Characterization of biosurfactants from rhizospheric pseudomonads and their utilization in development of bioformulations for biocontrol of Alternaria blight in *Brassica juncea* [L.]

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

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UTTAR PRADESH, INDIA

2021

Dedicated to My Dadi

&

My Parents

CERTIFICATE

This is to certify that the thesis titled **"Characterization of biosurfactants from rhizospheric pseudomonads and their utilization in development of bioformulations for biocontrol of Alternaria blight in *Brassica juncea* [L.]"** submitted by **Ms. Isha Mishra** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D) regulations-1999 as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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DECLARATION

This is to certify that I have worked on the research thesis entitled **"Characterization of biosurfactants from rhizospheric pseudomonads and their utilization in development of bioformulations for biocontrol of Alternaria blight in *Brassica juncea* [L.]".** The data mentioned in this thesis were collected and obtained during genuine work done by me. Data obtained from other agencies have been duly acknowledged. None of the findings pertaining to the work has been concealed. The result embodied in this report has not been submitted to any other University, Institution or Research Centre for the award of any degree. "The thesis is essential free from all kinds of plagiarism".

Place: Lucknow

Date: 31.12.2021

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Place: Lucknow

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LIST OF ABBREVIATIONS

%	Percent
$(NH_4)_2SO4$	Ammonium Sulphate
°C	Degree Celsius
°E	Degree East
°N	Degree North
µg	Micro Gram
µL	Micro Liters
µS/cm	Micro-Siemens per Centimeter
µS/m	Micro-Siemens per meter
ACC	1-Aminocyclopropane-1-Carboxylate
ACCD	1-Aminocyclopropane-1-Carboxylate Deaminase
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Sequence Tool
BNF	Biological Nitrogen Fixation
bp	Base Pairs
BSA	Bovine Serum Albumin
BTB	Bromothymol Blue
CAS	Chrome Azurol S
CFU	Colony Forming Unit
cm	Centimeters
CMC	Carboxymethylcellulose
DAS	Days After Sowing
DF	Dworkin and Foster
DMRT	Duncan’s Multiplicity Range Test
DNA	Deoxy Ribonucleic Acid
dS/m	Deci-Siemens per meter
EC	Electrical Conductivity
EDS	Energy Dispersive Spectroscopy
EL	Electrolyte Leakage

FT-IR	Fourier Transmission Infra-Red
g	Gram
GAs	Gibberellins
GC-MS	Gas Chromatography-Mass Spectrometry
GPA	Glucose Peptone Agar
H_2O_2	Hydrogen Peroxide
H_2S	Hydrogen Sulphide
ha	Hectare
HAM	Hofer's Alkaline Media
HCN	Hydrogen Cyanide
HDTMA	Hexadecyltrimethylammonium
hrs	Hours
IAA	Indole-3-Acetic Acid
K	Potassium
kDa	Kilo Dalton
Kg	Kilogram
KNO_3	Potassium Nitrate
m	Meter
mg	Miligram
Mha	Million Hectares
ml	Mili Liters
MR-VP	Methyl-Red and Voges-Proskauer
N	Nitrogen
N_2	Dinitrogen
Na^+	Sodium Ion
NaCl	Sodium Chloride
$NaNO_3$	Sodium Nitrate
NaOH	Sodium Hydroxide
NBAIM	National Bureau of Agriculturally Important Microorganisms
NH_3	Ammonia
NH_4Cl	Ammonium chloride
nm	Nanometer
OD	Optical Density
PBS	Phosphate Buffer Saline
PDA	Potato Dextrose Agar

PGP	Plant Growth Promoting
PGPB	Plant Growth Promoting Bacteria
PGPR	Plant Growth Promoting Rhizobacteria
PHB	Poly-P-Hydroxybutyrate
PIPES	Piperazine-N, N'-Bis (Ethane Sulfonic acid)
PSB	Phosphate Solubilizing Bacteria
PSI	Phosphate Solubilization Index
PSU	Percent Siderophore Unit
rpm	Rotation per Minutes
rRNA	Ribosomal Ribonucleic Acid
RWC	Relative Water Content
SD	Standard Deviation
SEM	Scanning Electron Microscopy
ST-PGPR	Salt-Tolerant Plant Growth Promoting Rhizobacteria
TCA	Trichloroacetic acid
UV	Ultra Violet
UV-Vis	Ultra Violet Visible
w/v	Weight by Volume
WHO	World Health Organization
XRD	X-Ray Diffraction
YEMA	Yeast Extract Mannitol Agar
Zn	Zinc
ZSI	Zinc Solubilization Index
α	Alpha
β	Beta
θ	Theta
ϒ	Gamma

Introduction

1. Introduction

Agriculture plays an imperative role in forming a significant segment of world's economy but in present era, this sector is continuously facing the challenge of increasing crop productivity and achieving food security. In the past few decades, the prevalence and outbreaks of fungal phytopathogenic diseases have increased drastically, mainly due to rapid pace of climate change and global warming (Almeida et al. 2019). Estimates suggest that worldwide plant pathogens are responsible to cause yield losses of about 10-40%, depending upon the crops before or after harvest and has caused losses of around $220 billion of global economy every year (FAO 2019). Soil borne pathogens pose a great challenge to crop production in several regions of the world, with fungi being the most aggressive of them (Garcia-Solache et al. 2010; Almeida et al. 2019). They can cause huge losses in terms of yield and quality of crops and are a global issue towards human health, economy as well as for arable lands (Thambugala et al. 2020). Hence, with increasing global population, it has become our concomitant need to optimize the yield of crops and minimize the losses caused by plant diseases.

Brassica juncea [L.] or Indian mustard is one of the major oilseeds crops of Indian subcontinent and contributes over 80% of the production rate of rapeseed-mustard in India (Bindhani et al. 2020). Central Asia is the main centre of origin of this crop and secondary centres include central and western China, eastern India, Burma, Iran and through the near east (Kumar et al. 2011). Globally, European Union is the largest producer of mustard followed by Canada, China, and India. (http://www.agriculture.rajasthan.gov.in/content/dam/agriculture/Rajasthan%20Agricultural%20Competitiveness%20Project/valuechainreport/RACP_VC_Mustard.pdf). Mustard seeds are widely used as a traditional pungent spice, edible oil and source of

protein and for medicinal purposes as well. Leaves of the plant are used in form of vegetables and has also attracted a lot of attraction for its health benefits for example presence of glucosinolates and isothiocyantes that possess cancer preventive properties (Ishida et al. 2014; Bassan et al. 2018). However, there are many fungal pathogens that disturb the physiological functions of the crop during its growth and developmental phase and thus have restrained its yield potential. Of them all, Alternaria blight caused by *Alternaria brassicae* is the most destructive disease and occurs more frequently than the one caused by other species*Alternaria brassicicola* and *Alternaria raphani* (Kumar et al. 2014; Saharan et al. 2016). It is a seed borne necrotrophic fungal pathogen that infects the aerial parts of the plant at all growth stages causing serious damage to oilseed Brassicas (Mandal et al. 2018). The disease symptoms appear as black or brown necrotic lesions in form of concentric rings on leaves, stems and even siliques followed by seed shrinking, decreased rate of photosynthesis, early ripening, defoliation, loss of flowers, buds and reduced levels of oil content (Kaushik et al. 1984; Sharma et al. 2002).

To overcome this problem, farmers mainly rely on synthetic chemicals such as fungicides in order to protect crops from fungal phytopathogens. Estimate suggests that global fungicide market accounted for USD 18.7 billion in 2019 and is projected to grow at rate of 4.6% to reach USD 24.5 billion by 2025 (https://www.marketsandmarkets.com/Market-Reports/fungicides-356.html#:~:text=%5B226%20Pages%20Report%5D%20The%20fungicides,USD%24.5%20billion%20by%202025). These agrochemicals being recalcitrant or xenobiotic in nature severely impact the health of soil and are also harmful to beneficial microbiomes leading to its deterioration (Arora et al. 2018). In addition, indiscriminate use of these synthetic compounds creates environmental concerns

causing pollution of land and water bodies, outbreak of resistant plant pathogens and once entered causes disruption in food chain (Wang et al 2019).

To supress the attack of phytopathogens and to reduce our dependence on agrochemicals, urgent strategic approach is needed which is sustainable in implementation, biological in origin and eco-friendly in nature (Arora 2018). In this context, use of plant growth promoting rhizobacteria (PGPR) have been extensively studied in the past few decades due to their promising nature towards agriculture and above all, the environment as a whole (Arora et al. 2020a). These beneficial microbes reside in the rhizosphere, the zone of soil that lies in the vicinity of plant root system and has potential to be used as green substitutes to agrochemicals in agricultural sector (Backer et al. 2018; Mustafa et al. 2019). PGPR directly influences plant health by producing growth regulators (in form of secondary metabolites), nutrient solubilizers, metal chelators, fixation of atmospheric nitrogen (Glick 2012; Ahemad and Kibret 2014; Arora 2018; Gouda et al. 2018). On the other hand, PGPR also indirectly help plants by acting as biocontrol agents against phytopathogens and inducing systemic resistance in plants by increasing their defensive machinery (Egamberdieva and Lugtenberg 2014; Egamberdieva et al. 2019; Mustafa et al. 2019). Several species of *Arthrobacter, Azospirillum, Bacillus, Burkholderia, Enterobacter, Pseudomonas, Rhizobium* are considered as the most potent PGPR candidates and have been reported to improve growth and yield of many agriculturally important crops (Gouda et al. 2018; Swarnalakshmi et al. 2020). Apart from the aforementioned roles that PGPR play to enhance plant productivity, they have also been investigated to synthesize surface active compounds known as biosurfactants (Banat et al. 2010; Santos et al 2016). These are amphipathic molecules having both hydrophilic and hydrophobic moieties and are comprised of wide range of chemical structures

including glycolipids, lipopeptides phospholipids, fatty acids and polymeric macromolecules (Rodrigues et al 2006; Santos et al. 2016). Microbially produced biosurfactants are being considered as next generation multifunctional biomolecules due to their biodegradable and biocompatible nature, non-toxicity, durability at range of temperatures and pH and biotic origin (Akbari et al. 2018; Fenibo et al. 2019; Mishra et al. 2020). From agricultural point of view, these green compounds have been reported to take part in root colonization (by PGPR) through swarming motility, induce biofilm formation and signalling molecules to protect plant from environmental stress, help in nutrient acquisition and metal chelation as well possess biocontrol activity against phytopathogens (Raaijmakers et al. 2010; Sachdev and Cameotra 2013; Mishra et al. 2020) (Fig. 4.). Biosurfactants are also well known for biodegradation of certain xenobiotic compounds such as polyaromatic hydrocarbons and hence are being successfully exploited as an environmental remediation technique (Pacwa-Plociniczak et al. 2015; Fenibo et al. 2019). Among the diverse range of PGPR, biosurfactants produced by *Pseudomonas* sp. are the most widely studied and have been reported to produce glycolipids and cyclic lipopeptide (CLP) type of biosurfactants (D'aes et al. 2010; Monnier et al. 2018). Biosurfactant produced by rhizospheric pseudomonads are particularly well known for their antifungal properties and have been documented to induce resistance in plants against phytopathogens by triggering their immune system (Vatsa et al. 2010; Schellenberger et al. 2019; Köhl et al. 2019). On direct application, these microbial surfactants cause zoospore lysis by forming channels into cell walls of fungal pathogens and produce structural perturbations affecting functions of membrane (Stanghellini and Miller 1997; Raaijmakers et al 2010). Hence, these properties of biosurfactants and their producing microbes make them suitable candidates to be used for agricultural applications that

could warrant effective results for biocontrol of phytopathogens and in increasing plant productivity in the present scenario of global warming and climate change.

The main objectives of the present study include isolation of biosurfactant producing rhizospheric pseudomonads, checking their PGP traits and then extraction and purification of biosurfactant to develop novel bioformulations from them at lab scale. Further, in order to check the efficacy of bioformulations as biocontrol and plant growth promoting agents, pot trials and field trials were carried out taking *B. juncea* as test crop and all the growth parameters of the treated plants were recorded (Fig. 1).

Objectives

1. Isolation and screening of biosurfactant producing PGPR from rhizosphere of *B. juncea*
2. Checking PGP traits of selected isolates of pseudomonads (Production of IAA, HCN, ammonia, siderophore and phosphate solubilisation)
3. Production of biosurfactant at lab scale from screened isolates of PGP pseudomonads.
4. Purification and chemical characterization of biosurfactant obtained from isolated strains PGP pseudomonads.
5. Development of bioformulations using biosurfactants from isolated strains.
6. Evaluating the efficacy of bioformulations in pot trials and field trials using *B. juncea* as test crop.

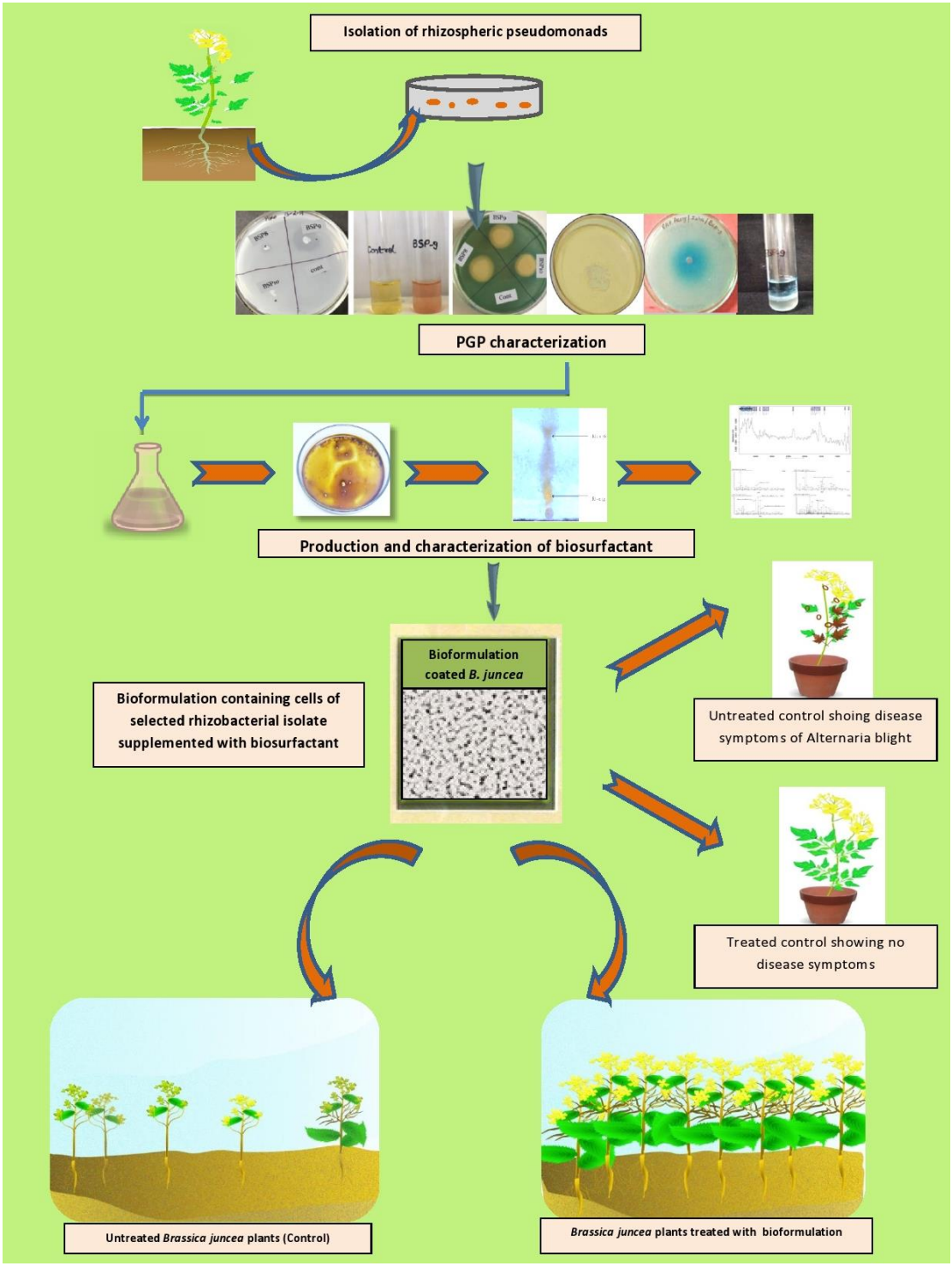


Fig. 1. Effect of inoculation of bioformulation under pot and field trial taking *B. juncea* as test crop

Review of Literature

2. Review of literature

2.1. *B. juncea* (L.) or Indian mustard and its global scenario

Oilseeds have been an integral part of farming system, especially in India and plays crucial role in agricultural economy. They are majorly grown for their edible oils and are also used in form of livestock meals, pharmaceuticals and biofuels (Sarwar et al. 2013). As the world population, urbanization and incomes are rising, need for high quality and high yielding varieties of oilseeds continue to grow. In India, demand for oilseeds has reached 13 million tons whereas production rate is only 7.5 million tons and to fulfil this gap, 42.24 million tons of oilseed output is required (Gupta and Gupta 2016). The major oilseeds produced in India are soybean, groundnut, rapeseed-mustard, sunflower, sesame, safflower and niger (edible oils), castor and linseed (non-edible oils) (Rai et al. 2016). Out of these, Rapeseed- mustard also referred as oilseed Brassicas is the second most important edible oil crop and European Union is the largest producer of this oilseed crop (Kumrawat and Yadav 2018; https://agriculture.rajasthan.gov.in/content/dam/agriculture/Rajasthan%20Agricultural%20Competitiveness%20Project/valuechainreport/RACP_VC_Mustard.pdf).

Globally, the estimated area, production and yield of rapeseed-mustard during 2018-19, was 36.59 million hectares (mha), 72.37 million tonnes (mt) and 1980 kg / ha, respectively (ICAR-DRMR 2018) (Fig. 2). In India, it is predominantly cultivated in the parts of eastern, northern and north western states. Rajasthan, Uttar Pradesh, Haryana, Madhya Pradesh and Gujarat are the major producer of rapeseed-mustard in the country (Bindhani et al. 2020). The production volume of rapeseed-mustard in India from financial year 2013-2019 has been shown in (Fig. 3).

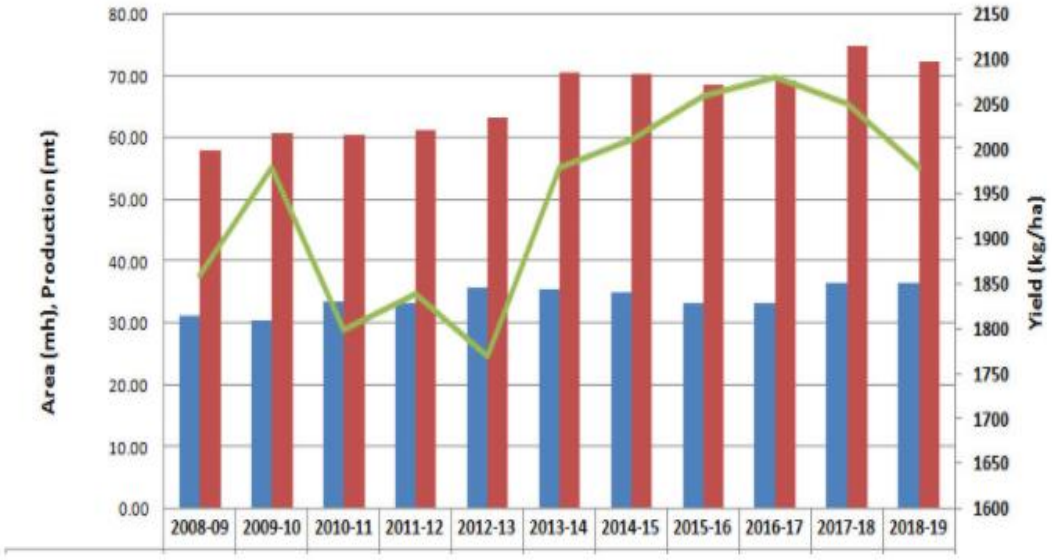


Fig. 2 Rapeseed-mustard production trend in the world

Data Source: ICAR-DRMR (2018)

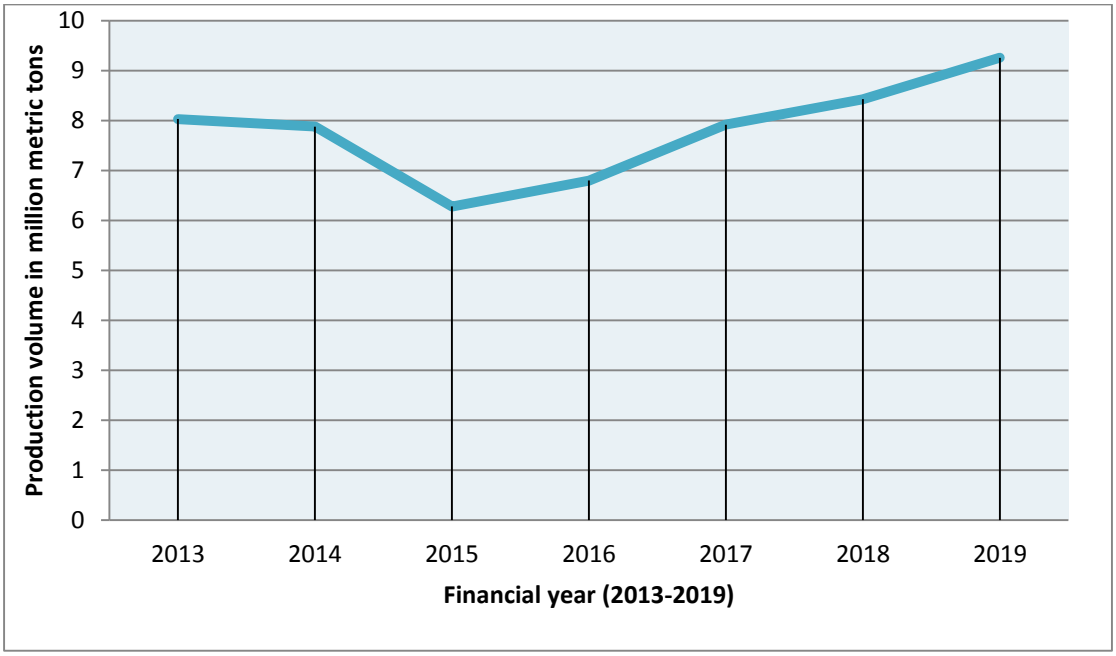


Fig. 3 Production volume of rapeseed-mustard in India from financial year 2013-2019. Data Source: Statista Research Department (2020) https://www.statista.com/statistics/769713/india-

The word "rape" is derived from the latin word "rapum" meaning turnip and "mustard" from the latin word "must" expressing mixing of old wine or grape juices with crushed seeds of black mustard to form a hot paste (Hemingway 1976; Chauhan et al. 2011a). Rapeseed-mustard comprises of six oilseeds crops belonging to family Brassicaceae (Cruciferae) namely Indian mustard [*B. juncea* (L.) Czern. & Coss.], toria (also known as brown sarson or yellow sarson) (*Brassica compestris* L. synonym *Brassica rapa*), gobhi sarson (*Brassica napus* L.), karan rai or Ethiopian mustard (*Brassica carinata* Braun), black mustard [*B.nigra* (L.) Koch], and taramira [*Eruca sativa/vesicaria* Mill.] (Chauhan et al. 2002; Chauhan et al. 2011b; Rai et al. 2016).

Indian mustard (*B. juncea* L.) is a amphidiploid (2n=36) rabi crop and grown in diverse agro-climatic conditions ranging from north-eastern/north western hills to down south under irrigated/rainfed, timely/late sown, saline soils and mixed cropping (ICAR-DRMR 2018). It can tolerate annual rainfall between 500 to 4200 mm, temperature ranging from 6 to 27°C and pH of 4.3 to 8.3 and requires well-drained sandy loam soil (Lal et al. 2021). There are conflicting views about its emergence, however, middle east is regarded as the probable place of origin as its putatative parents i.e. *B. nigra* (2n=18) and *B. rapa* (2n=20) belonged to this region (Kang et al. 2021). The plants are tall, heavily branched with height of 90-200 cm and have elongated corymbose raceme type of inflorescence with bright yellow flowers located terminally on the main stem and branches (Langer and Hill 1991; Rakow 2004). The foliage of the plant is dilated at the base, stalked, broad and pinnatifid whereas siliquae or pods are slender in shape and 2 to 5 cm long, erect with short and stout beaks (https://biosafety.icar.gov.in/wp-content/uploads/2016/10/1_Biology_of_ Brassica_juncea_Mustard.pdf.). Seeds are dark brown in colour and possess volatile oil content of 2.9% (Thomas et al. 2012). Oil quality is mainly governed by chemical

properties and fatty acid composition and is dependent on different varieties of plant including Sarson T42, Lahi T9, Varuna 59 and Rai Pusa Bold (Omidi et al. 2010; Mishra et al. 2017). Being a rich source of unsaturated fatty acids (about 7-10% α-linolenic and 17-21% linoleic acids) and low in content of saturated fats (5-7%), oil obtained from Indian mustard is considered nutritionally better than other oilseeds (Baux et al. 2008). It is also rich in protein content, iron (Fe), vitamin A and C and favourable amount of amino acid composition (essential sulphuric amino acids, methionine and cysteine), calcium, potassium, riboflavin, β-carotene and phenolic antioxidants (Cartea et al. 2010; Kapoor et al. 2014; Thakur et al. 2014; Frazie et al. 2017; Kwon et al. 2020). In addition, it also contains glucosinolates, isothiocyanates, allyl thiocyanates, phytosterols which possess medicinal and antimicrobial properties (Zhang 2010; Sharma et al. 2012; Tian and Deng 2020). The other important medicinal properties include antidiabetic, antitumor, antioxidant, goitrogenic activities (Tripathi et al. 2001; Kim et al. 2003; Sanlier and Guler Saban 2018). The above mentioned properties of *B. juncea* make it an important oilseed crop across the world and therefore it is needful to raise its production rate globally through sustainable agricultural methods.

2.2. *Alternaria brassicae:* A potent pathogen for Crucifers

Fungal phytopathogens have been recognised as the emergent threats to the food security around the globe. They are highly diverse, ubiquitous in nature and are critically endangering agricultural resources causing devastating yield and economic losses. Intensive farming practices (e.g. faulty irrigation, fertilization and application of monoculture technique), limited genetic diversity of crops, climate change, global warming are the major drivers of the global spread of plant diseases which has aggravated since past few decades (Brown and Hovmøller 2002; Fisher et al. 2012;

Santini et al. 2013; Dietzel. et al. 2019). Like many other crops, *B. juncea* is also challenged by many fungal pathogens including *A. brassicae, A. brassicicola, A. raphani, Alternaria japonica, Alternaria alternata* (Alternaria leaf blight), *Albugo candida* (white rust), *Sclerotinia sclerotiorum* (Sclerotinia stem rot), *Erysiphe cruciferarum* (powdery mildew), and *Hyaloperonospora parasitica* causative agent of downy mildew (Giri et al. 2013; Meur et al. 2015; Ali et al. 2017; Blagojević et al. 2020). Among all these diseases of *B. juncea*, Alternaria leaf blight caused by *A. brassicae* has been reported from all the continents of world which majorly affects the crop and causes yield losses of upto 70% (Singh et al. 2014; Gupta et al. 2020). The fungus affects the plant at all growth stages including seeds and forms dark concentric brown spots on leaves, stem and silique resulting in damping off and stunted growth of crops (Meena et al. 2010; Mandal et al. 2018). Disease symptoms generally appear in 45 days old plants and severity increases in 75 days old plants (Meena et al. 2004; Meena et al. 2010). Number of lesions formed on silique or pods increases the chance of seed infection and also results in reduced pod length, seeds per pod, weight of thousand seeds per plant, seed germination and thus low quality oil content (Singh et al. 2014; Fatima et al. 2019). The incidence of disease is greatly dependent on weather with severe outbreaks reported in wet seasons and in regions with high precipitation rate (Humpherson-Jones and Phelps 1989; Meena et al. 2010).

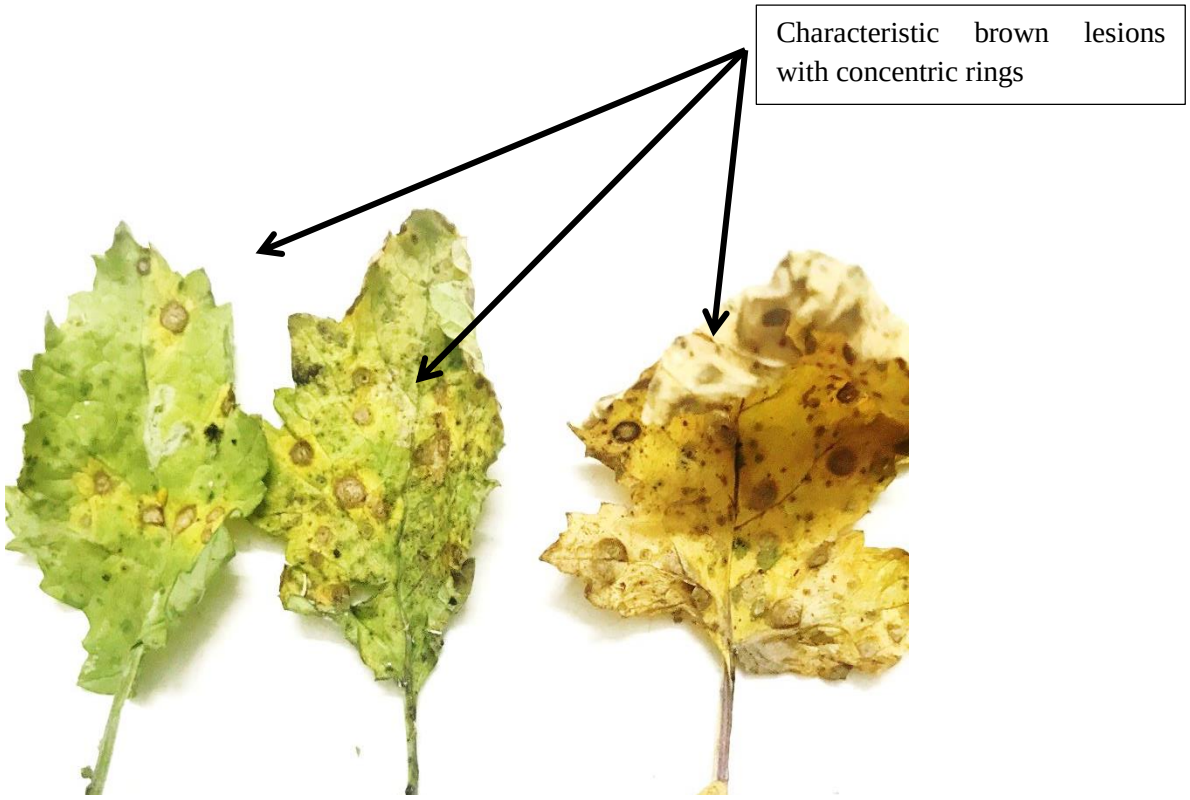


Fig. 4 Leaves of *B. juncea* showing symptoms of leaf blight disease caused by *A.*

Alternaria spp. is distinguished by formation of polymorphous conidia either singly or in short or long chains, sometimes in branching fashion and conidial size ranging from 148-184 x 17-24µm (Kumar et al. 2014; Singh et al. 2015). Spores are pale brown to brown in color, broad near base, typically ovoid to obclavate and have transverse or longitudinal septa which taper gradually to an elongated beak (Cho et al. 2001; Mamgain et al. 2013; Kumar et al. 2014). The fungus grows slowly on media and germinates at temperature ranging from 21-28^{0}C. Optimum temperature range for mycelial growth and sporulation is generally reported to be between 25-30^{0}C and 15-35^{0}C, respectively (Mamgain et al. 2013; Kumar et al. 2014). A continuous moisture and high relative humidity favours the sporulation. A relative humidity of 91.5% produces mature and large number of spores in 24 hours (Humpherson-Jones and Phelps 1989). The spores are disseminated through air, water, tools or animals and is

said to cause infection through their presence on the infected seeds and via the mycelium on seed coat. When stored at 0^0C, spores of *A. brassicae* are reported to be viable for as long as 20 months on seed coats or in form of mycelium in seed samples and when exposed to outdoor conditions (temperature ranging between -23^0C-30^0C), pathogenicity remains active for upto six months (Tripathi and Kaushik 1984; Thomma 2003). They can also survive as microsclerotia or chlamydospores when the infected leaves get partially decayed, continuing their infection for next cropping season (Tsuneda and Skoropad 1977; Humpherson-Jones and Maude 1982; Tripathi and Kaushik 1984; Jackson and Kumar 2019). It has also been reported to reside in alternative hosts during off season such as susceptible perennial crops or weeds and can cause transmission of the disease (Tripathi and Kaushik 1984; Verma and Saharan 1994; Mehta et al. 2002; Mishra et al. 2017). The spores after their arrival on host plant, adhere to its surface in form of conidia and penetrate inside by forming germtube through stomata which then enters into the epidermal cells through formation of appressorium mainly triggered by host signalling mechanism (Yang et al. 2005; Cho et al. 2006; Fatima et al. 2019). *Alternaria* spp. also produces number of host specific and non-specific toxins at its different pathogenic stages (e.g. ACR-toxin, AAL-toxin AM-toxin phytotoxins alternariol methyl ether, tenuazonic acid, zinniol, cyclic tetrapeptide tentoxin and so on) capable of causing number of disease symptoms in plants. They are all non-enzymatic compounds like phenols and peptides having low molecular weight and can cause necrosis in leaves (Kohmoto et al. 1987; Meena et al. 2017).

