Indian Institute of Technology Kanpur
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Contact No. 6178

Intellectual Property Disclosure Form

1. Title of the invention: Synthesis of Bio-Carbonoid-Metabolite Nanoparticles (BioDCM-NPs) using 6-pentyl-2H-pyran-2-one microbial metabolite for its Application in Bio-protection of Agriculture Crops

2. Innovator(s) who have contributed or conceived an essential element of the invention, either independently or jointly with others during evolution of the technology concept or reduction to practice:

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Phone:
E-mail:

*(*Students are requested to give their home address and email other than squirrel mail as well)*

3. Non-Confidential description of the invention in layman's Language:

A. Abstract in 100 words

The recent ecological and human health concerns about excessive use of chemical agents in the field of agriculture has led to development of biological alternatives in recent years. It has been reported that protective activity of certain microbial metabolites can be used to enhance crop protection against various diseases in different crops. But, due to less control on bioavailability, pre-mature degradation and absorption by the crops, makes it less suitable option in the age of high competition toward better crop productivity and makes it less feasible option for farmers. A Biodegradable-Carbonoid-Metabolic Nanoparticles (BioDCM-NPs) have been synthesized using a metabolite extracted from *Trichoderma* species which can avoid such shortcoming and can give better protection to rice crop against certain diseases. Preparation protocol of BioDCM-NPs is novel and found to be effective in laboratory conditions against Xanthomonas Oryzae pv. oryzae (Xoo)

which causes bacterial blight and Rhizoctonia solani (R. solani) known for causing sheath blight in rice.

B. Use Case

The developed protocol could be used to produce BioDCM-NPs having antimicrobial properties to provide better disease resistance in rice crops. The strategy can be used to impart enhanced temperature resistance to the otherwise temperature sensitive compounds. The product produced from the method can be used as:

(a) An antimicrobial bio-nano-formulation for agriculture crops esp. Rice
(b) Carbon enrichment source to the soil

Note: *Please Note that the above Information will be circulated to several agencies for Commercialization purposes.*

4. How does this invention relate to new processes, machines, compositions of matter, etc.? Please cover the following points:

(a) Describe the invention in detail for technical evaluation. Please use additional sheets for sketches, drawing, photographs and other materials that help to illustrate the description. (**See Annexure 1)**

(b) What is Novel in the invention?

Various literature reports include the use of various microbial and plant metabolites for the disease management in crops. Some of these previously developed biopesticides use the whole cell microorganisms to provide their antimicrobial activity and in addition for the enrichment of the soil. . In the current invention, a technology platform has been developed to produce biodegradable nanoparticle system of the secondary metabolite from the fungus *Trichoderma asperellum* strain TAIK1 using microwave technique which shows antimicrobial activity for the bio-protection of crops. The invention involves the use of 6-pentyl-2H-pyran-2-one, a microbial metabolite included in carbon nanoparticle system for better efficiency against agricultural crop diseases. The preparation of these Biodegradable-Carbonoid-Metabolic Nanoparticles (BioDCM-NPs) also results in the enhanced temperature sustenance to the microbial metabolite making it effective also at higher temperatures.

(c) What is the “inventive” step in your invention? Is the step non-obvious to a person from related fields?

The invention uses microwave radiation for the synthesis of CNPs using microbial metabolite as one of the components. The current strategy uses the microbe derived metabolite instead of whole cell live organism for the preparation of nano-formulation like BioDCM-NPs in this invention. The nano-formulation also gives better temperature stability to the metabolite without hampering its antimicrobial activity.

(d) What are the advantages of the present invention over comparable inventions available in patent literature? Please attach a summary of your patent search*.
The BioDCM-NPs have the following advantages:

1. Active at low concentration
2. Precise target action
3. Has similar advantages like chemical pesticides but safe and biodegradable (unlike chemicals)

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4. Multiple action (Biofertilizers, phyto-stimulants)
5. Fast in action since applied in bioactive forms
6. The bioformulation protects the active compound from high temperature

NOTE: The inventors should go through the Patent Search report carefully and write the difference between his invention and each content of the patent search. For Patent search please contact rpandey@iitk.ac.in

(e) Has the invention been tested experimentally? Are experimental data available?

Yes, the experimental data has been included in Annexure I.

(f) Technology Readiness Levels (**TRL**) description (mention the applicable stage of TRL given below). Please Mark as Appropriate

☐ **TRL-1**
Research Idea
(Potential Application/Basic Principles observed)

☐ **TRL-2**
Applied Research Idea
(Hypothesis testing and initial proof of concept is demonstrated in a limited number of trials)

☐ **TRL-3**
Project Plan
(Device Characteristics documents & project proposal completed, Proof-of concept phase)

☒ **TRL-4**
Design and Development
(POC & Safety of device demonstrated by prototype design)

☐ **TRL-5**
Standardization
(Validating the result of the prototype by testing in simulated environment)

☐ **TRL-6**
Preclinical Evaluation
(Clinical trials of functional prototype)

☐ **TRL-7**
Technology Transfer
(Technology transfer of the developed system)

☐ **TRL-8**
Clinical Evaluation
(Evaluation of the system by clinical trials or demonstration)

☐ **TRL-9**
Commercialization
(Commercialization & Post Market Surveillance)

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(g) Base Price of the Technology:

(h) Licensing Category:

- ☐ Material Transfer
- ☐ Prototype Transfer
- ☒ Intellectual Property Transfer
- ☐ Manufacturing Rights Transfer

(i) Technology Licensing Type:

- ☒ Exclusive
- ☒ Non-Exclusive

(j) Need and Demand

(Technology gaps addressed in domestic & international markets, pain points of Industry which are being resolved)

Bioformulations are increasingly becoming important in crop production due to its eco-friendliness in easy degradation and non-toxic nature to non-target organisms and low carbon inputs involved in its productions. The secondary metabolite that is encapsulated using the CNP formulation is extracted from the naturally occurring common soil fungi viz., *Trichoderma asperellum* and is established to be a potent natural chemical in suppressing the growth and development of soil plant pathogens. Natural products are in great demand for plant protection in organic agriculture and export-oriented products like Basmati rice.

