stored in sterile containers. Samples were collected with standard precautions required for microbiological analysis and brought to the laboratory in a portable icebox with-in two hours for further analysis.

2.2 Chitosan bead preparation

The one gram of chitosan was dissolved in acetic acid solution (pH = 4). The prepared solution was injected drop by drop using a syringe in a gelling solution (solution of sodium hydroxide 3M). The obtained solution was maintained for 6 hours at room temperature (25°C) to get the chitosan beads. Obtained beads were washed with sterile water and used the beads for antimicrobial treatment.

Fig 1 Chitosan Beads

Fig 2 Chitosan treated water

2.3. Sample analysis

2.3.1 Treatment Plant (Experimental Flask)

Chitosan beads were placed inside a sterile conical flask (experimental flask). Transferred 500ml of sewage water into the experimental flask under aseptic condition and was sealed by sterile cotton plug for 24 hours.

2.3.2 Control Plant

Five hundred ml of sewage water was poured into a sterile conical flask under aseptic condition and was sealed by sterile cotton plug for 24 hours. For the enumeration of pathogenic bacteria from untreated waste water sample, Serial Dilution Technique was followed.

2.2.3. Enumeration, isolation and identification of FIB

2.2.3.1. Plating methods and Bacteriological analysis

Immediately after arrival, control sample was inoculated in enrichment like Selenite F Broth (M052A Hi Media), BHI (M210, Hi Media) broth and alkaline peptone water (M618, Hi Media) and incubated at 37°C overnight. Simultaneously Nutrient agar (M 561, Hi Media) and MacConkey agar plates were used for streaking. After overnight incubation from the enrichment media the inoculums were sub-cultured using standard loop technique over the selective and differential media respectively, TCBS- Thiosulfate citrate bile sucrose agar (M189, Hi-Media), EMB- Eosin methylene blue agar (M317, Hi Media), MacConkey (M081, Hi-Media) agar, SS- Salmonella Shigella agar (M108, Hi-Media) and BHI- Brain Heart Infusion agar (M211, Hi-Media). Gram staining method was performed to differentiate gram positive and gram-negative bacteria.

The same procedure was used to analyze the bacteria from experimental sample (chitosan treated sewage). 0.1ml of treated sample from experimental flask was spread on the nutrient agar media by spread plate technique. Incubated the plate at room temperature for 48 hours.

2.2.3.2 Biochemical Analysis

Routine biochemical parameters were used to identify the bacteria and the tests were Indole, Methyl Red, Voges Proskauer, Citrate test (IMViC). TSI (Triple Sugar Iron) tests were used to identify acid/alkaline, gas production and H2S production. Motility of the bacteria was identified using the hanging broth method. Urease test was also performed. Catalase and oxidation tests were performed to differentiate enterococcus from other gram-positive bacteria. Biochemical tests were performed by using following media Peptone water, MR-VP broth (Methyl Red - Voges Proskauer Broth) Simmons Citrate Agar TSI Agar (Triple Sugar Iron) and Urease Agar.

2.2.3.3 Total Viable Count

The most common procedure for the enumeration of bacteria is the viable plate count. The bacterial populations from the samples were estimated via the spread plate method on nutrient agar media plates with 0.1 ml of suitable dilutions. All the media plates were incubated at 37^0C for 24–48 h and final counts of colonies were noted. The colonies on the individual plates were counted in the form of colony forming units (CFU). CFU is a measure of viable bacterial numbers that can replicate to form colonies.

3. Results

3.1 Enumeration of FIB in control and treated samples

A noteworthy difference was observed between the total count of bacteria in control and chitosan treated samples after the antibacterial treatment period of 24 hours. Total bacterial count recorded in control sample was $6.42x10^8$CFU/100ml and sewage water treated with chitosan beads showed no bacterial growth. (Table.1)

Table.1 Total viable count and isolated bacteria in control and experimental samples

Sl. No	Nature of samples	Total Viable Count CFU/100ml	Bacteria isolated	
			Gram negative	Gram positive
1	Control (Untreated sewage)	6.42x108	*E. coli*, *V.cholerae*, *Klebsiella* spp, *Aeromonas* spp, *Proteus* spp and *Salmonella* spp	*Enterococcus* spp
2	Experimental (Sewage treated with chitosan beads)	No growth	NA	NA