Traditionally, phytopathogenic fungal diseases are controlled by application of fungicides and have been considered the most reliable means of disease management. However, the unprecedented use of chemical inputs not only degrades environment

but is also harmful for the non-targeted microbes causing ecological imbalance (Gupta et al. 2020). Reports also claim that rigorous use of the synthetic compounds can also induce resistance in fungal population including *A. brassicae* (Varma et al. 2008). In addition, production cost of such agrochemicals is progressively greater and their toxic nature also causes detrimental health issues in humans (Nicolopoulou-Stamati et al. 2016; Tudi et al. 2021). Crop rotation practices were also used in past but the severity of disease was so huge that it could not reduce the devastating effects of disease on yield and productivity of crops. Another viable approach is the use of resistant varieties of the crops form host germplasm but despite of repeated attempts, little success has been achieved so far particularly during field trials which are mainly due to variability of virulence in *B. juncea* crops (Meena et al. 2010; Fatima et al. 2020). Therefore, to mitigate the impact of these soil borne fungal pathogens, there is need to adopt such a biological approach which is efficient, cost effective, eco-friendly and above all promises sustainability of environment (Arora et al. 2018). Application of PGPR in form of biocontrol agents is one of the excellent substitutes to chemical control strategies and can be used as a safer alternative to promote plant growth without affecting agroecosystem (Glick 2012;Tewari and Arora 2016; 2018;Fatima et al. 2020).

2.3. Plant growth promoting rhizobacteria

With the advent of green revolution, reliance on agrochemicals remarkably increased resulting in substantial increment of agricultural productivity and also reduced the damaging impacts of pathogens and pests (John and Babu 2021). Studies have revealed that more than 1,40,000 new chemicals and pesticides have been produced since 1950 showing exceptional growth in this area (Landrigan et al. 2017). However, injudicious use of these chemical inputs are reported to cause deleterious to

environment including loss of soil fertility, pollution of land and water bodies, reduction in soil microbial population and also to human health (Arora 2018). Moreover, these compounds are recalcitrant in nature and retain in the environment for a long period of time causing soil degradation (Backer et al. 2018; Mishra and Arora 2019). Hence, farming practices need to adopt a biotic and sustainable approach such as harnessing the role of beneficial soil microbes in order to obviate the damaging effects of plant pathogen and to increase the yield of crops.

Plants and soil microbes have an inextricable links and it is due to their symbiotic relationship as a result of which plants are benefitted in a number of ways leading to their improved growth and development. It is a complex, well-structured and regulated community and is termed as phytomicrobiome (Smith et al. 2017; Backer et al. 2018) and this phytomicrobiome plus the plant is referred to as holobiont (Berg et al. 2016; Theis et al. 2016; Backer et al. 2018). The huge bionetwork of this beneficial microbiota that thrive in rhizosphere, a narrow zone of soil surrounding the plant root, is called rhizomicrobiome. It is rich and diverse region of distinct microbial communities than the bulk soil and consists of 10^{10} bacteria per gram of soil (Gans et al. 2005; Roesch et al. 2007; Vacheron et al. 2013). These soil bacteria are known as plant growth promoting rhizobacteria (PGPR) which are either symbiotic or free living in nature and provide innumerous services to its host plant (Fig. 5) including decomposition of organic matter, nutrient acquisition, production of growth regulators and also acts as a bioontrol agents (Walker et al. 2003; Ahemad and Kibret 2014; Aeron et al. 2020).

Kloepper and Schroth (1978) first coined the term PGPR, the rhizobacteria that thrive in the vicinity of plant roots. Most of these bacteria mainly rely on root exudates for their survival as they serve as chemical attractants for a huge number of soil microbes

and in return, they favour plants by defending their health in an eco-friendly manner. PGPR consist of a variety of bacterial taxa including *Azospirillum, Azotobacter, Bacillus, Chromobacter Burkholderia Enterobacter, Klebsiella, Pantoea, Pseudomonas,* and *Rhizobium* (Glick 1995; Bhattacharyya and Jha 2012; Ahemad and Kibret 2014). There are number of ways by which PGPR help plants in promoting their growth and productivity and all processes take place either simultaneously or sequentially at all stages of plant growth. (Glick 1995; Bhardwaj et al. 2014; Gouda et al. 2018). Broadly, these mechanisms are divided into two categories i.e. direct and indirect growth promotion (Glick 2012). The direct mechanism by PGPR include nitrogen fixation, solubilization of minerals such as phosphorous (P), potassium (K) and zinc (Zn), production of phytohormones (indole acetic acid IAA, gibberallins and1-aminocyclopropane-1-carboxylase ACC deaminase), siderophores, an iron (Fe) chelating compound, exopolysaccharides (Zahran 2001; Khan et al. 2006; Zaidi et al. 2009; Ahemad and Khan 2012; Vacheron et al. 2013). On the other hand, they also promote plant growth by inhibiting the growth of phytopathogens and activities related to biotic and abiotic stresses i.e. indirect mechanism (Lugtenberg and Kamilova 2009; Beneduzi et al. 2012; Tewari and Arora 2016). They do so by producing antimicrobial metabolites like HCN, biosurfactants, antibiotics and also induce systemic resistance in plants which involves jasmonate and ethylene signalling. PGPR also induce production of metal chelators like siderophores for nutrient competition against phytopathogens (Lugtenberg and Kamilova 2009; Bhattacharyya and Jha 2012). Among all the PGPR, rhizospheric pseduomonads represent an excellent combination of traits that are useful for both plant growth promotion and disease control. They have emerged as the largest and most potent

candidate and possess both direct and indirect mechanisms for plant growth promotion and hence are ideal for development of bioinoculants (Islam et al. 2016).

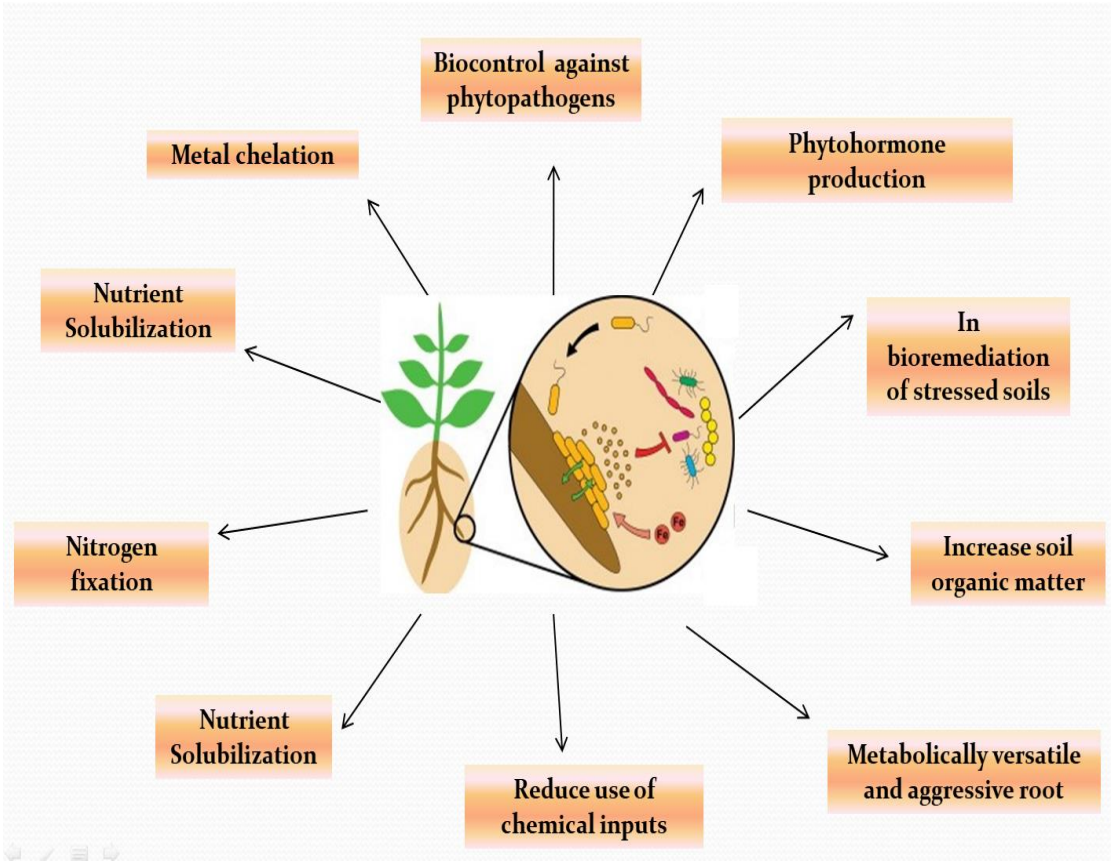


Fig. 5 Beneficial impacts of PGPR on plant growth promotion and in enhancing soil quality

2.4. Rhizospheric pseudomonads and their efficiency as biocontrol agents

Pseudomonas species are a large taxonomic group that are the member of γ subclass of Proteobacteria and collectively referred to their generic name 'pseuduomonads' (Palleroni 2010; Selvakumar et al. 2015). It was in the late nineteenth century when genus *Pseudomonas* was first given its name by Migula (1894) of the Karlsruhe Institute in Germany. However, it was the work of Stanier et al. (1966) that established the taxonomical basis of identification of this group which was largely determined by phenotypes and biochemical capabilities. Later, the genus was revised

and a subdivision of five groups was formed based on the results of DNA-DNA and rRNA-DNA hybridization and the description was inserted in the first edition of Bergey's Manual of Systematic Bacteriology (Palleroni 1984; Gomila et al. 2015). Further, pseudomonads caught everyone's attention worldwide during 1970s at the University of California, Berkeley, USA, when it was used for biocontrol research (Weller 2007; Dorjey et al. 2017). Inoculation of fluorescent pseudomonads (isolated from sugar beet root) with seeds or seed pieces demonstrated that certain strains were able to significantly improve the growth of potato sugar beet and radish by inhibiting growth of phytopathogens (Kloepper et al. 1980, 1988, 1991; Weller 2007). At present, there are 144 recognised and valid species of this genus including 10 subspecies and are mentioned in the List of Prokaryotic Names with Standing in Nomenclature (Parte 2014; Gomila et al. 2015).

Pseudomonas spp. are ubiquitous in agricultural soil, highly competent root colonizers and possess wide range of plant-beneficial properties (Lugtenberg et al. 2001; Haas and Défago 2005; Jain and Das 2016). Bacteria belonging to genus *Pseudomonas* are gram negative, strict aerobes, polarly flagellated rods (Palleroni 2010; Dorjey et al. 2017) and include mostly fluorescent species and few non-fluorescent ones (Botelho and Mendonça-Hagler 2006). Fluorescent pseudomonads comprise of i) phytopathogenic cytochrome c oxidase-positive species (e.g. *Pseudomonas cichorii, Pseudomonas. marginalis* and *Pseudomonas.tolaasii*) ii) and nonphytopathogenic, non-necrogenic strains (e.g. *Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas chlororaphis,* and *Pseudomonas aeruginosa*) iii) phytopathogenic necrogenic fluorescent *Pseudomonas* spp without cytochrome c oxidase (e.g. *Pseudomonas syringae*) (Young et al. 1992; Bossis et al. 2000). Non-fluorescent types represent species like *Pseudomonas stutzeri, Pseudomonas*

alcaligenes and Pseudomonas pseudoalcaligenes (Palleroni 1993; Holt et al. 1994b; Botelho and Mendonça-Hagler 2006). Rhizospheric pseudomonads have emerged as important plant health managers by showing potent phytostimulation and phytoprotection capabilities. Besides, their excellent properties as a biofertilizer (nitrogen fixation, phosphate solubilization, iron chelation, and phytohormone production), they are best characterized as promising biocontrol capabilities against range of phytopathogenic fungi. This is because they are capable of synthesizing antimicrobial compounds and metabolites like biosurfactants, pyocyanin and pyoverdin, siderophores, VOCs and hydrolytic enzymes and also elicit systemic responses in plants known as induced systemic resistance (ISR) (Lugtenberg and Kamilova 2009; Meyer et al. 2010; Bharucha et al. 2013; Pieterse et al. 2014; Berendsen et al. 2015). Among all these characteristics features shown by *Pseudomonas* species, biosurfactant production has gained special attention in recent times in context of disease suppression in plants. These microbial secondary metabolites are natural, eco-friendly and biodegradable that can either directly act on phytopathogens or can stimulate plant's immune response against invading microbes thus acting as a promising biocontrol strategy (Crouzet et al. 2020). In this way, potential of these biomolecules can be an essential alternative for crop protection and also to reduce the load of agrochemicals in agricultural sector.

Biosurfactants

Microbial surfactants or biosurfactants are amphiphillic compounds possessing both hydrophilic and hydrophobic moieties and are majorly synthesized by bacteria, fungi and yeast. They are broadly categorised as glycolipids, lipopeptides, phospholipids, neutral lipids, fatty acids and polysaccharide molecules (protein complexes, lipopolysaccharides, protein-like substances) (Van-Hamme et al. 2006; Nalini and

Parthasarathi 2018). Biosurfactants are known to reduce the interfacial and surface tension between two immiscible liquids and it is due to this tendency that makes them suitable agents for lubrication, foaming emulsification, wetting and dispersion (Nalini et al. 2021; Thakur et al. 2021). For this reason, numerous studies have been evaluated to determine the influence of biosurfactants in various industries such as food, cosmetic, petroleum, paint, pharamaceutical, and in areas like nanotechnology and environmental remediation (Paulino et al. 2016; Chong and Li 2017; Naughton et al. 2019; Singh et al. 2019). They have also been exploited in the field of biomedical sciences in form of modulators of human innate immunity by eliciting the production of antimicrobial compounds in body (Coelho et al. 2020; Edosa et al. 2020). In addition to their application in these areas, they also play a pivotal role in the field of agriculture as they serve as potent antifungal agents against vaious phytopathogenic fungi (Borah et al. 2016; Thakur et al. 2021). They also play crucial role in plant-microbial interaction through activities like swarming motility, signalling and biofilm formation (Sachdev and Cameotra 2013). They have also been reported to increase the availability of certain nutrients that are essential for plants (e.g., Cu, Fe, Mn, and Zn) (Singh et al. 2018).

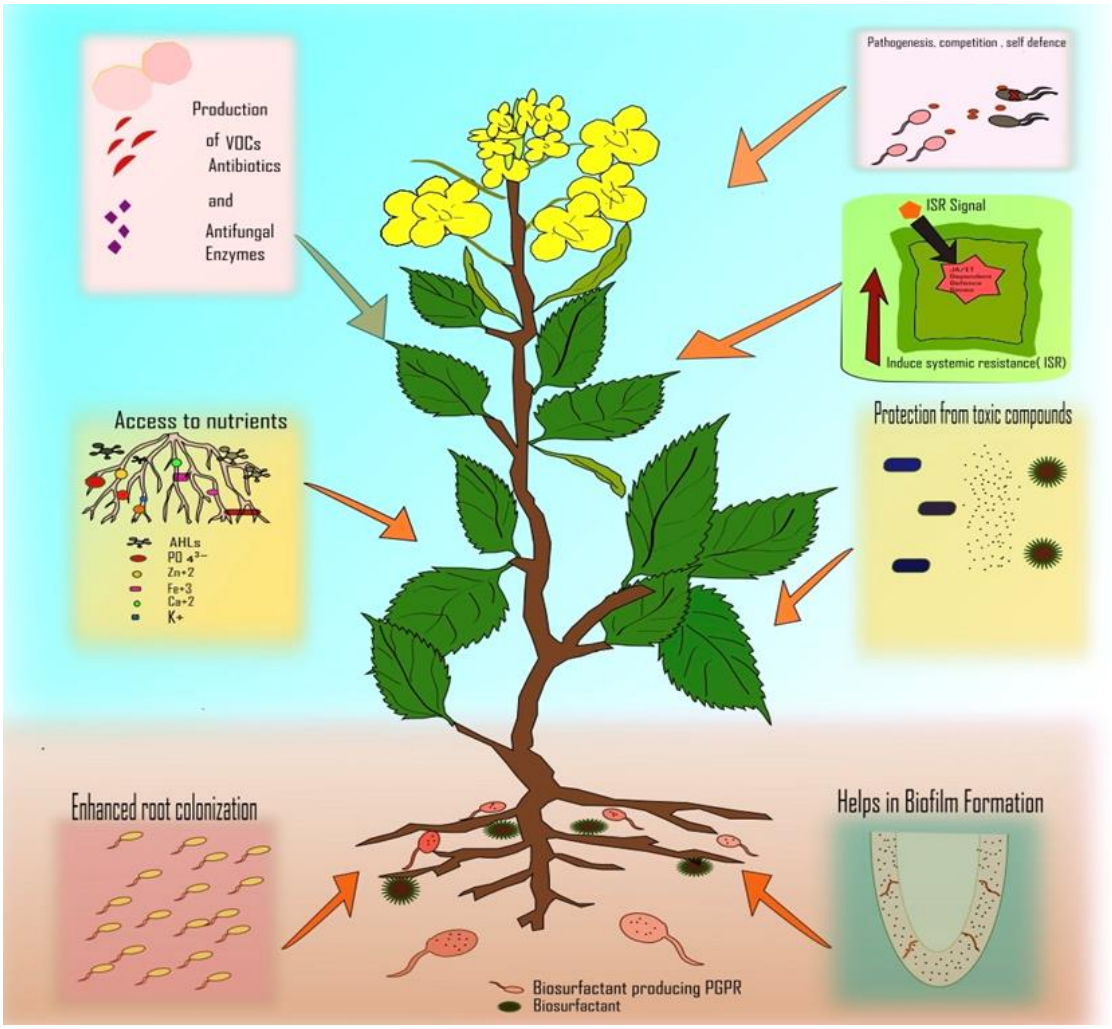


Fig. 6 Diverse roles exhibited by biosurfactant and its producing PGPR on plant health

Rhamnolipids are a type glycolipids which are one of the most widely studied biosurfactants over the years and were named as oily sludge by Bergstrom et al. (1946). These are composed of two moieties i) one or two (L) rhamnose congeners known as the glycan group which are hydrophilic in nature and attached together with α-1,2 glycosidic linkage ii) the lipid part known as the aglycan group which is hydrophobic in nature and made up of one or two β-hydroxy acid bonded together with an ester bond (Abdel-Mawgoud et al. 2010; Abbasi et al. 2012; Gudiña et al. 2015; Chong and Li 2017). The two moieties together are linked with a glycosidic bond (Chong and Li 2017). In addition to their biodegradability, lower toxicity, high

adaptability to range of pH, temperature and salinity, they also possess lower range in surface tension and critical micelle concentration (MIC), high emulsification index and high production in short time frame, and hence are being broadly exploited (Nitschke and Costa 2007; Gudiña et al. 2015; Liu et al. 2017). Rhamnolipids are predominantly produced by genus *Pseudomonas* and were first isolated and described by Jarvis and Johnson (1949) in *Pseduomonas aeruginosa.* However, due to the pathogenic nature of this bacterium, there were safety issues which were required to be addressed before its utilization in any field (Gunther et al. 2005). Other strains that are capable of producing rhamnolipids include *P. putida, P. chlororaphis, P. plantarii* and *P. fluorescens* (Randhawa and Rahman 2014).

Rhamnolipids are classified as mono and dirhamnolipids and levels of both rhamnolipids can be controlled in the production process (Randhawa and Rahman 2014). The concentration of rhamnolipid congeners greatly depend upon rhamnolipid-producing strains and any changes in the composition of the congeners can affect the physicochemical properties of the final compound (Abdel-Mawgoud et al. 2010; Hošková et al. 2015). The first patent on rhamnolipids produced from *P. aeruginosa* DSM 2659 was filed in 1984 by Kaeppeli and Guerra-Santos (US 4628030) which was received in 1986 (Kaeppeli and Guerra-Santos 1986). Meanwhile, Wagner et al in 1989 also filed a patent on rhamnolipids (US 4814272) which was based on its biotechnological production (Wagner et al 1989; Randhawa and Rahman 2014). However, research still needs to be focussed on identifying rigorous producing strains, use of cheap substrates and reduction in cost production to make it the possible front runner in multitude dimensions.

The main interest in the use of rhamnolipids in agriculture sector is their antagonistic behaviour against certain fungal phytopathogens which is an eco-friendly

and sustainable way to control plant diseases (Sha et al. 2011). The antifungal properties of rhamnolipids have been reported towards several species of fungi and oomycetes including *Botrytis* sp., *Rhizoctonia* sp., *Fusarium* sp., *Alternaria* sp., *Pythium* sp., *Phytophthora* sp., or *Plasmopara* sp. (Stanghellini and Miller 1997; Varnier et al. 2009). The main congeners responsible for their strong biocontrol properties are mono -rhamnose (Rha-C10-C10) and di-rhamnose (Rha-Rha-C10-C10). Rhamnolipids cause growth inhibition of fungal pathogen either by zoospore lysis, termination of spore germination or by preventing mycelial growth (Kim et al. 2000; Goswami et al. 2015; Yan et al. 2014, 2015). De Jonghe et al. (2005) reported that treatment of rhamnolipids caused zoospore lysis of *Phytophthora cryptogea* resulting in significant reduction of brown disease in *Cichorium intybus* var. *foliosum*. Similarly, antifungal activity of rhamnolipids was evaluated against *Fusarium verticillioides* in maize plants and results revealed considerable damage of mycelia under scanning electron microscopy (SEM). This resulted in improved plant biomass and suppression of disease symptoms in maize (Borah et al. 2016). Goswami et al. (2015) demonstrated the efficacy of rhamnolipids against *Colletotrichum falcatum* using microtitre plate assay. Results showed that 100 mg/ml concentration of rhamnolipids caused inhibition of spore germination by 86.6% and sugarcane plants treated with the biosurfactant exhibited reduced symptoms of red rot disease. The main mechanism by which rhamnolipids cause zoospore lysis is by inserting themselves inside its plasma membrane (which is not proetected by cell wall) and causing its disintegration (Stanghellini and Miller 1997; Oluwaseun et al. 2017). It is due to the amphipathic nature of rhamnolipids nature that they are easily able to interact with plasma membrane and their intercalation greatly depends on its composition (Aranda et al. 2007; Otzen 2017). Correspondinly, rhamnolipids have

been investigated to effect mycelial growth by its destabilization resulting in their lysis as well (Crouzet et al. 2020). In addition, rhamnolipids have also been identified to elicit immune response in plants which is another form of disease resistance from phytopathogens (Vatsa et al. 2010). They are reported to trigger signalling events like formation of ROS, synthesis of phytohormones, activation of defensive genes, calcium influx, and cascading of phosphorylation process (Varnier et al. 2009, Sanchez et al. 2012; Monnier et al. 2020). Yan et al. (2015) in their study demonstrated the effect of treatment of rhamnolipids with cherry tomatoes before and after inoculation of *A. alternata*. It was reported that activities of antioxidant enzymes were increased including superoxide dismutase and catalase in rhamnolipid treated plus *A alternaria* inoculated plants within 12 h while levels of ROS were found to be decreased. In the same way, Monnier et al. (2018) demonstrated that application of rhamnolipids triggered resistance in *B. napus* against phytopathogen by production of ROS, expression of defensive gene, stomatal closure and electrolyte leakage. Apart from disease resistance and inhibition of fungal phytopathogens, rhamnolipids are also involved in cell to cell communication by synthesizing quorum sensing molecules like acyl homoserine lactone (AHL) (Dusane et al. 2010). Furthermore, when applied externally, they have been reported to facilitate nutrient availability in plants by reducing the interfacial tension between metal and soil thus increasing its bioavailability (Pacwa-Plociniczak et al. 2015; Singh et al. 2018). The above studies provide concrete evidences about use of rhamnolipids in agricultural sector which could be exploited for protection of crops i.e. biopesticide as well as a phytostimulant or biofertilizer. Hence, their role must be harnessed in development of novel bioformulation in order to increase crop productivity in a sustainable way and minimize of reliance on chemical inputs.

Microbial Bioformulations

Microbe-based bioformulations are the biopreparations based on beneficial soil bacteria and/or their metabolites that are meant for enhancing crop productivity and inhibiting the growth of phytopathogens in a sustainable and eco-friendly way (Vessey 2003; Mishra and Arora 2016; Meftah Kadmiri et al. 2021). History of commercial bioformulation dates back a century ago when two German scientists, Nobbe and Hiltner (1896) registered the first patent for "Nitragin" as plant inoculation containing *Rhizobium* for providing nitrogen source to the crop. At present, a wide range of microbial genera including *Azospirillum*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Trichoderma,* and blue-green algae are being used in form these biobased products. They are as robust as the agrochemicals, even in small quantities and are being exploited commercially as biofertilizers and biopesticides (Sarma et al. 2015; Jiao et al. 2021). The market share of microbial bioinoculants was estimated around USD 452.07 million in 2021 which is expected to reach USD 751.51 million by 2028 at CAGR of 9.5% (https://www.marketdataforecast.com/market-reports/microbial-soil-inoculants-market)Some front runners commercial products in this category are s. Azo-Green™, Nutri-Life B.Sub™, Trichox WP, LIKUIQ® + ADD-IT AZUL (Arora et al. 2020b; Bejarano and Puopolo 2020). Bioformulations based on *Pseudomonas* spp. species are also commercially available in the market and are being used successfully in form of biofungicides e.g. Cerall, BioAgri, Proradix, Sourcon Padena and so on (Arora et al. 2020b).

Development of bioformulation is a multistep process and begins right after the isolation or selection of the active ingredient i.e. a potent strain for intended effect. Second and the crucial step is the mass production of the selected strain to determine the population density during harvesting time and also to maintain viability during

storage. Culture media, optimal temperature, dissolved oxygen, pH, and mixing speed are some crucial parameters that need to addressed during mass production (Morgan et al. 2006; Lobo et al. 2019). Further, they are mixed with a carrier and additive to produce an effective and high quality biobased preparation with stable shelf life (Bejarano and Puopolo 2020). A suitable carrier material plays a very important role as it assures the even distribution of the active ingredient (the PGPR) in and around the plant and should always be inert to support the growth of the living microbe (Mishra and Arora 2016). Some inert carrier materials include fine clay, peat, vermiculite, alginate, and polyacrylamide beads, diatomaceous earth, talc, vermiculite, carboxymethyl cellulose, and xanthan gum (Digat 1989). On the other hand, additives/adjuvants are meant for improving physical, chemical and nutritional properties of bioformulation (Schisler et al. 2004). Some common adjuvants or additives are clay, skim milk, gums, chitosan, humic acids, silica gel, methyl cellulose, and starch (Hynes and Boyetchko 2006; Mishra and Arora 2016; Vassilev et al. 2020). Once prepared, the bioformulation must ensure that it provides a microenvironment to the selected microbial strain, protect it from physical and chemical damage, maintain its viability during storage, support the microbial competition in soil with other microflora (Vassilev et al. 2020). Finally, a tripartite interaction between the microorganism, host plant and soil is of utmost importance in order to ensure the growth promoting traits towards the crop (Arora et al. 2020b).

Bioformulations are broadly classified into liquid and solid types based on carrier material (Burges and Jones 1998). Liquid formulations are aqueous solutions of microbial biomass in water, oil or emulsion (combination of water and oil) that improve viscosity, dispersion and stability of cell suspensions (Catroux et al. 2001; Schisler et al. 2004; Bashan et al. 2016). The drawback with this type of formulation

is that after they are introduced in the soil, the microbial population and their metabolic activity tend to decrease with course of time. Hence, more attention is being given to cell free broth filtrates having multiple properties of microbial metabolic products such as phytohormones, antibiotics, biosurfactants, siderophores, lytic enzymes (Thrane et al. 1997; Vassileva et al. 2015; Bashan et al. 2016; Vassileva et al. 2017).

Solid microbial formulations are based on different types of carrier material (organic or inorganic) prepared in granular or powder forms and are more preferred than wet formulation as they ensure extended shelf life and are more user friendly (Burges and Jones 1998). Size of granular inoculants varies between 0.1–2.5 mm (microgranules, 100–600 μm; fine granules, 0.3–2.5 mm) and sometimes can be produced in larger granular forms that are upto a size of 6mm. On the other hand, powder inoculants have the size of only of few hundreds of micrometers (Bejarano and Puopolo 2020). During recent years, more attention is being paid to polysaccharide-immobilized inoculants and is considered a better technological solution in order to assure the standardization and quality of the formulated inoculum. In cell immobilization system, encapsulation is particularly proven favourable for biocontrol agents where microbial cells are entrapped into a polymeric substance and are produced in beaded form that is permeable to nutrients, gases and metabolites to maintain viability of cells inside (John et al. 2011). Usually these capsules are made of dripping, emulsifying or solidifying liquid droplets using thermal and ionic gelation, coacervation and solvent extraction, spray drying, coating and sol-gel immobilization methods (Vemmer and Patel 2013). Among these, ionic-gelation using alginate is the most popular and are being exploited extensively for different bacterial genera including pseudomonads (Chang et al. 2007).

There is no denying the fact that progress has been made in development of bio based formulations using PGPR strains in form of biofertilizers and biopesticides but it is yet not satisfactory. A deeper insight is still needed regarding the relationship between the carrier-additives-microorganisms-soil-plant system to understand the functional properties and delivery systems of these biostimulants. Research efforts should also be oriented towards the use of cost effective carriers and additives especially in case of encapsulation technology. Evaluation of multiple field testing trials to determine the efficacy of bioformulations must be done and continuously revised before their marketization. Lastly, for successful commercialization of these sustainable green products in agricultural systems, effective guidelines must be framed and complex and time consuming registration process should be reformed worldwide. The global harmonization of bioformulations will undoubtedly lift the limitations and encourage the commercialization of microbial products to several extents.

Material and Methods

3. Material and Methods

3.1. Isolation of bacteria

Bacteria were isolated from rhizosphere of *B. juncea* from Lucknow region (26.8467° N, 80.9462° E) of Uttar Pradesh, India (Fig. 7). The samples were collected from the root surface into sterile polybags and stored at 4^0C for further use. For isolation, the soil sample was serially diluted upto 10^{-6} in distilled water (DW) and plated in triplicate on nutrient agar (NA; Hi-media). Petri plates were incubated on 28°C for 24 hours (Camargo et al. 2003) and isolates were selected on the basis of colony morphology and biochemical characterization. All the isolates were maintained in 25% glycerol at − 20 ^{0}C (Labocon, LUF-86-100) for further use.

3.2. Phenotypic characterization of isolates

Morphological characters of isolates were observed for growth pattern, color, shape, elevations and texture by observing their growth on NA (Khandelwal 2002). Also, morphology of cells was examined through Gram's staining (Gram 1884). Further, determination of motility of the isolates was detected by observing the growth pattern on semi-solid motility test-agar media (Tittsler and Sandholzer 1936).

3.3 Physiological and biochemical properties

Physiological and biochemical characters of isolates were examined and were referred to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994a) and Bergey's Manual of Systematic Bacteriology (Garrity 2005). Standard protocols were followed for each test.

(i) Generation Time

Isolates were checked for its growth kinetics following the protocol of Dubey and Maheshwari (2012). For this, the log phase culture of the isolates with OD 610 = 0.1 was inoculated in 50ml NB and incubated for 48 hours at 25 ± 2°C and 120 rpm. After every 4 hours the optical density of the samples was measured at 610 nm using Thermo Scientifc™ Evolution 201 UV–Vis spectrophotometer. The generation time was calculated using the formula:

Generation time = (T2–T1)/ 3.3 (log10 OD2- log10 OD1)

(ii) Growth at different temperature:

The NA plates were inoculated with bacterial isolates (using log phase culture; OD 610 adjusted to 1) and was subjected to various temperature conditions ranging from 4 to 40^0C and after incubation for about 3-5 days the growth was observed (Pandey et al. 2006).

(iii) Growth at different pH:

The pH tolerance of the selected isolates was evaluated by varying the range of pH from 5.0 to 12. NA was used for checking the tolerance ability and pH was adjusted by addition of HCl or NaOH. The inoculated (using log phase culture; OD 610 adjusted to 1) medium was incubated for 25 ± 2 ^{0}C for 3-5 days and observed for growth according to (Zablotowicz and Focht 1981).

(iv) Growth at different NaCl concentrations:

The salt tolerance assay of the isolates was done using NB supplemented with 2–8 % NaCl (w/v) and a non-saline control. The log phase culture of the isolates (OD610

adjusted to 0.1) were added to medium and incubated at 25 ± 2 °C with shaking at 120 rpm for 48 h. The optical density (at 610 nm) was measured using Thermo Scientific™ Evolution 201 UV–Vis spectrophotometer after every four hours up to stationary phase (Khare et al. 2011). The experiment was conducted in triplicates and under same physiological conditions.

