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Chitosan Incorporated Bio-Surfactant Formulation with Enhanced anti-Microbial Properties

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ABSTRACT

Over time the effects of antimicrobial resistance are natural, usually genetically modified. However, antimicrobial exploitation and overuse accelerate this process. Many nanoparticles have been developed with anti-microbial activity to combat aforementioned issues, but many of them are synthesized by chemical synthesis process. Preparing nanoparticles by combining natural substance would aid in achieving the targets without causing any environmental consequences. The present paper deals with developing a nanoparticles by combining a natural polymer and bio-surfactant i.e., chitosan and surfactin to boost the anti-microbial activity of the formulation against different bacterial species in order to be used in various fields (e.g. medical, food, Pharma). The prepared formulation was further characterized by using TEM, dynamic light scattering to determine morphology of nanoparticles. Further, performance evaluation of chitosan/surfactin nanoparticles were evaluated by conducting anti-microbial assay which preferably included Minimum inhibitory concentration, minimum bacterial count, biofilm assay and cytotoxicity assay. It was observed that the chitosan/surfactin NPs were potent in inhibiting anti-microbial activity of S.aureus, E.coli species at 95% and 98% respectively. Thus, chitosan/ surfactin NPs may be used as potent anti-microbial agents.

Keywords: Anti-Microbial, Chitosan, Nanoparticles, Surfactin

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INTRODUCTION

Antimicrobial resistance refers to alteration in nature of micro-organisms when subjected to anti-microbial medications, which preferably includes anti-fungal, anti-bacterial, anti-helmenthics, and anti-viral [1]. The micro-organisms used herein preferably include bacteria, fungus, virus and parasitic organisms. The 'superbugs' are often alluded to as pathogens with antimicrobial resistance. New methods for the treatment of common infectious ailments, which result in chronic illness, dysfunction and demise, are being established and spilled around the globe. Medical interventions like organ transplantation, medical oncology, diabetes control and major operation without adequate antimicrobial agents for infection deterrent and curing are very difficult to accomplish. Antimicrobial tolerance in recent studies is estimated to cause more than 10 million deaths by in 2050 thereby causing return towards an era of pre-antibiotics [2]. The clinical and economic problems due to hospital contracted bacterial infections, caused largely by multi-drug resistant contaminants have increased so far. The most prevalent contaminants in healthcare environments and in populations preferably include *Staphylococci* and *Enterobacteriaceae*, triggering invasive inflammation [1]. The opportunistic agents causing nosocomial an infection preferably includes *Staphylococcus aureus*, *S. epidermidis* and K. *oxytoca* [3].

Bacteria enters human body through typical routes of body which preferably include skin, gastrointestinal, and

urogenital infections, as well as implants like catheters and joints. More than 85% of human pathogenic infections and 60% of nosocomial infections have improved the extent of certain bacteria to produce biofilm [3]. Biofilm development is responsible for new way of living in contrast to planktonic state. Biofilms have important public health consequences since the resistance to antimicrobial agents in biofilm based micro-organisms has reduced significantly. This sensitivity could be inherent (as natural result of biofilm formation) or inherited (because of extra chromosomal factors transferred to fragile biofilm microbes) [4,5].

A normal micro-dilution experiment may not assess biofilms' sensitivity to antimicrobial agents, because such tests are based on reaction of planktonic species instead biofilm (surface-associated). Rather, resistance to biofilm-associated species should be assessed explicitly. Their eradication from production processes is problem associated with biofilm creation [6].

Surfaces in which agglomerates of bacteria are accumulated usually result in product toxicity and hinder significant processes which, in particular, cause issue in food sector. Traditional antimicrobial agents are typically attacking planktonic species, thereby leaving a void for plasma membrane in sessile cells. Therefore, there exists a requirement in the art for developing a tool with enhanced anti-microbial capabilities that particularly targets cellular structures in comparison to cellular methodologies.

Nanomaterial is proved to be a potent anti-microbial agent because of unique physiochemical characteristics which includes surface/volume ratios, that preferably enhances cellular contact, increased bioavailability and half-life and thereby decrease cytotoxicity [7]. The anti-microbial mechanism of action of nanoparticles is specifically defined as: oxidative stress induction, release of metallic ions, or non-oxidative protocol. Such three modes of mechanism may occur consequently.

