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

SUMMARY OF

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

LUCKNOW

BABASAHEB
BHIMRAO
AMBEDKAR
UNIVERSITY

LUCKNOW

प्रज्ञा शील करुणा

ESTABLISHED 1996

FOR THE DEGREE OF

Doctor of Philosophy

IN

ENVIRONMENTAL MICROBIOLOGY

Submitted By

Isha Mishra

(Enrolment No. 392/12)

Under the Supervision of

Prof. Naveen Kumar Arora

DEPARTMENT OF ENVIRONMENTAL MICROBIOLOGY

SCHOOL FOR ENVIRONMENTAL SCIENCES

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

(A CENTRAL UNIVERSITY)

VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW-226 025

UTTAR PRADESH, INDIA

2021

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.