1. Natural product with high shelf life and viability under varied storage and transport conditions
2. Easy for production to ensure timely availability and effective applications by farmers with less sophistications required in terms of applicators.
3. Encapsulated products are protected against vagaries of nature
4. Shall help in addressing the problem of spurious products in the market
5. Help in addressing the plant protection needs of organic agriculture

(k) Market Access Information

(Current Global & domestic Scenario, market size & CAGR)

(l) Future Developments

(Scope of future technology development and their application)

The developed BioDCM-NPs system can be used as an effective organic antimicrobial and organic fertilizer to provide protection against crop diseases and enrichment of soil, respectively. The methodology can also be used to produce products targeted against other crop diseases using other metabolites.

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(m) Application/s of the invention
(Please refer to appendix I)

(a) Use as an antimicrobial agent in agriculture crops
(b) Use as a carbon enrichment source to the soil

5. IPR Ownership

(a) Was the intellectual property created with the significant use of funds or facilities of IITK?

YES.

(b) Please describe any source of funding for the invention (Name of the funding agency and copy of agreement, letter of intent if any, must be enclosed with this form).

Indian Council of Agricultural Research- Indian Institute of Rice Research as part of institutional project and IOE, University of Hyderabad (Project no. RC-1-20-005)

School of Chemistry, University of Hyderabad

(c) What is the source of Salary/Remuneration of inventor/Co-inventor?

(i) Dr. Santosh K. Misra: Assistant Professor
(ii) Mr. Piyush Kumar IIT Kanpur institute fellowship
(iii) Dr. C. Kannan: Working as Principal Scientist, Plant Pathology in ICAR-IIRR, Hyderabad (172200+Level 14)
(iv) Dr. Rengarajan Balamurugan: Working as Professor, School of Chemistry, University of Hyderabad (144200+Level-14)
(v) Ms. Divya Mishra: ICAR-Senior Research Fellowship
(vi) Mrs. Mou Mandal: UGC-BSR

(d) Have you presented in any conference, seminar, etc., if yes, please give details?

No, the invention has not been presented at any conference, seminar etc.

(e) Have you published full/part of this invention, if yes, please give copy of publications?

No, the invention has not been published anywhere in full/parts.

(f) Was the intellectual property created in the course of or pursuant to a sponsored/consultancy research agreement with IITK? If yes, please enclose a copy of MOU with concerned project.

NO

(g) Was the intellectual property created as a part of academic research leading towards a degree or otherwise?

NO

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(h) **REVENUE SHARING AMONG INVENTORS:** Please disclose the extent of contribution of each inventor in the invention in percentage terms for revenue sharing.

NAME OF THE INVENTOR	% SHARE*	SIGNATURE
1. Dr. Santosh K. Misra	25	
2. Dr. C. Kannan	25	
3. Dr. R. Balamurugan	25	
4. Mr. Piyush Kumar	9	
5. Ms. Divya Mishra	8	
6. Ms. Mou Mandal	8	

* If this column is not filled and signed then it will be assumed that all inventor(s) have equal contribution

6. Commercial potential

Please provide as much information as possible; Attach extra sheets, if required

(i) Give brief description of potential commercialization by specifying

(a) Input (Financial) required taking it to best stage

(b) Break even capacity

(c) Can you identify possible end-users?

(d) Economic viability

(ii) Who are the Target companies, both in India or abroad?
(From customer point of view)

(iii) Please give specific list of companies and contact details of concerned person who can be contacted for initiating Technology Licensing

Sr. No.	Name of Companies	Name of the contact person	Contact no.
1.	ATGC Biotech Private Limited	Director	1800 121 2842
2.	T. Stanes and Company	Product Manager	+919080385602

(*Unsigned & Incomplete IPDF forms will not be accepted)

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(iv) Do you want to file Patent under PCT Route in other countries?

Yes ☐ No ☒

***The Institute shall file patent under PCT route only in those cases wherein industry/company has exhibited interest for commercialization.**

Disclaimer: *I/We declare that before the submission of this disclosure form or/and during the process of filing this invention as an IPR prospect, I/We will not publish the above information in public domain.*

I/We also give consent to IIT Kanpur being the applicant of this IPR prospect, that they may use this disclosure upon their discretion, which will not be limited to publication on e-auction website, Industry meets & different portals for promotional & licensing purpose.

02/11/21

Signature of Inventor with date

02/11/21

Signature of Inventor with date

05/11/21

Signature of Inventor with date

05/11/21

Signature of Inventor with date

08/11/21

Signature of Inventor with date

08/11/21

Signature of Inventor with date

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Annexure I

Title: Synthesis of Bio-Carbonoid-Metabolite Nanoparticles (BioDCM-NPs) using 6-pentyl-2H-pyran-2-one microbial metabolite for its Application in Bio-protection of Agriculture Crops

1. Extraction, purification, and characterization of metabolite

1.1 Isolation of microorganism

a. Soil sample was collected from rice fields in Rajendranagar, Hyderabad, Telangana to isolate *Trichoderma* species during *Kharif* 2017. The soil samples were taken randomly from five points in the field with the help of post - hole auger (Kisankraft) and homogenised. About 10 grams (weighed using Wensar precision balance) from the above homogenised soil sample was separated through quadrate method and used for serial dilution (Johnson and Curl, 1977). Soil was added and homogenised/made a suspension with 100 ml (Borosil make, borosilicate measuring cylinder) distilled water in 250 ml conical flask (Borosil). Samples were serially diluted at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} concentrations. With the help of a micropipette (20-200µl: Eppendorf), 100µl of soil suspension from 10^{-3} and 10^{-4} was inoculated in Petri plate (Borosil: 95 x 15mm) containing *Trichoderma* specific media (HiMedia make). The plates were incubated at 25 ± 2 °C in Biological Oxygen Demand (BOD) incubator (Bio Technics, India). Hyphal tip from three days old colonies of Trichoderma colonies were picked up with the help of sterile needle (Fisher scientific) and transferred to a Petri plate with sterile potato dextrose (PDA) for axenic cultures for further experiments.

