Antifungal compound from marine *Serratia marcescens* BKACT and its potential activity against *Fusarium* sp.

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Abstract
Ecofriendly biocontrol agents to control pathogenic fungi are in demand globally. The present study evaluated the antifungal potentials of marine bacteria *Serratia marcescens* BKACT against eight different *Fusarium* species. A highest 75.5 ± 0.80% of mycelial inhibition was observed against *Fusarium foetens* NCIM 1330. Structural characterization of the purified compound was analyzed by GC–MS and NMR techniques; based on the analysis, it is confirmed as 2, 4-di-tert butyl phenol (2, 4-DTBP) with chemical structure C14H22O. At 0.53 mM concentration, purified compound inhibited complete spore germination of *F. foetens* NCIM 1330. In vitro assay showed complete inhibition of *F. foetens* NCIM 1330 on the wheat seeds. Tested concentration does not show any toxic effect on germination of the seeds. By this study, we conclude that, 2, 4-DTBP is a suitable candidate to be used as biocontrol agent against *Fusarium* infection.

Keywords *Serratia marcescens* · Marine · 2, 4-di-tert butyl phenol · *Fusarium* sp

Introduction
Considerable interest has grown in finding an alternative solution to chemical pesticides for controlling of soil borne plant pathogens and parasitic nematodes. *Fusarium* is one of the most pathogenic genus of filamentous fungi which colonizes many host plants and crops worldwide. It includes around 70 species, which causes plant diseases such as wilt, seedling blight, rot and cankers. *Fusarium* is also known to produce mycotoxins in many economically important crops, resulting major loss in yields and quality of the crops (Munkvold 2017; Aoki et al. 2014). Microorganisms have always been regarded as a treasure source for useful compounds and are considered as green and sustainable alternatives for the chemical fungicides. Several studies have been carried out with respect to the use of soil and freshwater microorganism as a biocontrol agents. Relatively few of these antagonistic microbes have been commercialized as biocontrol agents due to their inconsistent performance in the fields, lack of broad-spectrum disease suppression activity, slower and less suppression when compared with chemical pesticides (Roberts et al. 2005). Despite having colossal diversity and potentials to produce a novel class of compounds, marine bacteria and their active compounds are not much explored for agricultural uses. Marine microorganism can produce a wide range of secondary metabolites (Imhoff et al. 2011) with potential antifungal activity. The Serratia species are endowed to produce a range of active compounds, making them exciting candidates for biocontrol activity. Many species, including *Serratia marcescens*, *Serratia plymuthica*, *Serratia liquefaciens* and *Serratia rubidaea*, are known for their biocontrol activity against various plant pathogenic fungi. Among these, *S. marcescens* is the dominant for the biocontrol activity against various plant pathogens. It produces prodigiosin and various bioactive metabolites, such as althiomycin, oocydin A, serrawettins, rubiwettin and carbapenem (Soenens and Imperial 2020). In the present study, we have demonstrated the detailed in vitro and in vivo antifungal potential of 2, 4-DTBP produced by marine *Serratia marcescens* against *Fusarium* species.
Materials and methods

Isolation, identification and antifungal screening

In the previous study, around 150 marine bacteria were isolated from Chorao Island, Goa, India, and preserved. In the present study, all these isolates were subjected for antagonistic activity against eight different *Fusarium* species. All the test fungi were procured from the NCIM-Resource Center, CSIR-National Chemical Laboratory Pune, India. All these strains were maintained on potato dextrose agar (PDA), pH-7.0 (Hi-media, Mumbai) throughout the study.

Screening of antifungal activity was carried out using the dual culture method on PDA (Tchameni et al. 2020). Test bacteria were streaked in a straight line at the center of a plate. Spot inoculation of fungi was made using a sterile loop and placed 1.0 cm away from the inoculated test bacteria. A plate inoculated with the fungal pathogen alone was used as a control. Three replicates of each test bacteria. A plate inoculated with the fungal pathogen alone was used as a control. Three replicates of each plate were incubated at 28 °C for 9 days, and the growth inhibition of fungal pathogens was observed at 3, 5, 7 and 9 days. The percent (%) inhibition growth of the test fungi was calculated by using the formula:

\[ \text{Growth inhibition percent} = \left( \frac{R - r}{R} \right) \times 100 \]

where \( R \) is the radial growth of fungal pathogens in the control plate and ‘\( r \)’ is the radial growth of fungal pathogens in the dual culture plate. All the experiments were performed in triplicates.

The effect of strain BKACT on the hyphal and conidial morphology of fungi was observed under a light microscope (Nikon, Japan) and scanning electron microscope (SEM). For SEM examination, samples were prepared as per the procedure mentioned with modification (Zhao et al. 2014). Briefly, the fungal hyphae was fixed in 2.5% glutaraldehyde (prepared in 0.1 M sodium phosphate buffer) at 4 °C for 24 h and subsequently rinsed three times with phosphate buffer (0.1 M). Then, hyphae was dehydrated in a graded series of ethanol concentrations (30, 50, 75 and 100%) for 5 min each, finally dried at 37 °C and mounted on stubs, sputter-coated with gold observed under FEI Quanta 200 3D dual-beam scanning electron microscope.