Few studies have proposed that silver NPs neutralizes surface electric charge of bacterial film and also alters its penetrability, consequently causing bacterial demise. According to current studies, key mechanisms underlying the impacts of NPs on antimicrobial cells are: (1) distortion of bacterial cell film; (2) ROS production; (3) bacterial cell permeability; and (4) causing intracellular antimicrobial action involving protein and DNA associations [7].

Due to all these, combination of two anti-microbial agents may leads to production of enhanced tool which may particularly increase its activity against pathogenic microorganism. Surfactin-centered lipopeptides made from Bacillus species are one of most crucial compound. They have a moderate Critical Micelle Concentration (CMC), balanced emulsification and outstanding moisture content. They also have high CMC content. Figure 1 refers to applications of surfactants in various fields.

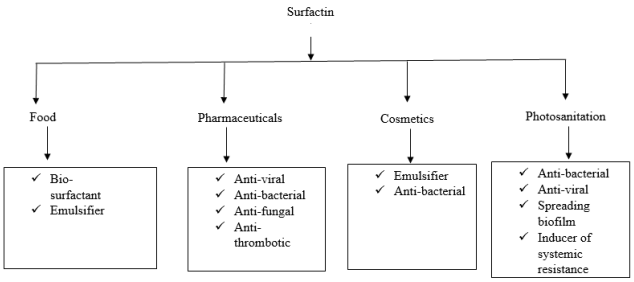

Figure 1: Applications of surfactants in different sectors which include food, pharmaceuticals, cosmetics and photo sanitation.

Surfactin is important bio surfactant developed by few strains of *Bacillus subtilis*. It contains seven amino acids and β-hydroxyl fatty acids. It is cyclic lipo heptapeptide [8]. Horse-saddle configuration reveals peptide loop of bio surfactants in aq. solution due to two amino acid compounds L-Glu and L-Asp, which are negatively charged. It has special surface, layout, and membranes active characteristics because of its amphiphilic composition. The suppression of cAMP phosphodiesterase and anti-HIV features is also cytoplasmic, anti-mutagenic, antibacterial and anti-tumoral [8]. These processes are linked to their bio macromolecular associations like enzymes and lipopolysaccharides. Surfactant features and the biological activity of surfactin aptamers tend to be very important both for the use in cosmetics and medicinal applications.

Chitosan is linear copolymer consisting of N-Acetyl - D-glucosamine, which is connected to β-(1-4), resulting from selective chitin de acetylation [9]. This compound is versatile and commonly used in biomedical, biotechnology applications, as its biological properties have antimicrobial activity on different microorganisms. Chitosan is mainly utilized to control obesity and Crohn's disease along with elevated cholesterol [10]. It is also utilized to treat conditions of dialysis, such as elevated cholesterol, "starved blood" (anemia), and lack of power, hunger and sleeping disorders. By interfering with cell wall polyanions (*i.e.* teichoic acid within gram-positive bacteria, and lipo-polysaccharide within gram-negative bacteria) and anionic phospholipids within cell membrane, chitosan destabilizes cell envelope. Chitosan nanoparticles are biocompatible, non-toxic, compostable, and are ideal for absorption of bio-active substances because of chemical features [9]. Due to its contact with bacterial membranes and its destabilization, chitosan nanoparticles are possible vectors for provision, by peptidoglycan and/or external mucous, of antimicrobials of low or limited solubility. Low toxicity and increased biodegradability are normal glycolipid surfactant.

It has also been observed that, many nanomaterial are composed of metals or synthetic surfactants. However, such synthetic surfactant is not very effective, are very costly and even lead to environmental pollution [11]. The accumulation of metals in the body leads to hazardous diseases which may be fatal in adverse situations.

Therefore to overcome from such situations the present research aims towards developing an anti-microbial tool lies within the principles of green synthesis and is also sustainable, bio-compatible and readily available.

REVIEW OF LITERATURE

Many studies have been conducted regarding the anti-microbial activity of nanoparticles against bacterial resistance. Such studies involves synthesis of nanoparticles by using toxic metals involving complex synthesis route [11,12]. This process of synthesis by using synthetic materials is not effective and caused health issues in addition to environmental degradation.

RESEARCH QUESTION

- Why does a need exist for sustainable formulation with enhanced anti-microbial activity?
- Why there is a need to combine natural polymer and bio-surfactant?
- What is the importance of green synthesis?