b. Colony morphology of *Trichoderma* species was studied in stereo binocular microscope (Olympus at 45X and 100X magnifications). Morphological identification of the fungi was confirmed at molecular level using sanger sequence methodology. 18S rRNA gene (partial sequence); internal transcribed spacer 1, 5.8S rRNA gene, and internal transcribed spacer 2 (complete sequence); and 28S rRNA gene (partial sequence) of *Trichoderma* were amplified by using the primer combinations ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and LR3R (5'-GGTCCGTGTTTCAAGAC-3') with fragment size of ~1200bp; using the following conditions viz. 1 min initial denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min primer, annealing at 50 °C, 90 sec extension at 74 °C, and a final extension period of 7 min at 74 °C. PCR amplicons were analysed on 1% agarose gel. PCR amplicon fragments were excised from the agarose gel and purified using Promega Wizard® SV Gel and PCR Clean - Up System kit and the purified products of about concentration 50-100 ng/µl was sequenced. The nucleotide sequences were subjected to Blast sequence similarity studies and accordingly the species was found to be *T. asperellum* and the isolate was named as *T. asperellum* IIRRCK1 (in short TAIK1). The sequences were submitted to NCBI GenBank database and NCBI accession number obtained (Accession number: MH825714)

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1.2. Evaluation of antagonistic and plant growth promotion activities of TAIK1 under *in-vitro* and *in-vivo* conditions

a. Antagonistic activity of TAIK1 was tested against two major pathogens *viz., Xanthomonas oryzae* pv. *oryzae* and *Rhizoctonia solani* causing bacterial blight and sheath blight disease in rice through dual culture assay *in-vitro* (Gangwar and Sinha, 2010; Sinclair and Dingra, 2017) and *in-vivo* under glasshouse conditions. Dual culture assay for determining the antagonistic efficacy of TAIK1 against *Xoo* was conducted as follows A loop of TAIK1 and *Xoo* were placed exactly opposite to each other on a petri plate maintaining equidistance from centre, containing modified Wakimoto media (HiMedia). Control plate was maintained by inoculating *Xoo* alone. Radial growth of *Xoo* was measured in treated as well as control plate and percent inhibition were calculated (Gangwar and Sinha, 2010)

   Dual culture assay for determining the antagonistic efficacy of TAIK1 against *R. solani* was studied as follows. Five mm mycelial discs of both TAIK1 and *R. solani* were placed opposite to each other at equidistance from centre of a petri plate containing PDA (HiMedia). Suppression of growth and development of *R. solani* by *Trichoderma* was observed as size of the inhibition zone, reduction in the growth and spread of *R. solani* colony and finally the hyper parasitic growth of TAIK1 over *R. solani*. Dual culture assay has showed ~51.8 and 84 percent growth inhibition of *R. solani* and *Xoo,* respectively.

b. *In-vivo* studies in potted plants grown under net house conditions for the purpose of estimating the antagonistic efficacy of TAIK1 against *Xoo* and *R. solani* were conducted in susceptible rice cultivar Taichung Native 1 (TN1). The seeds were soaked with sterile water for 24 hours and after draining the excess water, they were mixed with the bioagents suspension (10 ml/kg seeds, CFU ~2.26×10^7/ml) for 12 hrs. Treated seeds were then kept on blotting paper to test the germination percentage. About 25 seeds from the initial lot were then placed in nursery trays and monitored for 10 days to calculate morphological parameters like seedling length, seedling dry weight, vigour index-1 and vigour index-2.

   Vigour 1= Germination % X seedling length

   Vigour 2= Germination % X seedling dry weight

   The other lot of the seeds were placed in a different tray (60 × 30 cm) and maintained as nursery. About 25 days old seedlings from the nursery were transplanted in pots of size 30 cm height and 25 cm width carrying approximately 5-7 kg of soil. Thirty days after transplanting (DAT), soil application of TAIK1 was done at 10 g/kg of soil. The pathogens were inoculated at 40 DAT. *Xoo* was inoculated by leaf clipping method (Ke *et al.,* 2017). *Xoo* culture suspension was made by diluting with 10 mM $MgCl_2$ and maintaining the OD_{600} at 0.5. Inoculation of *R. solani* was done by placing about 0.5 mg of sclerotia in rice sheath and covering it by moist cotton swab (Singh *et al*., 2002). The observations were recorded at

different time intervals. The experiments were repeated during two seasons with three replications under controlled conditions in glass house and the values were averaged. Growth inhibition of *R. solani* and *Xoo* at glasshouse level was 58.35 and 67.56 respectively by TAIK1.

c. Growth promotion ability of TAIK1 was estimated by estimation of indole acetic acid (IAA) (Gravel *et al.,* 2007), Phosphate solubilisation (Saravanakumar *et al.,* 2013), siderophore production (Payne, 1994) was done. TAIK1 showed production of IAA (71.56 mg/ ml), PS (161.23 μg/ml), siderophores (95.02 % siderophore units). *In-vivo* studies were done by measuring change in root and shoot length. Percent increase in root and shoot length over control through TAIK1 was 54.59 and 35.94 respectively.

1.3. Extraction of crude metabolite from TAIK1

a. TAIK 1 was cultured in PDB by incubation at 26 ± 2 °C in a rotary shaker (150 rpm) for a time period of 15 days. Fungal biomass from the broth was separated and the active metabolites in crude form were extracted using ethyl acetate using a separating funnel.

2. Extraction purification and identification of active secondary metabolite compound as 6-pentyl-2H-pyran-2-one

a. Preliminary TLC (Thin Layer Chromatography) analysis of the extract using MERCK TLC 60-F_{254} plates revealed that several chemical components with varying polarities were present in it. For practical purposes, the extracted crude was first concentrated and separated into four fractions by column chromatography using silica gel of 100-200 mesh (2.4 cm diameter and 16 cm height). Fraction 1 was collected by eluting the column with 50 ml of 50% ethyl acetate/hexanes solvent system. Fraction 2 was collected by eluting with another 50 ml 50% ethyl acetate/hexanes solvent system. Then it was eluted with 150 ml ethyl acetate to collect fraction 3. Finally, the column was eluted with 50 ml 50% methanol/ethyl acetate solvent system to collect fraction 4.

