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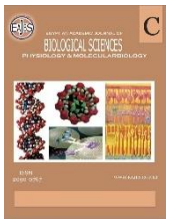

Ecofriendly System Based on Mesoporous Silica Nanoparticles Delivery of Chitosan to Control Soft Rot Disease and Maintain Postharvest Quality of Tuber Potato

Sodaf A. Maan[1] ; Gamar G. M. [2]; Abeer A. Faiesal[3] ; and Hoda R.A.El-Zehery[4]

[1]Dept. of Microbiology Fac. of Agriculture, Ain Shams Univ., Cairo, Egypt.
[2]Dept. of Life and Earth Sciences.Higher N, Djamene Institute For Training Teachers, Chad.
[3]Dept. of Basic and Applied Agriculture Sciences, Higher Institute for Agriculture Cooperation, Cairo, Egypt.
[4]Dept. of Microbiology, Fac. of Agriculture, Benha Univ., Egypt.
***E-mail:** sodaf@agr.asu.edu.eg ; abeerfaisal1523@yahoo.com ; gamarmahamat 1981@gmail.com ; **hoda.alzoheri@fagr.bu.edu.eg**

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ABSTRACT

Background: *Enterobacter cloacae* is a bacterium that leads to soft rot in many diverse types of vegetables & crops across the world. At this time nanotechnology is utilized as a nanomaterials strategy for controlling plant pathogenic bacteria and maintaining postharvest quality. **Methods:** The soft rotten tissues were collected from infected potato tuber and macerated in buffer saline solution. 50 uL of macerated sap was inoculated on selective King, B medium for isolated pathogenic bacteria. PCR was utilized to identify the isolated bacteria according to their morphology and the 16S rRNA gene. A randomized complete design was applied in the protective by Ch-MSNPs20nm@HTCC in postharvest. **Results**: Typical colonies on King, B medium were circular, moist, smooth, and entire margin, flat and fluorescent diffusible pigment in medium that identify *Enterobacter* sp. As well as phylogenetically it identified *Ent. cloacae* based on 16s RNA gene. Ch-MSNPs20nm @HTCC sprayed on infected potato tubers postharvest significantly reduced the disease severity, lowest HBS and AUDPC values and increased weight of healthy tuber tissues compared with Ampicillin as a positive control. In addition, it has the ability to prevent isolated *Ent. cloacae* from forming a biofilm. On the other hand, it has efficacy in increasing the longevity of potato tubers at postharvest.

INTRODUCTION

Potatoes, which are a member of the genus Solanum tuberosum L., are regarded as one of the most significant crops grown for human use. They are grown everywhere in temperate climates, but very occasionally in hotter ones. The potato is the 4th most widely cultivated edible crop on the planet, after wheat, rice, as well as maize. In Egypt, potato occupies a prominent position among all vegetable crops & is consumed by humans (Fao, 2008). The potato is widely regarded as the top crop that has gained significant economic significance for the entire planet (Chauhan *et al.*, 2022). With a global production of more than 360 million metric meters, the potato is recognized as one of the most vital crops. About 35 million tons of fruits & vegetables were grown in Egypt in 2018, contributing to the country's annual crop. Egypt exported approximately 724,200 thousand tons of potatoes in 2018, ranking sixth internationally, mostly to Russia and the EU.

This study examines the potato crop and land suitability for its development due to its importance and the various challenges and problems it faces in production and export. This paper also focuses on the agricultural practice of determining whether or not the land is suitable for the production of potatoes. (Mohammad *et al.*, 2022). Potato tubers are one of the postharvest crops and are attacked by different bacterial diseases in an open field and postharvest mainly, soft rot that results from *Pectobacterium carotovorum* (Eman, *et al.*, 2022 and Amira. *et al.*, 2022) & *Ent. Cloacae* (Eman, *et al.*, 2020) postharvest is considered the main causal agent of soft rot illness in potatoes and a wide diversity of vegetables & crops (Surman, 2011). It exists on the surface of tubers and penetrates through wounds or natural openings throughout transport and storage. If the invasion is successful, the pathogen will settle into the plant's intercellular space or vascular tissue, where it will create plant cell wall-degrading enzymes (PCWDEs) and spread illness. Maceration enzymes that are secreted from cells include polygalacturonase (Peh), cellulase (Cel), xylanase, pectate lyase (Pel), & protease (Prt). When pathogens invade plants, these enzymes can break down the tough cell walls made of cellulose, pectin & hemicelluloses, inducing plant cell death in addition to tissue maceration (Abbott & Boraston, 2008 and Czajkowski *et al.*, 2011).

The action of pectinolytic enzymes on the pectin middle lamella area of the cell wall is related to significant alterations in the characteristics of fruit & tuber materials during storage, microbial infection & processing (Benen *et al.*, 2003 and Jakób, *et al.*, 2009).

Numerous studies have led to the creation of Mobil Crystalline Material 41 (MCM-41) in an effort to fully use the benefits of mesoporous silica nanoparticles (MSNs) (Mohamed, et al.,2022). It has excellent dispersion, a porous structure that may be adjusted uniformly, & colloidal chemical properties (Farokhzad & Langer 2009; Shi *et al.*, 2016). Nevertheless, only in recent years has the focus of MSNs research and their applications in several Fields switched to agriculture and the treatment of pest diseases (Popat *et al.*, 2012; Wang *et al.*, 2014). MSNs are utilized as delivery mechanisms for biomolecules for example medicines (Zhang *et al.*, 2015), nucleic acids, (Keasberry *et al.*, 2017), urea, (Wanyika *et al.*, 2012) & protein (Guo *et al.*,2012), MSNs are distinguished by features such as their tunable size, their vast surface area of their pores & their biocompatibility, all of which allow them to be translocated into other cells. Chitosan is a biopolymer molecule that is often made by the de-acetylation process of chitin and has found widespread use in sustainable agriculture (Kashyap *et al.*, 2015). One of the most investigated chitosan derivatives is the quaternized form, namely N-(2-Hydroxyl) propyl- 3- tri-methyl-ammonium Chitosan chloride (HTCC). This is corroborated by the physiochemical features of N-(2-Hydroxyl) propyl-3-tri-methyl-ammonium Chitosan chloride, which include high water solubility, the preservation of a cationic charge at neutral pH, enlarged permeability, muco adhesiveness, & antibacterial activity (Rwei *et al.*, 2014). Therefore, electrostatic attraction is utilized to coat the positively charged HTCC layer over the negatively charged MSNs, making this the optimal technique for surface modification that allows MSNs to function as effective virucidal carriers. Finally, this research aims to add to the field of smart agro-nanotechnology for the managing of plant illnesses after harvest.

MATERIALS AND METHODS

Isolation of Bacteria:

Diseased tubers of the potato species (Solanum tuberosum cv. Spunta, with classic soft rot symptoms) were procured from the Plant Pathology Laboratory at the Agriculture Faculty at Ain Shams University in Cairo, Egypt. Soft rot tissue was inoculated on nutrient agar medium, and the bacteria were cultured for 48 hours at 37 degrees Celsius. A streak plate approach on Difco nutritional

agar medium was employed for the final purification.

Identification of Bacteria:

Crystal violet polypectate was applied as a selective medium, & then the isolated bacteria were grown on King B in addition to raffinose media to determine their identities. Isolates of bacteria were stored in a nutrient broth-glycerol solution (nutrient broth with 20 percent glycerol) at 4 degrees Celsius.

Identification of *Enterobacter* sp.