MATERIALS AND METHODS

Design

The method involved synthesis of polymer and natural bio surfactant nanoparticles wherein nanoparticles of chitosan and Surfactin were prepared by combining them followed by subjecting to several microbial analysis in order to determine its efficacy against bacterial species.

Materials

Surfactin (99.5%) was procured from Sigma Aldrich, Sodium TriPolyPhosphate (TPP) was procured from Synth, Chitosan was procured through Squid pens by performing deacetylation, depolymerization, deprotenization.

Sample

Samples include bacterial species on which anti-microbial analyses were performed. The bacterial species used herein is preferably *S.aureus* and *E.coli.*

Nanoparticles synthesis

Ion gelation techniques was adopted for synthesizing chitosan nanoparticles (C-NPs) and combination of chitosan/Surfactin nanoparticles (C/RL-NPs) wherein cross linking was performed with TPP as discussed by Calvo et al. [13] with slight modifications. The method of synthesis of chitosan/surfactin nanoparticles involves the following steps: firstly, 0.6 mg mL-1 of depolymerized chitosan was solvated in aq. CH_3COOH for overnight. Then 0.6 mg mL-1 prepared solution of Surfactin and TPP was filtered via filter paper, wherein the size ranged 0.40 µm for chitosan and surfactin, 0.20 µm for TPP. Finally chitosan and prepared solution of Surfactin and TPP was mixed together in equal ratio followed by continuous stirring for at least 1 day at 700 rpm and at room temperature to obtain a final mixture comprising chitosan/surfactin nanoparticles [3,13].

Chitosan nanoparticles were synthesized by same protocol as mentioned above by using distilled water. The nanoparticles were then subjected to centrifugation for at least 40 minutes at 15000 rpm at 4°C and were further dissolved in acetic acid solution at pH 4.5. Surfactin nanoparticles were prepared by solvating the Surfactin in double distilled water at 20X Critical Micelle Concentration (CMC) followed by filtering through 0.40 µm filter. The obtained nanoparticles were then kept stable at 4°C.

The chitosan surfactin nanoparticles synthesized were then subjected to characterization a study which includes: *In-vitro* release kinetics, morphological analysis and anti-bacterial assay.

In-vitro release kinetics

In order to determine release of chitosan and Surfactin through nanoparticles, 5 ml of chitosan NPs and Chitosan-surfactin NPs were put in dialysis packets, following which dialysis was carried out in 40 ml of CH_3COOH/sodium acetate buffer at pH 5.6 and incubated at 37 degree Celsius on thermostatic shaker [3]. After some duration, dialysis solution was replaced with fresh solution.

Anti-bacterial analysis

S.aureus DSM 1104, DSM 20045, *E.coli* were grown on their respective media *i.e.* Tryptic Soy Agar (TSA) and chromo select agar respectively, wherein the aforementioned microbes were streaked on their respective nutrient medium and were incubated overnight. After that, broth dilutions were prepared for the same and were subjected to incubation for overnight in Erlenmeyer flasks [3]. Finally cultures were diluted in fresh Mueller Hinton Broth (MHB, pH 5.6) at optical density OD600=0.5 in order to determine the exponential growth.

Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Count (MBC) Test

MIC is described as lowest inhibiting micro-organism development during a 24-hour incubation cycle. Estimation of minimum inhibitory, extracts amount was calculated by preparation within nutrient broth of specific extract concentrations of 500 μL^{-1} and then serial dilution was performed in a plat with 96 wells. In Muller Hinton agar reservoirs were seeded with uniform inoculum of sample bacteria, 0.5 ml amount of each dilution was applied aseptically. Triplicate tests were conducted. At 37°C, 18 hours agar plates have been incubated followed by counting optical density to determine growth. The lowest amounts of MIC were considered samples showing a strong inhibition region. Spread plating was conducted for analyzing anti-bacterial efficacy of the sample *i.e.* chitosan/surfactin NPs. The minimum sample amount with no growth was

considered as MIC and lowest amount with no detectable live cells was considered as MBC.

Determination of Minimum Biofilm Eradication Concentration (MBEC)

Biofilms were developed on peg lids to determine MBEC of chitosan/surfactin NPs. overnight cultures were prepared. Further, peg lids were inoculated for 2 hours at 37˚C in a plate with 96 wells. After incubating for 2 days the peg lids was then transported to naive plate comprising serial dilutions of chitosan/surfactin nanoparticles.