b. Fraction 1, which was found to show activity, was analyzed by TLC further. It was found to have a major compound (R_f = 0.41 in 20% EtOAc/hexanes). This compound was isolated by column chromatography using 20% ethyl acetate/hexanes solvent system. The purified compound was analyzed by ^{1}H and ^{13}C NMR techniques. The structure of the compound was found to be 6-pentyl-2H-pyran-2-one. It was further supported by FT-IR studies and HRMS analysis. The spectroscopic data matched perfectly with that reported in the literature (Syntheses of α-Pyrones Using Gold-Catalyzed Coupling Reactions; T. Luo, M. Dai, S.-L. Zheng, S. L. Schreiber; *Org. Lett.* **2011**, *13*, 2834–2836)

c. Analytical data for 6-pentyl-2H-pyran-2-one: Off-white gummy liquid; R_f = 0.41 in 20% EtOAc/hexanes; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.25 (dd, *J* = 9.3, 6.6 Hz, 1H), 6.12 (d, *J* = 9.3 Hz, 1H), 5.96 (d, *J* = 6.6 Hz, 1H), 2.45 (t, *J* = 7.6 Hz, 2H), 1.67-1.60 (m, 2H), 1.30 (s, 4H), 0.89 (t, *J* = 6.9 Hz, 3H); $^{13}C\{^{1}H\}$NMR (125 MHz, $CDCl_3$): δ 166.7, 162.9, 143.8, 112.9,

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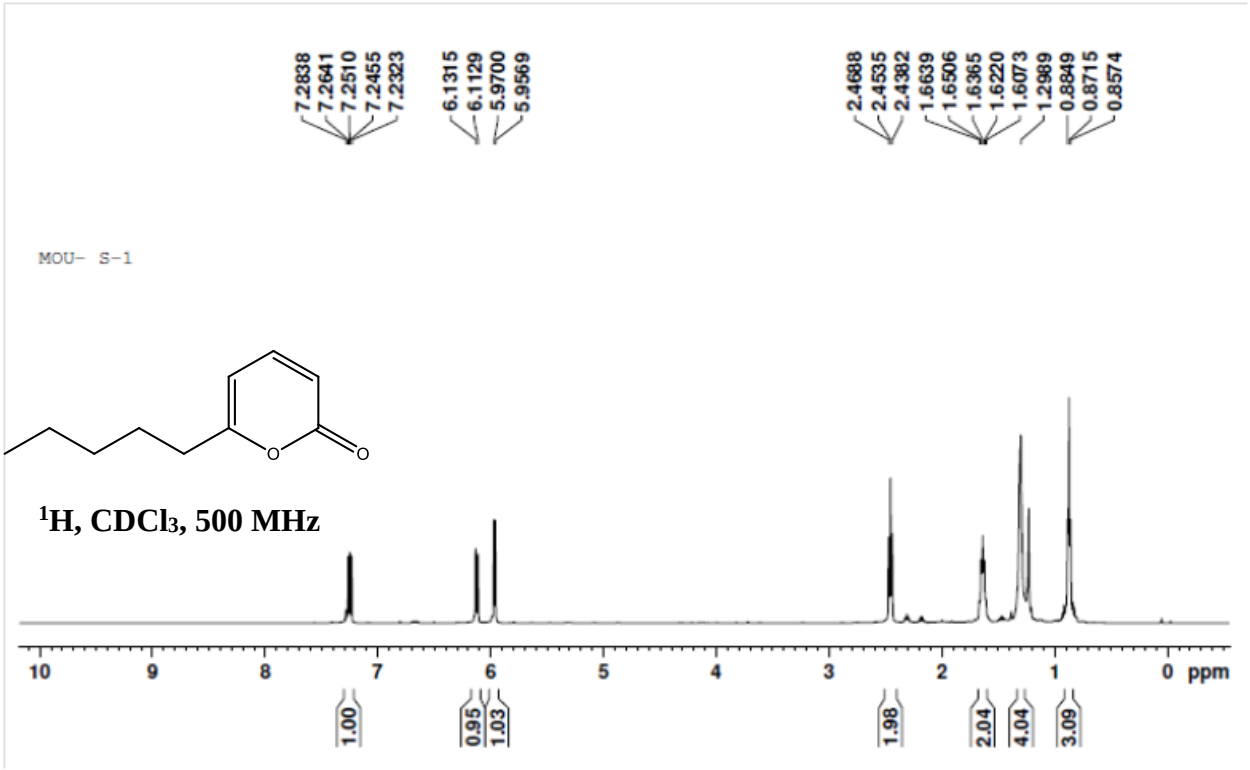
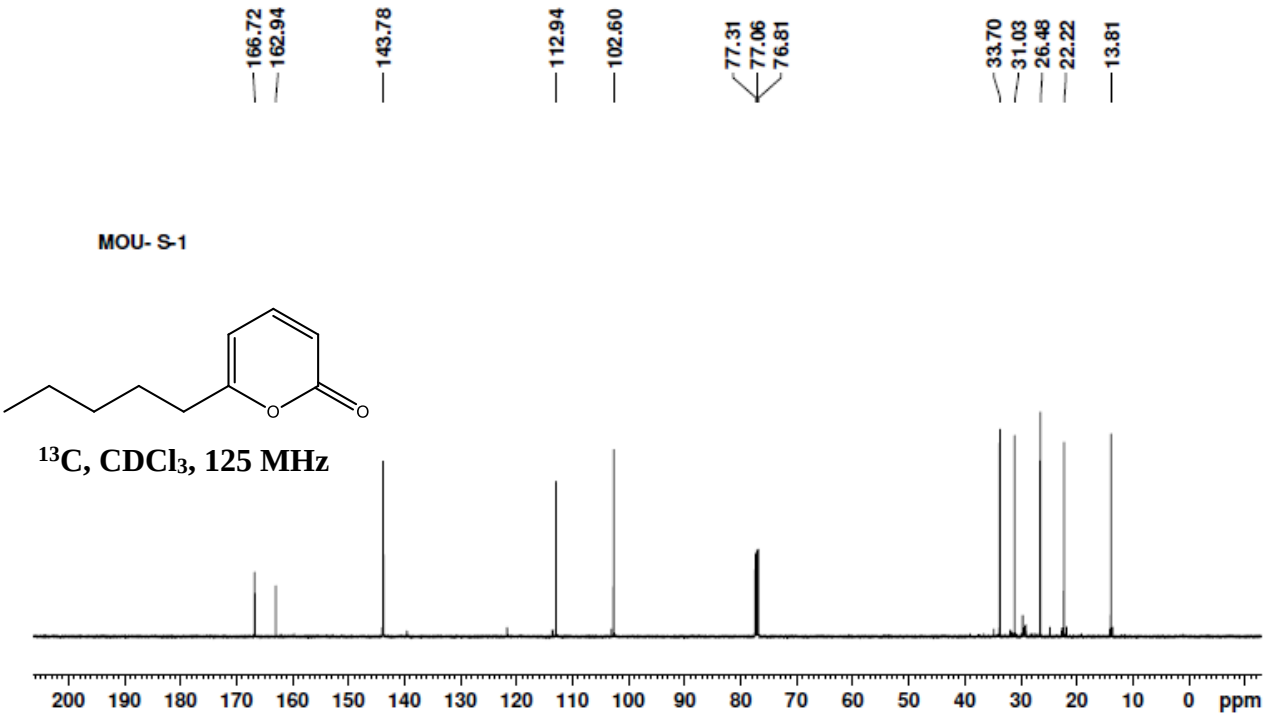
102.6, 33.7, 31.0, 26.5, 22.2, 13.8; IR (KBr, cm^{-1}): υ 2955, 1721, 1556, 1082, 798; HRMS (ESI-TOF) m/z: $[M + H]^+$ Calcd for $C_{10}H_{15}O_2$ 167.1067; found 167.1083.