For molecular characterization, genomic DNA was extracted from cell pellet of strain BKACT, using genomic DNA HiPurATM kit (Hi-Media, Mumbai). The 16S rRNA gene was amplified using universal primers 27F and 1492R. The PCR product was purified using an Exo SAP (New England Bio Labs) and sequenced by a 3500XL Genetic Analyzer (Applied Biosystems, USA). The identity of the strains with closest neighbors was determined by comparing the 16S rRNA gene sequence with available sequences in the NCBI Gene Bank using the BLASTn program. A phylogenetic tree was constructed using multiple alignments from closely related strains retrieved from the EzBioCloud database (http://www.ezbiocloud.net).

A neighbor-joining tree was constructed using MEGA (version 6.0) to determine the phylogenetic relationship of a strain BKACT. The topology of the phylogenetic tree was evaluated with bootstrap values based on 1000 replications.

Fermentation and extraction of antifungal compound

The production of the antifungal compound was accessed at flask level fermentation. A single colony of antagonistic bacteria from fresh nutrient agar plate were inoculated into the nutrient broth as a seed culture and kept on a rotary shaker at 28 °C with 140 rpm for 18 h. Subsequently, 5% inoculum was added to the 100 mL king’s modified broth ((g/L) glycerol 30, peptone, \( K_2HPO_4 \) 0.5, \( MgSO_4 \cdot 7H_2O \) 0.5 and pH 7.0) as a production medium and incubated for 5 days at 28 °C. Cells were harvested after 5 days of incubation by centrifugation at 10,000 rpm for 10 min. The resultant supernatant was acidified to pH 2.0 with 4 N HCl and subsequently extracted with an equal volume of ethyl acetate twice. The organic phase was collected and concentrated using a rotary evaporator. The crude extract was checked for its antifungal activity against all fungal strains. Based on the preliminary antifungal activity in crude extract, the antifungal compound was produced in a 14.0L lab scale fermenter (BioFlo/CellicGen 115) with 10.0-L working volume. Dissolved oxygen was maintained above 50% by adjusting agitation 250 to 350 rpm till the end of fermentation batch.

The antifungal activity of the crude extract from the fermentation batch was evaluated by well diffusion method. The spore suspension of all the test fungi was prepared in 0.01% Tween 80 solutions from a 7-day old grown culture. The final concentration of spore suspension was adjusted to have 1.0 × 10^8 CFU/mL by hemocytometer. To sterile PDA media spore suspension was added with proper mixing. Spore mixed media was poured into the sterile petri dishes. After solidification, 100μL of crude extract from the 10.0 mg/mL stock was loaded in each well, and 100μL of methanol was used as a solvent control; plates were incubated at 28 °C for 72–96 h, and antifungal activity was measured by the zone of inhibition in millimeter (mm).

Thin-layer chromatography (TLC) based bio-autography

Bio-autography was performed according to Grzelak et al. (2016) on TLC plate silica gel 60 (Merck, Darmstadt, Germany). The crude extract (30μL) was spotted 1.0 cm apart from the baseline into the silica gel plate and allowed to dry.
The plate was then developed with ethyl acetate:pet ether (70:30) in a previously saturated glass chamber at room temperature. The developed plate is dried at room temperature, and the spots were visualized in a UV chamber at 254 nm. Bio-autographic evaluation of the crude extract is performed to check the antifungal activity of the separated compounds on the TLC plate. TLC developed plates were UV sterilized for 30 min in laminar airflow and placed in a petri dish, and 20.0 ml of 0.8% semi-solid potato dextrose agar having 1.0 × 10^6 spore/mL of *F. foetens* NCIM 1330 is poured over the TLC plate placed in the petri dish. After proper solidification, the petri dishes were incubated at 28 °C for 72–96 h to observe the zones of inhibition of active compounds separated on the TLC plates.

### Purification and characterization

The crude extract of 9.6 g obtained from fermentation is subjected for purification by column chromatography using 45 × 7.5 cm column packed with 120–200 mesh silica. The column was eluted with dichloromethane (DCM) to ensure that the column was properly packed. The crude extract was mixed with 60–120 mesh silica bed and applied uniformly from the top of the column with the combination of dichloromethane and methanol (MeOH) (100 to 0% DCM with 0 to 100% MeOH ratio) in increasing order of the polarity. Forty-milliliter fractions were collected. A total of 126 fractions were collected and checked for TLC profiles. All the 126 tubes were pooled into nine fractions based on their Rf value; these pooled fractions were further confirmed for their antifungal activity. The active fraction no. 3 was partially purified using a smaller silica column (35 × 3.0 cm). Gradient elution of the column was carried out with a combination of pet-ether and ethyl acetate by increasing the order of the polarity with ethyl acetate. Twenty-five milliliters each of the fractions was collected in 86 tubes, TLC profiling was again based on their TLC similarity and all the tubes were pooled into five fractions. Subsequently, all five fractions were reconfirmed for their antifungal activity. The partially purified active fraction number F-3(II) was subjected to further purification by preparative thin-layer chromatography (PTLC) on pre-coated Silica Gel 60, 254 plates (20 × 20 cm, Merck). The PTLC plates were developed in pet ether:ethyl acetate (70:30), and after air-drying, the plates were visualized under UV light (254 nm). The desired band was scratched from the PTLC plate and extracted with ethyl acetate. The solvent was evaporated by vacuum, and the weight of a purified compound was recorded.

