Marine bacteria and its bioactive compounds for the agriculture applications

By

Bharat A. Kharat

AcSIR Registration No. 10BB17J26053

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Under the supervision of

Dr. Syed G. Dastager

Academy of Scientific and Innovative Research

AcSIR Headquarters, CSIR-HRDC Campus

Sector 19, Kamla Nehru Nagar,

Ghaziabad, U.P. -201 002, India

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Mr. Bharat A. Kharat

(Student)

Date: 03/03/2023

Dr. Syed G. Dastager

(Supervisor)

Date: 03/03/2023

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Name: Dr. Syed G. Dastager

Date: 03/03/2023.

Place: CSIR-NCL, Pune

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This Thesis is dedicated to

My dear parents

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Abbreviations

BCAs	Biocontrol agents
FHB	Fusarium Head Blight
Bt	*Bacillus thuringiensis*
GM	Genetically Modified
PIPs	Plant-incorporated protectants
VOCs	Volatile Organic Compounds
SAR	Systemic Acquired Resistance
ISR	Induced Systemic Resistance
PR	Pathogen Related proteins
NaCl	Sodium Chloride
PGP	Plant growth-promoting
IAA	Indole Acetic Acid
WGS	Whole Genome Sequencing
PDA	Potato Dextrose Agar
NA	Nutrient Agar
CMC	Carboxymethyl Cellulose
NaOH	Sodium Hydroxide
HCl	Hydrogen Cloride
MIC	Minimum Inhibitory Concentration

CFU	Colony Forming Units
PEG	Protein-encoding genes
RAST	Rapid Annotation and Subsystem Technology
ROS	Reactive oxygen species
2, 4-DTBP	2, 4-di-tert butyl-phenol
SA	Salicylic acid
TLC	Thin Layer Chromatography
DCM	Dichloromethane
HPLC	High Performance Liquid Chromatography
NMR	Nuclear Magnetic Resonance
$CDCl_3$	Deuterated chloroform
GC-MS	Gas Chromatography Mass Spectrometry
MFC	Minimum Fungicidal Concentration
DMSO	Dimethyl Sulfoxide
PSCI	Percent Seed Contamination Index
KOH	Potassium Hydroxide
PDB	Protein Data Bank
GPF	Grid Parameter File
GLG	Grid Log File
DPF	Docking parameter file

mM	Millimolar
PSGI	Percent Spore Germination Inhibition
SEM	Scanning Electron Microscope
µL	Microlitre
mg	Milligram
h	Hour

Chapter. 1:

Biocontrol bacteria and their bioactive compounds for the management of plant fungal diseases

General introduction

Global climate change has emerged as a major issue for the 21st century. Therefore, the agroecosystem continuously encounters the multiple abiotic and biotic stress combinations, which affects the crops growth, productivity, and quality. Biotic and abiotic stressors contribute nearly 30 - 50% loss in agricultural productivity worldwide (Kumar et al., 2018). Drought, salinity, and heavy metals are examples of abiotic stressors that have a deleterious impact on crop growth and yields. The abiotic stresses also influence the occurrence and spread of pathogens, insects, and weeds. Biotic stressors such as fungi, viruses, bacteria, nematodes, insects, and weeds cause various agricultural diseases which reduces crops yield (Chojak-Koźniewska et al., 2018).

In order to ensure global food security for the increasing human population in the 21st century, plant diseases are considered to be the most concerning barrier. They have catastrophic effects worldwide on the economic, social, and ecological levels. Countless lives were lost as well as migrated as a consequence of the most notorious plant diseases, such as the potato late blight and chestnut blight. (He et al., 2016). The "disease triangle" is a popular concept in plant pathology that mainly emphasizes on the interface of environment with pathogens and crops. Certain environmental factors promote diseases by providing optimum conditions to pathogens for their growth and development (Velasquez et al., 2018). Plant pathogens may exist for decades, and new ones are constantly emerging across the world. (Ramankutty et al., 2018). Fungi are the most common cause of plant diseases, responsible for the majority of agricultural loss (Fig. 1). Fungi use different strategies for colonization on the host plants. Necrotrophic fungi kills their hosts and feed on dead material, while bio-trophic gets colonized on living tissue. After the successful invasion of plant parts, fungi get colonized and produces specialized infectious structures to seek nutrients forcefully.

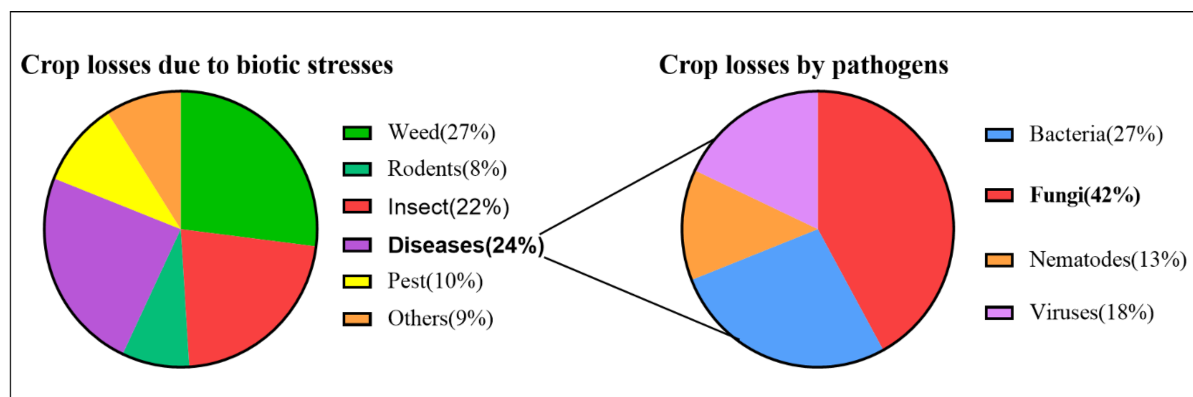


Figure. 1 Agriculture loss due to biotic stresses

Further, the disease is established, and many virulence factors are disposed of in the host. These factors perform various roles, like interference with primary plant defence and manipulating plant metabolism in favour of pathogens (Doehlemann et al., 2017). The agricultural productivity is seriously affected by epidemics like cereal rusts, smuts ergot, brown spot of rice, downy and powdery mildew of the grape, Sigatoka disease of the banana, and rubber leaf blight (Fig. 2) (Shuping et al., 2017). The top 10 fungal pathogens are ranked by plant pathologists as per their global destructiveness and propensity to infect crop plants (Table. 1) (Dean et al., 2012). Even single species of fungi can cause diseases in various host plants. For instance, *F. oxysporum* infects 100 distinct crops while *B. cinerea* infects 200 host plants. Besides these, *M. oryzae* is also a devastating fungus that produces huge grain loss. Occasionally, more than two pathogens can attack at once. For example, *Fusarium graminearum* and other *Fusarium* species often infect crops. In this case, the plant's immune response completely shuts down. Even after crop rotation, fungi like *Puccinia* sp. trigger frequent crop loss, which makes it difficult. (Shuping et al., 2017).

Table.1 Top ten fungal pathogens in agriculture

Sr. No	Fungi	Diseases
1	*Magnaporthe oryzae*	Rice blast disease
2	*Botrytis cinerea*	Botrytis bunch rot
3	*Puccinia* sp.	Stem black rust, Stripe yellow rust and leaf brown rust
4	*Fusarium graminearum*	Fusarium head blight (FHB)
5	*Fusarium oxysporum*	Vascular wilt disease
6	*Blumeria graminis*	Powdery mildews disease
7	*Mycosphaerella graminicola*	Septoria tritici blotch (STB) disease
8	*Colletotrichum* sp.	Anthracnose spots and post-harvest rots
9	*Ustilago maydis*	Corn smut disease
10	*Melampsora lini*	Flax rust disease

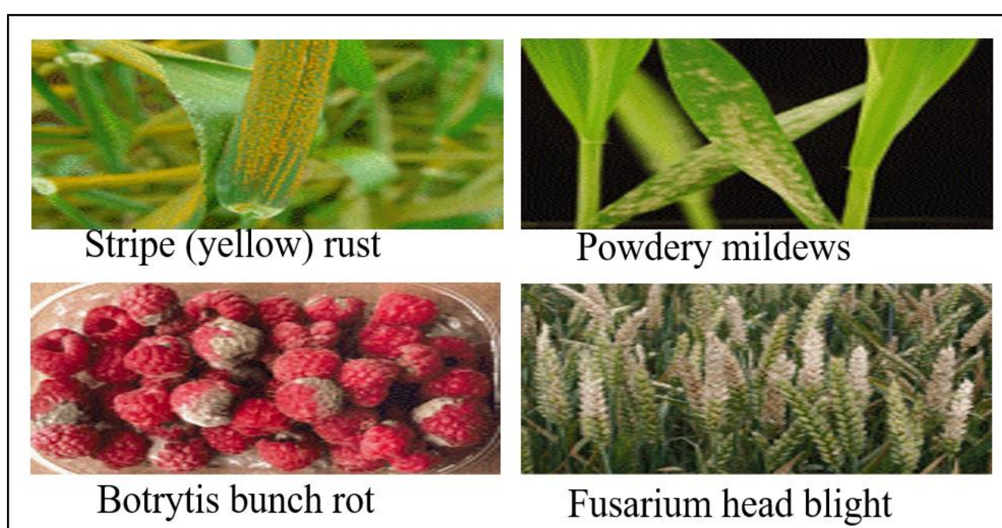


Figure. 2 Devastating fungal diseases in agriculture

Phytopathogenic fungi also causes post-harvesting infections and food deterioration during various processing steps such as harvesting, transportation, packing and storage (Agrios et al., 2009; Singh et al., 2018). The majority of postharvest infections are caused by hundreds of different fungus species, which can decrease up to 10 to 30% food production (Chakraborty et al., 2011; Tomar et al., 2015). For example, *B. cinerea* is one of the most destructive pathogen that may spoil valuable fruits including grapes, raspberries, kiwi fruit, strawberries, pears, cherries, and plums. *Penicillium* sp. also causes diseases including green rot and blue rot in apples and citrus, respectively (Moss et al., 2008). Moreover, *Aspergillus*, *Fusarium*, and *Geotrichum* are the most common pathogens of perishable fruits and vegetables, which causes huge economic loss. (Agrios et al., 2009).

Mycotoxins are hazardous, small molecules that are naturally produced by a certain type of fungus. They can infect and grow on several foodstuffs like fruits, cereals, spices, dried fruits and nuts before, after, or during storage under humid, damp, and warm conditions. The majority of mycotoxins are chemically resilient and remain active even after food processing. A number of mycotoxins have been discovered, even though only a small number of them, including ochratoxins, fumonisins, zearalenone, aflatoxins, and trichothecenes, pose health hazards to both humans and animals (Pitt et al., 2020). Mycotoxins can enter directly or indirectly through eating contaminated animal food like milk and meat. Even at small doses, they are carcinogenic, teratogenic, hepatotoxic and hemorrhagic to vertebrates (Eskola et al., 2020). Mycotoxin-related disorders are usually caused by *Aspergillus*, *Penicillium*, and *Fusarium* species. Annually, mycotoxins contribute to the destruction of almost 1 billion tonnes of agricultural products. This has a negative impact on the economy as well as food security (Bennet et al., 2019).

In order to combat plant fungal diseases, several synthetic compounds have brought much-needed respite to the farmers. Synthetic fungicides have played an important role in preventing

fungal attack and improving agriculture production in the past couple of decades. However, farmers are still facing problems because of the resistance that has emerged against fungicides which are currently available in the market. Fungicides are also hazardous to non-target species including microbes, earthworms, and mammals which cause imbalance in the ecosystem. Moreover, fungicides disintegrate gradually and are hard to eliminate, resulting in water pollution (Panth et al., 2020). As a reason, environmentalists, farmers, and consumers are becoming more inclined towards natural products. In the search of environment friendly and sustainable natural products, biocontrol microorganisms and their active compounds are recognized as the safest alternative to synthetic fungicides.

1. Biological control agents (BCAs)

Biological control agents (BCAs) are living organisms or their products used for controlling plant diseases. Plant diseases are occurred because of disproportion in the elements (environment, pathogens and host) of disease triangle. Biological control agents interact with the elements of the disease triangle to manage the disease (Velásquez et al., 2018). The ultimate aim of biocontrol is to control plant pathogens and minimize overuse of chemical pesticides. Natural BCAs must be utilized differently based on the life cycle of the target pest or pathogens, environmental conditions, and host plants. Biological control is the practical approach for controlling plant diseases because it has tremendous benefits, such as being safe and easy to use, eco-friendly, and cost-effective compared to chemical pesticides. Besides this, BCAs are self-established and sustained for an extended period of use.

1.1 History

In the 17th century, plant extracts of nicotine were used for the bio-control application against plum beetles. In 1835 white-muscadine fungus (*Beauveria bassiana*) was discovered against silkworms by Agostine Bassi. With the expansion of agricultural research during the early 20th

century, the number of studies on BCAs increased. In 1901, Japanese biologist Shigetane Ishiwata isolated *Bacillus thuringiensis,* the first most accepted BCA from the diseased silkworm. Then, in the early 1920s, the first commercial biocontrol product, sporeine from *B. thuringiensis*, was developed in France.

1.2 Classification of Biocontrol agents

Depending on the source of the organism, the active constituent, and the mechanism of action, the US Environmental Protection Agency (US-EPA) divides bio-control agents within three main groups.

1.2.1 Plant-incorporated protectants (PIPs)

Plant-incorporated protectants (PIPs) are pesticidal or antimicrobial substances produced in crop plants from the genetic elements inserted in the genome of plants. These plants are called transgenic or genetically modified (GM) crops, as their genome is integrated with biocontrol genes from natural organisms. Several PIPs are developed, but BT crops are the most famous example of GM crops in which a *cry* gene is inserted to control insect pests. Cry proteins are the first-generation PIPs, where GM crops consist of transgenes of soil bacterium *B. thuringiensis* (Basnet et al., 2022).

1.2.2 Biochemical agents

Biochemical agents are non-toxic natural bioactive compounds extracted from plants to control insect pests and other pathogens such as nematodes, fungi, bacteria, and viruses. These compounds or extracts can act as a repellents, attractants, antifeedants and growth inhibitors. Secondary metabolites such as alkaloids, terpenoids, and flavonoids have been discovered as a biochemical agents from several wild and herbal plants. Interestingly, some plant-based biochemical agents are mainly extracted from tobacco, pyrethrum, ryania, and neem plants. For example, pyrethrum extracts containing pyrethrin as the active compound are usually used

to control house flies. Biochemical agents are valuable tools for plant disease management as they have low toxicity and a low risk of disease resistance (Ngegba et al., 2022).

1.2.3 Microbial agents

Microbial agents consist of bacteria, fungi, viruses, and nematodes are used as the active ingredients. These microorganisms can suppress different kinds of pests and pathogens through various modes of action, such as antagonism, competition, or the production of pesticidal and antimicrobial compounds. Generally, live microorganisms are used to control target pathogens (Kohl et al., 2019). Some microbial agents such as *Trichoderma*, *Pseudomonas*, and *Bacillus* act as biofungicides, bioherbicides, and bioinsecticides. Microbial biocontrol agents are considered more specific, environment-friendly, sustainable, and safe for humans. Moreover, they break down quickly in the environment and do not leave toxic residue. Additionally, microbial biocontrol agents can increase biodiversity and improve soil health (Thomashow et al., 2019; Viterbo et al., 2002).

Nematodes

Nematodes are also considered biocontrol agents. They are tiny, worm-like organisms which can control many pests, including insects, mites, and certain types of nematodes themselves. Some nematodes have a mutualistic relationship with plants, protecting against pathogens in exchange for nutrients, while others are parasitic and can infect and kill specific pests. Entomopathogenic nematodes are the most commonly used nematodes for biocontrol. These nematodes specifically target insects and can be applied to crops as a spray or incorporated into seed treatments (Askary et al., 2010). Some examples of commercial biocontrol nematode products include *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*. These nematodes are effective against many insect pests, including cutworms, armyworms, and white grubs. *Heterorhabditis* and *Steinernema* are mutually associated with bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively. These symbiotic bacteria are also known to

produce antimycotic substances against a broad range of plant pathogenic fungi (Chen et al., 1994).

Viruses

Viruses consist of nucleic acid, which is generally encased in a protein or lipoprotein coat that can be replicated within a specific host. Among BCAs, a virus is an effective biocontrol agent which controls specific target pests and pathogens. Biocontrol viruses are mainly used against insect pests called entomogenous viruses. These biocontrol viruses generally found in nature belong to the baculovirus family, *Baculoviridae*. In addition, mycoviruses are also used as biocontrol agents against phytopathogenic fungi. These viruses can infect and kill specific fungi and are effective against pathogens such as *Botrytis cinerea* and *Sclerotinia sclerotiorum*. In addition, mitoviruses were also found to control two fungal species, such as *S. homoeocarpa* and *Ophiostoma novo-ulmi*. Furthermore, *the C. parasitica* hypovirus 1 (CHV1) virus has been reported to suppress the growth of several fungal species (Wagemans et al., 2022). At the same time, viruses as a biocontrol agents are still in the initial stages of research and development and not yet widely used in commercial agriculture.

Fungi

Fungi are known to infect and parasitize pests and pathogens. These fungi are used as biocontrol agents for plant disease management. Entomogenous fungi such as *Beauveria bassiana*, *Cordyceps fumosorosea*, *Akanthomyces muscarius*, *Purpureocillium lilacinum*, and *Metarhizium anisopliae* are known to control insect pests. In addition, fungi, including *P. lilacinum*, *Ampelomyces quisqualis*, *Clonostachys rosea*, *Trichoderma virens*, and *Trichoderma harzianum*, are also used as biocontrol agents against nematodes and fungal plant pathogens (Khan et al., 2012).

Bacteria

Biocontrol bacteria are microorganisms which can control plant pathogens and diseases. For the management of plant diseases, diverse bacterial species have been adopted as biocontrol agents, including *Arthrobacter*, *Bacillus*, *Agrobacterium*, *Pseudomonas*, *Alcaligenes*, *Serratia*, *Erwinia*, *Rhizobium*, *Enterobacter*, *Stenotrophomonas*, *Xanthomonas*, and *Streptomyces*. Some examples of biocontrol bacteria, such as *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Rhizobium leguminosarum*, inhibits the growth of fungal pathogens and induce systemic resistance in plants. These bacteria can be applied to crops as a spray, incorporated into seed treatments, or applied as a soil drench to protect plants from fungal infections. Biocontrol bacteria are the practical and sustainable alternative to chemical fungicides. There are several commercial biocontrol bacterial products, including Serenade (*Bacillus subtilis*), Actinovate® (*Streptomyces lydicus*), Howler™ (*Pseudomonas chlororaphis* strain AFS009), and EcoGuard® (*B. licheniformis* strain SB3086) are used safely and efficiently across the world to minimize the challenges posed by pathogens (Agrios et al., 2009).

In the global scenario, biocontrol agent use has been growing at a steady rate of 10% annually. Bio-control agents are available in different formulations, such as granules, dust, powders and liquid form. Among different types of bio-control agents, bacterial bio-control agents contribute the highest (60%), followed by fungal (27%) and viral (10%) (Kabaluk et al., 2010). The potential use of bacteria in treating plant fungal diseases are based on the antagonistic nature towards the fungal pathogens (Köhl et al., 2011). These bacteria can produce compounds that inhibit the growth of pathogens or outcompete them for nutrients. They may act directly or indirectly against pathogens to protect crop plants through one or a combination of mechanisms. They can also stimulate the plant's immune system, making it more resistant to infection.

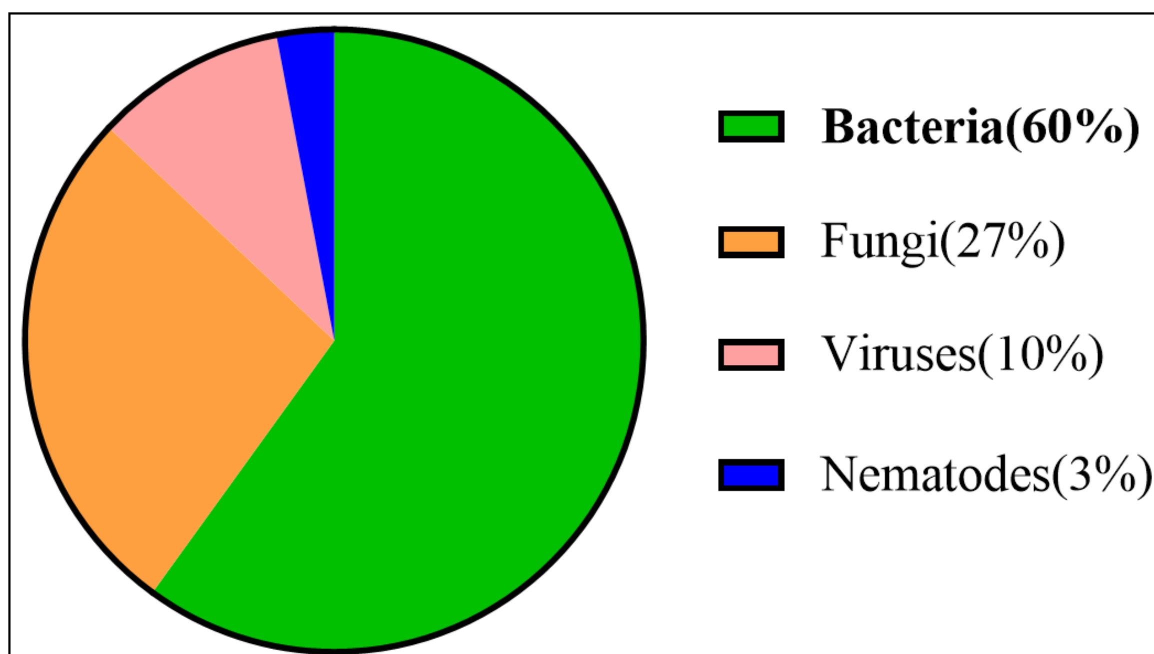


Figure. 3 Worldwide use of microbial biocontrol agents

2. Mechanism of biocontrol bacteria and their bioactive compounds

Table. 2 Understanding mechanism useful for the developing effective BCAs

Sr.no	Objectives of knowing mechanism	Importance of understanding mechanism
1	Selection of right bacteria	It is simple to choose competent microorganisms for a specific pathogen if we know the mechanism
2	Efficacy improvement	For best performance, other parameters can be optimised based on the mechanism
3	Avoiding resistance	Pathogen resistance can be avoided by adopting effective strategies
4	Combination strategies	Multiple biocontrol approaches can be combined for more comprehensive control
5	Regulations	Before commercialization, it is useful for assessing safety and efficacy

Understanding the mechanism of action is crucial in several aspects, as shown in (Table. 2), for the advancement of effective, sustainable, and safe biocontrol agents. The following section highlights several mechanisms used by biocontrol agents to manage plant diseases.

2.1 Hyperparasitism and predation

In parasitism, one organism gains nutrients from the other through direct competitive interaction. Hyper parasitism is another term utilized when the host itself is a parasite, as in the case of a plant pathogenic fungus serving as a host for biocontrol bacteria. The majority of the time, fungus display this sort of relationship. However, some rare examples, like *Bdellovibrio bacteriovorus*, act as predatory bacterium, using another microorganism's cytoplasm as a nutrient. The *B. bacteriovorus* in liquid co-cultures act as a BCA against phytopathogenic fungi (McNeely et al., 2017). The new BCA that prevents Fusarium wilt in cucumber plants is the *M. Corallococcus* sp. strain EGB. (Ye Luo et al., 2020)

2.2 Antibiosis

Secondary metabolites are organic small molecules produced by diverse bacteria which shows detrimental effect on the growth of pathogens. They are produced in small quantities and released into the environment. These antimicrobial metabolites are most potent against competitors, providing a competitive advantage to producing bacteria in resource-limited environments (Mullis et al., 2019). Several biocontrol bacteria are well-studied for antibiotics production and their role in plant fungal disease management. Some of them produce multiple compounds simultaneously; therefore, they can control a broad range of pathogens (Pal et al., 2006). Biocontrol bacteria from the *Serratia*, *Pseudomonas*, *Bacillus*, *Streptomyces*, *Pantoea*, *Stenotrophomonas*, *Agrobacterium* and other genera are known to produce broad range of antimicrobial metabolites. Surfactin, fengycin, and iturin are the most investigated lipopeptides in *Bacillus*. Whereas antibiotics such as phenazine, pyrrolnitrin, and 2, 4-diacetyl

phloroglucinol were explored in *Pseudomonas* against many phytopathogenic fungi. (Thomashow et al., 2019). Notably, *P. piscium* ZJU60 released phenazine-1-carboxamide, which inhibits *F. graminearum* growth, pathogenicity, and mycotoxin production in order to manage the head blight disease in wheat (Chen et al., 2018). The type strain of *B. amyloliquefaciens* subsp. plantarum FZB42 responds to *R. solani* by making different lipopeptieds in the rhizosphere of lettuce (Chowdhary et al., 2015). Conprimycin is the heat-stable ribosomally synthesized thiopeptied, produced in well-known BCA *Streptomyces griseus* S4-7, which has been controlling the devastating wilt disease on strawberries by inhibiting the growth of *F. oxysporum* f.sp.fragariae (Cho et al., 2017). Moreover, antibiotics like 2, 4-diacetyl phloroglucinol, phenazine carboxylic acid, and iturin have a role in the elimination of *Pythium* species, *B. cinerea*, and *Gaeumannomyces graminis* var. tritici infections (Gurusiddaiah et al., 1986; Nifakos et al., 2021; Notz et al., 2001).

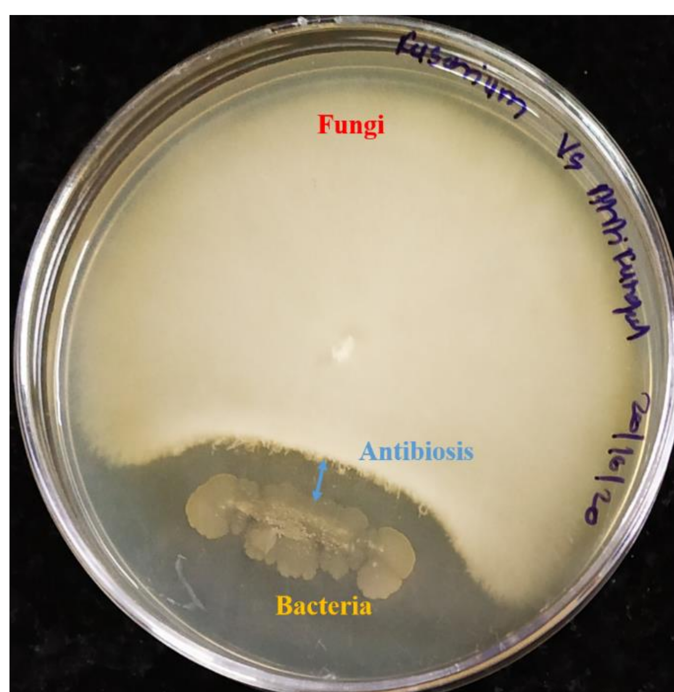


Figure. 4 Antibiosis mechanism between biocontrol bacteria and fungi

Table. 3 Selected examples of antibiotics produced by biocontrol bacteria against plant pathogenic fungi

Antibiotic	Source	Target pathogen	Reference
2,4-Diacetyl phloroglucinol	*Pseudomonas fluorescens* F113 and *Pseudomonas* sp.	*Pythium* sp., *Xanthomonas oryzae pv. Oryzae*	Almario et al., 2017
Phenazine and Phenazine-1-carboxamide	*P. fluorescens*, *P. chlororaphis*, and *P. aureofaciens*, *P. piscium* ZJU60	*Gaeumannomyces graminis var. tritici*, *F. oxysporum* and *Sclerotinia homeocarpa, Fusarium graminearum*	Chen et al., 2018; Jun Myoung et al., 2018
Pyoluteorin and pyrrolnitrin	*P. fluorescens* Pf-5 and *Burkholderia cepacia*	*P. ultimum, R. solani* and *Pyricularia oryzae*	Byung et al., 2002; Qing et al., 2017
Bacillomycin, fengycin	*B. amyloliquefaciens* FZB42	*Fusarium oxysporum*	Yu et al., 2018
Iturin A and surfactin	*B. subtilis*	*Rhizoctonia. solani*	Thomashow et al., 2019
Zwittermicin A	*B. cereus* UW85	*P. medicaginis* and *P. aphanidermatum*	Silo-Suh et al., 1994
Oligomycin A	*Streptomyces* sp. FX13	*B. cinerea*	Lin et al., 2021
Herbicolin	*Pantoea agglomerans* C9-1	*Erwinia amylovora*	Hwang et al., 2002
Xanthobaccin A	*Lysobacter* sp. strain SB-K88	*Aphanomyces cochlioides*	Islam et al., 2005

2.3 Volatile compounds

The volatile compounds produced in plants and microbes are in the spotlight. Further, they are classified into organic and inorganic molecules. Inorganic volatile compounds like CO_2, H_2, NH_3, H_2S, SO_2, SO_3 and HCN take a part in a variety of biological processes, from acting as interspecies communicators to electron acceptors and donors (Effmert et al., 2012). Volatile organic compounds (VOCs) are generally small carbon containing molecules, which have a low molecular weight, less water solubility and high vapour pressure that allows them to be exist in a gaseous state in normal ambient conditions (Schmidt et al., 2019). VOCs are

classified into various groups on the basis of their chemistry, which include hydrocarbons, thio-alcohols, aldehydes, alcohols, ketones, heterocyclic compounds, cyclo-hexanes, thioesters, benzene derivatives, and phenols (Tilocca et al., 2020). VOCs are generally effective at small concentrations as they can diffuse across long distances in the environment and exert antagonistic effect against target pathogens. In addition to control of plant pathogens, VOCs have also been involved in various biological processes. VOCs, for example, have the capacity to destroy plant-parasitic nematodes, restrict pathogens from colonization, boost plant growth, and generate defence mechanisms in plants (Bitas et al., 2013). Consequently, these compounds disperse quickly in the atmosphere and thus play essential biological/ecological roles in above and below-ground habitats. The *B. amyloliquefaciens* strain SQR-9 has been proven to release a blend of VOCs those are potent against *R. solanacearum* (Raza et al., 2016).

Table. 4 Selected examples of volatile compounds produced by biocontrol bacteria against plant pathogenic fungi

Volatile compounds	Producing bacteria	Target pathogen	References
Acetoin, 2,3-butanediol, and 2-methyl-1-butanol	*Paenibacillus* sp. strain (UY79)	*Botrytis cinerea, F. verticillioides, F. oxysporum, F. graminearum Phytophthora sojae, Rhizoctonia solani* and *Sclerotium rolfsii*	Andres et al., 2021
Hexadecanoic acid methyl ester and tetracosane	*Bacillus megaterium* BM344-1	*Aspergillus flavus, Aspergillus carbonarius, Penicillium verrucosum,* and *Fusarium verticillioides*	Saleh et al., 2021
Dimethyl di-sulfide and Dimethyl tri-sulfide	*Pseudomonas fluorescens* ZX	*Penicillium digitatum*	Wang et al., 2021
Caryolan-1-ol	*Streptomyces spp.*	*Botrytis cinerea*	Cho et al., 2017

2-ethyl-5-methylpyrazine and Dimethyl di-sulfide	*Streptomyces setonii* WY228	*Ceratocystis fimbriata*	Gong et al., 2022
Benzaldehyde and isoamyl alcohol	*Bacillus velezensis*	*Botrytis cinerea*, *Monilinia fructicola*, *M. laxa* and *Penicillium italicum*	Calvo et al., 2020
Isooctanol	*Corallococcus* sp. EGB	*Fusarium oxysporum* f. sp. *cucumerinum* (FOC) and *P. digitatum*	Ye et al., 2020
3-methyl-1-butanol	*Bacillus licheniformis* BL350-2	*Aspergillus* and *Penicillium*	Ul Hassan et al., 2019
2-phenethyl alcohol	*Aureobasidium pullulans*	*B. cinerea*, *Colletotrichum acutatum*, *P. expansum*, *P. digitatum* and *P. italicum*	Di Francesco et al., 2015
2,4-di-*tert*-butylphenol	*Pseudomonas monteilii*, PsF84	*Fusarium oxysporum*	Dharni et al., 2014

2.4 Production of lytic Enzymes

Fungal cell walls are typically composed of D-glucose polymers known as glucans and nitrogen-containing N-acetyl-D-glucosamine known as chitin. These glucans and chitins are major structural componant of the fungal cell wall which provide mechanical strength and rigidity to cell wall. Though, lytic enzymes attributed to degrade fungal cell wall that leads to cell lysis. Chitinases, glucanases, lipases, proteases, and other hydrolytic enzymes are examples of lytic enzymes (Viterbo et al., 2002). Biocontrol bacteria produce and release lytic enzymes which can digest a broad variety of natural polymers such as cellulose, hemicellulose, chitin, proteins, and DNA. In order to control plant pathogenic fungus, these extracellular enzymes are crucial. Biocontrol bacteria *Paenibacillus* sp., *Streptomyces* sp. and *S. marcescens* have been reported to produce chitinase against *B. cinerea, S. rolfsii*, and *F. oxysporum f.* sp. *Cucumerinum* (Veliz et al., 2017). Another hydrolytic enzyme β-1, 3-glucanase identified in

BCAs like *Arthrobacter, Streptomyces, Bacillus*, *Paenibacillus,* and *Burkholderia cepacia* against various plant pathogenic fungi (Wu et al., 2018).

Table. 5 Examples of lytic enzymes produced by biocontrol bacteria

Enzyme	Producing bacteria	Target phytopathogen	Reference
Chitinases	*Streptomyces luridiscabiei* U05	*Alternaria alternata*, *Fusarium oxysporum*, *F. solani* and *Botrytis cinerea*	Swiontek et al., 2019
	Bacillus pumilus RST25	*F. soloni* and *A. niger*	Gurav et al., 2017
	Chromobacterium sp. JH7	*Cylindrocarpon destructans*	Han et al., 2017
	Paenibacillus strain UKCH21	*R. solani*, *F. soloni* and *Sclerotium rolfsi*	Subbanna et al., 2016
	Pseudomonas fluorescens	*Fusarium oxysporum* f. sp. cumini	Rathore et al., 2020
	Serratia marcescens	*Alternaria alternata*	Narendrakumar et al., 2018
Glucanase	*Bacillus velezensis* strain HYEB5-6	*Colletotrichum gloeosporioides*	Huang et al., 2017
	Paenibacillus terrae strain NK3-4	*Magnaporthe oryzae* and *Rhizoctonia solani*	Yu et al., 2019
Cellulase and Protease	*P. aeruginosa* and *Bacillus subtilis* YJ1	*F. oxysporum* and *Rhizoctonia* sp.	Jadhav et al., 2017

2.5 Competition

Competition for nutrients and space is another mechanism to protect plants from pathogens. Biocontrol bacteria compete for nutrients in the phyllosphere and rhizosphere of plants. Therefore, biocontrol bacteria occupy the same niches and utilize existing nutrients from plants that restrict pathogens' initial establishment and growth (Köhl et al., 2019). Biocontrol bacteria can sequester iron from the environment by producing a siderophore as the iron-binding chelating agent. Biocontrol bacterial siderophores have a higher affinity for iron than fungal

pathogens. Therefore, the growth of fungal pathogens is suppressed due to the unavailability of iron (Patil et al., 2014).

2.6 Induced Systemic Resistance

Plants rapidly react to environmental cues such humidity, water, temperature, light, physical stress, and nutrition. Plants react simultaneously to chemical signals generated from bacteria in the environment. These inputs activate plant defences via chemical messengers that increase resistance to particular pathogen in future. According to the source, nature, and stimuli intensity, the activation of host response may be local or systemic (Pal et al., 2006). Defence response can be generated by using two different pathways. The first mechanism, known as systemic acquired resistance (SAR), is activated by salicylic acid (SA), a molecule often released in response to pathogen attack and stimulates the production of pathogenesis-related (PR) proteins. These proteins are enzymes that control pathogen growth by directly lyse invading pathogens or resisting pathogen entry by providing mechanical strength to plants. Jasmonic acid initiates a second mechanism known to induce systemic resistance (ISR) (JA).

Different microbes and pathogen attacks activate these resistance pathways to varying degrees in response to multiple stimuli. As a consequence, the intensity and length of host defence probably change over time (Pieterse et al., 2014). Biocontrol bacteria such as *Pseudomonas* sp., *Bacillus* sp. and *Serratia* sp; generate ISR in host plants (Kannojia et al. 2019). Also, they successfully manage several diseases including angular leaf spot, anthracnose, and Fusarium wilt. The beneficial bacteria may produce several elicitors such as siderophore, 2, 3-butanediol, salicylic acid, lipopolysaccharides, and other volatile molecules that elicit SAR and ISR mechanism against pathogens. In general, several microbial bioactive compounds have already been discovered to trigger host defences, showing that it probably activate plant defence throughout lifespan (Postel et al., 2009; Bakker et al., 2013).

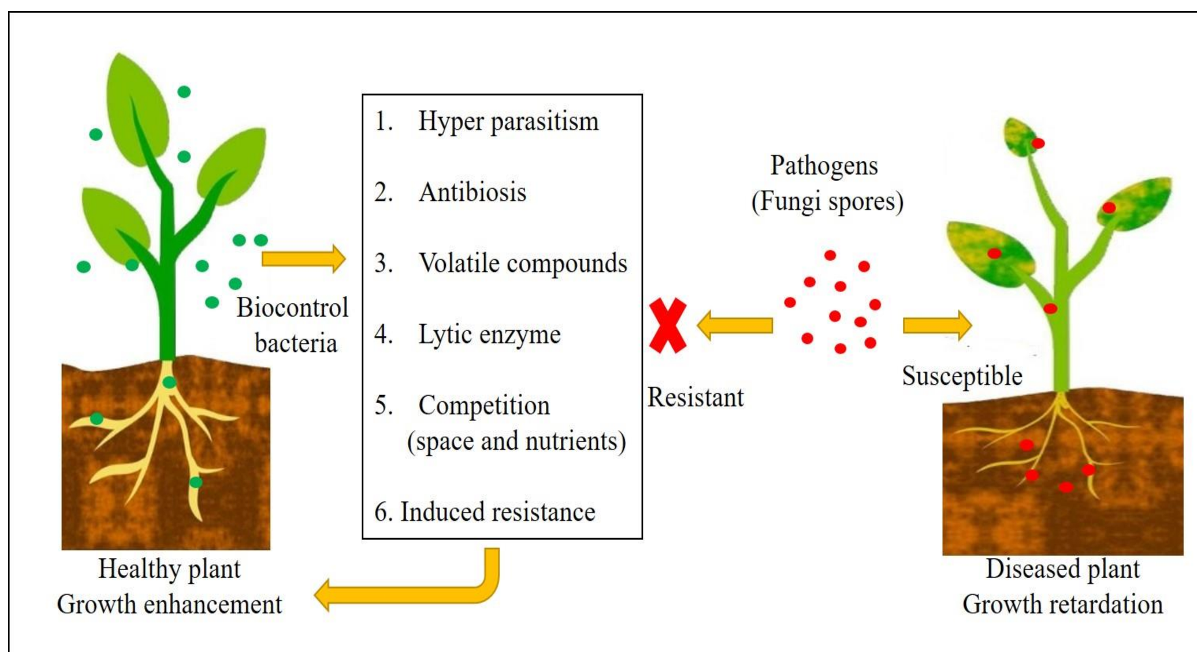


Figure. 5 Mechanism of action involved in biocontrol bacteria against plant pathogenic fungi

3. Conclusion and future direction

The effectiveness of biocontrol bacteria in comparison to chemical fungicides is now lacking, however they continue to have a bright future when diverse biotechnology approaches and expertise are applied. A thorough knowledge of biology, disease epidemiology, agroecosystems, population dynamics, ecology, and the relationships between all these variables is necessary for successful biocontrol. Scientific challenges in the biocontrol of plant diseases can be addressed using more advanced analytical and molecular techniques to study the genomic, proteomic, and metabolomics of biocontrol agents and plant pathogenic microorganisms in situ.

References

Askary, T. H. (2010). Nematodes as biocontrol agents. *In Sociology, organic farming, climate change and soil science* (pp. 347-378).

Agrios, G. N. (2009). Plant pathogens and disease: general introduction, *Encyclopedia of Microbiology*, 613-646.

Agriculture as an Alternative to Synthetic Pesticides. *Agriculture*, *12*(5), 600.

Basnet, P., Dhital, R., & Rakshit, A. (2022). Biopesticides: a genetics, genomics, and molecular biology perspective. *Biopesticides*, 107-116.

Bennett, J. W., & Moore, G. G. (2019). Mycotoxins. *Encyclopedia of Microbiology*, 267-273.

Bitas, V., Kim, H. S., Bennett, J. W., & Kang, S. (2013). Sniffing on microbes: diverse roles of microbial volatile organic compounds in plant health. *Molecular Plant-Microbe Interactions*, *26*(8), 835-843.

Bakker, P. A., Doornbos, R. F., Zamioudis, C., Berendsen, R. L., & Pieterse, C. M. (2013). Induced systemic resistance and the rhizosphere microbiome. *The plant pathology journal*, *29*(2), 136.

Chen, G., Dunphy, G. B., & Webster, J. M. (1994). Antifungal activity of two Xenorhabdus species and Photorhabdus luminescens, bacteria associated with the nematodes Steinernema species and Heterorhabditis megidis. *Biological Control*, *4*(2), 157-162.

Chakraborty, S., & Newton, A. C. (2011). Climate change, plant diseases and food security: an overview. *Plant pathology*, *60*(1), 2-14.

Cho, H. J., Kwon, Y. S., Kim, D. R., Cho, G., Hong, S. W., Bae, D. W., & Kwak, Y. S. (2017). wblE2 transcription factor in Streptomyces griseus S4-7 plays an important role in plant protection. *Microbiology Open*, *6*(5), e00494.

Chojak-Koźniewska, J., Kuźniak, E., & Zimny, J. (2018). The effects of combined abiotic and pathogen stress in plants: insights from salinity and *Pseudomonas syringae pv lachrymans* interaction in cucumber. *Frontiers in plant science*, *9*, 1691.

Chen, Y., Wang, J., Yang, N., Wen, Z., Sun, X., Chai, Y., & Ma, Z. (2018). Wheat microbiome bacteria can reduce virulence of a plant pathogenic fungus by altering histone acetylation. *Nature Communications*, *9*(1), 1-14.

Chowdhury, S. P., Hartmann, A., Gao, X., & Borriss, R. (2015). Biocontrol mechanism by root-associated Bacillus amyloliquefaciens FZB42–a review. *Frontiers in microbiology*, *6*, 780.

Doehlemann, G., Ökmen, B., Zhu, W., & Sharon, A. (2017). Plant pathogenic fungi. *Microbiology spectrum*, *5*(1), 5-1.

Dean, R., Van Kan, J. A., Pretorius, Z. A., Hammond‐Kosack, K. E., Di Pietro, A., Spanu, P. D., & Foster, G. D. (2012). The Top 10 fungal pathogens in molecular plant pathology. *Molecular plant pathology*, *13*(4), 414-430.

Effmert, U., Kalderás, J., Warnke, R., & Piechulla, B. (2012). Volatile mediated interactions between bacteria and fungi in the soil. *Journal of chemical ecology*, *38*, 665-703.

Eskola, M., Kos, G., Elliott, C. T., Hajšlová, J., Mayar, S., & Krska, R. (2020). Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate'of 25%. *Critical reviews in food science and nutrition*. *60*(16), 2773-2789.

Gurusiddaiah, S., Weller, D. M., Sarkar, A., & Cook, R. J. (1986). Characterization of an antibiotic produced by a strain of Pseudomonas fluorescens inhibitory to Gaeumannomyces graminis var. tritici and Pythium spp. *Antimicrobial agents and chemotherapy*, *29*(3), 488-495.

HE, Dun-chun, Jia-sui ZHAN, and Lian-hui XIE (2016). Problems, challenges and future of plant disease management: from an ecological point of view. *Journal of Integrative Agriculture*, *15*(4), 705-715.

Khan, S., Guo, L., Maimaiti, Y., Mijit, M., & Qiu, D. (2012). Entomopathogenic fungi as microbial biocontrol agent. *Molecular Plant Breeding*, *3*(7).

Kumar, A., & Verma, J. P. (2018). Does plant—microbe interaction confer stress tolerance in plants: a review? *Microbiological research*, *207*, 41-52.

Kabaluk, J. T., Svircev, A. M., Goettel, M. S., & Woo, S. G. (Eds.). (2010).The use and regulation of microbial pesticides in representative jurisdictions worldwide. *International Organization for Biological Control of Noxious Animals and Plants* (p. 99).

Köhl, J., Kolnaar, R., & Ravensberg, W. J. (2019). Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. *Frontiers in plant science*, 845.

Kannojia, P., Choudhary, K. K., Srivastava, A. K., & Singh, A. K. (2019). PGPR bioelicitors: induced systemic resistance (ISR) and proteomic perspective on biocontrol. In *PGPR amelioration in sustainable agriculture,* pp. 67-84.

Moss, M. O. (2008). Fungi, quality and safety issues in fresh fruits and vegetables. *Journal of Applied Microbiology*, *104*(5), 1239-1243.

McNeely, D., Chanyi, R. M., Dooley, J. S., Moore, J. E., & Koval, S. F. (2017). Biocontrol of Burkholderia cepacia complex bacteria and bacterial phytopathogens by Bdellovibrio bacteriovorus. *Canadian journal of microbiology*, *63*(4), 350-358.

Mullis, M. M., Rambo, I. M., Baker, B. J., & Reese, B. K. (2019). Diversity, ecology, and prevalence of antimicrobials in nature. *Frontiers in microbiology*, 2518.

Ngegba, P. M., Cui, G., Khalid, M. Z., & Zhong, G. (2022). Use of Botanical Pesticides in Wagemans, J., Holtappels, D., Vainio, E., Rabiey, M., Marzachì, C., Herrero, S., & Turina, M. (2022). Going viral: virus-based biological control agents for plant protection. *Annual Review of Phytopathology*, *60*.

Notz, R., Maurhofer, M., Schnider-Keel, U., Duffy, B., Haas, D., & Défago, G. (2001). Biotic factors affecting expression of the 2, 4-diacetylphloroglucinol biosynthesis gene phlA in Pseudomonas fluorescens biocontrol strain CHA0 in the rhizosphere. *Phytopathology*. *91*(9), 873-881.

Nifakos, K., Tsalgatidou, P. C., Thomloudi, E. E., Skagia, A., Kotopoulis, D., Baira, E., & Katinakis, P. (2021). Genomic analysis and secondary metabolites production of the endophytic Bacillus velezensis Bvel1. *Plants*, *10*(8), 1716.