^{1}H NMR of 6-pentyl-2H-pyran-2-one



^{13}C NMR of 6-pentyl-2H-pyran-2-one

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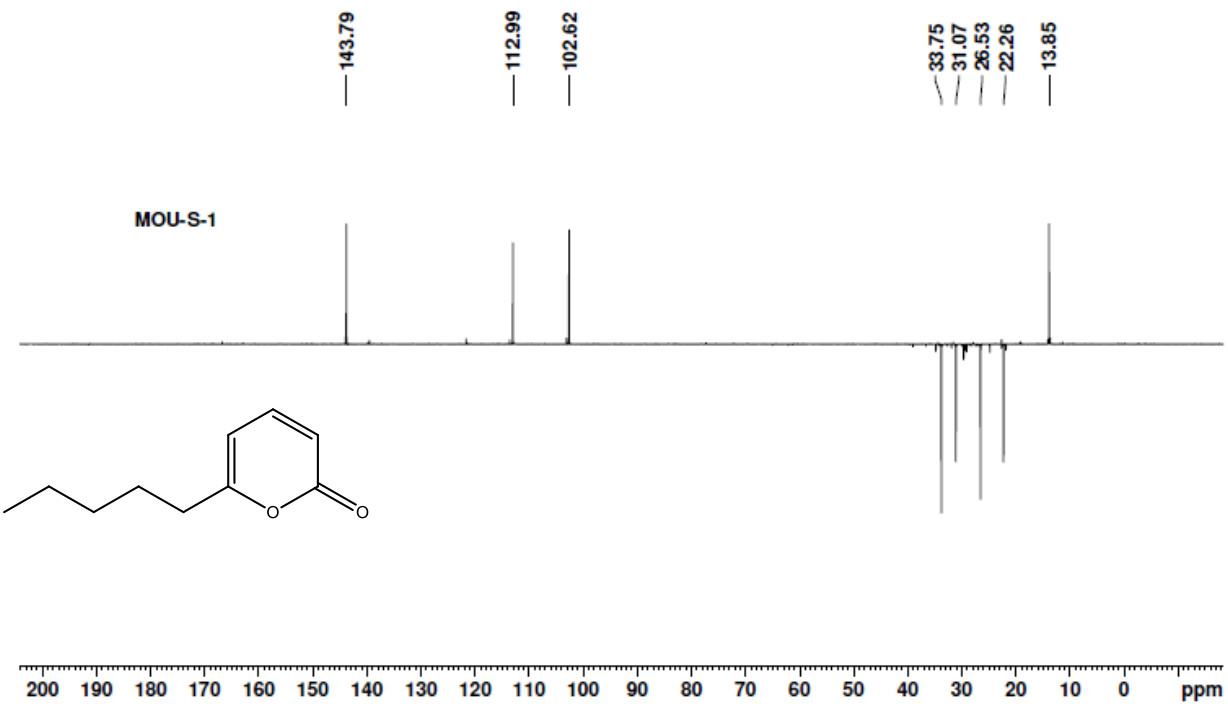
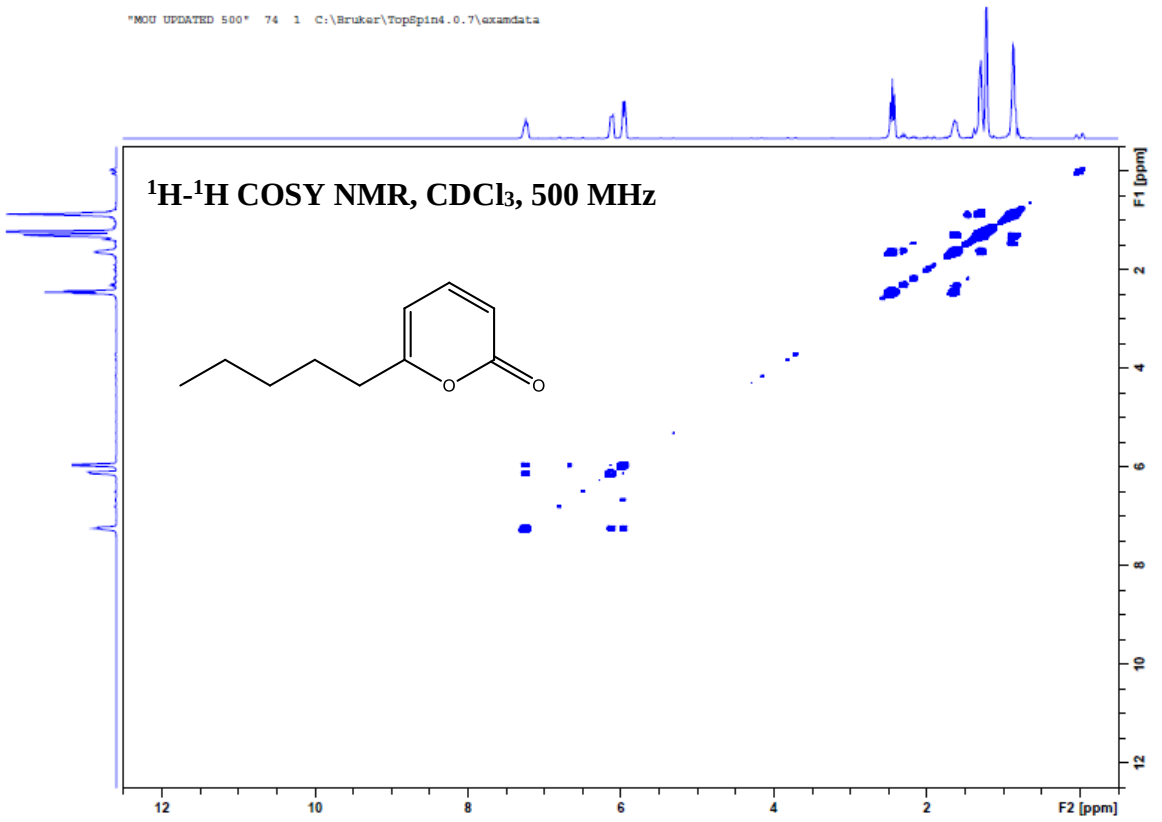