The structure of the purified compound was established by nuclear magnetic resonance (NMR). The 1H and 13C NMR spectra were recorded on Bruker AV 500 MHz and 125 MHz, respectively, in deuterated chloroform (CDCl3). The chemical shifts were given in δ, and ppm (parts per million) values referenced to the chloroform solvent at δ 7.27 in 1H and 77.00 ppm in 13C NMR.

In GC–MS grade methanol were prepared 1.0 mg/mL stock of purified compound and the crude extract. From the stock, 1.0 µL was injected, and GC–MS was carried out by using a 7890A gas chromatograph with a 5975C inert XL quadrupole mass spectrometer detector (MSD) (Agilent Technologies, USA) operated in electron ionization (EI) mode with a kinetic energy of the impacting electrons of 70 eV. The Restek Rtx®-5MS fused silica capillary column (30 m × 0.25 mm × 0.25 µm) with the non-polar stationary phase of 5% diphenyl/95% dimethyl polysiloxane was used. The data was analyzed with the ChemStation software and validated with the NIST mass spectral library (Agilent Technologies). The oven temperature for the column was programmed (total 51.71 min), starting from 40 °C with hold for 2 min, then rising with a ramp of 5 °C/min up to 180 °C. Then, it further increased with the ramp of 7 °C/min up to 220 °C and finally ramped with 10 °C per min up to 28 °C with having 10-min hold. Helium (99.9% pure) was used as a carrier gas with a constant flow of 1.0 mL/min. The inlet temperature was kept at 250 °C in split less mode. The auxiliary temperature was kept at 28 °C. The EI ion source and quadrupole temperature were kept at 230 °C and 15 °C, respectively. Mass spectra and reconstructed total ion chromatograms (TIC) were obtained after 4-min solvent delay by automatic scanning in the unified mass range of 50–600 u. The retention time and mass fragmentation pattern were compared with reference compounds identified as the possible compounds.

A standard of 2, 4-di-tert butyl phenol (2, 4-TBP, CAS: 96–76–4, Sigma-Aldrich, Switzerland) (1.0 mg/mL) and 0.1 mg/mL stock of purified compound were prepared in HPLC grade methanol for the comparative analysis. The purified compound and standard 2, 4-DTBP were analyzed by HPLC (Thermo Scientific Dionex Ultimate 3000) using a C18 column (4.6 × 250 mm, 5-µm particle size thermo hypersil gold) with methanol:water with 0.1% trifluoroacetic acid (TFA) as a mobile phase, flow rate of 1.0 mL/min and detection at 254 nm. The 5.0µL sample from both standard 2, 4-DTBP and purified compounds was injected in isocratic mode with 80:20 of methanol and water for 14 min. Further, both compounds were compared with TLC using ethyl acetate:pert ether 70:30 as the solvent system, and detection was completed at UV 254 nm to find the Rf values.

### Antifungal assay (MIC)

The minimum inhibitory concentration (MIC) values of 2, 4-DTBP were determined against *F. foetens* NCIM 1330 by well dilution technique using 96-well microtiter plate by following the guidelines of Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute 2008).
Ten microliters of spore suspension (1.0×10⁶ spores/mL) is added to the different concentrations of 2, 4-DTBP, and the total volume was adjusted to 100 µL/well with a sterile potato dextrose broth. The wells with only methanol served as a control, and micro plates were incubated for 72 h at 28 °C. The time killing rate was evaluated by OD at 595 nm for every 4-h interval up to 72 h. From each well, 10µL of broth is spread onto the PDA plates and incubated at 28 °C for 72 h. The minimum compound concentration with no detectable fungal growth on the PDA plate is considered the minimum fungicidal concentration (MFC).

**In vitro antifungal effect**

The compound 2, 4-DTBP is confirmed as a volatile in nature. Further, the compound is evaluated for its antifungal potential using the bi-compartment petri dish method (Mas-sawe et al. 2018). For the antifungal activity, pure compound with 20.0 mg/mL working stock was prepared in methanol (Hi-media, Mumbai). The fungal mycelial plug of *F. foetens* NCIM 1330 from 7-day old culture was inoculated on the one compartment of potato dextrose agar. In another compartment having different concentrations of compound, i.e. 0.07, 0.26 and 0.53 mM were loaded on the 9.0-mm size disc in individual plates. Methanol served as a control. Further, plates were sealed with paraffilm and incubated at 28 °C for 5, 7 and 9 days. The percent mycelial growth inhibition is calculated by using the below mentioned formula:

\[
\text{Growth inhibition percent} = \frac{R - r}{R} \times 100 \text{ where } R \text{ is the radial growth of fungal pathogens in the control plate and } 'r' \text{ is the radial growth of the fungal pathogen in the treated plate.}
\]

The experiment was conducted in triplicates.