(v)Utilization of different carbon sources:

The isolates were checked for utilization of various carbon sources including mannitol, glucose, fructose, lactose, dextrose, sucrose, maltose, malate, starch, glycerol, malate, trehalose and citrate. For this, minimal medium M9 (5X) was used to grow the isolates and different carbon sources were added to check for the utilization (Jovčić et al. 2010). Different carbon sources (sterilized by filtration through Millipore membranes pore size 0.22 μm) were added to medium at concentration of 10% (w/v). The plates were incubated for 28 to 48 hours at 25 ± 2 ^{0}C

(vi) Utilization of different nitrogen sources:

Different nitrogen sources including yeast extract, potassium nitrate (KNO_3), sodium nitrate ($NaNO_3$), tryptophan, ammonium chloride (NH_4Cl), glycine, ammonium sulphate ($(NH_4)_2SO_4$), lysine, glutamine, cysteine, and methionine were added to minimal medium M9 (5X) in place of ammonium chloride, source of nitrogen in the medium (Holt et al. 1994a). Streaked plates were incubated for 24 to 48 hours at 25 ± 2 ^{0}C.

(vii) Catalase test

For this test, a loopful of bacterial culture was placed on glass slide followed by addition of two to three drops of 3% hydrogen peroxide (H_2O_2). Slides were observed for presence of effervescence due to release of oxygen (Graham and Parker 1964).

(viii) Oxidase test

To perform this test, a whattman filter paper was taken and was cut into stripes and was soaked in tetra-methyl-p-phenylene-diamine di-hydrochloride. Then the paper was moistened with the help of few drops of water and bacterial culture was mixed with the substrate loaded filter paper. The stripe was observed for the change in color within 20-30 seconds of mixing (Shields and Cathcart 2010).

(ix) Urease test

Petri plates containing NA and added with 2% (w/v) urea and 0.012% (w/v) phenol red was used for the test. Bacterial isolates were spot inoculated on the plates and incubated for 48 hours at 25 ± 2^0C. Change in colour of the media from yellow to pink-red was observed for positive result (MacFaddin 2000).

(x) Gelatinase test

Test tubes filled with gelatinase medium was poured in equal amounts. Thereafter, tubes were stab inoculated using inoculating needle with bacterial cultures and incubated for 3-5 days at 25 ± 2^0C. After incubation the tubes were placed in refrigerator at 4^0C for 15 minutes. Liquefaction of the medium confirmed the positive result (McDade and Weaver 1959).

(xi) Amylase test

For this test, starch agar medium was prepared and isolates were streaked on the plates and incubated for 48 hours at 25 ± 2^0C. After incubation, petri-plates were flooded with Gram's iodine and development of clear zone around the bacterial colonies was observed (Lennette et al. 1985).

(xii) Lipase test

To perform this test, Tween 80 agar medium was prepared. Then isolates were inoculated and incubated at 25 ± 2^0C for 2-3 days. Opaque zone formation around the colonies was indicated positive (Samad et al. 1989).

(xiii) Protease test

Skim milk agar medium was used and prepared plates with media were inoculated with bacterial isolates. The plates were incubated at 25 ± 2^0C for 48 hours. Presence of clear zone around the colonies was observed (Kasana et al. 2011).

(xiv) Cellulase test

Prepared plates of Czapek's mineral salt agar were used for this test. Isolates were then inoculated and incubated for 2-3 days at 25 ± 2^0C After incubation the plates were stained with congo red dye for 15 minutes. Thereafter 1% NaCl was used for destaining for 15 minutes. Halo zone around the colonies was observed (Florencio et al. 2012).

(xv) Nitrate reductase activity

Bacterial isolates were inoculated in tryptone yeast extract (TYE) medium containing 0.1 % KNO_3 (Campbell 1999). After incubation of the medium for 48 hours at 25 ± 2^0C sulphanilic acid (8 g/l) in 5M acetic acid) and α-naphthylamine (5g/l in 5M acetic acid) was added to each tubes. Change in color was observed.

(xvi) Citrate utilization test

Plates containing Simmon's citrate agar medium were used and bacterial isolates were streaked and incubated for 24 hours at 25 ± 2^0C change in colur of the media from blue to green confirmed the positive result for the test (MacWilliams 2009).

(xvii) Ammonia production

Peptone broth was prepared and 5ml of the medium was added to the test tubes. Then bacterial culture was inoculated in each tube and incubated for 48 hours at 25 ± 2^0C. After incubation 0.5 ml of Nessler's reagent was added to each tubetube and observed for colour change along with slight precipitate formation (Cappuccino and Sherman 1992).

(xviii) Indole test

Tryptone broth was inoculated with bacterial culture and incubated for 24 hours at 25 ± 2^0C Further, 1 ml of Kovac's reagent was added to each tube and formation of "red color ring" on top of the broth was observed (Cheesbrough 1985).

(xix) Methyl Red-Voges Proskauer (MR-VP) test

MRVP broth was prepared and 10 ml was distributed in several tubes. Each tube was inoculated with bacterial culture and incubated for 28 hours at 25 ± 2 °C. After incubation, media was divided into two tubes one for MR and the other for VP test. For MR test 5 drops of methyl red and for VP test 12-15 drops of VP- I (5% α-naphthol) and VP- II (40% potassium hydroxide) was added to each set of tubes. Keeping the tubes for 10-15 minutes, color change was observed (Faddin and Jean 2000).

3.4 Screening of the bacterial isolates for biosurfactant production

Bacterial isolates were tested for biosurfactant production on minimal salt medium, (composition g/l $NaNO_3$: 2.5, $MgSO_4$: 0.5, $FeSO_4$: 0.01, KH_2PO_4: 1.0, Na_2HPO_4: 5.67, KCl: 0.1, $CaCl_2$: 0.1, $MnSO_4$: 0.002, NH_4NO_3: 0.39, dextrose: 15), incubated at 30°C for 72 hours with shaking at 160 rpm.

3.4.1 Foaming

Isolated bacteria showing foaming in the medium as compared to negative controls were observed for reflected light which scattered like rainbow colour. Contents of the test tubes of minimal salt medium with the test strains (bacterial strains) showing foaming were subjected to centrifugation at 10,000 rpm for 20 minutes (4°C). Cell-free supernatant (crude biosurfactant) was used for further experiments.

3.4.2 Oil displacement test

Oil displacement test was performed by following method described by El-Sheshtawy and Doheim (2014) for assessment of the surface activity of biosurfactant. It was done measuring the diameter of clear zone produced after adding drop of biosurfactant on thin film of oil on water. The experiment was carried out at room temperature by adding 20 ml of water in 10 cm diameter Petri plate. About 20 µl of oil was added above water to form thin film and then a 10 µl of biosurfactant was added on the surface of oil. The clear diameter zone diameter of oil was measured.

3.4.3 CTAB assay

To perform this test, method of Siegmund and Wagner (1991) was followed which is used for semiquantitative detection of extra cellular anionic surfactants. (MSA) media supplemented with 2% glucose was used and 0.5 g/l Cetyltrimethylammonium bromide (CTAB) and 0.005g/l methylene blue was added for detection of anionic biosurfactant. A loopful of bacteria were inoculated on agar plates and incubated at 37±2ºC for 48-72 hrs. Development of dark blue halo zone around bacterial colony was considered as positive test.

3.4.4 Emulsification activity

The Emulsification activity of biosurfactant was performed according to method of Cooper and Goldenberg (1987) with some modifications. 5 ml supernatant was mixed with 5ml crude and vegetable oil in a vial. The mixture was vortexed at high speed for 2 min and kept for 24 h. After 24 h, the emulsification activity was calculated by using formula:

Emulsification index (E_{24}) = height of emulsion formed/ total height x100

3.5 Plant growth promoting properties

3.5.1 Phosphate solubilisation

Solubilisation of phosphate was tested by spot inoculating the log phase cultures isolates on Pikovskaya medium. The plates were incubated at 28ºC for five days and solubilisation index (PSI) was calculated by measuring diameter of clear zone (Pikovskaya 1948) using formula

PSI = (Colony diameter + halo zone diameter)/ Colony diameter

3.5.2 Zinc solubilization

Solubilisation of zinc by isolates was checked on zinc media (dextrose, 1%; ammonium sulphate, 0.1 %; potassium chloride, 0.02 %; di-potassium phosphate, 0.01 %; magnesium sulphate, 0.02 %; zinc oxide, 0.1 %; agar, 3%). The petri plates containing media was spot inoculated by the bacterial culture and plates were incubated and incubated for 48 hours at 25 ± 2°C. Solubilisation index (ZSI) was calculated by measuring diameter of clear zone (Fasim et al. 2002) using formula

ZSI = (Colony diameter + halo zone diameter)/ Colony diameter

3.5.3 IAA production

IAA production was checked by colorimetric assay using Salkowski reagent according to the protocol of Ahmad et al. (2008). Firstly, isolates were grown on NB amended with 0.5% tryptophan with a control. After incubation, the supernatant was collected by centrifugation at 10,000 rpm (13,081 x g) for 30 min. Thereafter, Salkowaski reagent was prepared by addition of 1.0 ml of 0.5 M ferric chloride (FeCl3) to 50 ml of 35% perchloric acid. Next, 2-3 drops of orthophosphoric acid was added to 2ml of culture supernatant, followed by addition of 4ml Salkowaski reagent. The development of pink colour was quantified by measuring the optical density at 530 nm and the value was calculated using the standard IAA (concentration 10–100 µg/ ml; Hi-media).

3.5.4 Siderophore production

Siderophore production by isolates was checked using the standard CAS (chrome-azurol sulfonate) assay according to Schwyn and Neilands (1987). For this CAS reagent was prepared using CAS dye. 121 mg of CAS dye was dissolved in 100 ml distilled water and 20 ml of 1 mM ferric chloride ($FeCl_3.6H_2O$) solution was prepared in 10 mM HCl. Thereafter, the solution was added to 20 ml hexadecyl trimethyl ammonium bromide (HDTMA) solution (729 mg HDTMA in 400 ml distilled water) with continuous stirring and was sterilized before use. Both qualitative and quantitative estimation of siderophore production was estimated using following protocols:

Qualitative analysis: This was done on CAS plates that were prepared by addition of 100 ml of CAS reagent to 900 ml Luria Bertani (LB) agar medium (Hu and Xu (2011). The plates were spot inoculated with bacterial isolates and were incubated for 5-7 days at 25 ± 2 ^{o}C. Development of yellow to orange colored zone around the bacterial spot was indicative of siderophore production.

Quantitative analysis: For quantitative estimation of siderophore production CAS-Shuttle assay was performed (Arora and Verma 2017). Bacteria were already grown on LB broth and the supernatant was collected by centrifugation at 10,000 rpm (*13,081 x g*) (21^{0}C). Then, 0.5ml of supernatant of the isolate was mixed with equal amount of CAS reagent and after 20 min OD was taken at 630 nm (Thermo Scientific, Evolution 201). The amount of siderophore produced was calculated as percent siderophore unit (psu) using the formula:

Siderophore production (psu) = {(Ar-As)/ Ar} x100, where Ar = absorbance of reference (CAS solution and un-inoculated broth), As = absorbance of sample (CAS solution and cell free supernatant of sample)

3.5.5 ***Hydrogen cyanide production***

HCN production by bacteria was checked according to protocols of Bakker and Schippers (1987). The bacteria were grown on NA medium amended with glycine (4.4 g/ l). Filter paper (Whatman No. 1) (10 x 0.5 cm^{2}) dipped in alkaline picrate solution was placed on the lid of the petri plate contaiaining inoculated medium. The tubes were incubated for 48 h at 25 ± 2^{o}C and reduction of sodium picrate forming reddish-brown color was concluded as positive for HCN production.

3.5.6 Qualitative estimation of ACC deaminase activity

To check the ACCD activity by isolates, sterile minimal DF (Dworkin and Foster) salts media (DF salts per liter: 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 2.0 g glucose, 2.0 g gluconic acid and 2.0 g citric acid with trace elements: 1 mg $FeSO_4.7H_2O$, 10 mg H_3BO_3, 11.19 mg $MnSO_4.H2O$, 124.6 mg $ZnSO_4.7H_2O$, 78.22 mg $CuSO_4.5H2O$, 10 mg MoO_3, pH 7.2) amended with 3 mM ACC instead of $(NH_4)_2SO_4$ as sole nitrogen source was used (Dworkin and Foster 1958; Penrose and Glick 2003). Pre-grown bacterial pellets were prepared according to Penrose and Glick (2003) and were inoculated on DF media plates and incubated for 3 days at 28ºC. Growth was monitored and colonies showing growth on ACC plates were taken as positive**.**

3.5.6 Biocontrol activity

Antagonistic activity of the isolates was checked through dual culture assay according to standard protocol of Ikediugwu and Webster (1970). For this, a 5 mm mycelial disc of *A. brassicae* ITCC No. 2542 (procured from the division of Plant Pathology, Indian Type Culture Collection Identification/Culture Supply Services, IARI, New Delhi, India) grown on potato dextrose agar (PDA) (Hi-media, Mumbai) was excised and placed at the centre of fresh PDA plate. Thereafter, bacterial isolates were streaked on either side of the fungal disc equidistant from the periphery and incubated at 27°C for 5 days. After 5 days of incubation, radial growth of the fungus was calculated in relation to the control plate (growing fungus alone) by the following equation:

$$L = \frac{C - T}{C} \times 100$$

where L is the percentage inhibition of radial mycelial growth, C is radial growth of fungus in the control; T is radial growth of the fungus in the presence of bacterial isolate.

3.6 Identification of bacteria

3.6.1 Molecular analysis through 16S rRNA sequencing

The isolates were grown on NA plates at 25 ± 2 °C for 24 hours and DNA was extracted using standard protocols. Molecular identification of bacteria was done by sequencing of 1.5 kb 16S rRNA sequences using the universal primers 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5′-GGTTACCTTGTTACGACTT-3. Conditions for amplification reaction was maintained as following:

PCR conditions

Reaction Mixture (50 µl)		**Cycling Conditions**		
Template DNA	100 ng	Initial Denaturation	2 minutes at 95°C	35 Cycles
Forward Primer	0.3 µM	Denaturation	30 seconds at 95°C	
Reverse Primer	0.3 µM	Annealing	30 seconds at 52°C	
Master Mix	25 µl	Extension	2 minutes at 72°C	
Nuclease Free Water	Volume makeup 50 µl	Final Extension	15 minutes at 72°C	

Primer details for PCR:

S. No	Oligo name	Sequence (5'-3')	Tm (°C)	GC-content
1	27F	AGAGTTTGATCMTGGCTCAG	56.3	47.5%
2	1492R	CGGTTACCTTGTTACGACTT	55.3	45%

Primer details for sequencing:

On the basis of PCR results, single amplified fragment was used for sequencing of 16S rRNA gene from both the strands and the primers used are discussed below:

S. No	Oligo name	Sequence (5'-3')	Tm (°C)	GC-content
1	785F	GGATTAGATACCCTGGTA	56.3	47.5%
2	907R	CCGTCAATTCMTTTRAGTTT	55.3	45%

The resulting amplicons (5 µl) of the 16S rRNA genes were analyzed by gel electrophoresis and were mixed with loading buffer (2 µl). The product was then electrophoretically examined through 1% agarose gel stained with ethidium bromide (10 mg/ml) at 100 V for 60 min. The bands were observed under UV light and image was captured.

3.6.2 Phylogenetic tree analysis, sequence submission to GenBank and strain submission to culture collection center

The 16S rRNA sequences of the isolates were then run on NCBI-Basic Local Alignment Search Tool (BLASTn) programme (Altschul et al. 1990) and submitted in GenBank for accession number. Phylogenetic tree was constructed using neighbor-joining method (Saitou and Nei 1987) in MEGA X software (6.0 version) (Kumar et al. 2018). Finally the selected isolate was submitted to National Agriculturally Important Microbial Culture Collection (NAIMCC), National Bureau of

Agriculturally Important Microorganisms (ICAR-NBAIM), Mau, India, an international culture repository.

3.7 Production, extraction and purification of biosurfactant by isolate BSP9

Biosurfactant was extracted using 24 h old log phase culture of BSP9 (with OD 610 = 0.1 CFU 10^8/ml) inoculated in Bushnell Haas broth (Himedia, Mumbai) with following compositions (g/L): magnesium sulfate ($MgSO_4$) 0.2, calcium chloride ($CaCl_2$) 0.02, monopotassium phosphate (KH_2PO_4) 1.00, dipotassium phosphate (K_2HPO_4) 1.00, ammonium nitrate (NH_4NO_3) 1.00, ferric chloride ($FeCl_3$) 0.05 supplemented with 1% glycerol as carbon source and pH was adjusted to 7. After 72 hours of incubation, it was centrifuged at 10,000 rpm for 20 minutes. Supernatant was collected in a fresh tube, acidified to pH 2.0 using 6N HCl and incubated for overnight at 4^0C. Thereafter, biosurfactant was extracted using a mixture of chloroform:methanol in ratio 2:1 (v/v) in a separating funnel and was then centrifuged at 10,000 rpm for 15 minutes. Solvent extract was filtrated using 0.45mm Millipore membrane and evaporated to dryness in an oven (Fernandes et al. 2007). Finally, a dark brown coloured viscous residue was collected.

For purification, the crude precipitate was run on thin layer chromatography (TLC). Plate. Chloroform:methanol:water was used as the solvent system in the ratio 65:15:2 (v/v/v). The spots were produced by spraying distilled water and heating at 110^0C for five minutes and were scraped, re-extracted in chloroform methanol and evaporated to dryness (Tahzibi et al.2004)

3.8 Structural characterization of the biosurfactant

(i) Fourier Transformed-Infrared (FT-IR) spectroscopy

For identification of the functional groups in the purified biosurfactant, Fourier Transform Infra-Red spectroscopy (FTIR) analysis was done using Bruker Advance 600 instrument. To carry out this, 1 mg of freeze dried biosurfactant was mixed with 100 mg of KBr and a thin translucent pellet was placed to record the IR in the range 400 cm^{-1} and 4000 $cm^{-1.}$ (Ali et al. 2019).

(ii) Liquid Chromatography-Mass Spectroscopy (LC-MS) analysis

To further detect the structural composition of the purified biosurfactant, liquid chromatography–mass spectrometry (LC-MS) was performed according to Haba et al. (2003) with slight modifications using Waters UPLC-TQD Mass Spectrometer. C18 column (250 X 4.6mm, 5um pore size) was used with acetonitrile-water system at a flow rate of 0.3 ml/min. Specific rhamnolipid congeners were identified based on mass spectrum which ranged from m/z 150-100.

3..8.2 Structure morphology and elemental composition through SEM and EDS

The morphology and microstructure of dried purified biosurfactant was determined through scanning electron microscopy (SEM) (Model: JEOL, JSM6490LV) at USIC, BBAU, Lucknow. Further, the elemental composition of biosurfactant was obtained using Energy Dispersive Spectroscopy (EDS) (EDS 133, EV Dry Detector (INCA x-act) of OXFORD instruments, UK) at USIC, BBAU, Lucknow.

3.9 In vitro antifungal activity of purified metabolite against *A. brassicae*

To further assess the effect of purified metabolite against *A. brassicae*, the agar well diffusion method was used. For this, plates containing PDA were taken and 20 µl of

purified metabolite suspended in methanol in concentrations 0.1%, 1%, 2% and 5% was placed in wells at a distance of 1.0 cm from the edge of the plate. A mycelial disc of 5 mm in diameter was excised from the edge of an actively growing *A. brassicae* (on PDA plate) and placed at the centre of the same plate and incubated at 28ºC for 5 days. In control, only methanol was suspended in wells. Zone of inhibition was calculated.

3.9.1 Scanning Electron Microscopy (SEM) of *A. brassicae* mycelia treated with rhamnolipid

For this, a method devised by Torres et al. (2016) was applied. In brief, fungal mycelium from near the zone of inhibition produced by fractioned metabolite was taken. For control fungal mycelium grown without the presence of metabolite was taken. The sample were fixed with glutaraldehyde 2.5% v/v in a 0.1 M phosphate buffer (pH 7.2) for 24 h at 4◦C, then dehydrated using 30%, 50%, 70%, 90% and 100% of ethanol. Finally, the metallization of the dehydrated samples was carried out and seen under SEM (Model: JEOL, JSM6490LV) at USIC, BBAU.

3.10 Effect of biosurfactant and selected isolates on mustard growth, yield and disease inhibition of Alternaria blight caused by *A. brassicae*

3.10.1 Preparation of bioformulations

To prepare talc based bioformulations method of Nandakumar et al. (2001) was followed. For this, 1 kg of talc powder was mixed with 15g of calcium carbonate $CaCO_3$ to adjusted the pH to neutral. To this mixture, 1g of Carboxymethyl cellulose (CMC) was added so as to increase the adhesion property of bioformulation and subsequently sterilized by autoclaving. Further, preparation of bacterial inoculum

suspension was done by growing BSP9 in nutrient broth (NB) medium (Hi-Media) and incubating it on a rotary shaker at 150 rpm at 27 ± 2 °C. After growth, 400 ml of log phase cell suspension (OD 610 adjusted to ~ 1) was added to the sterilized carrier-cellulose mixture. Bacterial population density was measured by taking 1 g of bioformulation into 10 ml of distilled water and serially diluting it to 10^{-6} and 10^{-7} (Fatima et al. 2020). Bacterial density in the bioformulation mixture was recorded to be ~10^8 CFU/g. Varuna-T56 variety of *B. juncea* was selected and before sowing, seeds were washed and surface sterilized with 3% hydrogen peroxide followed by washing with sterilized distilled water 4 to 5 times. Surface sterilized seeds were dipped in the suspension and left overnight for proper coating. The bacterial population density on seeds was found to be ~10^7 CFU/seed. After this, biosurfactant was amended in various concentrations i.e. 0.1, 1.0, 2.0 and 5.0% weight/volume. The combination of metabolite and BSP9 was prepared by adding biosurfactant at the rate of 10 mg *w/v* (for 1% biosurfactant+ BSP9), 20 mg *w/v* (for 2% biosurfactant + BSP9) and 50 mg *w/v* (for 5% biosurfactant + BSP9) to 1000 ml of talc suspension containing BSP9 cells. Cell density of bioformulation containing cells and metabolite was measured and was found to be ~10^7 CFU/seed. The combination of these prepared bioformulations were then used for pot and field study according to treatments given as : 0.1% biosurfactant, 1.0 % biosurfactant, 2.0 % biosurfactant, 5.0 % biosurfactant, 1.0 % biosurfactant + BSP9, 2.0 % biosurfactant + BSP9, 5.0 % biosurfactant + BSP9, and untreated control.

In case of pot study each of these treatment sets were challenged by fungus *A. brassicae* (except the untreated control) and an additional negative control set was also added where seeds were only treated with *A. brassicae*.

a) ***Pot study***

The pot experiment was conducted in earthen pots (24×12×12 cm) in triplicates during *rabi season* for two consecutive years (2017 and 2018). The soil used in pots was sterilized in autoclave at 15 Psi at 121˚C for one hour for three consecutive days. The physicochemical parameters of soil i.e. pH, EC organic matter, available nitrogen (N), phosphorous (P) and available potassium (K) was analysed (Fernandes et al. 2007; Shreve et al 2019). Seeds of *B. juncea* treated with bioformulations were sown in pots and spore suspension of *A. brassicae* (10^5 spores/ml) was sprayed on adaxial or abaxial surface of leaves after seven days of seed germination. The plants were covered with polybags for 48 hrs to maintain moisture and development of disease incidence. The experiment was conducted in sterilized soil in following sets of treatments in triplicates (i) untreated control, C1 (ii) *A. brassicae* (negative control), C2 (iii) 0.1% biosurfactant + *A. brassicae*, T1 (iv) 1% biosurfactant+ *A. brassicae*, T2 (v) 2% biosurfactant+ *A. brassicae*, T3 (vi) 5% biosurfactant+ *A. brassicae*, T4 (vii) BSP9+1% biosurfactant+ *A. brassicae*, T5 (viii) BSP9+2% biosurfactant+ *A. brassicae*, T6 (ix) BSP9+ *A. brassicae*, T7. Observations were recorded 60 DAS for germination rate, root length (cm), shoot length (cm), fresh and dry weight (g), number of pods and 1000 seeds weight (g). Percentage disease incidence (PDI) was calculated as percentage of diseased plants out of the total number of plants (Tewari and Arora 2014).

b) Field study

Field trial was conducted during *rabi* season for two consecutive years i.e. 2017-2018 and 2018-2019 at an agricultural farm near Lucknow, Uttar Pradesh (U.P.) located at 26.8467° N, 80.9462° E. The maximum and minimum temperature in two years of

field trial ranged between 20-40^{0}C and 7-25.9^{0}C respectively, during crop growing season. The chemical properties of the soil including pH, electrical conductivity (EC), organic carbon, available nitrogen, phosphorous, and potassium were checked according to the standard protocols of (Fernandes et al. 2007; Shreve et al 2019). Field experiment was done in triplicate with completely randomized block design (CRD) (size of each block = 2.43 m x 2.43 m) taking *B. juncea* as test crop (Fig. 25).. Field was irrigated thrice by ground water between sowing and harvesting. Plants were uprooted 120 days after sowing (DAS) and growth parameters like root and shoot length, fresh and dry weight, number of pods, oil content.

3.10.2 Phytochemical parameters

For phytochemical analysis of plant leaves, the extracts were prepared in various solvents including methanol, ethanol, acetone, phosphate buffer (0.1 M, 7.6 pH) and distilled water at the concentration of 50 g/ 500 mL. The filtrate was collected by centrifugation and the desired extract was stored for further use (Das et al. 2014).

(i) Estimation of Total Chlorophyll

Chlorophyll content was estimated by using method described by Arnon (1949). About 0.5 gm of fresh leaf was crushed in 10 ml of 80% (v/v acetone/water) chilled acetone with the help of pestle mortar in dark and centrifuged at 5000 rpm at 10˚C for 15 min. The supernatant was taken and optical density was measured at wavelength 663 nm, 645 nm and 480 nm by spectrophotometer. 80% acetone was used as blank. Total chlorophyll (in mg/g fresh weight) were determined by following formula:

Total chlorophyll = 20.2 (A_{645}) + 8.02 (A_{663})

where $\mathbf{A_{645}}$ is absorbance at 645 nm and $\mathbf{A_{663}}$ is absorbance at 663)

(ii) Estimation of Total Flavonoid

The quantitative analysis of flavonoid content of leaves (aqueous extract) was determined by aluminium chloride colorimetric method according to Shubhangi et al. (2017) using quercitin (Hi-media, HPLC grade) solution in methanol as standard.

(iii) Phenolic content

Phenolic content of leaves was estimated according to Lee et al. (2015) using Folin-Ciocalteu reagent and the absorbance was read at 765 nm using gallic acid ((Hi-AR™/ACS) as standard.

(iv) Estimation of Protein content

Protein content of leaves was checked according to the standard method of Lowry et al. (1951) using BSA (Sisco-pH 6–7, fraction V) as standard.

(v)Estimation of production of defense-related enzymes

(a)Peroxidase (PO) – Peroxidase activity was estimated according to by Hammerschmidt et al. (1982). Leaf material was homogenized in 50mM sodium phosphate buffer at pH 6.8 (w/v, 1:1) and centrifuged at 10,000 rpm for 20 min at 4°C. The resulting mixture had 0.5mL of enzyme extract, 1.5mL of substrate 0.05M pyrogallol and 0.5mL 1% hydrogen peroxide. Absorption was read at 420nm was recorded at 30 seconds intervals for 2 minutes, Enzyme activity was expressed as ΔOD at 420/mg of protein/min.

(b) Polyphenol oxidase (PPO) - Polyphenol oxidase activity measured as described by Mayer et al. (1965). Leaf material was homogenized in 50mM sodium phosphate buffer at pH 6.8 (w/v, 1:1) and centrifuged at10,000 rpm for 20

min at 4°C. The mixture contained l00µL of enzyme extract, 50µL of substrate 60mM catechol and 2mL of 50mM sodium phosphate buffer (pH 6.8). The absorption was read at 290nm enzyme activity and was expressed as ΔOD at 420/mg of protein/ min.

(c) Phenylalanine ammonia lyase (PAL): PAL activity was estimated according to Lisker et al. (1983). The leaf material was homogenised in 25mM Tris-HCI buffer at pH 8 (w/v in 1:1) and centrifuged at 8000 rpm for 30 min at 4^0C. After incubation of 2h at 40°C, activity was stopped by the addition of 0.06mL 5N HCL and the absorption was read at 290nm. The enzyme activity was expressed as µmol of trance –cinnamic acid /mg of protein/h.

3.10.3 Statistical Analysis and heatmap clustering

All the data of plant growth parameters and phytochemicals (for both pot and field study) were statistically analyzed using one way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) at 5% level to compare difference between treatment means. Data was analyzed by software statistical package for the social science (SPSS) (2016) for windows. Heatmap was drawn using R-package (version 3.6.2) to visualize the effect of various treatments on plant growth parameters and phytochemicals through clustering analysis.

Results

4. Results

4.1 Isolation

In total, 15 bacterial isolates were obtained from rhizosphere of *B. juncea* and purified on NA medium (Fig. 8) and were checked for phenotypic, physiological and biochemical characters.

4.2 Phenotypic characterization

Morphological characters showed that all the isolates were Gram negative (Fig. 9, motile, rod shaped, forming smooth and shiny colonies with convex and entire margins. (Table 1),

4.3 Physiological characters and biochemical properties

(i) Generation time

From the result it was observed that most of the isolates were fast growers with generation time between 2-4.5 whereas some isolates were slow growers with generation time of 6 h (Fig. 10)

(ii) Growth at different temperature:

Checking growth of bacterial cultures for range of temperatures, it was observed that all the isolate were able to show positive growth at 15, 25 and 35^0C and at high temperature (40^0C) only PMI9 showed positive growth (Table 2)

(iii) Growth at different pH:

pH tolerance of the isolated bacteria were tested and it was depicted that all the bacteria showed growth at pH 6, 8 and 10 and only PMI9 could survive the pH of 4 and 12 (Table 2).

(iv) Growth at different NaCl concentrations:

Results of salt tolerance assay are shown in Table 4. From the table, it can be concluded that isolates BSP9, BSP51, were able to survive upto 4% of NaCl concentration whereas PMI9 was viable upto 8% NaCl concentration (Table 2).

(v)Utilization of different carbon sources:

Checking the utilization of various carbon sources, it was observed that the most preferred source of carbon for bacteria was glucose (100%) followed by mannitol (93.33%), sucrose (80%), and glycerol (73.33%) (Table 3).

(vi) Utilization of different nitrogen sources:

In case of nitrogen utilization, 100% isolates utilized yeast extract, KNO_3, $NaNO_3$, tryptophan and NH_4Cl (Table 4). The least utilized sources of nitrogen were cysteine and methionine with only 20% and 13.33% bacteria showing growth, respectively (Table 4).

(vii) Biochemical examinations revealed that 73.4% bacteria gave positive results for catalase, 80% oxidase, 60% for urease, 27.6% gave positive amylase, 40% for gelatinase, 40% for lipase, 33.45 for protease, 20%for cellulose, 805 for nitrate reductase, 100% for citrate utilization, 100% for ammonia production, 33.4% for indole and 13.4 % for MR-VP (Fig. 11, Table 5).

4.4 Screening of the bacterial isolates for biosurfactant production

Among 15 isolates, only BSP9 and BSP51 showed positive results for biosurfactant production including foam production with highest foam height of 52% shown by BSP9. Similarly, for oil displacement, it was observed that only BSP9 and BSP51 were able to displace the layer of vegetable oil in petri plate. BSP9 showed maximum

displacement of 4.8 cm as compared to BSP51 with 2.5 cm of oil displacement. In CTAB assay, again BSP9 and BSP51 were the two isolates that gave positive results by producing blue zones around colonies suggesting the anionic nature of biosurfactant. Appearance of blue zone is due to the formation of insoluble complex between biosurfactant and methylene blue agar.

Emulsification activity (E_{24}) was also calculated using crude and vegetable oil, and it was revealed that BSP9 showed 59.95% and BSP51 showed 48% emulsification in presence of vegetable oil. In presence of crude oil emulsification was increased and BSP9 gave 71% (E_{24}) while BSP51 showed (E_{24}) of 55%. All other isolates did not show any (E_{24}) activity. Screening of biosurfactant production tests is shown in Fig. 12 and Table 6

4.5 Plant growth promoting characterization

All the isolates were checked for PGP characterization and it was observed that amongst all, BSP9 gave most promising results and hence was selected for further study. All the PGP characters of the selected 15 isolates are shown in Table 7

4.5.1 Phosphate solubilisation

Amongst all 15 isolates, BSP9, BSP51 and PMI9 gave positive results for phosphate solubilisation BSP9 showed maximum solubilisation index of 3.6 PSI followed by BSP51 with 2.8 PSI. Results of phosphate solubilisation of BSP 9 is shown in Fig. 13 (a) and Table 8

4.5.2 Zinc solubilzation

Out of 15 isolates, only three isolates BSP9, BSP51 and PMI9 were able to solubilize Zn as evident by clear zone around spot inoculation. BSP9 showed maximum zinc solubilisation having 4.66 ZSI followed by BSP51 with 3.2 ZSI (Fig. 13 b, Table8).