Pitt, J. I. (2000). Toxigenic fungi and mycotoxins. *British medical bulletin 56*(1), 184-192.

Panth, M., Hassler, S. C., & Baysal-Gurel, F. (2020). Methods for management of soil borne diseases in crop production. *Agriculture,* 10: 16.

Pal, K. K., & Gardener, B. M. (2006). Biological control of plant pathogens. *The plant health instructor,* A-2006-1117-02

Patil, S., Bheemaraddi, M. C., Shivannavar, C. T., & Gaddad, S. M. (2014). Biocontrol activity of siderophore producing Bacillus subtilis CTS-G24 against wilt and dry root rot causing fungi in chickpea. *Journal of Agriculture and Veterinary Science*, 7.

Pieterse, C. M., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C., & Bakker, P. A. (2014). Induced systemic resistance by beneficial microbes. *Annual review of phytopathology*, *52*, 347-375.

Postel, S., & Kemmerling, B. (2009). Plant systems for recognition of pathogen-associated molecular patterns. *In Seminars in cell & developmental biology* (Vol. 20, No. 9, pp. 1025-1031).

Ramankutty, N., Mehrabi, Z., Waha, K., Jarvis, L., Kremen, C., Herrero, M., & Rieseberg, L. H. (2018). Trends in global agricultural land use: implications for environmental health and food security. *Annual review of plant biology*, *69*(1), 789-815.

Raza, W., Ling, N., Yang, L., Huang, Q., & Shen, Q. (2016). Response of tomato wilt pathogen Ralstonia solanacearum to the volatile organic compounds produced by a biocontrol strain Bacillus amyloliquefaciens SQR-9. *Scientific reports*, *6*(1), 1-13.

Shuping, D. S. S., & Eloff, J. N. (2017). The use of plants to protect plants and food against fungal pathogens: A review. *African Journal of Traditional, Complementary and Alternative Medicines*, *14*(4), 120-127.

Schmidt, R., Cordovez, V., De Boer, W., Raaijmakers, J., & Garbeva, P. (2015). Volatile affairs in microbial interactions. *The ISME journal*, *9*(11), 2329-2335.

Singh, Dinesh, and R. R. Sharma. (2018). "Postharvest diseases of fruits and vegetables and their management." *Postharvest disinfection of fruits and vegetables*. Academic Press, 1-52.

Tomar, M., & Raj, H. (2015). Use of native local bio resources and cow urine for the effective

Management of post-harvest diseases of apples in northwest Himalayan states of India. *Journal of Plant Pathology and Microbiology; l*, *6*, 316.

Thomashow, L. S., Kwak, Y. S., & Weller, D. M. (2019). Root-associated microbes in sustainable agriculture: models, metabolites and mechanisms. *Pest management science*, *75*(9), 2360-2367.

Tilocca, B., Cao, A., & Migheli, Q. (2020). Scent of a killer: Microbial volatilome and its role in the biological control of plant pathogens. *Frontiers in Microbiology*, *11*, 41.

Viterbo, A., Ramot, O., Chernin, L., & Chet, I. (2002). Significance of lytic enzymes from Trichoderma spp. in the biocontrol of fungal plant pathogens. *Antonie Van Leeuwenhoek*, *81*(1), 549-556.

Veliz, E. A., Martínez-Hidalgo, P., & Hirsch, A. M. (2017). Chitinase-producing bacteria and their role in biocontrol. *AIMS microbiology*, *3*(3), 689.

Velásquez, A. C., Castroverde, C. D. M., & He, S. Y. (2018). Plant–pathogen warfare under changing climate conditions. *Current Biology*, *28*(10), R619-R634.

Wu, Q., Dou, X., Wang, Q., Guan, Z., Cai, Y., & Liao, X. (2018). Isolation of β-1, 3-glucanase-producing microorganisms from Poria cocos cultivation soil via molecular biology. *Molecules*, *23*(7), 1555.

Ye, X., Li, Z., Luo, X., Wang, W., Li, Y., Li, R., & Zhang, R. (2020). A predatory myxobacterium controls cucumber Fusarium wilt by regulating the soil microbial community. *Microbiome*, *8*(1), 1-17.

Organization of the thesis

Present thesis is organized into five chapters. **The first chapter** introduces the plant pathogenic fungi and their devastating effect on agriculture loss. Further, it focuses on biocontrol agents and their classification. Simultaneously, the mechanism of biocontrol bacteria and their bioactive compounds against phytopathogenic fungi are explained in detail. **The second chapter** involves the screening and identification of the bioactive compounds producing marine bacteria. Further, potential antagonistic marine bacteria subjected to genome sequencing and gene analysis are associated with biocontrol activity and stress resistance.

The third chapter covers the optimization of the fermentation parameters at the flask level for bioactive compounds. Further, bioactive compounds are produced in a ten-liter lab-scale fermenter, and purification is carried out with chromatographic techniques. The characterization of the purified compounds were assessed with different methods such as HPLC, GC-MS, and NMR. **The fourth chapter** deal with the antifungal efficacy, toxicity, mechanism and agricultural application of the characterized bioactive compounds and producing bacteria individually. **The fifth chapter** summarises and concludes the overall results of the thesis, which also tells about the future direction of the work.

Statement of the problem

Over the last five decades, an extensive study was carried out on terrestrial bacteria and their bioactive compounds for agricultural applications. However, relatively few of these antagonistic bacteria have been commercialized as bio-pesticide and bio-fertilizer. Therefore, still need to improve efficacy and performance in open field conditions compared to chemical fertilizers and pesticides. In contrast, marine bacteria are gained attention as they adapt and produce unique bioactive compounds in extremely stressful habitats such as high saline, high pressure, minimum nutrients, fluctuating pH and varied temperature conditions. Aiming for

this, marine bacteria and their bioactive compounds need to explore for agricultural applications.

Objectives

1. Screening and identification of bioactive compounds producing marine bacteria
2. Fermentation, purification, and characterization of the bioactive compounds
3. Antifungal efficacy, cytotoxicity, mechanism, and applications

Chapter. 2:

Screening and identification of bioactive compound producing marine bacteria

Abstract

Microorganisms from severe environments, such as marine bacteria, are extremely important for crop protection against various biotic and abiotic challenges. Because they can produce bioactive molecules, which combat salt, heavy metals and infections. Therefore, the previously isolated marine bacteria were screened for antifungal activity against plant pathogens. Among these, two strains i.e., BKACT and NIO-1008 consider to be the best antagonistic candidates. Furthermore, both strains grew at a broad range of pH, high NaCl concentration and produced multiple plant growth-promoting (PGP) traits. However, NIO-1008 produces the most, 640 ±9 µg/mL of indole acetic acid (IAA), which is six fold greater than of strain BKACT. Finally, 16S r-RNA sequencing identified both strains, confirming BKACT as *Serratia marcescens* and NIO-1008 as *Arthrobacetr enclensis*. Furthermore, genome analysis validated the abovementioned characteristics in both strains by investigating genes related to stress tolerance, biocontrol activity, and plant growth-enhancing regulators.

1. Introduction

Plant diseases are projected to destroy at least 10% of world food production; the main culprits are nematodes, weeds and plant pathogenic fungi (Chakraborty et al., 2011). Conventional pesticides have provided numerous advantages to human in the agricultural production, while their hazard to humans and animals has long been an issue (Nicolopoulou-Stamati et al., 2016). To replace the conventional fungicides, novel bioactive compounds with excellent effectiveness and safety must be identified and produced (Velasquez et al., 2018). The data shows that most (35.7%) of newly introduced substances were registered with the Environmental Protection Agency (EPA) as natural products. In the past, microbial active molecules have been immensely effective as a significant component of NPs, including such products as isarolides, bialaphos, blasticidin, spinosyns, destruxins, and bassianolide (Huang et al., 2018). So far, after more than a 50 year of study on terrestrial microbes, the rate of novel molecules discovered has decreased, leading researchers to search for novel natural compounds.

In this context, the finding of new natural compounds has been inclined toward the world of fascinating marine microorganisms during the last two decades. The ocean, which occupies more than 70% of the Earth's surface, is a massive and diverse source of bacteria, particularly given that one millilitre of ocean water carries millions of microbial cells (Bhatnagar et al., 2010). There are 178000 marine species within 34 phyla, as per the UN Environment Program's World Biodiversity Assessment (Mitra et al., 2016). As a consequence, the marine biodiversity contributes about 50% of global biodiversity, offering marine organisms a prospective long-term source of new molecules with diverse bioactivity that really are helpful to humans (Ameen et al., 2021). The oceans constitute unique habitats which helps in bacterial adaptations at extreme variations such as pressure, salinity, temperature and nutrient loss conditions that can select unique biosynthetic pathways leading to new types of molecules. Several studies have reported a wide diversity of biological activities from marine bacteria, which makes these

microbial communities a valuable source of novel bioactive compounds (Bhatnagar et al., 2010).

Screening is the first step in the search for novel antagonistic bacteria and their bioactive compounds against the target pathogens. While selecting an efficient screening method, it should be simple, rapid, reproducible, and inexpensive (Raymaekers et al., 2020). Traditional approaches for screening efficient biocontrol microorganisms and their bioactive substances are still routinely employed. These methods are further categorised based on the antagonistic action.

Phenotype-based screening methods

In this method, the direct antagonistic action of bacteria is assessed based on the ability to restrict pathogen proliferation and harmful effects. The dual culture approach is one of the most widely used methods based on direct *in vitro* antagonistic activity. This method is simple to implement and has a wide range of applications. In this assay, bacteria and the target pathogen are co-cultured on a semisolid medium, and antagonistic activity is assessed based on the zone of pathogen growth inhibition (Zhang et al., 2017). The modified dual culture method is the most often used for screening volatile organic compounds (VOC) producing bacteria against pathogens. This experiment is carried out in a bi-compartment petri plate with the pathogen and antagonistic bacteria seeded in separate compartments. The antimicrobial effectiveness of the VOCs was assessed based on their capacity to impede pathogen development (Briard et al., 2016). In dual culture assay, a liquid medium can be used instead of semisolid to screen antagonistic bacteria and their bioactive compounds. In this assay, the pathogen and test compounds were inoculated in the liquid medium, and the effectiveness of the compounds was assessed based on pathogen growth suppression (Liu et al., 2019).

Furthermore, performing such experiments in regular microtiter plates enables high throughput screening of many bioactive compounds. In addition to *in vitro* screening, antagonistic bacteria can be tested directly *in vivo* on plant material for disease control. Rather than having certain practical limitations, these assays have advantages such as testing the antagonistic action of bacteria or bioactive substances under more natural conditions and being beneficial against pathogens that are difficult to develop *in vitro* (Mohammadi et al., 2017).

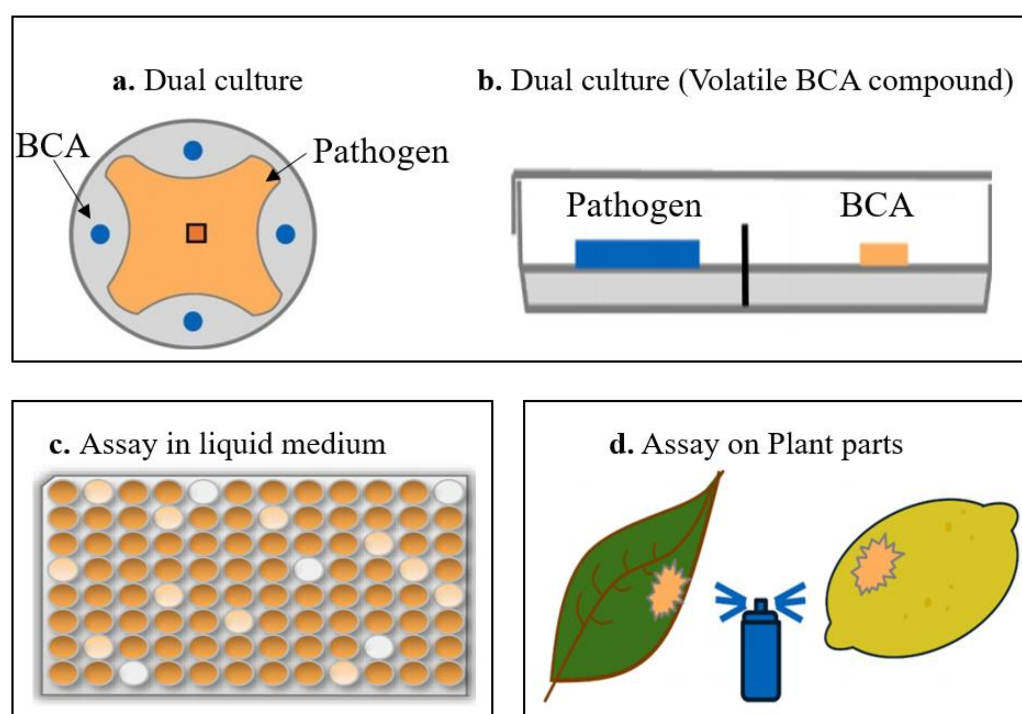


Figure. 1 Phenotype-based screening for the biocontrol agents (BCAs) (Adapted from Katrijn Raymaekers et al., 2020)

Marker-based screening

In marker-based screenings, the inhibitory Effect of the biocontrol agents is evaluated based on the markers that are closely involved in antagonism. These markers can be antimicrobial metabolites (siderophores) released by bacteria or lytic enzymes (chitinases and glucanases). Plant root colonization may also be used to screen biocontrol bacteria against pathogens based on competition for nutrients and space on the plant. Marker-based approaches are beneficial

for screening biocontrol agents at high throughput. The most crucial aspect of screening is the selection of a marker associated with antagonistic activity. (Raymaekers et al., 2020).

Whole-genome sequencing (WGS) is gaining popularity in the scientific community because it is a quick, low-cost, and highly efficient third-generation sequencing method that can offer a complete perspective of the genetic makeup. WGS is used to identify bacterial strains and screen for functional genes linked with specific tasks like competitiveness, nutrition uptake, stress response, and bioactive substances in the case of bacteria. This approach is also utilized to detect genetic markers associated with biocontrol capabilities, such as genes encoding enzymes that digest plant pathogens or the production of plant growth-promoting regulators. Furthermore, WGS is also used to determine genetic diversity among biocontrol bacteria, which can assist in the selection of strains with the highest biocontrol capability for a particular crop or disease. Specifically, WGS can be an effective technique for screening, identifying, tracking, and selecting microorganisms for specific roles (Sahu et al., 2020).

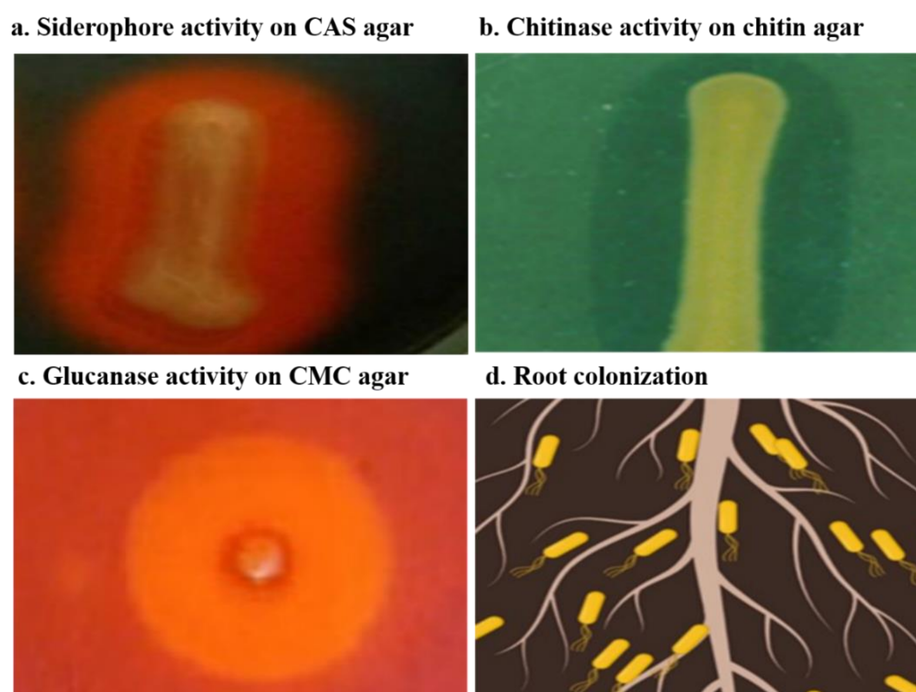


Figure. 2 Marker-based screening of biocontrol agents (BCAs) (Adapted from Katrijn Raymaekers, 2020)

2. Materials and methods

2.1 Reagents and kits

Genomic DNA HiPurA™ kit (Hi-Media), Exo SAP (New England Bio Labs), Ligation sequencing kit SQK-LSK 109 (Oxford Nanopore Technologies), Native barcoding kit NBD104 (Oxford Nanopore Technologies), Qubit® dsDNA HS Assay Kit (Life Technologies), Pikovskaya's agar medium (Hi-Media), Aleksandrov agar medium (Hi-Media), Carboxyl methyl cellulose (Sigma Aldrich), Glutaraldehyde (Sigma Aldrich), Sodium phosphate buffer (Hi-Media), Ethanol (Hi-Media), Peptone (Hi-Media), L-tryptophan (Sigma Aldrich), Indole acetic acid (Sigma Aldrich), Nessler's reagent (Hi-Media), Congo red (Hi-Media), Crab-shell chitin (Hi-Media).

2.2. Culture maintenance and preservation

Previous research identified 150 marine bacteria from Chorao Island in Goa, India (Dastager et al., 2013). Thirty marine strains (Table. 1) were chosen for further investigation based on their capacity to produce plant growth-promoting (PGP) characteristics. All the marine bacteria were grown on a nutrient agar medium (Hi-media, Mumbai), and their glycerol stocks were kept at -80°C. In this work, a total of 12 plant pathogenic fungi were used, of which six were field isolates and the remaining six were obtained from the National Collection of Industrially Important Microorganisms (NCIM) Resource Centre, CSIR-NCL Pune (Table. 2). Throughout the study, the fungal cultures were grown on potato dextrose agar (Hi-Media, Mumbai).

Table. 1 List of marine strains used in the study

Sr. No	Culture ID	Sr. No	Culture ID	Sr. No	Culture ID
1	1130	11	1011	21	AC-13
2	1013	12	1027	22	1002

3	1123	13	19a	23	1023
4	1009	14	NS 5.1	24	BKACT
5	1008	15	D-25	25	36-b
6	V-25	16	1016	26	M-37
7	1057	17	V-93	27	V-74
8	AC-8	18	1030A	28	V-88
9	M-29	19	1109	29	D-9
10	V-76	20	1051	30	R-2

Table. 2 List of test fungi used in the study

Sr.no	**Fungi**	**Strain**
1	*F. verticilliodes*	NCIM-1276
2	*Epicoccum pneumonia*	FS-1
3	*F. proliferatum*	NCIM-1101
4	*Colletotricum cobbittiense*	FS-2
5	*Fusarium* sp.	NCIM-1330
6	*F. oxysporum*	NCIM-1281
7	*Colletothrichum aeschynomens*	FS-3
8	*Acrophialophora levis*	FS-4
9	*Crinipellis tubtim*	FS-5
10	*Aspergillus niger*	NCIM-628
11	*Aspergillus flavus*	FS-6
12	*Aspergillus oryzae*	NCIM-1058

2.3. Screening of the marine strains for the bioactive compound production

2.3.1. Antagonistic activity

All the marine strains were screened against phytopathogenic fungi using the dual culture method (Tchameni et al., 2020). Marine 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 a test fungi alone was consider as the control. Both treatment and control plates were kept at 28°C, and fungal growth suppression was recorded on 3, 5, 7, and 9th day of incubation. The percent (%) inhibition growth of the test fungi was calculated by using the formula:

$$Growth\ Inhibition\ percent = \frac{(R-r)}{R} \times 100$$

R represents fungal pathogens radial growth on the control plate, while "r" represents fungal pathogens radial growth in the dual culture plate. All the experiments were performed in triplicates. The Effect of marine strains on fungi's hyphal and conidial morphology was examined in a light microscope (Nikon, Japan) and scanning electron microscope (SEM). For SEM examination, samples were prepared as per the procedure mentioned with modification (Zhou et al., 2011). In brief, the hyphae from the control and treatment plates were fixed for 24 hours at 4°C in 2.5% glutaraldehyde, and then washed three times with phosphate buffer (0.1 M). After that, hyphae were dehydrated in a graduated sequence of ethanol concentrations (30, 50, 75, and 100%) for 5 minutes each, dried at 37°C, pasted on stubs, and viewed in an FEI Quanta 200 3D dual-beam electron microscope.

2.3.2. Plant growth promoting (PGP) attributes

Furthermore, the multiple PGP traits of potential antagonistic marine strains such as BKACT and NIO-1008 were investigated.

Siderophore production

The production of siderophores were examined on a CAS-agar plate, and the composition of the CAS blue solution for this assay was made according to (Schwyn and Neilands et al., 1987). A pure colony of bacteria was inoculated on CAS agar plates with sterile toothpicks and cultured in the dark at 28ºC for seven days. The colony with an orange zone was proven to be producing siderophore.

Phosphate and potassium solubilisation

Phosphorus (P) and potassium (K) solubilisation activities were measured on Pikovskaya's agar medium and Aleksandrov's agar medium (Hi-media). Pure bacteria colonies were inoculated on solidified agar plates, and after five days of incubation, a clear zone surrounding the colony was considered a positive result.

Ammonia production

A freshly grown culture of marine strains were inoculated into peptone water and incubated for three days in a shaker incubator at 28ºC. The broth was centrifuged and the amount of ammonia in the supernatant measured by mixing 1mL of Nessler's reagent with 1mL of the supernatant and diluting the mixture to 10.0mL with distilled water. The transformation of brown to yellow colour was a favourable indicator of ammonia production.

Indole acetic acid production

Overnight grown cultures (1%) were inoculated in nutrient broth (Hi-media) supplemented with varied amounts of L-tryptophan (Sigma) (0%, 0.1%, 0.5%, 1.0, and 2.0%). The cells were cultured at 28º C for 1, 2, 3, 4, and 5 days. The medium is considered blank if bacteria are not inoculated at the specified quantity of tryptophan. After 5 minutes of centrifugation at 13000 rpm, 1.0mL of supernatant was combined with 1.0mL of Salkowski reagent. After 30 minutes

of incubation, the optical density of the solution was measured at 530 nm. The amount of indole acetic acid (IAA) produced was quantified by comparing it to a standard curve prepared with standard IAA.

Lytic enzymes activity

Chitinase activity was determined as per Roberts and Selitrennifoff method (Robert et al., 1998). Colloidal chitin was prepared by adding 10.0gm crab-shell chitin (Sigma) mixed with cold concentrated hydrogen chloride (175 mL) and placed at 4ºC for 24 h. The mixture was extracted with ice-cold 1000 mL ethanol and kept overnight 28ºC with stirring. The resulting chitin suspension was centrifuged at 10,000 g for 20 min. The chitin pellet were then continuously rinsed in water until the pH reached neutral, dried, and kept at 4 °C for further use. Colloidal chitin (20.0 gm/L), $MgSO_4$ (5.0 gm/L), $ZnSO_4$ (0.01 gm/L), K_2HPO_4 (7.0 gm/L), $FeSO_4$ (0.1 gm/L), KH_2PO_4 (3.0 gm/L), and Bacto agar (20.0 gm/L) were used to make chitin agar. The medium's pH was maintained at 7.0 prior to being autoclaved. The sterilised chitin agar was added into petri plates; when the agar solidifies, the marine strains were inoculated and then incubated at 28 °C for five days. The zone of clearing on the media was then visible surrounding the colony.

In the interest of assessing the glucanase activity, cultures were grown on carboxymethyl cellulose (CMC) agar that contained (g/l) $MgSO_4.7H_2O$ (0.5), NH_4NO_3 (0.3), K_2HPO_4 (0.5), NaCl (0.5), $MnSO_4.H_2O$ (0.01), $FeSO_4.7H_2O$ (0.01), CMC (10.0), and agar (15.0). Using 1 M NaOH, the pH was managed to 7 and plates were incubated at 28°C for five days. Following that, the CMC agar medium was washed with 1% Congo red solution. The creation of a clear zone showed cellulose breakdown.

2.3.3. Stress resistance ability of marine strains

2.3.3.1. Effect of pH and NaCl

The growth patterns were studied in nutrient broth at different pH and salt concentrations in 96 well plates using a SYNERGY 96 well plate reader. Growth media was adjusted at a pH of 0 to 14 using sodium hydroxide (NaOH) and hydrogen chloride (HCl). Salt concentrations were adjusted to 0 to 10% (w/v) using sodium chloride (NaCl). Eighteen hours old grown active culture were inoculated into sterile media with increasing salt and pH concentrations. The final colony-forming units of cultures were adjusted to 0.5 McFarland standards (10^8 CFU/mL) by a Densimat spectrophotometer and incubated for 48 h at 28ºC. Experiments were conducted separately in triplicates.

2.3.3.2. Effect of heavy metals

The heavy metal resistance ability of were studied in the presence of different heavy metals such as $CdCl_2$, $ZnCl_2$, $CuSO_4$, $CoCl_2$, and $NiCl_2$ at different concentrations. The metal resistant capacity was determined in 96 well microtiter plates using two-fold serial dilution concentrations ranging highest 500 to lowest 3ppm/Liter. The minimum inhibitory concentration (MIC) at which no visible colony forming units (CFU) were observed after 48 h incubation. All analysis was performed in triplicates.

2.4. Identification

For molecular characterization, genomic DNA was isolated from the cell pellet using a genomic DNA HiPurA™ kit (Hi-Media, Mumbai). The universal primers 27F and 1492R were used to amplify the 16S rRNA gene. Exo SAP (New England Bio Laboratories) was used to purify the PCR products before they were sequenced on a 3500XL Genetic Analyzer (Applied Biosystems, USA). The identity of the strains with closest neighbors was analyzed by comparing the 16S rRNA gene sequence with existing sequences in the NCBI Gene Bank using

the BLASTn program. Phylogenetic trees were generated using multiple alignments of closely related strains derived from the EzBioCloud database **(http://www.ezbiocloud.net)**. A neighbor-joining tree was constructed using MEGA (version 6.0) to determine the phylogenetic relationship. The phylogenetic tree's topology was examined using bootstrap values based on 1000 replications.

2.5. Whole genome sequencing, assembly, and Annotation

The pure and freshly grown culture of BKACT was used for the DNA extraction by using a genomic DNA HiPurA™ kit (Hi-Media, Mumbai) as per manufacturer instructions. The quality and quantity were evaluated using 1% agarose gel electrophoresis and a Nano-Drop spectrophotometer (Thermo Fisher Scientific, Q32853). The isolated DNA was kept at -20 °C until it was used. Afterward, extracted DNA was prepared for Nanopore sequencing. A DNA library was prepared by using 100 ng of extracted DNA with a ligation sequencing kit SQK-LSK 109 (Oxford Nanopore Technologies) and indexed using Native barcoding kit NBD104 (Oxford Nanopore Technologies). DNA concentration was determined during library preparation with Qubit® dsDNA HS Assay Kit (Life Technologies). The workflow combines enzymatic steps with minimal magnetic bead-based clean-ups. DNA samples were enzymatically degraded into ds DNA fragments, and then end repair cum A-tailing was carried out to generate 'end-repaired, 5'-phosphorylated, 3'-dA-tailed ds DNA fragments. Subsequently, each end-repaired DNA is given a unique identifier with the native barcodes. These indexed samples are then pooled to proceed with the adapter ligation step, followed by the library loading onto the MinION flow cell in an MK1C sequencing platform. Albacore version 2.0, (https://github.com/Albacore/albacore) was used for high-accuracy base calling with a minimum q-score of 8. The raw files obtained were processed using albacore to convert into fastq format, followed by another software, Porechop version 0.2.4 (https://github.com/rrwick/Porechop) for adapter trimming, followed by NanoPlot (De Coster

et al., 2018) for quality check. The genome assembly was done using Flye version 2.4 (Kolmogorov et al., 2019).

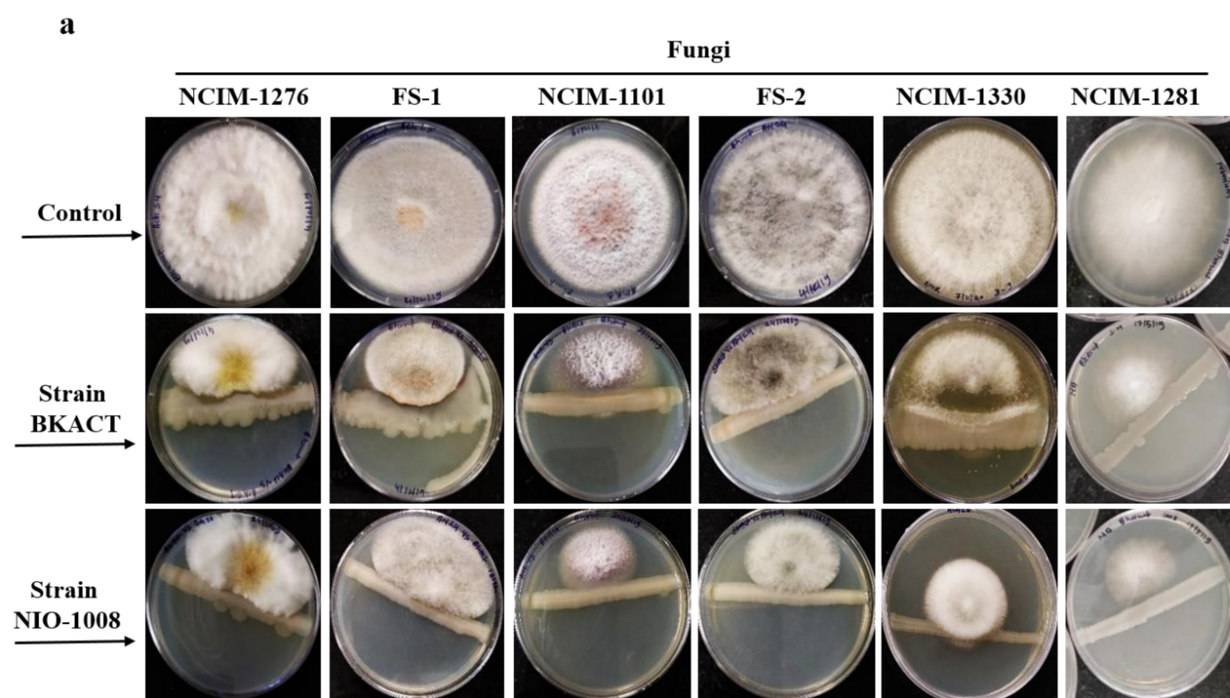
In the earlier study, a draft genome of *Arthrobacter enclensis* NIO-1008 was submitted under the accession number LNQM00000000 (Neurgaonkar et al., 2016). The draft genome sequence was retrieved from NCBI Gen Bank (https://www.ncbi.nlm.nih.gov/). The contigs of strain BKACT and NIO-1008 were further subjected to gene prediction and Annotation using the PATRIC web server (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3257917/), followed by functional genes identification using RAST-Rapid Annotation Subsystem Technology version 2.0 (https://academic.oup.com/nar/article/42/D1/D206/1062536), which confirm RNA genes, protein-encoding genes (PEGs) and repeat regions. The annotated genes were investigated to identify those associated in abiotic stress tolerance, pathogen suppression, PGP functions, and other relevant functions.

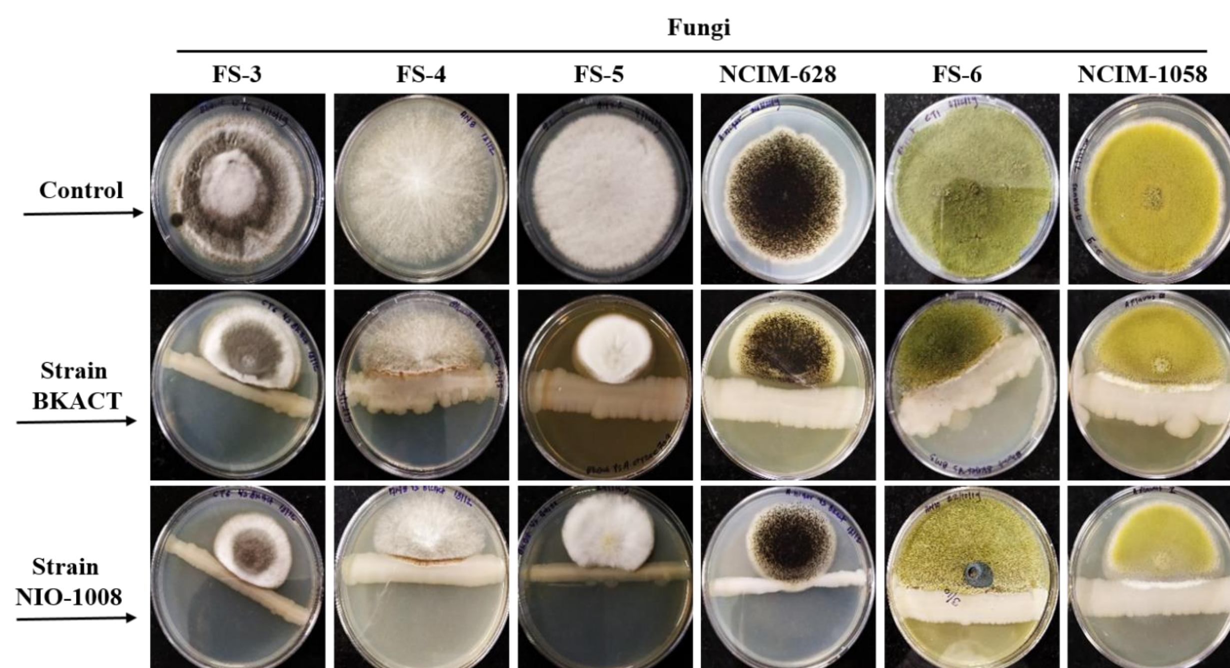
3. Results and discussion

3.1. Antagonistic activity

Abiotic and biotic stressors impair crop development and production by interfering with metabolic balance and nutrient absorption. However, beneficial bacteria from harsh habitats have the inherent potential to support and stimulate plant development under various biotic and abiotic challenges. As a result, bacteria from naturally harsh environments are gaining attention for plant growth promotion and stress control (Rima et al., 2018). Marine bacteria have attracted significant attention in this context because they have evolved to produce unique bioactive compounds in adverse conditions such as high salinity, high pressure, limited nutrient, fluctuating pH, and temperatures (Qin sheng et al., 2014). As a result, the previously isolated 30 marine strains were tested against phytopathogenic fungi in this study. Strains BKACT and NIO-1008 were validated as most promising antifungal candidates based on antagonistic activity displaying >50% mycelial growth inhibition against all test fungi except

A. flavus FS-6 (Fig. 3a). However, strains BKACT and NIO-1008 inhibited mycelial growth the most against *Fusarium* species such as *F. foetens* NCIM 1330 and *F. oxysporum* NCIM 1281, respectively (Fig. 3b).





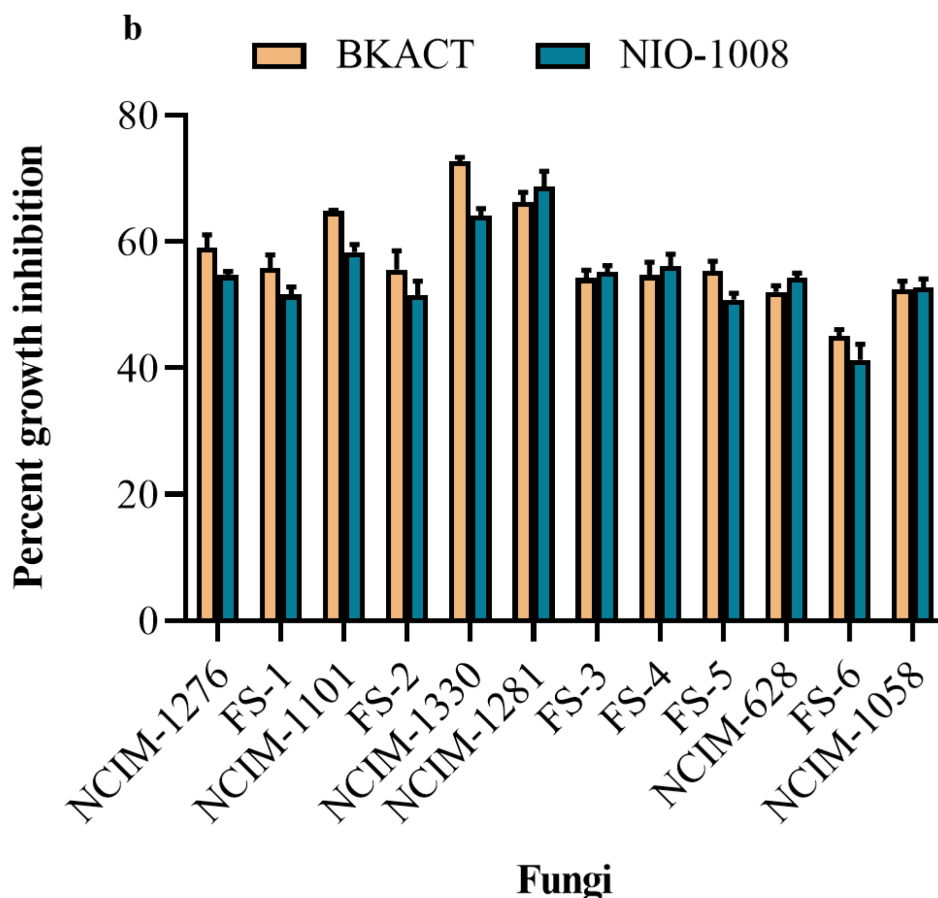


Figure. 3 Antagonistic activity of marine bacteria against fungi, **a.** Dual culture images and **b.** Percent mycelial growth inhibition

Mycelial growth of *F. oxysporum* NCIM 1281 was strongly suppressed in the presence of NIO-1008, with values of 43.6 ±2.2, 62.8 ±1.8, 66.2 ±2.3 and 68.4 ±1.1 percent at 3, 5, 7, and 9 days of incubation, respectively. At the same time; strain BKACT treated *F. foetens* NCIM 1330 mycelia were considerably suppressed, with values of 50 ± 2.0, 53.03 ± 2.14, 69.56 ± 1.22 and 75.56 ± 0.80 % on the third, fifth, seventh, and ninth days of incubation, respectively (Fig. 4a). Following that, the antifungal effects of strains BKACT and NIO-1008 against *F. foetens* NCIM 1330 and *F. oxysporum* NCIM 1281 were thoroughly investigated. Furthermore, its antifungal activity was observed using light and a scanning electron microscope. Compared to their control samples, the morphology of mycelia and spores in treated samples was thin, aberrant, deteriorated, and distorted (Fig. 4b).

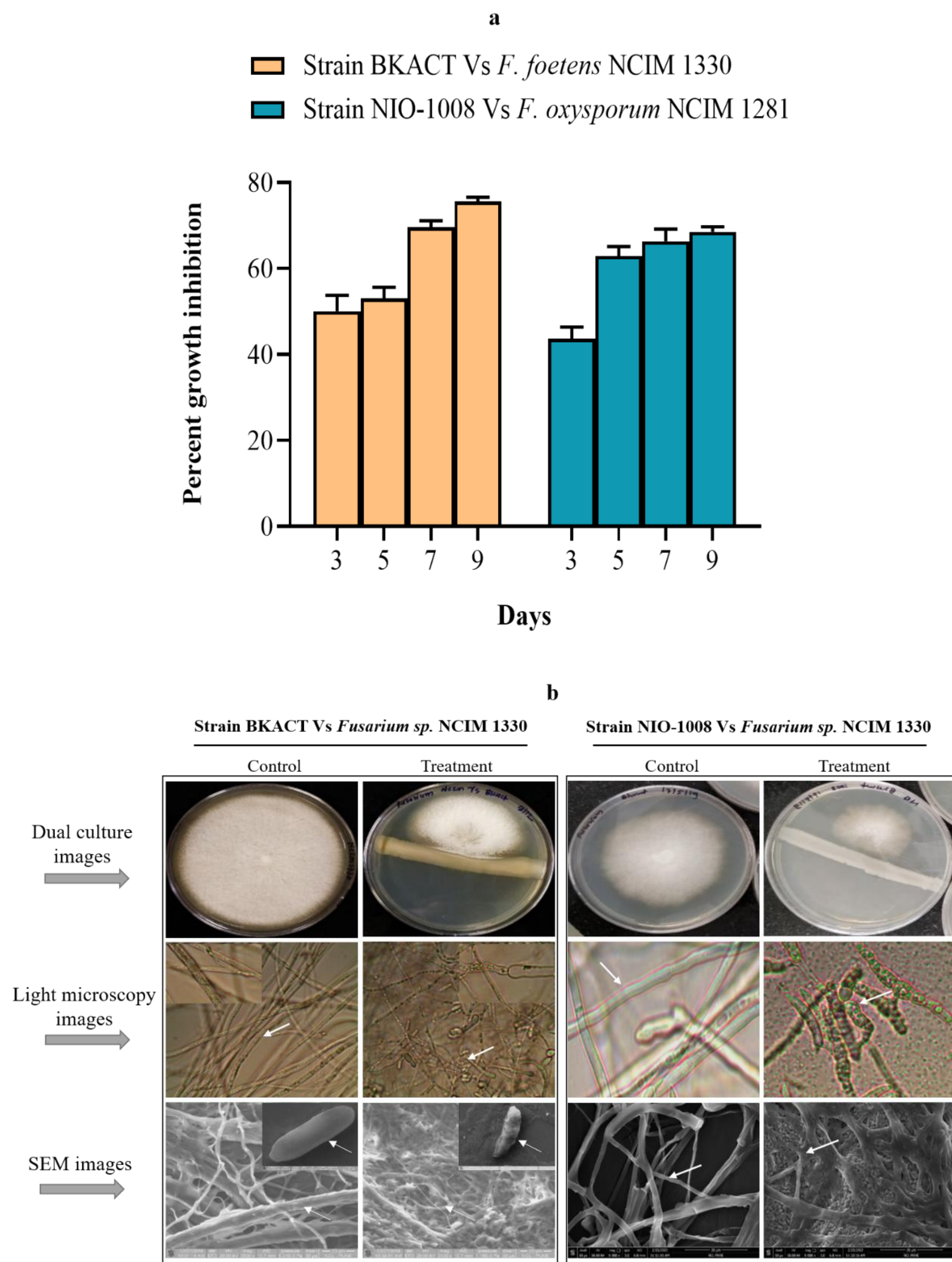
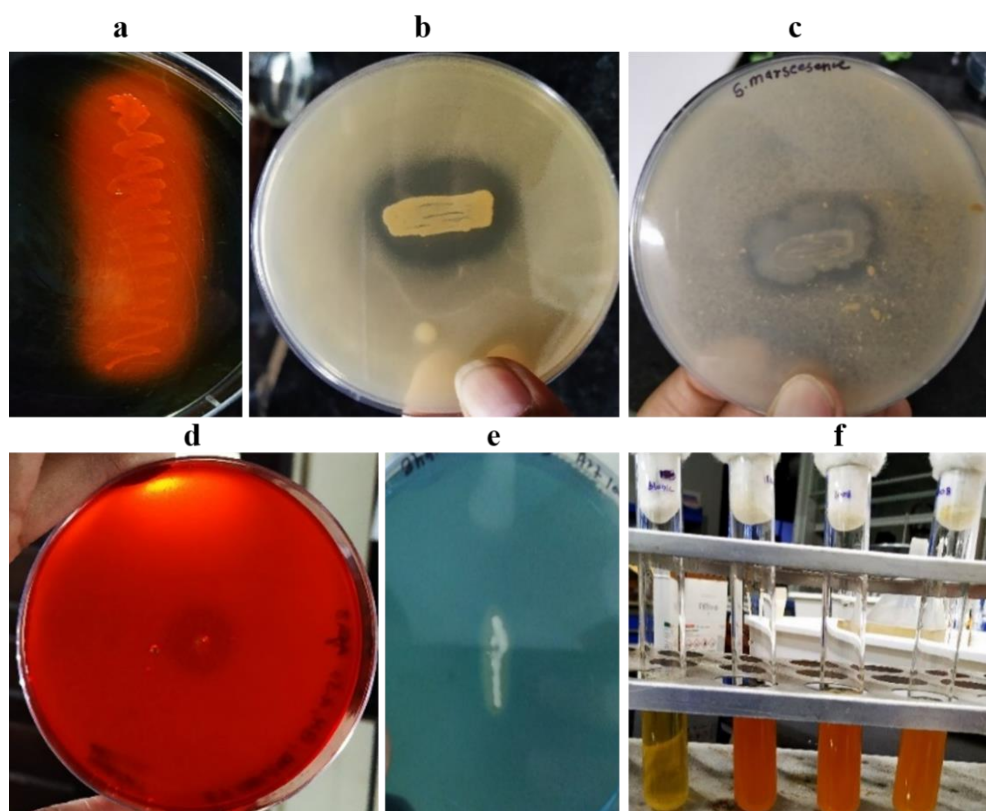


Figure. 4 Antagonistic effect of strain BKACT and NIO-1008 against *Fusarium spp.*, **a.** Percent mycelial growth inhibition and **b.** Antagonistic effect of marine strains evaluated under light microscope and scanning electron microscope

3.2. Plant growth-promoting (PGP) attributes

The antagonistic PGP bacterium can reduce crop damage by releasing plant hormones, siderophore, ammonia, lytic enzymes, and antimicrobial compounds (Kumar et al., 2018; Olanrewaju et al., 2017). As a result, both antagonistic strains were tested for plant growth-promoting (PGP) characteristics. Phosphate solubilisation, potassium solubilisation, and the formation of lytic enzymes (chitinase and glucanase) were thus verified in the strain BKACT (Fig. 5a-d). Simultaneously, another strain NIO-1008 was found to produce ammonia and siderophore (Fig. 5e & f). Both strains found indole acetic acid (IAA) common in the tryptophan-amended medium. However, NIO-1008 produces 640 ±9 µg/mL of IAA, which is six times more than BKACT in 2% tryptophan-modified nutrient broth (Fig. 5g &h). Even so, it is significantly higher than the 520 µg/mL reported by (Ozdal et al., 2017) under optimum circumstances employing immobilized *Arthrobacter agilis* A17 cells.