3.2 Isolation and identification of faecal indicator and pathogenic bacteria

Based on colony morphology and biochemical identification seven faecal indicator bacterial pathogens were isolated from the samples. *Enterococcus* spp was identified as gram positive and other six isolates were identified as gram-negative bacteria *(Escherichia coli (E. coli), Vibrio cholerae (V.cholerae), Klebsiella* spp, *Aeromonas* spp, *Proteus mirabilis* and *Salmonella typhi).*

Table 2. Biochemical Characteristics of Bacterial Isolates from sampling point

Media / Colony	Peptone water	MR VP	Simmons citrate	TSI	Urease agar	Organisms identified
Blue metallic sheen (EMB agar)	+	MR + VP -	-	alkaline/acid gas formation no H_2S	-	*E.coli*
Yellow colony (TCBS)	-	MR - VP -	-	acid/acid no gas no H_2S	-	*V.cholerae*
Black colony (SS agar)	-	MR + VP -	+	alkaline/acid gas formation H_2S present	-	*Salmonella*
Pink mucoidal colony (Mac Conkey agar)	-	MR - VP +	+	acid/acid no gas no H_2S	+	*Klebsiella*
Green colony (TCBS agar)	+	MR - VP +	+	acid/acid gas formation no H_2S	-	*Aeromonas*
swarming colony(Blood agar)	-	MR + VP -	+	alkaline/acid gas formation H_2S present	+	*Proteus vulgaris*
Pin point (BHI)	-	MR - VP +	-	alkaline/acid no gas no H_2S	-	*Enterococcus faecalis*

4. Discussion

Bacteria associated with sewage water which have been documented in Kanyakumari coast include pollution indicators such as *E. coli* and *Enterococcus faecalis* and human pathogens *Klebsiella* spp, *Aeromonas* spp, *Proteus mirabilis*, *V. cholera* and *Salmonella spp*. Among the seven bacterial species, *V. cholerae*, *Klebsiella* spp, *E. coli*, *Aeromonas* spp, *Proteus mirabilis* and *Salmonella spp.* belong to gram-negative group and *Enterococcus faecalis* belongs to gram-positive group (Table 1). High bacterial density ($6.42x10^8$ CFU/100ml) was observed in the control sample since it was collected from sewage outfall (Table 1). The high level of bacterial pathogens in the sewage outfall of Kanyakumari coastline was observed where wastewater from the coastal settlements, hotels, restaurants and lodges is directly disposed into the sea water without any treatment. Whereas the sewage water treated with chitosan beads showed no bacterial growth.

Although the exact mechanism of antibacterial activity is not yet fully understood, several hypotheses provide a different explanation. It has been established that the antimicrobial activity of chitosan is influenced by multiple factors that operate in a systematic and unconnected way. According to a widely accepted assumption, chitosan's antimicrobial activities are attributed to a positively charged amino group. The positively charged amino group interacts with the negatively charged microbial cell

membrane. This interaction causes the leakage of various proteins and other cellular components of the microbes (Benhabiles *et al*., 2012; Sahariah and Masson, 2017) causing disruption of the cell, thus altering the membrane permeability, followed by attachment to DNA causing inhibition of DNA replication and subsequently cell death (Nagy *et al*., 2011). Studies of Shahidi *et al*., 1999; Fu *et al*., 2005 and Jing *et al*., 2007 support this concept that interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents.

Another possible mechanism is that chitosan acts as a chelating agent that electively binds to trace metal elements causing toxin production and inhibiting microbial growth (Divya *et al*., 2017) One proposed mechanism for the bactericidal effect of chitosan is its direct blocking ability, which prevents nutrients and oxygen from entering the intracellular space. This mechanism is suitable for higher molecular weight chitosan, which forms a polymer membrane on the surface of the bacterial cell (Kong *et al*., 2010). However, due to the different composition of gram-positive and gram-negative cell walls, the interaction of chitosan with these two types of bacteria is different.