4.5.3. IAA production

10 out 15 rhizobacterial isolates gave positive result for IAA production and are shown in Table. Among them, BSP9 showed maximum yield for IAA i.e. 31.9 µg/ml of IAA Fig. 13c and Table 8 followed by BSP51 showing IAA production of 25.32 µg/ml.

4.5.4 Siderophore

Siderophore production on CAS agar was positively depicted by 2 out of 15 isolates namely BSP9 and BSP51 showing orange colour zone around spot inoculation Quantitative estimation of siderophore showed that maximum siderophore production was given by BSP9 with 44.74% psu of siderophore. BSP51, on the other hand showed 39.84 psu of siderophore (Fig. 13d, Table 8)

4.5.5 Hydrogen cyanide production

Qualitative analysis of HCN production revealed that only isolate PMI was able to produce HCN hence proving its potential as an antagonistic agent (Fig. 13e , Table 8)

4.5.5 Qualitative estimation of ACC deaminase activity

For ACC deaminase activity by isolates, the growth on DF medium with ACC as sole nitrogen source were checked. BSP9, alongwith 12 bacteria showed growth on both plates with ACC and ammonium sulphate as nitrogen sources as shown in Fig. 13f and Table 8

Biocontrol activity

Only BSP9 and BSP51 showed significant antagonistic activity against *A. brassicae*.. BSP9 was able to inhibit the fungus upto 70.44% while BSP51 showed inhibition of 61.11% (Fig. 13 (g and h) and Table 9).

4.6 Molecular identification of bacteria

On the basis of potent PGP characters, biosurfactant producing properties and biocontrol activity shown by BSP9, it was selected for molecular identification through Nucleotide-BLAST. The 16S rRNA sequence of the isolate was found to be clustered with *Pseudomonas putida* strain GQ-8 (Accession No. JX865419) and showed 99.6% similarity. The homology of the same was also evident from phylogenetic tree as shown in Fig. 14 The sequence data was submitted to GenBank with Accession No. LC489268. The strain was also submitted to National Agriculturally Important Microbial Culture Collection, an international culture collection center approved by International Depository Authority (IDA), with accession number *P. putida* NAIMCC-B-02326 (Table 10).

4.7 Production, extraction and purification of biosurfactant from BSP9

After production and extraction of biosurfactant from BSP9 using chloroform:methanol mixture and evaporating the organic layer, a dark brown coloured viscous residue was left behind weighing upto 2.5g/L (Fig. 15). This was the crude biosurfactant that was further subjected to purification through TLC analysis. After purification, it was observed that, two major spots were identified on TLC plate with retention factor Rf= 0.33 and Rf= 0.78 as shown in Fig. 16.

4.8.1 Structural characterization of the biosurfactant

(i) FT-IR

For structural characterization, first of all, FT-IR analysis was performed and from spectrum (Fig. 17), it was revealed that absorption bands ranging between 3650-3134 cm^{-1} indicates the presence of free -OH group arising as a result of H bonding of

polysachharides and/or –OH stretching of carboxylic acid. Two bands at wavenumber 1748 cm^{-1} and 1712 cm^{-1} denotes –C=O stretching arising from ester groups of lipids and fatty acids (-C=O in COOH). Wavenumber at 1396 cm^{-1} signifies C-H and OH deformation vibrations of carbohydrates. The range of spectra below 1000 cm−1 represents C-H, C-O and CH3 vibrations (Table 11)

(ii) LC-MS

On the other hand, result of LC-MS analysis (Fig. 18) showed that peak at m/z 529.3 with retention time 11.75 min corresponds to Rha-C12:1-C10, peaks at m/z 532.3 and 576.4 with retention time 12.49 min corresponds to Rha-C10-C12/Rha-C12-C10 and Rha-C10-C14:1/Rha-C12-C12:1, respectively. Peak at m/z 557.5 with retention time 13.24 min shows presence of Rha- C12-C12:1/Rha- C12:1-C12 congener while at m/z 503.4 with retention time 15 min, the peak reveals the presence of Rha-C10-C10. Lastly, peak at m/z 621.5 with retention time12.49 corresponds to Rha-Rha-C8-C10 or Rha-Rha-C10-C8 (Table 12)

4.8.2 SEM-EDS

The SEM-EDS analysis of the biosurfactant showed the presence of carbon, oxygen, sodium, chlorine and potassium in ratio of 67.14, 30.73, 1.02, 0.29, 0.62, and 0.19 %. in the scanned area. High amount of carbon and oxygen percentage in the biosurfactant confirms the presence of carbohydrate and lipid moieties in the sample (Fig. 19).

4.9.1 In vitro antifungal activity of purified metabolite against *A. brassicae*

Antifungal activity of purified rhamnolipid produced by BSP9 was determined by zone of inhibition against *A. brassicae* growth on PDA after 5 days of incubation.

Highest zone of inhibition was calculated around concentration 5% and 2% with nearly same values i.e 3.9 cm and 3.6 cm (Fig. 20). Rhamnolipid with concentration of 1% also showed zone of inhibition of 1.5 cm against fungi around the wells as compared to control. Least reduction was shown by 0.1% rhmanolipid concentration with only 0.5 cm of zone of inhibition.

4.9.2. SEM analysis of *A. brassicae* treated with rhamnolipid

Structural abnormality, hyphal coiling or mycelial deformity induced by purified metabolite produced by bacterial isolate were examined under SEM. Fig. 21 shows the disintegration in fungal hyphae treated with metabolite at places denoted by arrow.

4.10 Effect of biosurfactant and BSP9 on mustard growth, yield and inhibition of Alternaria blight caused by *A. brassicae*

4.10.1. Preparation of talc-based bioformulations

Talc based bioformulation were developed using isolate BSP9 amended with biosurfactant in various concentrations (0.1%, 1%, 2% and 5%) under aseptic conditions. These treatment were applied for pot and field study. However, in pot study fungal spore inoculation was also introduced to check the biocontrol activity of the treatments while during field trial PGP attributes of the bioformulations were analysed (both for two consecutive years) (Fig. 22)

(a)Pot study

The physicochemical analysis of the soil used in pot study revealed that the soil had pH=7.5, EC 1.5 dS/m, moderate in organic matter with 5.89 g/Kg, available nitrogen (N) 150.3kg/ha, phosphorous (P) as 18.0kg/ ha and available potassium (K) as 150kg/ha Under in vivo conditions, biocontrol activity and plant growth promoting

attributes of BSP9 and its biosurfactant was assessed (Fig. 23). Percent of disease incidence, plant growth parameters and phytochemicals were analysed (Table 13). Pot study showed that there was a gradual decrease in the incidence of disease caused by *A brassicae* with increase in concentration of biosurfactant as compared to negative control C2. The most notable reduction in disease incidence was observed in plants treated with 2% biosurfactant+BSP9 *P. putida* (11.12%) followed by 1% biosurfactant+BSP9 and only BSP9 treated plants (22.21% for both treatments). Treated plants also showed significant difference in germination rate as compared to control (negative and untreated). An increasing trend was observed in each treatment with increase in biosurfactant concentration. In treatments amended only with biosurfactant, the rate of germination was highest in 2% and 5% (T3 and T4) 68.20% and 60.47% respectively as compared to control. In case of addition of bacterial cells BSP9, an improved germination rate of 78.42% was achieved by plants with treatment having 2% biosurfactant+BSP9 in presence of *A brassicae* i.e. treatment T6, followed by T5 (1%biosurfactant+BSP9+*A. brassicae*) with 75.25% germination rate (Table 14.

Checking the growth parameters of mustard, it was observed that significant improvement was observed in all growth parameters of plants treated with treatment T6 (BSP9+2% biosurfactant + fungus) In case of root length, an increase of 273% was observed in plants treated with treatment T6 as compared to only fungus inoculated negative control C1. While with untreated control C2, the same treatment showed an increment of 121% in root length. In the same way, BSP9 plus 2% biosurfactant in presence of fungus (treatment T6) brought about 72% and 20% increment in shoot length as compared C1 (negative control) and C2 (untreated control) respectively. Highest values in fresh weight content (70%) and dry weight (453%) was also observed in T6 treatment when compared to negative control C1. On

the other hand, in case of untreated control C2, 59% increase for fresh weight and 266% for dry weight was displayed. Number of pods in plants treated with T6 treatment were highest (241%) than C1 (negative control) and 72% more than untreated control (C2). An increase of 14 fold was observed for 1000 seed weight as compared to negative control and 5.5 fold increase was shown when compared to untreated control. All the data of growth parameters of mustard under pot trial are shown in Table 14. It was also observed that in case of higher concentration of biosurfactant, i.e. 5%, values of all growth parameters slightly dipped and hence further concentration was not checked. Least values were observed in case of plants treated with 0.1% biosurfactant.

In case of phytochemicals (Fig. 24), maximum chlorophyll (468%) and flavonoid (147%) content was recorded in T6 treatment (BSP9+2% biosurfactant+ *A.brassicae*) as compared to *A. brassicae* infested control (C1). When compared to untreated control C2, an increment of 112% was observed for chlorophyll content and for flavonoid there was an increase of 100%. Similar trend was noted in case of phenolic and protein content where all the treated plants showed a linear spike in values of these phytochemicals. Highest increase in phenolic and protein content was again shown by combination of 2% bacteria and metabolite (T6) with 186% and 589%respectively when compared to negative control C1. All the data of the phytochemical parameters of mustard under pot trial are shown in Table 15

In case of defence related enzymes, an increase in concentration of PPO, PO and PAL enzyme were noted in plants treated with combination of bacteria and metabolite. In treatment 2%biosurfactant+BSP9 challenged by *A. brassicae*, 508%, 536% and 47% higher activity of PPO PO and PAL were recorded than their respective negative controls (C1). Overall, all treated plants showed a significant increase as compared to control counterparts (Table 15)

(b) Field study

To check the efficacy of rhamnolipids and BSP9 as a bioinoculant using nine different treatments which included metabolite (purified biosurfactant), only cells (BSP9), combination of both (biosurfactant and cells of BSP9) and an untreated control The soil of the field site had pH=7.5, EC 1.5 dS/m, moderate in organic matter with 5.4 g/Kg, available nitrogen (N) 148.3kg/ha, phosphorous (P) as 18.2kg/ ha and available potassium (K) as 153kg/ha. The field trial revealed that significant enhancement in germination rate (82.3%), root length (100.8%), shoot length (81.0%), fresh weight (66.3%), dry weight (79.0%), number of pods (69.3%), oil content (28.6%) and phytochemical contents i.e chlorophyll (94.2%) and flavonoid (112.1%) was observed in plants treated with 2% biosurfactant plus BSP9 with reference to control (Fig. 26). The results of 5% biosurfactant plus BSP9 were significantly similar hence not shown. Results of growth parameters of treatment containing only BSP9 cells also showed significant improvement but were lower as compared to biosurfactant and BSP9 together. Interestingly, when only biosurfactant was used in the bioformulation, an increasing trend in growth parameters was observed with increase in its concentration (0.1%, 1% and 2%) and the values dipped after 2% increase, although slightly. All the data of the growth and phytochemical parameters of mustard under field trial are shown in Table 16

4.10.2 Statistical analysis and Heatmap clustering

Statistical analysis of all the growth parameters of *B. juncea* in both pot and field is shown in Table 14, 15 and 16.

Heatmap generated to determine distance and clustering and showing the effect of biosurfactant and BSP9 on various growth parameters of Indian Mustard (Distance measure using Euclidean and clustering algorithm using Ward's method). In case of

pot trial (Fig. 27 a), the heatmap generated to determine the hierarchy of treatments through Euclidean and clustering algorithm using Ward's method. The effect of various treatments on growth of mustard was analyzed through dendrogram and compared to annotate the best treatment. From the dendrograms it was noted that the treatments were divided into two main classes: one showing no or almost negligible stimulation in plant growth including fungal treated plants (C1), untreated plants (C2) and 0.1% biosurfactant treated plants; the other class included plants treated with higher concentration of biosurfactant and BSP9 alone and in combination. Only fungus infected plants were showing least plant growth parameters while among the treated plants, 0.1% biosurfactant (T1) was nearing the effect of untreated sets. The second class of treated plants analyzed through dendrograms highlighted that in first subclass best treatments were categorized including 2% biosurfactant plus BSP9 (T6) as the most outstanding treatment and the following treatments were 1% biosurfactant plus BSP9 (T5) and only BSP9 (T7). The latter two treatments were closely clustered showing similar effect on growth and stress mitigating properties of mustard. Similarly, 2% biosurfactant (T3) and 5% biosurfactant (T4) treated plants were showing insignificant difference and were assigned in subclass (subclass of treated plants) of lesser effective treatments along with 1% biosurfactant (T2).

Similarly, dendrogram generated from field trial values revealed that there are two major classifications, one including the untreated control, 0.1% and 1% biosurfactant treated plants with least growth parameters. The other major classification included higher concentration of BS (2% and 5%), bacteria only and metabolite and bacterial combination treatments. The treatments further showed two class of variations, first the combination (1% and 2% BS with BSP9) applied to plants with highest impact on all growth parameters and second, bacteria alone and higher concentrations of metabolite (2% and 5%) application with comparatively lesser impact (Fig. 27 b)

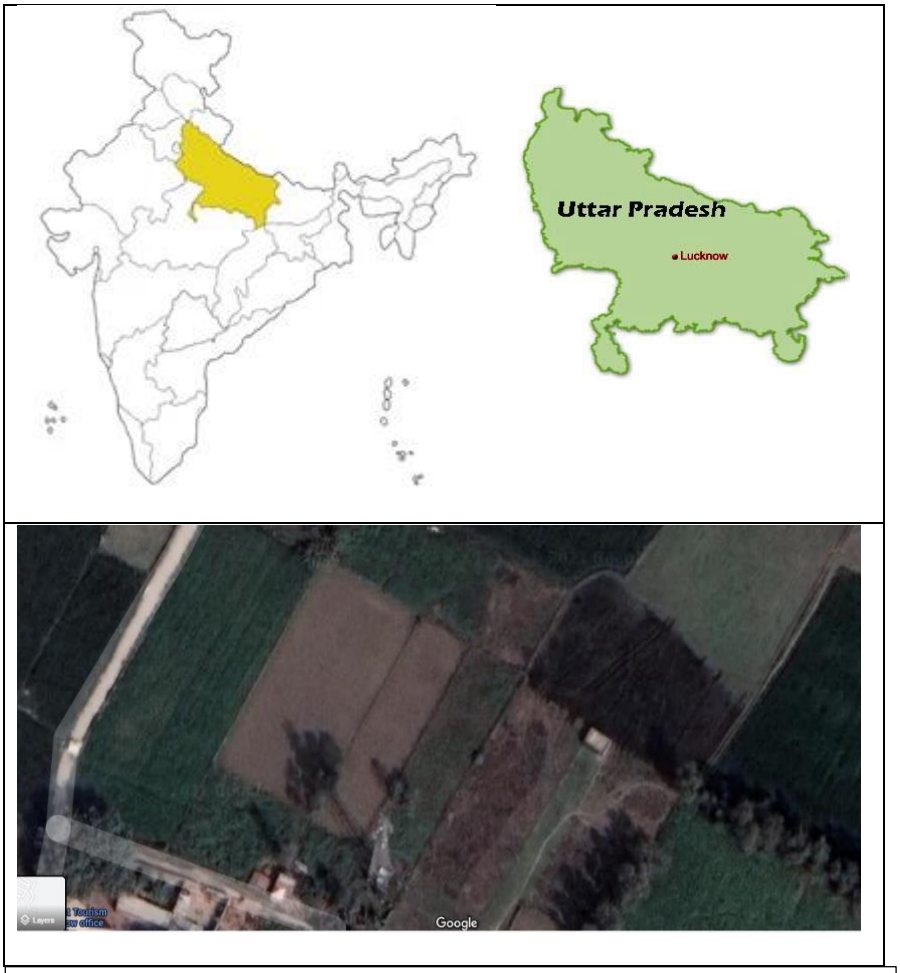


Fig. 7 Sampling site of Lucknow region, Uttar Pradesh, India (26.84° N, 80.9462° E)

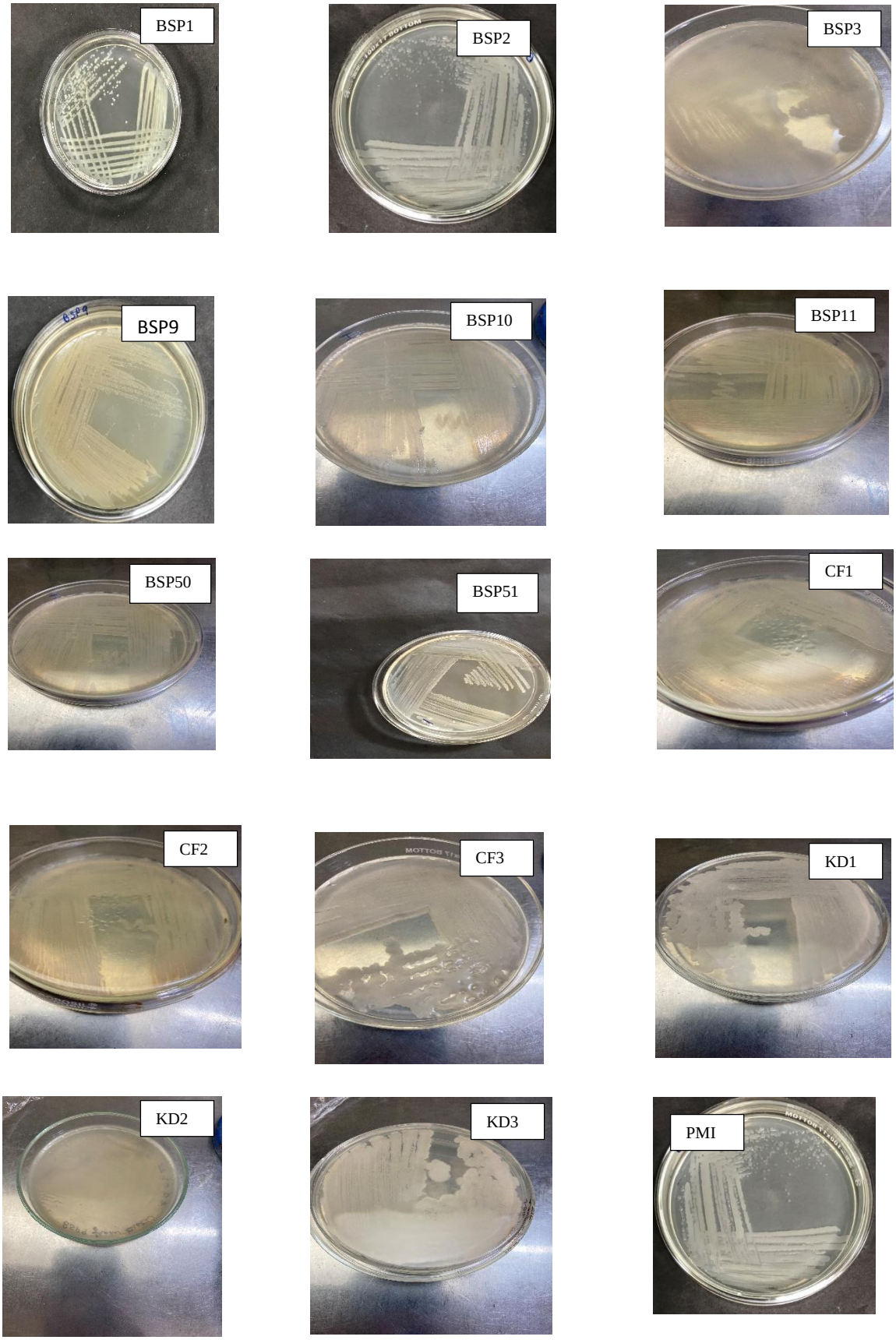
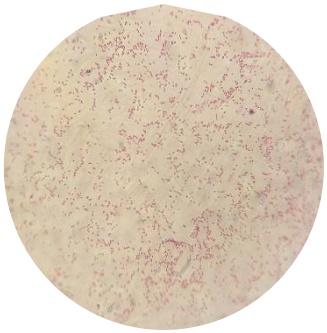


Fig. 8 Growth of selected isolates on Nutrient agar media



Gram Negative bacteria with pale pink colonies

Fig. 9 Gram staining of bacterial isolates

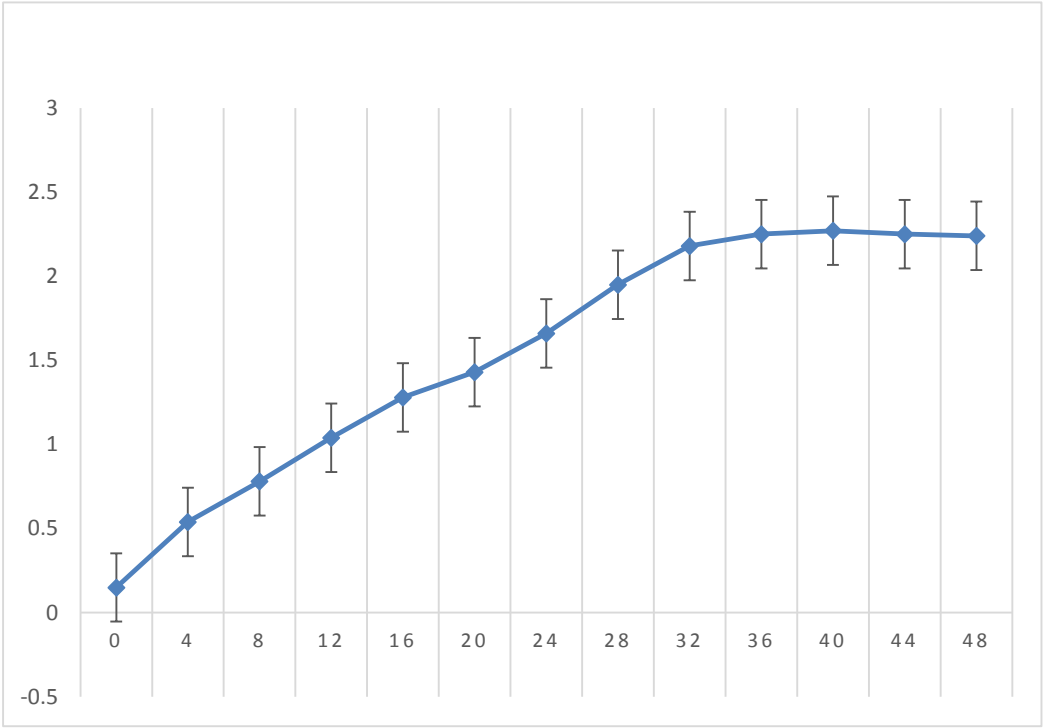
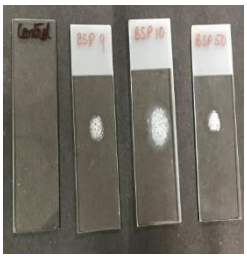
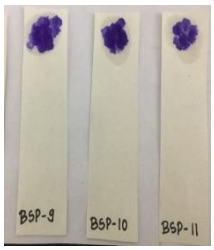


Fig. 10 Growth curve of isolate BSP9

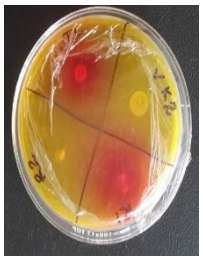
Data are represented as mean values (n = 3); error bars represent standard deviation



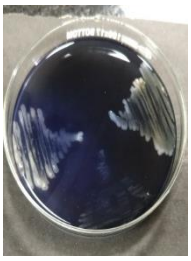
(a) Catalase



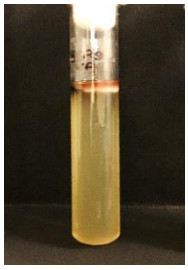
(b)Oxidase



(c) Urease

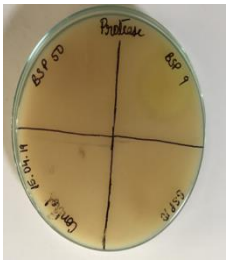


(d) Amylase

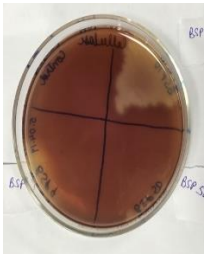


(e) Gelatinase

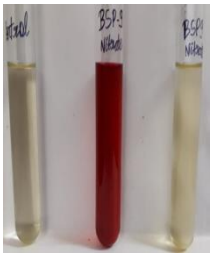
(f) Lipase



(g) Protease

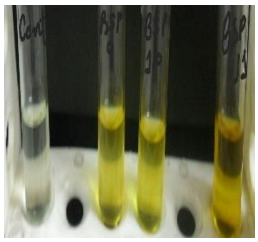


(h) Cellulase

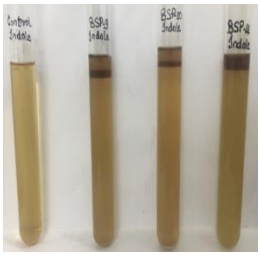


(i) Nitrate reductase

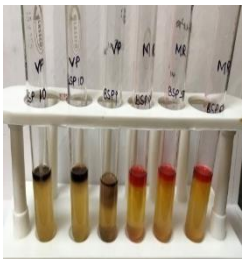
(j)Citrate utilization



(k) Ammonia

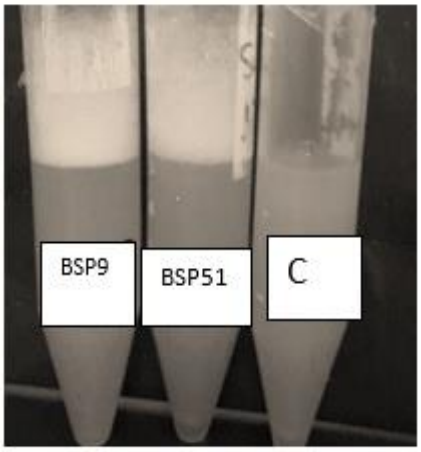


(l) Indole

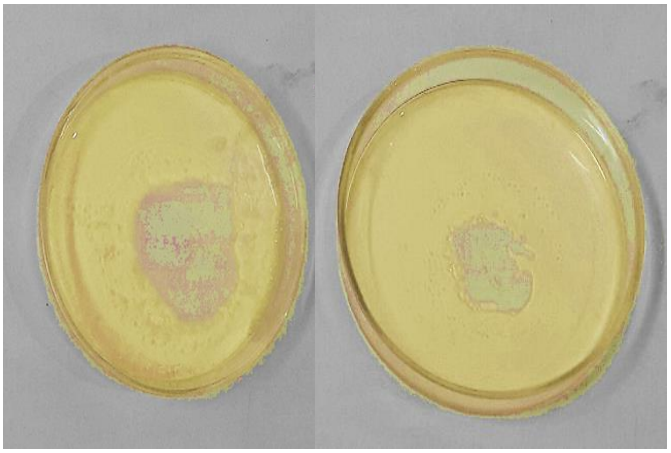


(m) MR-VP

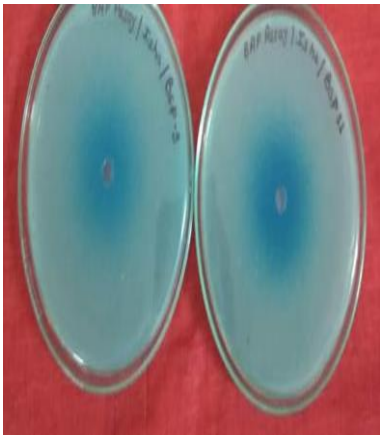
Fig. 11 Biochemical characterization of selected isolated bacteria



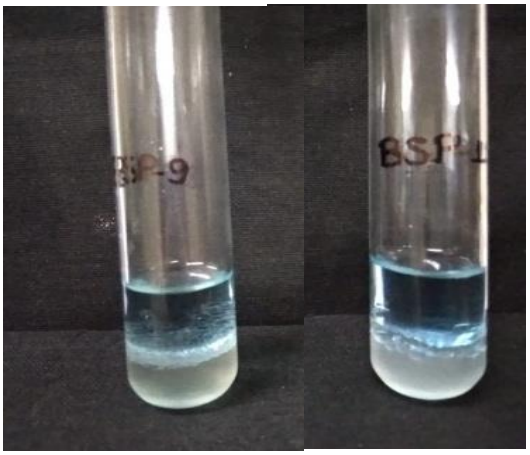
(A) Foam Production



(B) Oil displacement test

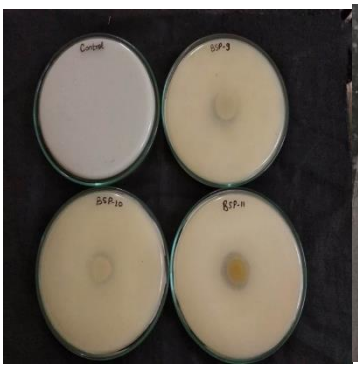


(c) CTAB assay

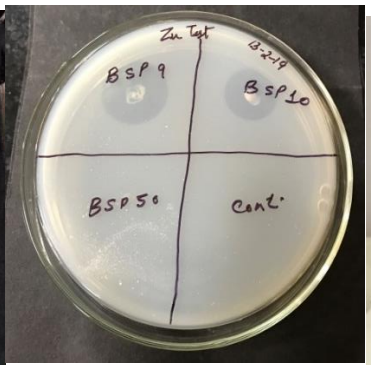


(D) Emulsification activity E_{24}

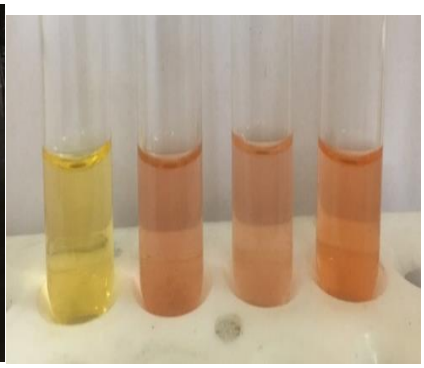
Fig. 12 Screening tests for biosurfactant production