DNA Extraction:

About 520 CFU of isolated bacteria was suspended in 2 mL of PrepMan™reagent in a new microcentrifuge tube and vortexed for 30 sec. then centrifuged for five minutes at 6,000 rpm. The pellet was resuspended in 200 uL of sterile distilled water and boiled at 100 degrees Celsius for 20 minutes. It was then cooled to room temperature for 2 min to release the DNA. Tubes were centrifuged at 6000 rpm/2min. 5µL of supernatant was added to 495µL nuclease-free water to obtain a dilution ratio of 1:100 and saved for PCR.

Measurement of DNA Concentration By Using Nanodrop:

The nanodrop (thermo2000C) was applied to measure the absorbance at 260/280nm, which provided information on DNA concentration and purity. Total DNA analysis and agarose gel visualization: Each isolate's genomic DNA was analyzed using agarose gel electrophoresis (Sambrook and Russel, 2001).

Detection of 16S Ribosomal DNA (16S rDNA):

Using the universal bacterial 16S rDNA primers indicated in Table(1), we were able to use polymerase chain reaction (PCR) to identify the bacterial isolates.

Table 1: Universal primers sequence of 16S rDNA used in polymerase chain reaction.

Primers	Sequence	AT	Length
27F	5'-AGAGTTTGATCCTGGCTCAG-3'	60ºC	20
1492R	5'AAGGAGGTGATCCAGCCGCA-3	60ºC	20

AT = Annealing temperature, F = Forward primer, R = Reverse primer.

Reagents:- PCR amplification reagents & amounts utilized are listed in Table (2).

Table 2: Reagent as well as volume (50µl) utilized in PCR amplification for 16s rDNA

No	Reagent		Volume per reaction
1	Primers	27 Forward	2.5 µl
2		1492 Reverse	2.5 µl
3	Taq DNA polymerase		1 µl
4	DNA template		1 µl
5	dNTP mix		4 µl
6	Sterile dH2O		30 µl
7	10×PCR buffer		5 µ1
8	MgCl2		4 µl

PCR Amplification of 16S rDNA:

Table (3) detailed the thermal cycling protocol for amplification of *Enterobacter sp.* 16S rDNA.

Table 3:Program utilized in PCR amplification for 16S rDNA

Steps	Temperature	Time	No. of cycles
Initial denaturation	95ºC	5min	1
Denaturation	95ºC	30 sec	35
Annealing	60ºC	40 sec	
Extension	72ºC	120 sec	
Finial Extension	72ºC	10 min	1

The Preparation of Agarose Gel & DNA Loading Is As Follows:

Gel Electrophoreses:

10 ul of PCR product was electrophoresed on 2 percent agarose gel for determining the size of the product via RF of DNS ladder (100 to 1000bp) and developed by ethidium-bromide staining (0.5µl/ml). The fragments were photographed using a UV lamp in gel-documentation. The negative control consisted of all PCR components except for the template DNA

Preparation of DNA Fragment.

16S rDNA PCR fragment (677 bp) was purified using Gene All Combo reagent (Korea) consistent with the manufacturer's instructions for sequencing. After cycle sequencing, excess dye terminators were removed and the purification product was purified using MicroSEQ™ ID purification combo kit, with clean-up cartridges (Including ExoSAP-IT™ Reagent).

Microbiology Lab., Department of Agriculture, Ain Shams University, Cairo, Egypt, analyzed 16s rDNA data via MicroSEQ™ ID ID Analysis Software. After obtaining sequence chromatograms from the macro gene firm in South Korea, the data were manually altered in chromas version 2.01, which allowed them to be sent to the sequencer. On the homepage of the National Center for Biotechnology Information (NCBI), the BLASTn software was utilized to analyze sample similarity (http:// www .ncbi .nlm.nih.gov) and Ribosomal database project II (Cole *et al.*,2005). Sequences with similarity percentages above 97 percent were selected as a species (Stackebrandt *et al.*,2002). Alignment of sequences constructed with Clustal W http:// www.ebi.ac.uk/clustalW (Thompson *et al.*,2002) & utilized for phylogenetic analysis in PAUP4 in Geneious program (Drummond *et al.*,2011).

Source of Nanoparticles:

Preparation of Chitosan:

Native chitosan (2-Amino-2-deoxy-(1,4)–B-D-glucopyranan, M.W 10000 KD) (Fluka AG, CH-9470 Buchus chemical company) was dissolved in 0.05% (W/V) acetic acid & pH was adjusted to 5.5 with NaOH. The average deacetylation degree of the products was 85%.

The applied nanoparticles mesoporous silica (MSNPs) delivery, (Fig.,1) water-soluble chitosan (Ch) derivative and HTCC called N-(2-Hydroxyl) propyl-3- tri-methyl-ammonium chloride were formulated Ch- MSNPs @ HTCC were kindly obtained for scientific research purposes from Nanotechnology & Advanced nanomaterials laboratory "NANML", Plant Pathology Research Institute, Agricultural Research Center (ARC), Egypt. During the vegetative growth phase, the plants were foliar sprayed with Ch- MSNPs at HTCC 50 mg/l until the spray started dropping. This was done with a modest pressure pump after Tween 20 (0.5 percent) was added as a wetting agent. The results were based on labs. of "NANML" ARC.

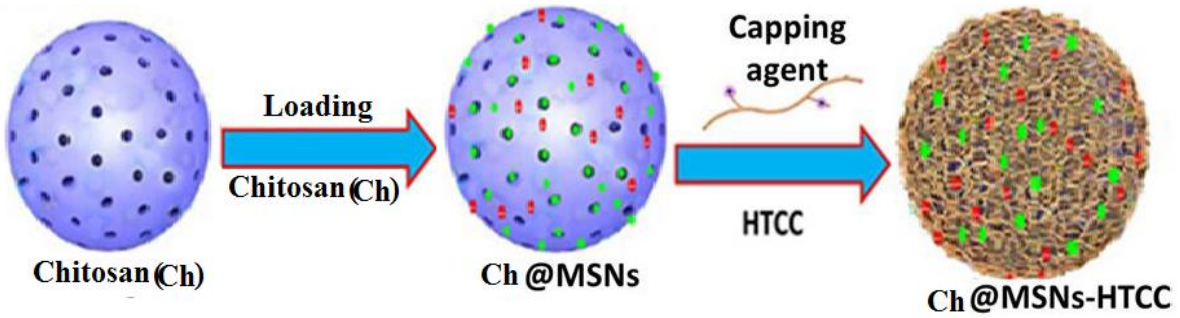

Fig. 1: An illustration of the loading process of chitosan onto mesoporous silica nanoparticles as well as the creation of Ch@MSNPs20nm-HTCC.

Transmission Electron Microscope (TEM):

A few drops of nanoparticles were dropped on a carbon-coated copper grid and negative staining with urinal acetate. The grids were examined by TEM (Joel JEM-1400 TEM machine, El-Azher Univ.).

Antibiotic, 1 mg/ml ampicillin (Sigma, St. Louis, MO) Placing the material on suitable agar dishes confirmed in the experiments that the absence of live four bacteria causes soft rot in potato tuber.