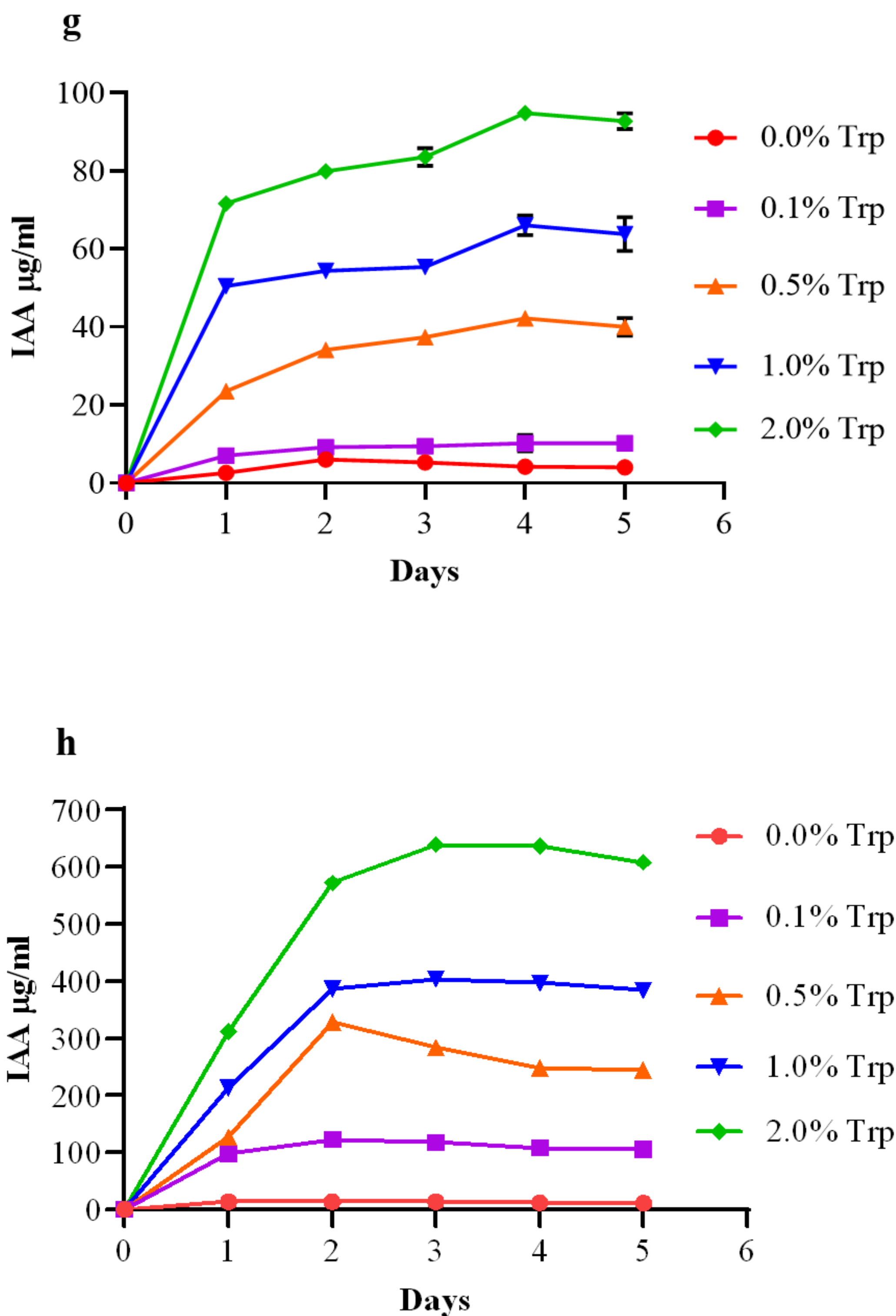
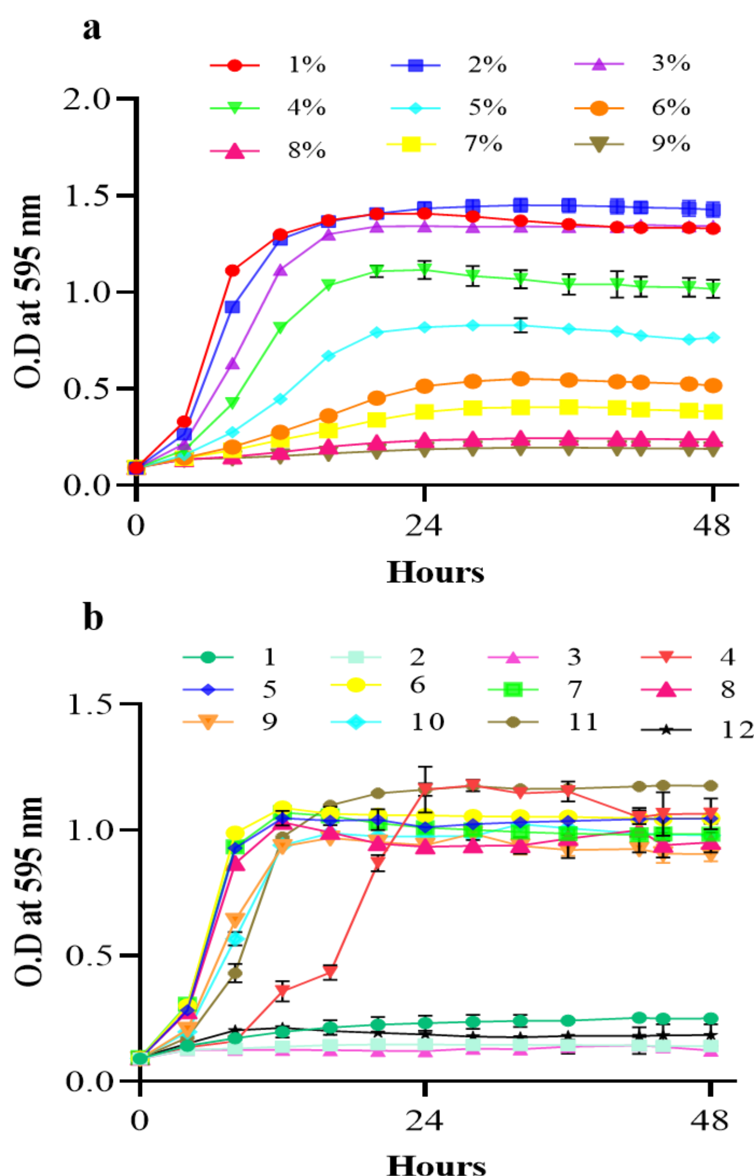


Figure. 5 Plant growth promoting (PGP) traits of strain BKACT and NIO-1008, **a.** Zone of potassium solubilisation on Aleksandrov's agar, **b.** Zone of phosphate solubilisation on Pikovskaya's agar, **c.** Chitinase activity on chitin agar, **d.** Glucanase activity on CMC agar**, e.** Siderophore activity on CAS agar, **f.** Ammonia production, **g.** Indole acetic acid (IAA) production in BKACT **h.** IAA production in NIO-1008

3.3. Effect of pH and NaCl on the growth of strain NIO-1008 and BKACT

Along with the antagonistic activity and PGP characteristics, stress tolerance is most important for efficient performance and endurance in open-field situations. The stress resistance ability of strain NIO-1008 and strain BKACT was investigated at various pH and NaCl concentrations. In this context, marine strain NIO-1008 grew best at pH levels ranging from 4 to 11, with a tolerance of up to 6% NaCl content (Fig. 6a &b). Simultaneously, strain BKACT grew well at 7% NaCl and a pH range of 3 to 11. (Fig. 6c &d). This finding suggests that bacteria from natural saline environments have salt and pH tolerance mechanisms.



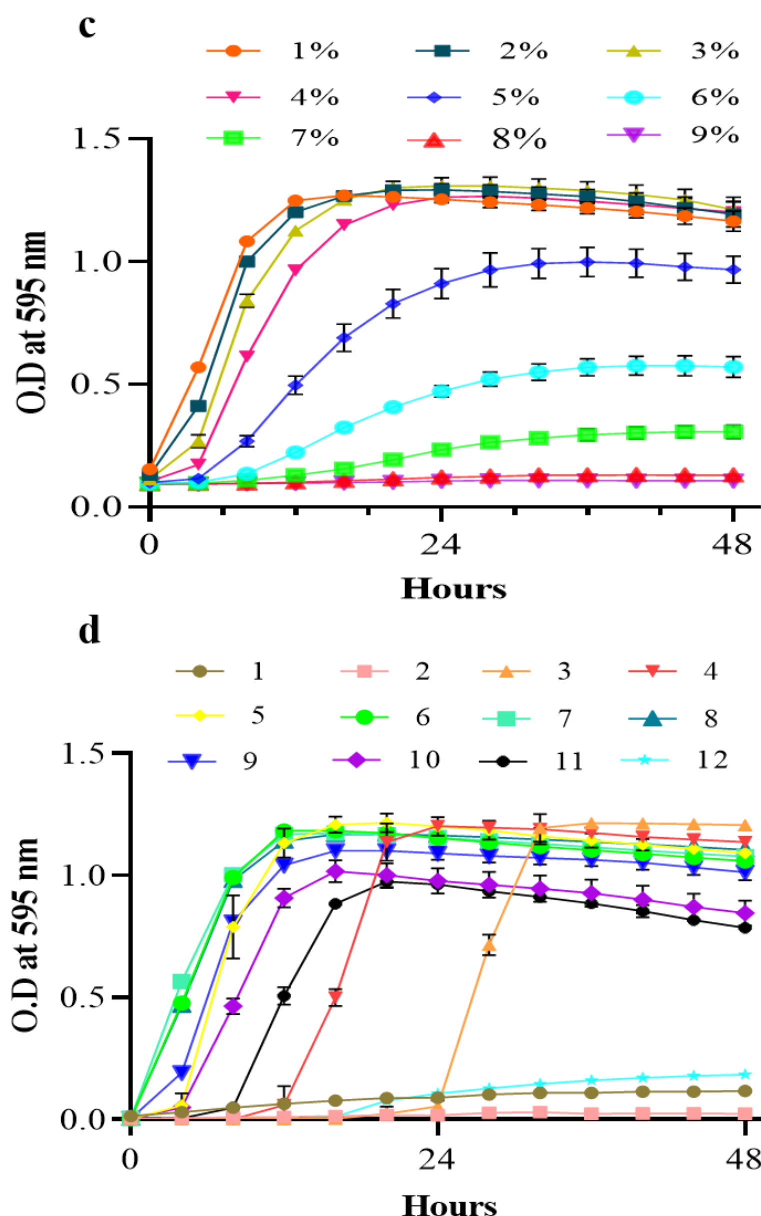


Figure. 6 Stress tolerance ability of marine strains BKACT and NIO-1008 at different pH and NaCl concentrations, **a.** NIO-1008 at different concentrations of NaCl, **b.** NIO-1008 at various pH, **c.** BKACT at different concentrations of NaCl and **d.** BKACT at various pH

3.4. Effect of heavy metal stress on the growth of strain NIO-1008 and BKACT

Heavy metals are also a significant abiotic stressor for plants and microorganisms (Rizvi et al., 2020). In heavy metal resistance, plant growth-promoting bacteria play a crucial role through various mechanisms, including phytostabilization, chelation, bioaccumulation, and the

synthesis of antioxidant enzymes (Alves et al., 2022). In this investigation, we tested both strains stress tolerance ability against various concentrations of heavy metal salts such as $CdCl_2$, $ZnCl_2$, $CuSO_4$, $CoCl_2$, and $NiCl_2$. Except for cadmium, marine strain NIO-1008 was resistant to all the heavy metals examined. The MICs of the heavy metals $NiCl_2$, $CoCl_2$, and $ZnCl_2$ were determined to be 500, 250, and 62ppm, respectively. Even at 500ppm concentration, $CuSO_4$ had no inhibitory impact (Fig. 7a-e).

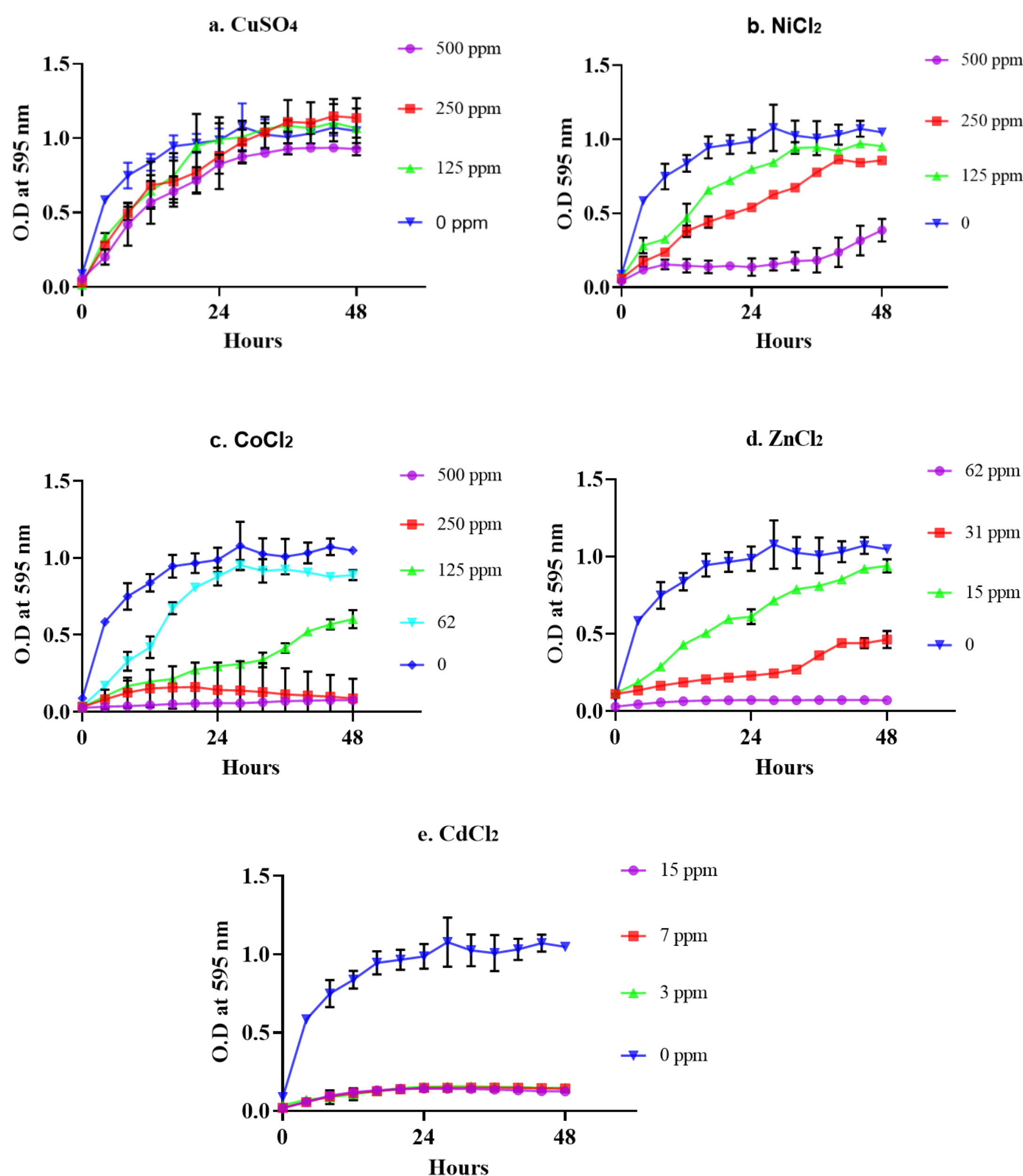


Figure. 7 Metal stress resistance study of *A. enclensis* NIO-1008 in presence of $CuSO_4$, $NiCl_2$, $ZnCl_2$, $CoCl_2$ and $CdCl_2$

At the same time, the MIC of all heavy metals tested against strain BKACT was 500 PPM, with the exception of $CoCl_2$ (125 PPM) (Fig. 8a-e). In compared to strain NIO-1008, strain BKACT demonstrated superior resistance to all metals tested, including cadmium.

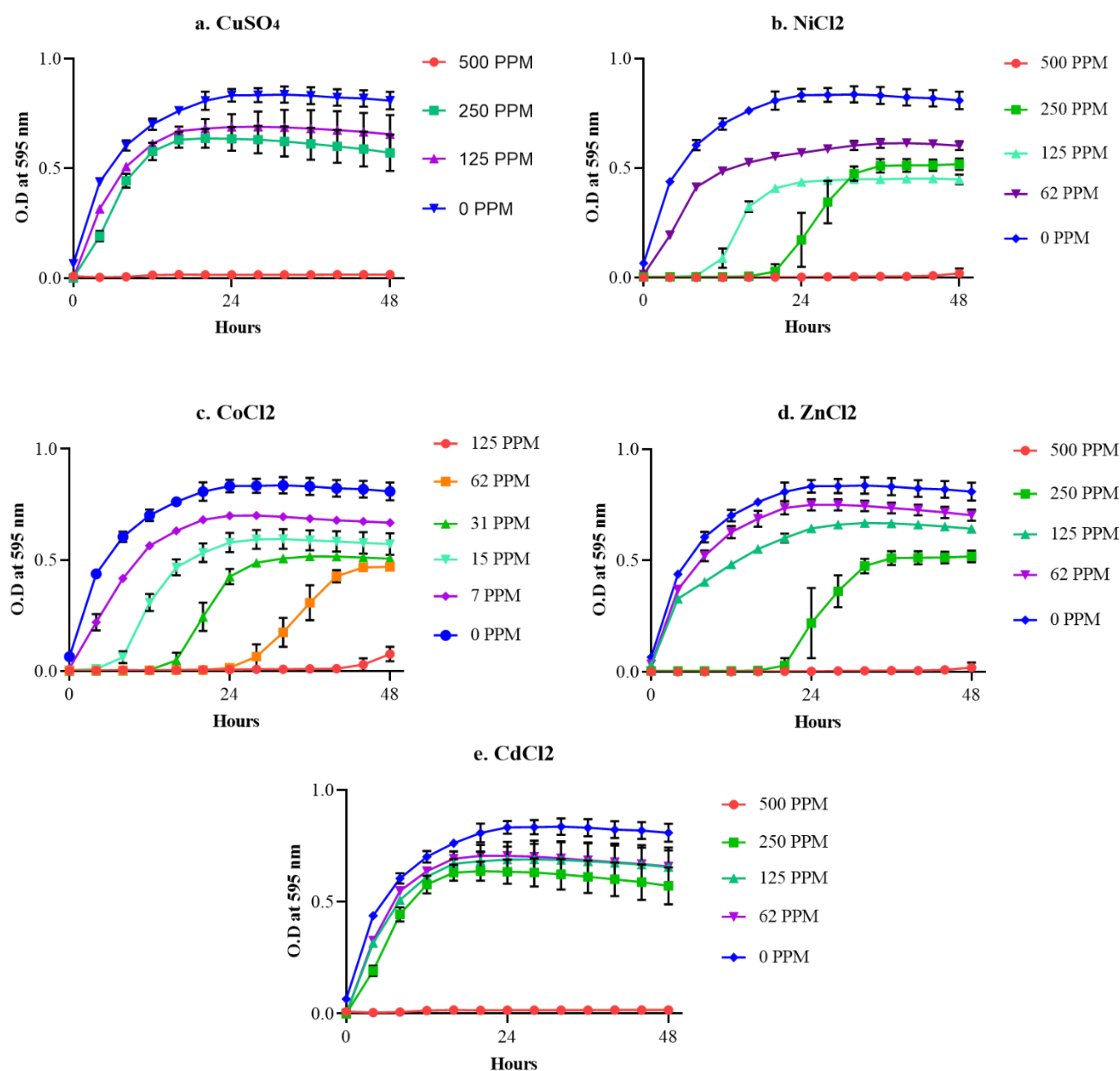
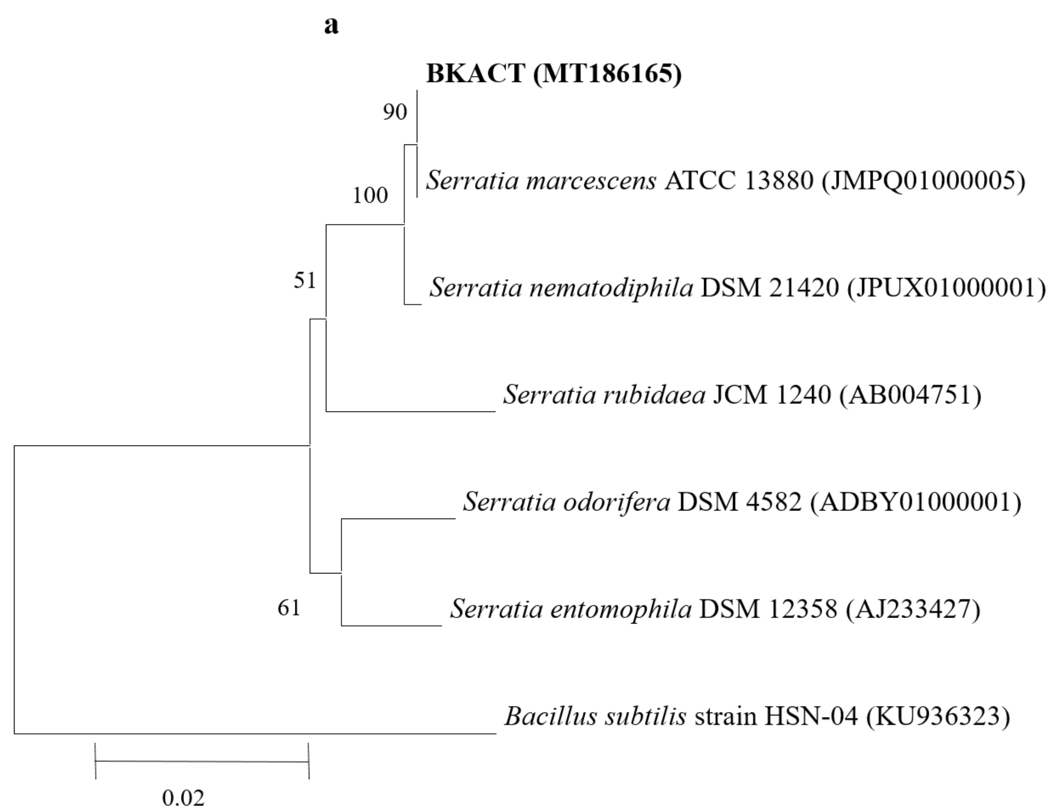


Figure. 8 Metal stress resistance study of strain BKACT in presence of $CuSO_4$, $NiCl_2$, $ZnCl_2$, $CoCl_2$ and $CdCl_2$

3.5. Molecular characterization

Based on the antagonistic activity, PGP traits, and stress tolerance ability, strain BKACT and NIO-1008 are potential candidates for further study. Identification of antagonistic strains based

on 16S rRNA gene sequencing analysis and NCBI-BLASTn confirmed strain BKACT belongs to the genus *Serratia*, and strain NIO-1008 belongs to the genus *Arthrobacter*. The strain BKACT showed the highest sequence similarity to *Serratia marcescens* sub sp. ATCC 13880 and its phylogenetic analysis also confirm by forming a similar clade with *Serratia marcescens* sub sp. ATCC 13880 (Fig. 9a). The 16S rRNA gene sequence of strain BKACT has been deposited in NCBI GenBank under accession number MT186165. At the same time, generated phylogenetic tree of strain NIO-1008 is very closely related to *Arthrobacter enclensis* NIO-1008 which was previously reported as a novel species (Dastager et al., 2014) (Fig. 9b). Several marine species of *Serratia* and *Arthrobacter* genus have been already known for the production of some bioactive compounds (Qin et al., 2014; Krishnan et al., 2016; Olanrewaju et al., 2017). Though, bacteria from marine habitats are rarely studied for plant growth promotion and crop protection. Therefore, both marine strains were further subjected to genome sequencing and validation of functional genes associated with the above attributes.



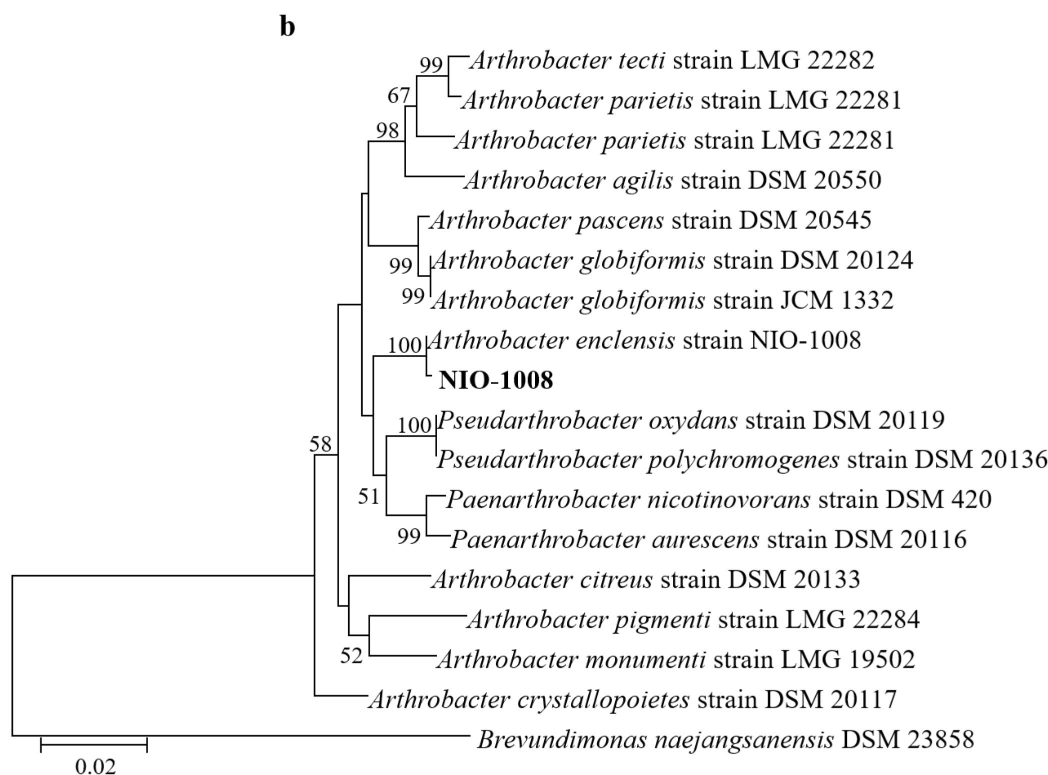


Fig. 9 A neighbour joining phylogenetic tree showing the relationship of marine antagonistic strains and their closest neighbours. The numerals at the branching points indicate the bootstrap values (%) obtained after 1000 replications. Bar, 0.02 substitutions per nucleotide position

3.6. Whole genome sequencing, assembly, and gene prediction

The Nanopore genome sequencing platform produced 174,396 processed strain BKACT reads with an average read length of 4,463bp. The mean coverage and read length N50 were 142 and 9,879, respectively. Both strains' genomes were assembled, as shown in (Table. 3), with the BKACT genome eventually completed in two contigs totaling 5,342,465 bp (N50 of 5233659). Strain NIO-1008 genome comprises 18 contigs with a total length of 4,226,231 bp (N50 of 528663). Based on the sequencing data, the estimated genome size for *Serratia marcescens* BKACT is 5.3 Mb and for *Arthrobacter enclensis* NIO-1008 4.2 Mb. On the other hand, when raw data was examined via Rapid Annotation and Subsystem Technology (RAST) version 2.0, 8526 and 3862 protein-coding genes were observed for BKACT and NIO-1008, respectively.

Table.3 Genome statistics of antagonistic marine strains

Genome features	*S. marcescens* BKACT	*A. enclensis* NIO-1008
Size	5,342,465	4,226,231
GC Content	59.8 %	67.1 %
N50	5233659	528663
L50	1	3
Number of Contigs (with PEGs)	2	18
Number of Subsystems	383	408
Number of Coding Sequences	8526	3862
Number of RNAs	108	60

The number of RNA genes in these two bacteria was 108 in strain BKACT and 60 in strain NIO-1008 (Table. 3). Furthermore, the genomes of both strains are represented in a Circos plot (Fig. 10), with GC content represented by the innermost circle, non-coding genes represented by the second circle from the inside, coding genes on the negative strand represented by the third circle from the inside, coding genes on the positive strand represented by the fourth circle, and contigs represented by the outermost circle.

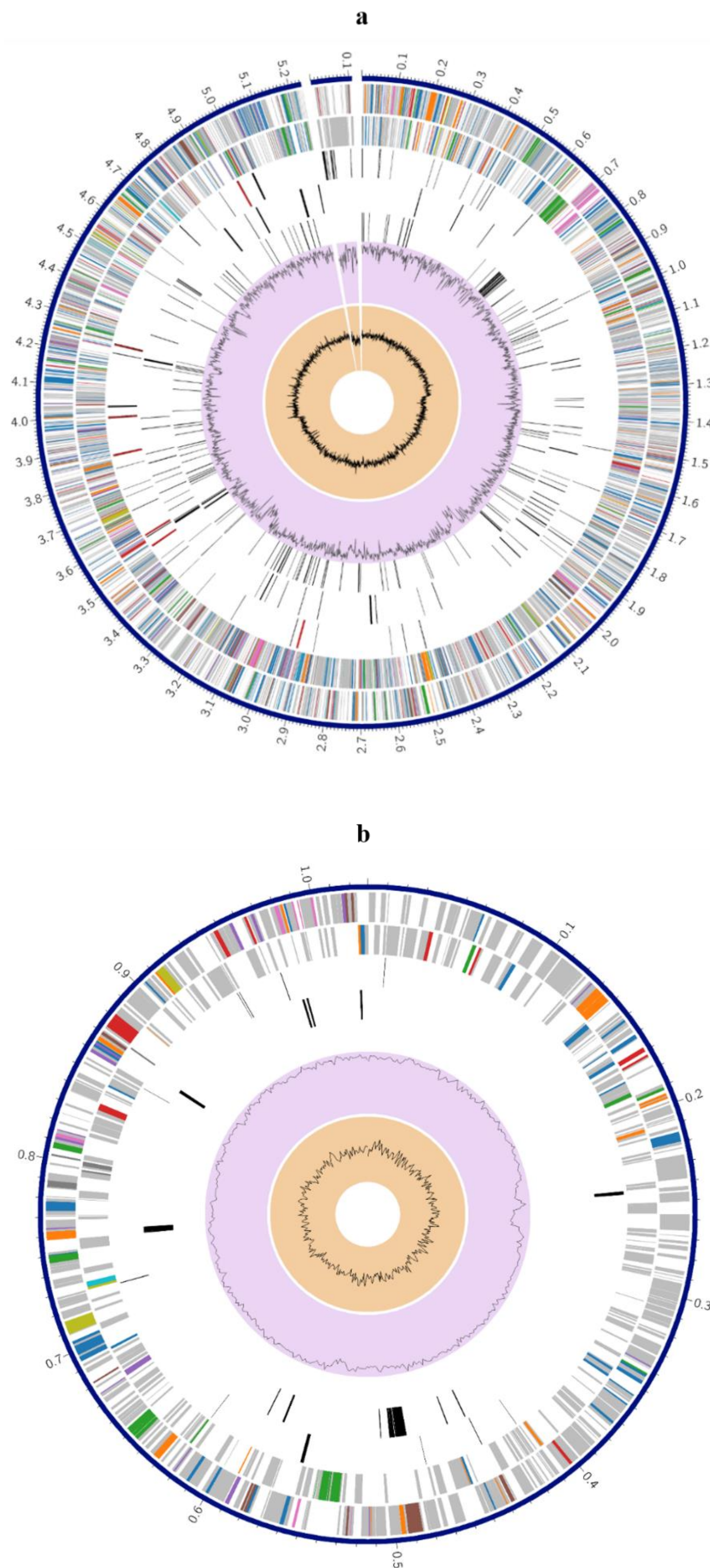
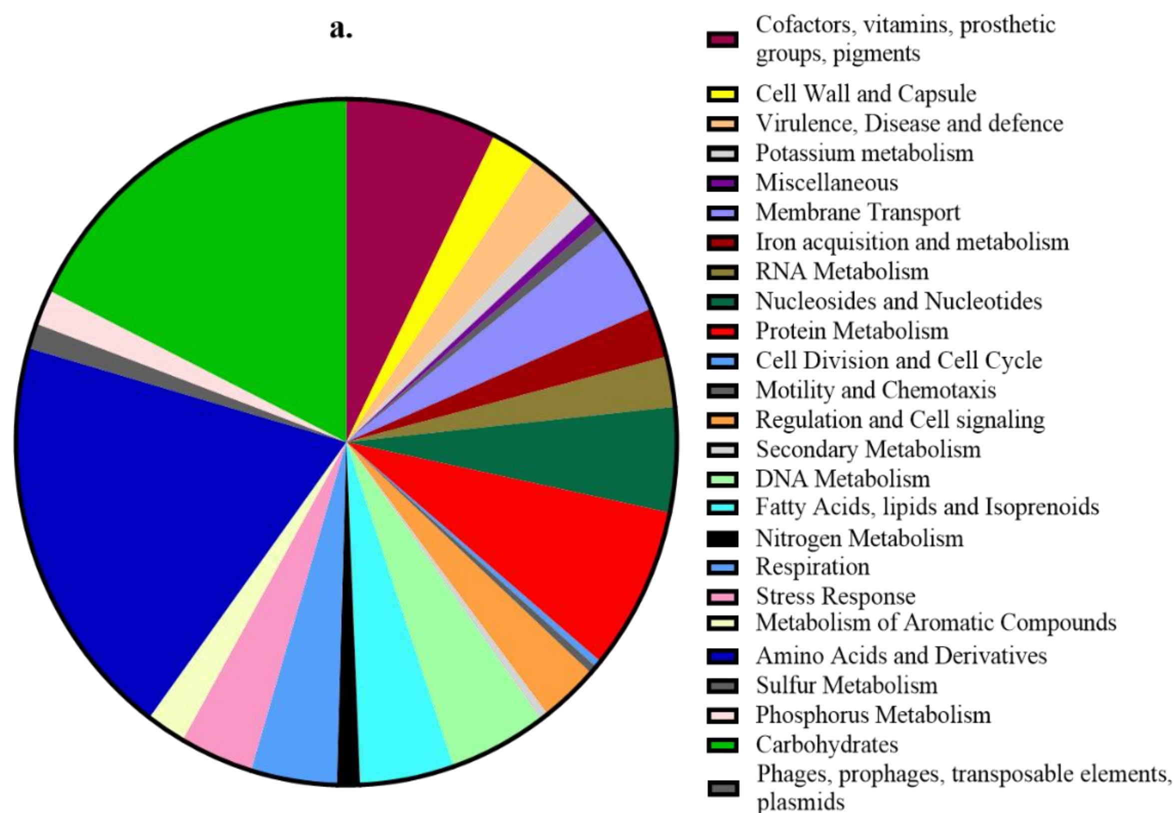


Figure. 10 Circos plot representing the genomes of, **a.** *S. Marcescens* BKACT and **b.** *A. enclensis* NIO-1008

Both bacteria SEED-annotated genomes were analysed with hundreds of other genomes retained in the SEED integration, and the RAST annotation platform also generated a pie chart that shows 25 functional categories. The pie chart was constructed using the RNA genes, repeat regions, and protein-encoding genes (PEGs) recognized by the RAST annotation system (Fig. 11). In both bacteria, it was found that certain genes and metabolic pathways were abundant, including those involved in the production of carbohydrates, amino acid derivatives, proteins, vitamins, pigments and cofactors. However strain BKACT has more genes for secondary metabolites, nutrition uptake, and amino acid derivative and carbohydrate pathways than strain NIO-1008.



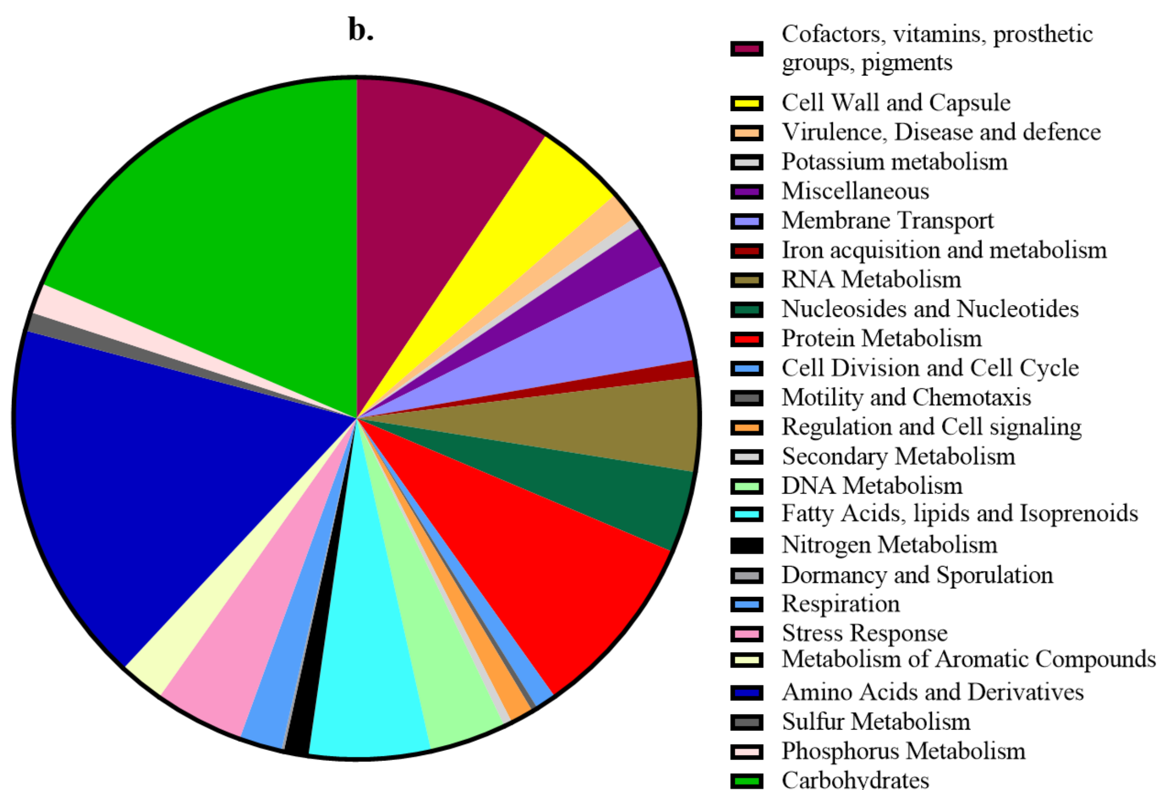


Figure. 11 SEED classification of strain BKACT and NIO-1008 genomes. Pie chart depicting 25 functional categories in (a) strain BKACT genome, (b) strain NIO-1008 genome

Genes associated with plant growth promotion, biocontrol activity, and stress tolerance

The genomes of both marine strains have been annotated and classified into 25 functional categories. Several protein-encoding genes are involved in a variety of biochemical processes, including nutrition acquisition, metabolism, respiration, stress tolerance, disease resistance, and many more. These genes are essential in plant development and stress control, either directly or indirectly. Abiotic stress caused by salinity is the most significant because it reduces soil water potential and makes it harder for plants to absorb nutrients and water (Qin et al., 2014; Khan et al., 2021). In response to osmotic stress, PGP bacteria activate osmosensitive K+ channels via the Kdp-ATP pathway, which maintains K+ ion homeostasis (Polarek et al., 1992). Furthermore, osmolytes and osmoprotectants such as trehalose, glycine-betaine, proline, choline, and periplasmic glucan are synthesized to maintain cell turgidity for plant growth and

development (Kumar et al., 2018 Soni et al., 2021). In our study, genes for osmoregulation (*Opg*, *Bet*, *Pro*, *tre* and *Kdp)* and polyamine biosynthesis (*AUH*, *AIH*, *SpdS*, *ArcD*) are identified in both strains BKACT and NIO-1008 (Table. 4).

Heavy metal-related soil pollution has recently raised severe concerns around the globe. Heavy metals are a leading cause of abiotic stress for plants and microorganisms. Reactive oxygen species (ROS) are generated at high concentrations of heavy metals and impede cell function by causing damage to DNA, RNA, and proteins. Uncontrolled ROS generation negatively impacts respiration rates, which hinders plant development (Rizvi et al., 2020; Kumar et al., 2018). On the other hand, the PGP bacterium generates antioxidant defense enzymes such as catalase, superoxide dismutase, peroxidase, and glutathione spermidine synthase to quench excess ROS and thereby prevent oxidative damage. Simultaneously, PGP bacteria dealt with heavy metal stress by employing a variety of effective mechanisms, including exclusion, active metal transport away from the cell, intracellular and extracellular sequestration, and enzymatic degradation into less toxic forms. For the reduction of heavy metal stress in bacteria and plants, heavy metals resistance proteins such as P-type ATPase, Cd(II) Pd(II) responsive transcriptional regulator, cobalt-zinc-cadmium protein, arsenic efflux pump protein, arsenate reductase, zinc uptake regulation protein, and chromate transporter are crucial (Li et al., 2019 and Soni et al., 2019). The aforementioned protein-encoding genes for heavy metal stress resistance (*CopA*, *CopD*, *CopC*, *czcD, TRCd, czcR, arsR, arsB, arsC, ChrA*, and *Znu*) and oxidative stress resistance (*sodC, KatE, KatG*, and *gss*) are likewise validated in both strains, according to genome analysis (Table. 4).

A further challenge in agriculture is nutrient deficiency, which lowers crop productivity. In this condition, plants rely primarily on helpful bacteria, which encode proteins in nutrient uptake. Nitrogen is abundant in the atmosphere, yet it is inaccessible to plants, thus beneficial bacteria transform the atmospheric nitrogen to its reduced form, such as ammonia, nitrate, and nitrite,

which is readily available to plants (Olanrewaju et al., 2017; Richardson et al., 2009). The nitrogen fixation potential of the bacteria was proven by protein-encoding genes in the genome such as *nif*, *NiR*, *NRT*, and *NAS* (Hallin et al., 2018). Except for the *nif* gene, all of these genes were found in both strains in our investigation (Table. 5). Despite the missing nif gene in the genome still, atmospheric nitrogen was fixed in some bacteria (Franco-Sierra et al., 2020).

Phosphorus is another vital soil nutrient, but it is complex with metal ions that plants cannot use (Richardson et al., 2009; Olanrewaju et al., 2017). The genomes of both strains BKACT and NIO-1008 were examined for genes-producing products such as alkaline phosphatase, phosphate starvation inducible protein, and transporters. At the same time, genes involved in sulphur absorption and transport were found in the genome (Table. 5). Iron is another essential mineral for plant growth and development since it participates in critical metabolic processes such as photosynthesis and respiration. It is the fourth most plentiful element on the planet, yet it is still limited to plants since it exists in an insoluble complex form. PGP bacterium released siderophore as an iron chelating agent in response to iron deficit, scavenging Iron from the soil for its host plants (Richardson et al., 2009 and Olanrewaju et al., 2017). The present investigation found genes for siderophore production and metabolism in both strains' genomes. A few common and separate genes for different kinds of siderophores were found in both strains. As a result, genes encoding siderophores such as (Enterobactin and Aerobactin) in BKACT and (Deferoxamine) in NIO-1008 were confirmed in the genome (Table. 5).

Phytohormones are key growth regulators produced in plants and bacteria. Indole-3-acetic acid, one of the most common naturally occurring auxin, plays an essential role in plant growth and stress management (Olanrewaju et al., 2017). IAA is synthesized in bacteria with either tryptophan dependant or tryptophan-independent pathways. The gene-encoding enzymes include aromatic L- amino acid decarboxylase, anthranilate phosphoribosyl transferase, phosphoribosyl anthranilate isomerase, monoamine oxidase, and auxin efflux carriers are

involved in IAA production and transportation (Zhang et al., 2021 and Soni et al., 2021) were identified in the genome. In addition, marker gene *ipdC* (Indole-3-pyruvate decarboxylase) was present in BKACT but missing in NIO-1008 (Table. 5).

Volatile organic compounds (VOCs) are key signaling molecules in PGP bacteria that play an essential role in plant disease management via induced systemic resistance (ISR) and indirectly improve plant development (Olanrewaju et al., 2017). Plant-beneficial bacteria such as *Pseudomonas*, *Bacillus*, *Serratia*, and *Arthrobacter* are known to produce 2, 3-butanediol and acetoin, which play critical roles in crop growth promotion and ISR (Farag et al., 2013). Genes for 2, 3-butanediol, and acetoin were found in the genomes of BKACT and NIO-1008 (Table. 2). In addition to VOCs, both strains were found to have the genes for lytic enzymes (chitinase and glucanase), and antifungal compounds (phenazine and salicylic acid) (Table. 5). Major lytic enzymes like chitinase and glucanase destroy the fungal cell wall, leading to cell lysis and death (Olanrewaju et al., 2017). Antifungal compounds like phenazine and salicylic acid in PGP bacteria are widely investigated for crop protection by inhibiting a broad spectrum of phytopathogens. The results above imply that marine strains can produce antifungal compounds in addition to stress tolerance and plant growth regulators.

Table. 4 The genome analysis of BKACT and NIO-1008 revealed genes associated to abiotic stress tolerance.

Gene Id of NIO-1008	Gene Id of BKACT	Gene name	Product name or function
Osmoregulation			
2032	3019	*OpgC*	Synthesis of osmoregulated periplasmic glucans
2082	3607	*BetA*	Choline dehydrogenase
2081	3608	*BetB*	Betaine aldehyde dehydrogenase
1995	3612	*BetT*	High-affinity choline uptake protein
1840	7638	*BetU*	Glycine betaine transporter
591	-	*ProW*	L-proline glycine betaine ABC transport system
1797	1700	*ProP*	L-Proline/Glycine betaine transporter
2071	-	*TPP6*	Trehalose-6-phosphate phosphatase
-	5447	*treC*	Trehalose-6-phosphate hydrolase
2106	-	*treP*	Trehalose phosphorylase
2801	5360	*treS*	Trehalose synthase
1227	4103	*KdpD*	Osmosensitive K+ channel histidine kinase

Oxidative stress			
1804	2437	*sodC*	Superoxide dismutase [Cu-Zn] precursor (Protection from ROS)
1175	344	*KatE*	Catalase
1819	637	*KatG*	Catalase-Peroxidase
39	-	*gss*	Glutathionyl spermidine synthase
Polyamine Production and Modulation of Ethylene Levels			
3533	7469	*AUH*	Agmatinase
2507	1997	*AIH*	agmatine deiminase
3078	2171	*SpdS*	Spermidine synthase
2381	1804	*ArcD*	arginine/ornithine antiporter
Metal resistance			
1513	4282	*CopA*	Lead, cadmium, zinc and mercury transporting ATPase
3303	-	*CopZ*	Copper chaperone
2101	2882	*CopD*	Copper resistance protein D
3051	2883	*CopC*	Copper resistance protein C
296	1935	*czcD*	Cobalt-zinc-cadmium resistance protein
1902	-	*TRCd*	Cd(II) Pd(II)- responsive transcriptional regulator
296	1935	*czcR*	Cobalt-zinc-cadmium resistance protein
2392	-	*arsR*	Arsenical resistance operon repressor
389	3827	*arsB*	Arsenic efflux pump protein
2390	8267	*arsC*	Arsenate reductase
180	2240	*ChrA*	Chromate transport protein
776	6865	*ZnuR*	Zinc uptake regulation protein
773	4340	*ZnuA*	Zinc ABC transporter, substrate binding protein
774	1444	*ZnuC*	Zinc ABC transporter, ATP-binding protein
775	1442	*ZnuB*	Zinc ABC transporter, inner membrane permease protein

Particular genes absent in genome denoted with (-)

Table. 5 The genome analysis of BKACT and NIO-1008 revealed genes associated to plant growth promotion and disease resistance.

