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Immobilization and characterization of *Bacillus subtilis* in PVA-chitosan composite Nanofiber

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Abstract
Bacillus subtilis is a plant growth promoting bacteria that is employed in agriculture for increasing crop productivity. However, maintaining the viability of microorganism in formulations is a major problem during application. In this work, a new methodology has been developed for immobilizing bacteria in PVA/CS nanofiber and evaluated the suitability as carrier for *B. subtilis*. The morphology and functional group of bacteria loaded in nanofiber were characterized by Scanning Electron Microscope (SEM) and Fourier Transform Infra-Red Spectroscopy (FTIR). After encapsulation of bacteria, the nanofiber size was increased and confirmed by SEM and the functional groups of *Bacillus subtilis* were profiled in nanofiber through FTIR spectral finger printing. The bacterial viability was maintained in the nanofiber throughout the study period and also the cells remained effective and infective. The results confirmed that the encapsulation of bacteria in nanofiber could be a cost effective and eco-friendly methodology for the efficient delivery of beneficial microbes in soil and plant eco systems to enhance the agricultural productivity.

Keywords: Electrospinning, encapsulation, Nanofiber, bacteria, composite

1. Introduction
Bacillus subtilis is a gram-positive plant growth promoting bacteria which provide broad variety of ecological services like, the acquisition of nutrients, prevention of diseases, increases crop yield and decreases pest damage to plants (Garcia-Fraile *et al.*, 2015) [6]. The free cells of bacteria were prone to various physical damage and environmental stresses like temperature, pressure and humidity changes which cause instability in cellular content and damage them. To avoid these consequences caused to cells, they can be encapsulated in the carriers' which acts a barrier between environment and cell content, and also store cells at dry condition. Encapsulation is an effective technology that improves shelf life, protection, handling, and controlled release of microbes (John *et al.*, 2011) [8]. Several polymers can be used for cell encapsulation by exploiting various methods like, freeze drying, extrusion, spray drying, and emulsion. These methods have major disadvantages: high fabrication cost, less microbial survival, low upscaling for industrial scale, lack of multiple strain encapsulation and ambiguity in root colonization (Bashan *et al.*, 2014) [2].
Electrospinning is a simple eco-friendly and cost-effective method to yield solid nanofibers by various polymer solutions using high voltage as driving force. These nanofibers have unique properties *viz*., high mechanical/thermal stability, porosity, tunable sustained delivery and high surface to volume ratio (Persano *et al.*, 2013) [13]. Nanofibers can be used for encapsulation of mammalian cells (Canbolat *et al.*, 2011) [5], bacterial strains (Zussman, 2011) [18], spores (Spasova *et al.*, 2011) [14], yeast cells (Letnim *et al.*, 2015) [10], nanoparticles (Zhang *et al.*, 2014) [17], antibiotics, and plasmids (Lee *et al.*, 2014) [9]. Now, electrospun nanofibers are gaining attention in agriculture as a new delivery system for seed coating of agrochemicals and also carrier of microbial cells.
In the present assignment, poly vinyl alcohol (PVA) and chitosan (CS) have been selected for fabrication of encapsulating material due to their good electro spinnability, biocompatibility, nontoxicity and biodegradability (Teodorescu and Bercea, 2015) [15]. Chitosan polymer also has additional benefits as biostimulant and bio fungicide.

Fabrication of nanofiber using pure chitosan is difficult due to its high viscosity (Abdelgawad *et al.*, 2014) [1] and to overcome this disadvantage, PVA is blended with chitosan to improve nanofiber forming characteristics and also to reduce crystallinity of chitosan (Li and Hsieh, 2006) [11]. In the present study, PVA/CS composite solution was mixed with *Bacillus subtilis* cells and the microbe encapsulated composite nanofiber was formed. The concept is to preserve the viability of bacteria for long time by encapsulation and to evaluate the encapsulation efficiency, microbe viability and confirming the presence functional group analysis.

2. Material and Methods

2.1 Materials

Medium molecular weight Chitosan (Product no. 448877) and Glacial acetic acid (Product no. 695092) were purchased from Sigma-Aldrich, and Partially hydrolysed PVA (Product no. GRM6170) was obtained from HI media. Freeze dried culture of Bacillus *subtilis* was obtained from MTCC (Accession number: 1305).