The Antivirulence Activity of Ch-MSNPs@ HTCC:

1-Assessment of Biofilm Formation:

Tissue culture plates are the best biofilm detection method. Christensen and colleagues (1995) described this method as a quantitative test. It was determined whether or not the isolated *Enterobacter sp* has the ability to generate biofilm. In a nutshell, 10 (ml) of nutritional broth containing two percent glucose was infected with 10^8 CFU of bacteria that had been previously isolated, as well as the mixture was then heated to 37 degrees Celsius for 24 hours. After that, the cultures were diluted with fresh medium at a ratio of 1:100. Diluted cultures calibrated to 0.5 McFarland standards were placed in sterile 96-well flat-bottom polystyrene tissue cultures (Sigma-Aldrich, Costar, USA) at 200 L per well. This was done so that the cultures could be measured against the standard. Negative control wells were injected with a volume of sterile broth of 200 L. The plates were stored in an incubator for 24 hours at a temperature of 37 degrees Celsius. After incubation, an excess of free-floating bacteria in each well was gotten rid of by tapping on the sides of the well. Four times, 0.2 milliliters of phosphate buffer saline with a pH value of 7.2 was used to wash the wells. Bacteria that had formed a biofilm & were sticking to the wells were preserved in two percent sodium acetate & stained with 0.1 percent crystal violet. Deionized water was used to wash away the remaining discoloration, and the dishes were set aside to dry. The absorbance at 570 nm was measured with an ELISA microtiter-plate reader (Sun Rise-TECAN, Inc. ®, USA) to determine the optical density (O.D.) of the stained adherent biofilm. The procedure was run three times, each time with identical results. Biofilm formation was interpreted utilizing the criteria of (Stepanovic *et al*., 2007)

2- Biofilm Inhibition Assay :

The **Ch-MSNPs@HTCC** potential to prevent biofilm formation of ***Enterobacter* sp** isolates was tested at sub-MIC concentrations of 50mg/L.

2-1- In the Microtiter Plate: Bacterial suspensions (50µL; 5×10^6 cfu/ml, final concentration) were then transferred into the plate. 50µL nanoparticles at 50 mg/L concentration were added to the well microtiter plate containing Bacterial suspensions and broth with two percent glucose. Nutrient broth containing distilled water was employed as a (-ve control). After an incubation period of one day at 37 degrees Celsius. Microplate reader readings at 640 nm for optical density & 570 nm for adherent biofilm were used to determine the influence of nanoparticles on bacterial growth (Lin *et al*., 2011).

2-2- On Potato Tuber Slices: Twenty potato tuber slices were weighed and divided into two groups. 1st group sprayed with Ch-MSNPs@ HTCC at 50 mg/L concentration. 2nd group was inoculated with bacterial suspension (50µl,5×10^6 cfu/ml, conc.) using a hand-hold plastic sprayer until completely wet for overnight then sprayer with buffer phosphate, 3thgroup inoculated with bacterial suspension (50µl,5×10^6 cfu/ml, conc.) by a hand-hold plastic sprayer until completely wet for overnight then sprayer with Ch-MSNPs@ 25 nm HTCC at 50 mg/L concentration. 4th group was inoculated with bacterial suspension (50µl,5×10^6 cfu/ml, conc.) by a hand-hold plastic sprayer until completely wet then sprayed with ampicillin 1mg/ml following and incubated for 7 days at room temperature. After incubation, excess free-floating bacteria were removed by gentle tapping & washed with 0.2(ml) of phosphate buffer saline (pH=7.2) four times. Bacterial

biofilms got on the slices & were fixed in two percent sodium acetate and then stained with 0.1% crystal violet. Deionized water was used to wash away the remaining discoloration, as well as the dishes were set aside to dry. The effect of Ch-MSNPs@ 25 nm HTCC at 50 mg/L concentration on the bacterial growth was evaluated in the reduction of the colour violate degree on the tuber slices surface.

Control of Soft Rot on Potato Tubers by Ch-MSNPs@HTCC:

Virulence Test on Potato Tubers:

Healthy potato tubers cv. Spunta (Ministry of Agriculture) was applied in the control experiment. Sixty Potato tubers cv. spunta were divided into six group treatments as follows:

1-Ten tubers were sprayed with phosphate buffer

2-Ten tubers were sprayed with *Ent. cloacae* isolate *Ent-4* suspension

3-Ten tubers were sprayed with Ch-MSNPs@HTCC.

4-Ten tubers were sprayed with ampicillin

5-Ten tubers were inoculated with *Ent. cloacae* isolate *Ent-4* suspension and sprayed with Ch-MSNPs@HTCC

6-Ten tubers were inoculated with *Ent. cloacae* isolate *Ent-4* suspension and sprayed with ampicillin.

The potato tubers were inoculated with *Ent. cloacae* isolate *Ent-4* suspension (10^8 CFU/ml) using a hand-held plastic sprayer until totally wet. The Ch-MSNPs@HTCC, 0.5 mg /ml were sprayed with potato tubers until completely wet. The ampicillin 1 mg/ml was sprayed on potato tubers until completely wet. The potato tubers were incubated for up to one month at room temperature.

1-Determination of Disease Severity :

Weller (2007) used rotting weight as well as the calculation of the soft rot areas under the disease progress curve (AUDPC) (Shaner & Finney, 1977) to assess the severity of the disease twice during the time of incubation for potato tubers sprayed with Ch-MSNPs20nm@HTCC & Ampicillin.

2-Determination of the Bacteria Count :

The weight of the tubers was measured both prior to and following the diseased parts were removed, as described by (Balogh and Jones 2003). We weighed the decaying tissue, poured 100 cc of deionized water into plastic bags, and stored them in the refrigerator's cold storage. The Ent. Cloacae titer was determined in terms of colony-forming units (CFU) per gram of potato tubers using a plate count assay.

$$\text{Bacteria titer} = \frac{\text{CFU x 1.000}}{\text{we. of sample bag} - \text{we. of empty bag.}}$$

We = weight

Extraction of Total Proteins:

Total proteins were extracted according to Lanna *et al.*, (1996). The infected tubers with bacterial suspensions at 2 weeks under stirred conditions as described by Tadashi (1975). The tuber tissue was determined total cellular proteins by the Bradfored method (1976) using bovine serum albumin (BSA) as a standard protein and pectinase activity

Extraction of PME From Potato Tuber :

Pectinase methyl ester (PME) was extracted according to (Pires & Finardi-Filho, 2005). Potato tubers infected with soft rot were homogenized & suspended in frozen water, where they were agitated for an hour. We next centrifuged the samples for 2 minutes at 12000g to get rid of the supernatants, which had no detectable enzyme activity. The sediment was re-stirred for 1 hour in 1 M ice-cold NaCl. After centrifuging the samples for 2 minutes at 12000g, the PME-containing supernatant was collected. The temperature for all of the processes was set to 4◦C.

Measurement of Enzyme PME **Activity:**

Using a modified version of the pH-colourimetric approach developed by Hagerman & Austin (1986), in which a rise in pH is measured spectrophotometrically with bromothymol blue, the pectinase activity was calculated. This test required 50–500 μL of tuber juice, 50 g L1 of tuber tissue in distilled water, & 0.1 g L1 of bromothymol blue in

0.003 M phosphate buffer. These solutions had a pH of 6.0, thus 2 M NaOH was added to bring it up to 7.5. Distilled water was initially utilized to establish the zero absorbance value. The initial absorbance at 620 nm was measured after combining the pectin and bromothymol blue solutions in a thermostated (25◦C) cuvette. After adding the juice, a drop in absorbance was measured to indicate that the reaction had begun. A decrease in absorbance of 0.1 per minute was considered 1 U of PME activity.