Auxin			
3186	-	*Aad*	Aromatic-L-amino-acid decarboxylase
1371	-	*AprT*	Anthranilate phosphoribosyl transferase
1874	990	*PraI*	Phosphoribosyl anthranilate isomerase
1132	3912	*Aec*	Auxin Efflux Carrier
1960	1960	*MAO*	Monoamine oxidase
-	4659	*ipdC*	Indole-3-pyruvate decarboxylase
Nitrogen			
2770	6656	*NiR1*	Nitrite reductase large subunit
2774	6655	*NiR2*	Nitrite reductase small subunit
2782	1248	*NRT*	Nitrate/nitrite transporter
2781	-	*NAS*	Assimilatory nitrate reductase large subunit
-	1243	*NarL*	Nitrate/nitrite response regulator protein
-	1244	*NarX*	Nitrate/nitrite sensor protein NarX
Phosphorus			
2581	7241	*SAP*	Secreted alkaline phosphatase
-	2590	*PhnX*	Phosphonoacetaldehyde hydrolase
2711	4143	*PhoH*	phosphate starvation-inducible protein
2253	6156	*PstB*	Phosphate transport ATP-binding protein

2254	6159	*PstA*	Phosphate transport system permease protein
2255	6161	*PstC*	Phosphate transport system permease protein
2256	6162	*PstS*	Phosphate ABC transporter
2816	6155	*PhoU*	Phosphate transport system regulatory protein
Sulphur			
3290	3179	*SsuE*	FMN reductase
2843	5297	*Prc*	ABC-type nitrate/sulfonate/bicarbonate transport system
2842	6693	*TauD*	Alpha-ketoglutarate-dependent taurine dioxygenase
Siderophore biosynthesis and Iron acquisition			
2122	-	*DesD*	Siderophore synthetase component, ligase
2123	7344	*DesC*	Siderophore biosynthesis protein, monooxygenase
2124	7345	*DesB*	Siderophore biosynthesis protein, monooxygenase
2125	2108	*DesA*	Siderophore biosynthesis L-2,4-diaminobutyrate decarboxylase
897	-	*Pch*	ABC iron siderophore transporter
2917	1926	*PitA*	Ferric iron ABC transporter iron-binding protein
2918	7507	*PitC*	Ferric iron ABC transporter, permease protein
2919	1923	*PitD*	Ferric iron ABC transporter, ATP-binding protein
-	5689	*entC*	Isochorismate synthase
-	5686	*entB1*	Isochorismatase
-	5687	*entB2*	Isochorismatase
-	5688	*entE*	2,3-dihydroxybenzoate-AMP ligase
-	5694	*entF*	Enterobactin synthetase
-	5697	*ybdZ*	Enterobactin biosynthesis operon
-	5692	*entS*	Enterobactin exporter
-	7344	*lucD*	L-lysine 6-monooxygenase
-	7453	*FhuA*	Ferric hydroxamate outer membrane receptor
-	7456	*FhuC*	Ferric hydroxamate ABC transporter
3444	1214		Iron-chelator utilization protein
-	4774	*IutA*	Aerobactin siderophore receptor
Volatile compounds (Biosynthesis Acetoin and butanediol)			
3534	4988	*ALSg*	Acetolactate synthase large subunit
878	4987	*ALSs*	Acetolactate synthase small subunit
2585	-	*BDH*	2,3-butanediol dehydrogenase
-	79	*aldB*	Alpha-acetolactate decarboxylase
Antifungal compounds			
3289	2222	*PhzF*	Phenazine biosynthesis protein
697	2679	*crsM*	Chorismate mutase I
1475	6854	*pchB*	Isochorismate pyruvate-lyase
1913	-	*Hbh*	n-hydroxybenzoate hydroxylase
Lytic enzymes			
1944	1542	*ChiC*	Chitinase
2194	5967	*EgH*	Endoglucanase H

Particular genes absent in genome denoted with (-)

Conclusion

Due to global warming plant pathogenic fungi has emerging as the biggest problem in agriculture, hence crop production and quality is lost. The impact of fungal diseases varies with respect to the crop and the severity of the infection; in certain situations, diseases can cause full crop failure, leading to significant economic loss. To deal with fungal infections synthetic fungicides are frequently used, instead of having severe impact on the environment and human health. Thus, there is a need to develop sustainable and eco-friendly approaches for the management fungal diseases. In this regard, biocontrol bacteria are recognized as the safest alternative to chemical fungicides. In our investigation, marine strains BKACT and NIO-1008 were shown to be the more efficient antagonistic bacteria. Moreover, both strains are able to tolerate stress and produce a variety of PGP traits. Strains BKACT and NIO-1008 were identified as *Serratia marcescens* and *Arthrobactr enclensis*, respectively. The genes involved in the aforementioned activities (stress tolerance, plant growth promotion, and antifungal activity) were located in the genome of both strains. Based on these results, we propose that marine strains BKACT and NIO-1008 must be studied further for bioactive compound production and biocontrol applications.

References

Alves, A. R., Yin, Q., Oliveira, R. S., Silva, E. F., & Novo, L. A. (2022). Plant growth-promoting bacteria in phytoremediation of metal-polluted soils: Current knowledge and future directions. *Science of the Total Environment*, 156435.

Ameen, F., AlNadhari, S. Al-Homaidan, (2021). Marine microorganisms as an untapped source of bioactive compounds. *Saudi Journal of Biological Sciences*, *28*(1), 224-231.

Bhatnagar, I., Kim, S.K., 2010. Immense essence of excellence: Marine microbial bioactive compounds. *Mar. Drugs,* 8, 2673–2701.

Briard, B., Heddergott, C., & Latgé, J. P. (2016). Volatile compounds emitted by *Pseudomonas aeruginosa* stimulate growth of the fungal pathogen *Aspergillus fumigatus*. *MBio*, *7*(2), e00219-16.

Chakraborty, S., & Newton, A. C. (2011). Climate change, plant diseases and food security: an overview. *Plant pathology*, *60*(1), 2-14.

Dastager, S. G., & Damare, S. (2013). Marine actinobacteria showing phosphate-solubilizing efficiency in Chorao Island, Goa, India. *Current microbiology*, *66*, 421-427.

Dastager, S. G., Qin, L., Tang, S. K., Krishnamurthi, S., Lee, J. C., & Li, W. J. (2014). *A. enclensis* sp. nov., isolated from sediment sample. *Archives of microbiology*, *196*, 775-782.

De Coster, W., D'hert, S., Schultz, D. T., Cruts, M., & Van Broeckhoven, C. (2018). NanoPack: visualizing and processing long-read sequencing data. *Bioinformatics*, *34*(15), 2666-2669.

Franco-Sierra, N. D., Posada, L. F., Santa-María, G., Romero-Tabarez, M., Villegas-Escobar, V., & Álvarez, J. C. (2020). *Bacillus subtilis* EA-CB0575 genome reveals clues for plant growth promotion and potential for sustainable agriculture. *Functional & integrative genomics*, *20*(4), 575-589.

Hallin, S., Philippot, L., Löffler, F. E., Sanford, R. A., & Jones, C. M. (2018). Genomics and ecology of novel N2O-reducing microorganisms. *Trends in Microbiology*, *26*(1), 43-55.

Huang, R.H., Gou, J.Y., Zhao, D.L., Wang, D., Liu, J., Ma, G.Y., Li, Y.Q., Zhang, C.S., 2018. Phytotoxicity and anti-phytopathogenic activities of marine-derived fungi and their secondary metabolites. RSC Adv. 8, 37573–37580.

Khan, M. A., Sahile, A. A., Jan, R., Asaf, S., Hamayun, M., Imran, M., & Lee, I. J. (2021). Halotolerant bacteria mitigate the effects of salinity stress on soybean growth by regulating secondary metabolites and molecular responses. *BMC plant biology*, *21*(1), 1-15.

Kumar, A., & Verma, J. P. (2018). Does plant—microbe interaction confer stress tolerance in plants: a review? *Microbiological research*, *207*, 41-52.

Kolmogorov, M., Yuan, J., Lin, Y., & Pevzner, P. A. (2019). Assembly of long, error-prone reads using repeat graphs. *Nature biotechnology*, *37*(5), 540-546.

Liu, W., Sun, B., Yang, M., Zhang, Z., Zhang, X., Pang, T., & Wang, S. (2019). Antifungal activity of crude extract from the rhizome and root of Smilacina japonica A. Gray. *Evidence-Based Complementary and Alternative Medicine*, *2019*.

Li, L., Liu, Z., Meng, D., Liu, X., Li, X., Zhang, M., & Yin, H. (2019). Comparative genomic analysis reveals the distribution, organization, and evolution of metal resistance genes in the genus *Acidithiobacillus*. *Applied and environmental microbiology*, *85*(2), e02153-18.

Mitra, A., Zaman, S., Mitra, A., & Zaman, S. (2016). Threats to marine and estuarine ecosystems. *Basics of marine and estuarine ecology*, 365-417.

MohaMMadi, P., Tozlu, E., KoTan, R., & KoTan, M. Ş. (2017). Potential of some bacteria for biological control of postharvest citrus green mould caused by *Penicillium digitatum*. *Plant Protection Science*, *53*(3), 134-143.

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., & Hens, L. (2016). Chemical pesticides and human health: the urgent need for a new concept in agriculture. *Frontiers in public health*, *4*, 148.

Olanrewaju, O. S., Glick, B. R., & Babalola, O. O. (2017). Mechanisms of action of plant growth promoting bacteria. *World Journal of Microbiology and Biotechnology*, *33*(11), 1-16.

Ozdal, M., Ozdal, O. G., Sezen, A., Algur, O. F., & Kurbanoglu, E. B. (2017). Continuous production of indole-3-acetic acid by immobilized cells of *A. agilis*. *3 Biotech*, *7*(1), 1-6.

Polarek, J. W., Williams, G., & Epstein, W. (1992). The products of the kdpDE operon are required for expression of the Kdp ATPase of *Escherichia coli*. *Journal of bacteriology*, *174*(7), 2145-2151.

Qin, S., Zhang, Y. J., Yuan, B., Xu, P. Y., Xing, K., Wang, J., & Jiang, J. H. (2014). Isolation of ACC deaminase-producing habitat-adapted symbiotic bacteria associated with halophyte *Limonium sinense* (Girard) Kuntze and evaluating their plant growth-promoting activity under salt stress. *Plant and soil*, *374*(1), 753-766.

Raymaekers, K., Ponet, L., Holtappels, D., Berckmans, B., & Cammue, B. P. (2020). Screening for novel biocontrol agents applicable in plant disease management–a review. *Biological Control*, *144*, 104240.

Rima, F. S., Biswas, S., Sarker, P. K., Islam, M. D., & Seraj, Z. I. (2018). Bacteria endemic to saline coastal belt and their ability to mitigate the effects of salt stress on rice growth and yields. *Annals of Microbiology*, *68*(9), 525-535.

Rizvi, A., Zaidi, A., Ameen, F., Ahmed, B., AlKahtani, M. D., & Khan, M. S. (2020). Heavy metal induced stress on wheat: phytotoxicity and microbiological management. *RSC Advances*, *10*(63), 38379-38403.

Richardson, A. E., Barea, J. M., McNeill, A. M., & Prigent-Combaret, C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and soil*, *321*(1), 305-339.

Roberts, W. K., & Selitrennikoff, C. P. (1988). Plant and bacterial chitinases differ in antifungal activity. *Microbiology*, *134*(1), 169-176.

Sahu, J., Vaishnav, A., & Singh, H. B. (2020). Insights in Plant-Microbe Interaction through Genomics Approach (Part IV). *Current Genomics*, *21*(7), 479.

Schwyn, B., & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical biochemistry*, *160*(1), 47-56.

Soni, R., Rawal, K., & Keharia, H. (2021). Genomics assisted functional characterization of *Paenibacillus polymyxa* HK4 as a biocontrol and plant growth promoting bacterium. *Microbiological Research*, *248*, 126734.

Tchameni, S. N., Cotârleț, M., Ghinea, I. O., Bedine, M. A. B., Sameza, M. L., Borda, D., & Dinică, R. M. (2020). Involvement of lytic enzymes and secondary metabolites produced by *Trichoderma* spp. in the biological control of *Pythium myriotylum*. *International Microbiology*, 23(2), 179-188.

Velásquez, A.C., Castroverde, C.D.M., He, S.Y., 2018. Plant–Pathogen Warfare under Changing Climate Conditions. *Current biology*, *28*(10), R619-R634.

Zhang, D., Gao, T., Li, H., Lei, B., & Zhu, B. (2017). Identification of antifungal substances secreted by Bacillus subtilis Z-14 that suppress *Gaeumannomyces graminis* var. tritici. *Biocontrol Science and Technology*, *27*(2), 237-251.

Zhang, B. X., Li, P. S., Wang, Y. Y., Wang, J. J., Liu, X. L., Wang, X. Y., & Hu, X. M. (2021). Characterization and synthesis of indole-3-acetic acid in plant growth promoting Enterobacter sp. *RSC advances*, *11*(50), 31601-31607.

Zhao, Y., Selvaraj, J. N., Xing, F., Zhou, L., Wang, Y., Song, H., & Liu, Y. (2014). Antagonistic action of Bacillus subtilis strain SG6 on *Fusarium graminearum*. *PloS one*, *9*(3), e92486.

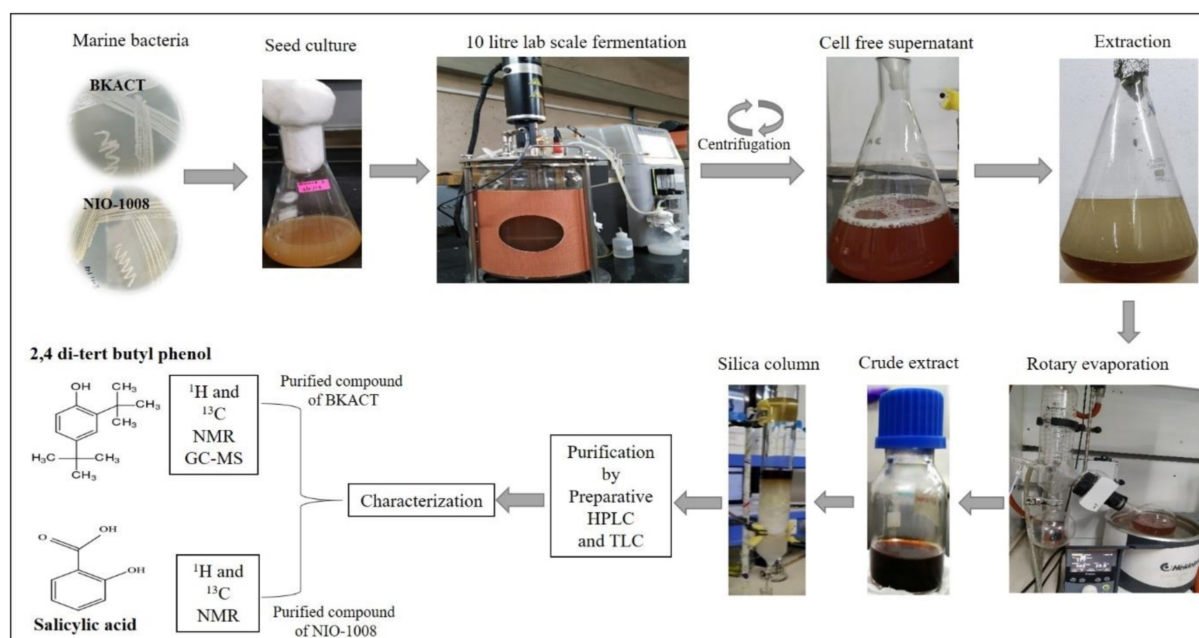
Chapter. 3:

Fermentation, purification and characterization of the bioactive compounds

Abstract

Based on genome analysis for antifungal compound associated genes, both marine isolates were investigated at flask level and lab scale fermentation. Fermentation yields, 9.6gm and 12.0gm crude extracts of strain BKACT and NIO-1008, respectively, and antifungal activity against *Fusarium* species was verified in their crude extracts. The crude extracts were purified using chromatographic methods such silica column, Prep HPLC, and Prep TLC. Simultaneously, NMR and GC-MS techniques were used for the structural characterisation. Based on structural elucidation, the active molecules from strains BKACT and NIO-1008 were confirmed as 2, 4-di-tert-butyl phenol and salicylic acid, respectively.

Graphical abstract

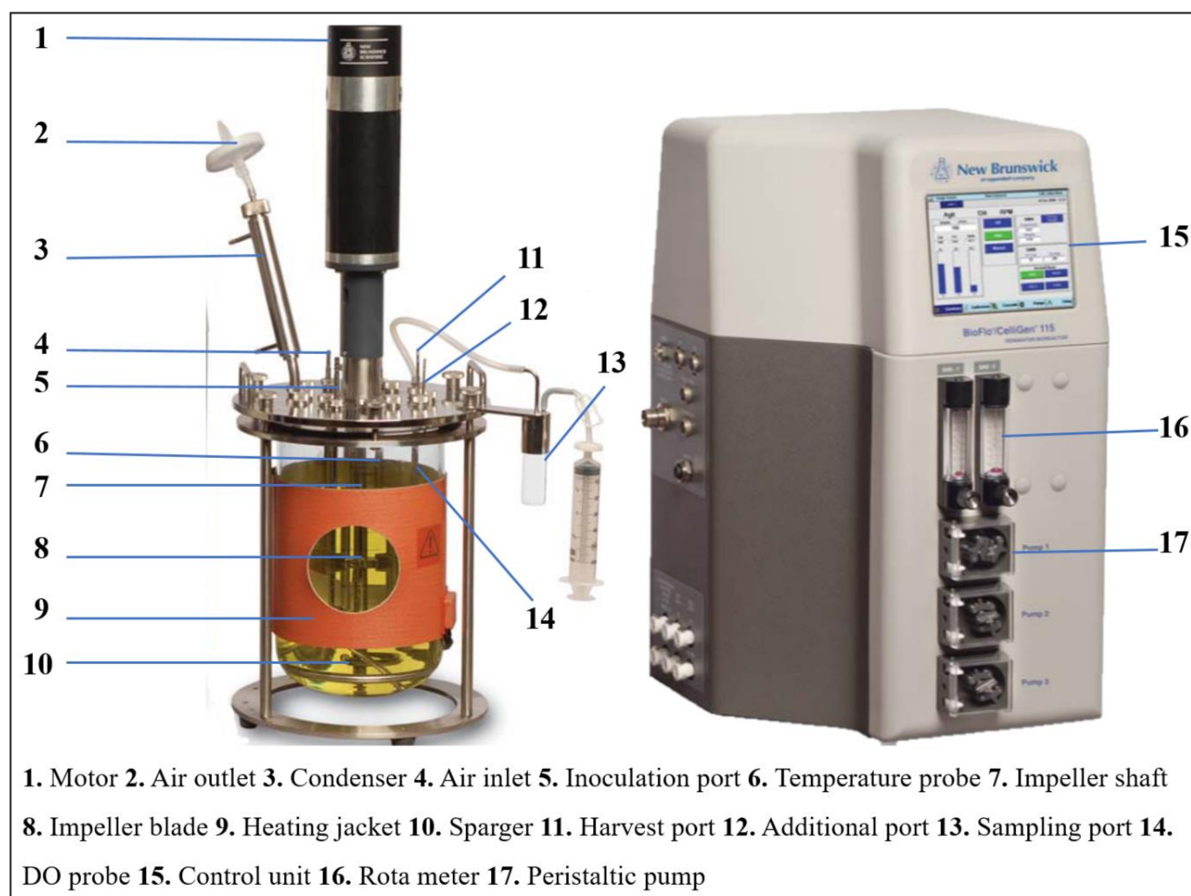


1. Introduction

Natural products are secondary metabolites produced by a variety of species, including plants, animals and microorganisms. Approximately half of all pharmaceutical drugs which are in use are directly derived or inspired by natural products. These chemically and physiologically diverse molecules are extremely important in the therapeutic, food, and agriculture sectors (Cragg et al., 2013). In the early 1900s, roughly 80% of active compounds for human benefit had been obtained from plant sources (Dias et al., 2012). Following the discovery of penicillin by Alexander Fleming in 1928, microorganisms started to replace plants as the primary source of need for active compounds. Since then, microbial bioactive molecules have been exploited in research, agriculture, medicine, and the food. Microbial natural products, which are distinct in their structural make-up and exhibit a range of action against a variety of diseases. Consequently, they always have advantages over competing organisms in terms of environmental resilience (Demain et al., 2009).

Earlier, the commercial supply of natural products were based on extraction from natural sources such as plants, which has several constraints such as seasonal and geographical changes in plant chemical content and difficulty in quality control (Wu X et al., 2020). Thus, reliable and sustainable contemporary biotechnological approaches are required to obtain bioactive molecules. In this context, genetically modified organisms or wild-type organisms produce target molecules in fermenter under strictly regulated conditions. The purpose of a fermenter is to provide an environment where microorganisms or cells can transform substrate into the desired product. Typically, it is made up of several components, including a vessel, agitation system, aeration system, temperature control, foam control, and pH control (Fig. 1). All of these components are required for the bioreactor to function properly. For instance, agitation promotes gas mixing, air dispersion, heat transfer, facilitates oxygen transfer, and maintains a homogeneous environment in the vessel (Nienow et al., 2014). Additionally, in order to reduce

the possibility of contamination, a foam control system is also crucial. Furthermore, a temperature and pH control system is essential for the optimum growth of microorganism and cells.



1. Motor **2.** Air outlet **3.** Condenser **4.** Air inlet **5.** Inoculation port **6.** Temperature probe **7.** Impeller shaft **8.** Impeller blade **9.** Heating jacket **10.** Sparger **11.** Harvest port **12.** Additional port **13.** Sampling port **14.** DO probe **15.** Control unit **16.** Rota meter **17.** Peristaltic pump

Figure. 1 Bioreactor and its components

2. Materials and methods

2.1. Chemical and media

Agar powder, Potato dextrose media, Hexane, Pet ether, Ethyl acetate, Dichloromethane, Methanol, Antifoam agent, Hydrochloric acid, KH2PO4 buffer, Acetonitrile, Trifluoroacetic acid, Deuterated chloroform, Salicylic acid, 2, 4-di-tert butyl-phenol, and Tween-80 solution. The majority of the chemicals and solvents were ordered from Hi-media and Sigma Aldrich, as aforementioned.

Table. 1 List of production media and their compositions

Sr. no	Media	Composition (g/L)
1	Nutrient broth	Standard ready to use media (Hi-media,Mumbai)
2	Zobell marine broth	Standard ready to used media (Hi-media, Mumbai)
3	King's broth	Glycerol 30.0, Peptone 10, K_2HPO_4 0.59 and $MgSO_4.7H_2O$, 0.5
4	Pigment producing medium	Glycerol 10.0, Peptone 10.0 and yeast extract 10.0
5	Medium 5294	Starch 10, Yeast extract 2.0, Glucose 10, glycerol 10, corn steep liquor 2.5, peptone 2.0, NaCl, 1.0 and $CaCO_3$, 3.0
6	Medium 5254	Glucose 15.0, Soymeal 15.0, corn steep liquor 5.0 , NaCl 5.0 and $CaCO_3$ 2.0
7	Medium 5333	Starch 15.0, Yeast extract 4.0, K_2HPO_4 1.0 and $MgSO_4.7H_2O$ 0.5

2.2. Flask level fermentations and parameters optimization for the bioactive compounds

Based on antagonistic activity, two promising marine strains BKACT and NIO-1008 were investigated for the production of bioactive compounds initially at the flask level. A single colony of antagonistic strains from a fresh nutrient agar plate was inoculated into the nutrient broth as a seed culture and incubated on a rotary shaker at 28°C and 140 rpm for 18 hours. Following that, 5% inoculum was added to 100 mL of each of the seven different production mediums listed in Table.1 and incubated at 28°C for five days. The cells were removed by centrifugation at 10,000 rpm for 10 minutes after five days of incubation. The antifungal efficacy of the cell-free supernatants was evaluated against *Fusarium* species.

The best antifungal activity in the supernatant was the criteria for choosing the optimal production medium for further studies. Following that, nutrient broth for strain NIO-1008 and king's broth for strain BKACT were chosen, while the other parameters remained the same as previously described. After five days of fermentation, the cell-free supernatants were extracted with an equal volume of organic solvents such as hexane, ethyl acetate and dichloromethane. Using a rotary evaporator, the organic phase was concentrated and the crude extracts were tested against *Fusarium* species

Simultaneously, the percent inoculum of both strains were standardised by adding 1.0 to 10.0% active seed culture. Furthermore, applying all of the standardised parameters, incubation periods for the production of bioactive compounds were optimised. Both strains were grown for varying lengths of time, ranging from day one to day eight. Based on the activity in the crude extract, the ideal inoculum and incubation time was selected.

2.3. Ten-litre lab-scale fermentation

Based on preliminary antifungal activity in their crude extracts, the active compounds were produced in a 14.0L lab-scale fermenter (BioFlo/CelliGen 115) with a 10.0L working volume. Production medium, percent inoculum, incubation time and incubation temperature was remained same as per optimization. Other condition, such as dissolved oxygen (pO2), were maintained above 50% during fermentation by applying 250 to 350 rpm agitation until the completion of the batch. After five days of incubation, cells were separated via centrifugation at 10,000 rpm for 10 min. The recovered supernatant was then acidified to pH 2.0 with 4N HCl and extracted two time with an equal amount of ethyl acetate. At the same time, the organic phase was collected and concentrated in a rotary evaporator, and the crude extracts was then processed for purification.

2.4. Antifungal activity of the crude extracts

The antifungal activity of the crude extracts of both strains were assessed using the well diffusion method (Magaldi et al., 2004). The test fungal spore suspension was prepared in 0.01% Tween 80 solution from a 7-day-old culture. The final concentration of spore suspension was adjusted to 1.0 x10^6 CFU/mL in sterile semisolid PDA and added in sterile petri dishes after proper mixing. Following solidification, 100 µL of crude extract was added into each well, and solvent was serve as a control. The plates were incubated at 28°C for 72–96 hours, and the zone of inhibition was measured in order to assess antifungal activity in millimetres (mm).

2.5. Thin-layer chromatography (TLC) based Bio-autography

According to (Grzelak et al., 2016), bio-autography was carried out on a TLC plate using silica gel 60. (Merck, Darmstadt, Germany). The crude extract (30µL) from both strains was spotted 1.0 cm apart from the baseline into a separate silica gel plate and allowed to dry. The plate was then developed in a previously saturated glass chamber at room temperature with a 70:30 mixture of ethyl acetate and pet ether. The developed plate is dried at room temperature, and the spots are examined in a UV chamber at 254 nm. The crude extract is bio-autographed to assess the antifungal activity of the separated compounds on the TLC plate. Further, TLC plates were sterilized by UV light for 30 min in laminar airflow and placed in a petri dish. Following that, 20.0 mL of 0.8% semisolid potato dextrose agar was mixed with 1.0 x10^6 spores/mL of *Fusarium* species and poured over the TLC plates in the petri dishes. After proper solidification, the plates were incubated for 72-96 h to detect the zones of inhibition of active fractions separated on TLC plates.

2.6. Purification

2.6.1 Bioactive compounds purification from BKACT

The crude extract of 9.6 gm was purified using column chromatography on a (45× 7.5 cm) column filled with 120-200 mesh silica. To confirm that the column was packed properly, it was eluted with dichloromethane (DCM). The crude extract was combined with a 60-120 mesh silica bed and applied uniformly from the top of the column with a mixture of Dichloromethane and methanol (100 to 0% DCM with a 0 to 100% MeOH ratio) in increasing polarity order. From a 40 mL volume, 126 fractions were collected, and the TLC profiles were examined for all the fractions. Based on their Rf values, all 126 fractions were combined into nine fractions, and their antifungal activity was evaluated. The partially pure active fraction number-3, was used for further purification via smaller silica column (35×3.0 cm). The column was eluted using a gradient of pet-ether and ethyl acetate, with increasing polarity with ethyl acetate. The fractions were collected in 86 tubes, each holding 25mL, and the tubes were pooled into five fractions based on the similarity in TLC profiles. Following that, the partially pure active fraction number F-3(II) was subjected to further purification using preparative thin-layer chromatography (PTLC) on pre-coated Silica Gel 60_{254} plates (20×20 cm, Merck). The PTLC plates were developed in a 70:30 mixture of pet ether and ethyl acetate, and after air drying, they were examined under UV light (254 nm). The desired band was extracted with ethyl acetate after being scratched off the PTLC plate. The solvent was vacuum evaporated, and the weight of a pure compound was measured.

2.6.2 Bioactive compounds purification from NIO-1008

The column was packed with 120-200 mesh silica, and a blend of 12 gm crude extract and 60-120 mesh silica was placed properly onto the top of the column, without using vacuum uniform band generated. Gradient elution of the column was carried out with the combination of Dichloromethane and methanol with increasing order of polarity. A total of 105 fractions were

collected in glass tubes and merged into seven fractions based on similarity in TLC profiles. Subsequently, all seven fractions were tested against *F. oxysporum* NCIM 1281. Further, the active fractions 2 and 3 merged in one and were subjected to preparative HPLC (Thermo Scientific Dionex Ultimate 3000) coupled with a fraction collector and UV detector. The RP-C18 (symmetry 300^{TM} 19 × 100 mm) column was used for the gradient of elution from 60% water for 15 minutes, 0% for 5 minutes and 90% for the final 5 minutes with acetonitrile over 30 min, with a flow rate of 10 mL/min and total eight fractions were collected manually based on peak profile. Among the collected peak, the active peak numbers 7 and 8 merged based on similarity in the TLC profile and were further subjected to purification in a small silica column. Gradient elution of the column was carried with a mixture of pet ether and ethyl acetate in increasing order of polarity. A total of five fractions were collected; among these, active fraction number two was purified by preparative TLC.

2.7. Characterization

2.7.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

The structure of the purified compounds was established by nuclear magnetic resonance (NMR). In deuterated chloroform ($CDCl_3$), the ^1H and ^{13}C NMR spectra were obtained on Bruker AV 500 MHz and 125 MHz spectrometer. The chemical shifts were provided in δ and parts per million (ppm) values that compared to the chloroform solvent at δ 7.27 in ^1H and 77.00 ppm in ^{13}C NMR.

2.7.2 Gas Chromatography-Mass Spectrophotometry (GC-MS)

Purified compound was prepared as a 1.0 mg/mL stock in methanol of GC-MS grade. From the stock, 1.0 µL of sample 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 were 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 withhold for 2 min, then rising with a ramp of 5°C/ min up to 180°C. Then 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 splitless mode. The axillary 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 compared with reference compounds were identified as the possible compounds.

2.7.3 High-Performance Liquid Chromatography (HPLC)

For the comparative analysis, a 1.0 mg/mL stock of purified compounds, standard salicylic acid (CAS: 69-72-7, Sigma-Aldrich, Switzerland), and standard 2, 4-di-tert butyl-phenol (2, 4-TBP, CAS: 96-76-4, Sigma-Aldrich, Switzerland) were prepared in HPLC grade methanol. Thermo Scientific Dionex Ultimate 3000 HPLC was used to analyse the pure compound of strain BKACT and its reference chemical 2, 4-DTBP by using C18 column (4.6×250mm, 5µm particle size thermo hypersil gold). The mobile phase was a mixture of methanol, water, and 0.1% trifluoroacetic acid (TFA), with a flow rate of 1.0 mL/min, and the detection wavelength was 254 nm. The 5.0µL samples were injected in isocratic mode with 80:20 of methanol and water for 14 minutes.

The purified compound of strain NIO-1008 and standard salicylic acid were analysed simultaneously on the same system using distinct mobile phase consisting of KH2PO4 buffer,

methanol, and acetonitrile (20:30:50) with a flow rate of 1.0 mL/min. In the isocratic mode, a 5.0 µL samples were injected on C18 column for 15 minutes.

2.7.4 Thin Layer Chromatography (TLC)

Additionally, the Rf values for both compounds were determined by comparing them using TLC in ethyl acetate: pet ether 70:30) as the solvent system.

3. Result and discussion

3.1. Fermentation parameters optimization

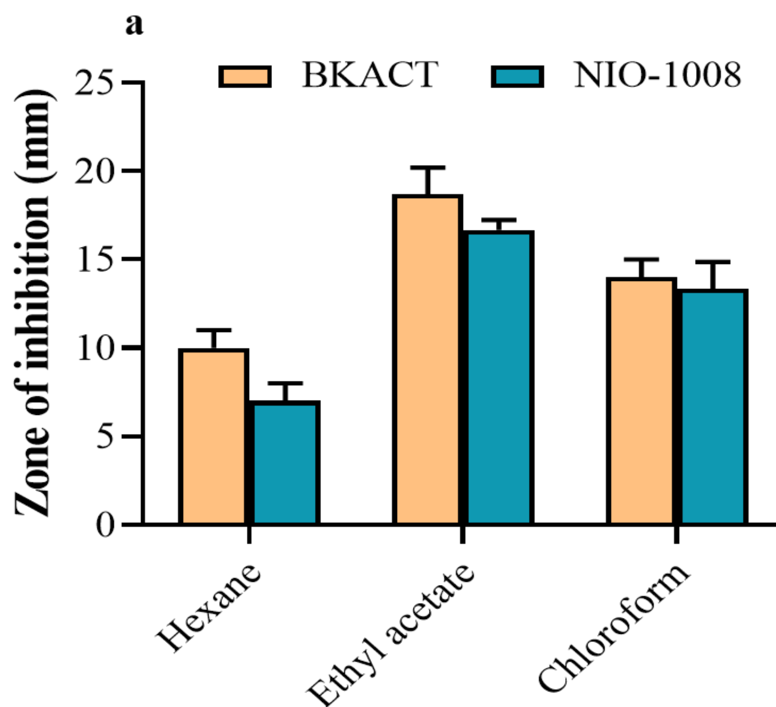
Fermentation parameters were optimised at the flask level in order to produce bioactive compounds from prospective marine strains. The king's and nutrient broth media were proven as the best production mediums for the strains BKACT and NIO-1008, respectively, among the seven different mediums tested (Table. 2). Simultaneously, ethyl acetate was found as the ideal organic solvent for antifungal compound extraction in both strains (Fig.2a). The strains BKACT and NIO-1008 had the maximum antifungal activity in the crude extract at 5% and 6% inoculum, respectively (Fig.2b). Furthermore, the highest antifungal activity in both strains were reported on the fifth day of incubation (Fig.2c).

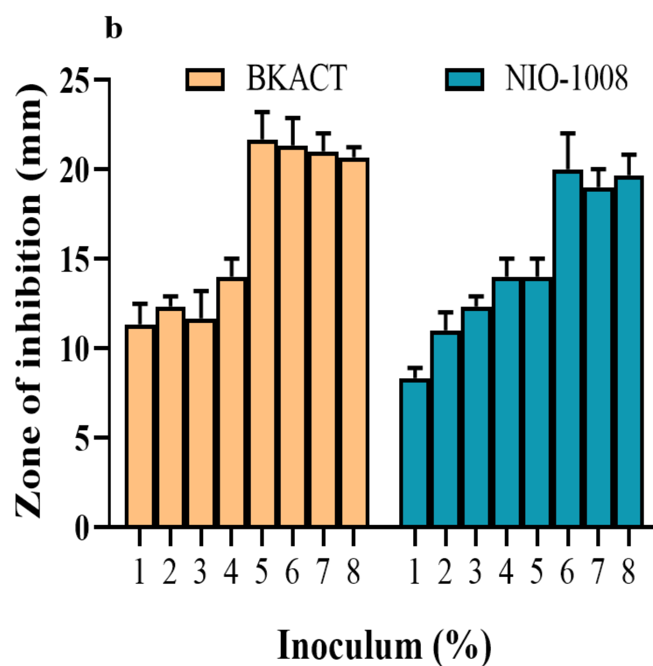
Table. 2 Selection of production medium based on the antifungal activity

Sr. No	Production media	Strain BKACT Vs Fusarium sp. NCIM 1330	Strain NIO-1008 Vs Fusarium sp. NCIM 1281
1	**Nutrient broth**	-	++
2	Zobell marine broth	+	-
3	**King's broth**	++	+
4	Pigment producing medium	+	-

5	Medium 5294	-	-
6	Medium 5254	+	-
7	Medium 5333	-	+

Zone of inhibition millimetre (mm) diameter (+ <10), (++ >10) and (- absent)





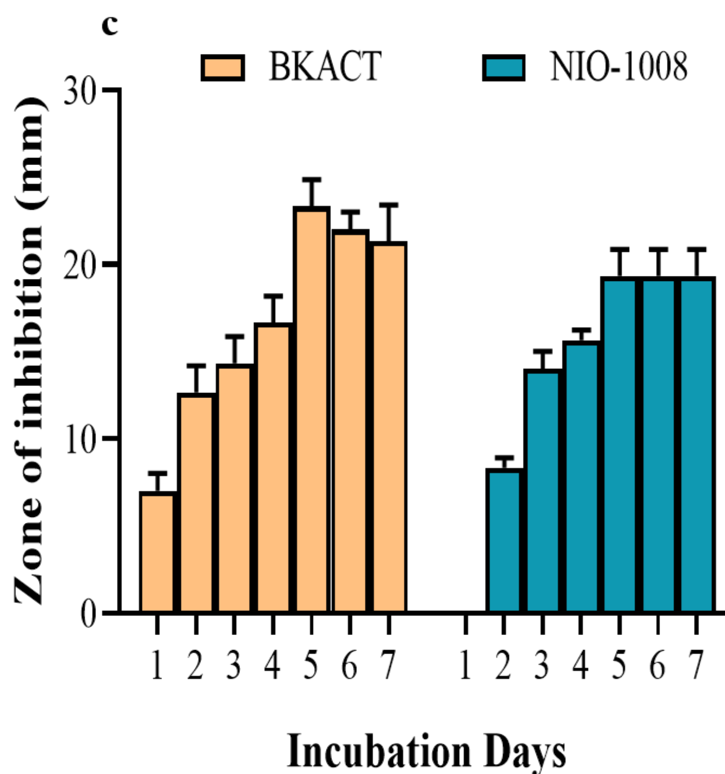


Figure. 2 Optimization of the fermentation parameters for bioactive compounds **a.** Extraction of bioactive compounds in different solvents, **b.** Percent inoculum optimization and **c.** Incubation time selection

3.2. TLC-based Bio-autography

TLC bio autography was used to detect antifungal fractions in the crude extracts. Under UV 254 nm, six bands in strain BKACT and five bands in strain NIO-1008 crude extracts were observed on the TLC. Among these, band number 5 in BKACT and band number 3 in NIO-1008 were identified as active fraction against *Fusarium* species (Fig. 3a &b).

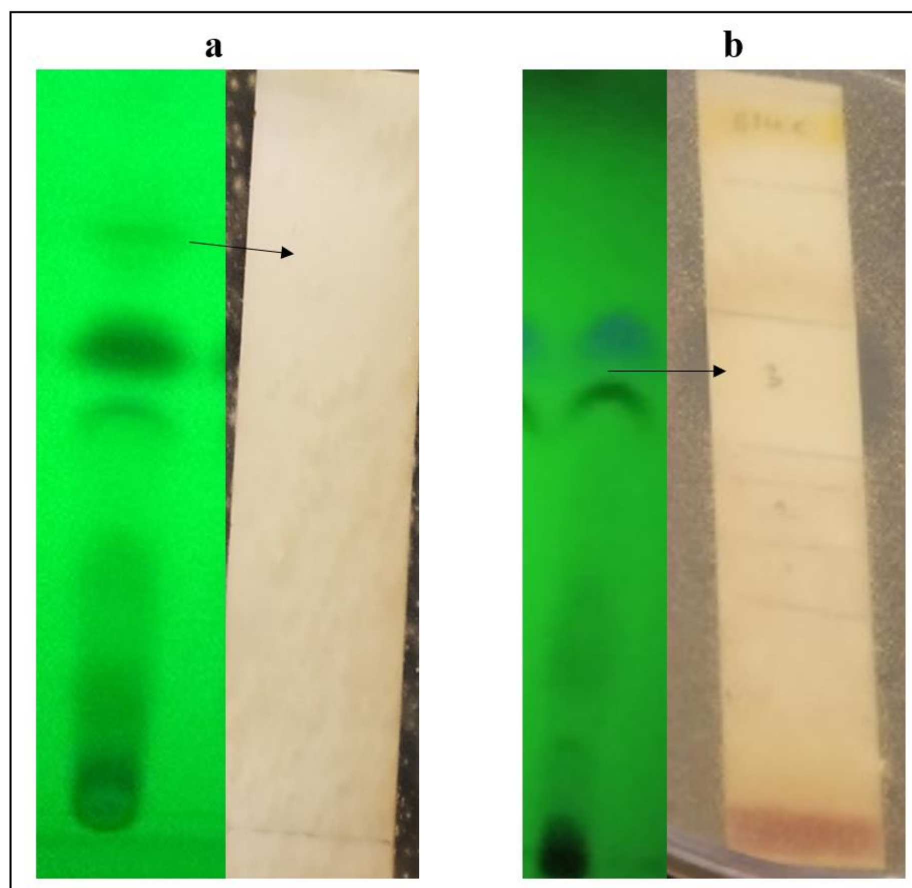


Figure. 3 TLC profile and its bio autography images **a.** Crude extract of the strain BKACT against *F. foetens* NCIM 1330 and **b.** Crude extract of the strain NIO-1008 against *F. oxysporum* NCIM 1281

3.3 Fermentation, purification and characterization

3.3.1 Bioactive compounds from strain BKACT

The marine isolate *S. marcescens* BKACT was identified as the possible antifungal candidate in the current investigation to inhibit *Fusarium* species. Previously, the antifungal activity of *S. marcescens* was mostly described due to its indigenous chitinase-producing competence (Ordentlich et al., 1988; Someya et al., 2001; Dhar Purkayastha et al., 2018). Although it was recently shown that the chitinase mutant *S. marcescens* still has the capacity to destroy fungi (Hover et al., 2016). As a result, we speculated that compounds other than the chitinase enzyme may be involved in an antifungal action.

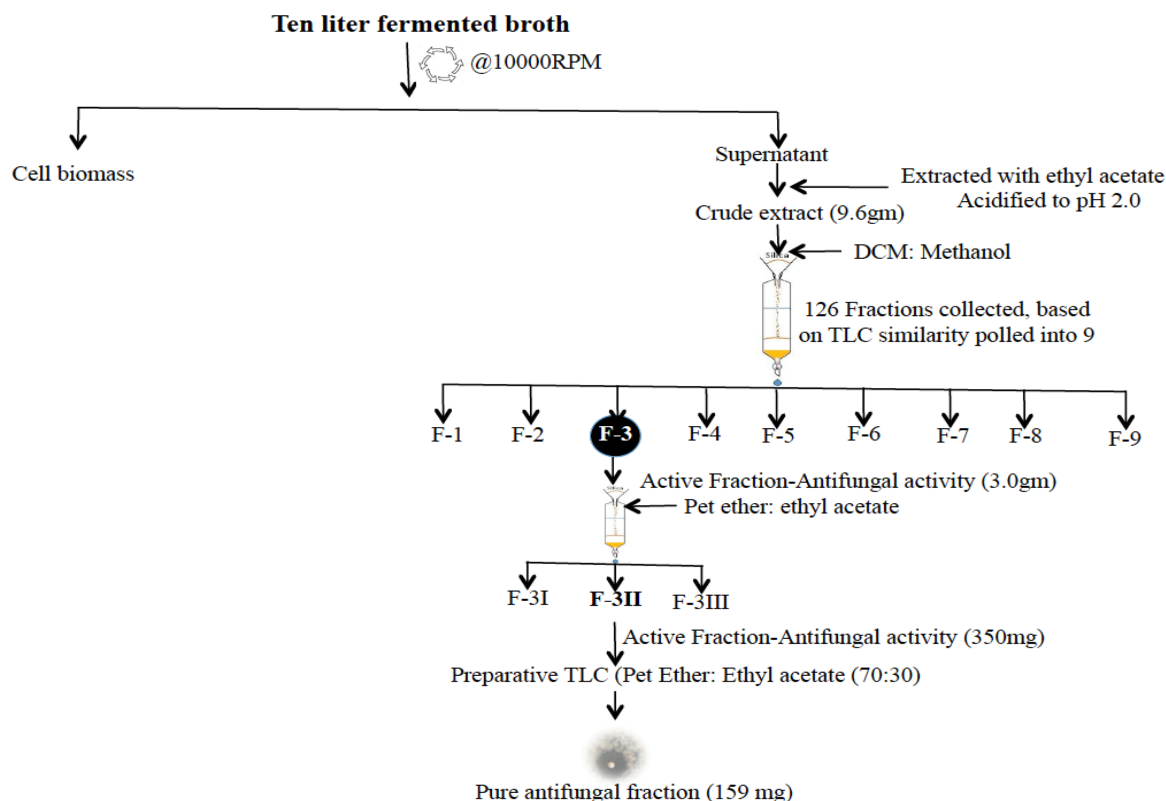


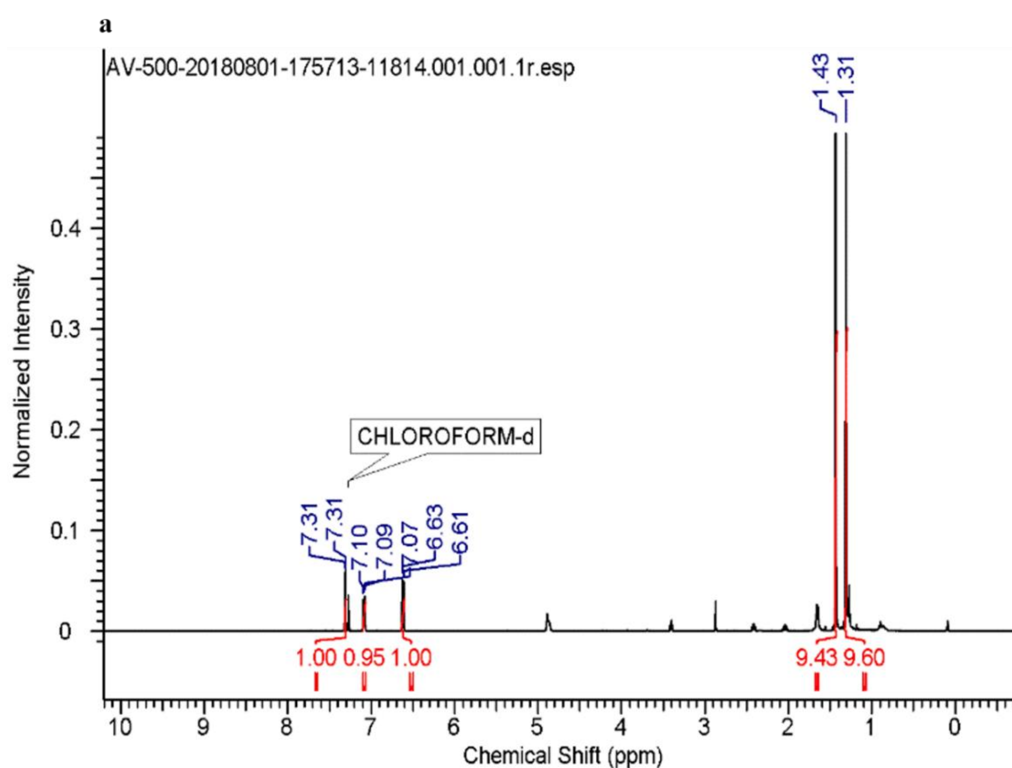
Figure. 4 Schematic diagram for the purification of bioactive compound from crude extract of BKACT

As a consequence, strain BKACT is challenged to bioactive compound production in a 10.0L lab-scale fermenter. From the 10.0L fermentation batch, 9.6 gm of crude extract was extracted, and the antifungal activity was reconfirmed in the crude extract. The detail concerning the purification of an antifungal compound is schematically presented in (Fig. 4). Finally, the isolated antifungal compound was studied using HPLC, NMR, and GC-MS methods.