Visualization of live cells in biofilms

To assess the impact of chitosan/surfectin within biofilms, *S.aureus* was utilized as model. Overnight cultures of S.aureus were developed as mentioned before and then transferred to plate with 96 wells. The bacterial suspension were then properly eradicated by aspiration and replaced with MHB, followed by incubating at 37˚C for 24 hours followed by replacing the supernatant and again incubating for next 24 hrs. After 48 hours of incubation the supernatant was again replaced and again incubated for 24 hours at 37°C. Finally the plates were observed by utilizing CLSM (Zeiss LSM800, 40 × Plan-Apochromat (NA 1.5) objective.

RESULT AND DISCUSSION

Physio-chemical properties of chitosan-surfactinnanopartilces

Physiochemical properties of chitosan-surfactin nanoparticles are assembled in Table 1.

Table 1: Representation of physio-chemical properties of chitosan-surfactin nanoparticles.

Physiochemical properties of Chitosan/Surfactin nanoparticle	
Hydrodynamic diameter	285.5±0.65
Polydispersity index	0.19±0.04
Zeta potential (mV)	+59.6±1.9
Concentration µg/mL	73.5

The size of the chitosan/surfactin nanoparticles were observed to be spherical (Figure 2) and were found to be stable. It was observed that the nanoparticles were stable below pH 6.5. This may be due to chitosan's protonating nature of amino group. At pH above 13 formations of agglomerates were observed. DLS was used for measuring approx. dimensions and zeta potential. The chitosan/surfactin nanoparticles were also stable at 4°C for duration of six months. There are many pathways to aid in development of minute and stable nanoparticles in presence of surfactants [14]. Development of nanoparticle does not have any effect on just polymer, surfactant structure, but also on process utilized for synthesis [14]. During cavitation, hydrophobic tail decreases surface stress, leading to more compact nanoparticles [15].

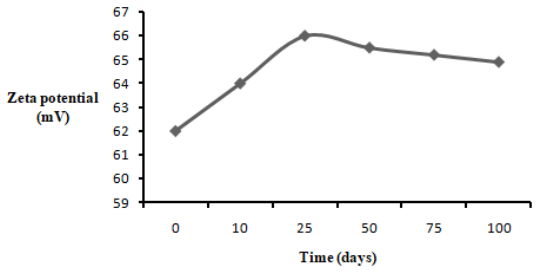

Figure 2: Zeta potential of chitosan/surfactin NPs as a function of storage time at 4°C. Data procured from DLS.

Anti-bacterial analysis

Chitosan and Surfactin both are known for inhibiting bacterial cell membrane but through different mechanism. By taking into account that the target is cellular in nature, it can be concluded that the chitosan/ surfactin formulation are effective for both planktonic and slow-developing bacteria over biofilms. For this MIC and MBC was calculated against E.coli and two different strains of S.aureus (Table 2).

A clean result was obtained wherein it was observed that the nanoparticles was effective for both gram positive and gram negative bacteria, however, slightly differed in terms of efficacy.

This difference may be due to covering over gram negative bacteria which may potentially provide a barrier to Surfactin permeation within the cell membrane.

Chitosan is widely known to inhibit the bacterial cell, but combination of chitosan and Surfactin is proved to be more potent towards inhibiting the cell envelope. Table 2 represents the MIC and MBC count of the chitosan/Surfactin nanoparticles.

Table 2: MIC and MBC count of chitosan/surfactin nanoparticles.

Strains	MIC	MBC
S.aureus	15	30
S.aureus	30	39
E.coli	15	20

Minimum Biofilm Eradication Concentration (MBEC)

The effectiveness of antimicrobials is much reduced in removal of biofilm-growing bacteria. It may be that fact due to the absence of (or delayed) antimicrobial penetration and enhanced bacterial tolerance within biofilm. Bacterium chitosan promotes nanoparticle aggregation and antimicrobial phase of biofilm chitosan and rhamnolipid activity is also effective against bacteria species that are biochemically dormant. Thus anti-biofilm was calculated for chitosan/ surfactin nanoparticles by demonstrating CFU/ml in aforementioned bacterial species biofilms after treating it for 24 hours. It was observed that the combination of chitosan/surfactin was effective against said bacteria in comparison to chitosan and Surfactin alone. Also no. of viable cells was below assessment level of chitosan/ Surfactin amount of 60/73 µg/mL for both bacterial strains. Thus the chitosan/Surfactin was able to remove biofilms in aforementioned bacterial species. The potency of biofilm biocides may also be evaluated by determining level necessary to remove all sustainable cells within biofilms or Minimum of Biofilm Eradication Concentration (MBEC).