Determination of Pectinase Isozymes :

Pectinase isozyme analysis was carried out using native polyacrylamide gel electrophoresis (PAGE). (Smith, 1969). A liter of the sample buffer was added to the protein extracts, and the mixture was stirred. Submerged protein extract samples were put into the gel wells prior to electrophoresis on a 10% (W/V) slab gel for polyacrylamide gel electrophoresis (DISC-PAGE). Two gels were electrophoresed at a voltage of 150 volts until the marker dye BPB reached the end of the gels. Complete staining and pectin solution substrate incubation occurred when the gel was heated to 37 degrees Celsius and then kept in the dark. Alpha Ease FC 4.0 was used to evaluate the gels. After a run, the gel was dyed using the results from Hofelmann *et al.* (1983).

Statistical Analysis:

The experimental design consisted of a randomized whole block setup with three different copies of everything. Using a program known as the Statistical Package for the Social Sciences (SPSS for Windows 16.0), The LSD Tests provided by SPSS were utilized so that a comparison could be made amongst the various means at a significance level of P under 0.05.

RESULTS

Bacterial Identification:

The Enterobacter cloacae isolate has a creamy, round, smooth, convex also complete border on nutrient agar. They form cream-colored colonies without fluorescent pigment on King B medium, pits on CVP media, and small dark red colonies on Raffinose. The rod-shaped, gram-negative, motile, spore-free bacterium had no color.

Nucleotide Sequence & Phylogenetic Analysis 16s gene *Ent.cloacae*:

Ent.cloacae Egyptian isolate RT-PCR products were purified & sequenced (Fig,1). The following accession numbers represent the partially sequenced 16s gene of the isolate that was submitted to the NCBI-GenBank: CP040827.1, CP039318.1 CP039303.1 CP039311.1 & CP020089.1 (Fig.2 And Table,4).

***Enterobacter cloacae* Strain (KY400130):**

GATGTCAATGTGTATGTGGGGCCCTTGAGAGCGTGGCTGCCGGAGCTAACGCGT
TAAATCAACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGGTTTAATTCGATGCAACGCGAAAAACC
TTACCTGGGCTTGACATCCTGAAAACTTTCCAAAAATGGATTGTTGCCTTCGGGA
ACTGTGACACAGGTGCTGGCTGGCTGTCTTCCGCTCGTGTTGTGAAATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCTGCGGTTAGGCCGGGA
ACTCATAGGAGACTGCCGGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGT
CATCATGGCCCTTACTACCAGGGCTACACACGTGCTACAATGGTGCATACAAAA
GAAGCGACCTCCCGAGAGCAAGCGGACCTCCTAAAGTGCGTCGTAGACCGGATT
GTAGTCTGCAACTCTACTCCGTGAAATCGGAATCTCTATTAATCTTAAATCAAAA
TGCTACGGTGAATACTTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGA
GTGGGTTGCACAAAAAAAAGGTAGCTTAACCTTCGGGAGGGAGCTTACCACTTT
GTGATTCATGACTGGG GTGAA

Fig. 2 : Partial nucleotide sequence (677 nts) of 16SrRNA gene of BK-27 of isolate**.**

The 16SrRNA gene using PCR was amplified with a fragment of about 650 bp corresponding to the C-terminal region of the 16SrRNA gene (Fig. 3).

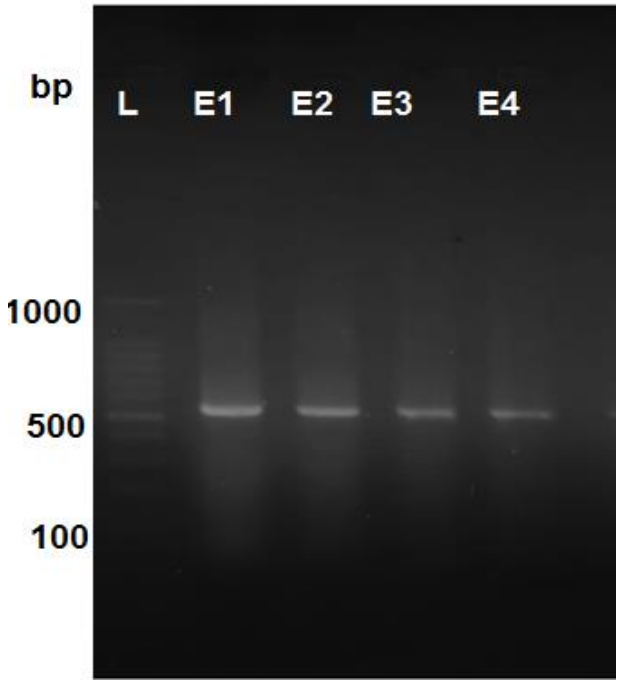

Fig. 3: Gel electrophoresis 1.5% agarose showing of 16s gene PCR amplified products (677 bp fragment) using universal primers for ***Ent. cloacae*** isolated from soft rot (Lane, E1&E2) and rhizosphere soil (Lane, E3&E4) cultivated with potato. Lane M : 100 bp DNA Ladder RTU (GenDirex).

The phylogenetic tree data (Fig. 4) showed that there was little genetic variation across the various Ent. cloacae isolates and the sequences of other isolates accessible in NCBI, which had been isolated from various *Eht. cloacae* as well as various geographical locations. One cluster consisting entirely of Egyptian isolates was identified with 93.35% bootstrap support. The Egyptian strain of *Enterobacter cloacae* was given the entry number (KY400130) in the NCBI - GenBank database.

Ent. cloacae KY400130) and five different isolates including CP040827.1, CP039318.1 CP039303.1 CP039311.1 and CP020089.1 grouped together in a cluster with 93.35% bootstrap support. The Egyptian isolates had a very high degree of nucleotide sequence identity, at 93.35 percent. Sequence comparisons in databases indicated a significant degree of similarity among the various *Ent. cloacae* isolates (over 94%) (Table 4).

Table 4: Identities of isolated *Ent. cloacae* compared to five similar bacteria strains documented in GenBank based on 16SrRNA gene sequencing.

Description of *Ent. cloacae* strains	**Identity**	**Accession**
Ent. cloacae strain NH77 chromosome, complete genome	93.35%	CP040827.1
Ent. cloacae strain Effluent_2 chromosome	93.35%	CP039318.1
Ent. cloacae strain Effluent_4 chromosome	93.35%	CP039303.1
Ent. cloacae strain Effluent_3 chromosome	93.35%	CP039311.1
Ent. cloacae strain PIMB10EC27 chromosome, complete genome	93.35%	CP020089.1

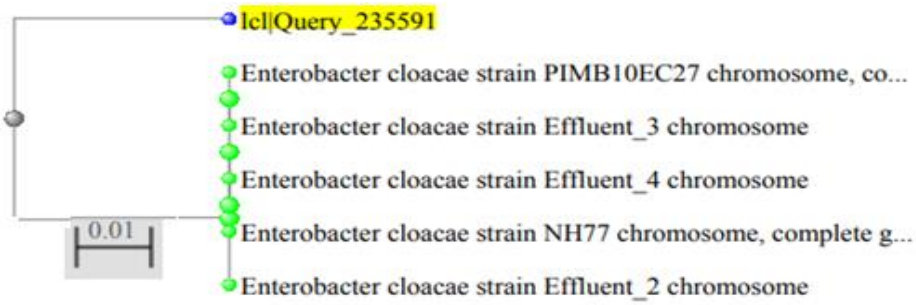

Fig. 4 : Phylogenetic tree based on the partial nucleotide sequence of 16SrRNA gene of BK-27 of isolate compared to five similar bacterial strains documented in GenBank The 16SrRNA gene sequence was utilized as an out-group. Bootstrap values generated from 1000 iterations are indicated on the tree.