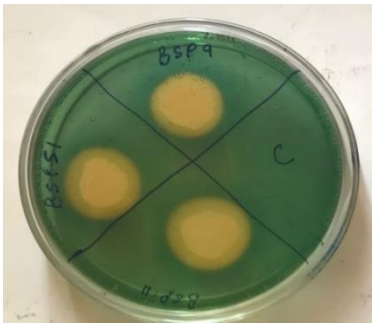
(a)Phosphate solubilization

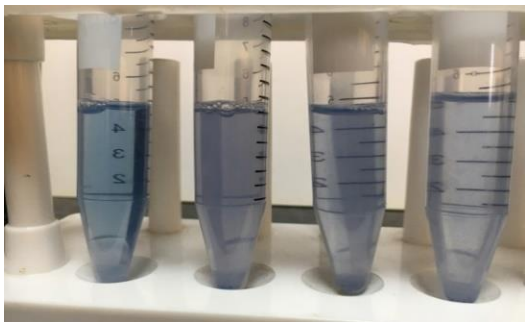


(b) Zinc solubilization

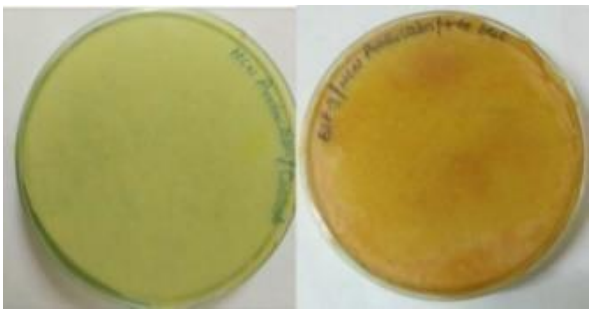


(c) IAA production

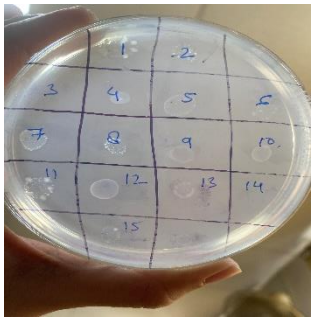




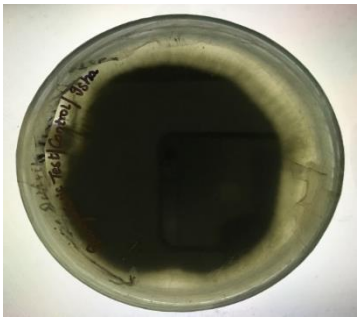
(d) Siderophore production



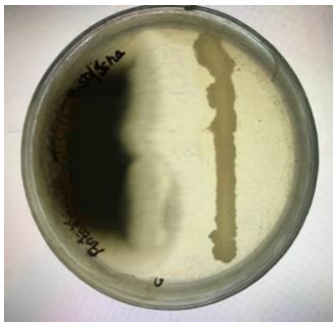
(e) HCN production



(f) ACC deaminase activity



(g) Control of *A.brassicae*



(h) Dual culture assay using BSP9 against

Biocontrol Activity

Fig. 13 PGP characterization of the isolates

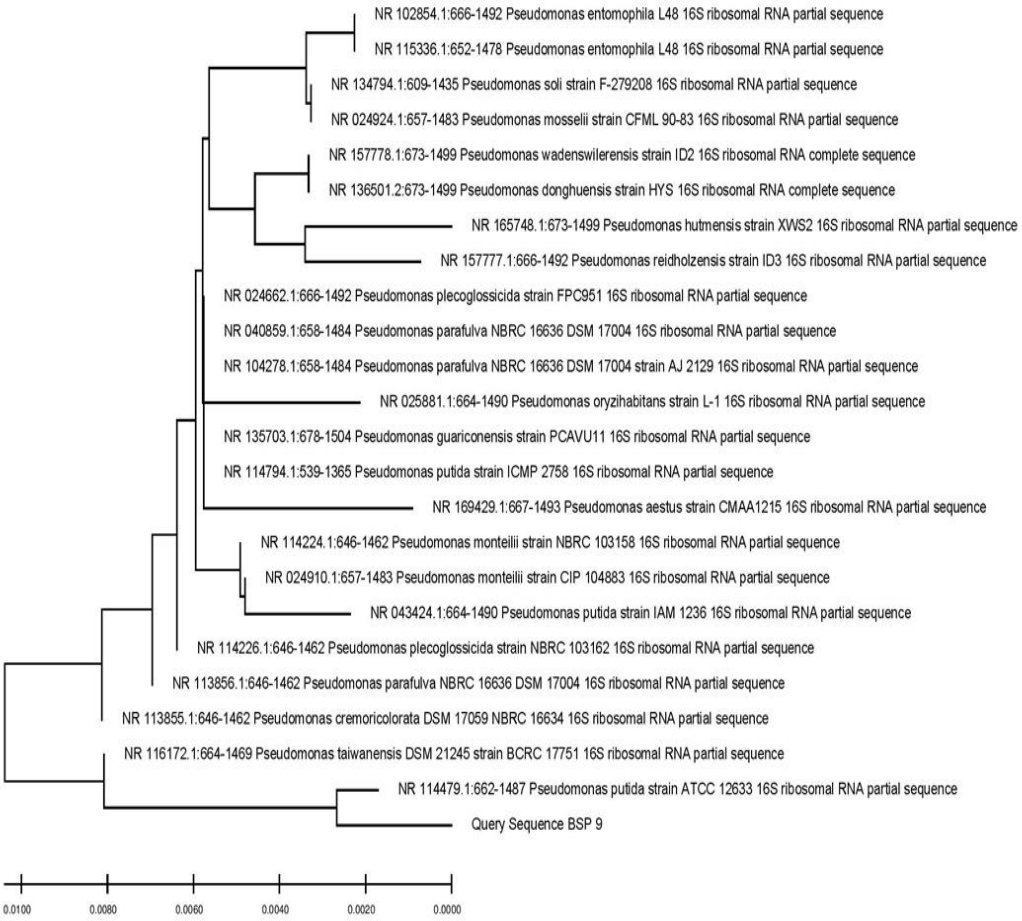
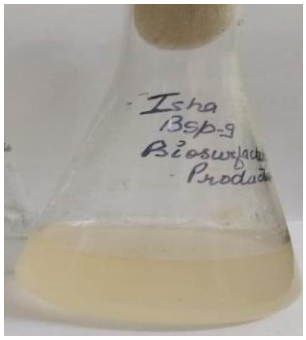
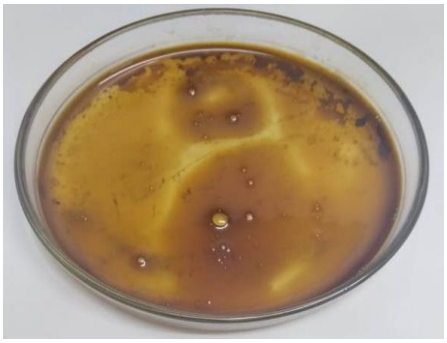


Fig. 14 Phylogenetic tree analysis of isolate BSP9





2.5 g/L of crude biosurfactant

Fig. 15 Production of biosurfactant by BSP9 isolate at labscale

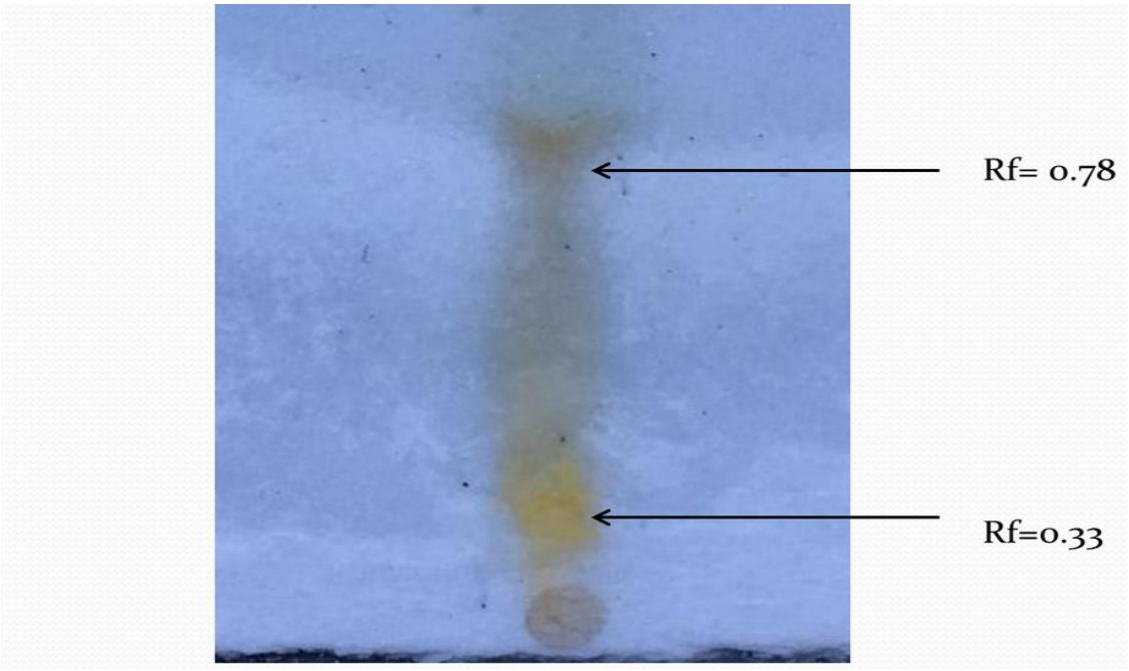


Fig. 16 Purification of the biosurfactant through TLC method

Where TLC plate shows two major spots with retention factor Rf 0.78 and 0.33 confirming presence of glycolipids

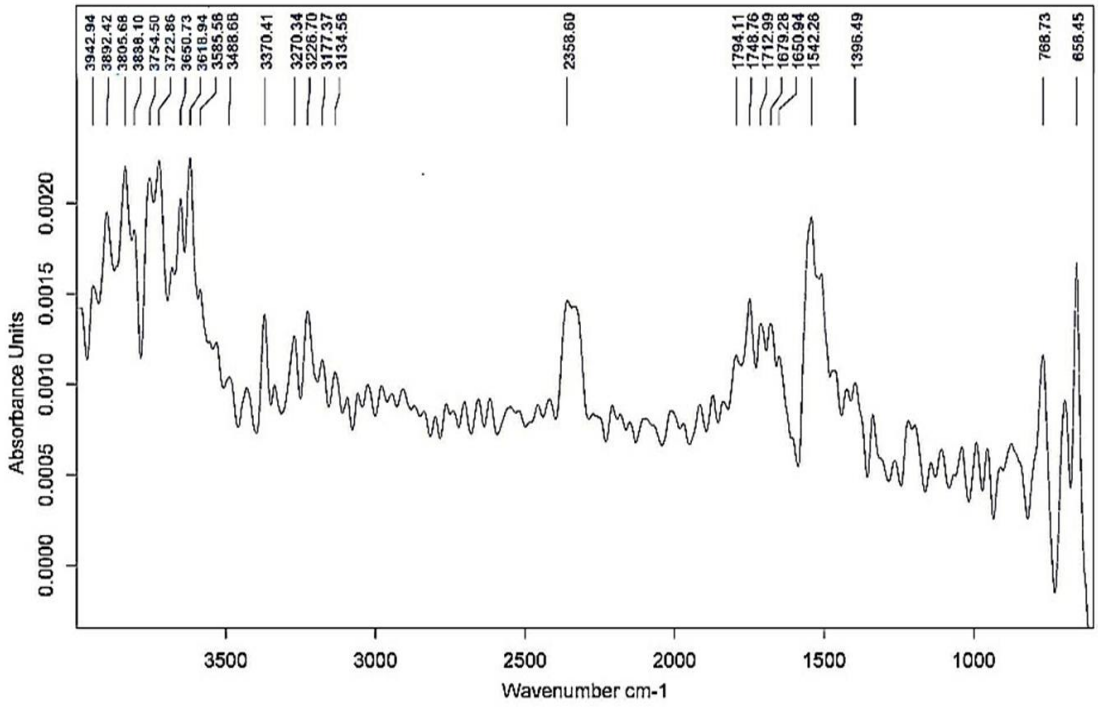
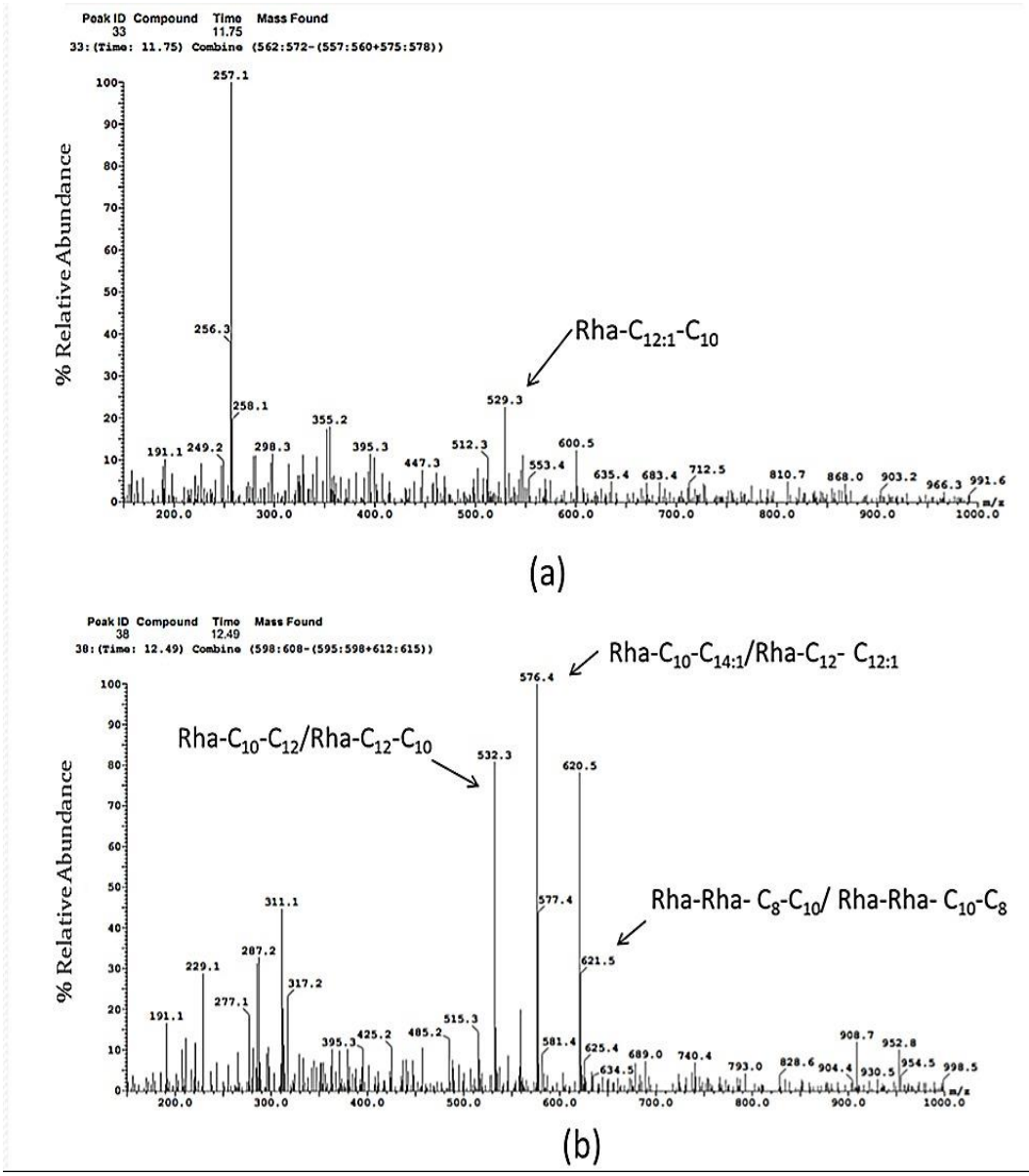


Fig. 17 FTIR spectrum of the functional groups present in purified biosurfactant produced from BSP9


Peak ID Compound Time Mass Found
33 11.75
33:(Time: 11.75) Combine (562:572-(557:560+575:578))
% Relative Abundance
Rha-C12:1-C10
(a)
Peak ID Compound Time Mass Found
38 12.49
38:(Time: 12.49) Combine (598:608-(595:598+612:615))
% Relative Abundance
Rha-C10-C14:1/Rha-C12- C12:1
Rha-C10-C12/Rha-C12-C10
Rha-Rha- C8-C10/ Rha-Rha- C10-C8
(b)

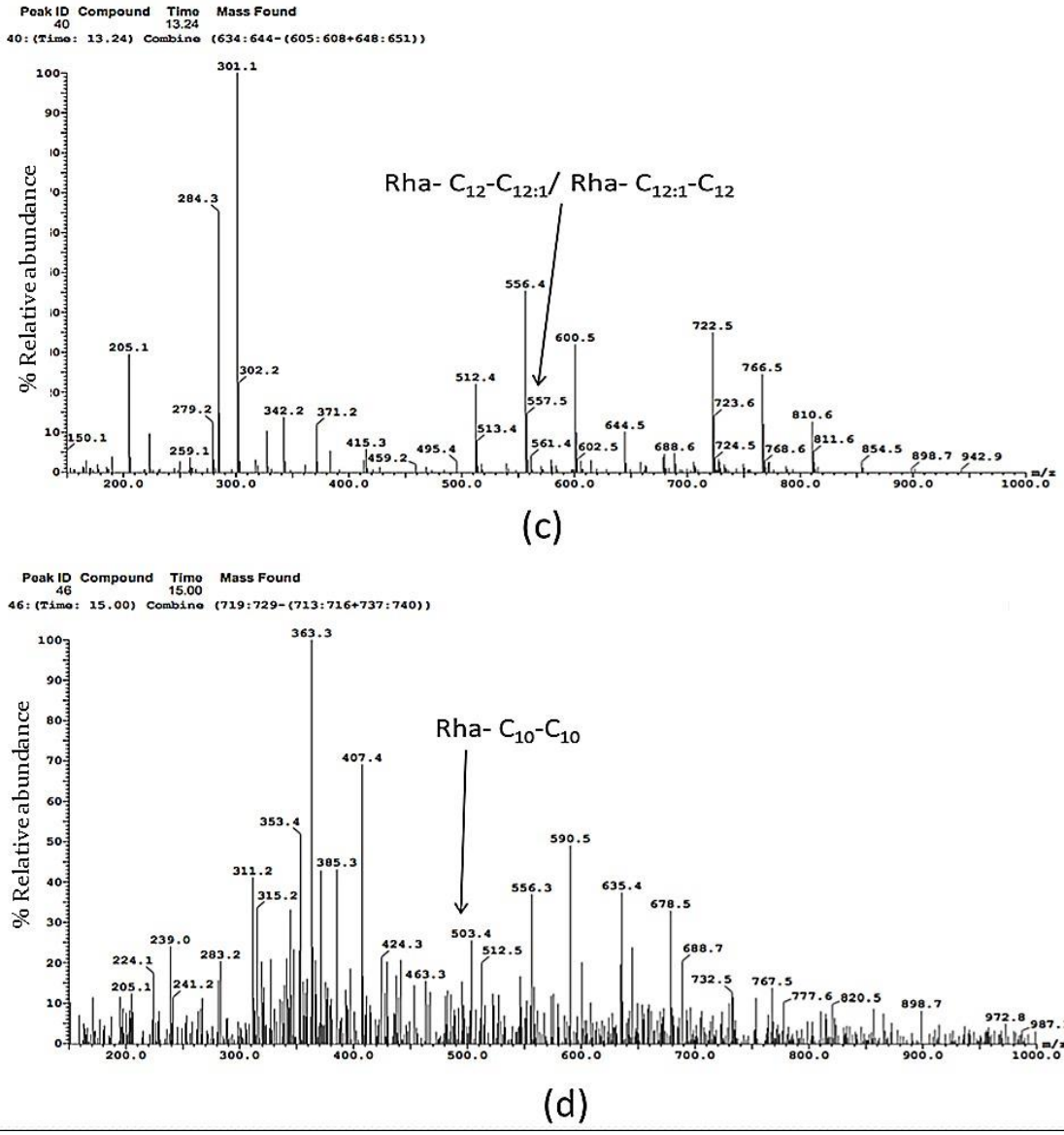


Fig. 18 LC-MS analysis of the biosurfactant produced from BSP9 showing specific rhamnose congeners.

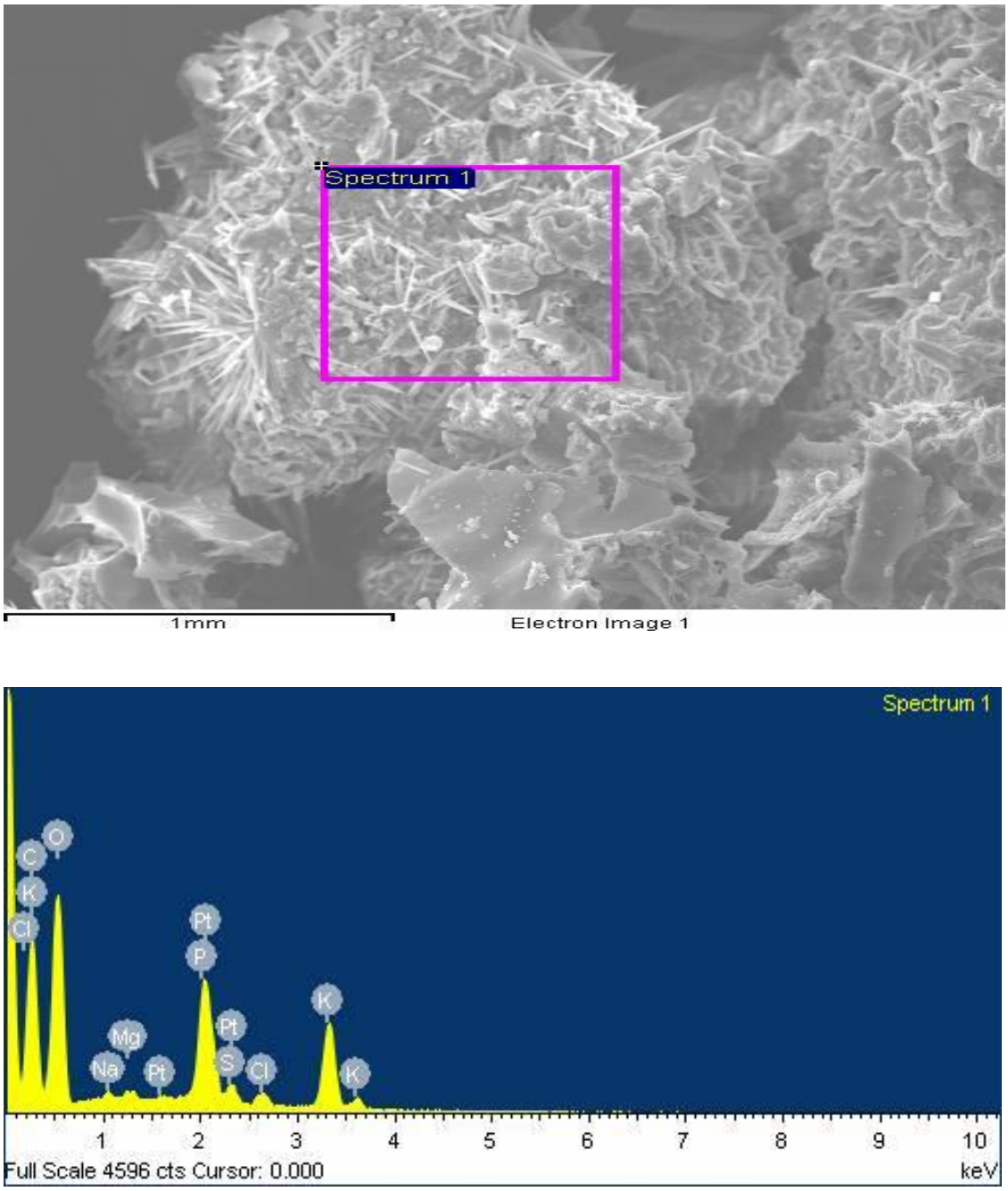


Fig. 19 SEM-EDS analysis illustrating the elementary composition of extracted and purified biosurfactant

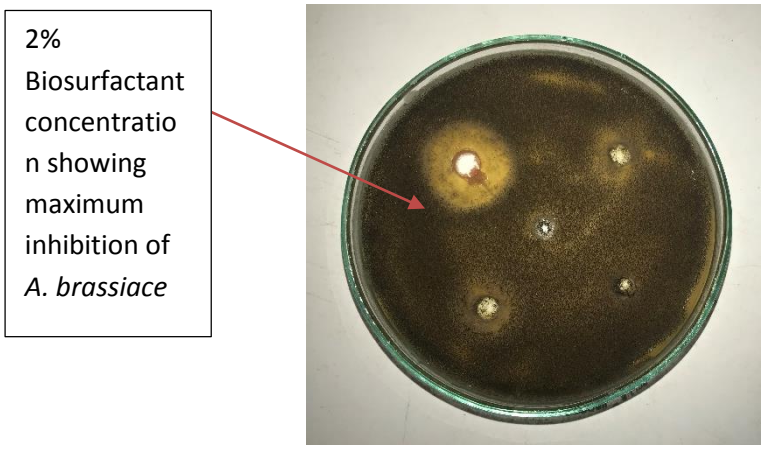
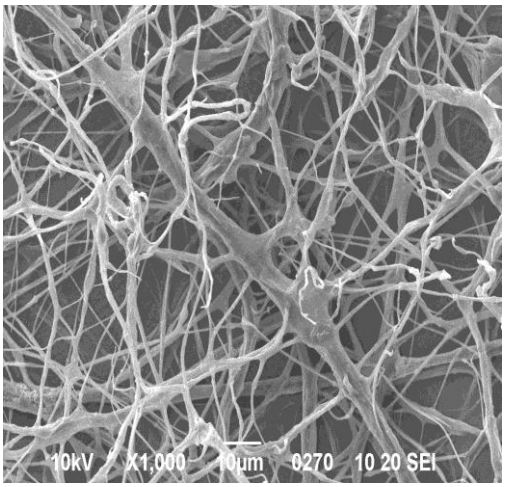
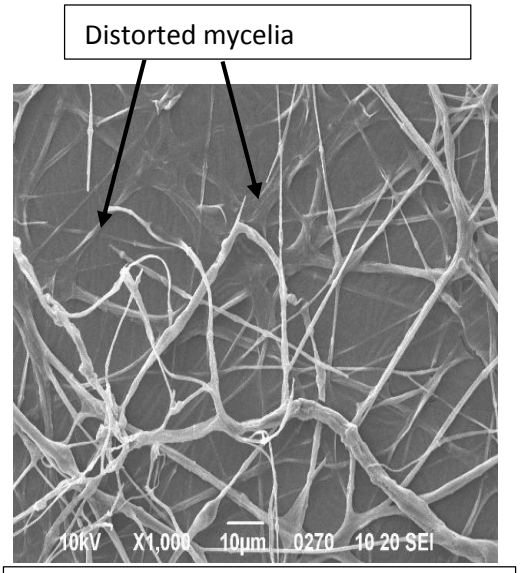


Fig. 20 In vitro Antifungal activity of purified biosurfactant against *A.brassicae* using agar well diffusion method



(b) Untreated Control of *A. brassicae*



(c) Effect of biosurfactant on *A. brassica* mycelia

Fig. 21 SEM analysis of *A. brassiace* showing distortion in mycelia due to interaction of purified biosurfactant

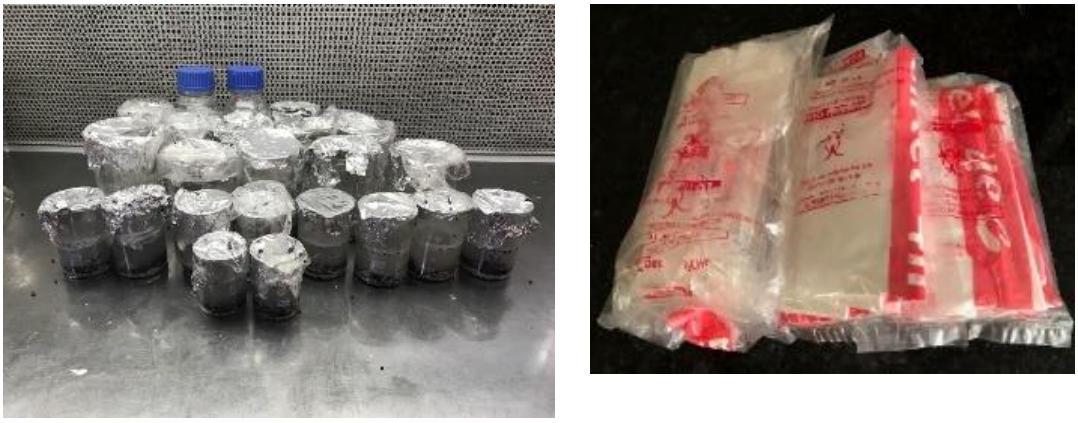


Fig. 22 Development of talc based bioformulations at labscale

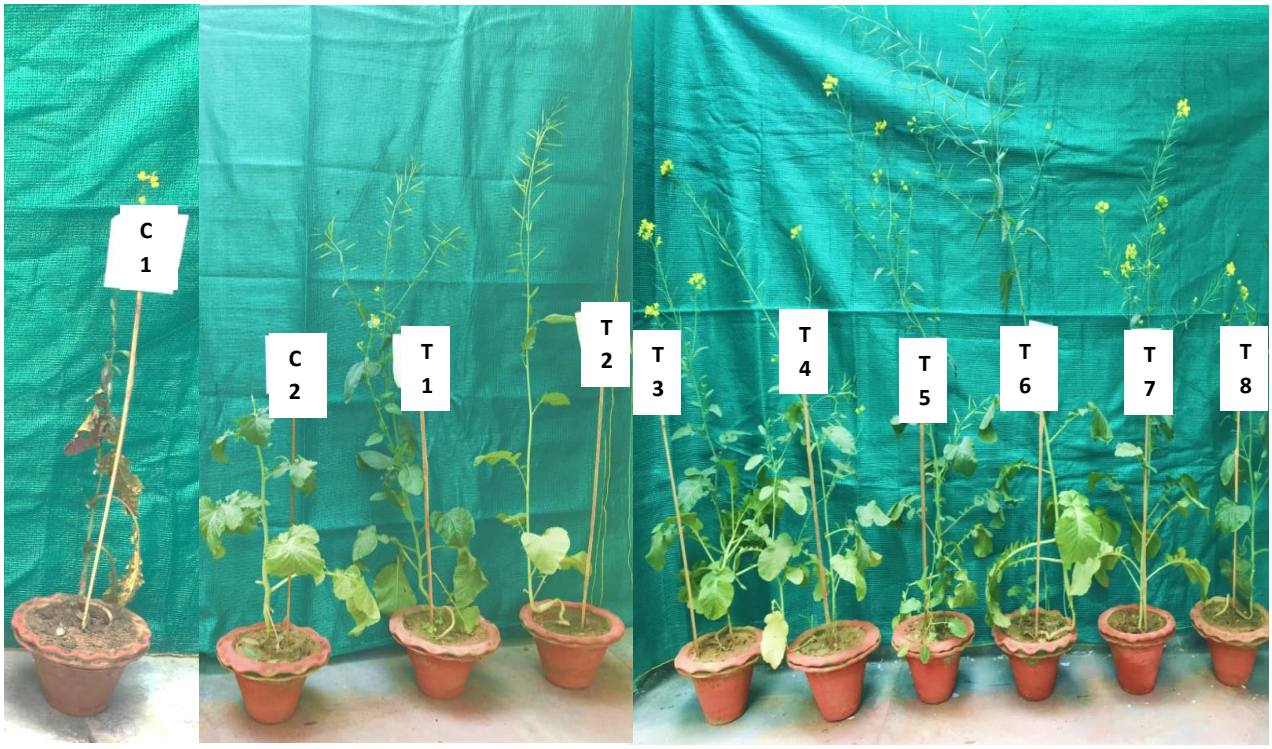
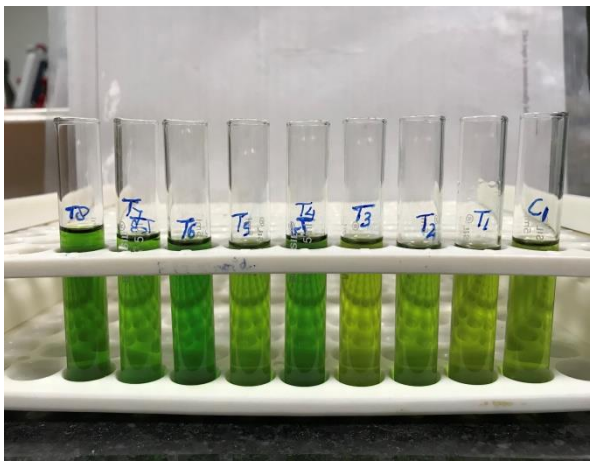
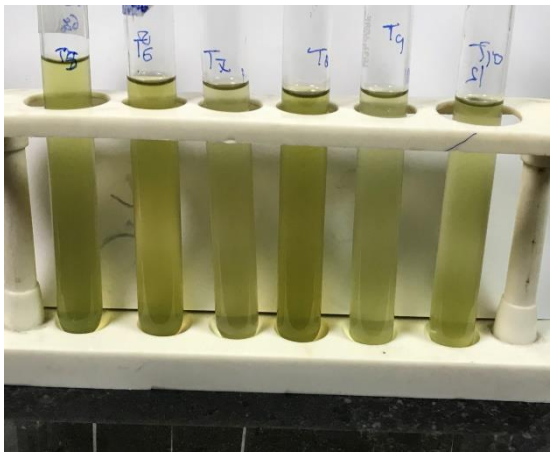


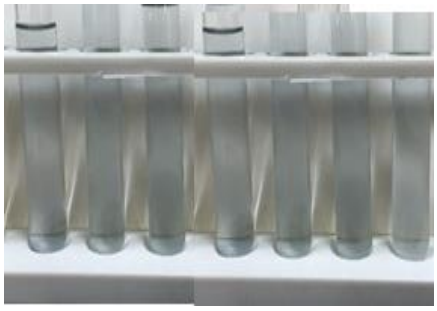
Fig. 23 Effect of various concentrations of biosurfactant, isolate BSP9 (alone) and in combination with its biosurfactant, on growth of Mustard plants (pot study)



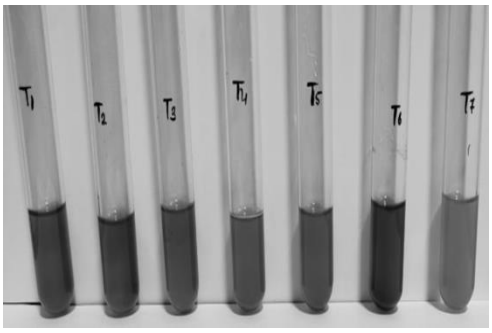
Chlorophyll content



Flavonoid content



Phenolic content



Protein content

Fig. 24 Phytochemical analysis of the plants treated with bioformulations

Fig. 25 Experimental field, block preparation, seed sowing and yield of mustard crops

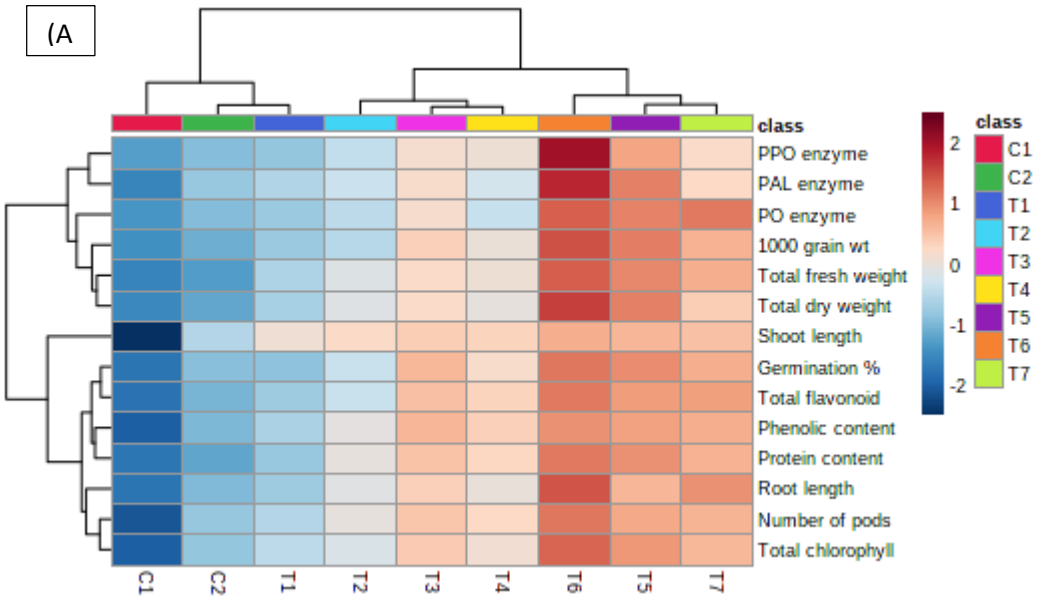
Plants treated with 2% biosurfactant alone

Plants treated with 2% biosurfactant+BSP9a

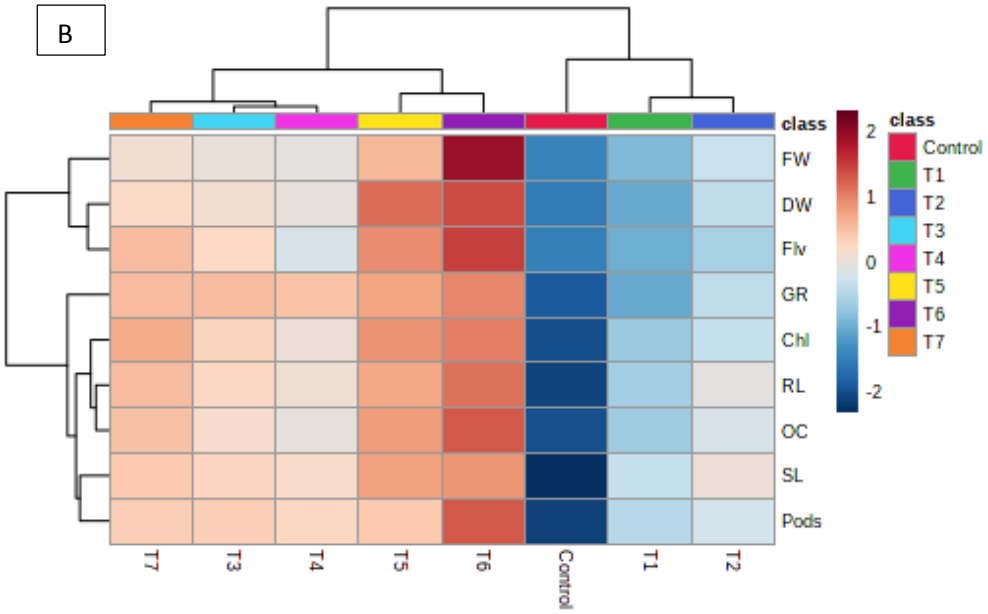
Plants treated with only BSP9

Untreated control

Fig. 26 Effect of various concentrations of biosurfactant , isolate BSP9 (alone) and in combination with its biosurfactant on growth of *B. juncea* plants



where, C1- negative control with only *A. brassicae,* C2-untreated control**,** T1- 0.1% biosurfactant + *A. brassicae* (B.S), T2- 1.0 % B.S+ *A. brassicae*, T3- 2.0 % B.S+ *A. brassicae*, T4- 5.0 % B.S+ *A. brassicae*, T5- 1% B.S + BSP9+ *A. brassicae* , T6-2% B.S +BSP9+ *A. brassicae*, T7- only BSP9 cells + *A. brassicae*



where, T1- 0.1% biosurfactant (B.S), T2- 1.0 % B.S, T3- 2.0 % B.S, T4- 5.0 % B.S, T5- 1% B.S + BSP9, T6-2% B.S +BSP9, T7- only BSP9 cells and Control = untreated control, FW- fresh weight, DW- dry weight, Chl- chlorophyll content, Flv- flavonoid content, GR- germination rate, SL- shoot length, Pods- Number of pods, RL- root length, OC- oil content

Fig. 27 Heatmap generated to determine distance and clustering and showing the effect of biosurfactant and BSP9 on various growth and phytochemical parameters of Indian Mustard (pot and field study) (Distance measure using Euclidean and clustering algorithm using Ward's method).