Spore germination assay was carried according to Wang et al. (2020). Spore suspension of 1.0×10⁶ spores/mL was prepared in 0.01% tween 80 solution; 20 µL was added to potato dextrose agar smear (diameter—12 mm) on the glass slide for spore germination assay. Slides were placed in a petri dish and moist filter paper at the bottom to maintain 90–92% humidity. A sterile paper disc containing 2, 4-DTBP (0.07, 0.26 and 0.53 mM) was placed on the other side of the plate. Water and methanol served as the negative and solvent control respectively. All the plates were prepared in triplicate and sealed with paraffilm and were incubated at 28 °C for 3, 6, 12 and 24 h. At least 200 spores of each sample were examined at 40x under light microscopy (Nikon, Japan). Observation was made on germination by checking the germ tube length that exceeded half of the diameter of spores.

**In vivo antifungal effect**

Mature and healthy wheat seeds were purchased from the local market and used for the experiment. The assay was carried out according to the method of Zhang et al. (2021). Seeds were cleaned by washing and soaked in water for 1.0 h and autoclaved. All the seeds were spiked with a 1.0×10⁶ conidia/mL suspension. In each petri dish, 20 seeds were placed at one corner of the plate. The antifungal volatile effect of 2, 4-DTBP is evaluated by different concentrations, i.e. 0.07, 0.26 and 0.53 mM, whereas water and methanol were used as negative and solvent control. All the plates were sealed with paraffilm and incubated at 28 °C for 7 days. All the experiments were performed in three experimental replications. The protection of fungal infection on the wheat seeds was quantified based on infected seed counts as:

\[
\text{Percent seed contamination index (PSCI)} = \frac{(\text{Negative control} - \text{Treatment})}{\text{Negative control}} \times 100.
\]

Mold count in wheat seeds is carried out after 7 days of 2, 4-DTBP treatment. Ten seeds (1.0 g) were taken out and mixed with 9.0 mL of sterile saline solution from each treatment group along with control and blank. The sample is mixed by shaking for 30 min at 300 rpm. The samples were diluted by the standard serial dilution method up to 10⁸. Each dilution of 100µL sample is placed on PDA and incubated further at 28 °C for 72 h. The number of colony forming units (CFU) of mold per gram of wheat sample is calculated as.

\[
\text{Colony forming unit (CFU)/gram of sample} = \frac{\text{number of colony count} \times \text{dilution factor}}{\text{volume of culture plate}}.
\]

**Toxic effect of 2, 4-DTBP on the wheat seeds**

The toxic effect of 2, 4-DTBP on the germination of wheat seeds is performed according to Zhang et al. (2021). All the seeds were washed with tap water and surface sterilized in 1% sodium hypochlorite for 2 min, then rinsed twice with sterile distilled water for 1 min and air-dried. Two hundred seeds were added per plate keeping the sterile disc containing 2, 4-DTBP at lowest 0.07 and highest of 1.06 mM concentration; methanol was used as solvent control and incubated for 7 days at 28 °C. A hundred seeds were randomly withdrawn after 7 days of incubation and placed on a filter paper (pre-soaked in sterile water) and cultivated at 28 °C. The germination percentages of wheat seeds were calculated. Germination was considered, when the radicle protruded by 2 mm from the seeds.

**Understanding the mechanism of mycelial growth inhibition**

**Cytoplasmic leakage assays**

The effect of 2, 4-DTBP on the cytoplasmic leakages of *F. foetens* NCIM 1330 was evaluated according to Wang et al. (2020).
et al. (2020). Briefly 7-day-old mycelial plug of 6 mm was inoculated onto the PDA plates. Subsequently, it was fumigated with different concentrations of the 2, 4-DTBP, and methanol was used as a solvent control. All the plates were sealed with parafilm and incubated for 7 days. Afterwards, the mycelia were suspended in 50 mL sterile distilled water and incubated for 0, 30, 60 and 120 min at 28 °C on a rotary shaker. Extracellular conductivity was measured using an electrical conductivity meter, and nucleic acid was quantified at OD260 using (NANODROP LITE Spectrophotometer). Protein concentration was determined according to Bradford’s method.

**HPLC analysis of the ergosterol**

The ergosterol content of the *F. foetens* NCIM 1330 was determined according to Wang et al. (2020). One hundred milligrams each of mycelia from all the treatment and control samples were suspended in 20 mL methanol, 5 mL absolute ethanol and 2 g KOH respectively. After mixing for 5 min, it was incubated at 70 °C for 40 min. Later, 5 mL water was added and centrifuged at 1735 × g for 15 min. Further supernatant was extracted with an equal volume of n-hexane. The organic phase was collected and evaporated in a rotary vacuum evaporator, and the crude residues were suspended in 1.0 mL methanol. All the samples were analyzed on a C18 column (4.6 × 250 mm, 5 µm particle size thermo hypersil gold) using Thermo Scientific Dionex Ultimate 3000 model HPLC with the mobile phase 95% ethanol:acetonitrile (1:1), at a flow rate of 1.0 mL/min, and Ergosterol was detected at 280 nm.