DEPT spectra of 6-pentyl-2H-pyran-2-one



COSY spectra of 6-pentyl-2H-pyran-2-one

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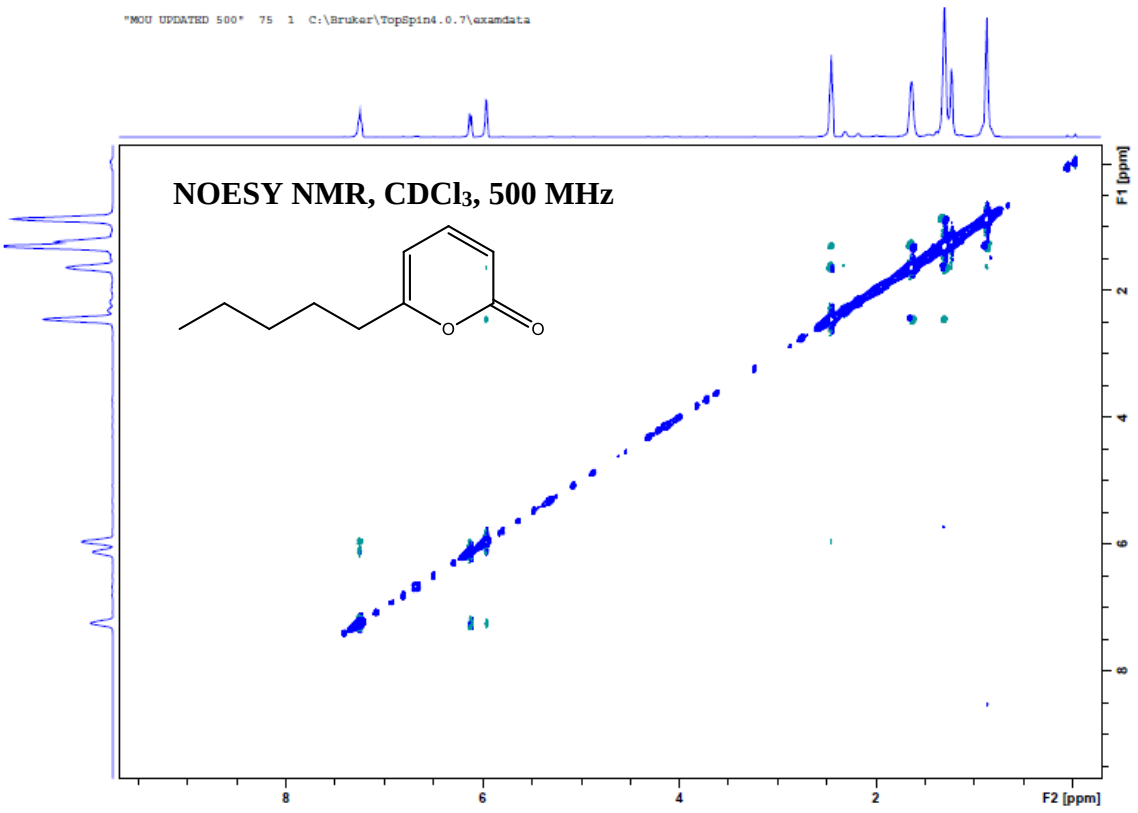
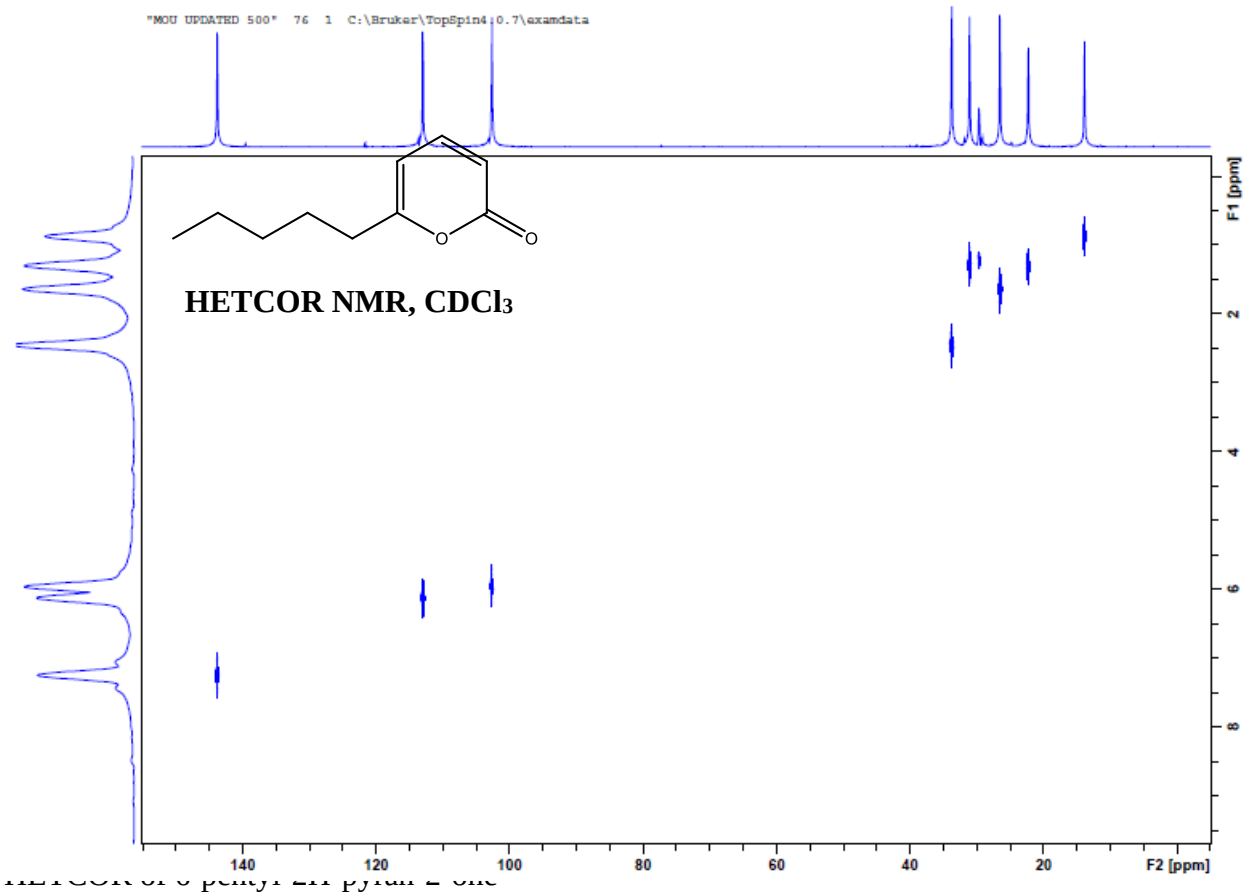