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 methyl's at δ 1.43 (9H, s, 3 × CH_3) and 1.31 (9H, s, 3 × CH3) 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, confirming the benzene ring's presence. The 151.6 ppm of phenol substitution on the benzene

ring and 29.6 and 31.6 ppm indicate 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 (Fig. 5).

GC-MS analysis identified the purified compound of the BKACT strain as 2, 4-di-tert butyl-phenol. The mass spectra of the identified peaks (16.5) of purified compound was compared with peaks from the NIST mass spectral data to validate the chemical structures of the detected compound as 2, 4-di-tert butyl-phenol. The Ion [M] + at m/z 206 and the fragment ion [M - CH3] + at m/z 191 are signature spectra of 2, 4-di-tert butyl-phenol in GC-MS (Fig.6).



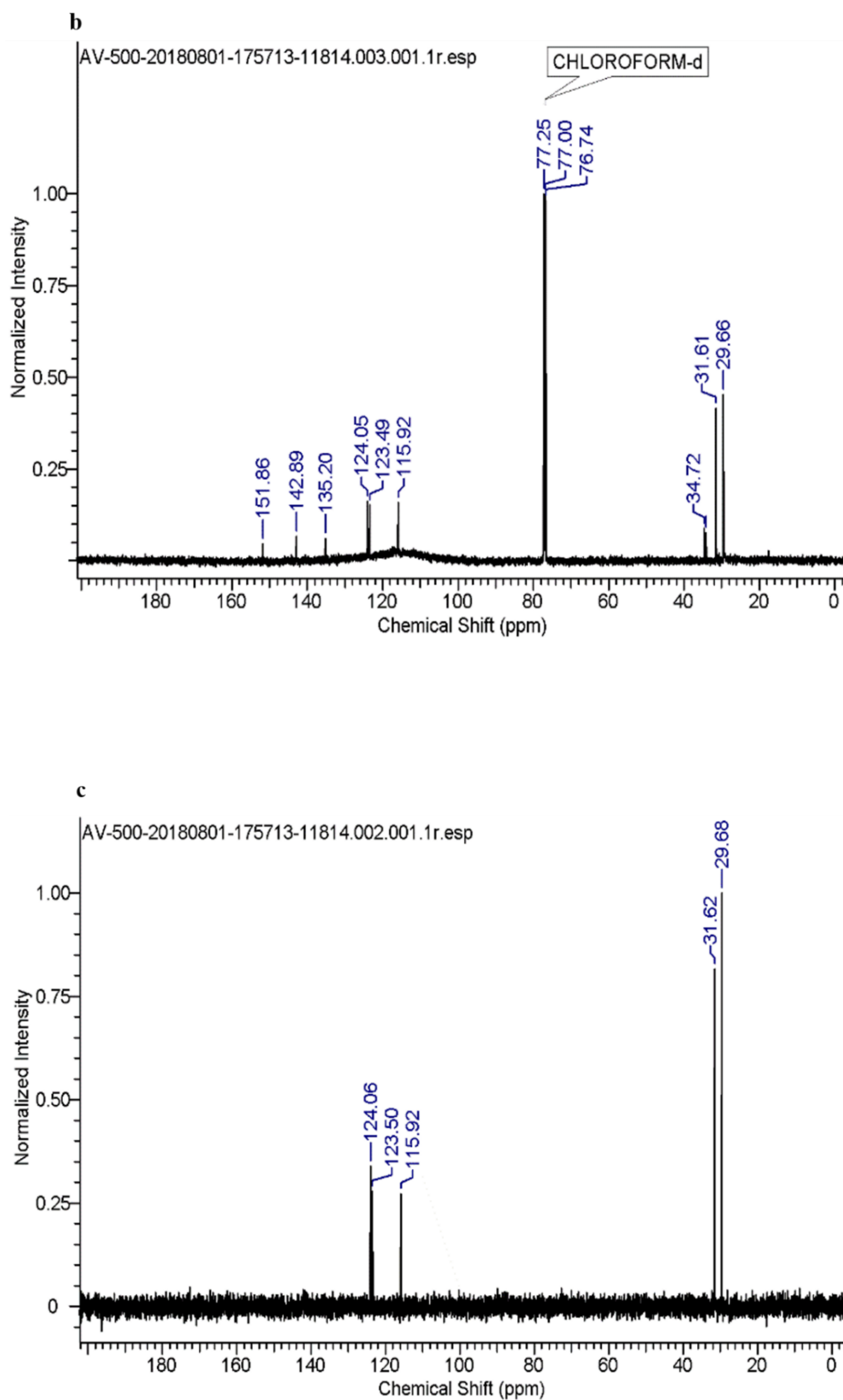


Figure. 5 Structure elucidation of purified compound from strain BKACT by NMR, **a.** ^{1}H NMR spectrum, **b.** ^{13}C NMR spectrum, **c.** DEPT NMR spectra

In HPLC and TLC analysis, the purified compound was validated against reference compound. Peak profiles with a similar retention time of 7.8 min in HPLC and the identical Rf on TLC plate verified the purified compound as the 2, 4-di-tert butyl-phenol once compared to the standard molecule (Fig.7a &b). Several studies have indicated that 2, 4-DTBP from *B. subtilis* CF-3, *P. monteilii*, and *Lactococcus* sp. is efficient against fungi that are pathogenic to plants (Dharni et al., 2014; Varsha et al., 2015; Wang et al., 2021). There have been no investigations on the purification and characterisation of 2, 4-DTBP from *S. marcescens*. To the best of our knowledge, this is the first study to purify and characterize 2, 4-DTBP from the marine *S. marcescens*.

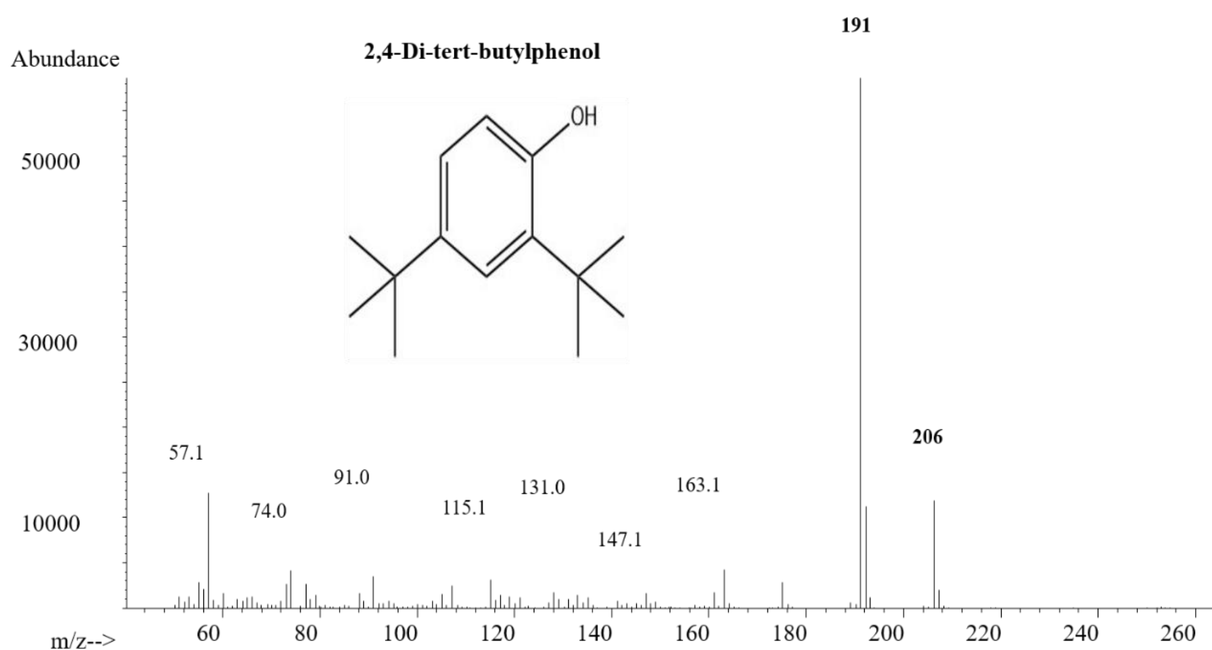


Figure. 6 GC-MS profile of purified compound, Ion [M] + at *m/z* 206, the fragment ion [M –CH3] + at *m/z* 191 are characteristic for 2, 4 di-tert butyl phenol

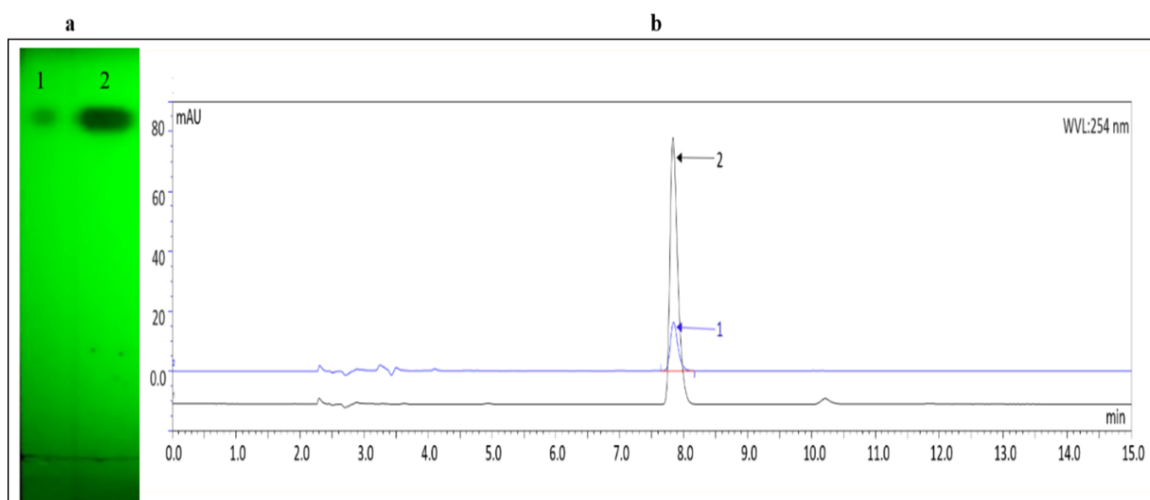


Figure. 7 Identification of purified compound by HPLC and TLC **a.** TLC image shows the compound 1 &2 has the same Rf value (1. purified compound, 2. Standard 2, 4 di-tert butyl phenol) and **b.** Chromatogram showing the compound 1 &2 eluted at same retention time 7.847

3.3.2 Bioactive compounds from strain NIO-1008

In addition to strain BKACT, the strain NIO-1008 was also proven as a potent antagonistic bacterium against *Fusarium* species. Besides antifungal activity in crude extract, genes related with antifungal compounds such phenazine, benzoic acid, and salicylic acid were also revealed in the genome of strain NIO-1008. Bioactive compounds were produced in a lab-scale fermenter. As a result, 12.0gm of crude extract was extracted from the cell-free broth, and its further purification was schematically shown in (Fig.8.). Structural elucidation of the purified compound was completed by NMR spectroscopy. The ^{1}H NMR spectra of a purified compound showed four signals of aromatic protons at 7.95 (d, J = 7.9 Hz, 1 H), 7.54 (t, J = 7.8 Hz, 1 H), 7.03 (d, J = 8.4 Hz, 1 H), 6.96 (t, J = 7.5 Hz, 1 H) which confirms the di-substituted benzene. The peak at 10.37 (br. s., 1 H) in ^{1}H NMR and 174.9 in ^{13}C NMR shows the presence of the acid functional group. The ^{13}C NMR spectra show that three quaternary carbon and four methines were present. Also, in ^{13}C, NMR d162.2 indicate the presence of the phenolic OH group (Fig. 9). Based on NMR data and literature reports, the structure was confirmed as the salicylic acid.

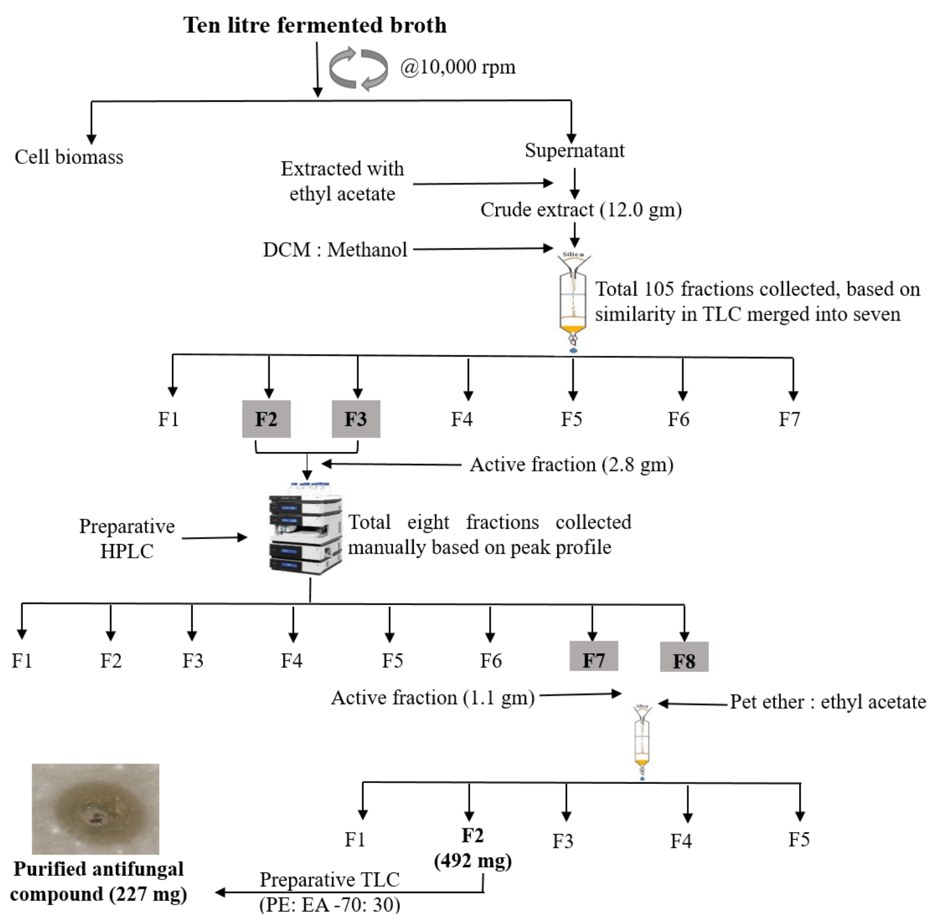
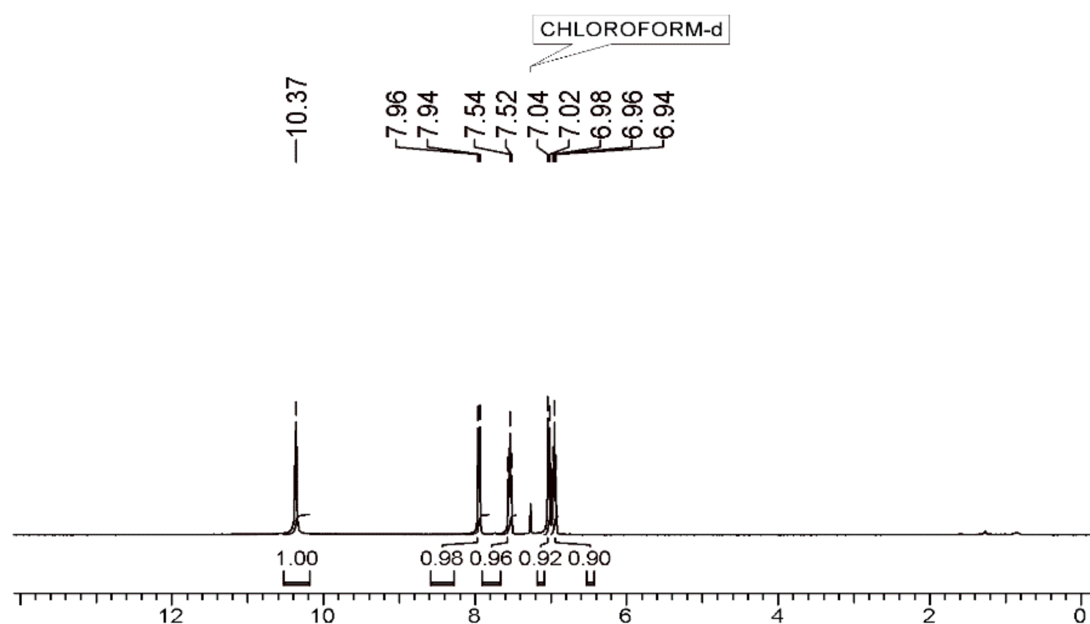


Figure. 8 Schematic diagram for the purification of antifungal compound from crude extract of *A. enclensis* NIO-1008



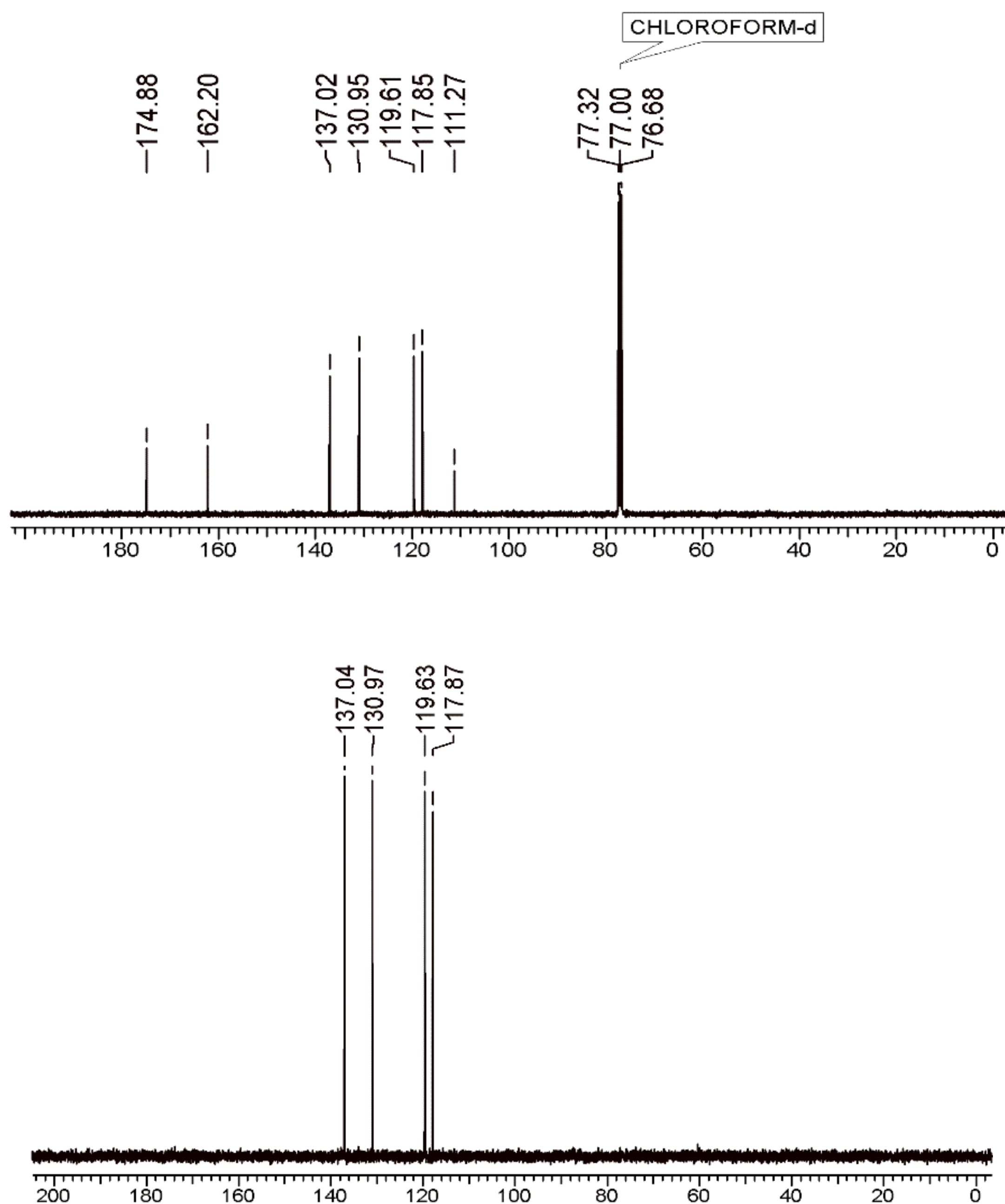


Figure. 9 Structure elucidation of purified compound from NIO-1008 by NMR spectroscopy

Following structural confirmation, the purified compound was verified via HPLC and TLC by comparing it to the reference salicylic acid. The TLC image and HPLC chromatogram (Fig. 10a and b) demonstrated that the Rf and Rt (2.9 min) value of purified compound and the standard salicylic acid are remain the same. Similar compounds have predominantly been reported from bacteria that have been isolated from terrestrial habitats, including *P.*

fluorescence, *S. marcescens*, *B. pumillus*, *Citrobacter*, *Klebsiella*, and *Achromobacter xylosoxidans* (Bakker et al., 2014). However, this is the first investigation to describe the salicylic acid from a marine *Arthrobacter* species.

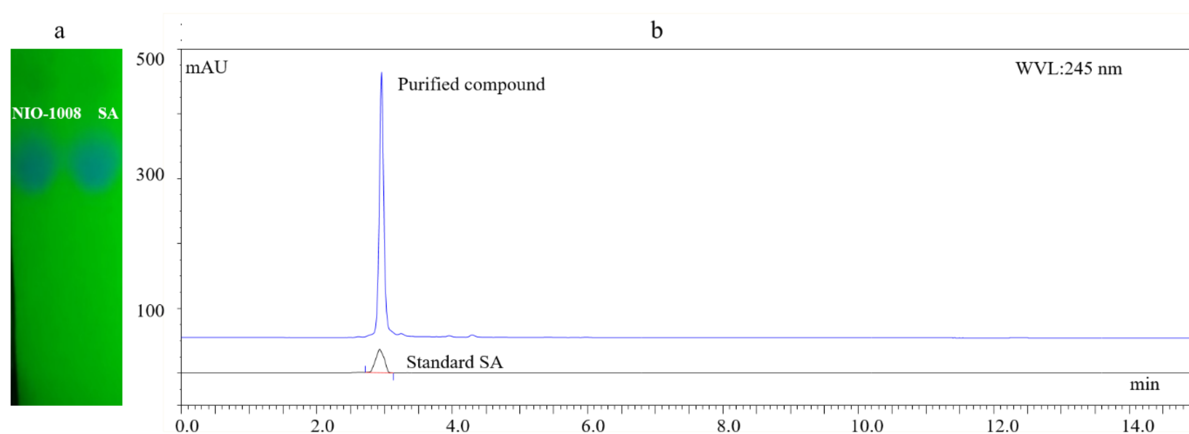


Figure. 10 Comparative analysis of purified compound of *A. enclensis* NIO-1008 with salicylic acid (SA) **a.** TLC image and **b.** Chromatogram showed in HPLC the peaks identified at the same retention time

4. Conclusion

Marine microorganisms are known to be a rich source of bioactive molecules. Because of their ability to survival in harsh marine environments that trigger them to produce structurally distinct compounds with a spectrum of biological actions. In the current study, the promising antagonistic marine strains BKACT and NIO-1008 were investigated for the production of bioactive compounds. As a result, the antifungal compounds from BKACT and NIO-1008 were isolated, purified and characterized as 2, 4-di-tert-butylphenol and salicylic acid, respectively. To the best of our knowledge, 2, 4-DTBP in *Serratia* sp. and salicylic acid in *Arthrobacter* sp. were isolated and characterised for the first time from the entire genus. Interestingly, both compounds are beneficial in crop protection against plant pathogens. This study suggests that marine microorganisms and their active compounds could be green and environmentally safe approach towards plant disease management.

References

Bakker, P. A., Ran, L., & Mercado-Blanco, J. (2014). Rhizobacterial salicylate production provokes headaches! *Plant and soil*, *382*(1), 1-16.

Cragg, G. M., & Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. *Biochimica Biophysica Acta (BBA)-General Subjects*, *1830*(6), 3670-3695.

Dias, D. A., Urban, S., & Roessner, U. (2012). A historical overview of natural products in drug discovery. *Metabolites*, *2*(2), 303-336.

Demain, A. L., & Sanchez, S. (2009). Microbial drug discovery: 80 years of progress. *The Journal of antibiotics*, *62*(1), 5-16.

Dharni, S., Maurya, A., Samad, A., Srivastava, S. K., Sharma, A., & Patra, D. D. (2014). Purification, characterization, and in vitro activity of 2, 4-di-tert-butylphenol from *Pseudomonas monteilii* PsF84: conformational and molecular docking studies. *Journal of agricultural and food chemistry*, 62(26), 6138-6146.

Dhar Purkayastha G, Mangar P, Saha A, Saha D (2018) Evaluation of the biocontrol efficacy of a *Serratia marcescens* strain indigenous to tea rhizosphere for the management of root rot disease in tea. *PloS one*, 13(2), e0191761

da Rosa, L. M., Koerich, D. M., & Della Giustina, S. V. (2019). Bioreactors operating conditions. *Essentials in Fermentation Technology*, 169-212.

Grzelak, E. M., Hwang, C., Cai, G., Nam, J. W., Choules, M. P., Gao, W., & Jaki, B. U. (2016). Bioautography with TLC-MS/NMR for rapid discovery of anti-tuberculosis lead compounds from natural sources. *ACS infectious diseases*, *2*(4), 294-301.

Hover T, Maya T, Ron S, Sandovsky H, Shadkchan Y, Kijner N, Osherov N (2016) Mechanisms of bacterial (*Serratia marcescens*) attachment to, migration along, and killing of fungal hyphae. *Appl Environ Microbiol*, 82(9):2585–2594

Karthikeyan, A., Joseph, A., Subramanian, R., & Nair, B. G. (2022). Fermenter Design. In *Industrial Microbiology and Biotechnology,* (pp. 129-167).

Lizardi-Jiménez, M. A., & Hernández-Martínez, R. (2017). Solid state fermentation (SSF): diversity of applications to valorize waste and biomass. *3 Biotech*, *7*(1), 1-9.

Nienow, A. W. (2014). Stirring and stirred-tank reactors. *Chemie Ingenieur Technik*, *86*(12), 2063-2074.

Ordentlich, A., Elad, Y., & Chet, I. (1988). The role of chitinase of Serratia marcescens in biocontrol of Sclerotium rolfsii. *Phytopathology*, *78*(1), 84-88.

Singh, J. S., Koushal, S., Kumar, A., Vimal, S. R., & Gupta, V. K. (2016). Book review: microbial inoculants in sustainable agricultural productivity. *Front. Microbiol,* Vol. II.

Someya, N., Nakajima, M., Hirayae, K., Hibi, T., & Akutsu, K. (2001). Synergistic antifungal activity of chitinolytic enzymes and prodigiosin produced by biocontrol bacterium, Serratia marcescens strain B2 against gray mold pathogen, Botrytis cinerea. *Journal of General Plant Pathology*, *67*(4), 312-317.

Subramaniyam, R., & Vimala, R. (2012). Solid state and submerged fermentation for the production of bioactive substances: a comparative study. *Int J Sci Nat*, *3*(3), 480-486.

Varsha, K. K., Devendra, L., Shilpa, G., Priya, S., Pandey, A., & Nampoothiri, K. M. (2015). 2, 4-Di-tert-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated Lactococcus sp. *International journal of food microbiology*, *211*, 44-50.

Wu, X., Zha, J., & Koffas, M. A. (2020). Microbial production of bioactive chemicals for human health. *Current Opinion in Food Science*, *32*, 9-16.

Wang, K., Qin, Z., Wu, S., Zhao, P., Zhen, C., & Gao, H. (2021). Antifungal mechanism of volatile organic compounds produced by Bacillus subtilis CF-3 on Colletotrichum gloeosporioides assessed using omics technology. *Journal of Agricultural and Food Chemistry*, *69*(17), 5267-5278.

Chapter. 4:

Antifungal efficacy, cytotoxicity, mechanism and their applications

Section 4A. 2, 4-di-tert-butylphenol as a natural biocontrol agent against *F. foetens* NCIM 1330

Abstract

Fusarium species are complex group of plant pathogenic fungi with substantive agricultural relevance. The extensive use of chemical fungicides to control *Fusarium* infection in plant diseases has harmed both the environment and human health. Finding eco-friendly biocontrol agents is needed to address the *Fusarium* infection. In this regard, the 2, 4-DTBP is a natural volatile organic compound (VOC) isolated from *Serratia marcescens* strain BKACT and evaluated against *F. foetens* NCIM 1330. As a result, spore germination was completely inhibited using 2, 4-DTBP at 0.53mM fumigation concentration, and mycelial growth was suppressed by 86.6%, 86.9%, and 82.8% on the 5, 7, and 9th days of incubation, respectively. Simultaneously, at 1.0 mM concentration the growth of the test fungi on wheat seeds was restricted. Furthermore, the compound would have no adverse effects on wheat seed germination at the same dose. In addition to antifungal efficacy, blocking ergosterol formation elucidated the mechanism of mycelial growth inhibition. Interestingly, docking analysis revealed that 2, 4-DTBP has the best binding conformation with Sterol 14-alpha demethylase (CYP51) and has a binding energy of -6.29 Kcal/mol. This study found that 2, 4-DTBP is an efficient biocontrol agent against *Fusarium* infection.

Introduction

Fusarium is devastating pathogen in agriculture which cause severe loss of economically important plants such as wheat, maize, banana, tomato, sugarcane etc. Different species *Fusarium*, including *F. proliferatum*, *F. graminearum*, *F. tricinctum*, *F. verticillioides, F. moniliforme* and *F. foetens* are the major pathogens in agriculture. They are also known to produce mycotoxins like trichothecenes, zearalenone, deoxynivalenol and fumonisins (Aoki et al., 2014). Among them, *F. foetens* has recently emerged as a destructive fungus in ornamental crops, particularly 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 et al., 2017). The production of mycotoxins such beauvericin and fusaric acid by *F. foetens* in the maize plant was also highlighted (Gonzalez-Jartn et al. 2019). Despite harming 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 has acquired resistance to the existing fungicides. In searching for a safe, eco-friendly and sustainable alternative, biocontrol bacteria and their natural volatile compounds are to be considered as the best choice for the control of fungi (Kohl et al., 2019).

Volatile compounds of microbial origin are gaining popularity in the scientific community. They play a different biological roles, from electron acceptors/donors to interspecies communication and warfare. Volatile organic compounds (VOCs) are carbon-based small molecules have a less water solubility and a high vapour pressure, allowing them to exist in gaseous form under normal circumstances (Tilocca et al., 2020). VOC's are generally effective at low concentrations; they can diffuse easily in the environment and spread over large distances. Therefore, they can exert inhibitory effect on the target organisms despite having direct or physical contact of producing microorganism. Besides, the mycelia suppression and the adverse effects on fungal spore germination, microbial VOCs have also been involved in

various processes. Moreover, VOCs have a critical role in supporting plant growth and disease resistance. Consequently, these compounds disperse quickly in the atmosphere and thus play essential biological/ecological roles in above and below-ground habitats. Based on chemical nature, VOCs are categorized into different molecular classes, such as thio-alcohols, hydrocarbons, ketones, alcohols, aldehydes, thioesters, cyclo-hexanes, heterocyclic compounds, benzene derivatives and phenols (Schmidt R et al., 2015; Tilocca et al., 2020)

Phenolic compounds are distinguished by the presence of a hydroxy (-OH) group connected to a benzene ring or other intricate aromatic ring configurations (Bruneton et al., 1999). The hydrophobic properties of phenolic compounds allow their preferential diffusion into the lipid membrane. The mechanism of the antifungal compounds is mostly based on their ability to disrupt cellular lipoprotein membrane function, damage cellular ionic homeostasis, acidify the vacuole and cytosolic pH, and even compromise cellular integrity (Xu et al., 2008; Zabka et al., 2013).

2, 4-Di-tert-butylphenol, also known as 2, 4-bis (1, 1-dimethylethyl)-phenol, is a prominent secondary metabolite produced in various species. 2, 4-DTBP is a lipophilic phenol found in at least 169 different species of bacteria, fungi, plants, and mammals. It exhibits antimicrobial, antioxidant, anticancer, pesticide, and weedicide activities (Fig. 1). Among these properties, it is widely known because of its antifungal and antioxidant activities. It is a volatile organic molecule found in the essential oils of several plants. By suppressing spore germination and hyphal development, it proved effective against major agricultural fungi (Zhao et al., 2020). Despite being highly effective against a variety of plant pathogenic fungi, antifungal mechanism of 2, 4-DTBP is still not well understood. In the current work, the antifungal mechanism of 2, 4-DTBP is investigated in addition to its biocontrol potential against *F. foetens* NCIM 1330.

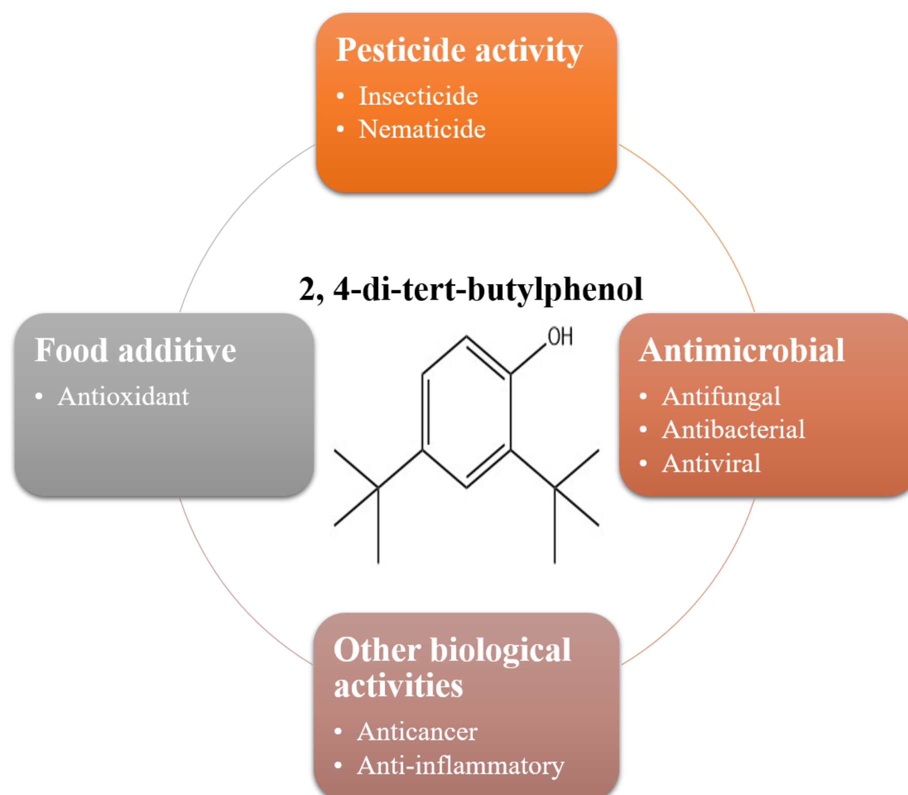


Figure. 1 Biological activities of 2, 4-DTBP as natural compound

2. Materials and methods

2.1 Chemicals

Kits and chemicals were ordered from Hi-media, Mumbai, Sigma Aldrich and Invitrogen (Gibco) as mentioned below.

Dulbecco's modified Eagle's medium (Gibco), Fatal bovine serum (Gibco), Dimethyl sulfoxide (Hi-media), 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (Sigma), Sodium hypochlorite (Hi-media), Protein quantification kit (Sigma), Ergosterol (Sigma), KOH (Hi-media), NaCl (Hi-media), and Ethanol (Hi-media)

2.2 Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) assay

According to the Clinical and Laboratory Standards Institute, the broth dilution method determined the MIC of 2, 4-DTBP against *F. foetens* NCIM 1330 (CLSI et al., 2008). The

spore suspension (1.0 x 10^6 spores/mL) was prepared in sterile PD broth. Different concentrations of 2, 4-DTBP were added to the PD broth by serial dilution, and the final volume was maintained to 100 µL/well. In control well, methanol was added in place of 2, 4-DTBP and incubated at 28°C for 72h. The time-killing rate was evaluated by O.D at 595 nm for every four hours interval up to 72h. After 72h incubation, MFC was assessed by spreading a 10µL sample from each concentration on PDA plates and incubating at 28°C for another 72h. The minimum concentration at which no detectable fungal growth consider is the MFC.

2.3 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay

The Vero cell lines were used to test 2, 4-DTBP cytotoxicity using the method described by (Tong et al., 2021). American Type Culture Collection supplied the Vero cell lines, which were grown in Dulbecco's modified Eagle's media supplemented with 10% FBS. Trypsinized cultured Vero cell lines were seeded into 96-well flat-bottomed plates at a density of 1.0×10^4 cells in 100 µL of the medium. After that, the plates were incubated for 16 hours at 37 °C in a 5% CO2 environment to test for adherence. The 2, 4-DTBP different concentrations were adjusted in DMEM. After 24 h incubation, media was removed, and 100 µL of MTT (0.5 mg/ml) was added and incubated in a 5% CO_2 atmosphere at 37 °C. After four hours, DMSO was applied to solubilize the formazan crystal, and absorbance at 570 nm was measured in each well using a SYNERGY 96 well plate reader. Blanks were subtracted from all data, and results were analysed using Prism software (Graph Pad 8.0.2).

2.4 *In-vitro* antifungal volatile effect

The plate enthalpy approach, with some modifications, was used to assess the 2, 4-DTBP volatile effect (Arrebola et al., 2010). In a brief, the experiment was carried out in a bi-compartment Petri plate where *F. foetens* NCIM 1330 was inoculated on PDA in one compartment. There, another empty side was loaded with the test compound 2, 4-DTBP; it can

only transfer as vapours from one side to the other. Treatment was given at concentrations of 0.07, 0.26, and 0.53 mM, while methanol served as the solvent control. After being parafilm-sealed, all the plates were incubated at 28°C for 5, 7, and 9 days. Using the previous formula, the percentage of mycelial growth inhibition was calculated.

For spore germination assay, 1.0×10^{6} spores/mL suspension was prepared in 0.01% tween 80 solutions, and 20µL was loaded above the PDA smear (diameter-12mm) on sterile glass slides. The slides were kept in one compartment of a petri dish containing moist filter paper with 90–92% humidity. As above mentioned, varying 2, 4-DTBP concentrations were applied to another compartment and incubated for 3, 6, or 24 hours. Further, all the samples were examined under 40x light microscopy (Nikon, Japan). At least 200 spores were observed per sample. The germination was confirmed when the germ tube length exceeded half of the diameter of the spores.

2.5 *In-vivo* volatile 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 (Zhang et al., 2021). Briefly, seeds were cleaned and surface sterilized with 2% sodium hypochlorite, then soaked in sterilized water for one hour. Subsequently, seeds were spiked with a 1.0×10^{6} conidia/mL suspension. Twenty seeds were added to one corner of each Petri plate. The treatment of 2, 4-DTBP at 0.07, 0.26, 0.53 and 1.0mM concentration was given as above mentioned and incubated at 28°C for seven 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 according to formula as mentioned below.

$$\text{Per cent seed contamination index (PSCI)} = \frac{\text{Negative control} - \text{Treatment}}{\mathit{Negative\ control}} \times 100$$

Simultaneously mould count in wheat seeds was carried out after seven days of treatment. Ten seeds (1.0 gm) were taken out from treatment and control samples and mixed with 9.0 mL of sterile saline solution by shaking at 300 rpm for 30 minutes. The samples were diluted by the standard serial dilution method. A 100μL sample was placed on PDA from each dilution and incubated further at 28°C for 72h. The formula calculated the number of moulds per gram of wheat samples,

$$\text{Colony forming unit (CFU) per gram of sample } = \frac{\text{Number of colony} - \text{Dilution factor}}{Volume\ of\ culture\ plate}$$

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

The toxic effect of 2, 4-DTBP on wheat seed germination was performed according to (Zhang et al., 2021). All the seeds were washed with tap water and surface sterilized in 2 % sodium hypochlorite for 2 minutes, then rinsed twice with sterile distilled water for one minute and air-dried. Two hundred seeds were added to one corner of Petri plates, and 0.07 and 1.0 mM concentrations of 2, 4-DTBP were added to another corner. Methanol was used as the solvent control. All of the plates were parafilm-sealed and kept at 28°C for 7 days. A hundred seeds were randomly withdrawn after seven days of incubation, placed on a filter paper (pre-soaked in sterile water), and cultivated at 28°C. The germination percentages were calculated based on the seeds' radicle protruding by 2 mm.

2.7 Understanding the mechanism of mycelial growth inhibition

2.7.1 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). A 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 seven 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 O.D 260nm (NANODROP LITE Spectrophotometer). Protein concentration was determined according to Bradford's method.

2.7.2 HPLC analysis of the Ergosterol

The ergosterol content of the *F. foetens* NCIM 1330 was determined according to (Wang et al., 2020). Each sample in the treatment and control groups received 100 mg of mycelia suspended in a mixture of (Methanol 20mL, ethanol 5mL and KOH 2.0gm). After mixing for 5 minutes, it was incubated at 70 °C for 40 minutes. Afterwards, 5 mL of water was added, and the mixture was centrifuged at 1735 × g for 15 minutes. Then, a same volume of n-hexane was used to extract the supernatant. 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 analysed on a C-18 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.

2.8 Molecular Docking

Ligand and protein preparation: The natural antifungal compound 2, 4-di-tert-butylphenol (Fig. 8) was obtained by using PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Canonical ligand smiles were gathered and transferred to protein data bank format. Then, for molecular docking, OpenBabel v 2.4.1. was used. Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (https://www.rcsb.org/) was used to get the three-dimensional structure of ergosterol biosynthesis enzymes with PDB ID 6ay4, 6c6p, 4quv, and 6oht. Among these, the best target 6ay4 beta sheets are shown in yellow, the twists and

loops in green, and the alpha helices are shown in red (Fig. 9). Before docking, protein's water molecules, inhibitors, native ligands, and other heteroatoms were removed by using PyMOL v.1.74.

AutoDock 4.2.6: AutoDock 4.2.6 and additional supporting tools, namely Python 2.5.2 and MGLTools 1.5.6, were installed from the Scripps Research Institute's official website (http://autodock.scripps.edu/). These are free and acknowledged by the scientific community to be the most trustworthy molecular docking software (Ravindranath et al., 2015).

Preparation of PDBQT files: The starting directory, along with the autodock and autogrid applications, were all set to the appropriate folder prior to docking. The processed protein molecule from PyMOL v. 1.74 was loaded into the AutoDock 4.2.6 workspace. The polar hydrogen atoms are inserted after all of the water molecules have been withdrawn, and the protein's Kollman and Gasteiger charges are determined. The protein was then stored in PDBQT format and used as the target. The ligand was imported into the software, the root of the torsion tree was chosen to define it, the number of rotatable bonds was calculated, and the data was saved in PDBQT format. The workspace was uploaded with the ligand and protein as PDBQT format for further simulation.

Grid parameters: Setting the grid dimensions is an important step in molecular docking as it directs the ligand to the designed protein binding site. The 0.597 Å grid spacing was used as the default. With offset values of 1.944, 3.417, and 0.583, the values for the centre grid box get fix to x ¼ _18.553, y¼_0.043, and z¼_19.273. The grid settings were set to 126 equal points in the x, y, and z axis. There were total 2048383 grid points upon every map. These parameters comprised the whole three-dimensional active site of the protein. The grid parameter file (GPF) format was used to save the output. The specified coordinates and grid dimensions were relatively identical to those described by (Odhar et al., 2020; Yu et al., 2020).

Running AutoGrid and AutoDock: The executable and GPF files were uploaded in order to execute the AutoGrid, after which the grid log file (GLG) was transformed into an output file. Then, the AutoGrid was initiated. Once AutoGrid executed correctly, the genetic algorithm was adjusted to its default settings, which are as shown (10 GA runs; 150 individual's population; 2.5 million energy assessments; 2.0 clustered tolerance and 27000 generations). The docking parameter file (DPF) format was used to save the outcomes of the Lamarckian genetic algorithm. Docking was started once the DPF and AutoDock executable files were submitted as input and transformed to the docking log file (DLG). Valuable data, including the top 10 free binding energies for each run and the inhibitory constant, were present in the final DLG file. According to their binding energies, the results were analysed and documented in PDBQT format; the complex with the lowest binding energy was then stored in PDB format for continued information.