**Statistical analysis**

Statistical analysis was carried by one-way analysis of variance (ANOVA) followed with post hoc Tukey’s (HSD) honestly significant difference test. The p values < 0.05 were considered as statistically significant. Statistical analysis is performed using SPSS software version 26.0 (SPSS Inc., USA). Graph Pad Prism 8.0.2 software is used for plotting the graphs. Detailed statistical analysis data has been provided in the supplementary information. All experiments were carried out in triplicates, and data were presented as mean ± standard deviation.

**Results**

**Screening for antagonistic activity**

Among one hundred and fifty marine bacteria were screened for antifungal activity; strain BKACT was identified as the potential antifungal candidate which inhibited > 50% of mycelial growth against all the *Fusarium* species tested (Table 1). Under light and scanning electron microscopy, the hyphal morphology of treated fungi showed an abnormal, degraded and deformed shape in comparison to the control test (Fig. 1 and Supplementary Fig. 1). The highest percentage of mycelial growth inhibition of 75.56 ± 0.80 was observed against *Fusarium foetens* NCIM 1330 (Table 1). Further, strain BKACT demonstrated a significant mycelial growth inhibition of 50 ± 2.0, 53.03 ± 2.14, 69.56 ± 1.22 and 75.56 ± 0.80% on 3rd, 5th, 7th and 9th day of incubation (Fig. 1).

**Molecular characterization**

Identification of antifungal strain BKACT based on 16S rRNA gene sequencing analysis and NCBI-BLASTn confirmed it belongs to genus *Serratia*. The strain BKACT showed the highest sequence similarity to *Serratia marcescens* sub sp. ATCC 13,880, and its phylogenetic analysis also confirms by forming similar clade with *Serratia marcescens* sub sp. ATCC 13,880 (Fig. 2). The 16S rRNA gene sequence of strain BKACT has been deposited in NCBI GenBank under accession number MT186165.

**Antifungal activity and thin layer chromatography based bio-autography.**

The crude fermented extract of strain BKACT showed a significant antifungal activity when compared with solvent

| Tested fungal strains                  | % growth inhibition | Inhibition zone (mm) |
|---------------------------------------|---------------------|----------------------|
| *Fusarium verticilliodes* NCIM 1276   | 59.3 ± 1.0          | 29 ± 0.8             |
| *Fusarium nivale* NCIM 1191           | 64.1 ± 2.6          | 37 ± 0.8             |
| *Fusarium tricinctum* NCIM 1189       | 66.7 ± 2.7          | 31 ± 0.81            |
| *Fusarium proliferatum* NCIM 1101     | 65.0 ± 2.2          | 28 ± 0.9             |
| *Fusarium moniliforme* NCIM 1100      | 55.5 ± 2.4          | 31 ± 0.8             |
| *Fusarium oxysporum* NCIM 1281        | 67.5 ± 2.0          | 37 ± 0.8             |
| *Fusarium* sp. NCIM 1383              | 55.8 ± 2.8          | 24 ± 0.9             |
| *Fusarium* foetens* NCIM 1330         | **75.56 ± 0.80**    | **40 ± 1.2**         |

Bold letter indicated the 1.3-1.6 fold increased significance of inhibition against *F. foetens* NCIM 1330 in comparison with other test organisms.
control against all the Fusarium species tested. The highest zone of inhibition (40 ± 1.0 mm) was observed against Fusarium foetens NCIM 1330 (Table 1). For further identification and confirmation of antifungal fraction from the crude extract, thin layer chromatography (TLC) based bioautography is performed. At UV 254 nm, total six bands were observed on the TLC plate; out of these six, band number 5 showed antifungal activity against F. foetens NCIM 1330 (Supplementary Fig. 2).

Pilot scale (10L) production, purification and characterization

Based on its preliminary antifungal activity in the crude extract and further presence of active fraction were confirmed by TLC bioautography. Strain BKACT is further subjected for pilot scale production at a 10.0L lab scale fermenter. From the 10.0L fermentation batch, 9.6 g of crude extract was extracted, and the antifungal activity is reconfirmed against the test fungi. The detailed information...
on purification of antifungal compound is schematically presented in Supplementary Fig. 3. The purified antifungal compound was further subjected for structural elucidation by HPLC, NMR and GC–MS analysis.