2.2 Immobilization of Bacillus subtilis in composite nanofiber

For the electrospinning of bacteria loaded composite nanofiber, 10% of PVA solution was prepared by dissolving in distilled water and 2% chitosan solution was prepared by dissolving in 2% acetic acid. These solutions were stirred separately at 100rpm at room temperature until complete dissolution was achieved. The composite of PVA and chitosan solution was prepared by blending them at the ratio of 9:1, and stirred at 100rpm for obtaining homogenous solution. The freeze-dried culture of *Bacillus subtilis* was inoculated in nutrient broth and incubated at 37ºC for 24 hrs. The log phase microbial cells were centrifuged at 2000xg for 15 mins and *Bacillus subtilis* pellets with a cell load of 10^{16} CFUs was added to the PVA/CS composite solution, and stirred at 80rpm for 3 hrs to obtain well dispersed bacterial mix composite solution. This solution was filled in the syringe equipped with metal needle and electrospinning was done under the following parameters *viz*., voltage, 17kV; tip to collector distance, 15cm and flow rate, 0.5ml/hr. The bacteria loaded nanofiber was collected over the aluminium foil fixed over the nanofiber collector. The bacteria loaded nanofiber was stored at room temperature for future studies.

2.3 Characterization of bacteria loaded nanofiber

2.3.1 Morphological structure analysis

The surface morphology and diameter of *Bacillus subtilis*, composite nanofiber and *B.subtilis* loaded nanofiber were studied with the help of Scanning Electron Microscope (SEM)(FEI QUANTA 250). The samples were mounted on the sample stub and gold sputter coating was done to avoid sample damage from electrons while scanning. The samples were scanned at different magnifications to confirm the immobilization of bacteria in composite nanofiber.

2.3.2 Functional group analysis

The functional group of *Bacillus subtilis*, composite nanofiber and *B.subtilis* loaded nanofiber was analysed by Fourier Transform Infrared (FTIR) spectroscopy to obtain the variation in the spectral finger prints of samples. The samples were analyzed in FTIR spectrophotometer-6800 type A (M/s. Jasco, Japan) equipped with Attenuated Total Reflectant Unit (ATR) sensor. TGS detector was used to analyse the sample and the spectral scanning was done in mid-range IR spectra ranges from 400 cm^{-1} to 4000 cm^{-1}.

2.4 Viability of bacteria

The cell viability of *Bacillus subtilis* in nanofiber was assessed by spread plate technique. The bacteria loaded nanofiber was dissolved in phosphate buffer and serial diluted, and inoculated in nutrient agar medium. The inoculated petriplates were incubated at 37ºC for 24 hours and colonies were counted. The viability of bacteria loaded in nanofiber was tested for six months, and colony forming units were represented as $\log_{10}$ CFU gm^{-1} of nanofiber with standard error.

3. Result and Discussion

3.1 Electrospinning of bacteria loaded nanofiber

The nanofiber is an one dimensional structure with special properties like high surface area, porosity and safety when compared to other nanostructures. In this study, the PVA/CS composite nanofiber was fabricated by blending them at the ratio of 9:1. This composite nanofiber has outstanding properties like biocompatibility, biodegradability and nanofiber forming capacity. The beneficial properties of nanofiber made them as wonderful delivery system of cells and extends its' viability. The encapsulation of microbes in nanofiber offers numerous advantages in comparison to free cells (Loh *et al.*, 2020) [12] and provides stability, viability and long-term reusability without loosing its beneficial activity (Costa *et al.*, 2018) [3]. Also, nanofiber increases the sustainability, protection from toxicants and toxic substances, and increased plasmid stability of the cell under different conditions (Spasova *et al.*, 2011) [14]. The current work discovers the electrospinning process to encapsulate *B.subtilis* in PVA/CS composite nanofibers and confirmed its' successful immobilization through morphological and structural studies by using SEM and FTIR. The nanofiber has improved the viability of cells by enhancing barrier properties against temperature, pressure, moisture and physical damage.