Docking analysis: The final DLG file contain essential data, including the top 10 conformations for each run, which included rank, free binding energy (Kcal/mol), mean rmsd, and inhibitory constant (M). Using the autodock tools analysis option, the outcomes were evaluated according to their binding energies, ligand efficiency, internal molar energies, Vander wall: desolvation energies, total internal energies, torsional energies, and unbound energies. The ideal pose of the ligand-protein complex with the lowest binding was chosen for the study by using the Docking option from the analyse parameter. The interactions were also investigated in order to determine the active sites of the protein.

Visualizing interactions: PyMOL v.1.74, Autodock tools 4.2.6, and Discover Studio v20.1.0.19295 from Biovia were used to investigate and display the 3-dimensional, 2-dimensional, and surface annotation of ligand interaction with the protein.

Statistical analysis

All experiments were carried out in triplicates, and data were presented as mean ± standard deviation. Different letters above bars indicate a significant difference between groups as demonstrated by one way ANOVA followed with Post hoc Tukey's (HSD) honestly significant difference test. The p-values<0.05 were considered statistically significant. Statistical analysis was performed using SPSS software version 26.0 (SPSS Inc., USA). Graph Pad Prism 8.0.2 software was used for plotting the graphs.

3. Result

3.1. MIC, MFC and cytotoxicity study

The minimal inhibitory and minimal fungicidal concentrations of 2, 4-DTBP against *F. foetens* NCIM 1330 were determined. The 2, 4-DTBP inhibited the growth of fungi at MIC and MFC values of 0.3 and 0.6 mM, respectively. The findings indicated that 2, 4-DTBP had antifungal action (Fig. 2). At the same time, it demonstrated cytotoxicity on the Vero cell line at 0.6 mM concentration (Fig. 3).

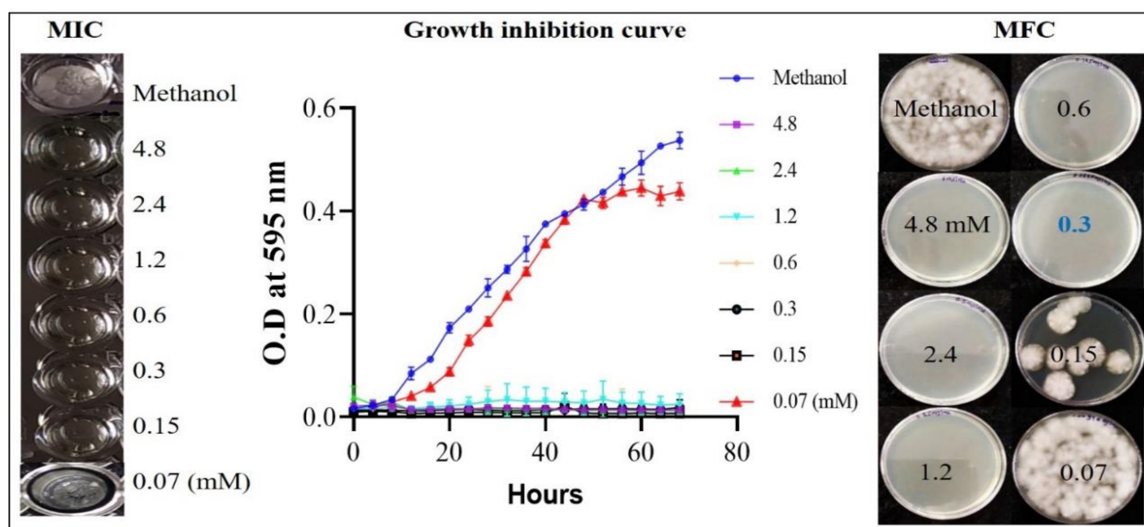


Figure. 2 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) assay of 2, 4-DTBP against *F. foetens* NCIM 1330

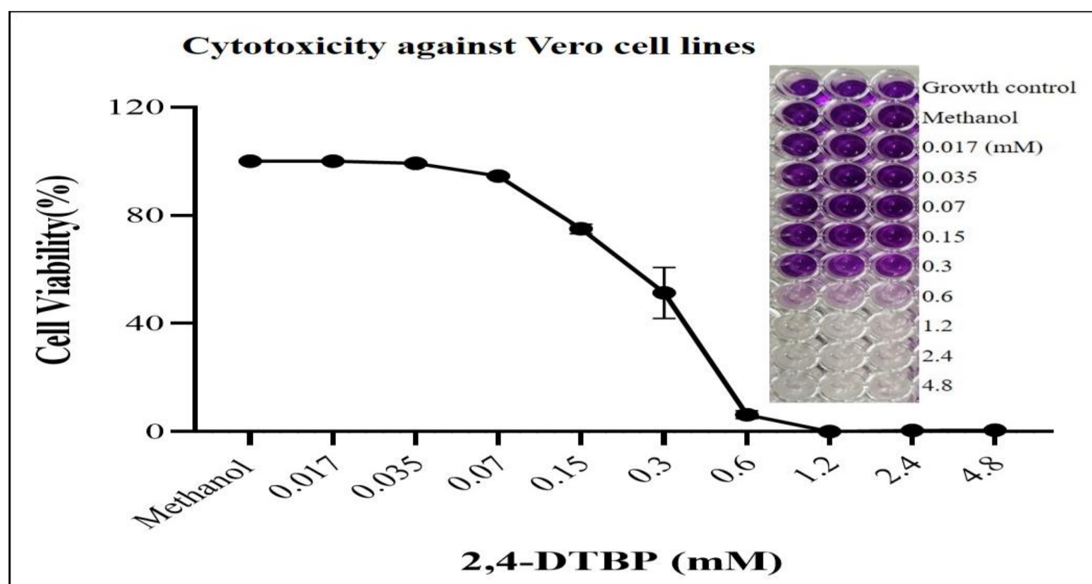


Figure. 3 Cytotoxicity activity of 2, 4-DTBP against Vero cell lines

3.2. *In vitro* antifungal volatile effect

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

The volatile effect of the 2, 4-DTBP was qualitatively analysed on spore germination. In solvent control and a lower concentration of 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 than the control, but it decreased as the incubation time increased.

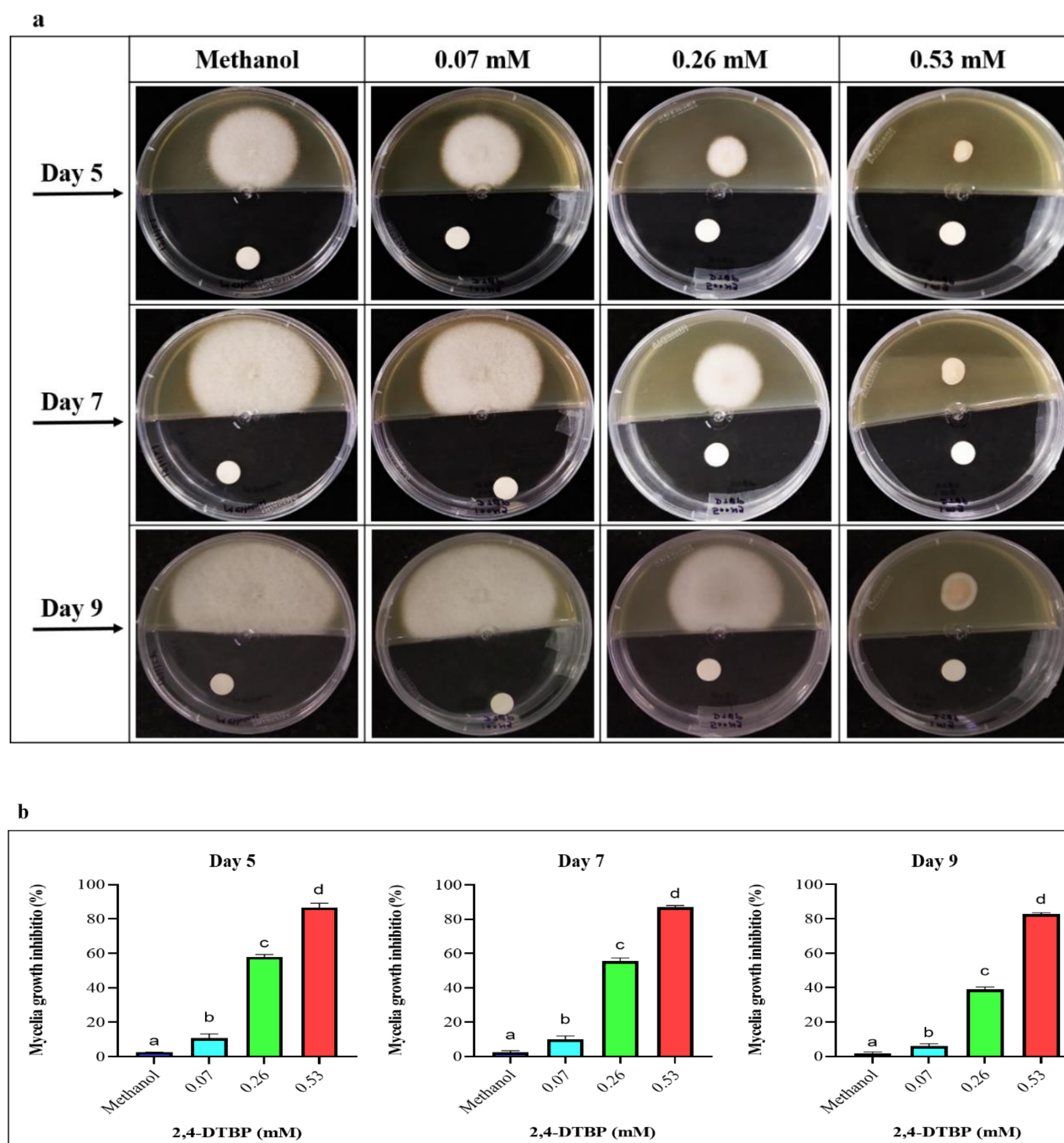
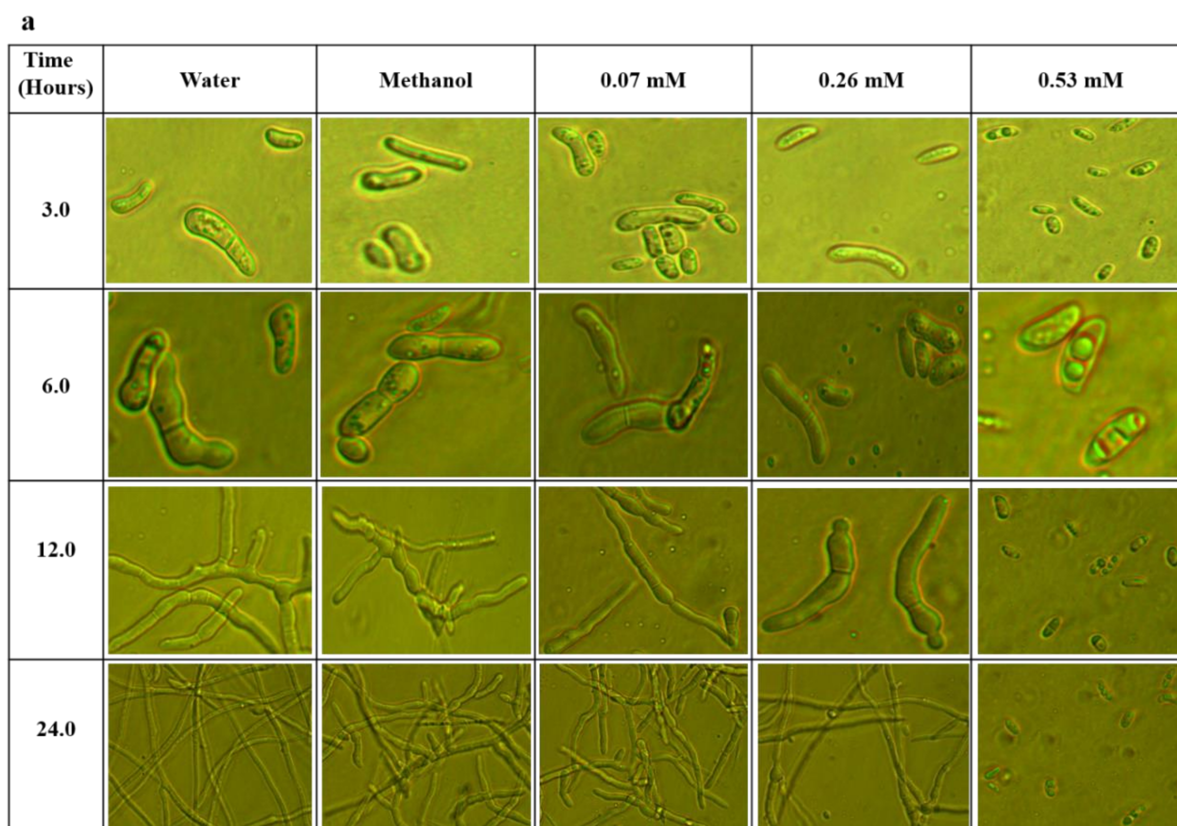


Figure. 4 Effect of the 2, 4- DTBP on the growth of *F. foetens* NCIM 1330, **a.** Antifungal effect of the 2, 4-DTBP on the mycelial growth and **b.** Percent mycelial growth inhibition

At 0.53 mM concentration of 2, 4-DTBP spore germination was utterly (100%) suppressed at all the incubation times (Fig. 5a). The per cent 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 three hours of incubation. As the time increases, the inhibition rate decreases to zero at 24 h. Moreover, 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. 5b).



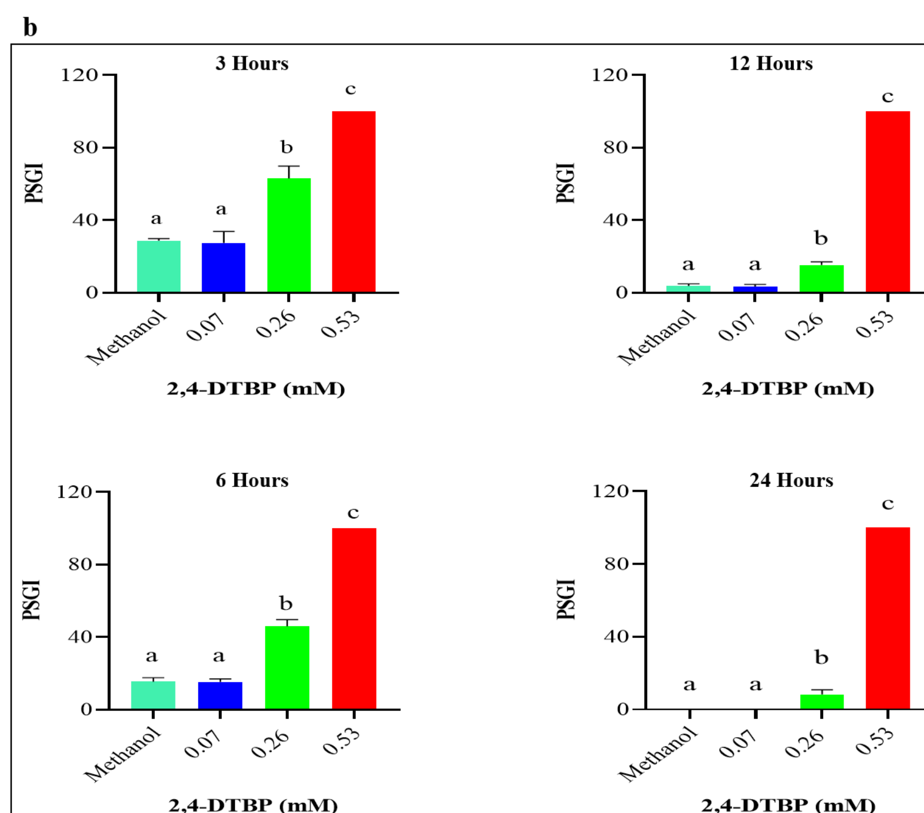


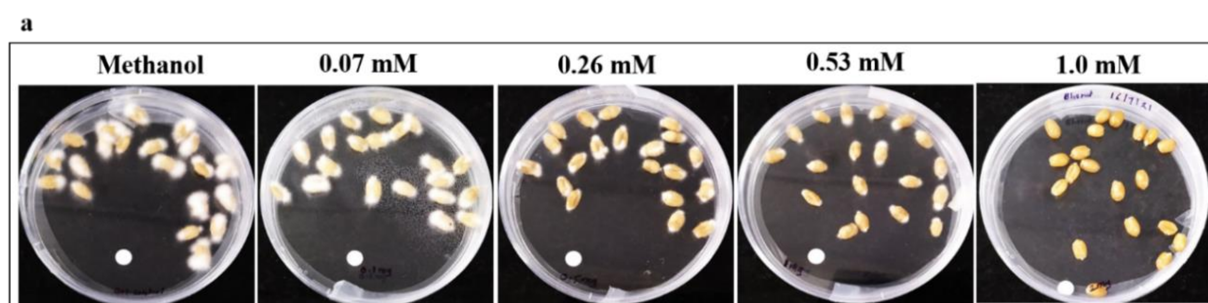
Figure. 5 Effect of the 2, 4- DTBP on spore germination of *F. foetens* NCIM 1330, **a.** Qualitative analysis of spore germination at different time and concentration and **b.** Quantitative analysis of percent spore germination inhibition (PSGI)

3.3. *In vivo* volatile antifungal 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 suppressed entirely even after seven days of incubation (Fig. 6a). The per cent 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, confirmed as the effective concentration for protecting the wheat seed from *F. foetens* NCIM 1330 (Fig. 6b). After treating 2, 4-DTBP at different concentrations, the colony-forming unit (CFU) of mould was also determined in wheat seeds. The CFU of mould was identified to decrease significantly ($p < 0.05$) at 0.26 and 0.53, and 1.0mM concentration compared with control. At 1.0mM concentration, not a single CFU was detected in the treated sample (Fig. 6c).

3.4. 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 when compared with the solvent control ($p < 0.05$) (Fig.6d &e).



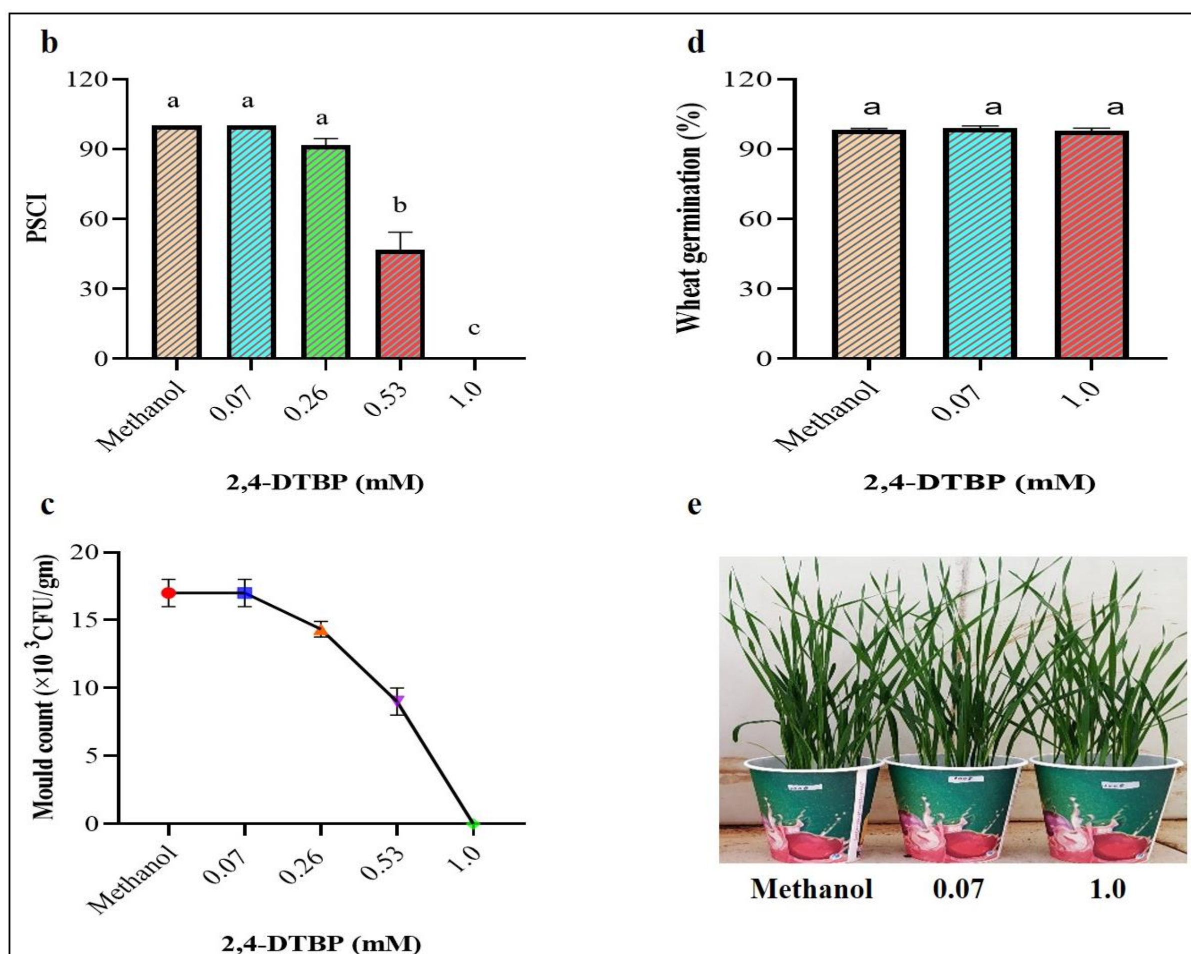


Figure. 6 Biocontrol efficacy of 2, 4-DTBP against *F. foetens* NCIM 1330 on the wheat seeds, **a.** Qualitative analysis of fungal contamination on wheat seeds, **b.** Quantitative analysis of the wheat seed contamination index (SCI), **c.** Fungicidal effect of DTBP on the wheat seeds, **d.** Germination percentage of wheat grains after DTBP fumigation and **e.** Evaluation of adverse effect on wheat plant growth

3.5. Mechanism of mycelial growth inhibition

The mechanism of mycelial growth inhibition was studied by measuring ergosterol concentration in mycelia after treatment of 2, 4-DTBP. Ergosterol content in mycelia-treated samples was not detected in HPLC at 0.26 and 0.53mM fumigation concentrations. However, at lower concentrations (0.07mM), and control sample showed a peak at 2.98 retention time, which matches to the standard ergosterol peak (Fig. 7a). Following that, cytoplasmic leakage was also analysed in 2-DTBP-treated and control mycelia. Extracellular conductivity, protein and nucleic acid leakages were substantially increased in the treated mycelia at 0.26 and 0.53

mM concentrations compared to the control (Fig. 7b). This findings suggest that 2, 4-DTBP triggers cell membrane disruption via inhibiting ergosterol biosynthesis.

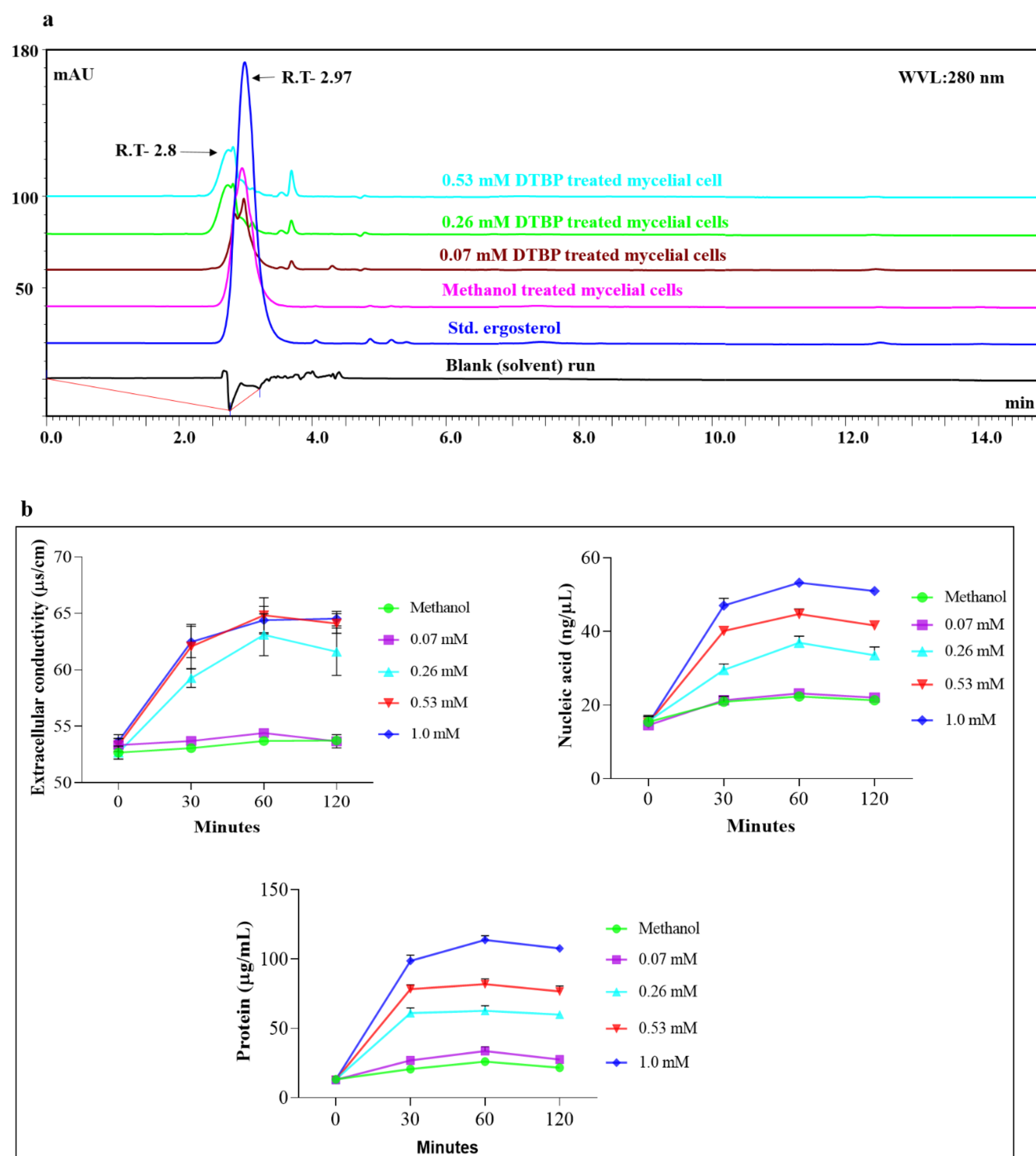


Figure. 7 2, 4- DTBP fumigation effect on cell wall integrity of *F. foetens* NCIM 1330, **a.** Ergosterol detection in HPLC, and **b.** Cytoplasmic leakages measurement in mycelia

3.6. Molecular Docking

The interaction of the key enzymes of ergosterol biosynthesis with the naturally occurring antifungal compound 2, 4-di-tert-butylphenol was examined in docking. At a binding energy of -6.29 Kcal/mol, the ligand seemed to have the optimum binding shape with Sterol 14-alpha demethylase (CYP51). Other enzymes, like sterol-14 reductase, sterol-8 isomerase, and squalene epoxidase, in comparison, have higher binding energies of -6.14, -5.32, and -4.63 Kcal/mol, respectively. Descriptions of the ligand with the highest conformations are provided in (Table. 1). Best conformation of natural antifungal compound 2, 4-di-tert-butylphenol was further analysed using AutoDock and ranked based on their binding energies (-6.29), ligand efficiency (-0.42), internal molar energy (-7.19), Vander wall: desolvation energy (-7.16), total internal energy (-0.55), torsional energy (0.89), and unbound energy (-0.55). Ligand (2, 4-di-tert-butylphenol) with protein (Sterol 14- alpha demethylase (CYP51)) interactions denoting the amino acids binding sites (PRO A:474, PRO A:460, ARG A:476, ILE A:456, THR A:475, TYR A:455, LYS A:458, GLY A:457, SER A:472) as shown in (Fig. 10 &11). Non bond interactions are also being studied for the ligand and the protein denoting the (name, distance between binding in Armstrong, category and type of interactions) as mentioned in (Table. 2). Out of 10 runs, 2 multi-member conformational clusters were identified.

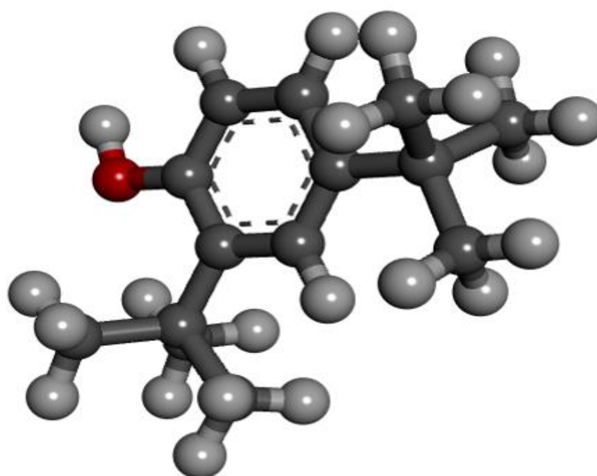


Figure. 8 2, 4-di-tert-butylphenol, PubChem CID 7311

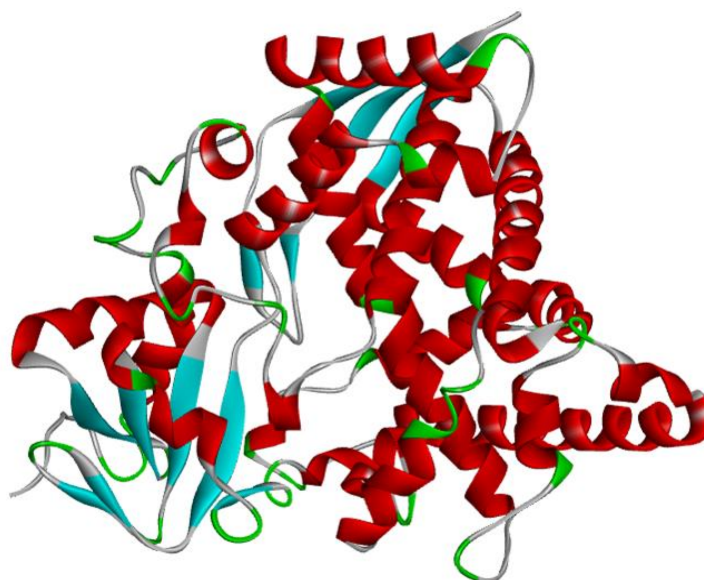


Figure. 9 Sterol 14- alpha demethylase (CYP51) Protein Processed

Table. 1 RMSD (Root mean square deviation) table from Lamarckian log file (dlg file) generated by AutoDock 4.2.6 showing top ten binding poses of protein and ligand docked

Rank	Sub-rank	Run	Binding Energy	Cluster RMSD	Reference RMSD	Grep Patterns
1	**1**	**5**	**-6.29**	**0.00**	**11.67**	**RANKING**
1	2	10	-6.29	0.11	11.63	RANKING
1	3	9	-6.05	1.58	11.59	RANKING
2	1	3	-6.12	0.00	12.23	RANKING
3	1	6	-5.33	0.00	20.09	RANKING
4	1	2	-5.03	0.00	32.66	RANKING
4	2	7	-4.99	1.66	32.73	RANKING
5	1	1	-4.99	0.00	31.52	RANKING
6	1	8	-4.59	0.00	44.24	RANKING
7	1	4	-4.43	0.00	34.37	RANKING

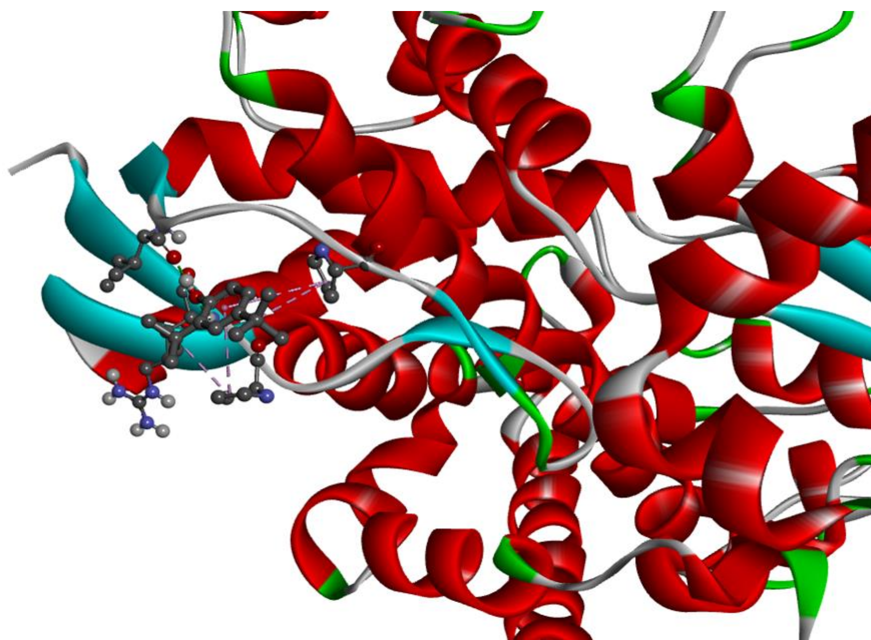


Figure. 10 Ligand (2, 4-DTBP) with Protein (CYP51)) interactions

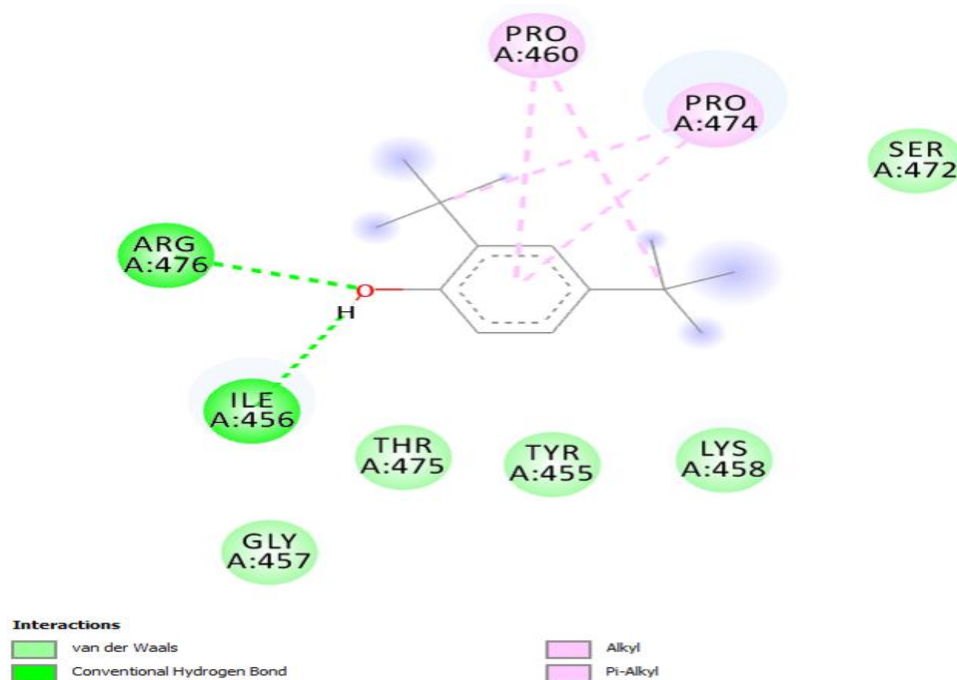


Figure. 11 Two Dimensional diagram showing interactions between ligand (2, 4-DTBP) with Protein (CYP51))

Table. 2 Non bond interactions of the protein and ligand

Name	Distance Å	Category	Type	From	To
:UNL1:O-A:ARG476:HN	2.16742	Hydrogen Bond	Hydrogen Bond	A:ARG476:HN	:UNL1:O
:UNL1:H-A:ILE456:O	1.82761	Hydrogen Bond	Hydrogen Bond	:UNL1:H	A:ILE456:O
:UNL1:C-A:PRO474	4.05146	Hydrophobic	Alkyl	:UNL1:C	A:PRO474
:UNL1:C-A:PRO460	4.70839	Hydrophobic	Alkyl	:UNL1:C	A:PRO460
:UNL1 -A:PRO460	5.01091	Hydrophobic	Pi-Alkyl	:UNL1	A:PRO460
:UNL1 -A:PRO474	4.75965	Hydrophobic	Pi-Alkyl	:UNL1	A:PRO474

4. Discussion

Microbial volatile organic compounds (VOCs) have a key role in pathogen control, particularly in the suppression of plant pathogenic fungi. Even at very low concentrations, the VOCs have the ability to travel long distances in the atmosphere and exert inhibitory effect without being in direct contact with the target pathogens. In addition, VOCs are easily degradable and have excellent permeability and diffusion efficiency (Schmidt et al., 2015). As a result, the mechanism of action of VOCs with microbial origins is safer and more effective compared to chemical fungicides. In this regard, 2, 4-DTBP obtained from *S. marcescens* BKACT as the natural VOC that has been explored for the suppression of *F. foetens* NCIM 1330 infection on wheat seeds.

The 2, 4-DTBP is a common natural product that has been extensively studied in a variety of species for a wide range of biological functions. Among all the functions studied, the molecule is commonly recognised for its antifungal and antioxidant properties (Zhao et al., 2020). Despite being involved in several activities, the volatile nature of 2, 4-DTBP has not yet fully investigated for agricultural applications. However, (Wang et al., 2021) revealed that one mole

per litre of 2, 4-DTBP is an effective volatile concentration against *Colletotrichum gloeosporioides*. In comparison to the study mentioned above, our results show that *F. foetens* growth was substantially suppressed at a minimal (0.53 mM) volatile concentration of 2, 4-DTBP. Mycelial growth inhibition was significantly higher when compared to control, even at below 0.53 mM concentrations. The highest 86.6 ±2.0 percent mycelial growth was suppressed at 0.53 mM concentration. At the same concentration, 100 percent spore inhibition was also observed. The reason for the significant variation in inhibitory concentration might be due to the 2, 4-DTBP produced by various bacteria and the differences between DMSO (Wang et al., 2021) and methanol (used in the current investigation) as solvents for the 2, 4-DTBP's dissolution.

Following confirmation of *in vitro* antifungal volatile capability, we assume 2, 4-DTBP has the potential to combat *F. foetens in vivo* on wheat seeds. According to (Lamprecht et al., 2017), the *F. foetens* is known to cause the damping-off in rooibos seedlings as well as to produce mycotoxins such beauvericin and fusaric acid on maize (Gonzalez-Jartn et al. 2019). As a result, for the first time, our study demonstrated that 2, 4-DTBP fumigation has the ability to suppress *F. foetens* infestation on the wheat seeds. The most efficient dose for completely controlling the growth of the test fungi on the wheat seeds was found to be 1.0 mM fumigation treatment. The percent seed contamination index (PSCI) was significantly reduced compared to the control, even with a lower (0.53 mM) fumigation treatment. Interestingly, at a higher (1.0 mM) fumigation treatment, the compound has not displayed any detrimental effects on the germination of wheat seeds.

Antifungal activity of 2, 4-DTBP against a wide spectrum of phytopathogenic fungi was demonstrated in several studies. However, the mechanism of mycelial growth inhibition remained unclear. The majority of antifungal agents are known to target the fungal cellular membrane and cell wall. Due to their structural and physicochemical properties, the fungus

phospholipid bilayer and internal cellular organelles are more permeable to them. According to a recent study (Wang K et al., 2021), gene expression and proteins involved in cell membrane fluidity and cell wall integrity were downregulated in *C. gloeosporioides* treated with 2, 4-DTBP compared to untreated mycelia. Based on results, we predict that 2, 4-DTBP, due to its non-polar volatile nature, it may weaken cell wall integrity and disrupt the cytoplasmic balance by leaking internal components. Ergosterol is a major component of the fungal cell wall. Usually, a reduction in the amount of ergosterol causes an osmotic imbalance and disrupts cell growth (Sant et al., 2016). As a consequence, ergosterol was not identified in mycelia treated with 2, 4- DTBP at 0.26 and 0.53mM concentrations in our investigation. Moreover, cytoplasmic leakage was also noticed in the 2, 4-DTBP-treated samples. Conversely, at lower concentration (0.07mM) and control samples ergosterol was produced in mycelia. This results indicate that 2, 4-DTBP suppressed mycelial growth by blocking ergosterol biosynthesis. In order to validate the mechanism of the inhibition of ergosterol biosynthesis even further, a molecular docking approach was used. In docking analysis 2, 4-DTBP showed best binding conformation with the key ergosterol biosynthesis enzyme i.e. Sterol 14- alpha demethylase (CYP51) with a binding energy of -6.29 Kcal/mol. To our knowledge, this is the first investigation to demonstrate the 2, 4-DTBP mycelial growth inhibition mechanism by both silico docking analysis and experimental validation.

Conclusion

Agriculture have challenges not only with food production for humans, but also regarding environmental protection. As a reason, there is a growing demand to minimize pesticide use in order to avoid possible environmental harm. Thus, biocontrol bacteria and their active compounds are the best alternative for synthetic pesticides. In present work, we attempted to control the *Fusarium* infection on the wheat seeds through fumigation of 2, 4-DTBP. As a result, test fungus growth was potentially decreased in the presence of 2, 4-DTBP at both *in*

vitro and *in vivo* assays. Moreover, the test compound had no deleterious effect on wheat seed germination. We showed a safe and efficient strategy for controlling *Fusarium* contamination on wheat seeds at a minimal concentration. Interestingly, the mechanism of 2, 4-DTBP inhibition of mycelial growth has been identified for the first time. This work is significant since it provides important results that may be beneficial in agricultural research. We conclude that active compounds derived from marine strains has promising biocontrol potential for plant disease management.

References

Aoki, T., O'Donnell, K., & Geiser, D. M. (2014). Systematics of key phytopathogenic Fusarium species: current status and future challenges. *Journal of General Plant Pathology*, *80*(3), 189-201.

Arrebola, E., Sivakumar, D., & Korsten, L. (2010) Effect of volatile compounds produced by *Bacillus* strains on postharvest decay in citrus. *Biological control*, 53(1), 122-128.

Clinical and Laboratory Standards Institute (2008) Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard- CLSI document M38-A2. *Clinical and Laboratory Standards Institute.*

González-Jartín JM, Alfonso A, Sainz MJ, Vieytes MR, Aguín O, Ferreiroa V, Botana LM (2019) First report of *F. foetens* as a mycotoxin producer. *Mycotoxin Res,* 35(2):177–186.

Gautret, P., Lagier, J. C., Parola, P., Meddeb, L., Mailhe, M., Doudier, B., & Raoult, D. (2020). Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. *International journal of antimicrobial agents*, *56*(1), 105949.

Hasim, S., & Coleman, J. J. (2019). Targeting the fungal cell wall: current therapies and implications for development of alternative antifungal agents. *Future medicinal chemistry*, *11*(08), 869-883.

Köhl J, Kolnaar R, Ravensberg WJ (2019) Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. *Frontiers in plant science*, 845.

Lamprecht SC, Tewoldemedhin YT (2017) Fusarium species associated with damping-off of rooibos seedlings and the potential of compost as soil amendment for disease suppression. *South Afr J Bot*, 110:110–117.

Ma, Y. Q., Ye, X. Q., Fang, Z. X., Chen, J. C., Xu, G. H., & Liu, D. H. (2008). Phenolic compounds and antioxidant activity of extracts from ultrasonic treatment of Satsuma mandarin (Citrus unshiu Marc.) peels. *Journal of agricultural and food chemistry*, *56*(14), 5682-5690.

Odhar, H. A., Ahjel, S. W., Albeer, A. A. M. A., Hashim, A. F., Rayshan, A. M., & Humadi, S. S. (2020). Molecular docking and dynamics simulation of FDA approved drugs with the main protease from 2019 novel coronavirus. *Bioinformation*, *16*(3), 236.

Ravindranath, P. A., Forli, S., Goodsell, D. S., Olson, A. J., & Sanner, M. F. (2015). AutoDockFR: advances in protein-ligand docking with explicitly specified binding site flexibility. *PLoS computational biology*, *11*(12), e1004586.

Sant, D. G., Tupe, S. G., Ramana, C. V., & Deshpande, M. V. (2016). Fungal cell membrane—promising drug target for antifungal therapy. *Journal of Applied Microbiology*, *121*(6), 1498-1510.

Schroers HJ, Baayen RP, Meffert JP, De Gruyter J, Hooftman M, O'Donnell K (2004) *Fusarium foetens*, a new species pathogenic to begonia elatior hybrids (*Begonia hiemalis*) and the sister taxon of the *Fusarium oxysporum* species complex. *Mycologia,* 96(2):393–406.

Schmidt R, Cordovez V, De Boer W, Raaijmakers J, Garbeva P (2015) Volatile affairs in microbial interactions. *ISME J*, 9(11):2329–2335.

Tilocca, B., Cao, A., & Migheli, Q. (2020). Scent of a killer: Microbial volatilome and its role in the biological control of plant pathogens. *Frontiers in Microbiology*, *11*, 41.

Tong, Y., Zhang, J., Wang, L., Wang, Q., Huang, H., Chen, X., & Zhang, L. (2021). Hyper-synergistic antifungal activity of rapamycin and peptide-like compounds against Candida albicans orthogonally via tor1 kinase. *ACS Infectious Diseases*, *7*(10), 2826-2835.

Varsha, K. K., Devendra, L., Shilpa, G., Priya, S., Pandey, A., & Nampoothiri, K. M. (2015). 2, 4-Di-tert-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated *Lactococcus* sp. *International journal of food microbiology*, 211, 44-50.

Wang, K., Qin, Z., Wu, S., Zhao, P., Zhen, C., & Gao, H. (2021). Antifungal mechanism of volatile organic compounds produced by Bacillus subtilis CF-3 on Colletotrichum gloeosporioides assessed using omics technology. *Journal of Agricultural and Food Chemistry*, *69*(17), 5267-5278.

Wang, T., Li, Y., Bi, Y., Zhang, M., Zhang, T., Zheng, X., & Huang, Y. (2020). Benzyl isothiocyanate fumigation inhibits growth, membrane integrity and mycotoxin production in *Alternaria alternata*. *RSC Advances*, 10(3), 1829-1837.

Zabka, M., & Pavela, R. (2013). Antifungal efficacy of some natural phenolic compounds against significant pathogenic and toxinogenic filamentous fungi. *Chemosphere*, *93*(6), 1051-1056.