The $^1$H NMR spectra of a purified compound showed three signals of aromatic protons at 7.31 (1H, d, $J = 2.29$ Hz), 7.09 (1H, dd, $J = 8.39, 2.29$ Hz) and 6.62 (1H, d, $J = 8.39$ Hz) which confirms the trisubstituted benzene and six methyls at $\delta = 1.43$ (9H, s, $3 \times $ CH$_3$) and 1.31 (9H, s, $3 \times $ CH$_3$) confirmed the two di-tert butyl groups present on the benzene ring. The $^{13}$C NMR spectra indicate the ten carbon signals in which six signals were in the aromatic region, which confirms the presence of the benzene ring. The 151.6 ppm of phenol substitution on the benzene ring and 29.6 and 31.6 ppm indicates the methyl signals of the tertiary butyl group. Using NMR and literature reports, we confirmed that the purified compound structure is 2, 4-di-tert butyl phenol (Supplementary Fig. 4).

The purified compound from BKACT strain was further identified as the 2, 4-di-tert butyl-phenol by analyzing at GC–MS. The mass spectra of the identified peaks using pure standard substances were compared with peaks of the NIST mass spectral data to confirm the chemical structures of the detected compound as 2, 4-di-tert butyl-phenol (16.5 min) ion [M]$^+$ at m/z 206; the fragment ion [M–CH$_3$]$^+$ at m/z 191 is characteristic for 2, 4-di-tert butyl-phenol (Fig. 3). The same compound has been confirmed in the crude extract of the BKACT (Supplementary Fig. 5).

Based on the structure of a purified compound by NMR and GC–MS, the same compound was analyzed with TLC and HPLC. The purified compound was again confirmed as the 2, 4-di-tert butyl-phenol compared to the synthetic compound by peak profiles at the same retention time of 7.8 min in HPLC and the same Rf on TLC plates (Supplementary Fig. 6a & b).

**MIC and MFC**

The minimum inhibitory and fungicidal concentrations of the 2, 4-DTBP were determined against *F. foetens* NCIM 1330. The 2, 4-DTBP has demonstrated inhibitory effects against tested fungi with 0.03 and 0.06 mg/mL concentrations for MIC and MFC, respectively. The results showed that 2, 4-DTBP has potential antifungal activity (Supplementary Fig. 7). Further, the toxicity of the compound was checked against producer *S. marcescens* BKACT. Even at the highest concentration of 1 mg/mL, 2, 4-DTBP does not show any toxic effect on BKACT (Supplementary Fig. 8.).

**In vitro antifungal volatile effect**

The volatile effect of 2, 4-DTBP showed a strong inhibitory effect on mycelial growth of *F. foetens* NCIM 1330 compared to the solvent control group. The diameter of mycelia at different incubation time decreased significantly ($p < 0.05$) with the increasing concentration of the compound (Fig. 4). At 0.53 mM concentration 2, 4-DTBP was identified to show strong antifungal effect. At the same concentration, 86.6 ± 2.0, 86.9 ± 0.89 and 82.8 ± 0.70% mycelial growth inhibition was reported on the 5, 7 and 9th day of incubation (Fig. 4).

The volatile effect of 2, 4-DTBP was qualitatively analyzed on spore germination. At the presence of solvent control and lower concentration 0.07 mM, spores germinated normally and formed visible white mycelia as the incubation time increased. Even at 0.26 mM concentration, the spore germination inhibition rate was initially significantly higher as compared to the control, but it decreases as the incubation time increases. At 0.53 mM concentration of 2, 4-DTBP spore germination was completely (100%) suppressed at all the incubation times (Fig. 5). The percent germination...
inhibition rate of the spores in the control and 0.07 mM treatment groups was 28.74 ± 0.85 and 27.5 ± 5.14 respectively after 3 h of incubation. As the time increases, the inhibition rate decreases to zero at 24 h. And at the 0.26 mM concentration of compound, the rate of spore germination inhibition was 63.01 ± 5.6, 46.05 ± 2.94, 15.35 ± 1.4 and 8.33 ± 2.05 at 3, 6, 12 and 24 h of incubation, respectively (Fig. 5).

In vivo antifungal volatile effect

The volatile antifungal effect of the 2, 4-DTBP was checked against *F. foetens* NCIM 1330 to protect wheat kernels. As the concentration of the compound increases, the visual growth of *Fusarium foetens* NCIM 1330 decreases. At 1.0 mM concentration, the growth was completely suppressed even after 7 days of incubation (Fig. 6). The percent seed contamination index (PSCI) was analyzed at a different compound concentration. The seed contamination index (SCI) in control and 0.07 mM concentration was 100% and 91.66 ± 2.35 at 0.26 mM concentration. At 0.53 mM concentration, the SCI was 46.66 ± 6.23, significantly less than the control. The PSCI was zero at 1.0 mM, which is confirmed as the effective concentration for protecting the wheat seed from *F. foetens* NCIM 1330 (Fig. 6). After treating 2, 4-DTBP at different concentrations, the colony-forming unit (CFU) of mold was also determined in wheat seeds. The CFU of mold was identified to decrease significantly (*p* < 0.05) at 0.26 and 0.53 and 1.0 mM concentration compared to control. At 1.0 mM concentration, not a single CFU was detected in the treated sample (Fig. 6).