Characterization of Chitosan Capped @MSNs-HTCC:

Chitosan-loaded MSNs capped HTCC were constructed and their creation was simply depicted. Figure 5 displays the porous structure, regular spherical shape, and relatively smooth surface of the produced .

CH-MSNs@HTCC. Although somewhat larger at roughly 33.9 nm in size, the particles remained spherical or sub-spherical in shape. MSNs@HTCC and CH-MSNs@HTCC with size (20 nm) were chosen to proceed with further development

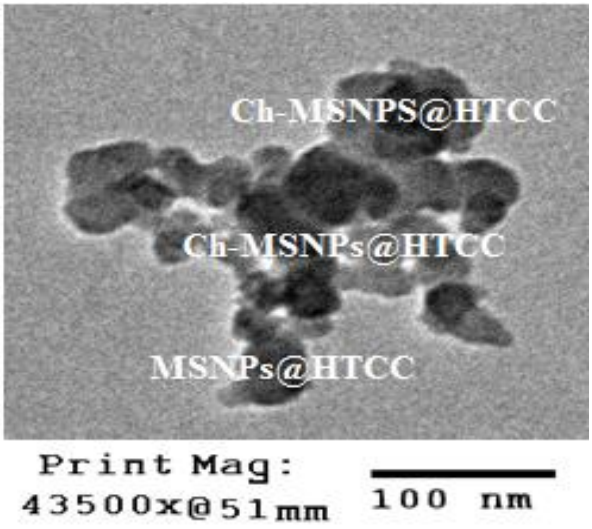

Fig. 5: TEM images, showing Characterization of Mesoporous silica nanoparticles (MSNPs@HTCC) and loaded chitosan (Ch-MSNPs@HTCC) of size **(≈20.9nm).**

Antibacterial Activity:

Susceptibility of *Ent. cloacae* isolates (Ent-1, Ent-2, Ent-3, & Ent-4) for MSNPs@ HTCC and Ampicillin were examined using inhibition zone disc assay. *Ent. cloacae* isolates have a broad sensitivity to MSNPs@ HTCC while showing resistance to antibiotics. Fig,5).

The Ch-MSNPs@ HTCC and ampicillin reduced the growth of *Ent. closcae* Isolates ranging from 4.2 to 14.5 and 2.2 to 3.5 mm inhibition zone respectively. It was found that Ch-MSNPs@HTCC had a significant increase in the inhibition zone area. On the other hand, *Enterobacter* Isolate Ent-4 was more sensitive to Ch-MSNPs@HTCC and it was used for experiment control (Table,5 and Fig. 6).

Table 5: Antibacterial activities of Ch-MSNPs@ HTCC compared to antibacterial drug (Ampicillin) by inhibition zone.

Treatments / *Enterobacter* Isolates	Ampicillin		Ch- MSNPs@ HTCC						LSD
			0.1. mg/mL		0.5. mg/mL		1.0mg/mL		
	Inhibition zone area		Inhibition zone area		Inhibition zone area		Inhibition zone area		
	MM	%	MM	%	MM	%	MM	%	
Ent-1	2.6	**9.3**	4.2	15	4.8	17.1	7.2	25.7	2.5
Ent-2	3.5	12.5	4.4	15.7	5.4	19.3	8.4	30.0	2.8
Ent-3	2.4	8.6	7.4	26.4	8.4	30.0	10.4	37.1	3.2
Ent-4	2.2	7.8	5.4	19.3	7.4	26.4	14.5	51.8	3.5
LSD	0.3		0.4		1.2		2.4		

The inhibition zone area was as a mean of three replicates.

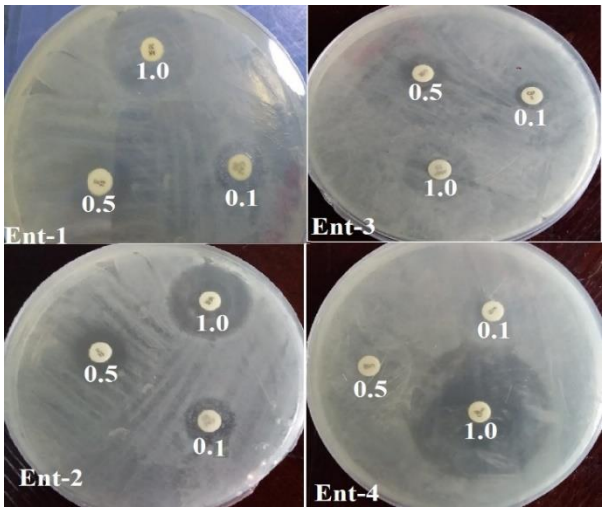

Fig. 6: Disc diffusion test showing inhibition zone of Ent.cloacae isolates (Ent-1 ,Ent-2, Ent-3 & Ent-4) on NA plates supplemented with Ch-MSNs20nm@HTCC at concentrations (0.1, 0.5 and 1.0 mg/mL) after 48 d of incubation.

Antibacterulence Activity of MSNPs@ HTCC:

ELISA Microplate:

Biofilm Formation: *Ent. cloacae* isolates (code, Ent-1, Ent-2, Ent-3 & Ent-4) have the potential for formed biofilm with different values OD 1.4, 1.2, 1.6, 1.9 respectively **(**Table, 6).

Biofilm Inhibition:

MSNs20nm@HTCC was significantly reduced biofilm formation 0.12, 0.08, 0.15, 0.18 with 98.6, 93.3, 90.6 &90.5 % than Ampicillin 0.72, 0.45, 0.65, 0.72 with percent 48.5l 62.5, 59.4, 62.1% against *Ent. cloacae* isolates codes, E1, E2, E3 & E4 respectively (table,6 and figure,7).

Table 6: Biofilm and biofilm reduction of *Ent. cloacae* isolates treated with MSNs20nm@HTCC and Ampicillin using Tissue culture plate method by ELISA reader.

Bacterial isolates		*Ent*-1	*Ent*-2	*Ent*-3	*Ent*-4
Biofilm (OD, 570nm)		1.4	1.2	1.6	1.9
Biofilm (OD, 570nm)	Ch-MSNPs20nm@HTCC	0.12	0.08	0.15	0.18
	Ampicillin	0.72	0.45	0.65	0.72
% Biofilm reduction	Ch-MSNPs20nm@HTCC	98.6%	93.3%	90.6%	90.5%
	Ampicillin	48.5%	62.5%	59.4%	62.1%

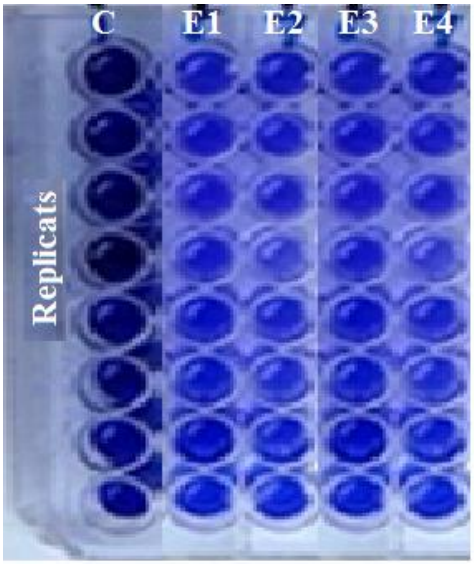

Fig.7: ELISA Micro plate showing biofilm inhibition of E1, E2, E3, E4 *Ent cloacea* isolates treated with Ch-MSNs20nm@HTCC and (C) untreated *Ent.cloacea* (control) grown overnight in polystyrol microtiter wells in TSB supplemented with 2% glucose. Crystal violet was used to highlight the cells that had remained attached to the plate following washing.