Table 1 Phenotypic characterization of isolates

Isolates	Generation time (hours)	Motility	Appearance	Microscopic morphology	
				Gram nature	Shape
Bsp1	2.30	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
BSP2	2.20	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
BSP3	4.50	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
BSP9	4.66	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
BSP10	6.10	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
BSP11	4.10	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
BSP50	4.50	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
BSP51	4.70	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
CF1	5.10	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
CF2	4.50	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
CF3	4.96	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
KD1	5.80	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
KD2	2.40	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
KD3	6.23	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod
PMI9	4.50	+	Shiny, Smooth, Convex with entire Margin	Negative	Rod

+ indicates positive result

Table 2 Growth pattern of isolates at various pH, temperature and NaCl Concentration

Isolates	pH variants					Temperature (° C)					Salt Tolerance Assay (%)			
	4	6	8	10	12	5	15	25	35	40	2	4	6	8
BSP1	-	+	+	+	-	-	+	+	+	-	-	-	-	-
BSP2	-	+	+	+	-	-	+	+	+	+	-	-	-	-
BSP3	-	+	+	+	-	-					-	-	-	-
BSP9	-	+	+	+	-	-	+	+	+	-	+	+	+	-
BSP10	-	+	+	+	-	-	+	+	+	-	-	-	-	-
BSP11	-	+	+	+	-	-	+	+	+	-	-	-	-	-
BSP50	-	+	+	+	-	-	+	+	+	-	-	-	-	-
BSP51	-	+	+	+	-	-	+	+	+	-	+	+	+	-
CF1	-	+	+	+	-	-	+	+	+	-	-	-	-	-
CF2	-	+	+	+	-	-	+	+	+	-	-	-	-	-
CF3	-	+	+	+	-	-	+	+	+	-	-	-	-	-
KD1	-	+	+	+	-	-	+	+	+	-	-	-	-	-
KD2	-	+	+	+	-	-	+	+	+	-	-	-	-	-
KD3	-	+	+	+	-	-	+	+	+	-	-	-	-	-
PMI9	+	+	+	+	+	-	+	+	+	+	+	+	+	+

+ indicates growth, - indicates no growth

Table 3 Different carbon sources utilized by isolates

S.No	Isolates	Carbon sources												
		Mannitol	**Glucose**	**Dextrose**	**Lactose**	**Sucrose**	**Galactose**	**Maltose**	**Starch**	**Citrate**	**Malate**	**Trehalose**	**Fructose**	**Glycerol**
1	BSP1	-	+	+	-	+	-	+	-	-	-	+	+	-
2	BSP2	+	+	+	-	-	-	-	-	-	-	-	-	+
3	BSP3	+	+	-	+	+	+	-	-	+	+	-	+	-
4	BSP9	+	+	-	+	+	+	-	-	+	+	-	+	+
5	BSP10	+	+	+	-	-	-	-	-	-	-	-	-	+
6	BSP11	+	+	-	+	+	+	-	-	+	+	-	+	+
7	BSP50	+	+	+	-	+	-	-	-	-	-	-	-	+
8	BSP51	+	+	-	+	+	+	-	-	+	+	-	+	+
9	CF1	+	+	-	+	-	+	-	-	+	+	-	+	+
10	CF2	+	+	+	-	+	-	+	-	-	-	+	+	+
11	CF3	+	+	-	+	+	+	-	-	+	+	-	+	+
12	KD1	+	+	-	-	+	+	+	-	-	+	-	+	-
13	KD2	+	+	-	-	+	+	+	-	-	+	-	+	-
14	KD3	+	+	-	+	+	+	-	-	+	+	-	+	+
15	PMI9	+	+	-	-	+	+	+	-	-	+	-	+	+

+ indicates utilized

- indicates not utilized

Table 4 Different nitrogen sources utilized by isolates

S.No	Isolates	Nitrogen sources										
		yeast extract	**Potassium nitrate (KNO3)**	**Sodium nitrate (NaNO3)**	**Tryptophan**	**Ammonium Chloride (NH4CI)**	**Glycine**	**Ammonium sulphate (NH4SO4)**	**Lysine**	**Glutamine**	**Cysteine**	**Methionine**
1	BSP1	+	-	-	+	+	-	+	+	+	-	-
2	BSP2	+	+	+	-	+	-	+	-	-	-	
3	BSP3	+	-	+	-	-	+		+	+	-	-
4	BSP9	+	+	+	+	+	-	+	+	+	+	+
5	BSP10	+	+	-	-	+	+	-	-	+	-	-
6	BSP11	+	-	+	-	-	-	-	+	-	-	-
7	BSP50	+	-	+	-	+	+	+	-	-	-	-
8	BSP51	+	+	+	+	+	-	+	+	+	+	
9	CF1	+	-	-	+	+	-	+	+	-	-	-
10	CF2	+	-	-	+	-	+	-	-	-	-	-
11	CF3	+	+	-	+	+	-	+	+	-	-	-
12	KD1	+	+	+	+	-	+	-	+	-	-	-
13	KD2	+	-	+	+	-	+	-	+	-	-	-
14	KD3	+	+	+	+	+	-	+	+	+	-	-
15	PMI9	+	+	+	+	-	-	+	-	-	+	+

+ indicates utilized

- indicates not utilized

Table 5 Biochemical characterization of the isolates

Isolates	Catalase	Oxidase	Urease	Amylase	Gelatinase	Lipase	Protease	Cellulase	Nitrate Reductase	Citrate utilization	Ammonia	Indole	MR-VP
BSP1	+	+	+	-	+	+	+	-	+	+	+	-	-
BSP2	-	-	+	+	+	-	+	-	+	+	+	-	-
BSP3	+	-	-	-	-	+	-	-	+	+	+	+	-
BSP9	+	+	-	-	-	+	-	+	+	+	+	+	-
BSP10	+	+	-	-	-	-	-	-	-	+	+	-	-
BSP11	-	+	+	-	+	-	-	-	-	+	+	-	-
BSP50	+	-	-	+	-	-	-	-	+	+	+	-	+
BSP51	+	+	-	-	-	+	-	+	+	+	+	+	-
CF1	-	+	+	+	+	-	+	-	-	+	+	-	-
CF2	+	+	+	-	-	-	+	-	+	+	+	-	-
CF3	+	+	+	-	-	-	+	-	+	+	+	-	+
KD1	-	+	+	-	+	-	-	-	+	+	+	+	-
KD2	+	+	+	-	+	-	-	-	+	+	+	-	-
KD3	+	+	+	+	-	+	-	-	+	+	+	-	-
PMI9	+	+	-	-	-	+	-	+	+	+	+	+	-

+ indicates positive result, - indicates negative result

Table 6 Biosurfactant screening tests

Parameters	BSP9
Oil displacement test	4.8 cm
Blue-agar Plate Assay	+
Emulsification activity E24 with vegetable oil as C source With crude oil	 59.95% 71%

+ indicates positive result

Table 7 PGP characters of the isolates

Isolates	**Phosphate solubilization**	**Zinc s0lubilization**	**IAA production**	**Siderophore production**	**ACC deaminase**	**HCN production**	**Biocontrol activity**
BSP1	+	+	-	-	+	-	-
BSP2	-	-	++	-	+	++	-
BSP3	+	+	+	+	-	-	
BSP9	+++	+++	+++	+++	+++	+++	+++
BSP10	++	+	++	-	+	-	-
BSP11	++	+	++	+	-	-	+
BSP50	+	+	+	+	-	+	-
BSP51	++	+++	++	++	+++	++	++
CF1	+	+	+++	+	+	+	-
CF2	-	-	+	-	-	-	-
CF3	-	-	+++	-	-	-	-
KD1	+	++	++	+	+	-	-
KD2	-	-	-	-	-	-	-
KD3	++	+	+	+	-	-	-
PMI9	-	+	+++	++	-	-	-

Table 8 Plant growth promoting properties of BSP9

Properties	BSP9
Phosphate solubilization index (PSI)	3.6± 0.2
Zinc solubilization index (ZSI)	4.66±2.1
IAA production (µg/ml)	31.90± 2.1
Siderophore production (percent siderophore unit)	95.28 ± 1.4
ACC deaminase	+
HCN production	+

Table. 9 Biocontrol activity of the isolate by dual culture technique

Name of the isolate	Radial growth inhibition of fungus
BSP9	70.44±0.04

Table 10 Identification of selected rhizobacterial isolate

Isolate	Identified as	Percentage similarity	Accession number (NAIMCC, Mau)	Isolation source	Geographical locations
BSP9	*Pseudomonas putida*	99.60%	(NAIMCC-B-02326)	Rhizosphere of *B. juncea*	Lucknow (26.8467° N, 80.9462° E)

Table 11 FTIR analysis of the purified biosurfactant

Frequency range	Functional group
3650-3134 cm^{-1}	-OH group
1748 cm^{-1} and 1712 cm^{-1}	–C=O stretching
1396 cm^{-1}	C-H and OH deformation
2358 cm^{-1}	phosphines (P-H_3)
1000 cm^{-1}	C-H, C-O and CH_3 vibrations

Table 12 LC-MS analysis of the purified biosurfactant

Peaks at m/z	**Rhamnose congeners**	**Retention time (in min)**
529.3	Rha-C12:1-C10	11.75
532.3 and 576.4	Rha-C_{10}-C_{12}/Rha-C_{12}-C_{10} and Rha-C_{10}-$C_{14:1}$/Rha-C_{12}-$C_{12:1}$, respectively	12.49
557.5	Rha- C_{12}-$C_{12:1}$/Rha- $C_{12:1}$-C_{12}	13.24
503.4	Rha-C_{10}-C_{10}	15
621.5	Rha-Rha-C_8-C_{10} or Rha-Rha-C_{10}-C_8	12.49

Table 13 Percent disease incidence in *B. Juncea* after different treatments of bioformulation

Mustard plant treated with following treatments	Percent Disease Incidence (PDI) (%)
C1 (negative control i.e. infected with *A. brassicae*	100
C2 (untreated control)	0
T1 (0.1% biosurfactant + *A. brassicae*)	80.88
T2 (1% biosurfactant + *A. brassicae*)	77.77
T3 (2% biosurfactant + *A. brassicae*)	44.44
T4 (5% biosurfactant + *A. brassicae*)	33.31
T5 (1% biosurfactant + BSP9 +*A. brassicae*)	22.21
T6 (2% biosurfactant + BSP9 +*A. brassicae*)	11.12
T7 (BSP9 +*A. brassicae*)	22.21

Where number of plants n= 3 per pot per treatment

Table 14 Effect of biosurfactant (at various concentrations) and selected isolate *Pseudomonas putida* BSP9 (alone and in combination with biosurfactant) on growth parameters and productivity of *B. juncea* (pot study)

Sample	% Germination	Root length (cm)	Shoot length (cm)	Total fresh weight (g)	Total dry weight (g)	Number of pods	1000 seeds wt (gm)
C1	25.20±0.60a	5.48±0.26a	70.15±0.05a	49.13±0.05a	4.11±0.01a	25.81±0.01a	0.21±0.02a
C2	40.77±0.67b	9.26±0.23b	100.56±0.07b	52.55±0.05b	6.22±0.02b	50.52±0.02b	0.51±0.02b
T1	41.30±0.99b	10.31±0.27c	110.65±0.15c	60.52±0.02c	9.29±0.05c	55.23±0.01c	0.81±0.03c
T2	50.66±0.29c	13.20±0.10d	113.11±0.10d	65.82±0.02d	12.50±0.05d	65.34±0.10d	1.02±0.02d
T3	68.20±0.26e	15.43±0.07f	115.53±0.25f	70.21±0.02f	14.49±0.03f	74.99±0.05f	1.81±0.02f
T4	60.47±0.19d	13.73±0.20e	114.40±0.10e	68.42±0.03e	13.06±0.10e	70.15±0.04e	1.51±0.03e
T5	**75.25±0.34g**	**16.65±0.15g**	**119.25±0.50h**	**79.62±0.03h**	**19.76±0.05h**	**80.21±0.02h**	**2.48±0.02h**
T6	**78.42±0.27h**	**20.46±0.07i**	**120.48±0.02i**	**83.54±0.05i**	**22.76±0.05g**	**88.10±0.10i**	**2.80±0.05i**
T7	70.04±0.69f	18.21±0.03h	117.50±0.05g	75.81±0.02g	15.62±0.02g	78.11±0.09g	2.08±0.14g

Data are means of three replicates with standard error. Same letters denote statistically insignificant difference while treatments with different letters have significant difference in the values (P < 0.05). C1- negative control with only *A. brassicae, C2*-untreated control**,** T1- 0.1% biosurfactant + *A. brassicae* (B.S), T2- 1.0 % B.S+ *A. brassicae*, T3- 2.0 % B.S+ *A. brassicae*, T4- 5.0 % B.S+ *A. brassicae*, T5- 1% B.S + BSP9+ *A. brassicae* , T6-2% B.S +BSP9+ *A. brassicae*, T7- only BSP9 cells + *A. brassicae*

**data means of three pots. Values in bold indicate the most effective treatment*

Table 15 Effect of biosurfactant (at various concentrations) and selected isolate *Pseudomonas putida* BSP9 (alone and in combination with biosurfactant) on phytochemical parameters of *B. juncea* (pot study)

Sample	Total Chlorophyll (mg/gm)	Total Flavanoid (mg/gm)	Phenolic content (µg/gm)	Protein (mg/gm)	PPO Enzyme activity ΔOD at 420/mg of protein/min	PO enzyme activity ΔOD at 420/mg of protein/min	PAL enzyme activity (trans-cinnamic acid/mg of protein/min
C1	2.38±0.20a	3.95±0.05a	80.51±0.02a	2.75±0.05a	0.62±0.10a	1.22±0.02a	8.51±0.01a
C2	6.37±0.25b	4.88±0.01b	133.02±0.26b	5.81±0.20b	0.95±0.05b	2.21±0.15b	9.44±0.05b
T1	7.53±0.17c	5.53±0.02c	150.70±0.02c	8.08±0.14c	1.04±0.17c	2.64±0.11c	9.68±0.05c
T2	8.51±0.01d	6.35±0.02d	178.65±0.15d	12.22±0.20d	1.43±0.11d	3.32±0.10d	9.97±0.23d
T3	10.53±0.05f	8.36±0.23f	214.07±0.06f	15.14±0.05f	1.97±0.20f	4.89±0.10f	10.59±0.01e
T4	9.49±0.01e	7.90±0.01e	200.28±0.15e	13.89±0.05e	1.89±0.10e	3.59±0.10e	10.07±0.02d
T5	**12.15±0.05h**	**9.13±0.17h**	**224.05±0.08h**	**17.68±0.26h**	**2.58±0.26h**	**7.05±0.20h**	**11.69±0.16g**
T6	**13.53±0.01i**	**9.77±0.15i**	**230.39±0.01i**	**18.70±0.11i**	**3.77±0.17i**	**7.77±0.13i**	**12.51±0.32h**
T7	11.23±0.26g	9.05±0.35g	218.24±0.17g	16.09±0.79g	2.05±0.20g	6.26±0.17g	10.68±0.26f

Data are means of three replicates with standard error. Same letters denote statistically insignificant difference while treatments with different letters have significant difference in the values (P < 0.05). C1- negative control with only *A. brassicae, C2*-untreated control**,** T1- 0.1% biosurfactant + *A. brassicae* (B.S), T2- 1.0 % B.S+ *A. brassicae*, T3- 2.0 % B.S+ *A. brassicae*, T4- 5.0 % B.S+ *A. brassicae*, T5- 1% B.S + BSP9+ *A. brassicae* , T6-2% B.S +BSP9+ *A. brassicae*, T7- only BSP9 cells + *A. brassicae*

**data means of three pots. Values in bold indicate the most effective treatment*

Table 16 Effect of biosurfactant (at various concentrations) and selected isolate *Pseudomonas putida* BSP9 (alone and in combination with biosurfactant) on growth and phytochemical parameters of *B. juncea* (field study)

Treatment	% Germination	Root length (cm)	Shoot length (cm)	Total Fresh weight (gm)	Total dry weight (gm)	Number of pods	Total Oil content (%)*	Total Chlorophyll Content (mg/g)	Total Flavonoid Content (mg/g)
C	50.1 ±0.5^{a}	15 ±0.4^{a}	119.3 ±0.5^{a}	290.8 ±1.4^{a}	114 ±1.02^{a}	284.2 ±8.7^{a}	35.00%	16.6 ±0.1^{a}	6.4 ±0.2^{a}
T1	62.3 ±0.9^{b}	21.9 ±0.1^{b}	179.7 ±1.3^{b}	323.4 ±4.2^{b}	129.2 ±1.9^{b}	379.8±11.3^{b}	39.00%	23.4 ±0.16^{b}	7.5 ±0.2^{b}
T2	71.5 ±0.6^{c}	24.6 ±0.3^{c}	191.3 ±1.4^{c}	354.5 ±2.9^{c}	149.2 ±0.8^{c}	394 ±4.7^{b}	40.50%	25.2 ±0.1^{c}	8.5 ±0.1^{c}
T3	84.9 ±0.6^{d}	26.1 ±0.5^{de}	198.8 ±0.9^{e}	374.2 ±2.1^{d}	164.5 ±1.1^{d}	426.8 ±1.2^{c}	41.50%	28.5 ±0.2^{e}	10.5 ±0.2^{e}
T4	83.9 ±0.9^{d}	25.2 ±0.4^{cd}	195.4 ±0.5^{d}	371.2 ±1.9^{d}	161±1.4^{d}	421.6 ±2.8^{c}	41.00%	27.2 ±0.2^{d}	9.5 ±0.2^{d}
T5	**88.2 ±0.7^{e}**	**28.1 ±0.1^{f}**	**213.8 ±1.1^{g}**	**405.5 ±1.7^{e}**	**197.0 ±1.2^{e}**	**429.4 ±6.6^{c}**	**43.50%**	**31.4 ±0.2^{f}**	**12.2 ±0.2^{g}**
T6	**91.3 ±0.2^{f}**	**30.1 ±0.1^{g}**	**216 ±1.8^{g}**	**483.4 ±5.3^{f}**	**204±4.6^{e}**	**481.2 ±2.1^{d}**	**45.00%**	**32.3 ±0.1^{h}**	**13.5 ±0.1^{h}**
T7	85.2 ±1.1^{d}	27.4 ±0.5^{ef}	202.8 ±0.7^{f}	379 ±3.2^{d}	168.6 ±1.3^{d}	427±4.2^{c}	42.50%	30.4 ±0.1^{g}	11.2 ±0.1^{f}

Results expressed as mean ± S.D (n =10 of two years data (2017-18 and 2018-19) Same superscript letters denote no significance difference ($P < 0.05$) determined by Duncan's multiple range test (DMRT)

T1- 0.1% biosurfactant (B.S), T2- 1.0 % B.S, T3- 2.0 % B.S, T4- 5.0 % B.S, T5- 1% B.S + BSP9 , T6-2% B.S +BSP9, T7- only BSP9 cells and C= untreated control

-* Collective mean values oil content has been shown. *Values in bold indicate the most effective treatment*

Discussion

Discussion

Brassica juncea is a rabi crop and is one of the richest source of protein, iron (Fe), vitamin A and C and also contains calcium, potassium, riboflavin, β-carotene and phenolic antioxidants like sinapic acid and sinapine (Cartea et al. 2010; Thakur et al. 2019). Due to its low amount of saturated fatty acids (5-7%) and high level of unsaturated fatty acids (about 7-10% α-linolenic and 17-21% linoleic acids), it is considered as a healthier option in comparison to other edible oils (Baux et al 2008). In addition, it possesses glucosinolates, isothiocyanates, allyl thiocyanates, phytosterols, which have medicinal properties and antimicrobial characters (Zhang 2010; Mazumder et al. 2016). Therefore, in order to meet the ever growing demands of oilseed in the country, it is important to increase the production of Indian mustard. For this, use of PGPR and their metabolites in form of bioinoculants is the best possible substitute to their chemical counterparts. They can improve plant health, productivity and also protect them from abiotic stresses minimizing reliance on synthetic inputs.

In the present study, 15 isolates were obtained from rhizosphere of *B. juncea*. The isolates were examined on basis of colony morphology, physiological and biochemical characters as described in Bergey's Manual of Systematic Bacteriology (Garrity 2005). All the isolates were Gram negative, rod shaped with shiny smooth colonies having convex and entire margins. Most of the isolates showed they were fast growers with generation time 2-4.5 h and some also showed slow growth with 6h of generation time. Isolates were checked for biochemical and physiological properties according to Bergey's Manual of Systematic Bacteriology. All the 15 isolates were able to utilize glucose and yeast extract as their carbon source and nitrogen source respectively. Bacterial communities in the rhizosphere is the most

diverse and helps in proper functioning of plant health. Rhizosphere is the most active zone around plant root that harbours microbial communities which is essential for plant functioning. Given the fact that root exudates differ with different plants, hence plant-specific microbial communities could also be isolated (Bakker et al. 2013). Isolates BSP9 and BSP51 displayed ability to survive at 4% salt concentration and only isolate PMI9 showed growth at 8% NaCl. Such rhizobacteria are termed as salt tolerant PGPR and in last few years research showed that the use of salt-tolerant plant growth promoting rhizobacteria (ST-PGPR) in saline agriculture can be harnessed for enhancing productivity and improving soil fertility as well (Egamberdieva et al. 2019; Arora et al. 2020a). At pH 6, 8 and 10, all isolates showed positive growth and below pH 4 only PMI9 was capable of growing. Most bacteria grow over a broad range of external pH values, from 5.5–9.0, and maintain a cytoplasmic pH that lies within the narrow range of pH 7.4–7.8, hence, they are able to acidify or alkalinize the cytoplasm relative to the external environment (Krulwich et al. 2011). Similarly all the isolates showed growth at temperatures range of 15, 25 and 35^0C and one isolate also showed growth at 40^0C. Temperature tolerance is a good parameter for isolating competitive and diverse PGPR in fluctuating temperature conditions in fields (Hungria and Vargas 2000; Getahun et al. 2020). Studies suggested that rhizobacterial isolates RR-1, GGP-1, and GNR-1 were tolerant to high temperature (45°C) and also exhibited multiple beneficial plant growth-promoting activities (Manasa et al. 2017).

Isolates were further checked for biosurfactant production properties. In this context, all the 15 isolates were screened for biosurfactant production, of which, only two i.e. BSP9 and BSP51 gave positive results. The isolates were checked for foam production and two isolates BSP9 and BSP51 gave positive results by producing foam

in the MSM media. Foam production by a biosurfactant producing isolate is a simple and important technique to indicate the presence of the metabolite. The foam produced was stable for 48 h. Stable foam indicates that the produced biosurfactant can be used as a good foaming agent (Saikia et al. 2012). Next, isolates were checked for oil displacement test. BSP9 and BSP51 both were able to displace crude oil layer but isolate BSP9 showed maximum displacement of 4.8 cm than BSP51 with 2.5 cm. Oil displacement property of a biosurfactant is indicative of its surface wetting behavior (Youssef et al. 2004; Elazzazy et al. 2015). Further, isolates were tested for CTAB assay and BSP9 and BSP 51 gave prominent blue zones around the bacterial inoculation due to binding of cetyl tri ammonium bromide (CTAB) with biosurfactant. Similar results were depicted by *Pseudomonas fluorescens* in a study conducted by Meylheuc et al. (2001) The positive result of this test confirms the anionic nature of the biosurfactant(Siegmund and Wagner 1991; Bajpai et al. 2020). Formation of blue halos around bacterial colony on methylene blue agar can be considered as the presence of rhamnolipid biosurfactant (Eslami et al. 2020). Emulsification assay was another screening test performed for biosurfactant. From the result it was revealed that BSP9 showed better emulsification activity with 59.95% (E_{24}) in presence of vegetable oil as carbon source. On the other hand, BSP51 showed 48% of emulsification with same carbon source. However in presence of crude oil, an improved emulsification of 71% by BSP9 was witnessed while BSP51 showed 55% (E_{24}). According to Abouseoud et al. (2008), diesel oil and kerosene (55 %) were defined as the best substrates for emulsification whereas sunflower oil (45 %) was found to be less efficient substrate for emulsification. This property defines the ability of the isolated strains to emulsify hydrocarbons including crude and vegetable oil and hence can be applied against recalcitrant hydrocarbons (Singh and Tiwary 2016). All

these aforementioned screening methods confirmed the production of biosurfactant by BSP9 and BSP51.

Alongwith exploring the biosurfactant producing traits, isolates were also screened for PGP attributes. It was revealed that out of 15 isolates, BSP9 showed significant results for PGP characterization followed by BSP51. There are numerous species of soil bacteria which flourish in the rhizosphere of plants and stimulate plant growth by a plethora of mechanisms such as water and nutrient uptake, phytohormone production as well as abiotic and biotic stress tolerance (Vejan et al. 2016; Backer et al. 2018, Arora et al. 2020b). Among all PGPR, *Pseudomonas* are known for their excellent PGP properties and have proved their potential to enhance crop yields (Ahemad and Khan 2012; Qessaoui et al. 2019; Fatima and Arora 2021). From the results of PGP activities, it was revealed that BSP9 showed best result for phosphate solubilisation with 3.6 PSI. Phosphorous (P) is one of the essential macronutrient for plants and contributes in approximately 0.2% of plants dry weight (Maharajan et al. 2018). It serves as a crucial element for various metabolic processes such as photosynthesis, cell division, respiration, energy storage, nutrient uptake and synthesis of proteins in plants (Siedliska et al. 2021). There are numerous soil microbes that are capable of releasing P from soils through solubilisation and mineralization and are known as phosphate solubilizing microbes (PSM) (Bhattacharyya and Jha 2012; Alori et al. 2017). In a study by Wang et al. (2021), phosphate solubilizing bacteria (PSB) *Burkholderia cepacia* ISOP5, *P. putida* ISP-1 and a mixed inoculation of these two bacteria (MB) on peanut yield was evaluated. From the results, it was revealed that five years of inoculation with *B. cepacia* ISOP5, *P. putida* ISP-1, and MB treatments caused an increment in total yield of peanut by 8.1%, 12.5%, and 19.5%, respectively. In another study, four phosphate

solubilizing *Psedomonas* spp. were isolated from high altitude of Himalayas namely GBPI_506 (*Pseudomonas* sp.), GBPI_508 (*Pseudomonas palleroniana*), GBPI_Hb61 (*Pseudomonas proteolytica*), and GBPI_CDB143 (*Pseudomonas azotoformans*). GBPI_CDB143 showed highest solubilisation efficiency to tri-calcium phosphate (110.50 ± 3.44 μg/mL). Further, these isolates were inoculated in *Arabidopsis thaliana* to check its growth promotion and it was revealed that isolate GBPI_Hb61 showed significant enhancement in all growth parameters of the plant including total rosette diameter (1.9-fold increment), leaf area (4.25-fold increment), shoot and root (1.53 and 1.79-fold, respectively) as compared to untreated control (Adhikari et al. 2021).

Zn is another vital micronutrient which is imperative in small concentrations for optimum growth and reproduction of plants. Zn deficiency in plants can cause impaired integrity of cell membrane and reduced synthesis of carbohydrates, auxins, nucleotides, cytochrome and chlorophyll along with susceptibility to heat stress (Singh et al. 2005; Goteti et al. 2013). In the present study, maximum Zn solubilization was shown by BSP9 isolate having 4.6 ZSI. There are various studies that show the ability of PGPR to solubilize Zn. In a study by Kamran et al. (2017), Zn solubilizing *Pseudomonas fragi* EPS1 was inoculated in wheat seedlings using zinc carbonate ($ZnCO_3$) as Zn source. It was observed that inoculated plants showed significant root dry weights and improved zinc content as compared to uninoculated plants. In the same context, *Pseudomonas* sp. VBZ4 and *P. stutzeri* VBZ17 was screened from vermicompost and its PGP efficiency was evaluated using tomato plant. Results showed that isolate VBZ4 solubilized 26.8 $mg{\cdot}L^{-1}$ followed by isolate VBZ17 with 22.2 $mg{\cdot}L^{-1}$ solubilization on ZnO enriched media and 18.3 $mg{\cdot}L^{-1}$ (isolate VBZ4), 15.6 $mg{\cdot}mL^{-1}$ (isolate VBZ17) on $ZnCO_3$ supplemented

media. It was also observed that tomato plants inoculated with VBZ4 improved plant growth and maximum Zn content in fruits (2.87 mg/100 g) followed by isolates VBZ4+ VBZ17 and isolate VBZ17 with 2.17 mg/100 g and 2.06 mg/100 g Zn, respectively (Karnwal 2021).

BSP9 also showed significant result for production of IAA producing 31.9 µg/ml of the phytohormone. IAA is one of the most common plant auxins that plays multitude of important physiological processes including cell enlargement and division, tissue differentiation, and senescence (Lambrecht et al. 2000; Bharucha et al. 2013; Nicastro et al. 2021). According to Patten and Glick (1996), about 80% of rhizospheric microbes have ability to synthesize and release IAA have potential to change any of above mentioned processes by altering the plant auxin pool (Ali et al. 2010; Olanrewaju et al. 2017). In this context, a rhizospheric *P. putida* strain AF137 isolated from soils of rice cultivar in Afghanistan produced 92.30 µg mL^{-1} of IAA and its inoculation increased dry weights of root and shoot in rice plants (Habibi et al. 2019). In another study, production of IAA by *P. aeruginosa* UPMP3 was evaluated and its effect was observed on oil palm seedling growth. The maximum produced IAA by the isolate was upto 52 µg/ml. The extracted IAA also showed positive effect on seedling growth in respect to average root and leaf number, root, shoot, and leaf length compare to the synthetic IAA and the control (Parvin et al. 2020).