Toxic effect of DTBP on the wheat seed germination

The toxic effects of 2, 4-DTBP on seed germination were tested on wheat kernels. The results revealed that 0.07 and 1.06 mM concentrations of 2, 4-DTBP had no significant adverse toxic effects on the seed germination compared with the solvent control (*p* < 0.05) (Fig. 6).

Cytoplasmic leakage assays

The cellular membrane and cell wall are the leading target sites for the antifungal compounds. The structural and
Fig. 5 Effect of the 2, 4-DTBP on spore germination of *F. foetens* NCIM 1330. a. Qualitative analysis of spore germination at different concentration of DTBP and without treatment as the control and b. Quantitative analysis by counting percent spore germination inhibition (PSGI) at the 0.07, 0.26 and 0.53 mM/L treatment of DTBP with compared to control. Data presented mean ± s. d. (n = 3)

Fig. 6 The fumigation effect of the 2, 4-DTBP at 0.07, 0.26, 0.53 and 1.06 mM/L concentrations against *F. foetens* NCIM 1330 on the wheat seeds. a. Qualitative analysis of *F. foetens* contamination on wheat seeds, b. quantitative analysis of the wheat seed contamination index (SCI), c. fungicidal effect of DTBP on the wheat seeds and d. germination percentage of wheat grains after DTBP fumigation. Data presented mean ± s. d. (n = 3)
physicochemical properties are essential to penetrate and act upon phospholipid bilayer and internal cellular organelles. The non-polar compounds, due to their nature, easily penetrate through the phospholipid bilayer. The 2, 4-DTBP is the non-polar volatile compound. We assume it may damage cell wall integrity first and disturb cytoplasmic balance by leaking internal components. Ergosterol is a major component of the fungal cell membrane. Generally, a decrease in the Ergosterol content that results in osmotic imbalance, disruption of the cell growth and proliferation.

In this study, Ergosterol was not detected in mycelia treated with 2, 4-DTBP at 0.26 and 0.53 mM concentration. However, at 0.007 mM treatment and control samples showed peak at 2.98 retention time, which was compared with standard Ergosterol (Fig. S9A). Further cytoplasmic leakage was observed after fumigation treatment of the 2, 4-DTBP. The extracellular conductivity, protein and nucleic acid leakage of the treated mycelia at 0.26 and 0.53 mM concentration were significantly increased compared to the control sample (Fig. 7A–C). The result indicates that the 2, 4-DTBP damages the cell membrane integrity.

Our study (Fig. 5) showed that at 0.53 mM treatment, the outer membrane of the spore was observed to be damaged and abnormal compared to control in the light microscope.

Discussion

Fusarium species is a devastating pathogen in agriculture which causes severe loss of economically important plants such as wheat, maize, banana, tomato, sugarcane. It is also known to produce mycotoxins such as fumonisins, zearalenone, deoxynivalenol, fusaric acid and trichothecenes. Different species such as Fusarium graminearum, Fusarium proliferatum, Fusarium tricinctum, Fusarium moniliforme, Fusarium verticillioides and F. foetens are known as the plant pathogens (Aoki et al. 2014). The F. foetens has been reported as a pathogen in ornamental crops, specifically Begonia plants (Schroers et al. 2004). It causes damping-off of rooibos seedlings and destructive vascular wilt disease that leads to the plant’s death (Lamprecht and Tewoldemedhin 2017). Recently, González-Jartín et al. (2019) reported production of mycotoxins such as beauvericin and fusaric acid from F. foetens in the maize plant.

Despite having negative impact on humans and the environment, many chemical fungicides are being used to control Fusarium attacks. However, due to its excessive and frequent use, the phytopathogenic fungi are able to acquire resistance to the existing fungicides. In the sense of searching safe, eco-friendly and sustainable alternative, biocontrol bacteria and their active components are considered the best choice (Köhl et al. 2019). In the past few decades, extensive study has been conducted on terrestrial bacteria and their active compounds against various plant pathogenic fungi. Still, the discovery of potential organisms and their novel metabolites is diminishing. Oceans are the most diverse, adverse and competitive ecosystem. To survive in such a unique environment, marine bacteria have developed adaptation mechanisms to produce unique biomolecules. Consequently, marine bacteria can produce bioactive compounds generally not found in terrestrial environments (Imhoff et al. 2011; Dionisi et al. 2012).