Slices of Potato Tuber :

Biofilm formation of *Ent. cloacae* isolates (code, Ent-1, Ent-2, Ent-3 & Ent-4) on slices of potato tuber have potential with different OD values 8.5, 8.45, 8.12, 8.35 respectively (Table, 7).

Biofilm reduction on tuber slices stained by crystal violet.

Ch-MSNs20nm@HTCC was significantly reduced biofilm formation 1.24, 1.09, 1.15, 1.18 with 85.4, 87.1, 85.8 &86.9 % than Ampicillin 3.75, 3.35, 3.24, 3.26 with percent 55.9, 60.4, 60.1 60.9% against *Ent. cloacae* isolates codes, E1, E2, E3 & E4 respectively **(**Table,7**)**.

Table 7: Biofilm and biofilm reduction area of *Ent cloacae* isolates against Ch-MSNs20nm@HTCC & Ampicillin on tuber slices stained by crystal violet.

Treatments \ Bacterial isolates		Reduction of colour violates degree on tuber slices							
		Ent-1		*Ent*-2		*Ent*-3		*Ent*-4	
		DCV	AVC	DCV	AVC	DCV	AVC	DCV	AVC
Biofilm (*Ent cloacea*) +	Control	-	8.5	-	8.45	-	8.12	-	8.35
	Ch-MSNPs20nm@HTCC	+++	1.24	+++	1.09	+++	1.15	+++	1.18
	Ampicillin	++	3.75	++	3.35	++	3.24	++	3.26
Biofilm reduction	Ch-MSNPs20nm@HTCC	85.4		87.1		85.8		86.9	
	Ampicillin	55.9		60.4		60.1		60.9	

DCV = Degree of violet color

(–) colorless , (+) weak density color , (++) low density color and (+++) high density color

AVC = Area of violet color.

The total count of *Ent. cloacae* isolates (code, Ent-1, Ent-2, Ent-3 & Ent-4) on slices of potato tuber have potential with different Log count values 6.4, 6.5, 6.2, 6.5 log respectively **(**Table, 8**)**. The same results significant reduction in total count of *Ent .cloacea* treated with Ch-MSNs20nm@HTCC with 1.2, 1.4, 1.5, 1.2 log with 81.2, 78.5, 75.8 &81.5 % than Ampicillin 3.5, 3.3,3.4, 3.6 log with percent 45.3, 49.2, 45.2, 44.6 % against *Ent. cloacae* isolates codes, E1, E2, E3 & E4 respectively **(**Table, 8**)**.

Table 8: Reduction of total count for *Ent cloacae* isolates against Ch-MSNs20nm@HTCC and Ampicillin on tuber slices stained by crystal violet.

Treatments \ Bacterial isolates		*Ent .cloacea* count on tuber slices (Log)			
		Ent-1	*Ent*-2	*Ent*-3	*Ent*-4
Biofilm(*Ent cloacea*) +	Control	6.4	6.5	6.2	6.5
	Ch-MSNPs20nm@HTCC	1.2	1.4	1.5	1.2
	Ampicillin	3.5	3.3	3.4	3.6
Biofilm reduction	Ch-MSNPs20nm@HTCC	81.2	78.5	75.8	81.5
	Ampicillin	45.3	49.2	45.2	44.6

Nano-Control of Soft Rot Disease on Potato Tubers:

Potato Tuber Assay (Bacterulence assay):

Whole potato tuber was used to check whether the application of Ch-MSNPs20nm@HTCC would protect potato tubers against soft rot infection caused by *Ent. cloacae*, code (*Ent-4*). Spraying of potato tuber with Ch-MSNPs 20nm@ HTCC showed a significant reduction with 87 % of disease severity 95% rotting diameter and 97% of rotting weight. compared with Ampicillin were 66, 78 and 87 % respectively (table .6 and Fig 6&7). In addition, Ch-MSNPs20nm@HTCC showed a significant reduction with 0.2 Log1 compared with Ampicillin where 66 of *Ent.cloacea* count compared with Ampicillin 0.5 Log2 related to control 1.2Log7 CFU/g rotting.(Table,9and Fig.,8) .

Table 9: Disease severity, diameter, and weight of soft rot on potato tubers sprayed with antibiotic and MSNPs .

Ent. cloacae infectivity \ Treatments		*Ent. cloacae*	*Ent.cloacae* +Antibiotic	*Ent. cloacae* + MSNs20nm@HTCC	LSD
Disease severity	% **Disease severity**	94c	32 b	12	12.5
	% Reduction	-	66	87	
Diameter of soft rot	Diameter(cm)	5.5c	2.2 b	0.3	1.4
	% Reduction	-	78	95	
Weight of rotting	Weight (g)	65c	21.2b	1.5	4.5
	% Reduction	-	67	97	
Ent. cloacea	Count (g)	1.2Log 7	1.5 Log 3	1.2 Log1	Log2
	% Reduction	-	57	86	

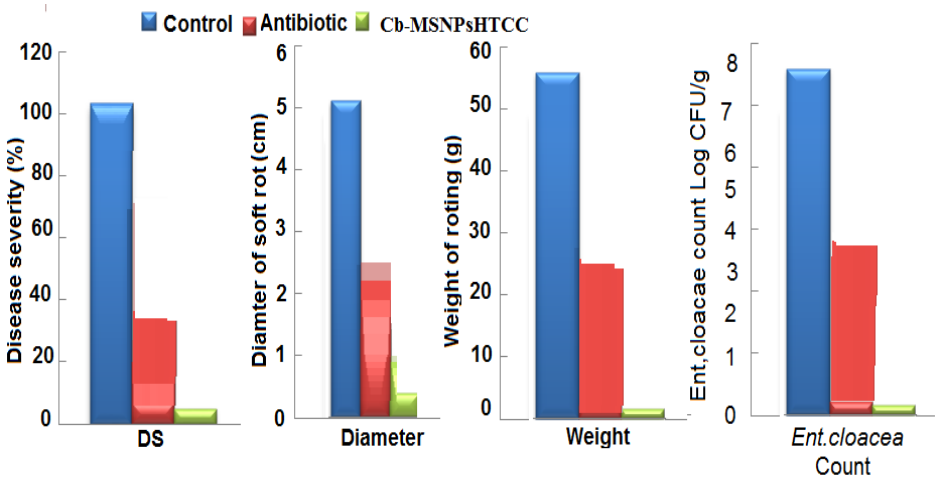

Fig. 8 : Histogram showing a reduction in Disease severity, diameter, weight of soft rot symptoms and *Ent.cloacea* count on potato tubers sprayed with ampicillin and ChMSNPs@HTCC.