NOESY spectra of 6-pentyl-2H-pyran-2-one



HETCOR of 6-pentyl-2H-pyran-2-one

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3. Preparation of Bio-Degradable Carbonoid-Metabolite Nanoparticles (BioDCM-NPs)-

a. Materials and Methods:

a.i. Reagents

- Sucrose (Merck, cat. no. DB8D680233)
- Metabolite (stock concentration: 5000 ppm)
- Milli-Q water (Millipore Milli-Q Direct-Q3® ultrapure water (Type 1); Resistivity: 18.2 MΩ.cm)

a.ii. Equipments

- NuWav-ProMaster (NuTech, Microwave frequency: 2450 MHz)
- Magnetic stirrer (Remi 2 MLH)
- Weighing balance (Aczet, model no, CY224)
- Zetasizer Nano ZS90 (Malvern Panalytical)
- Platinum temperature probe (Pt100; NuTech)
- 25 ml round bottom flask with 4 necks (NuTech)
- Round bottom flask neck closure caps
- 50 ml conical flask
- Reflux condenser
- Clamp and stand
- 10 ml measuring cylinder
- Magnetic stirring bar (10 × 30 mm; Tarsons, cat. no. 4141)
- Aquarium water pump
- Water container
- Plastic tubes
- cuvette

a.iii. Equipment Setup

- The reflux condenser was mounted onto the NuWav-ProMaster microwave synthesizer using a clamp and stand.
- The condenser was connected to the aquarium pump using the plastic tubes.
- Appropriate amount of water was filled in the water container and the aquarium pump was placed in it. The pump was switched ON after the setup to make circulation of water through the condenser.

b. Procedure

b.i. Synthesis of BioDCM-NPs

1. 1 g sucrose was weighed and transferred into a 50 ml conical flask.

2. 5 ml of Milli-Q water was added to conical flask and stirring was performed using magnetic bar at room temperature (25 °C) with 200 rpm on magnetic stirrer till the sucrose was completely dissolved.
3. Added 1 ml of metabolite (5000 ppm) to the solution and stirred it for 5 min at 200 rpm.
4. Transferred the solution into a 10 ml measuring cylinder and the volume was made up to 10 ml by adding 4 ml of Milli-Q water.
5. Transferred the solution into a 25 ml round bottom flask with more than one necks.
6. The flask was securely placed into the NuWav-ProMaster and was connected one neck of the flask to the reflex condenser. The Pt100 temperature probe was connected to the machine using the connection port provided inside the reaction chamber of the machine. The probe was secured using the treads provided on the probe.
7. The solution was heated at 110 °C using microwave power of 200 W for 60 min. The water was kept flowing through the reflux condenser during the reaction. The level of solution in the round bottom flask was continuously observed during the reaction, as required, 1-2 ml of prewarmed Milli-Q water was added through the condenser to maintain the volume of the solution.
8. A golden yellow to brownish black solution was formed after the completion of the reaction. The solution was collected, and the final volume was made up to 10 ml by adding Milli-Q water.
9. The as synthesized **BioDCM-NPs** were transferred into a 15 ml centrifuge tube and stored it at 4 °C till further use.
10. Prepared **BioDCM-NPs** were analyzed for hydrodynamic size by using Dynamic Light Scattering (DLS) experiments.
11. Control nanoparticles (Bio-Degradable Carbonoid Nanoparticle; **BioDC-NP**s) to compare the functional properties were produced using same method but metabolite was not added in the step #3 and 5 mL of Milli-Q water was added in step #5 to make up the total volume of 10 ml.

b.ii. Hydrodynamic size measurement using DLS

1. Took 1 ml of **BioDCM-NP** suspension and vortexed it for 1 min.
2. Transferred 500 µl of solution into a cuvette and diluted to 1 mL. Suspension was mixed properly during pipetting while bubbles were avoided.
3. Placed the cuvette in the Zetasizer nano ZS90 machine and adjusted the cell temperature to 25 °C.
4. Took the measurement with an equilibrium time of 10 sec.
5. Step 4 was repeated three times.

c. Observations

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A golden yellow to brownish black colored suspensionn was formed indicating the oxidation of carbohydrates and generation of **BioDCM-NPs** during reaction progression. The protocol described here resulted in particles having a hydrodynamic diameter in the range of 295 nm. Further changes in reaction time influenced the size of the particles. In general, the increase in reaction time resulted in increase in particle size. Hydrodynamic size of the prepared control particle **BioDC-NP**s was found to be ~227 nm.