3.2 Morphological and Internal structure analysis

Scanning Electron Microscope (SEM) image showed the morphology of *B. subtilis* before and after loading in the nanofiber (Figure 1). The average size of rod-shaped *B. subtilis* was 540±120 nm in width and 2498±510nm in length (Figure 1a) and the composite nanofiber have smooth surface with average diameter of 128±11nm (Figure 1b). After embedding *Bacillus* cells, the average diameter of the nanofiber has been increased (2653±653nm in length and 635±139nm in width), and cell distribution resulted in widening of the nanofiber (Figure 1c). Similar trend of results recorded by Diep and Schiffman (2021), in which they observed the length and width of nanofiber was increased from 2.44±0.57µm to 2.52±1.14µm and 0.65±0.07µm to 0.86±0.05µm respectively after impregnating alginate-based nanofiber with *Escherichia coli*. The SEM results showed entire embedment of *B. subtilis* in PVA/CS composite nanofiber.

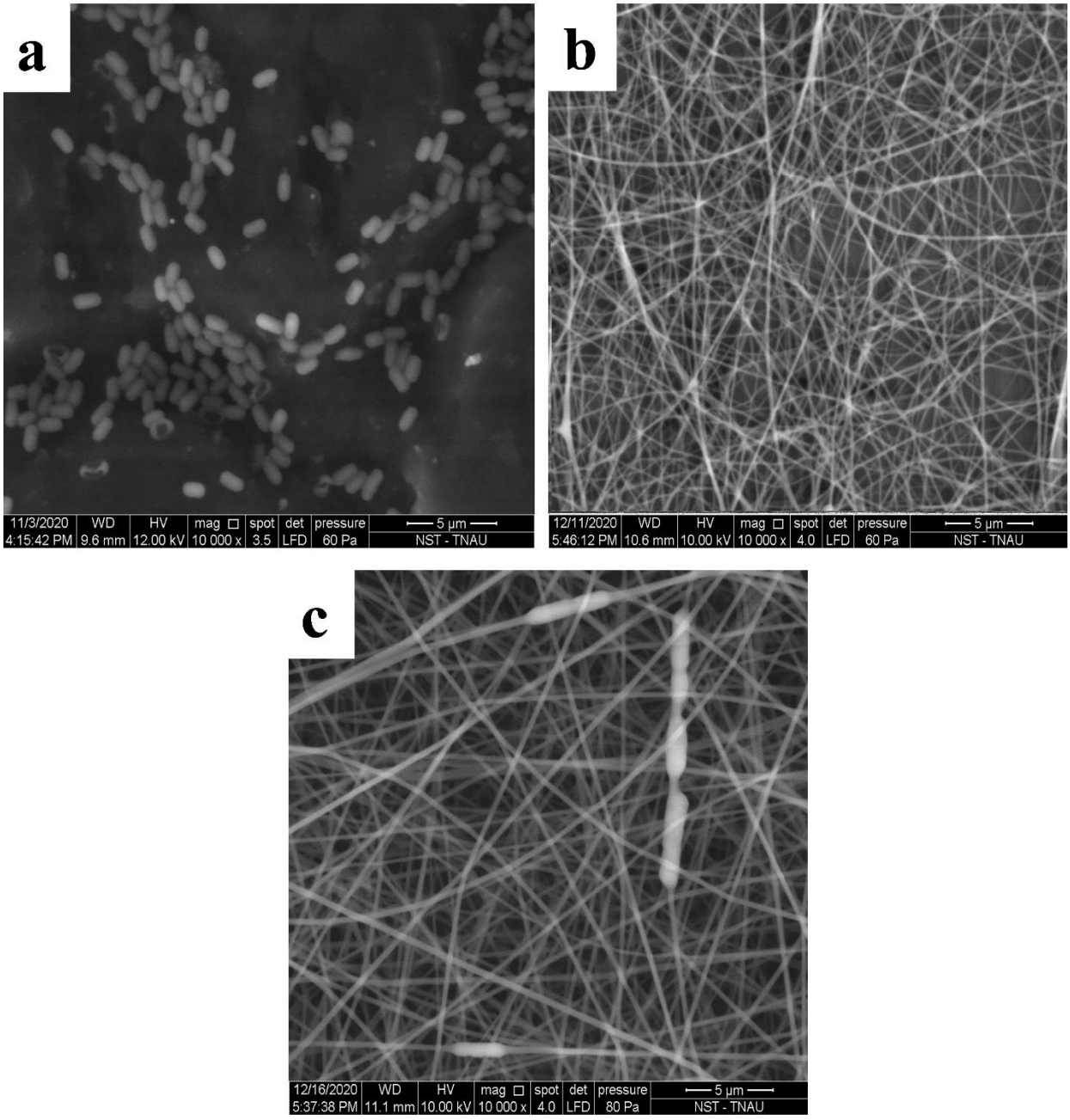

Fig 1: Scanning Electron Microscope images of (a) *Bacillus subtilis* cells (b) PVA/CS composite nanofiber, and (c) *Bacillus subtilis* loaded PVA/CS composite nanofiber

3.3 Functional group analysis

FTIR analysis showed the bonding between samples by using infrared absorption spectrum and confirm the successful encapsulation of cells in the carrier. In general, the bacterial cell wall consists of lipoproteins, proteins, phospholipids and lipopolysaccharides which were made by the functional