Zhang S, Zheng M, Zhai H, Lyu Y, Hu Y, Cai J (2021) Effects of hexanal fumigation on fungal spoilage and grain quality of stored wheat. *Grain & Oil Science and Technology,* 4(1):10–17.

Zhao, F., Wang, P., Lucardi, R. D., Su, Z., & Li, S. (2020). Natural sources and bioactivities of 2, 4-di-tert-butylphenol and its analogs. *Toxins*, 12(1), 35.

Section 4B. Salicylic acid and its producer novel strain *Arthrobacter enclensis* NIO-1008 for the mitigation of salinity stress and fungal infestation

Abstract

Microorganisms from extreme habitats are having significance for crop protection against abiotic and biotic stresses. These bacteria can produce growth regulators, which fight against salinity, heavy metals and pathogens. In the present work, a novel species *A. enclensis* NIO-1008 and its isolated salicylic acid was investigated for biotic and abiotic stress mitigation. In the *in-vitro* antifungal experiment, salicylic acid demonstrated a significant inhibitory effect on spore germination as well as mycelial growth and weight of *F. oxysporum* NCIM 1281. Further, in the greenhouse pot trials, the disease incidence was considerably decreased to 70% in presence of strain NIO-1008 and 45.7% in salicylic acid treated wheat plants when compared to the pathogen control. Also, strain NIO-1008 and salicylic acid both enabled wheat plants resist up to 100 mM salt stress. These results indicate that a marine strain and its active compound potentially increases plant growth under normal and stress conditions.

1. Introduction

The agriculture sector is facing multiple challenges, including abiotic and biotic stressors which significantly lowers the crop productivity. Worldwide it has contributed approximately 30 to 50% crop loss (Kumar et al., 2018). Temperature, drought, heavy metals, and salinity are the major abiotic stressors. Among them, soil salinity is most destructive environmental stress that has an adverse effect on plant growth and which leads to significant economic loss (Qin et al., 2014). Approximately 20% of the irrigated land is affected by high salt content, and around 50% of cultivable land will be salinized by 2050 (Khan et al., 2021).

Salinity directly affects the plant physiology and modulates its defence responses, and increasing the risk of susceptibility to plant pathogens (Chojak-Kozniewska et al., 2018; Eydoux et al., 2020). Fusarium wilt is a common vascular plant disease, mainly driven by *Fusarium oxysporum*, which reduces the significant yield in various crop plants by inducing necrosis and wilting (Rocha et al., 2017 and Ye et al., 2020). The majority of the farmers use the chemical fungicides, especially against *Fusarium*. These fungicides are proven for their detrimental effect on humans and the environment. Another biggest challenge is fungicide resistance in agriculture. Hence, safe and effective new strategies for fighting soil-borne pathogens are urgently required.

In this context, beneficial microorganisms are supposed to be a better alternative to synthetic agrochemicals. Plant growth-promoting bacteria (PGPB) include several genera *Klebsiella*, *Pseudomonas*, *Bacillus*, *Variovorax*, *Burkholderia*, *Serratia*, *Azospirillum*, *Azotobacter*, and *Arthrobacter*, which support plant growth under normal and stressful conditions (Qin et al., 2014; Krishnan et al., 2016; Olanrewaju et al., 2017). The mechanisms established by PGPB's are widely known and understood, whereby a nutrients such as nitrogen, iron and phosphorus are acquired for plant growth development via direct mechanisms. In order to improve plant growth and fight pathogens, the indirect mechanism is mainly used by producing antibiotics,

lytic enzymes, volatile chemicals, and siderophores. (Richardson et al., 2009; Olanrewaju et al., 2017). Moreover, PGPB regulates plant growth via releasing a several of phytohormone, such as auxin, abscisic acid, cytokinin, ethylene, gibberellin and salicylic acid.

Salicylic acid (2- hydroxyl benzoic acid) is the natural phenolic compound mostly produced in plants and some of bacteria. However, phenolic compounds are well known for essential processes, including lignin and pigment biosynthesis, allelopathy, plant-microbe interaction, and management of abiotic and biotic stresses (Fig. 1). SA is a key plant hormone that plays direct or indirect roles in plant growth and development by regulating many aspects of thermogenesis, osmoregulation and disease resistance (Singh et al., 2016). Beyond its role in plants, SA and its acetylated derivate (aspirin) are major pharmacological agents for human being. In clinical sector SA often used to treat psoriasis, warts, and acne, beside this aspirin is one of the most commonly prescribed medications all over the globe; for the treatment of fever, pain, swelling and inflammation (Dempsey et al., 2017).

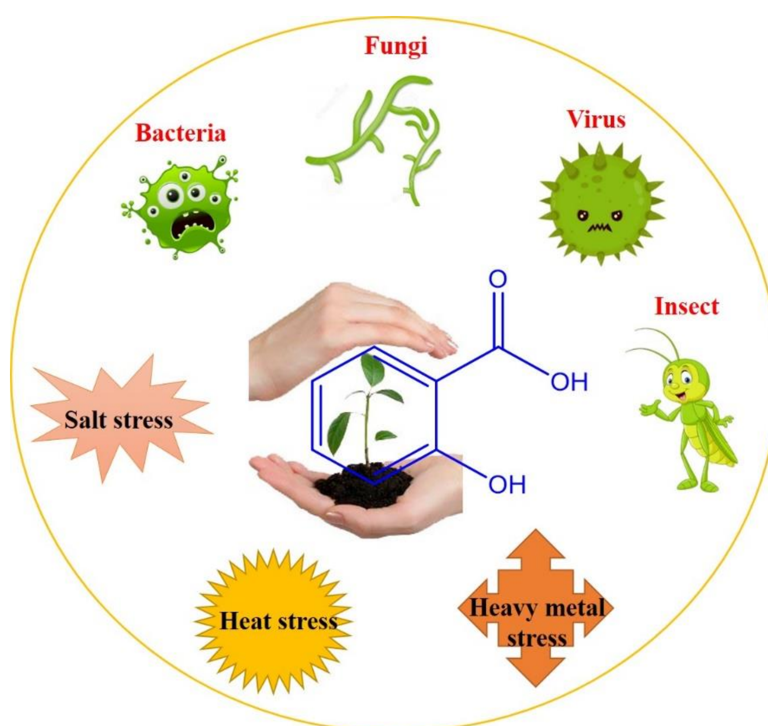


Figure. 1 Salicylic acid for the mitigation of biotic and abiotic stresses in plants (Adapted from Wani et al., 2017)

2. Materials and methods

2.1 MIC, MFC and MTT assay

According to the Clinical and Laboratory Standards Institute (CLSI) guidelines, the minimum inhibitory concentration (MIC) of SA against *F. oxysporum* NCIM 1281 was assessed in 96-well plates. The spore suspension was adjusted to 1.0 x 10^6 spores/mL with sterile potato dextrose broth. Different concentrations of compound were added into the wells with 100 µL as the final volume by serial dilution. Methanol was added as solvent control and incubated at 28°C for 72 h. From all the treated and control wells, a 10µL sample was inoculated onto the potato dextrose agar (PDA) plates for the next 72 h. The minimum concentration of SA where spores did not produce mycelia on the PD agar plate was considered the minimum fungicidal concentration (MFC). Cytotoxicity study of the salicylic acid was tested against Vero cell lines in Dulbecco's modified Eagle's medium supplemented with 10% FBS as already discussed.

2.2 Antifungal effect of salicylic acid on *F. oxysporum* NCIM 1281

2.2.1 Effect on spore germination

For the spore germination assay, spore suspension of test fungus was prepared in 0.01 percent Tween 80. Then, 1.0 × 10^6 spores /mL was adjusted with sterile potato dextrose broth and treated with the 0.9, 1.8 and 3.6 mM concentrations of SA. Methanol was used as the solvent control and incubated at 28° C. Sample was examined under 40x light microscopy (Nikon, Japan) at 0, 3, 6, 12 and 24 h of incubation. Spore germination was evaluated by counting at least 200 spores in each sample. Germination was considered when the germ tube length exceeded half of the diameter of the spores.

2.2.2 Effect on mycelial growth

The effect of SA on mycelial growth of test fungi was evaluated by using (Zou et al., 2022) method. The final concentrations of 1.8 and 3.6 mM of SA were adjusted with sterile molten PD agar. In the negative and solvent control plates, respectively, water and methanol were used in place of SA. Further, all test and control plates were inoculated with a 6 mm mycelial plug

from a 7-day-old culture. After 3rd, 5th, and 7th days of incubation, the colony size was measured, and the formula previously given was used to calculate percentage mycelial growth inhibition.

2.2.3 Effect on mycelial weight

The test fungus spore suspension was adjusted to 1.0×10^6 CFU/mL in 100 mL of sterile PD broth. Following that, the effect of SA on mycelial biomass was evaluated by adding final concentrations of 1.8 and 3.6mM. In the control flask, methanol was used instead of SA, and it was shaken at 140 rpm for 0, 3, 5, and 7 days at 28 °C. The mycelium was separated by using muslin cloth, dried in a dry oven at 70 °C for three days. The dried hyphae weight was taken and compared to the control.

2.3 Greenhouse pot experiment

The effect of strain NIO-1008 and SA on the wheat (*Triticum aestivum*) plants were studied at normal and stress conditions. The biotic stress was *F. oxysporum* NCIM1281, whereas the abiotic stress was soil salinity. The experiments were carried out separately in triplicates. During the green house studies, paper pots (8.0cm x 11.0cm x 5.0cm) were used and filled with 200 g of sterilised soil. Soil sterilization was carried out in an autoclave for three days to eliminate already available microorganisms in the soil. Wheat seeds were disinfected for 2 minutes with 2% sodium hypochlorite and 70% ethanol, then rinsed twice with sterile water. Following that, five seeds per pot were added, and treatments of salicylic acid and NIO-1008 were applied in the specified treatment groups. For the treatment, a 1.0 O.D inoculum was prepared using an 18-hour active culture, and a salicylic acid working solution was made in water. Instead of culture and SA, water was given to the control pots.

2.3.1. Effect of salicylic acid on the wheat plant growth

The growth-promoting action of SA on wheat plants was investigated at various concentrations. Immediately after seed sowing, 0.9, 1.8, and 3.6 mM 40mL solutions of salicylic acid were

applied as a treatment. The optimal concentration was chosen among these for the subsequent experiments.

2.3.2. Biocontrol effect of the strain NIO-1008 and SA on *F. oxysporum* NCIM 1281

A spore suspension of the fungus was prepared in sterile 0.01 percent tween 80 solution using a seven-day-old culture. The spore solution was then combined with sterile soil and incubated in a plastic bag with rising humidity for 48 h in the dark. Following that, 200 gm of *F. oxysporum* infested soil was added among all of the treatment and control pots once it reached 1.0×10^6 colony forming unit (CFU) per gram of soil (Rossi et al., 2001). After seed sowing, 40mL NIO-1008 suspension and 0.9mM SA solution were added to respective treatment groups. After that, the same volume of water was added every three days throughout the experiment. For 25 days, plants were cultivated in a greenhouse at 16:8 hours of light to darkness. Plants were taken out of the pots following incubation, rinsed under clean water, and their roots examined for signs of wilting. Disease severity was assessed based on percent of roots affected and the length and weight of roots and shoots (Sinha et al., 2019). The effect on chlorophyll content was estimated according to (Arnon et al., 1949) method.

2.3.3. Mitigation of salinity stress by NIO-1008 and SA

For this experiment salinity of sterile soil was adjusted to 0, 50 and 100 mM with the addition of sodium chloride (NaCl) (w/w). After seed sowing, 40mL treatment of 1% bacterial suspension and salicylic acid solutions were added to respective treatment groups. In the control group, 40 ml of water was added. From the second watering same volume of water was added every three days during the experiment in both the control and treatment groups. In a greenhouse, plants were kept for 25 days at a 16:8 light: dark proportion. After the experiment, plant growth was evaluated based on shoot root length, biomass, and chlorophyll content. The plant's dry weight was noted after oven-drying at 60°C for 48 h.

Statistical analysis

All experiments were carried out in triplicates, and data were presented as mean ± standard deviation. Different letters above bars indicate a significant difference between groups as demonstrated by one way ANOVA followed with Post hoc Tukey's (HSD) honestly significant difference test. The p-values<0.05 were considered statistically significant. Statistical analysis was performed using SPSS software version 26.0 (SPSS Inc., USA). Graph Pad Prism 8.0.2 software was used for plotting the graphs.

3. Result

3.1 MIC, MFC and MTT assay

The antifungal activity of SA was assessed based on the MIC and MFC value. The (Fig. 2) demonstrated that the mycelial growth of test fungus was not visible at a MIC value of 1.8 mM. At the same time, MFC is 3.6 mM, at which fungi cannot grow on the PD agar plates. Interestingly, salicylic acid did not showed cytotoxic effect on Vero cell lines, even at highest (7.2mM) concentration (Fig. 3).

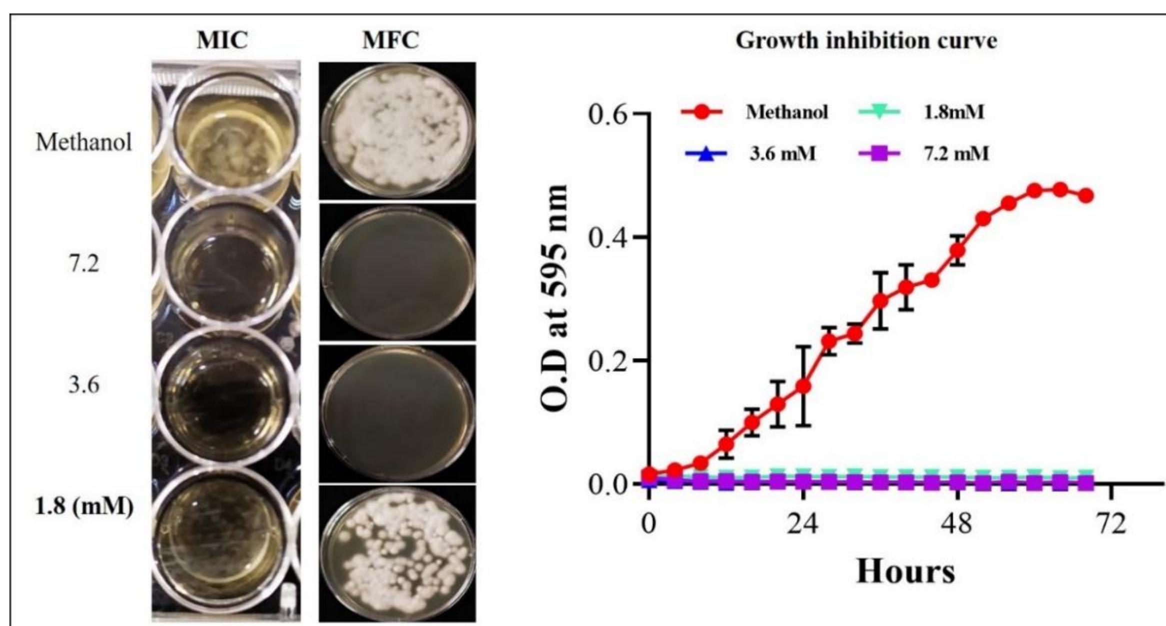


Figure. 2 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration activity of SA against *F. oxysporum* NCIM 1281

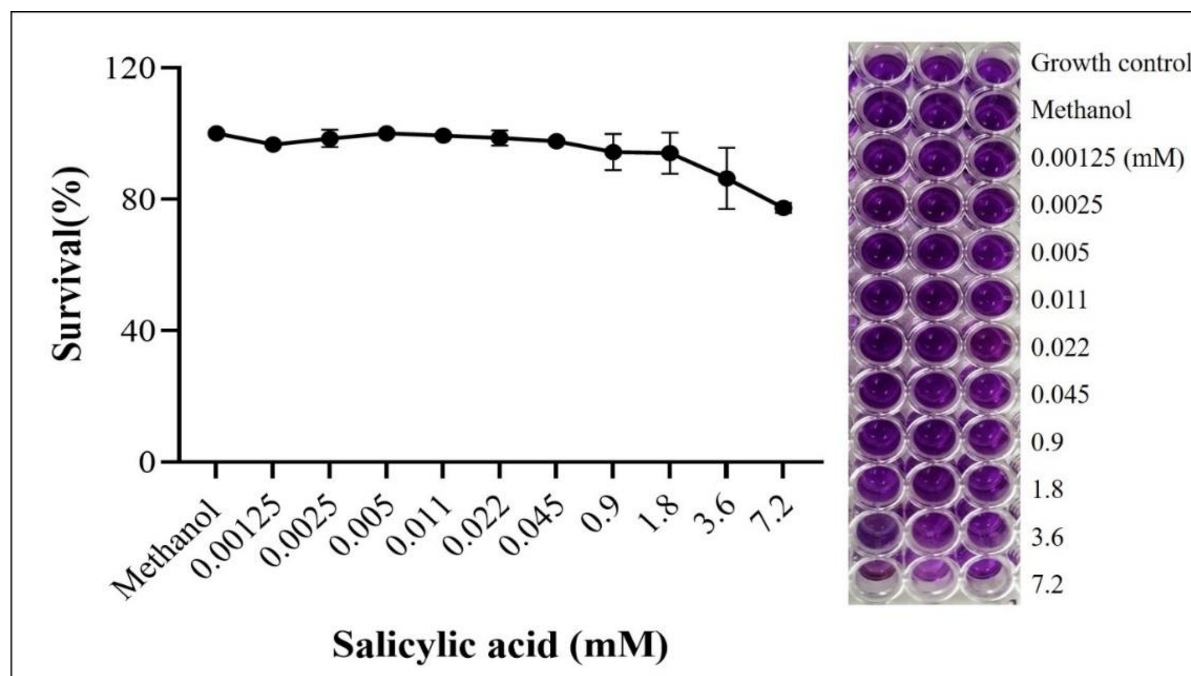


Figure. 3 Cytotoxic activity of salicylic acid against Vero cell lines

3.2 Antifungal effect of salicylic acid on *F. oxysporum* NCIM 1281

The inhibitory effect of SA on the spore germination was analysed at a different incubation time. The rate of per cent spore germination inhibition (PSGI) was significantly greater with the increasing concentrations of SA compared to solvent control (Fig. 4a). The rate of PSGI was declined with rising incubation time at 0.9 mM of SA. Simultaneously, SA at 1.8 mM demonstrated 100, 92.4, 86.1, and 81.7 PSGI after 3, 6, 12, and 24 hours of incubation, respectively. At 3.6 mM concentration, spore germination was completely inhibited throughout the incubation period (Fig. 4b). Moreover, the effect of SA on the mycelial growth of test fungus was also investigated. Compared to the solvent control, the SA demonstrated a significant inhibitory action on mycelial growth. The diameter of the mycelia drastically reduced with increasing compound concentration, as shown in (Fig. 5a). At 3.6 mM, the SA was found to be very efficient, suppressing mycelial growth by 100%, 88.4%, and 77.8% on the 3, 5, and 7th days, respectively (Fig. 5b). (Fig. 5c).

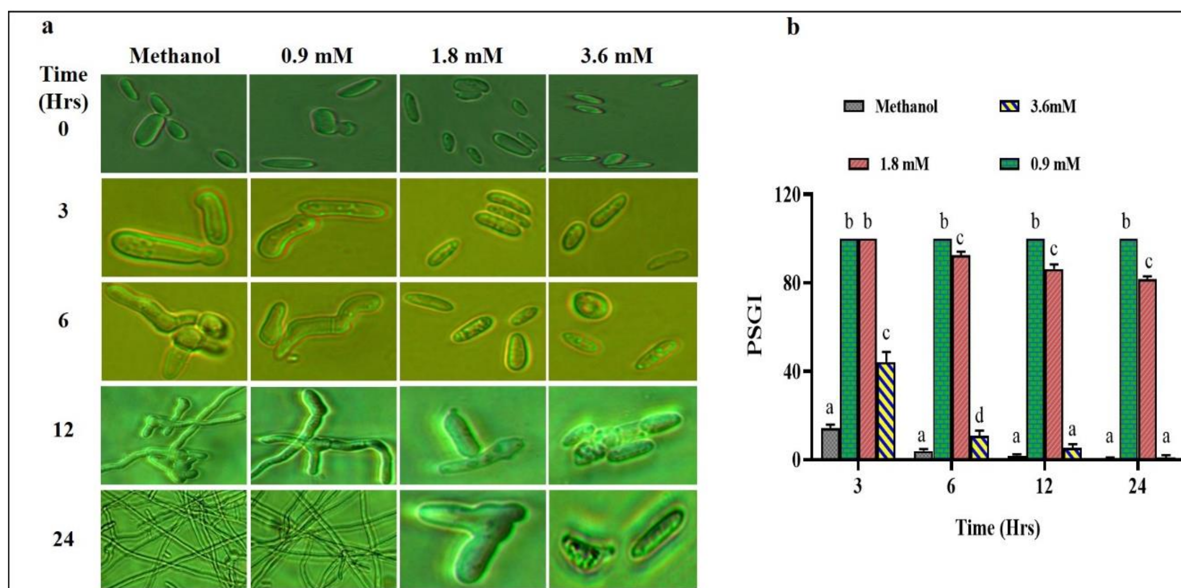


Figure. 4 Effect of SA on the spore germination of *F. oxysporum* NCIM 1281, a. Qualitative analysis of spore germination and b. Quantitative analysis of the percent spore germination inhibition (PSGI)

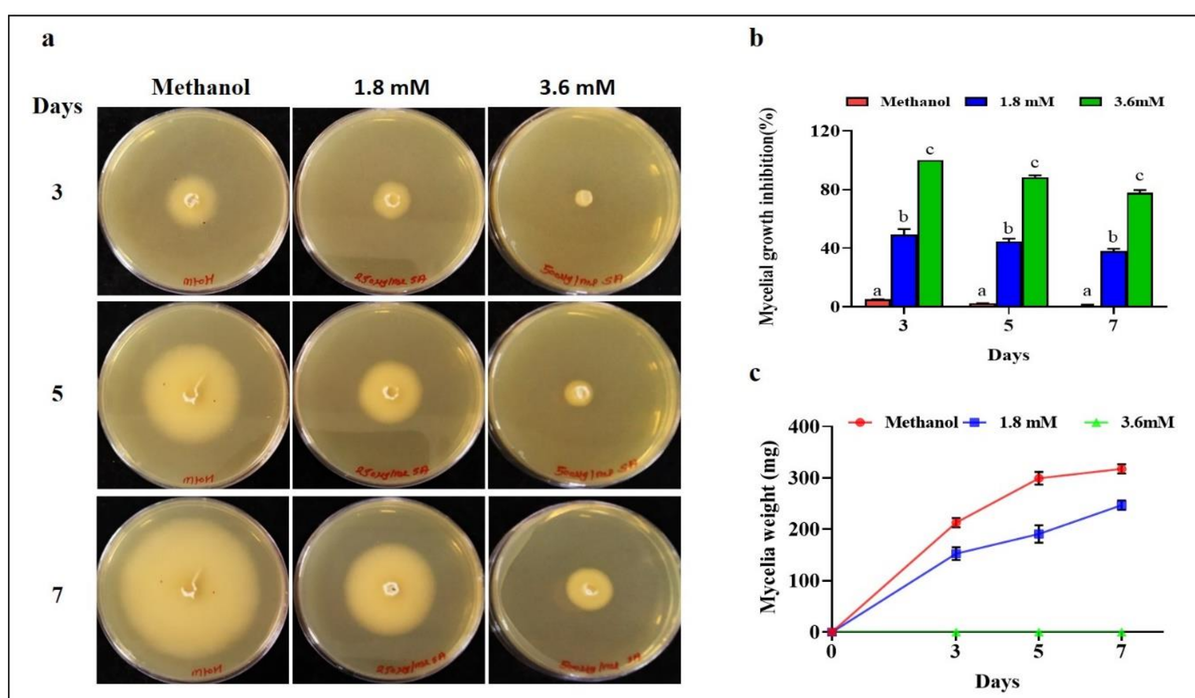


Figure. 5 Effect of SA on the growth of *F. oxysporum* NCIM 1281, **a.** Qualitative analysis of the percent mycelial growth inhibition, **b.** Quantitative analysis of the percent mycelial growth inhibition and **c.** effect on the weight of mycelia

Beside this, the mycelial biomass was also remarkably reduced in SA-treated samples compared to solvent control. At 1.8 mM of SA, mycelial weight was drastically reduced to 28.6, 35.4, and 22.0 percent on the third, fifth, and seventh days of incubation, respectively, compared to solvent control. Similarly, even on the seventh day of incubation, mycelial weight was reduced to zero at 3.6 mM SA concentration

3.3 Effect of SA on the wheat plant growth

In the greenhouse pot experiment, wheat plants were treated with different concentrations of SA in the soil. The higher concentrations treatment of SA at 3.6 mM exhibited a negative effect compared to untreated control. The 1.8 mM treatment did not have any noticeable effects on the plants, either positive or negative. It's interesting to observe that, in comparison to untreated controls and higher concentrations, low concentration (0.9 mM) had a significant impact on the root shoot length and overall growth of wheat plants (Fig. 6a-c).

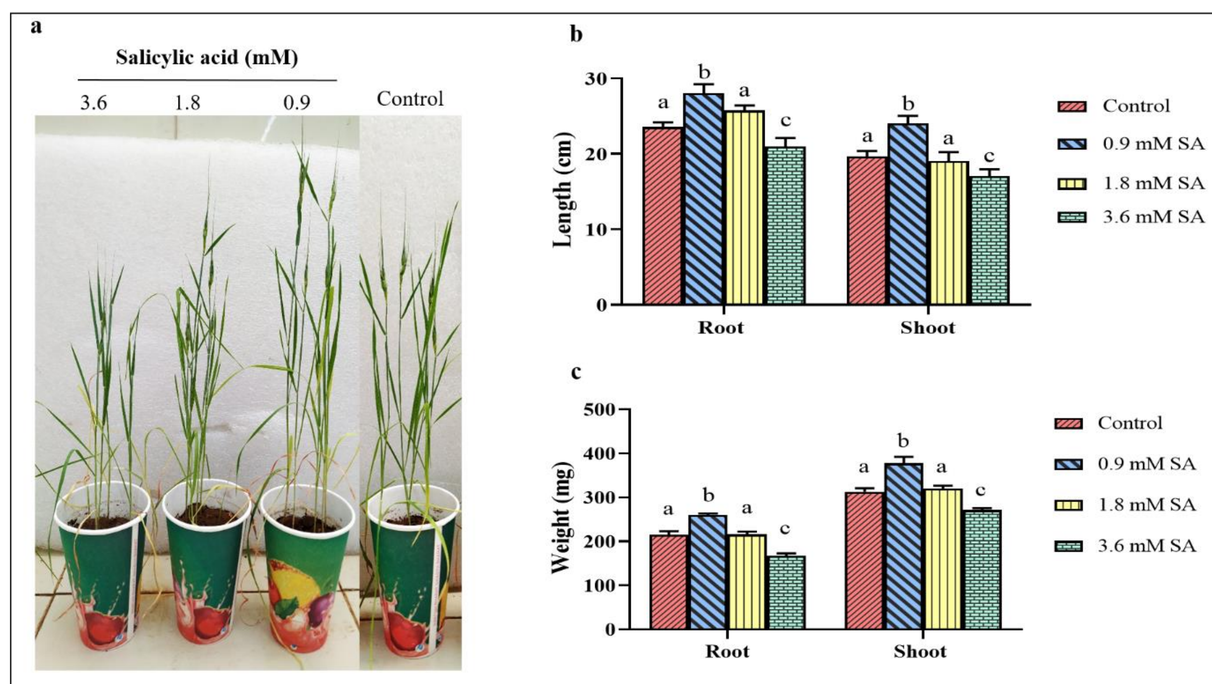


Figure. 6 Effect of salicylic acid (SA) treatment on the wheat plants growth at 0.9, 1.8 and 3.6 mM concentrations and untreated control, **a.** Representative image showing the effect of SA on the overall growth of plants, **b.** effect on root shoot length and **c.** Root shoot weight

3.4 Biocontrol effect of strain NIO-1008 and SA on *F. oxysporum* NCIM 1281

The effectiveness of strains NIO-1008 and SA to stimulate plant growth in the presence of *F. oxysporum* infestation was investigated on wheat plants. In comparison to pathogen control, the results presented in (Fig. 7a) demonstrated a decrease in disease severity and an improvement in the overall quality of plants treated with strain NIO-1008 and SA. Relative to pathogen control, the incidence of disease was decreased substantially to 70% in NIO-1008 and 45.7% in SA treated wheat plants (Fig.7b). Further, the plant growth-promoting parameters such as the root length was improved by 27.6% in NIO-1008 and &15.1% in SA-treated plants. In addition, shoot length improved by 45.9% and 41.9%, respectively, in plants treated with NIO-1008 and SA (Fig. 8a). The fresh and dry weights were also significantly increased to 76.1% and 50.2% in NIO-1008 and 72.3% and 35.5% in plants treated with SA, respectively. (Fig. 8b & c). Also, plants treated with NIO-1008 and SA had more chlorophyll a and b than control plants (Fig. 8d).

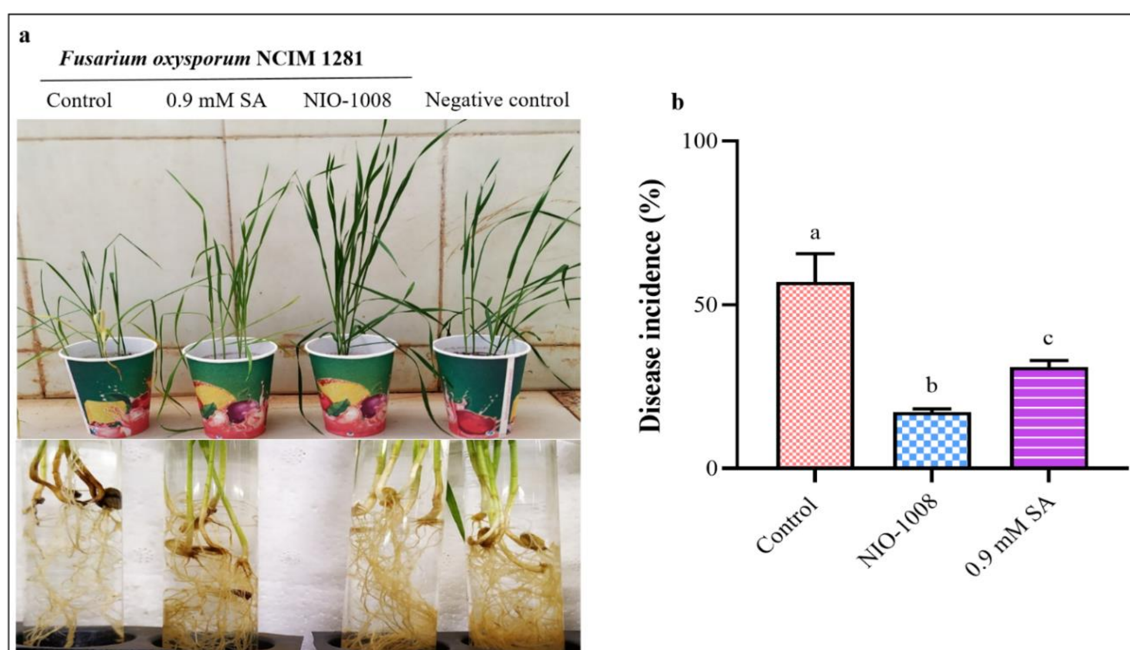


Figure. 7 Biocontrol activity of NIO-1008 and SA to suppress effect of *F. oxysporum* (F.O) NCIM 1281 on the wheat plants at green house pot experiment, **a.** Representative image of wheat plant to show the overall growth and root quality adversely affected in control plants while minor symptoms observed in plants treated with SA and NIO-1008 **b.** Disease incidence

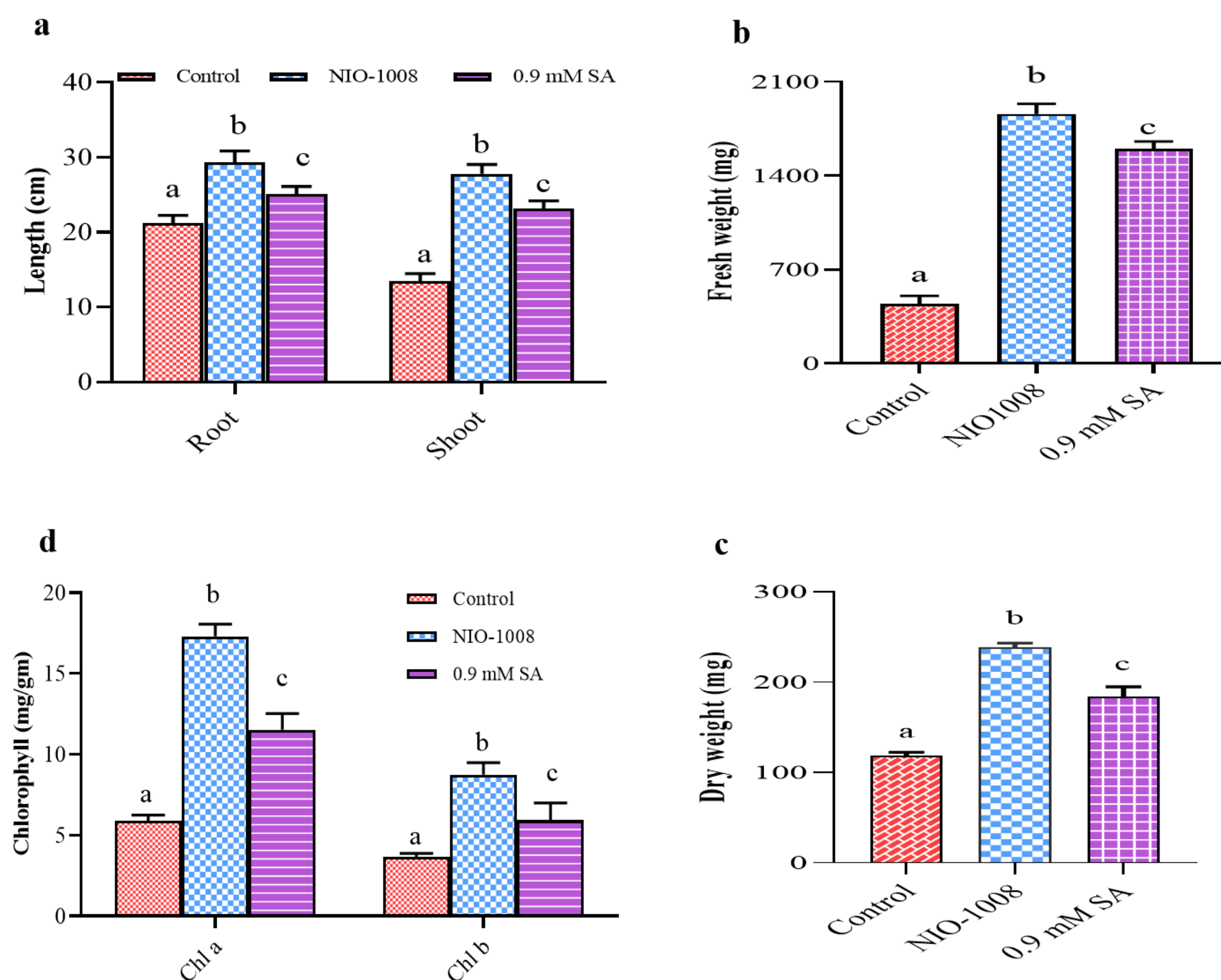


Figure. 8 Evaluation of growth parameters in wheat plants treated (NIO-1008 & SA) and untreated control under *F. oxysporum* (F.O) NCIM 1281 stress, **a.** Root shoot length **b.** Fresh weight, **c.** Dry weight and **d.** Chlorophyll content

3.5 Mitigation of salinity stress by strain NIO-1008 and salicylic acid

The influence of the strain NIO-1008 and SA on wheat plants grown in salinated and non-salinated soil was investigated. In 50 and 100 mM salinity, untreated control plants overall growth and root length were drastically reduced compared to non-saline. Plants treated with NIO-1008 and SA at 50 and 100 mM salinity conditions showed decreased salt stress, as shown in Figure. 9. To further investigate the effects of NIO-1008 and SA on the wheat plants, essential growth-promoting characteristics such as root and shoot length, plant biomass content, and chlorophyll levels were examined. When compared to the untreated control, the

plants treated with NIO-1008 and SA had relatively long roots. In 50 and 100 mM of salt stress, inoculation with NIO-1008 enhanced root length by 43.3% and 41.5%, respectively. At the same time, plants treated with SA induced root length by 36.7% in 50 mM and 36.5% in 100 mM (Fig.10a). Shoot length was substantially shortened in untreated control plants as salt stress increased. At all doses of salt stress, the plant treated with NIO-1008 and SA improved considerably in shoot length. NIO-1008 extended shoot length by 25.3% in 50 mM salt stress, and 35.5% in 100 mM salt stress. The shoot length of the SA-treated plant improved by 16.5% at 50 mM, and 22.5% at 100mM. (Fig.10b). Apart from root shoot length, increasing salt concentration has a negative impact on plant biomass and chlorophyll content. Results shown in (Fig.10c) indicate that under 100 mM salinity stress, fresh weight improved by 56.4% in NIO-1008 and 53.8% in SA-treated plants as compared to controls. The dry weight of plant improved by 34.3% in NIO-1008 and by 30.5% in SA treated plants (Fig. 10d). Following that, chlorophyll a and b content was found to be significantly higher in the plant treated with NIO-1008 and SA compared to the control at all of the tested saline stress (Fig.10e & f).

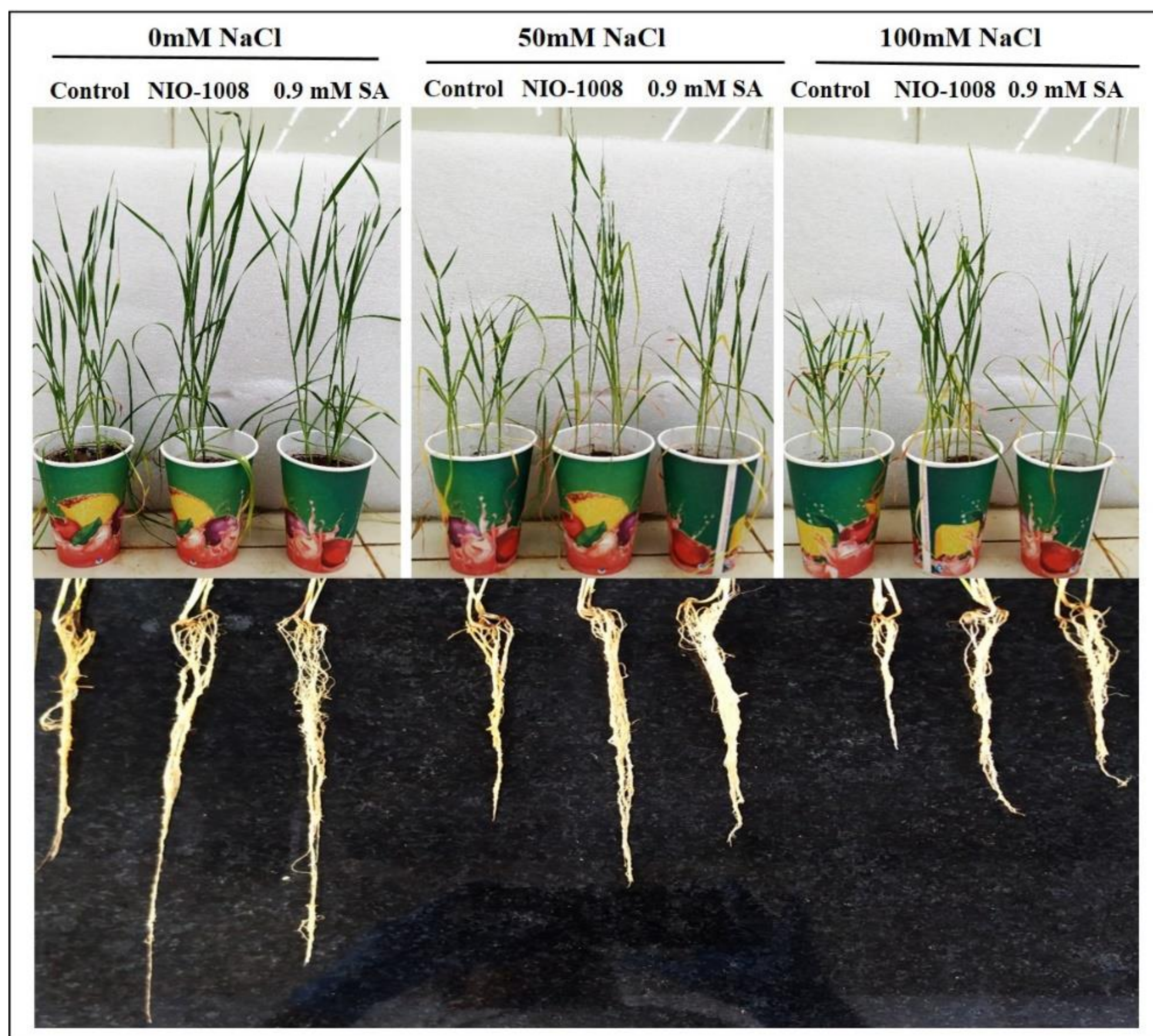


Figure. 9 Growth promoting effect of NIO-1008 and SA on the wheat plants under normal and salinity stress conditions

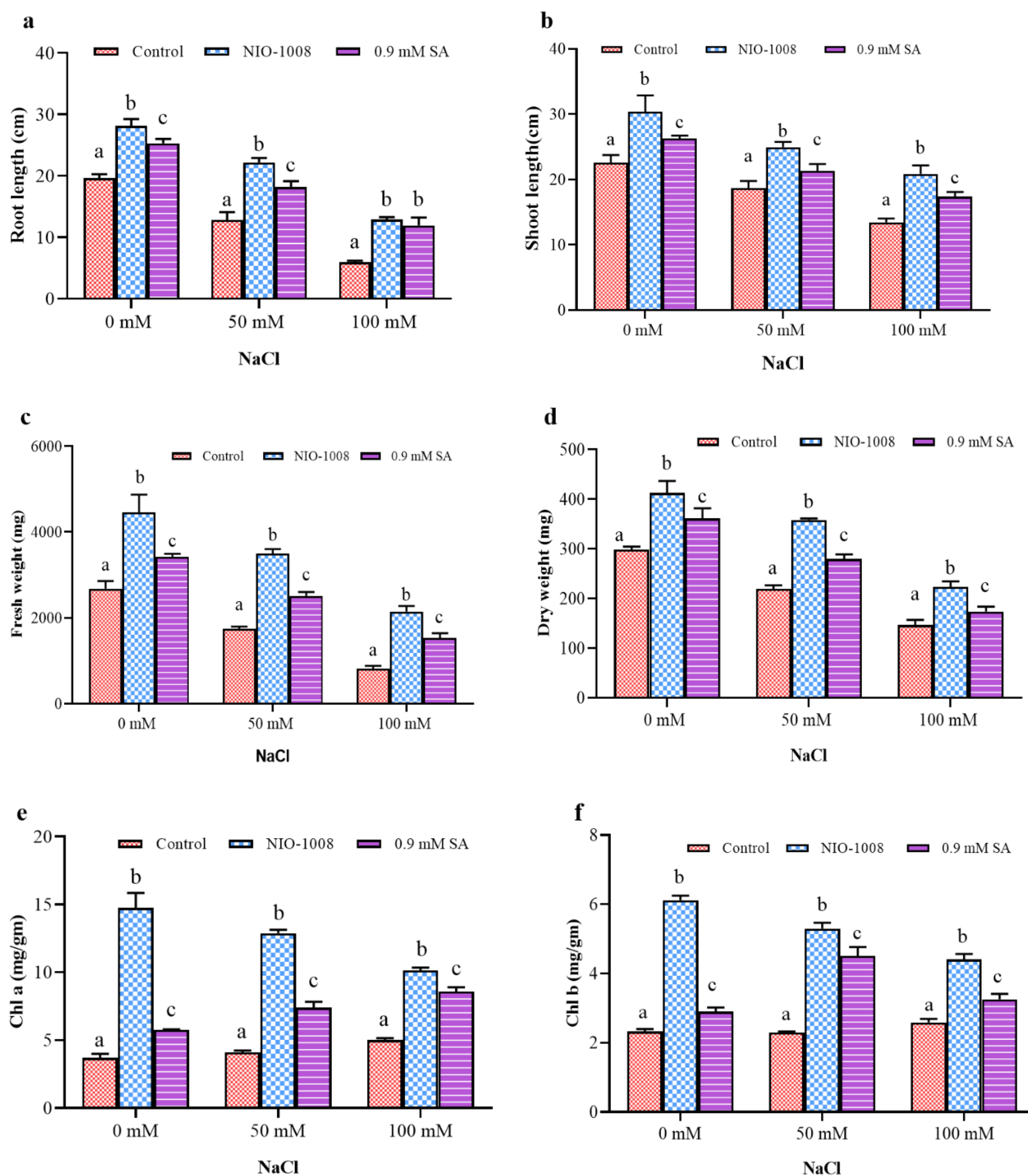


Figure. 10 Effect of NIO-1008 and SA treatment on the growth parameters of wheat plants under normal and salinity stress conditions, **a.** Root length **b.** Shoot length, **c.** Fresh weight **d.** Dry weight, **e.** Chlorophyll a content and **f.** Chlorophyll b content

4. Discussion

Salicylic acid is a key signalling molecule, which induces plant immunity via systemic acquired resistance (SAR) and protects against a variety of abiotic and biotic stressors (Bakker et al., 2014; Koo et al., 2020). Its significance in plant growth and development has been extensively studied in different stress conditions. As a result in our investigation, the lowest tested concentration of SA (0.9 mM) was found to be more useful to the wheat plant than the higher tested doses (Fig. 6). This is quite similar to (Koo et al., 2020), which revealed that >1mM SA adversely affected plant development and growth. This results indicated that natural active molecules like as salicylic acid are very effective even at low concentration that can be advantageous in reducing the chemical load on agriculture.

Phytopathogenic fungi are among the worst biotic stressor in agriculture, causing a reduction in crop quality and productivity. *F. oxysporum* is one of the most destructive pathogen that infects economically valuable crops including wheat, banana, maize, cotton, and tomato by causing wilt disease (Rocha et al., 2017). As a consequence, prior studies (Rocha et al., 2017; Ye et al., 2020) revealed that plants infected with *F. oxysporum* had reduced root shoot length, plant biomass, and chlorophyll content. However, in our study, SA and the marine strain NIO-1008 treatments on wheat plants significantly reduced disease incidence generated by *F. oxysporum* (Fig. 7a & b). Also, root shoot length, plant biomass, and chlorophyll content were substantially greater in the treated plants compared to the control (Fig. 8a-d).