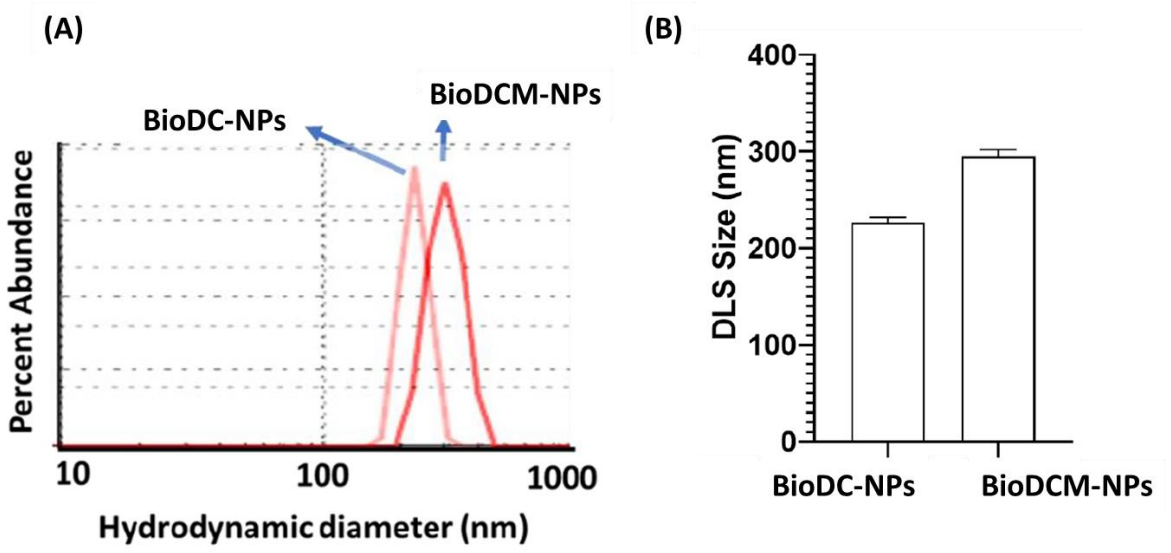

Figure. Hydrodynamic size of prepared BioDC and BioDCM NPs. (A) Comparative histogram of % distribution of particle size and (B) change in DLS size across samples.

d. Storage

Both type of particles could be stored at room temperature in brown bottles without showing any visible coagulation of particles for 4 months (still being observed). A much longer storage might be achieved probably by reducing the storage temperature.

e. Precautions

- The level of water in the round bottom flask was carefully observed as lower volume might cause damage to the reaction vessel and machine.
- In order to maintain the volume during the reaction, prewarmed water should be added as cold water might result in cracking of glass condenser.

f. Troubleshooting

- If no color change was observed after the reaction time, the reaction temperature was checked. The temperature probe was reinstalled, and protocol was rerun. If probe is not properly dipped in reaction liquid, desired temperature is not obtained to start the reaction.

*PCT/ International filing is subject to support from the Project funds of the Inventor.

4. Evaluation of Functional activity of BioDCM-NPs

a. The compound was dissolved in 17.25 ml of DMSO (HiMedia) and used for testing antagonistic activity of *Trichoderma* against selected pathogens. Selective medium for both the pathogen was prepared, autoclaved and poured in the plates with the volume of 20 ml along with test compound at different ppm concentration (50, 150, 250 and 500). Pathogen were inoculated in the media and then the plates were incubated at 25 ± 2 °C for 3 days. The diffusion of secondary metabolites into agar inhibited the growth of the test pathogen and the diameter of zone of inhibition was measured (Brown et al., 1975). The compound showed 100% growth inhibition of both the pathogen at 150 ppm concentration.

b. The BioDCM NPs formulation was diluted in sterile distilled water in a ratio of (1:1) to make a stock suspension. The suspension was transferred in two separate 2 ml centrifuge tubes one containing a loop full of *Xoo* and other *R. solani* culture. From 0 min up to 90 mins after every 30min intervals, 100µl of suspension was pipette out and inoculated in the plate containing media. The plates were incubated at 25 ± 2 °C and the time at which growth of pathogen got stopped was noted. The efficiency of formulation was also tested at different temperatures *viz.*, 20 °C, 32 °C and 45 °C. Efficacy of BioDCM NP formulation in reducing the growth of pathogens was seen after 30 mins of incubating period of formulation and pathogen in the suspension. This signified that the compound was getting released from the protected coat after 30 mins. The formulation BioDCM NPs co-prepared at 110 °C was successful in complete reduction of pathogens growth in plate at all the used temperatures.

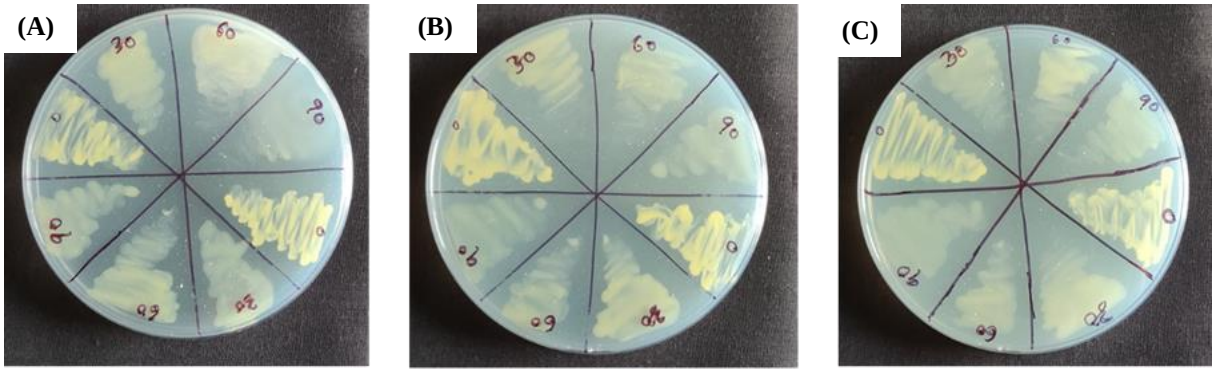

Figure. Representative images of BioDCM NP formulation treated agar plates for growth inhibition studies of *Xoo* across 0, 30, 60 and 90 min time points and working temp of (A) 20, (B) 30 and (C) 45 °C.

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