groups like amide, phosphatic, hydroxyl and carboxyl. The functional group analysis confirmed the positive loading of *B. subtilis* in the PVA/CS composite nanofiber (Figure 2). FTIR spectra of *Bacillus subtilis* had transmittance peaks at 2943cm^{-1}, 1658 cm^{-1}, 1573 cm^{-1}, 1239 cm^{-1}, 1079 cm^{-1} and 562 cm^{-1} due to C-H stretching of cell wall, C=N stretching (amide I), amide II, amide III, asymmetric stretching of C-O-C, and P-O-C bonding of phospholipids. The PVA/CS composite nanofiber have characteristic peaks of PVA at 1090 cm^{-1} (C-O stretching) and 2934 cm^{-1} (C-H stretching) and also have chitosan characteristic peaks at 1550 cm^{-1} and 1649 cm^{-1}due to N-H bending and C=O stretching. After loading bacteria in nanofiber, the amide groups at 1686 cm^{-1}, 1567 cm^{-1} and 1259 cm^{-1} were slightly shifted and deformed in their intensity due to binding of amides with the nanofiber. The other transmittance peaks at 2934 cm^{-1}, 1089 cm^{-1} and 610 cm^{-1} were due to C-H stretching, C-O stretching and P-O-C bonding of phospholipids represented the presence of bacteria in nanofiber. Similar results were reported by Chun *et al.* (2021) [16] in which they observed peaks of phospholipids, amide (I,II,and III), and polysaccharide groups of bacteria in alginate film.

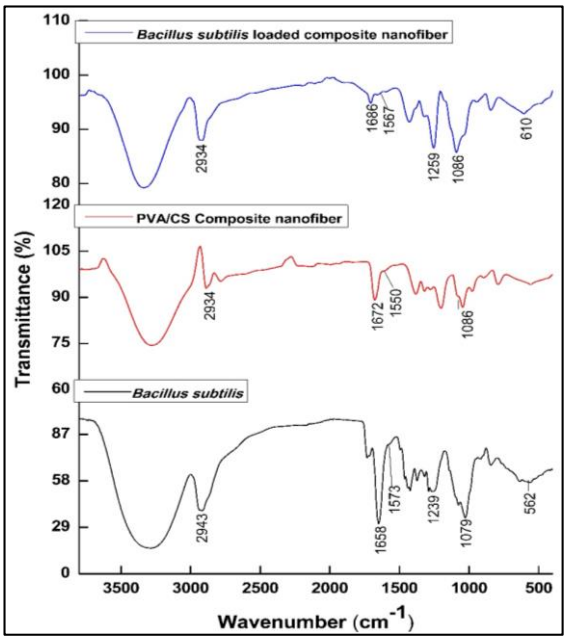

Fig 2: FTIR analysis of (a) *Bacillus subtilis* cells (b) PVA/CS composite nanofiber, and (c) *Bacillus subtilis* loaded PVA/CS composite nanofiber