In general, the antimicrobial effectiveness of chitosan and its derivative against gram-positive and gram-negative bacteria is somewhat controversial. Some studies reported that the bactericidal effect of chitosan is stronger in gram-negative bacteria than in gram-positive bacteria, due to the higher affinity of amino groups for anionic radicals in the cell wall (Hussain *et al*., 2014; Al-Hassan, 2016). In other studies, gram-positive bacteria were thought to be more sensitive to the antimicrobial activity of chitosan, which is due to the gram-negative outer membrane barrier. In our study both gram negative (*V. cholerae, Klebsiella spp, E. coli, Aeromonas spp, Proteus mirabilis and Salmonella spp.*) and gram positive bacteria (*Enterococcus faecalis*) were killed in chitosan beads treated sample. Gram-positive and gram-negative bacteria exhibit remarkable differences in their cell wall structure, in which gram-positive bacteria have thicker peptidoglycans and gram negative bacteria are enriched in lipopolysaccharide (Pasquina *et al*., 2020; Rohde, 2019; Gan *et al*., 2008).

Differences in the cell surface structure of these types of bacteria isolated from sewage sample could have led to distinct susceptibilities to chitosan in our study. For example, gram-negative bacteria present a more negative charge than gram-positive bacteria because lipopolysaccharide is often attached to phosphorylated groups (Raetz *et al*., 2007; Kraus and Peschel, 2006). Although the electrostatic interaction between positively charged chitosan groups and negatively charged sites on microbial cell is assumed as the main antimicrobial mechanism (Rabea *et al*., 2003), the thickness of the peptidoglycan layer can play an important role in providing a rigid structure which can act as a barrier against chitosan interactions (Zheng and Zhu, 2003).

Numerous studies indicated that chitosan and its products have shown to have a greater influence on the cell wall degradation (Packirisamy *et al*., 2019; Eaton *et al*., 2008; Moon *et al*., 2007; Rabea *et al*., 2003). Increased antimicrobial activity has been witnessed against various strains of gram-positive and gram-negative bacteria, especially for *S. aureus* and *E. coli* (Verbeeck *et al*., 1977). Lee *et al*., 2009 and Abd and Niamah, 2012 reported that no faecal coliform and *Vibrio* spp were found in the samples treated with chitosan. They agreed with the hypothesis of Qin *et al*., 2006, in which chitosan molecule has the ability to interact with bacterial surface and is adsorbed on the surface of the cells and stacks on the microbial cell surface and forming an impervious layer around the cell, leading to the block of the channels. Vital antibacterial activities have been observed against various types of bacteria like *B. cereus, Staphylococcus aureus, Lactobacillus plantarum, Bacillus megaterium, L. bulgaris, Salmonella typhymurium, E. coli, Pseudomonas fluorescens* and *Vibrio parahaemolyticus* (Jeon *et al*., 2001; Coma *et al*., 2003; Dutta *et al*., 2009). Tsai *et al*., 1999 studied the mechanism of chitosan antibacterial action involves ionic interaction between the chitosan and the bacterial surface that changes the membrane permeability also caused leakage of glucose and lactate dehydrogenase from *E. coli* cells. Taha *et al*., 2002 observed the chitosan affected growth and haemolysin production of *Aeromonas* spp.

The antimicrobial effect of chitosan has been reported in many studies, and our study validates this mechanism by suppressing bacteria's growth. Chitosan's unique properties can be used to treat polluted environments in remarkable ways. Sewage-related coastal problems are still present globally,

and the challenges of the future are more complicated than those of the past. Chitosan and its derivatives can be utilized to treat sewage water before it is disposed of into any water bodies.

5. Conclusion

Chitosan is widely used in various fields, especially in environmental protection, due to its unique biological properties, including antimicrobial activity. The removal of various pollutants from the environment can be achieved through the use of chitosan and its derivatives. In this study, mechanically stable chitosan beads were prepared and used to eliminate the faecal indicator and pathogenic bacteria isolated from waste waters from the sewage outfall of Kanyakumari Coast where untreated was sewage is dumped directly in to the coast. The antibacterial effect of Chitosan against faecal indicator and pathogenic bacteria was reported in this study by inhibiting the growth.

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