In certain studies, marine bacteria such as Paenibacillus sp. PNM200, Bacillus marinus B9987 and Pseudomonas aeruginosa were reported against various plant pathogenic fungi (Vinchira-Villarraga et al. 2021; Zhang et al. 2010; Manwar et al. 2004). The dominant genera Bacillus, Pseudomonas, Streptomyces and Serratia are well known for their biocontrol potential against several plant pathogenic fungi. In the current study, the marine isolate BKACT showed significant antifungal activity by reducing over 50% mycelial growth against all tested Fusarium spp. The highest 75.56 ± 0.80% of mycelial growth inhibition was observed.
against *F. foetens* NCIM 1330. To date, there are no reports highlighting use of biocontrol bacteria against *F. foetens*. To the best of our knowledge, this is the first study to feature the antagonistic activity of marine *S. marcescens* against *F. foetens*. The biocontrol potentials of *S. marcescens* has mainly focused on its indigenous chitinase producing ability (Ordentlich et al. 1988, Someya et al. 2001 and Dhar Purkayastha et al. 2018). A recent study by Hover et al. (2016) reported the chitinase mutant *S. marcescens* which retained fungal killing ability and suggested that antifungal compounds, along with chitinase enzyme, together help the antifungal activity. In the present study, isolation, purification and characterization of the antifungal compound from marine *S. marcescens* BKACT are carried out. The antifungal compound was characterized as 2, 4-DTBP by GC–MS and NMR spectroscopy. The results were validated using TLC and HPLC by comparing the retention time and Rf value of purified compound with the standard reference compound (Sigma). Additionally, GC–MS analysis of the crude extract from strain BKACT also ascertained the compound to be 2, 4-DTBP. Several previous studies (Dharni et al. 2014, Varsha et al. 2015 and Wang et al. 2021) have reported 2, 4-DTBP from *Pseudomonas monteilii*, *Lactococcus* sp. and *Bacillus subtilis* CF-3 for the control of plant pathogenic fungi. However, there are no reports detailing the purification and characterization aspects of 2, 4-DTBP from marine *Serratia marcescens*. To the best of our knowledge this is the first study to purify, characterize and experimentally validate in vitro and in vivo antifungal activity of 2, 4-DTBP from the marine *S. marcescens*.

Regardless of antifungal and antioxidant activity, the 2, 4-DTBP has great volatile property. The microbial, volatile organic compounds (VOCs) have a significant role in disease management, especially for controlling the plant pathogenic fungi. VOCs are generally effective at minimal concentration, and they are capable of spreading in the atmosphere over the large distances. VOCs exert their inhibitory activity without direct physical contact with target pathogens (Schmidt et al. 2015). In the recent study, 2, 4-DTBP at one mole per liter was reported as having the effective volatile concentration against *Colletotrichum gloeosporioides* (Wang et al. 2021). However, in our present study, we found that 2, 4-DTBP from *S. marcescens* showed a great volatile antifungal activity against *F. foetens* NCIM 1330 at minimal concentration of 0.53 mM as compared to reported values. Mycelial growth inhibition was significantly higher when compared to control, even at lower concentrations. The highest 86.6 ± 2.0% mycelial growth was inhibited at 0.53 mM concentration. At the same concentration, 100% spore inhibition was also observed. A significant difference in the inhibitory concentration could be attributed to the 2, 4-DTBP produced by different microorganisms and the solvent variation of DMSO (Wang et al. 2021) and methanol (used in the present study) for dissolution of 2, 4-DTBP.

Varsha et al. (2015) in their study coated a 25 mg/ml concentration of 2, 4-DTBP on wheat seed which identified to protect from Aspergillus niger, *Fusarium chlamydosporum* and *F. moniliforme* infections. Considering its antifungal activity and volatile property, we believe that this molecule could control *F. foetens* on the wheat seeds. To date, there is only one report highlighting *F. foetens* to produce mycotoxin such as beauvericin and fusaric acid in the cereal like maize (González-Jartín et al. 2019). Here, for the first time we observed in the absence of compound *F. foetens* infect wheat seed and grew easily at above 90% relative humidity. At 0.53 mM concentration of 2, 4-DTBP, percent seed contamination index (PSCI) was significantly lowered when compared to control. Hundred percent controls of *F. foetens* NCIM 1330 were observed at 1.0 mM concentration, and it was identified as the effective treatment. Interestingly, the compound has not shown any adverse effect on the germination of wheat seeds at 1.0 mM concentration.

**Conclusion**

In the present study, we have found potential antagonistic marine *S. marcescens* BKACT, which produces an antifungal compound against *Fusarium* spp. This is the first study that emphasizes on detailed purification and characterization of the 2, 4-DTBP from a marine *S. marcescens* strain BKACT. Additionally, the potential volatile antifungal effect on the growth of mycelia and spore germination of *F. foetens* NCIM 1330 was observed at 0.53 mM concentration. For the first time, *F. foetens* NCIM 1330 was identified to infect wheat seeds, and the 1.0 mM concentration of 2, 4-DTBP determined an effective concentration for controlling *F. foetens* NCIM 1330. At the same concentration, no toxic effect was observed on seed germination. The current study is important as it provides important observations that may be instrumental in the agricultural research. We conclude that the marine bacteria *S. marcescens* strain BKACT and its purified compound have promising antifungal potentials for the control of *Fusarium* spp. This study also suggested that the antifungal activity of 2, 4-DTBP acts through the disturbance of membrane integrity. However, further evaluation of 2, 4-DTBP is required for various formulations utilizing its bioactive potentials and volatile characteristic in plant disease control.

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Author contribution BK: conceptualization, data curation, methodology, formal analysis, writing—original draft. MSS: methodology, molecule purification, structural elucidation, data curation. SGD: conceptualization, resources, supervision, project administration, writing—manuscript and editing.

Declarations

Competing interests The authors declare no competing interests.

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