Soil salinity is yet another major agricultural challenge that decreases the yeild of important crops like wheat, rice, maize and others. During salt stress, plant roots are unable to absorb nutrients and water, which hinders plant growth and development and limiting the length of the roots shoots and the amount of biomass in the plant (Qin et al., 2014; Bacilio et al., 2016; Khan et al., 2021). In a similar manner, we found that increasing salinity stress had a detrimental effect on wheat plant development in the un-inoculated control group (Fig. 9). However as compared to un-inoculated control plants, the plant treated with NIO-1008 and SA

showed a significant rise in the length of its roots, shoots, biomass, and chlorophyll content (Fig. 10a-f). Interestingly, our results revealed that the marine strain NIO-1008 is more effective than salicylic acid for alleviating salt stress and controlling *F. oxysporum* infection. These results suggest that growth regulators such salicylic acid, siderophore, and indole acetic acid (IAA), which were identified in strain NIO-1008, may be working together to alleviate plant stress. Previous research has shown that a number of PGP bacteria can promote plant growth under several of abiotic and biotic stresses, although even the most of those examined their effect under a single stress. In our study, the marine strain NIO-1008 showed plant growth improving effect on wheat plant under fungal infestation as well as salinity stress. For the goal of increasing crop productivity, the competence of microorganisms to deal with multiple stresses is extremely important in agriculture.

5. Conclusion

The relevance of microorganisms in plant growth promotion and stress tolerance has long been acknowledged, however marine bacteria have been largely neglected in agricultural applications as compared to terrestrial bacteria. In the current study, marine strain NIO-1008 and its active molecule salicylic acid were tested for stress mitigation efficacy on wheat plants in a greenhouse pot experiment. As a result, the deleterious effects of salinity and *F. oxysporum* stress were greatly decreased by strain NIO-1008 and its active compound. Interestingly, whole cells of strain NIO-1008 showed better activity on wheat plants than salicylic acid. It suggests that the bacteria produce a variety of growth regulators under controlled manner, which act together to regulate plant stress. Adopting primitive microbial culture is a more economical and environmentally beneficial approach for sustainable agriculture instead of using pure compound. Based on the results, we conclude that marine bacteria and their bioactive compounds have great potential for sustainable agriculture and need to be addressed further as bio-fertiliser and bio-pesticides.

References

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. *Plant physiology*, *24*(1), 1.

Bakker, P. A., Ran, L., & Mercado-Blanco, J. (2014). Rhizobacterial salicylate production provokes headaches! *Plant and soil*, *382*(1), 1-16.

Bacilio, M., Moreno, M., & Bashan, Y. (2016). Mitigation of negative effects of progressive soil salinity gradients by application of humic acids and inoculation with *Pseudomonas stutzeri* in a salt-tolerant and a salt-susceptible pepper. *Applied Soil Ecology*, *107*, 394-404.

Chojak-Koźniewska, J., Kuźniak, E., & Zimny, J. (2018). The effects of combined abiotic and pathogen stress in plants: insights from salinity and *Pseudomonas syringae pv lachrymans* interaction in cucumber. *Frontiers in plant science*, *9*, 1691.

Eydoux, L., & Farrer, E. C. (2020). Does salinity affect lifestyle switching in the plant pathogen *Fusarium solani*? *Access Microbiology*, *2*(6), e000114.

Kumar, A., & Verma, J. P. (2018). Does plant—microbe interaction confer stress tolerance in plants: a review? *Microbiological research*, *207*, 41-52.

Koo, Y. M., Heo, A. Y., & Choi, H. W. (2020). Salicylic acid as a safe plant protector and growth regulator. *The plant pathology journal*, *36*(1), 1.

Khan, M. A., Sahile, A. A., Jan, R., Asaf, S., Hamayun, M., Imran, M., & Lee, I. J. (2021). Halotolerant bacteria mitigate the effects of salinity stress on soybean growth by regulating secondary metabolites and molecular responses. *BMC plant biology*, *21*(1), 1-15.

Limonium sinense (Girard) Kuntze and evaluating their plant growth-promoting activity under salt stress. *Plant and soil*, *374*(1), 753-766.

Olanrewaju, O. S., Glick, B. R., & Babalola, O. O. (2017). Mechanisms of action of plant growth promoting bacteria. *World Journal of Microbiology and Biotechnology*, *33*(11), 1-16.

Qin, S., Zhang, Y. J., Yuan, B., Xu, P. Y., Xing, K., Wang, J., & Jiang, J. H. (2014). Isolation of ACC deaminase-producing habitat-adapted symbiotic bacteria associated with halophyte Limonium sinense (Girard) Kuntze and evaluating their plant growth-promoting activity under salt stress. *Plant and soil*, *374*(1), 753-766.

Rocha, F. Y. O., de Oliveira, C. M., da Silva, P. R. A., de Melo, L. H. V., do Carmo, M. G. F., & Baldani, J. I. (2017). Taxonomical and functional characterization of *Bacillus* strains isolated from tomato plants and their biocontrol activity against races 1, 2 and 3 of *Fusarium oxysporum f. sp. Lycopersici*. *Applied soil ecology*, *120*, 8-19.

Richardson, A. E., Barea, J. M., McNeill, A. M., & Prigent-Combaret, C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and soil*, *321*(1), 305-339.

Rossi, V., Ravanetti, A., Pattori, E., & Giosue, S. (2001). Influence of temperature and humidity on the infection of wheat spikes by some fungi causing Fusarium head blight. *Journal of Plant Pathology*, 189-198.

Sinha, R., Irulappan, V., Mohan-Raju, B., Suganthi, A., & Senthil-Kumar, M. (2019). Impact of drought stress on simultaneously occurring pathogen infection in field-grown chickpea. *Scientific Reports*, *9*(1), 1-15.

Singh, P. K. (2016). Role of Salicylic Acid in Survival Strategy of Plants under Changing Environment. *International Journal of Plant and Environment*, *2*(1 and 2), 79-86.

Wani, A. B., Chadar, H., Wani, A. H., Singh, S., & Upadhyay, N. (2017). Salicylic acid to decrease plant stress. *Environmental Chemistry Letters*, *15*, 101-123.

Ye, X., Li, Z., Luo, X., Wang, W., Li, Y., Li, R., & Zhang, R. (2020). A predatory myxobacterium controls cucumber Fusarium wilt by regulating the soil microbial community. *Microbiome*, *8*(1), 1-17.

Zou, X., Wei, Y., Jiang, S., Xu, F., Wang, H., Zhan, P., & Shao, X. (2022). ROS stress and cell membrane disruption are the main antifungal mechanisms of 2-Phenylethanol against Botrytis cinerea. *Journal of Agricultural and Food Chemistry*, *70*(45), 14468-14479.

Chapter. 5:

Summary and Future Perspectives

1. Overall summary

The agriculture sector is tackle with a several challenges, such as biotic and abiotic stresses that decrease crop production substantially. As a reason, synthetic agrochemicals have been extensively utilized all over the world. The scientific community is striving to find eco-friendly substitutes to synthetic agrochemicals. Beneficial microorganisms and their active compounds are believed to be the best alternative to synthetic agrochemicals in this aspect. Terrestrial microorganisms and their bioactive molecules have been thoroughly examined for agricultural use during the past 50 years. Unfortunately, only a small number of these microbes have been successfully marketed as bio-pesticides and bio-fertilizers. But, when compared to chemical fertilisers and pesticides, they still fall short in terms of efficacy and performance in open fields. The main reason of failure in open field condition is that they are unable to endure and perform in the presence of several abiotic and biotic stressors. On the other hand, harsh environment microorganisms like marine bacteria are gaining more attention due to their ability to produce bioactive compounds that assist to function and survive in situations involving elevated salinity, high pressure, nutrient scarcity, variable pH, and high temperatures.

In our study, marine bacteria and their bioactive compounds were studied for agricultural applications. To assess antagonistic activity against plant pathogenic fungi, the previously isolated 30 marine bacteria were screened. Among these, the strains BKACT and NIO-1008 were selected as the leading antagonistic candidates. As well, both strains were confirmed to grow at a broad range of pH, high NaCl concentration and produced multiple plant growth-promoting (PGP) traits. In the strain BKACT, phosphate solubilization, potassium solubilization, and the production of lytic enzymes were observed. Another strain, NIO-1008, was also found to release siderophore and ammonia. Indole acetic acid (IAA) was also detected

in both strains, although NIO-1008 reported the most, 640 ±9 µg/mL IAA, which is six fold greater than BKACT. The identification of both strains were completed by 16S r RNA sequencing, which confirmed that NIO-1008 is *Arthrobacetr enclensis* and BKACT is *Serratia marcescens*. Moreover, genome study revealed the aforementioned traits in both strains by examining genes related to stress tolerance, biocontrol activity, and plant growth promotion. After the finding of antifungal compound genes in the genomes, crude extracts of 9.6 gm from strain BKACT and 12 gm from strain NIO-1008 were produced at lab scale 10.0L fermentation, and their antifungal activity against *Fusarium* species was proven. The crude extracts further purified using a silica column, preparative HPLC, and TLC. Also, the structures of purified compounds were established to be salicylic acid from strain NIO-1008 and 2, 4-di-tert-butyl phenol from strain BKACT using NMR and GC-MS techniques. Interestingly, this is the first attempt to purify and structurally characterise these compounds from their respective marine bacteria.

Since phenolic compounds seem to be involved in a wide range of biological processes, both purified molecules were investigated further for potential uses. As a result, employing 2, 4-DTBP at 0.53mM fumigation, spore germination was totally inhibited, and mycelial growth was also significantly decreased. Simultaneously, the test fungus growth on wheat seeds was completely inhibited at 1.0 mM concentration, while the same concentration had no detrimental effects on wheat seed germination. Besides antifungal activity, inhibiting ergosterol biosynthesis highlighted the mechanism of mycelial growth inhibition. Docking study found that 2, 4-DTBP had the best binding conformation with Sterol 14-alpha demethylase (CYP51), with a binding energy of -6.29 Kcal/mol. This is the first study to use 2, 4-DTBP fumigation to understand the mechanism of ergosterol inhibition of mycelia.

Salicylic acid, another phenolic compound, inhibited spore germination, mycelial growth, and weight of *F. oxysporum* NCIM 1281 *in vitro*. On the other hand, even at higher concentrations

(7.2 mM), it was not toxic to Vero cell lines. Salicylic acid and its producing strain NIO-1008 were investigated at the greenhouse pot experiments with the purpose of managing stress and promoting plant growth. As a response, in SA and strain NIO-1008 treated wheat plants, the disease incidence was significantly decreased to 70% and 45.7%, respectively. Moreover, SA and NIO-1008 allowed wheat plants to withstand salt stress of up to 100mM.

2. Future Perspective

Serratia marcescens BKACT and *Arthrobacter enclensis* NIO-1008 were found to be promising marine bacteria via comprehensive screening that exhibit PGP traits and stress tolerance. Also, the genomes revealed genetic markers linked to the above mentioned attributes. In addition to conventional methods, advanced techniques like as genome sequencing should be used, since it will aid in the screening of more efficient bacteria against a specific pathogen. We isolated and characterised 2, 4-DTBP and salicylic acid from marine bacteria, which has greater agricultural relevance. Based on results, we conclude that marine bacteria have a tremendous capability of producing bioactive compounds that could be useful in the agriculture. Natural VOC 2, 4-DTBP has been identified from a number of species with a wide spectrum of biological activity. Though, the biosynthetic pathways and its mechanism of action against pathogens are still undiscovered, and therefore it should be addressed in future study. So far, first ever, we discovered the mechanism of 2, 4-DTBP, which targets the ergosterol production in fungus. Due to the absence of ergosterol in other pathogens (bacteria, viruses, nematodes, and insects), the mechanism of 2, 4-DTBP may be different and it should be discovered. In addition to protecting wheat seeds from *Fusarium* infection, the 2, 4-DTBP at 1.0mM fumigation treatment had no detrimental effects on seed germination. Our suggestion is to apply 2, 4-DTBP as a seed protective agent in this context. Moreover, this molecule volatile properties need to be investigated for the prevention of postharvest fungal diseases.

Salicylic acid is well accepted for its involvement in treating plant stress. Thus, the SA-producing marine strain *A. enclensis* NIO-1008 relieves salt stress and *F. oxysporum* infection in wheat plants. It suggests that bacteria from harsh environments produce molecules like salicylic acid that enable bacteria in managing plant stress. In order to develop bio-fertilizers and bio-pesticides for sustainable agriculture, further research has to be done on marine bacteria and their active compounds.

ABSTRACT

Student Name: Bharat A. Kharat **Registration No:** 10BB17J26053

Faculty of Study: Biological science **Year of Submission:** 2023

AcSIR Academic Centre/CSIR Lab: CSIR- NCL **Supervisor Name:** Dr. Syed G. Dastager

Thesis Title: Marine bacteria and its bioactive compounds for the agriculture applications

In order to find alternatives to conventional pesticides, 30 previously identified marine bacteria were screened against plant pathogenic fungi. The two strains BKACT and NIO-1008 were recognized as the top antifungal candidates among them. They were also found to grow in a wide pH range, with a high NaCl concentration, and to produce a number of plant growth-promoting (PGP) features. Using 16S r RNA sequencing, both strains BKACT and NIO-1008 were subsequently identified as *Serratia marcescens* and *Arthrobacetr enclensis*, respectively. Furthermore, genome mining has highlighted genes that contribute to stress tolerance, biocontrol activity, and plant growth regulators, validating the above-mentioned attributes in both strains. Afterwards, 9.6 gm and 12 gm of crude extract from the strains BKACT and NIO-1008, respectively, were produced in a lab scale 10.0L fermentation, and their antifungal activity against *Fusarium* species was verified. The crude extracts were purified via chromatographic techniques such silica column, preparatory HPLC, and TLC. Purified molecules from strains BKACT and NIO-1008 were confirmed as 2, 4-di-tert-butyl phenol and salicylic acid, respectively, using NMR and GC-MS. Both compounds had a strong inhibitory effect on spore germination and mycelial growth of *Fusarium* species in an *in-vitro* antifungal experiment. Also, the mechanism of 2, 4-DTBP suppressing ergosterol production in mycelia was elucidated experimentally and further validated by in silico docking. Besides this, at 1.0 mM concentration, 2, 4-DTBP was found to be an effective biocontrol agent that preventing the growth of the test fungus on wheat seeds. Meanwhile, in greenhouse pot experiments, another molecule salicylic acid and its producing strain NIO-1008 reduced the disease incidence of *F. oxysporum* as well as tolerate up to 100 mM salt stress in wheat plants.

List of Publications

1. **Kharat B. A,** Said, M. S., & Dastager, S. G. (2022). Antifungal compound from marine Serratia marcescens BKACT and its potential activity against Fusarium sp. ***International Microbiology*, 1-12.**
2. **Kharat B. A,** Madhukar S. Said, Dastager S. G. Mitigation of salinity stress and *F. oxysporum* NCIM 1281 infestation by salicylic acid producing novel plant growth promoting *Arthrobacter enclensis* NIO-1008 (**Manuscript under preparation**).

Conference

1. Best Rapid presentation and poster award

Conference: International Conference on BREEECH jointly organized by CSIR-IIP and BRS, at Dehradun, India.

Conference date: December 1-4, 2021

Title: Characterisation of the antifungal compound from antagonistic *Serratia marcescens* BKACT: Its application for the control of postharvest infection of *Fusarium* sp.

Authors: Bharat A. Kharat [ab*], Madhukar S. Said [bc], Syed G. Dastager [ab]

Abstract:

A marine bacteria *Serratia marcescens* BKACT isolated from the Chorao Island Goa, India, showed a potential antifungal activity against *Fusarium sp*. The antifungal compound further purified through chromatographic techniques and characterisation carried by ^{1}H NMR, ^{13}C NMR and GC/MS analysis to confirm its chemical structure. Marine isolate *Serratia marcescens* BKACT demonstrated antagonistic activity against all the tested fungi. The highest per cent growth inhibition up to 69.56 ± 1.22 was recorded against *Fusarium spp*. A total of 9.6 gm of crude extract was obtained from the 10L fermentation batch. The final purified compound was characterised and identified as the 2, 4, di-tert butyl phenol. The MIC and MFC value 0.0625 and0.03125 mg/ml respectively was recorded against *Fusarium sp*. The fumigation effect of the compound was tested in vitro against the *Fusarium sp*. At the 0.533mM/L fumigation concentration the spore germination almost completely suppressed and up to 86.6 ± 2.0, 86.9 ± 0.89 and 82.8 ± 0.70 per cent mycelial growth inhibition reported on the 5, 7 and 9th day of incubation time. Further fumigation effect was checked directly on wheat seeds and the *Fusarium sp* contamination was completely restricted at 1.065mM/L concentration. The fumigation treatment of the compound observed safe for the seed germination. Our study suggests that, for the first time the antifungal compound 2, 4 di- tert-butyl phenol purified and characterized from the biocontrol marine bacteria *Serratia marcescens* BKACT and demonstrated the fumigation mechanism for the control *Fusarium sp* on wheat seeds.

2. Oral presentation

Conference: "International Virtual Conference on ET-FPIC 2021" organised by CSIR-Central Food Technological Research Institute, Mysuru, India

Conference date: February 24-25, 2021

Title: Antifungal volatile compound produced from a marine *Serratia marcescens* BKACT: purification, characterization and its fumigation potential against *Fusarium* species.

Authors: Bharat A. Kharat [ab*], Madhukar S. Said [bc], Syed G. Dastager [ab]

Abstract:

Outbreaks of new diseases in the agriculture due to global climate change has become a challenging to counter back. There is a need to search a new biocontrol agents and its bioactive compounds, which can replace the chemically synthesized toxic pesticides. Marine bacteria are a very important and essential source of bioactive molecules, having potential applications in the agriculture sector. The present study is designed to explore the antifungal potentials of marine bacteria, which were isolated from Chorao Island Goa, India. These bacteria were screened for antifungal activity against *Fusarium* sp. NCIM 1330. Among the screened bacteria, the marine isolate BKACT was identified to be produces the antifungal volatile compound. For the isolation of antifungal VOC, the total of 9.6 gm crude extract was extracted from the 10-litre fermentation batch. The purification of antifungal VOC was carried out with help of chromatographic techniques. The final purified compound was characterised by GC-MS and NMR spectroscopy. Based on the analysis the compound is identified as 2, 4-di-tert butyl phenol. The mycofumigation potential of the purified compound was identified to inhibit the mycelial growth of *Fusarium* sp. NCIM 1330 on the wheat and maize seeds as well as on the divide plate assay. Thus, our study suggests that the marine isolate *Serratia marcescens* BKACT has good biocontrol potential as well as its purified molecule 2, 4 DTBP can be used for mycofumigation in agriculture and food sector to control the fungal infection.

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ORIGINAL ARTICLE

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

Bharat A. Kharat[1,3] · Madhukar S. Said[2,3] · Syed G. Dastager[1,3]

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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 $C_{14}H_{22}O$. 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.

✉ Syed G. Dastager
sg.dastager@ncl.res.in

[1] NCIM-Resource Center, Biochemical Science Division, CSIR-National Chemical Laboratory, Dr Homi Bhabha Road, Pune-411008, India

[2] Division of Organic Chemistry, CSIR-National Chemical Laboratory, Dr Homi Bhabha Road, Pune-411008, India

[3] Academy of Scientific and Innovative Research (AcSIR), Ghaziabad-201002, India

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 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:

$Growth\ inhibition\ percent = \frac{(R-r)}{R}\times 100$.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 HiPurA™ 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, $MgSO_4.7H_2O$ 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/CelliGen 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 1.0×10^6 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 ^{13}C NMR spectra were recorded on Bruker AV 500 MHz and 125 MHz, respectively, in deuterated chloroform ($CDCl_3$). 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 ^{13}C 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 axillary 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:pet 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 (1.0×10^6 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 (Massawe 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 parafilm and incubated at 28 °C for 5, 7 and 9 days. The percent mycelial growth inhibition is calculated by using the below mentioned formula: $Growth\ inhibition\ percent = \frac{(R-r)}{R}\times100$.where R is the radial growth of fungal pathogens in the control plate and 'r' 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 1.0×10^6 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 parafilm and were incubated at 28 °C for 3, 6, 12 and 24 h. At least 200 spores of each sample were examined at 40× 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 1.0×10^6 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 parafilm 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.

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

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 10^8. 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.

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

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). Briefly 7-day-old mycelial plug of 6 mm was inoculated on to 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 OD_{260} 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.

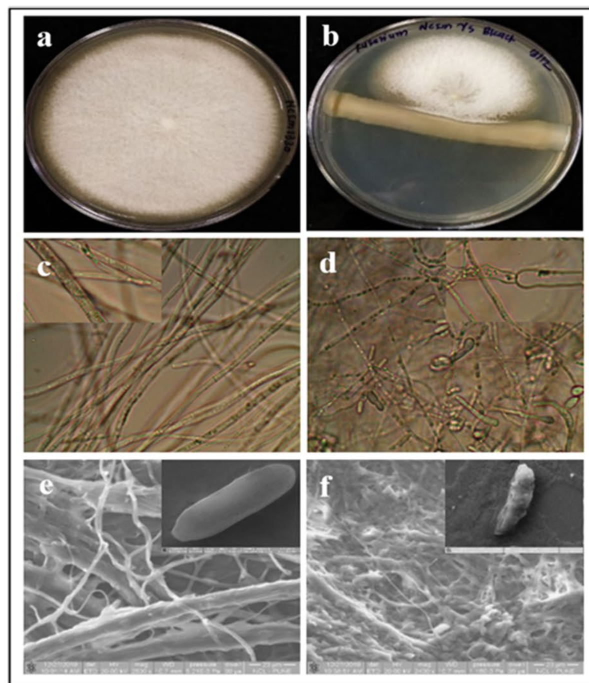
Antifungal activity and thin layer chromatography based bio-autography .

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

Table 1 Antagonistic activity of marine isolate BKACT against *Fusarium* spp. Data presented mean ± s. d. ($n = 3$) (Fungal strains used in the present study were procured from NCIM Resource Center)

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 comparision with other test organisms



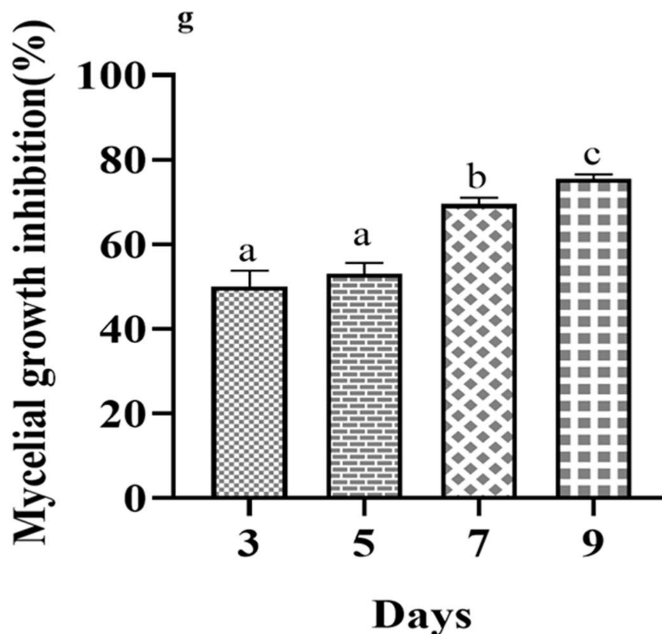


Fig. 1 Antagonistic effect of BKACT against *Fusarium foetens* NCIM 1330. **a**) Control image of *F. foetens*, **b**) treated image of *F. foetens* in the presence of BKACT, **c**) light microscopy image of control *F. foetens*, **d**) light microscopy image of treated *F. foetens* in the presence of BKACT, **e**) SEM image of control *F. foetens*, **f**) SEM image of treated *F. foetens* in the presence of BKACT and **g**) percent mycelial growth inhibition. Data presented mean ± s. d. ($n = 3$)

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 bio autography 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

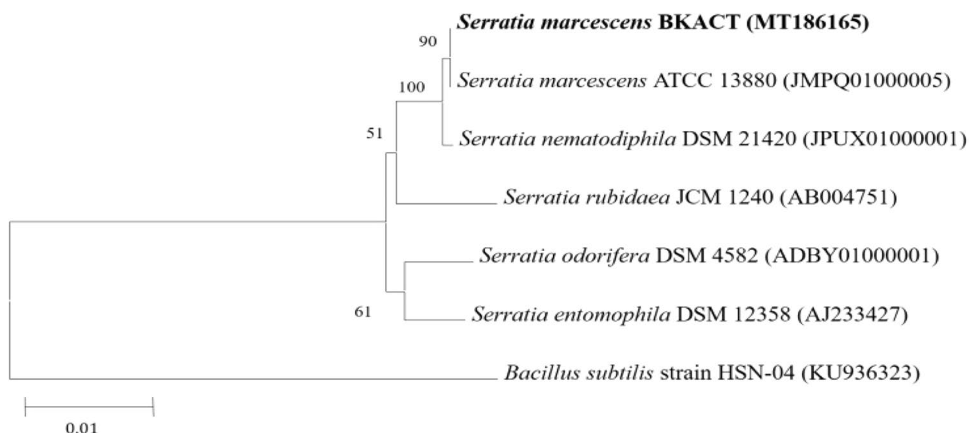


Fig. 2 A neighbor joining phylogenetic tree showing the relationships between BKACT and their closest neighbor among *Serratia* genus. The numerals at the branching points indicate the bootstrap values (%) obtained after 1000 replications. Bar, 0.02 substitutions per nucleotide position

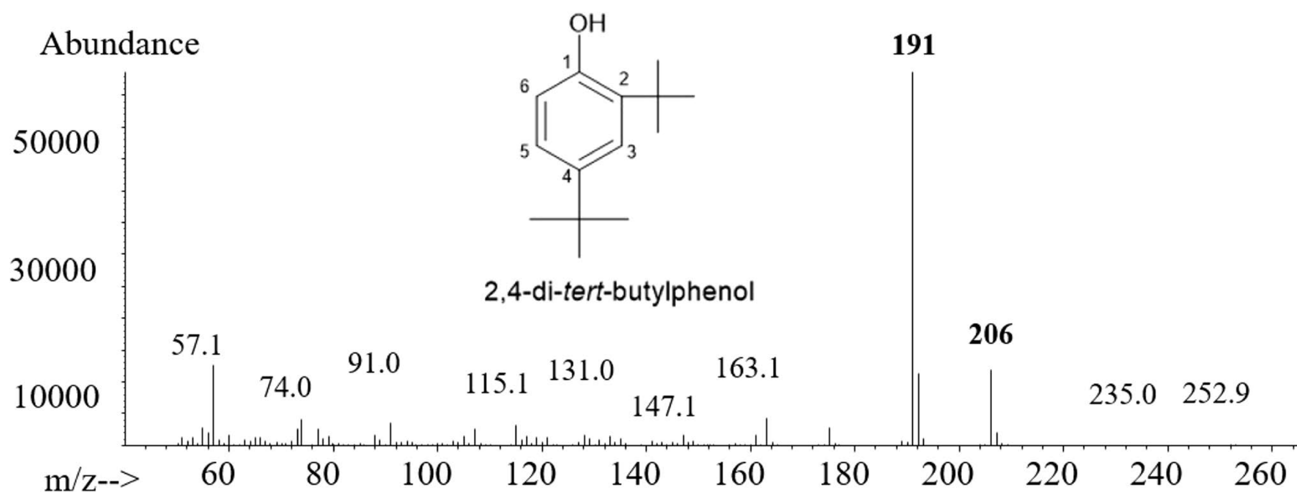


Fig. 3 GC–MS profile of purified compound, ion [M] $^+$ at m/z 206, the fragment ion [M–CH_3] $^+$ at m/z 191 are characteristic for 2, 4-DTBP

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 δ 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 the 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

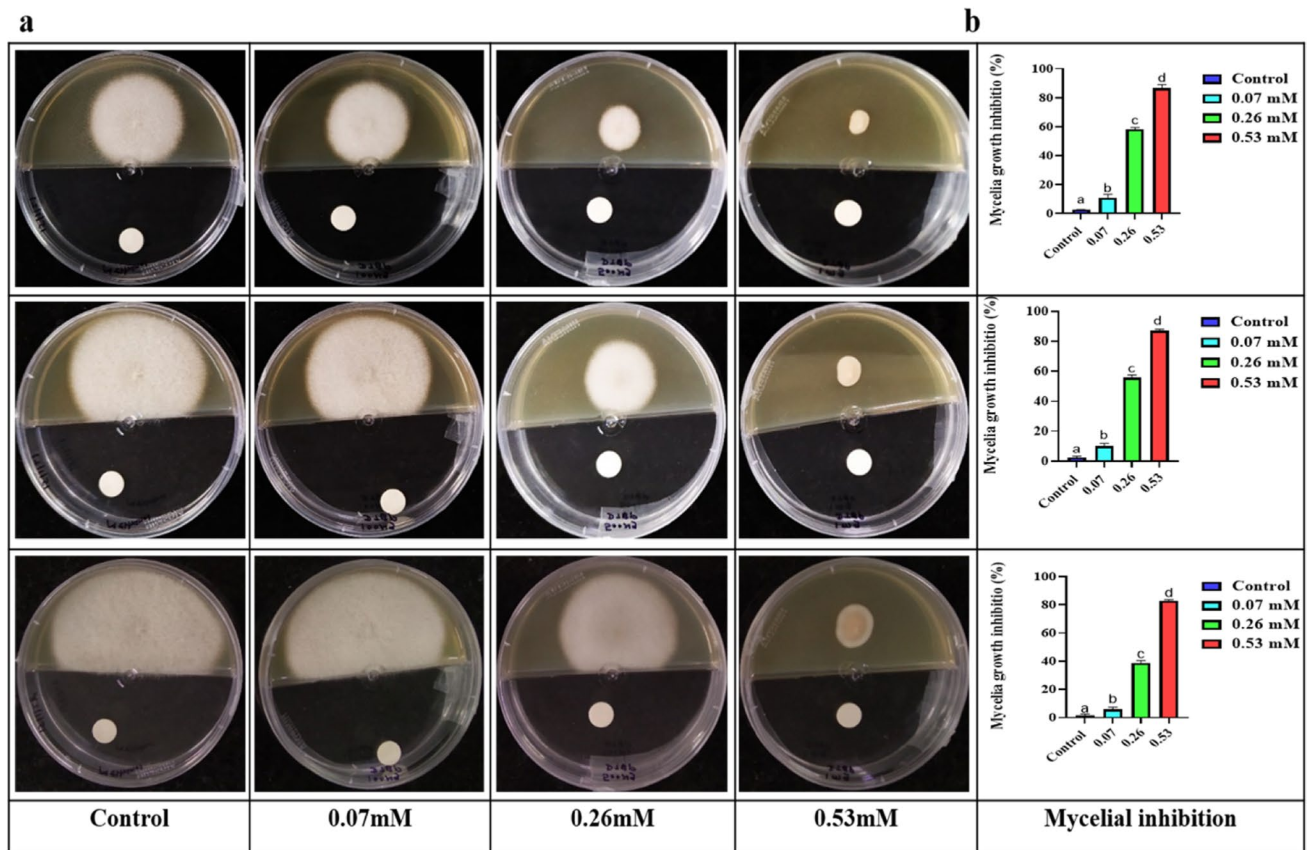


Fig. 4 Effect of the 2, 4-DTBP on the growth of *F. foetens* NCIM 1330, **a**. Antifungal effect of the DTBP at 0.07, 0.26 and 0.53 mM/L concentration on the mycelial growth with reference to methanol as the solvent control on 5, 7 and 9th day of incubation and **b**. Percent mycelial growth inhibition at respective time intervals. Data presented mean ± s. d. ($n = 3$)

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 when 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

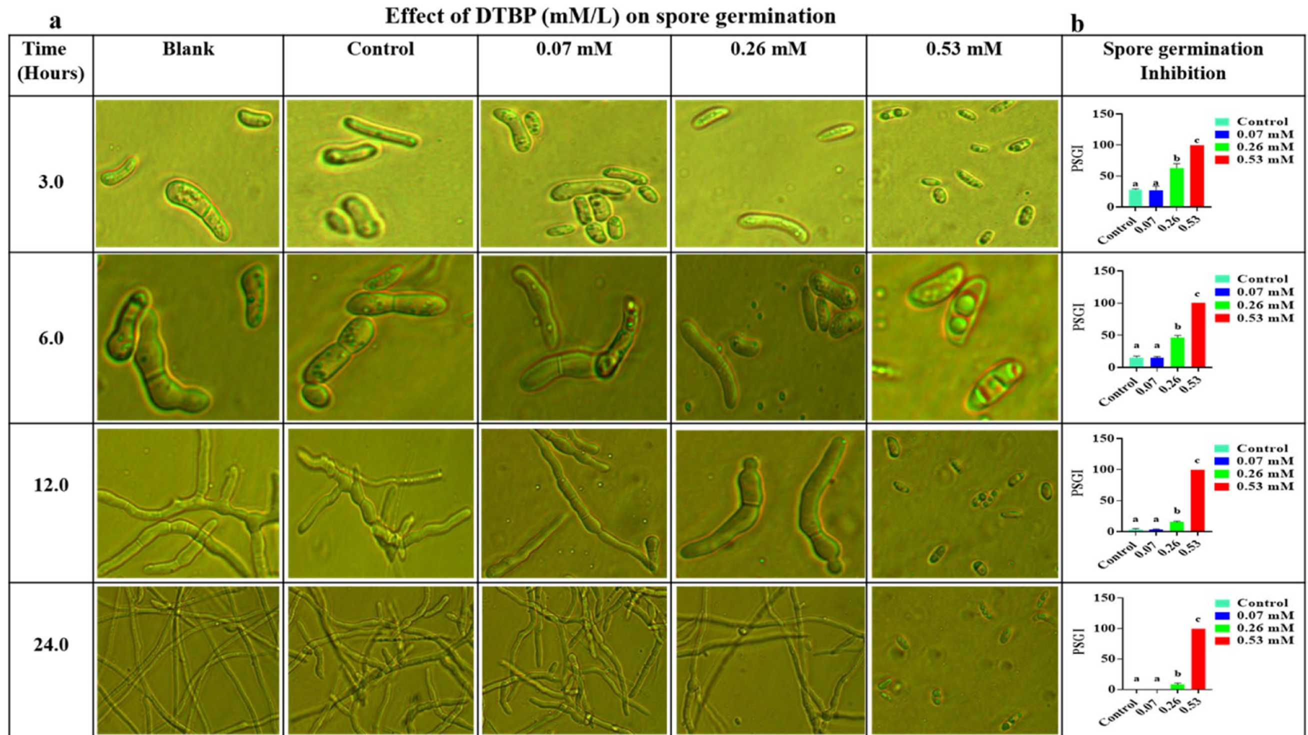


Fig. 5 Effect of the 2, 4-DTBP on spore germination of *F. foetens* NCIM 1330, **a**. Qualitative analysis of spore germination at different concentration of DTBP and without treatment as the control and **b**. Quantitative analysis by counting percent spore germination inhibition (PSGI) at the 0.07,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

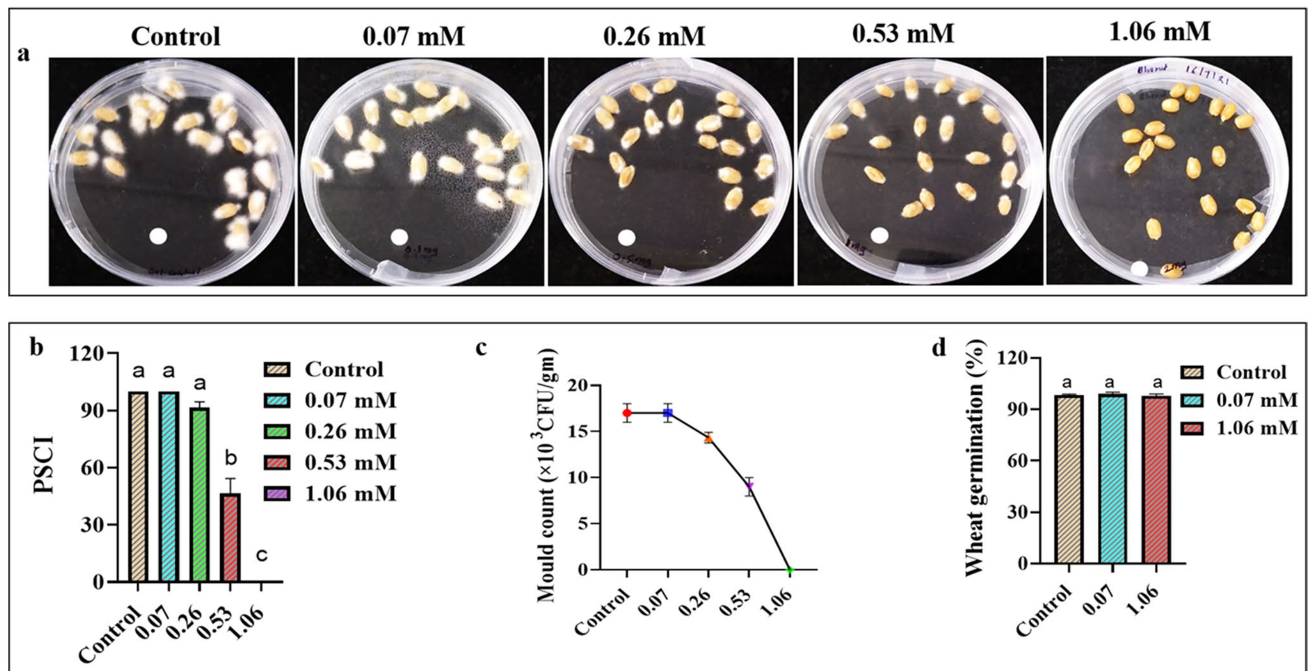


Fig. 6 The fumigation effect of the 2, 4-DTBP at 0.07, 0.26, 0.53 and 1.06 mM/L concentrations against *F. foetens* NCIM 1330 on the wheat seeds, **a**. Qualitative analysis of *F. foetens* contamination on wheat seeds, **b**. quantitative analysis of the wheat seed contamination index (SCI), **c**. fungicidal effect of DTBP on the wheat seeds and **d**. germination percentage of wheat grains after DTBP fumigation. Data presented mean ± s. d. ($n = 3$)

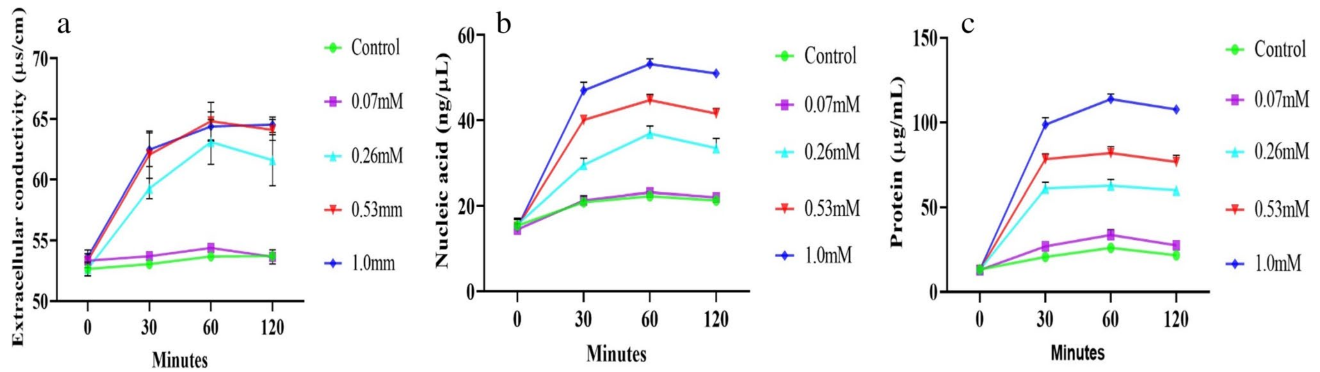


Fig. 7 Fumigationeffect of the 2, 4-DTBP on the cytoplasmic leakages of *F. foetens* NCIM1330, **a**. Extracellular conductivity, **b**. Nucleic acid content and **c**. Protein content

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 $75.56 \pm 0.80\%$ 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 $86.6 \pm 2.0\%$ 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.

References

Aoki T, O'Donnell K, Geiser DM (2014) Systematics of key phytopathogenic Fusarium species: current status and future challenges. J Gen Plant Pathol 80(3):189–201

Clinical and Laboratory Standards Institute. (2008). Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard—CLSI document M38-A2. *Clinical and Laboratory Standards Institute*.

DharPurkayastha G, Mangar P, Saha A, Saha D (2018) Evaluation of the biocontrol efficacy of a Serratia marcescens strain indigenous to tea rhizosphere for the management of root rot disease in tea. PLoS ONE 13(2):e0191761

Dharni S, Maurya A, Samad A, Srivastava SK, Sharma A, Patra DD (2014) Purification, characterization, and in vitro activity of 2, 4-di-tert-butylphenol from Pseudomonas monteilii PsF84: conformational and molecular docking studies. J Agric Food Chem 62(26):6138–6146

Dionisi HM, Lozada M, Olivera NL (2012) Bioprospection of marine microorganisms: biotechnological applications and methods. Rev Argent Microbiol 44(1):49–60

González-Jartín JM, Alfonso A, Sainz MJ, Vieytes MR, Aguín O, Ferreiroa V, Botana LM (2019) First report of Fusarium foetens as a mycotoxin producer. Mycotoxin Res 35(2):177–186

Grzelak EM, Hwang C, Cai G, Nam JW, Choules MP, Gao W, Jaki BU (2016) Bioautography with TLCMS/NMR for rapid discovery of anti-tuberculosis lead compounds from natural sources. ACS Infect Dis 2:294–301

Hover T, Maya T, Ron S, Sandovsky H, Shadkchan Y, Kijner N, Osherov N (2016) Mechanisms of bacterial (Serratia marcescens) attachment to, migration along, and killing of fungal hyphae. Appl Environ Microbiol 82(9):2585–2594

Imhoff JF, Labes A, Wiese J (2011) Bio-mining the microbial treasures of the ocean: new natural products. Biotechnol Adv 29(5):468–482

Köhl J, Kolnaar R, Ravensberg WJ (2019) Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. Front Plant Sci 10:845

Lamprecht SC, Tewoldemedhin YT (2017) Fusarium species associated with damping-off of rooibos seedlings and the potential of compost as soil amendment for disease suppression. S Afr J Bot 110:110–117

Manwar AV, Khandelwal SR, Chaudhari BL, Meyer JM, Chincholkar SB (2004) Siderophore production by a marine Pseudomonas aeruginosa and its antagonistic action against phytopathogenic fungi. Appl Biochem Biotechnol 118(1):243–251

Massawe VC, Hanif A, Farzand A, Mburu DK, Ochola SO, Wu L, Gao X (2018) Volatile compounds of endophytic Bacillus spp. have biocontrol activity against Sclerotinia sclerotiorum. Phytopathology 108(12):1373–1385

Munkvold GP (2017) Fusarium species and their associated mycotoxins. Methods Mol Biol 1542:51–106

Ordentlich A, Elad Y, Chet I (1988) The role of chitinase of Serratia marcescens in biocontrol of Sclerotium rolfsii. Phytopathology 78(1):84–88

Roberts DP, Lohrke SM, Meyer SL, Buyer JS, Bowers JH, Baker CJ, Chung S (2005) Biocontrol agents applied individually and in combination for suppression of soil borne diseases of cucumber. Crop Prot 24(2):141–155

Schmidt R, Cordovez V, De Boer W, Raaijmakers J, Garbeva P (2015) Volatile affairs in microbial interactions. ISME J 9(11):2329–2335

Schroers HJ, Baayen RP, Meffert JP, De Gruyter J, Hooftman M, O'Donnell K (2004) Fusarium foetens, a new species pathogenic to begonia elatior hybrids (Begonia hiemalis) and the sister taxon of the Fusarium oxysporum species complex. Mycologia 96(2):393–406

Soenens A, Imperial J (2020) Biocontrol capabilities of the genus *Serratia*. Phytochem Rev 19(3):577–587

Someya N, Nakajima M, Hirayae K, Hibi T, Akutsu K (2001) Synergistic antifungal activity of chitinolytic enzymes and prodigiosin produced by biocontrol bacterium, Serratia marcescens strain B2 against gray mold pathogen, Botrytis cinerea. J Gen Plant Pathol 67(4):312–317

Tchameni SN, Cotârleț M, Ghinea IO, Bedine MAB, Sameza ML, Borda D, Dinică RM (2020) Involvement of lytic enzymes and secondary metabolites produced by Trichoderma spp. in the biological control of Pythium myriotylum. Int Microbiol 23(2):179–188

Varsha KK, Devendra L, Shilpa G, Priya S, Pandey A, Nampoothiri KM (2015) 2, 4-Di-tert-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated *Lactococcus* sp. Int J Food Microbiol 211:44–50

Vinchira-Villarraga DM, Castellanos L, Moreno-Sarmiento N, Suarez-Moreno ZR, Ramos FA (2021) Antifungal activity of marine-derived Paenibacillus sp. PNM200 against Fusarium oxysporum f. sp. lycopersici, the causal agent of tomato vascular wilt. Biol Control 154:104501

Wang T, Li Y, Bi Y, Zhang M, Zhang T, Zheng X, Huang Y (2020) Benzyl isothiocyanate fumigation inhibits growth, membrane integrity and mycotoxin production in Alternaria alternata. RSC Adv 10(3):1829–1837

Wang K, Qin Z, Wu S, Zhao P, Zhen C, Gao H (2021) Antifungal mechanism of volatile organic compounds produced by Bacillus subtilis CF-3 on Colletotrichum gloeosporioides assessed using omics technology. J Agric Food Chem 69(17):5267–5278

Zhang DJ, Liu RF, Li YG, Tao LM, Tian L (2010) Two new antifungal cyclic lipopeptides from Bacillus marinus B-9987. Chem Pharm Bull 58(12):1630–1634

Zhang S, Zheng M, Zhai H, Lyu Y, Hu Y, Cai J (2021) Effects of hexanal fumigation on fungal spoilage and grain quality of stored wheat. Grain Oil Sci Technol 4(1):10–17

Zhao Y, Selvaraj JN, Xing F, Zhou L, Wang Y, Song H, Liu Y (2014) Antagonistic action of Bacillus subtilis strain SG6 on Fusarium graminearum. PloS one 9(3):e92486

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