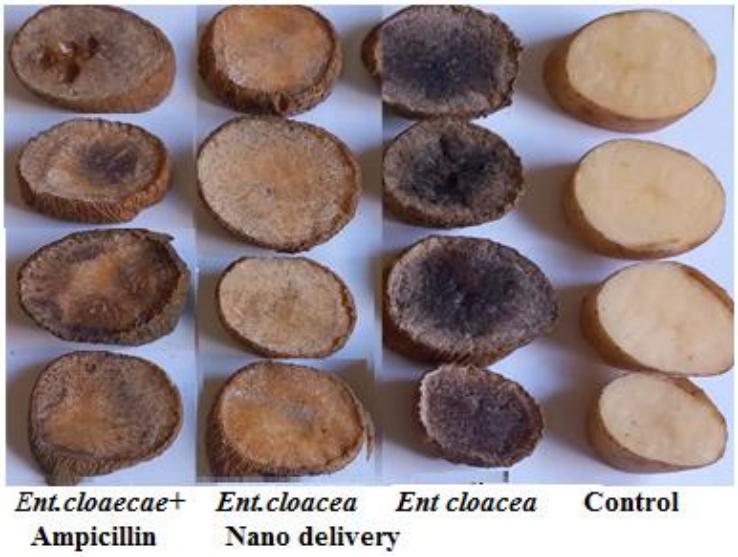

Fig. 9: Photograph showing soft rot disease severity on potato tubers infected with *Ent.cloacea* (Ent-4) and treated with Ch-MSNPs@HTCC and Ampicillin.

Protein Content and Pectinase Activity:

The total protein, pectinase activity and isozymes were determined in infected potato tuber and treated with antibiotic & Ch-MSNPs@HTCC. There was a detectable increase in protein, pectinase activity and isozyme in sprayed infected tubers with antibiotic and Ch-MSNPs@HTCC than in sprayed healthy tuber (Table 10). It was revealed that the total protein was significantly increased in the infected tuber by 20.2 mg/g tuber and in the infected tuber treated with antibiotic and MSNPs 16.4 and 17.4 mg/g tuber compared significantly increased in the healthy tuber and non-treated Phosphate buffer**,** Antibiotic and Ch-MSNPs@HTCC ones, 12.4, 13.5 and 15.8 mg/g respectively. As well as pectinase activity was 1520, 135, 146 µ and Specific activity by 75.2472, 8.2317, 8.3908 (µ/mg) respectively compared significantly decreased in healthy tuber and treated Phosphate buffer**,** Antibiotic and MSNPs@HTCC ones, 0.24, 0.31and 0.52 µ and Specific activity by 0.0193, 0.0229, 0.0329 (µ/mg) respectively (Table 10).

Table 10 : Protein content & pectinase activity in infected potato tubers and sprayed with antibiotic and MSNPs@HTCC.

Protein and Pectinase / Treatments	Total Protein (mg/g tuber)	Pectinase activity	
		Total activity UmL−1	Specific activity (µ/mg)
Sprayed healthy tuber with phosphate buffer	12.4	-	-
Sprayed infected tuber with phosphate buffer	20.2	2.0	0.099
Sprayed infected tuber with amplicine	16.4	1.6	0.097
Sprayed infected tuber with Ch-MSNPs@HTCC	17.4	1.3	0.075
LSD	2.1	0.4	0.055

Pactinase Isozymes:

The pectinase isozymes analysis was determined in infected potato tuber and treated with ampicillin & Ch-MSNPs@HTCC basis of the intensity, relative mobility and reproducibility of bands

(table 11& fig.11).. Disc-PAGE analysis displayed 6 bands of Polymorphism variable in density whereas two common bands (monomorphic) with 33.3 and 4 variable bands (polymorphic) with 66.6 %. The number of isozymes for each tuber treatment was 2 (10 %), 6 (30%), 6 (30%) and 6 (30%) for Healthy, Infected, Infected and Sprayed ampicillin and Infected and Sprayed *Ch*-MSNPs@HTCC respectively. (Table 11 & Fig.10).

Table 11: Disc-PAGE bonding patterns of pectinase isozymes of four Ent. Cloacae isolates.

Rf			Sprayed infected tubers						Polymorphism
	Healthy tuber		Infected tuber		Ch-MSNPs@HTCC		Ampicillin		
	Density	%	Density	%	Density	%	Density	%	
1.7	++++	65	++++	29	++++	15	++++	25	Monomorphic
1.9	++++	35	++++	10	++++	9	++++	9	Monomorphic
2.5	-	-	++	32	++	24	++	21	Polymorphic
3.0	-	-	+	12	+	25	+	15	Polymorphic
3.4	-	-	+	7	+	10	+	18	Polymorphic
Total	2 (10)	2	6 (30%)		6 (30%)		6 (30%)		20

Density of pectinase isozymes bands: - Absent band, + Weak band, ++ Moderate band, +++ Strong band and ++++ Very strong band

Polymorphism: Monomorphic = Common polypeptide,
Polymorphic = Specific polypeptide

Fig. 10 : DISC-PAGE (10%) illustrating pectinase isozymes of 4 *Ent. cloacae* isolates E-a, E-b, E-c & E-d..

DISCUSSION

Bacterial soft rot disease caused by *Enterobacter cloacae* (Atia, A.A.,2019 & Eman, et al.2022) & *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) (Eman, *et al.*, 2021) has been a considerable problem in Egypt. Potatoes, peppers, lettuce, cabbage & carrots all fall prey to the soft rot disease caused by Enterobacter cloacae (Misic et al., 2009). Characters of colonies were comparable to the type strain of Ent.r cloacae described in Bergy's Manual of Systematic Bacteriology (Brenner *et al.*, 2007; De Vos *et al.*, 2009), as determined by morphological & biochemical assays. In addition to being gram-negative, spore-free, rod-shaped, motile via peritrichous flagella, as well as colorless, it was discovered that *Ent. cloacae* belonged to the family Enterobacteriaceae and held the aforementioned characteristics. Consistent with previous research, we observe that when

grown in CVP media, when grown in King B medium, colonies form pits; colonies are a creamy color & lack fluorescent pigment; as well as when grown in raffinose medium, colonies are tiny & dark red. (Kettani-Halabi *et al*., 2013 and Mohamed & Selman, 2013). The 16S ribosomal RNA gene sequence is the "gold standard" for determining the evolutionary history of prokaryotes (Nayak *et al.*, 2011). The ribosomal small subunit is the most utilized housekeeping genetic marker because it is present in all bacteria. The 16S rRNA gene is an excellent resource for studying bacterial ecology, phylogeny, and taxonomy because it contains both hypervariable areas with species-specific variability where sequences have diverged over evolution and conserved regions (Vetrovsky and Baldrian, 2013). Bacterial isolates from the environment and hospitals are recognized & classified using this molecular method (Clarridge, 2004). Ribosomal RNA genes are highly conserved amongst bacteria, and the presence of hypervariable sequences within one species allows for fine-scale taxonomic classification. Crop yields can be negatively impacted by phytopathogens that infect economically significant plant species. At now, these phytopathogens are managed in order to reduce their impact. Nanotechnology's ability to inhibit and destroy microorganisms without selecting them specifically is an important one. Proper supply to affected plants is also required to guarantee (Davidsson, *et.al.,* 2013).

Nanotechnology provides efficient, non-specific phytopathogen attacks, a broad host range, and safe delivery of biomolecules and agrochemicals like virucidal and insecticides (Mosa and Youssef 2021), with the purpose of reducing or diminishing the indiscriminate usage of conventional chemical pesticides as above ninety percent of used pesticides usually fail to reach their targets into plants for many reasons involving evaporation (Nuruzzaman *et al.,* 2016), leaching (El Bahri and Taverdet 2007), hydrolysis (Nuruzzaman *et al.,* 2016), deposition (Yin *et al.,* 2018), aerobic & photodegradation (Ravier *et al.,* 2005).