Iron (Fe) is another key determinant for plant's growth but since, its bioavailability is largely dependent on pH and redox potential, it is limiting factor for plants (Lemanceau et al. 2009; Lurthy et al. 2020). Production of iron chelating compounds known as siderophores by rhizospheric bacteria is another important PGP trait (Abdallah Hussein and Joo 2019). Iron sequestration helps plant in synthesis of chlorophyll, involved in electron transfer, helps in synthesis of DNA, RNA as well as

their repair and also acts as antagonistic agents against phytopathogens (Sayyed et al. 2010; Ferreira et al. 2019). In the present study isolates were checked for siderophore production both qualitatively and quantitatively. Out of 15 isolates, BSP9 and BSP51 showed positive result for siderophore production by showing orange zones around bacterial colonies. Quantitative estimation revealed that BSP9 was a better siderophore producer with 44.74% psu of siderophore than BSP51 with 39.84 psu of siderophore. In a study by Arya et al. (2018), two strains of *P. fluorescens* SPs9 and SPs20 were evaluated for siderophore production and checked for inhibition of Fusarium wilt in tomato plants. Results revealed that both strains i.e. SPs9 and SPs20 produced 59.30 and 52.74 µg ml^{-1} siderophore and inhibited fungal growth by 3.4 and 3.8 cm respectively. Inoculation of both bacterial strains, SPs9 and SPs20, showed disease suppression in tomato plants by 100% and 75% respectively as well as increased their fresh and dry weights. Another iron chelating *P. putida* MPJ6 produced 85.3% siderophore under iron deficient condition and its biofortification increased iron content and other vegetative parameters in mungbean (Patel et al. 2018).

In addition, some PGPR including pseudomonads have ability to control pathogens and inhibit the incidence of disease in plants by production of a volatile metabolite called HCN. Studies have reported that HCN is a powerful inhibitor of many metal enzymes, especially copper containing cytochrome C oxidases and is formed from glycine through the action of HCN synthetase enzyme, which is associated with the plasma membrane of certain rhizobacteria (Martínez-Viveros et al. 2010). Selected isolates in the present study was checked for HCN production qualitatively and it was observed that except for one isolate i.e. PMI9, no other isolate gave positive test for this metabolite.

Production of ACC deaminase enzyme by PGPR has been widely reported by pseudomonads under stress conditions to reduce levels of ethylene (a stress hormone) via hydrolyzing ACC (Gupta and Pandey 2019). ACC is the precursor of the hormone ethylene and ACC deaminase has the ability to degrade ACC into ammonia and α-ketobutyrate, thereby reducing levels of ethylene in plants. Such PGPR have the potential to alleviate stress caused by abiotic factors and plant pathogens. Characterizing the selected isolates for ACC deaminase production, it was found that BSP9, BSP51 and PMI9 were able to grow on plates containing ACC as sole N-source hence confirming the presence of ACC deaminase enzyme. There are several studies that have exploited the role of ACC deaminase producing PGPR under stressed conditions. In this regard, roots of tomato seedling when dipped in the cell suspension of *Pseudomonas putida* UW4 inhibited tumor formation in plants caused by *Agrobacterium tumefaciens* by four- to fivefold (Toklikishvili et al. 2010). In another study, ACC deaminase-producing *Pseudomonas thivervalensis* SC5 when inoculated in cucumber plants showed significant improvement in its growth under salt stress and also inhibited in vitro growth of the pathogen *Botrytis cinerea* and *Pseudomonas syringae* DC3000 indicating active biological control activities (Nascimento et al. 2021).

Studies have shown that pseudomonads are known for their biocontrol activity as they are capable of producing antimicrobial compounds (antibiotics, proteolytic and cellulolytic enzymes, volatile compounds) including biosurfactant (Chopra et al. 2020). Referencing to the context, all the 15 isolates were checked for their biocontrol activity against *A. brassicae* using dual culture technique. The best results were shown by isolates BSP9 and BSP51 having 70.44% and 61.11% antagonistic activity, respectively, against *A. brassiace*. In this context, a fluorescent pseudomonad strain

PF17 having PGP properties showed considerable biocontrol activity against *Macrophomina phaseolina* and also promoted growth of sunflower (Tewari and Arora 2016). Similarly, biocontrol activity of rhizospheric *Pseusdomonas fluorescenes* strains B3 and B12 were evaluated against *A. brassicae* and it was observed that the two strains showed reduced disease incidence i.e. 32% and 35% for B2 and B12 respectively as compared to control with disease incidence of 62.8% (Gupta et al. 2020).

On the basis of potent PGP characters and biosurfactant producing abilities, isolate BSP9 was selected for molecular characterization. Nucleotide homology revealed that isolate BSP9 was 99.6 % similar to *P. putida* and culture was submitted to NAIMCC, an International Depository Authority (IDA) with accession number *P. putida* NAIMCC-B-02326. Studies reveal that *P. putida* are ubiquitous bacteria and are well known for its metabolic versatility, adaptability and PGP properties. Hence, these bacteria are considered as excellent candidates for development of bioinoculants and as soil enhancers to increase crop yields (Molina et al. 2020).

Production and extraction of biosurfactant by BSP9 was further carried out by solvent extraction method. Among various other extraction methods such as acid precipitation, foam fractionation, ultrafiltration, ion-exchange chromatography, solvent extraction method is regarded as the most effective method for recovery of biosurfactant (Eslami et al 2020). After evaporating the organic layer, a dark brown-coloured viscous residue was left over (crude biosurfactant) and weighed 2.5 g/L. Study has reported that water soluble carbon sources such as glucose, glycerol, mannitol ethanol aliphatic and aromatic hydrocarbons are recommended for rhamnolipid by *Pseudomonas* spp. (Silva et al. 2010; Occhipinti et al. 2018). Although the use of low-cost materials is usually considered to solve the cost

problem, the selection of a substrate compatible with cell growth is very important. Studies have shown that biosurfactant production tend to increase in presence of oil based carbon sources like that of glycerol. It has been noted that glycerol can increase production of rhamnolipids upto 9.3 g/L with increase in its concentration (Tan et al. 2018).

After extraction, biosurfactant was purified using TLC method and the analysis of the extracted residue showed two major spots (retention factor Rf= 0.33 and Rf= 0.78) having mobility similar to those of glycolipids where the higher spot denoted the presence of monorhamnolipids and lower spot is of dirhamnolipids. The result of TLC analysis were in line with the findings of Eraqi et al. (2016) where TLC biosurfactant from *P. aeruginosa* also showed two prominent spots. Higher one corresponded to the mono rhamnose and the lower spot corresponds to dirhamnose moieties with reference to commercially available rhmanolipids.

Structure morphology and elemental composition through SEM-EDS was analysed. Result of SEM-EDS showed ratio of carbon, oxygen, sodium, potassium, chlorine as 50.64: 38: 0.70:2.66: 0.97% denoting profound presence of carbon and oxygen ratio as compared to sodium, potassium and chlorine in the scanned area. The result was in line with the study of Patowary et al. (2017) where SEM-EDS of the biosurfactant produced by *P. aeruginosa* PG1 showed carbon, oxygen, sodium, phosphorus, chlorine, and potassium in ratio 67.14, 30.73, 1.02, 0.29, 0.62, and 0.19 %. This illustrates the existence of carbohydrate and lipid congeners in the biosurfactant sample (Patowary et al. (2017)

Next, the purified biosurfactant was structurally characterized by FTIR and LC-MS analysis. From the IR spectrum, it can be illustrated that absorption bands ranging

between 3650-3134 cm^{-1} indicates the presence of free -OH group arising as a result of H bonding of polysachharides and/or –OH stretching of carboxylic acid. Two bands at wavenumber 1748 cm^{-1} and 1712 cm^{-1} denotes –C=O stretching arising from ester groups of lipids and fatty acids (-C=O in COOH); wavenumber at 1396 cm^{-1} signifies C-H and OH deformation vibrations of carbohydrates that may be arising due to the presence of rhamnose units in the biosurfactants (Leitermann et al. 2008; Mishra et al. 2020). A small peak at wavenumber 2358 cm^{-1} is likely due to the presence phosphines (P-H_3) in the biosurfactant which was also revealed in the study by (Singh et al. 2016). The range of spectra below 1000 cm^{-1} represents C-H, C-O and CH_3 vibrations (Günzler and Gremlich 2003). Glycolipids are among the most promising biosurfactants and have shown high productivity and extreme biochemical versatility and hence are being used in wide range of industries including agriculture (Mnif and Ghribi 2016; Akbari et al. 2018). These type of biosurfactant possess carbohydrate moieties linked to fatty acid (as evident from the present FTIR data) and are largely produced by *Pseudomonas* sp. (Günzler and Gremlich 2003).

Further, identification of rhamnose moieties in purified biosurfactant was done by LC-MS analysis. From the result it was observed that rhamnose analogues detected at m/z 529.3 corresponds to Rha-$C_{12:1}$-C_{10}. The same mass range was identified in the LC-MS analysis of rhamnolipids produced by *P. aeruginosa* strain ATCC 9027(Zhang et al. 2012). Likewise, ionization at m/z 532.3 and 576.4 with retention time of 12.49 minutes corresponds to Rha-C_{10}-C_{12}/Rha-C_{12}-C_{10} and Rha-C_{10}-$C_{14:1}$/Rha-C_{12}-$C_{12:1}$. Bajpai et al. (2020) in their study have reported similar rhamnose congeners produced by *Pseudomonas protegens* strain BNJ-SS-45 at similar mass range. Molecular ions at *m/z* 557.5 (retention time = 13.24 minutes) and 503.4 (retention time = 15.00 minutes) showed the presence of Rha- C_{12}-$C_{12:1}$/Rha- $C_{12:1}$-C_{12}

and Rha-C_{10}-C_{10} respectively. The LC-MS study of rhamnolipids produced by *Pseudomonas gessardii* showed similar rhamnolipidic moieties having same mass range (Buonocore et al. 2020). The present LC-MS analysis revealed the abundance of six compositionally different homologues of mono-rhamnolipid congeners. However, a di-rhamnose congener i.e. Rha-Rha-C_8-C_{10} or Rha-Rha-C_{10}-C_8 was also obtained at *m/z* 621.5 with retention time of 12.49 minutes which was also evident in the study by Sabturani et al. (2016). Rhamnolipids are predominantly produced by *Pseudomonas* sp. containing mono, di rhmanolipidic congeners or a mixture of both and there are more than 100 known homologues of rhamnolipids produced by *Pseudomonas* sp. which have been reported till now (Loiseau et al. 2018). Earlier findings suggest that presence of mono-rhamnolipidic moeities act as a wetting agent for bacteria, while, on the hand other, a di-rhamnolipidic congener have been found to work as a self-produced chemotactic attractants by some *Pseudomonas* sp. (Abdel-Mawgoud et al. 2010; Loiseau et al. 2018).

The purified biosurfactant i.e. rhamnolipid was also checked for its biocontrol activity using agar well diffusion method in 0.1%, 1%, 2% and 5% concentrations. Interestingly, it was found that biosurfactant with 2% and 5% concentration showed almost similar values for zone of inhibition against *A. brassicae* (hence not shown in figure), which was maximum among other concentrations. The result of biocontrol activity of rhamnolipids were subjected to SEM analysis and it was observed that there was hyphal deformity in *A brassicae* mycelia at specific sites where fungus was in contact with the biosurfactant. A similar deformity and morphological alterations in fungal hyphae of *A alternata* was observed when it was treated with rhamnolipid biosurfactant showing antifungal property against the phytopathogen (Yan et al. 2015). A large range of rhamnolipid concentrations from 0.005 to 1 mg/mL have been

used to induce immunity on various plant species including Brassicas (Varnier et al. 2009; Sanchez et al. 2012; Monnier et al. 2018, 2020). These activities were mainly targeted to fungi and oomycetes including *Botrytis* sp., *Rhizoctonia* sp., *Fusarium* sp., *Alternaria* sp., *Pythium* sp., *Phytophthora* sp., or *Plasmopara* sp. species. Among all the congeners that are present in the rhamnolipids, purified mono-rhamnolipids (Rha-C_{10}-C_{10}) generally possess the strongest antifungal activity (Crouzet et al. 2020). This structure is responsible for antimicrobial effect such as zoospore lysis, spore germination abortion and mycelial growth inhibition. In the LC-MS analysis of BSP9 biosurfactant, presence of mono-rhamnolipid congener (Rha-C_{10}-C_{10}) was confirmed and could be a strong reason for its biocontrol activity against *A. brassicae*. Rhamnolipids and lipopeptides have been extensively studied in the context of crop protection (Crouzet et al. 2020). Rhamnolipids are known to affect mycelial cells resulting in their destabilization or lysis. It was studied that purified mono and di-rhamnolipids are able to intercalate into phosphatidylcholine and phosphatidylethanolamine bilayers, notably altering their packing (Abbasi et al, 2012, 2013; Crouzet et al. 2020).

To check the efficacy of rhamnolipids and BSP9 as a bioinoculant, talc based bioformulations were developed and were checked under pot and field trial conditions in different combination of treatments using *B. juncea* as test crop. Pot experiment conducted to screen biocontrol activity of BSP9 and its biosurfactant were found to be effective for each treatment set. Results of pot trials showed that plants treated with combination of cells and metabolite proved to be best in controlling disease incidence. 2% biosurfactant in combination with *P. putida* BSP9 in presence of fungus inhibited the occurrence of disease and hence the reduced disease incidence of Alternaria blight by (11.12%) followed by plants treated with 1%biosurfactant+BSP9combination and

only BSP9 treated plants (22.21% for both treatments). *Pseudomonas putida* has been reported as an effective biocontrol agent against several plant pathogens such as *Rhizoctonia solani* in cucumber, *Fusarium oxysporum* f.sp. *radicis-lycopersici* in tomato and also *Alternaria* spp. in crucifers (Abo-Elyousr et al. 2021). Referencing to the context, rhizospehric and biosurfactant producing *Pseudomonas putida* caused lysis of zoospores of the oomycete pathogen *Phytophthora capsici*; causative agent of damping-off of cucumber (Kruijt et al. 2009). In a study by Joseph et al. (2017), biocontrol activity of *P. putida* was evaluated against *A. solani* and it was observed that tomato plants treated with bacteria inhibited disease severity by 71%. Similarly, Sharma et al. (2018) conducted a study on biocontrol of Alternaria blight using antagonistic *P. putida* HMR70, HMR48. From the study it was concluded that two strains HMR70 and HMR48 caused 80% and 85% disease inhibition in mustard plant showing plant growth and biocontrol potential of the isolates. In the present pot study, addition of biosurfactant in treated plants served as a strong parameter for inhibition of Alternaria blight. Studies have shown that rhamnolipid biosurfactants acts a natural biopesticides for plants and they are very interesting candidates in biocontrol strategies (Otzen 2017). In a study conducted by Millioli et al. (2009) application of rhamnolipids together with *R. glutinis* was evaluated against a serious pathogen *A. alternate* that causes post-harvest black rot in certain cultivars of plants showed bioactivity against. Borah et al.(2016) in their study, have showed treatment of maize seeds with a rhamnolipid concentration of 50 mg/L resulted in improved biomass and fruiting compared to those of healthy control plants and complete suppression of characteristic disease symptoms and colonization of maize by *F. verticillioides*. From the pot study, it was also observed that there was an increment in growth parameters of plants including germination rate, root and shoot length, fresh and dry weight,

number of pods, seed weight (1000seeds), seed yield, chlorophyll and flavonoid content, in all treated plants as compared to both negative and untreated control.

A similar trend in the increment was also noted during field trial where all treated plants showed same spike in each growth parameter of plants as above. Among all the treatments, the best results were again displayed by plants treated with *P. putida* BSP9 plus 2% biosurfactant (T6 treatment). It was also observed that BSP9 alone could bring about significant enhancement in each growth parameter of plants in both pot and field trials. Multifarious PGP traits of BSP9 (production of IAA, siderophore and solubilization of phosphate could largely be the reason for growth promotion of Indian mustard. In addition to these properties, its potential to produce biosurfactant, has conferred added benefits to the plants. All these parameters help plant tolerate various biotic and abiotic stress and provide specific immunity to plants (Sinumvayo and Ishimwe (2015).

Defence related enzymes were also recorded to increase in presence of *A. brassicae* challenged plants under pot study. Observations revealed that maximum concentrations of PPO, PO and PAL was noted in presence of BSP9 and biosurfactant together and were least in negative control. Studies show that inoculation of PGPR including *P. putida* and external metabolite like rhamnolipids are elicitors of defence related antifungal enzymes in plants (Monneir et al. 2018; Pršić and Ongena 2020). In this context, a biocontrol strain of *P. putida* BTP1 isolated from barley roots was able to increase levels of hydrolytic enzymes in presence of fungus *Botrytis cinerea* in tomato plant after its inoculation (Akram et al. 2008). When applied alone, rhamnolipids induce antioxidative reactions in cherry tomato fruit, leading to a significant reduction of fungal disease (Yan et al. 2015).

In both pot and field trial, it was observed that with increase in biosurfactant concentration, there was a linear increase in all growth parameters of plant but after 2% concentration i.e. at 5% , values either dipped or were almost similar to former concentration. For this a heatmap was generated to determine distance and clustering and showing the effect of biosurfactant and BSP9 on various growth parameters of Indian Mustard. From the dendrograms of both pot and field study, it was noted that bacteria and metabolite synergistically are outstanding growth promoters, yet 2% and 5% BS (treatment alone) can show almost similar impact as BSP9. Therefore, it can be addressed that metabolites like biosurfactant are capable of directly increasing the plant growth and yield in addition to their remediation and biocontrol activities. Also analyzed from clustering algorithm, it can be summarized that lower concentration of BS (0.1% and 1% BS) are incapable of stimulating significant plant growth promotion as compared to other treatments. The study also highlights that there is a threshold of metabolite (biosurfactant) concentration required to enhance productivity and yield of plants due to causes unknown which can be the future scope. Relatively, explaining the effect of concentration, Chaprão et al. (2015) reported that application of more than 40 mg/ml biosurfactant to soil reduced diesel biodegradation efficiency. Confirming the fact, Fenibo et al. (2019) concluded that if optimum concentration of biosurfactant is exceeded, the microbial biomass decreases and degradation process is also negatively affected. However, there are no previous reports about optimized biosurfactant concentration for direct plant growth promotion under field conditions and hence the present work is a novel report extending the knowledge about the biopolymer.

The present study, thus, confirms that use of *P. putida* BSP9 and its biosurfactant can be an effective strategy to increase yield and growth of *B. juncea*. Its ability to

produce bisurfactant, displaying range of PGP characters including solubilization of phosphate and zinc, production of IAA, siderophore and HCN confirms it to be a potent PGPR that could be utilized for development of bioformulations. BSP9 and its purified biosurfactant inhibited the phytopathogen significantly that was confirmed in the SEM analysis where mycelia could be seen in a distorted state after interacting with bacteria and biosurfactant. From the pot and field study, it was further confirmed that combined effect of BSP9 and biosurfactant can increase the growth parameters of *B. juncea* in a significant manner. This could be attributed due to the added effects of BSP9 as PGPR and biosurfactant that acts as wetting, signalling and antifungal agent conferring benefits to its host plant. Also, presence of Rha-C_{10}-C_{10} moiety that was revealed in LC-MS may have inhibited fungal pathogen. It was also observed from the pot and field trial that 2% biosurfactant concentration gave best results both singly and in combination with bacteria and after a threshold the values in growth parameters did not change. Hence, optimization of biosurfactant can be an important prerequisite that for development of biosurfactant based bioformulation. In this way, use of BSP9 and its rhamnolipid biosurfactant can be a sustainable and ecofriendly approach to increase yield of *B. juncea*

Conclusion

5. Conclusion

From the study, it can be concluded that use of plant growth promoting strain *P. putida* BSP9 and its rhamnolipid biosurfactant is a novel technique for enhancing productivity of *B. juncea.* Owing to its excellent biocontrol property against *A. brassicae,* multiple PGP properties, biosurfactant producing ability, and non-pathogenic nature, use of *P. putida* BSP9 and its rhamnolipid biosurfactant can serve as a potential substitute which can overcome the use of agrochemicals and can serve as a gateway for future commercialization of biofertilizers across the globe.

Summary

6. Summary

B. juncea or Indian mustard is an important oilseed crop that belongs to family Brassicaceae (Cruciferae) and is predominantly grown in India under wide range of agro-climatic conditions. It is known for its rich contents of protein, iron, vitamin A and C, riboflavin antioxidants and has gained attention as important medicinal, therapeutic and antimicrobial agent. However, this oilseed is susceptible to certain fungal phytopathogens and as a result suffers great loss of yield across the world. One such fungal phytopathogen is *A. brassicae* which is among the most destructive fungi of Brassicas and causes Alternaria blight that appears as black or brown necrotic lesions in form of concentric rings on leaves, stems and even siliques. In order to obviate this issue and increase the growth and yield of mustard, we need to tend towards sustainable agricultural methods like use of PGPR and their metabolite instead of relying on agrochemicals as they are recalcitrant in nature and toxic to environment. Application of PGPR and their metabolites such as biosurfactants is an eco-friendly, biotic and green approach that will maximize the productivity of *B. juncea* without hampering the environment.

In present study, 15 bacterial isolates were obtained from rhizosphere of *B. juncea* near Lucknow region of Uttar Pradesh. Rhizosphere is regarded as the most active zone for isolation of range of beneficial microbes. Microbial communities in this zone are the most diverse in nature and have ability to produce wide range of metabolites and plant growth regulators that helps in proper functioning of their host under abiotic and biotic stresses. All 15 Isolates were characterized on the basis of morphological, physiological, biochemical and molecular basis. The colonies of the isolates were smooth, shiny and convex with entire margins. The isolates were also checked for utilization of various carbon and nitrogen sources. It was observed that glucose and

yeast extract were the most common carbon and nitrogen sources, repectively that were utilized by all 15 isolates. Further, isolates were checked for their growth over different temperatures, pH, and salt stress conditions. It was observed that maximum number of isolates showed growth at range of temperatures (4, 10, 15, 25 and 35^0C), pH (6, 8 and 10). However, in case of salt stress, only BSP9 and BSP51 showed growth at 4% saline condition and PMI9 even showed growth till 8% NaCl concentration. Biochemical tests revealed that most of the bacterial isolates (80%) showed similar results as those displayed by genus *Pseudomonas* and hence could be suggetsted that they belonged to the same category. Next, the isolates were screened for biosurfactant production by oil displacement, BAP test and emulsification activity (E24). Results revealed that BSP9 and BSP51 were the only two isolates that displayed positive tests for biosurfactant screening. Further, characterization on the basis of PGP attributes was done. It was observed that BSP9 and BSP51 showed maximum positive results for all the PGP tests that include solubilisation of phosphate, zinc, production of IAA, siderophore and ACC deaminase. Biocontrol activity of the isolates were determined by dual culture technique against *A. brassicae* fungus and it was found that BSP9 displayed maximum antagonism against the phytopathogen i.e. up to 70.44% followed by BSP51 that showed 61.11% inhibition of the fungus. Out of the two potent isolates, BSP9 was selected for further study as it showed best results for biosurfactant production, PGP characterization as well as bioocontrol activity.

BSP9 was subjected to 16S rRNA gene sequence analysis, and was found to be aligned with *P. putida* strain GQ-8 (Accession No. JX865419) with 99.6% similarity with BSP9 . The sequence was submitted in the GenBank with Accession No. LC489268. The isolate was also deposited in National Agriculturally Important

Microbial Culture Collection (NAIMCC) with *P. putida* NAIMCC-B-02326, an international culture collection centre approved by International Depository Authority (IDA).

For production and extraction of biosurfactant, BSP9 was subjected to solvent extraction method using chloroform:methanol mixture. After evaporation of organic layer, a dark coloured viscous liquid was left behind that weighed 2.5g/L using crude oil as carbon source. After extraction of the metabolite, its purification was carried out using TLC method. The analysis showed the presence of two major spots on TLC plate having same mobility as that of glycolipids and with retention factor Rf= 0.33 and Rf= 0.78. The higher spot (with Rf 0.78) denoted the presence of monorhamnolipids and the lower one (with Rf 0.33) denoted dirhamnolipids.

The TLC purified biosurfactant was then structurally characterized using SEM-EDS, FT-IR and LC-MS analysis. In case of SEM-EDS, the scanned area showed the presence of ratio of carbon, oxygen, potassium, sodium, chlorine as 50.64: 38: 0.70:2.66: 0.97% respectively . High ratio of carbon and oxygen denotes the presence of carbohydrate and lipid content in the sample.

In FT-IR analysis, the spectrum showed the of functional groups including –OH in the absorption range 3650-3134 cm^{-1}, -C=O at wavenumbers 1748 cm^{-1} and 1712 cm^{-1}, C-H and O-H deformations at wavenumber 1396 cm^{-1} and a small peak of phosphine ($P\text{-}H_3$) at wavenumber 2358 cm^{-1}. All these functional groups are specific to the glycolipid category which confirms that the purified biosurfactant belonged to the same group.

To identify the specific congeners in the purified biosurfactant, LC-MS analysis was performed. The result of analysis showed that six monorhamnose congeners i.e. Rha-

$C_{12:1}$-C_{10}, Rha-C_{10}-C_{12}/Rha-C_{12}-C_{10} and Rha-C_{10}-$C_{14:1}$/Rha-C_{12}-$C_{12:1}$, Rha- C_{12}-$C_{12:1}$/Rha- $C_{12:1}$-C_{12} and Rha-C_{10}-C_{10}, and a dirhamnose congener Rha-Rha-C_8-C_{10} or Rha-Rha-C_{10}-C_8 in their specific *m/z* range were present in the biosurfactant. This confirms that the purified product belonged to rhamnolipid category, a kind of glycolipid which is exclusively produced by genus *Pseudomonas*.

The purified sample of biosurfactant was checked for its antagonistic behavior against the phytopathogen *A. brassicae* by agar well diffusion method in concentrations ranging from 0.1%, 1%, 2% and 5%. From the results, it was observed that 2% and 5% concentration showed maximum and almost similar range of zone of inhibition of the fungus around agar well containing metabolite as compared to control. Least inhibition was shown by 0.1%. This indicated that both 2% and 5% have same inhibitory effect on *A. brassicae* and biosurfactant with concentration of 2% could be applied for biocontrol analysis of this phytopathogen. The result was further confirmed by SEM analysis of the fungus treated with purified biosurfactant. Result of SEM analysis showed deformity and curling of mycelia at certain places where fungus was in contact with the metabolite. This reveals that purified biosurfactant was effective in inhibiting the growth of this phytopathogen.

To further assess the biocontrol and plant growth promoting potential of the selected isolate BSP9 and its bisurfactant, pot and field trials were conducted using *B. juncea* as test crop for two consecutive years. For this, talc based bioformulations were developed at labscale using *P. putida* BSP9 and amended with different concentrations of biosurfactant i.e. 0.1%, 1%, 2% and 5%. Sterilized seeds of *B. juncea* were treated with prepared bioformulation and sown according to different set of treatment both pot and field trial in completely randomized block design (RBD). The treatment for pot study were as follows (i) untreated control, C1 (ii) *A. brassicae* (negative control), C2 (iii) 0.1% biosurfactant + *A. brassicae,* T1 (iv) 1%

biosurfactant+ *A. brassicae*, T2 (v) 2% biosurfactant+ *A. brassicae*, T3 (vi) 5% biosurfactant+ *A. brassicae*, T4 (vii) BSP9+1% biosurfactant+ *A. brassicae*, T5 (viii) BSP9+2% biosurfactant+ *A. brassicae*, T6 (ix) BSP9+ *A. brassicae*, T7. In case of fungus, the seeds were subjected to infection with *A brassicae* after 6-7 days of seed germination by spraying spore suspension of spore suspension 10^5 spores/ml on adaxial and abaxial leaf surfaces and covered in polybags for 2 days to check incidence of disease. In case of field study treatment were given as 0.1% biosurfactant (T1), 1.0 % biosurfactant (T2), 2.0 % biosurfactant (T3), 5.0 % biosurfactant (T4), 1.0 % biosurfactant(T5) + BSP9, 2.0 % biosurfactant + BSP9 (T6), 5.0 % biosurfactant + BSP9 (T7), and untreated control (T8). All the treatments both in pot and field trial was applied in triplicates.

Pot study revealed that disease incidence of fungal phytopathogen *A. brassicae* was reduced with increase in concentration of biosurfactant in prepared bioformulations. Plants treated with combination of bacteria BSP9 and 2% and 5% concentration of biosurfactant showed almost no symptoms of the leaf blight as compared to negative control followed by treatment having BSP9 and 1% biosurfactant. Overall, when compared to negative control (plants treated only with *A. brassicae),* all the treated sets showed less disease symptoms. Similarly, all plant growth parameters including germination rate, root and shoot length, fresh and dry weight, number of pods, weight of 1000seeds per plant, chlorophyll and flavonoid content showed significant enhancement in their values as compared to untreated and negative control. Not only this, fungal enzymatic activity were observed to be reduced in case of treated plants in presence of fungus. Maximum enhancement was observed in case of plant treated with combination of BSP9 and 2% biosurfactant.

Like in case of pot study, plant growth parameters in field trial including germination rate, root and shoot length, total fresh and dry weight, number of pods, total oil,

chlorophyll and flavonoid content showed similar trend i.e metabolite and bacterial cell showing best results. Again, 2% biosurfactant combined with *P. putida* BSP9 treated plants showed maximum improvement in values of all categories of plant growth parameters compared to untreated control. It was also observed that all the treated plants were showing growth enhancement whether treated singly or combination when compared to control confirming the positive effect of bioformulation over treated plants.

A heatmap was also drawn from the above results showing hierarchal clustering of the treatments and its associated effects on each parameter. From the dendrogram it was revealed that with increasing concentration of biosurfactant, value of growth parameters increased and was highest at 2% after which i.e. at 5% a threshold was achieved. It was observed that plants treated with 2% and 5% biosurfactant both in combination and singly, showed almost similar values in every growth parameter as well as in phytochemical analysis. This is a significant conclusion that optimization of biosurfactant could be an important parameter for development of bioformulation based. Moreover use of 2% biosurfactant will be more cost effective in comparison to 5%.

From above study, it can be concluded that use of PGPR *P. putida* BSP9 and its biosurfactant for development of bioformulations could be an effective technique to mitigate the occurrence of Alternaria blight in *B. juncea*. The combination of bacteria and metabolite not only reduced incidences of disease symptopms (up to 80%) in plants but also significantly enhanced growth parameters of all treated plants both in pot and field trial. This could be a novel technique that can be utilized for development of biosurfactant based bioinoculants to increase the yield of mustard across the workd in sustainable manner and decrease our reliance on agrochemicals.

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Appendix

Appendix

CZAPEK'S MINERAL SALT

Ingredients	**gms/liter**
Carboxymethyl cellulose	5.0
Ammonium nitrate	1.0
Dipotassium hydrogen phosphate	1.0
Magnesium sulphate	0.5
Potassium chloride	1.0
Yeast Extract	0.5
Glucose	1.0
Agar	17
Distilled water	1000 ml

SIMMON'S CITRATE AGAR

Ingredients	**gms/ liter**
Ammonium dihydrogen phosphate	1.0
Dipotassium hydrogen phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulphate	0.2
Bromothymol blue	0.08
Agar	20.00
Distilled water	1000 ml

UREA AGAR

Ingredients	**gms/liter**
Peptone	1.0
Sodium chloride	5.0
Potassium monohydrogen (or dihydrogen phosphate) phosphate	2.0
Glucose*	1.0
Phenol red solution**	6.0 ml

Urea (20% aqueous solution)	100 ml
Agar	20.00
Distilled water	1000 ml

*Add glucose and phenol red to the molten base and steam for 1 hour, cool to 50°C.
**Add 100 ml urea (filter standardized solution) to the basal medium.