3.4 Viability of bacteria

The nanofiber was an excellent carrier for immobilization and maintaining the viability of microbes and the polymers used for nanofiber fabrication protected cellular integrity while exposed to external environment. The average load of *B. subtilis* cell suspended in the spinning solution was 10^{16} CFUs. After spinning, 14.06±0.3 $\log_{10}$ CFUs were loaded in the nanofiber and the remaining cell load was lost due to mechanical stress and pressure caused by solvent evaporation while applying high voltage to bacterial polymer mix. The viability of *B. subtilis* loaded in nanofiber was checked monthly wise up to six months, which was stored at the room temperature. Figure 3 showed only gradual decrease of cell count in nanofiber from 14.06±0.3 $\log_{10}$CFUs to 7.96±0.8 $\log_{10}$CFUs between 1st and 6th months, while storing at room temperature. This loss in viability was due to heat transfer through nanofiber from external environment. Hussain *et al.*, 2019 noticed that the viability of microbial consortium (*Bacillus subtilis and Serratia marcescens*) loaded in hybrid nanofiber was decreased from 6.15±0.05 $\log_{10}$CFUs to 4.12±0.06 $\log_{10}$CFUs, while storing at the ambient condition. Thus, the result proved that PVA/CS composite nanofiber could protect and safeguard the microbes from external stresses.

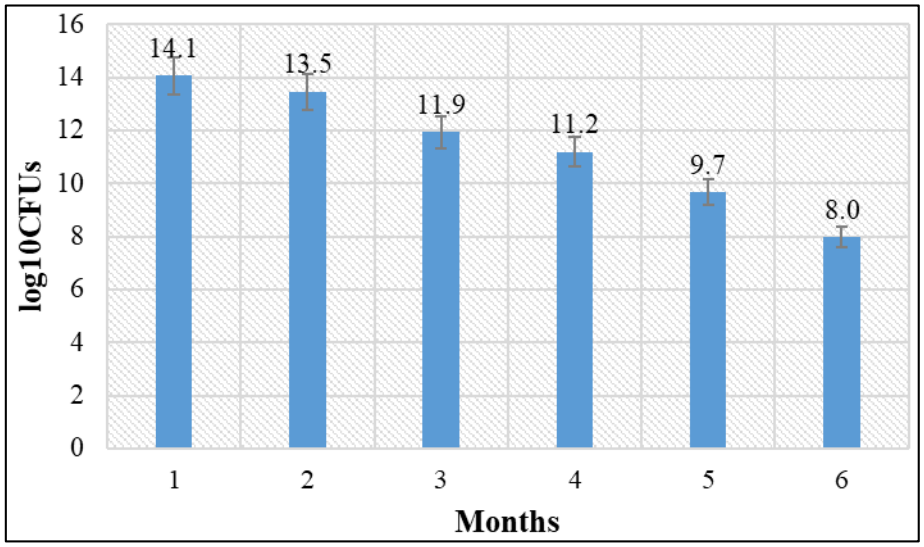

Fig 3: Viability of *Bacillus subtilis* immobilized in PVA/CS composite nanofiber for 6 months period stored at ambient condition. The cell count represented in (Log_{10} CFU/gm of nanofiber).

4. Conclusion

The current work emphasized the methodology for the fabrication of bacteria loaded PVA/CS composite nanofiber. SEM results revealed the presence of microbial cells through enhancement of the diameter of nanofiber after immobilizing *Bacillus subtilis.* The FTIR analysis confirmed the presence of amide and phospholipid functional groups of bacteria in cell immobilized nanofiber. The viability of *Bacillus subtilis* impregnated in nanofiber exhibited a survival rate of 7.96±0.8 $\log_{10}$CFUs during 6th month of storage at ambient temperature. The improved storage of microbes in nanofiber paved the way for wide applications in seed coating and in the biofertilization programme. High load of microbial cells can be infused in the nano fibre, resulting in better field efficacy and cell performance. Further research on polymer composition and methodology in fibre formation would certainly improve the bio-efficacy and release pattern of microbes from nanofiber.

5. Acknowledgement

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