Surface charges measured with a Zeta Sizer Nano ZS Analyzer revealed that the manufactured MSNPs in aqueous solution were negatively charged, whereas the HTCC layers were positively charged. This was demonstrated by the fact that the HTCC layers had a zeta potential of -20 mV. The driving forces of both the electrostatic and hydrogen bonding interactions were responsible for efficiently wrapping those layers onto the surface of the delivered MSNPs. Curiously, and after the loading of the pesticide, it was discovered that Ch-MSNs20nm-HTCC had a structure, size, as well as dispersion that were reasonably comparable to one another. Because the majority of the pesticide molecules penetrated the mesoporous structure of the silica nanoparticles, which is thought to be imprisoned inside the pores of the nanoparticles, the virucidal loading method did not significantly alter the size of the nanoparticles in this study (Mosa, *et al.,*2022).

The 1075 cm1 absorption band was also linked to stretching vibrations in the siloxane linkage (Si-O-Si). According to Lim & Hudson (2004), the existence of a quaternized amine group in the chitosan structure can be inferred from the presence of an absorption peak at 1450 cm 1 wavelength in the spectra of HTCC layers, which is likely due to the aliphatic C-H bending vibration of -trimethylamine (N(CH3)3) +. The CTAB as a surfactant was completely removed from the produced MSNs since there were hardly any detectable C-H aliphatic stretch peaks (Kankala *et al.,* 2015).

The natural shells that polymers provide make them ideal for enclosing biologically active substances. Their superiority in biological processes was due to their low toxicity, exceptional biocompatibility, & multi-physiochemical characteristics. De-acetylation of chitin is a typical method of producing chitosan (CS), a biopolymer molecule utilized extensively in sustainable agriculture (Kashyap *et al.,* 2015).

One of the most researched forms of chitosan is the quaternized form, specifically N-(2-Hydroxyl) propyl-3-tri-methyl-ammonium CS chloride. Physiochemical features of HTCC, including cationic charge retention at neutral pH, permeability-enhancing capabilities, high water solubility, muco adhesiveness, & antibacterial activity, lend credence to this claim (Rwei *et al.,* 2014). For MSNs to function as efficient pesticide carriers, electrostatic attraction among the HTCC layer and the MSNs is the optimal technique for surface modification because it allows the positively charged HTCC layer to be coated over the negatively charged MSNs without the need for complex chemical grafting.

The research presented here describes the development of a smart nano-delivery system (Ch-MSNs20nm-HTCC) to administer chitosan as a virucidal model for the treatment of viral diseases. The current study's ultimate goal is to contribute to the field of smart agro-nanotechnology for the control of plant diseases.

The development of antibiotic-resistant Enterobacter isolates over the past decade has sparked growing interest in nanotechnology-based treatments. Biofilm formation & soft rot disease control are two areas where Ch-MSNPs@HTCC have been investigated as possible agents.

Several plant pathogenic bacteria, including *Ent. cloacae,* may have a virulent component in common: bacterial biofilm. Four different *Enterobacter* isolates were shown to be biofilm formers in this investigation. The current findings are consistent with the studies of, although Ent-3 demonstrated moderate biofilm development, (Hossain and Tsuyumu, 2006).

Pathogenicity and biofilm growth, both virulent variables for Pectocaterium carotovorum, are controlled by cell-cell communication (QS), a cell density-dependent process that regulates genes in Prokaryotes. QS uses small signal molecules. Bacteria can accumulate autoinducers inside and outside the cell to adapt gene expression for enormous cell densities (Mieszkin *et al*., 2013; Castillo-Juárez *et al*., 2015 and Wu *et al*., 2015).

In this research, Ch-MSNPs@HTCC potential was discovered to reduce biofilm formation by Enterobacter isolates at doses of 0.1, 0.5, and 1.0 mg/ml (Sub dilution endpoint). The current findings are consistent with those of Pei & Lamas-Samanamud (2014) and El Dougdoug (2016). Using phage combinations of varying doses, they discovered that the proportion of Staphylococcus aureus biofilm inhibition ranged from 54.55 to 95.45 percent.

Interfering with bacterial cell-cell communication to stop biofilm development is an intriguing new approach because QS is essential for biofilm formation. Interfering with the synthesis, identification, or transport of QS is a common way to stifle bacterial cell-cell communication (quorum quenching, QQ).

According to these findings, Ch-MSNPsHTCC has been tested as antimicrobials, showing efficacy against pathogens like *Enterobacter*; they may also be useful as anti-biofilm agents. This means that Ch-MSNPsHTCC can be used as a source of antimicrobial-derived proteins in the fight against pathogenic bacteria, in addition to being a bactericidal agent in and of itself. PME in *Penicillium expansum*-infected apple & tomato fruits was effectively assayed using a pH-colourimetric approach for assaying pectinase activity based on the absorbance shift of a pH indicator, bromothymol blue, at 620 nm during hydrolysis of pectin methyl esters. (Miedes & Lorences, 2006) . Pectinase activity was measured in crude soft tissue from infected potato tubers using this method. The tubers were sprayed with ChMSNPsHTCC & a control antibiotic, ampicillin. After adding ampicillin or ChMSNPsHTCC, the indicator's color changed, indicating the presence of chemicals whose absorbance spectra substantially interact with bromothymol blue.

The detailed characterization of Ch-MSNPsHTCC led to the discovery that they are suitable for use in nano therapy. Ch-MSNPsHTCC was used at a concentration of

0.5 mg/ml on infected potato tuber cv. spunta with isolate Ent-4 at a level of 108 CFU/ml. Potato tuber cv. spunta was infected with strain Ent-4 (108 CFU/ml), and subsequently, Ch-MSNPsHTCC was administered at a concentration of 0.5 mg/ml. Potato tubers that had been inoculated with isolate Ent-4 exhibited severe cases of soft rot, as well as a large diameter of the disease's affected area, intense disease severity, as well as loss of healthy potato tissue. The application of Ch-MSNPsHTCC (0.5 mg/ml) resulted in a stunning significant reduction in soft rot, with a score of at least 97 compared to ampicillin's score of 67 percent. the severity of the disease, the diameter of the soft rot, as well as the need to prevent the formation of mold in the tubers with healthy potato tissues either before or After injury (Ocsoy. et al.,2013). These outcomes are consistence with the findings of (Abdelghany, *et al.*, 2022, Wei, *et al.*,2022 & Soltani, *et al.*,2022) biosynthesized silver nanoparticles from oak fruit exudates for the treatment of postharvest soft rot disease in vegetables caused by Pectobacterium carotovorum subsp. carotovorum (Ayisigi *et al.*, 2020). Furthermore, spraying tubers with Ch-MSNPsHTCC extended their shelf life much more than ampicillin did. After three weeks, the amount of Ent.r cloacae in potato tubers was determined. After three weeks, the concentration of Ch-MSNPs@HTCC & ampicillin were both significantly lower than their starting concentrations of 1.5 log 7 reducing the efficacy of disease control, with 1.2 log 1 and 1.5 log 3 CFU/g for *Ent. cloacae*, respectively. The present findings are in agreement with the study of Boyraz *et al.*, 2006 Davidsson, *et. al.*, 2013, Cao., *et. al.*, 2017 & Hossain, *et al.*,2019) who developed Bacterial leaf & peduncle soft rot triggered by *Pectobacterium carotovorum* on tulips in Konya, Turkey.

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