On conducting analysis, amounts after 72 hours, it was observed that none of compounds had been able to eliminate biofilm (data not displayed). Therefore, although viable cells cannot be detected in CFU counting, it infers to biofilm comprise few sustainable cells which repopulate media when nanoparticles are excised it can thus be concluded that, adsorption of biofilms may be due to chitosan but may not be able to permeate within biofilm due to its extra dimensions and interactions with negatively charged particles within biofilm's matrix. However, if some other anti-microbial agent may be able to penetrate within matrix then it could become possible to reach within biofilm. Therefore the present study shows that since nanoparticles which strongly communicate with biofilm matrix couldn't approach bacteria far into biofilm they are an efficient way of providing certain antimicrobials that spread In biofilm. These findings support the prospect of utilizing antimicrobial nanoparticles along with other ways to improve penetration of biofilm into illnesses.

Cytotoxicity

Bacterial toxicity's selectiveness is crucial for designing naive anti-microbial therefore evaluation of cytotoxicity of chitosan/Surfactin is against human BJ cells. Human BJ cells culturing was performed in serially diluted amounts of test samples. The range of concentration tested was then selected to increase biocidal amount for bacteria and should lie within range evaluated for cytotoxicity in other studies. The chitosan/surfactin nanoparticles at varying concentrations (0, 10, 25, 50, 75,100 µg/mL) after 48 hours did not show any cytotoxicity (Figure 3).

Thus smaller size of the formulated nanoparticles, enhanced surface charge, ameliorated stability are general points that are important for surged antimicrobial efficacy. Also larger amount of free amino groups of chitosan in the nanoparticle formulation creates greater charge density thereby boosting antimicrobial activity of said chitosan, which is specifically due to electrostatic interactions. Thus it may be concluded that higher electrostatic interactions of the chitosan/surfactin nanoparticles leads to disruption of bacterial cell covering. The surface interactions also leads to release of Surfactin in proximity of bacterial cell thereby aiding in accession of Surfactin to cell envelop.

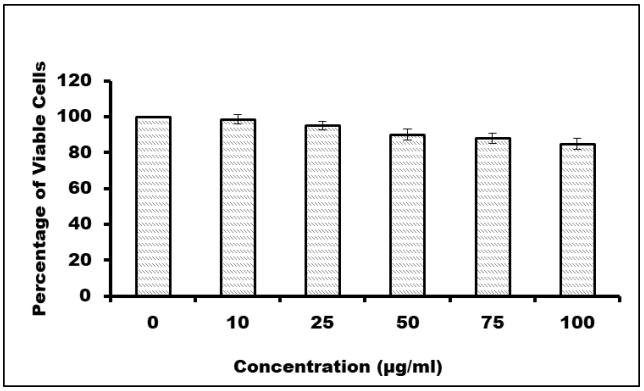

Figure 3: Cell viability %age of chitosan/Surfactin NPs. The chitosan/Surfactin nanoparticles at varying concentrations (0, 10, 25, 50, 75,100 µg/mL) after 48 hours did not show any cytotoxicity.

CONCLUSION

Chitosan and Surfactin, both possess anti-microbial activity; however their mechanism of action is different. But still their single effect on microorganism was not much effective. Therefore the present paper dealt towards combination of both of said components i.e. chitosan and surfactant and evaluate their anti-microbial activity towards gram positive and gram negative bacterial species. It was observed that nanoparticles formed were of small size with high charge density that specifically leads to increased anti-microbial efficacy against *S.aureus* strains, *E.coli* and *S. epidermis*. The present study demonstrates that, chitosan/surfactin nanoparticles boosted the anti-microbial activity against bacterial species which clearly infers that this application can be widely applied in medical and food related problems thereby preventing contamination of the same. Also by application of the present study novel compounds can be formulated in order to gain different outcomes and potentialities.

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