TRYPTONE WATER

Ingredients	**gms/liter**
Tryptone	10.0
Sodium chloride	5.0
Calcium chloride- Previously sterilized $CaCl_2$ was added after autoclaving	1.0 ml
Distilled water	1000 ml

SKIM MILK AGAR

Ingredients	**gms/liter**
Skim milk powder	100
Peptone	5.0
Agar	15.0

STARCH AGAR

Ingredients	**gms/liter**
Starch	20.0
Peptone	5.0
Beef Extract	3.0
Agar	15.0
Distilled water	1000ml

MR-VP BROTH

Ingredients	**gms/liter**
Peptone	7.0
Potassium phosphate	5.0
Dextrose	5.0
Distilled water	1000.00 ml

TWEEN 80 AGAR

Ingredients	**gms/liter**
Peptone	10.0
Sodium chloride	5
Calcium chloride dihydrogen	0.1
Tween 80	10
Agar	20
Distilled water	1000 ml
pH	6

GLUCOSE PEPTONE AGAR

Ingredients	**gms/liter**
Glucose	40
Peptone	5
Agar	20
Distilled water	1000 ml

YEAST EXTRACT GLUCOSE AGAR

Ingredients	**gms/liter**
Glucose	10
Dipotassium hydrogen phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Calcium carbonate	4
Yeast extract	1
Agar	20
Bromothymol blue	25 mg

PEPTONE WATER

Ingredients	**gms/liter**
Peptone	10.0
Sodium chloride	5.0

PIKOVSKAYA'S AGAR

Ingredients	**gms/liter**
Sodium chloride	0.500
Potassium dihydrogen phosphate	3.000
Magnesium sulphate	0.120
Calcium chloride dihydrate	0.013
Yeast extract	3.000
Disodium hydrogen phosphate	6.000

STAINS, INDICATORS, AND REAGENTS

a) Gram staining

Crystal violet solution: Dissolved 2.0 gm crystal violet in 20.0 m of 95% ethyl alcohol; Gram's Iodine solution: Mixed 1.0 gm Iodine, 2.0 gm potassium iodide in 300.0 ml of distilled water; Safranin: Dissolved 10.0 ml of safranin into 100.0 ml of distilled water

b) Indole production test

Kovac'sreagent: Dissolved the 5.0 gm of diaminobenzaldehyde in the 75.0 ml of amyl alcohol. Then added 25.0 ml of hydrochloric acid to the above preparation. Stored the reagent in the refrigerator.

c) MR-VP test

Methyl red indicator: Dissolved methyl red (0.1 gm) in 500 ml of 95% ethyl alcohol. Added distilled water and filtered the preparation.

VP reagent I: 5.0 gm alpha-naphthol was weighed and dissolved in 95.0 ml of absolute ethyl alcohol; VP reagent II 40% potassium hydroxide (KOH)

d) IAA production

Salkowski reagent : 2 ml 0.5M $FeCl_3$ and 49 ml water and 49 ml 70% perchloric acid.

e) Ammonia production

Nessler'sreagent: Dissolved 50.0 gm of potassium iodide in 35 ml of distilled water and added saturated solution of mercuric chloride. Added 400 ml of potassium hydroxide. Diluted to 1000 ml by addition of distilled water. Allowed to settle for one week. Stored in tightly stopper brown bottles.

f) HCN production

2% Sodium carbonate solution: Dissolved 2 gm of $NaCO_3$ in 100 ml of distilled water. 0.5% Picric acid solution: Mixed 0.5 gm picric acid in 100 ml of distilled water.

g) Amylase production

Gram's Iodine: 1.0 gm Iodine and 2.9 gm of Potassium iodide was mixed and added water to make total of 300 ml.

Research Publications

Research Publications

1. **Mishra I,** Fatima T, Egamberdieva D, Arora NK (2020) Novel bioformulations developed from *Pseudomonas putida* BSP9 and its biosurfactant for growth promotion of *Brassica juncea* [L.]. *Plants* 9(10):1349 DOI: 10.3390/plants9101349. **I.F-3.93**

2. Fatima T, **Mishra I**, Verma R, Arora NK (2020) Mechanisms of halotolerant plant growth promoting *Alcaligenes* sp. involved in salt tolerance and enhancement of the growth of rice under salinity stress. *3Biotech* 10(8). DOI: 10.1007/s13205-020-02348-5. . **I.F-2.40**

3. Kumari S, **Mishra I,** Mishra J, Prakash J, Arora NK (2020) Secondary metabolites from halotolerant plant growth promoting rhizobacteria for ameliorating salinity stress in plants. *Frontiers in Microbiology* 11:567768 DOI: 10.3389/fmicb.2020.567768/full. . **I.F-5.64**

4. Arora NK, Fatima T, Mishra J, **Mishra I**, Verma S et al. (2020) Halo-tolerant plant growth promoting rhizobacteria for improving productivity and remediation of saline soils. *Journal of Advanced Research* 12:69-82 DOI: 10.1007/s13205-020-02348-5. . **I.F-10.47**

5. Arora NK, Egamberdieva D, Mehnaz S, Li W-J, **Mishra I** (2021) Editorial: Salt tolerant rhizobacteria: for better productivity and remediation of saline soils. *Frontiers in Microbiology* 12:660075 DOI: 10.3389/fmicb.2021.660075. **I.F-5.64**

6. Arora NK., Fatima T, **Mishra I**, Verma M, Mishra J (2018) Environmental sustainability: challenges and viable solutions.

Environmental Sustainability 1: 309–340. DOI: 10.1007/s42398-018-00038-w

7. Arora NK, **Mishra I** (2021) COP26: more challenges than achievements. *Environmental Sustainability* 4:585–588 (2021).

8. Arora NK., **Mishra I** (2019) Ocean sustainability: essential for blue planet. *Environmental Sustainability* 3:1–3 DOI 10.1007/s42398-020-00100-6.

9. Arora NK., **Mishra I** (2019) United Nations Sustainable Development Goals 2030 and environmental sustainability: race against time. *Environmental Sustainability* 2: 339–342DOI:10.1007/s42398-019-00092-y.

Book Chapters

1. **Mishra I**, Arora NK (2019) Rhizoremediation: A Sustainable Approach to improve the Quality and Productivity of Polluted Soils. In: Arora NK (ed) Phyto & Rhizo Remediation, Springer, Singapore pp.33-66. DOI: 10.1007/978-981-32-9664-0_2
2. Arora NK, Fatima T, **Mishra I**, Verma S (2020) Microbe-based Inoculants: Role in Next Green Revolution. In: VKN Shukla (eds) Environmental Concerns and Sustainable Development, Springer, Singapore, pp.191-246, DOI 10.1007/978-981-13-6358-0_9

Awards

- Awarded as **"Young Scientist" by IUCN-CEM Agroecosystem Specialist Group Special Session conducted during International Conference on "Environmental Sustainability: Innovations, Translational Dimensions and Way Forward"** organized on 10th & 12th February, 2020 at BBA University, Lucknow, India.

- Awarded **Second prize in theme I ePoster presentation on "Role of rhizospheric pseudomonad BSP9 and its biosurfactant as a green approach to increase yield of *Brassica juncea*** held virtually at Department of Microbiology, Barkatullah University, Bhopal held during 3rd and 4th September , 2021.

plants

MDPI

Article

Novel Bioformulations Developed from *Pseudomonas putida* BSP9 and its Biosurfactant for Growth Promotion of *Brassica juncea* (L.)

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Abstract: In this study, *Pseudomonas putida* BSP9 isolated from rhizosphere of *Brassica juncea* was investigated for its plant growth promoting and biosurfactant producing activities. The isolate showed the ability to produce indole acetic acid, siderophore, phosphate solubilization activity and was an efficient producer of biosurfactant. Purification (of the biosurfactant) by thin layer chromatography (TLC) and further characterization by Fourier transform infrared spectroscopy (FTIR) revealed that biosurfactant produced by the isolate belonged to the glycolipid category, which is largely produced by *Pseudomonas* sp. In addition, liquid chromatography-mass spectroscopy (LC-MS) analysis showed the presence of a mixture of six mono-rhamnolipidic and a di-rhamnolipidic congeners, confirming it as a rhamnolipid biosurfactant. Bioformulations were developed using BSP9 and its biosurfactant to check their impact on promoting plant growth in *B. juncea*. It was noted from the study that bioformulations amended with biosurfactant (singly or in combination with BSP9) resulted in enhancement in the growth parameters of *B. juncea* as compared to untreated control. Maximum increment was achieved by plants inoculated with bioformulation that had BSP9 plus biosurfactant. The study also suggested that growth promotion was significant up to a threshold level of biosurfactant and that further increasing the concentration did not further enhance the growth parameter values of the plant. The study proves that novel bioformulations can be developed by integrating plant growth promoting rhizobacteria (PGPR) and their biosurfactant, and they can be effectively used for increasing agricultural productivity while minimizing our dependence on agrochemicals.

Keywords: *Pseudomonas putida*; PGPR; biosurfactant; bioformulation; glycolipids; rhamnolipids; *Brassica juncea*; sustainable agriculture

1. Introduction

Agricultural productivity is a worldwide concern and there is continuous pressure on this sector to meet the rising food demands of the ever-growing population. A report by the Food and Agricultural Organization (FAO) suggests that the world population will reach nine billion by 2050, and hence global agricultural production must increase by 70% [1]. In the race to enhance crop productivity, humans are adversely impacting the environment by applying high amounts of fertilizers and using intensive agronomic practices. Climate change coupled with natural disasters

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ORIGINAL ARTICLE

Mechanisms of halotolerant plant growth promoting *Alcaligenes* sp. involved in salt tolerance and enhancement of the growth of rice under salinity stress

Tahmish Fatima[1] · Isha Mishra[1] · Renu Verma[1] · Naveen Kumar Arora[2]

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Abstract
In the present study halotolerant bacteria were isolated from saline soil (EC ~ 11.9). Based on salt tolerance and plant growth promoting characteristics isolate AF7 was selected for further study. It was identified as *Alcaligenes* sp. on the basis of protein profiling and 16S rRNA sequence homology. Interestingly, AF7 showed diverse PGP characters at different salinity levels. While phosphate solubilization activity was expressed up to 300 mM NaCl, siderophore production was shown up to 700 mM, zinc solubilization up to 1000 mM and indole acetic acid (IAA), gibberellic acid (GA) and exopolysaccharides (EPS) production were depicted till 1400 mM. Correlative and regression analysis suggested positive relation between IAA, GA, EPS, siderophore production and zinc solubilization capability of AF7 and salinity up to 300 mM NaCl. EPS was found to be the most significant response and there was 263% increment in presence of 300 mM NaCl when compared to non-saline control. Analysis also showed that while growth promoting attributes were significant up to a threshold salinity level, further increasing the stress deviates the mechanism towards survival involving proline, antioxidant and hydroxyl scavenging activities. Combination of halotolerant AF7 and EPS showed more than twofold increase in the vegetative growth parameters of rice at ~ 170 mM NaCl (EC 9 dS/m). The study shows the mechanisms/metabolites of the plant growth promoting bacterium (PGPB) AF7 prominently involved during the salinity stress. Study also proves that novel bioformulations can be developed by integrative use of EPS and salt tolerant-PGPB which can be effective for saline soils.

Keywords Salinity stress · Plant growth promoting bacteria · Exopolysaccharides · Rice · *Alcaligenes*

Introduction

Soil salinity is a global menace which has challenged the food security and has affected the agricultural sustainability. Soil salinization is the second leading cause of land degradation and desertification (only after soil erosion) affecting several countries across the globe (Orhan 2016). Currently, almost 1 billion hectares of land on Earth is degraded due to salinity (FAO 2015). Arora et al. (2018) highlighted the direct relationship between anthropogenic activities and increase in saline lands. Saline soils are characterized with electrical conductivity (EC_e) greater than 4 dS/m at 25 °C and excess of accumulated salts of Na^+, Ca^{+2}, Mg^{+2}, Cl^{-1}, SO_4^{-2}, CO_3^{-2} (Shahid et al. 2018). Salinity negatively impacts the physical structure and nutrient status of soil and thereby the growth and productivity of crops. The presence of high salt concentration restricts the uptake of minerals, nutrients, and water (Machado and Serralheiro 2017). Plant physiology is also impacted by salt toxicity resulting in reduced photosynthesis rate, lower germination and growth rate along with impaired yield. The hyperosmotic conditions cause loss of water in plant cell sap, and subsequent ionic imbalance results in reduction of enzyme activities, disrupted membrane structure and cell plasmolysis (Munns and Tester 2008; Rahneshan et al. 2018).

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MINI REVIEW
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Secondary Metabolites From Halotolerant Plant Growth Promoting Rhizobacteria for Ameliorating Salinity Stress in Plants

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Soil salinization has emerged as one of the prime environmental constraints endangering soil quality and agricultural productivity. Anthropogenic activities coupled with rapid pace of climate change are the key drivers of soil salinity resulting in degradation of agricultural lands. Increasing levels of salt not only impair structure of soil and its microbial activity but also restrict plant growth by causing harmful imbalance and metabolic disorders. Potential of secondary metabolites synthesized by halotolerant plant growth promoting rhizobacteria (HT-PGPR) in the management of salinity stress in crops is gaining importance. A wide array of secondary metabolites such as osmoprotectants/compatible solutes, exopolysaccharides (EPS) and volatile organic compounds (VOCs) from HT-PGPR have been reported to play crucial roles in ameliorating salinity stress in plants and their symbiotic partners. In addition, HT-PGPR and their metabolites also help in prompt buffering of the salt stress and act as biological engineers enhancing the quality and productivity of saline soils. The review documents prominent secondary metabolites from HT-PGPR and their role in modulating responses of plants to salinity stress. The review also highlights the mechanisms involved in the production of secondary metabolites by HT-PGPR in saline conditions. Utilizing the HT-PGPR and their secondary metabolites for the development of novel bioinoculants for the management of saline agro-ecosystems can be an important strategy in the future.

Keywords: salinity, sustainable agriculture, secondary metabolites, halotolerant PGPR, exopolysaccharides, bioinoculants

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INTRODUCTION

The anthropocene era is facing rigorous environmental stress due to aggravated human activities combined with rapid pace of climate change that is negatively affecting agricultural production and its sustainability. Soil salinity is one of the major global issues which is undermining crop yield and jeopardizing productive capacity of soils (Arora et al., 2018). The declining soil fertility and inadequate crop productivity has raised serious concerns for food security of ever rising human population. Reports claim that soil salinity has affected several countries of the world up

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Halo-tolerant plant growth promoting rhizobacteria for improving productivity and remediation of saline soils

Naveen Kumar Arora [a,*], Tahmish Fatima [b], Jitendra Mishra [c], Isha Mishra [b], Sushma Verma [b], Renu Verma [b], Maya Verma [d], Ankita Bhattacharya [a], Priyanka Verma [a], Priya Mishra [a], Chanda Bharti [a]

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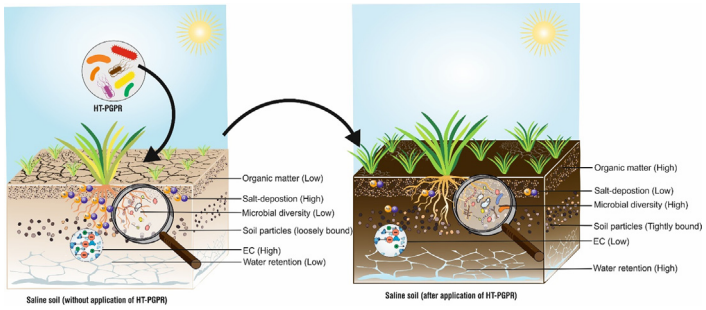
GRAPHICAL ABSTRACT



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ABSTRACT

The collective impact of climate change and soil salinity is continuously increasing the degraded lands across the globe, bringing agricultural productivity and food security under stress. The high concentration of salts in saline soils impose osmotic, ionic, oxidative and water stress in plants. Halo-tolerant plant growth promoting rhizobacteria (HT-PGPR) are emerging as efficient biological tools to mitigate the toxic effects of high salt concentrations and improve the growth of plants, simultaneously remediating the degraded saline soils. HT-PGPR are involved in alleviating the salinity stress in plants through a number of mechanisms evoking multipronged physiological, biochemical and molecular responses. These include changes in expression of defense-related proteins, exopolysaccharides synthesis, activation of antioxidant machinery, accumulation of osmolytes, maintaining the Na^+ kinetics and improving the levels of phytohormones and nutrient uptake in plants. The modification of signaling by HT-PGPR inoculation under stress conditions elicits induced systemic resistance in plants which further prepares them against salinity stress. The role of microbial-mechanisms in remediating the saline soil through structural and compositional improvements is also important. Development of novel bioinoculants for saline soils based on the concepts presented in the review can be a sustainable approach in improving productivity of affected agro-ecosystems and simultaneously remediating them.

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frontiers in Microbiology

EDITORIAL
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Editorial: Salt Tolerant Rhizobacteria: For Better Productivity and Remediation of Saline Soils

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Keywords: saline soils, salt tolerant rhizobacteria, plant growth promoting rhizobacteria, bioremediation, agro-ecosystems

Editorial on the Research Topic

Salt Tolerant Rhizobacteria: For Better Productivity and Remediation of Saline Soils

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Soil salinity has been recognized as a major issue particularly in arid and semi-arid areas of the world and is one of the main constraints that undermine plant growth and agricultural productivity. The pace at which saline soils are increasing around the globe has posed a serious threat to food security, the environment, and biodiversity. The Research Topic entitled "Salt Tolerant Rhizobacteria: For Better Productivity and Remediation of Saline Soils" is focussed on reviews and research articles on major challenges caused by soil salinization in agroecosystems and its remediation using salt-tolerant rhizobacteria as sustainable solutions to increase the productivity of these degraded lands.

The electrical conductivity (EC) of saline soils is either equal to or exceeds 4dS/m. Saline soils have a higher concentration of the salts Na^+, Cl^-, Ca^{2+}, HCO^{3-}, Mg^{2+}, NO^{3-}, and SO_4^{2-}, which prove detrimental to the microbial communities of the soil. The problems associated with salt accumulation are visible in several vital functions of soil such as poor water holding capacity and structural stability, reduced infiltration rate, disturbed pH, decreased levels of nutritional content, and lower organic matter. A study by Wang et al. explored the soil factors determining the structure and composition of bacterial communities in saline soils of Songnen Plain, China to dig out the potential of microbial resources. Systemic analysis using high throughput sequencing (Illumina MiSeq sequencing) revealed that the EC of the soil is one of the direct environmental factors that control the distribution of bacterial communities. The above concept was also explained in another research article by Yang et al., in which a metagenomic approach and *NifH* Illumina sequencing has been carried out for the characterization of rhizosphere and nodule microbiomes of wild salt-tolerant soybean growing in saline-alkaline soils of China. The study provides a systematic and functional understanding of the plant root microbiome under saline-alkaline conditions. Yang and Sun, have presented a similar correlation by showing how changes in soil properties regulate soil fungal communities and affect their distribution patterns and ecological functions.

There are myriad impacts on crops grown in salt stress conditions which have been interestingly detailed in a review by Kumar et al.. Such crops show signs of nutrient deficiency, ionic toxicity, oxidative stress, reduced photosynthetic activity, and decreased germination rate resulting in lower

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REVIEW

Environmental sustainability: challenges and viable solutions

Naveen Kumar Arora[1] · Tahmish Fatima[2] · Isha Mishra[2] · Maya Verma[2] · Jitendra Mishra[3] · Vaibhav Mishra[4]

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Abstract
Since last century or so anthropogenic activities have intensely metamorphosed the earth's ecosystem and resulted into major environmental changes. Widespread interference of human related activities have resulted in major problems including environmental pollution, land degradation, global warming/climate change, paucity of potable water supply and biodiversity loss. These issues have directly affected the quality and sustainability of the ecosystems. In addition, these activities have resulted in loss of habitats resulting in mass extinction of species which in itself is a matter of great concern. Studies and data clearly show that if present trends continue the conditions are expected to worsen in the coming time and human civilization itself will be in trouble. To minimize this crisis, possible green solutions like use of microbes and biotechnological tools are gaining importance and need further attention in order to lessen or remediate the harmful effects of anthropogenic activities thus ensuring environmental sustainability.

Keywords Environmental sustainability · Anthropogenic activities · Climate change · Greenhouse gases · Pollution · Microorganisms · Bioremediation · Biotechnology · Biofuels · Sustainable agriculture

Introduction

Earth is the only celestial body in the universe where life is known to exist and that too with such a huge diversity. However, it is now becoming more and more inhabitable for most of the species as indicated by steep decline in diversity of flora and fauna. The main reasons behind the worsening conditions for life on earth are the anthropogenic activities. The human interference has resulted in increase in concentration of greenhouse gases (GHGs), climate change, degradation of land, pollution of air water and soil, depletion of non-renewable resources, loss of biodiversity, accumulation of harmful recalcitrant chemicals and several related issues.

Since the last few decades the impact of environmental problems has been highlighted at several forums. In 1960s, the United Nations (UN) discussed the environmental costs of growth-centered or conventional development. In the report 'Our Common Future' (known as the Brundtland Commission), released in 1987 by the World Commission on Environment and Development (WCED), it was stated that development is only 'sustainable' if it 'meets the needs of the present without compromising the ability of future generations to meet their own needs' (WCED 1987). WCED global agenda was an initiative to work on sustaining the planet for better future. Similarly, the UN Conference on the Environment in Stockholm and on Environment and Development (UNCED) in Rio de Janeiro (1992) accepted that the un-sustained human activities towards environment will cause huge, irreversible damage to life on earth (http://www.un.org/geninfo/bp/enviro.html). The UN Agenda 21 was to promote sustainable development undertaken at the Earth Summit held at Rio de Janeiro. The agenda formed the basis for a "global partnership" to encourage cooperation among nations for sustaining life on earth by protecting the environment while simultaneously resulting in growth. For reducing the climate change, countries of the world adopted the Paris

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EDITORIAL

COP26: more challenges than achievements

Naveen Kumar Arora[1] · **Isha Mishra**[2]

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"Conference of the Parties" or COPs is a global climate summit which was started by United Nations (UN) to bring all the countries of the world together in order to escalate goals of Paris Agreement (2015) and the UN Framework Convention on Climate Change (UNFCCC) (a treaty that came into force in 1994) (https://ukcop26.org/wp-content/uploads/2021/07/COP26-Explained.pdf). This year (2021) was the 26th annual summit of COPs, hence given the name COP26 and was held at Glasgow, United Kingdom (UK) in partnership with Italy. COP26 is one of the biggest and most significant summits on climate change since the Paris Agreement in 2015) (https://www.nationalgrid.com/responsibility/environment/cop26). Paris Agreement was an international treaty on climate change and was taken up by 196 Parties at COP21where countries were determined to put forward the plans on how to reduce their emissions, known as Nationally Determined Contributions, or NDCs. The main target was to restrict global warming to below 2 °C, preferably 1.5 °C when compared to pre-industrial era (https://unfccc.int/process-and-meetings/the-paris-agreement/the-paris-agreement). In COP26 nearly 200 countries participated to adopt the 'Climate Pact', which is directly related to Paris Agreement rulebook and is a way forward to keep the aims of the treaty alive in order to spike the decarbonisation of the global economy (https://ec.europa.eu/commission/presscorner/detail/en/ip_21_6021). The Glasgow meeting made few progresses, such as cutting down on emissions, declaration on zero-emission vehicles to promote greener transport and acknowledging deforestation, but looking at the outcome of the conference, the evaluation reveals that the targets have not been achieved and overall the roadmap is not very clear. The signatories do not seem to be unified against the environmental menace, just like the nations have failed to show united front to COVID-19.

There are four major goals that have been set up to achieve at COP26. First is to secure global net zero carbon by mid-century, keeping 1.5 °C within reach (https://ukcop26.org/cop26-goals/). To keep the first target within reach global emissions must be reduced by 45% by 2030 in comparison to levels of 2010 (https://www.nature.com/articles/d41586-021-03431-4). Climate change is no more a low-level issue but has become a life threatening global emergency. According to a study by Climate Action Tracker, even if the pledges announced at the COP26 meeting are enforced, still temperatures are predicted to rise by 2.4 °C up to 2100. This is much above the target of 1.5 °C accepted at the Paris Agreement and the effects of this are likely to be catastrophic in future (https://www.nature.com/articles/d41586-021-03433-2). In fact, in the latest report of Intergovernmental Panel on Climate Change (IPCC), it has been revealed that the planet is going to witness the limit of 1.5 °C in early 2030s itself, triggering irreversible changes in our environment (such as flooding, drought, bushfires, and destruction of species) and called it 'code red for humanity' (https://www.ipcc.ch/sr15/). According to a global climate report published by World Meteorological Organisation (WMO) (2021), despite the fact that the world was stalled due to pandemic caused by novel corona virus (COVID-19) for last 2 years, yet the global mean temperature in 2021 i.e. between January and September was 1.08 ± 0.13 °C more than the 1850–1900 (pre-industrial era). The report also suggests that as a result of rising temperatures, global sea levels have also risen by 4.4 mm/year between 2013 and 2021 (State of the Global Climate 2021 https://library.wmo.int/doc_num.php?explnum_id=10859). Oceans are known to produce 50% of atmospheric oxygen, absorb 25% of anthropogenic carbon dioxide (CO_2) emissions and trap 90% of excess heat in the climate system. To highlight the important connect between oceans and climate change, European Union (EU) called for 'EU Ocean Day' at COP26. However, acidification of oceans due to unprecedented rate of global warming and overexploitation of its resources remains to be

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EDITORIAL

Ocean sustainability: essential for blue planet

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Oceans cover over 70% of earth's area which is more than two thirds of the planet, providing huge services to mankind and other life forms. But since recent past, these natural treasures are facing increasing constraints due to unprecedented anthropogenic activities which have negatively hampered their sustainability. Oceans are now being over-exploited for food supply, as transport of goods and humans, and a convenient dumping zone for various types of wastes. With technological advancements humans have accessed even the remotest parts of marine ecosystems. This has caused severe degradation of oceans, resulting in lack of replenishment and a catastrophe in making. Rising sea levels due to rapid pace of global warming, acidification due to increasing carbon emissions, unsustainable overfishing, and ocean pollution due to industrialization, agricultural wastes and oils spills are some of the major threats faced by marine life (Arora and Mishra 2019).

Reports claim that global mean sea level have risen by 8–9 inches since 1880, mainly due to melting of ice sheets and glaciers and thermal expansions in sea water. It has also been reported that the year 2018 witnessed sea level rise of 3.2 inches which is the highest recorded (for a single year till that year) (https://www.climate.gov/news-features/understanding-climate/climate-change-global-sea-level). The Intergovernment Panel on Climate Change (IPCC) has predicted that even if the current greenhouse gas (GHG) emissions are reduced, the global mean sea level could still rise by 30 to 60 cm by 2100 (https://www.ipcc.ch/2019/09/25/srocc-press-release/). Similarly, Kwok (2018) reported that thickness of Arctic ice sheet has been lost by 50% during 1977–2017. In the same context, loss of Antarctic ice sheet has tripled between 2007 and 2016 (IPCC 2019). The rise in sea level will threaten the human population and infrastructure, particularly in coastal regions, and especially during deadly cyclones/storms or tsunamis, which can cause havoc in the mainland and especially in Small Island Development States (SIDS) which have low resilience to such extreme weather events. On the other hand, high levels of carbon emissions are causing acidification of oceans. Unchecked pace of deforestation, burning of fossil fuels, shrinkage in available land due to population explosion, industrialization, are some of the key factors responsible for increasing CO_2 levels. Oceans are regarded as sinks of CO_2, and as it dissolves in water, formation of carbonic acid takes places decreasing the pH. Ocean acidity is projected to increase by up to 0.042 pH units by 2100 (Gaines et al. 2019). These alterations in chemistry of ocean systems adversely affect sea creatures such as corals, oysters, sea urchins, sea shells and other calcareous phytoplanktons. Acidification also affects the behaviour, growth and reproduction process in marine life. This can have profound effects on markets based on marine products. Moreover, global warming is also interrupting the circulation pattern of the ocean known as 'global conveyor belt' which is responsible for maintaining equilibrium in heat distribution on the globe.

The injudicious amount of pollution faced by marine environment has adverse effects on the biology of oceans which has entered at several levels of food chain. The human activities on land are the major drivers of pollution in water bodies. Waste runoff from agricultural farms (e.g. fertilizers) trigger the formation of algal blooms, resulting in eutrophication of oceans, suffocating the marine life. Moreover, release of contaminants such as heavy metals, petroleum hydrocarbons, pesticides, sewage and radioactive disposal get absorbed by tiny planktons, which when consumed by other small fishes enter the food chain and get biomagnified at different trophic levels. Consumption of these infected sea foods by humans cause genetic disorders, cancer, and infertility, to name a few. Plastic pollution is another menace playing havoc in oceans which has resulted in great loss of ecosystem services on the whole. A report by Our World in Data estimates that about 80% of plastic pollution in oceans

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EDITORIAL

United Nations Sustainable Development Goals 2030 and environmental sustainability: race against time

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United Nations Sustainable Development Goals (SDGs), established on September 15, 2015 entitled as "Transforming our world: the 2030 Agenda for Sustainable Development", are a follow up of Millennium Development Goals (MDGs) but with some major differences. On one hand, where MDGs were mainly focussed on poverty and hunger (largely of less developed countries), SDGs have 17 agendas covering around 169 targets and is applicable to all the countries and regions of the globe. The goals are much broader in their terms, extending to social and economic aspects of human society and their dimensions with natural environment, by putting sustainability at the centre. The two key features i.e. economic security and environmental sustainability have been mainly highlighted in the wide range of categories. There is no denying the fact that to achieve the SDGs by 2030 is an ambitious project and its success will definitely obviate various issues related to sustainability of life on earth. Several of the targets set in SDGs were to be achieved by 2020, which means only a year to go. At this point of time it becomes important to review the scenario on what has been achieved and what is lacking.

In a very recent report by Global Sustainable Development Report (2019), UN Secretary-General António Guterres has stated, "Our world as we know it and the future we want are at risk. Despite considerable efforts these past 4 years, we are not on track to achieve the Sustainable Development Goals by 2030". A recent report on SDGs index based on the monitoring of Sustainable Development Solutions Network (SDSN) has revealed that no country is in line to achieve targets of 2030 and slowest progress has been witnessed mainly on goals focussed on environment (Sachs et al. 2019). Only a very few countries (Sweden, Denmark and Finland) have achieved three quarters of the UN goals. Organization for Economic Co-operation and Development (OECD) which includes the best performers, also needs to do better in controlling climate change and sustainable consumption (https://www.bertelsmann-stiftung.de/en/topics/latest-news/2019/june/long-in-words-but-short-on-action-un-sustainability-goals-are-threatened-to-fail/). In the same way, another report by Global Opportunity Project and United Nations Global Compact (UNGC) based on a publication of 2016 titled as "The Future of Spaceship Earth" explains that there would surely be improvements in some areas in some regions of the globe but overall most of the goals are in red in most of the areas of the planet and will definitely miss the targets. Among the targets, environmental issues lack the most and will nullify other achievements if not tackled on priority.

Goals 6, 7, 12, 13, 14 and 15 are directly related to environmental sustainability. The targets in these goals are mainly linked to natural environment but are lagging far behind and seem impossible to achieve in due time frame. The aforementioned goals are devoted for clean water and sanitation, affordable and clean energy, sustainable consumption of natural resources, climate change, life below water and on terrestrial ecosystems, halting biodiversity loss and combating land degradation and desertification. Clean water and sanitation is the basic human right but alarmingly it has been denied to billions of people across the globe. World Water Development Report (2019) explains that since 1980, water consumption has been increasing at a rate of 1% per year and is expected to grow up to 3% by 2050 (https://www.unwater.org/publications/world-water-development-report-2019/). As per the analysis of Water Aid (2017), it was found that one in ten people still has no access to clean water and 31% lack basic sanitation facilities (https://washmatters.wateraid.org/sites/g/files/jkxoof256/files/How%20to%20get%20water%20and%20sanitation%20to%20eve

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Microbe-based Inoculants: Role in Next Green Revolution

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Naveen Kumar Arora, Tahmish Fatima, Isha Mishra, and Sushma Verma

Abstract

Increasing food demand, with growing population, has been a major concern throughout the globe. The aim can only be achieved with the onset of next green revolution being much defined by sustainable approaches. The past green revolution had its negative impact due to excessive use of agrochemicals contaminating the environment and further challenging the food security. Henceforth, designing the blueprint of next green revolution requires the application of effective and sustainable approaches which enhance the yield of plants while still maintaining the decorum of sustainability. In this regard, microbes have been concluded as the best players finding their roles in plant growth promotion and also stress management. Currently, there are several bacterial-, fungal-based inoculants available in the market along with genetically modified organisms, forming the base of upcoming green revolution. Thus, the future of sustainable agriculture is related to the efficiency and action of these microbes.

Keywords

Microbial inoculants · Green revolution · Environmental sustainability · Plant growth-promoting rhizobacteria (PGPR) · Stress

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Soils

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Chapter
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Abstract

Soil contamination is a widespread problem which has been causing deleterious changes in the biology, structure, and also its productivity. Various recalcitrant and xenobiotic compounds, due to rapid pace of anthropogenic activities, have accumulated in soil resulting in its degradation and infertility. In addition to this, persistent nature of these pollutants allows them to enter into the food chain posing serious threats to living beings. Therefore, a holistic and sustainable approach which is ecofriendly, cost-effective, and organic in nature, is the need of the hour. Rhizoremediation is one such method which could obviate the problem of such hazardous compounds from soils. The co-evolutionary relationship between plant and their associated microbiota is being successfully used to reclaim and restore degraded soils without causing any harmful by-products unlike conventional methods. Not only this, application of rhizoremediation technique is also reported to improve soil organic matter (SOM), nutrient cycling, bioavailability of insoluble compounds, which in turn enhances biomass production rendering the soil fertile and productive for better agronomic purposes. Recent advances in genetic engineering and “omics” techniques have further strengthened our knowledge in this area which when exploited in future could be used to alleviate the problem of soil contamination with precision and in shortest possible time. In this way, this green technology along with amalgamation of biotechnological tools could be envisaged as an excellent substitute to chemical and physical methods to remediate contaminated soil making it fertile and productive.

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