Summary

The chemical pesticides have been conventionally used for controlling the pathogens causing pre and post-harvest losses of agricultural produce. The extensive use of these chemicals over the years has resulted in emergence of resistant variants of pests, thereby reducing their efficacy and restricting their further use. Therefore, studies to develop BCAs, using natural micro-organisms or their products to control pests as environmental friendly alternative to chemical pesticides, have gained considerable attention. The effective control of pests causing post harvest damage to cereals (wheat, rice and maize) and fruits (tomatoes, mangoes, apples) had been reported by use of bacterial isolates or their biomolecules. The different mechanisms reported for suppression of pathogens includes competition for nutrients; production of antimicrobial metabolites such as glycolipids, lipopeptides, phospholipids and fatty acids; and induction of systemic resistance in host plant to these pathogens. The molecules *viz.* rhamnolipids, massetolide A, surfactin and fengycin produced by bacterial isolates are generally known to elicit specific microbe associated molecular patterns. Massetolide A produced by *Pseudomonas* spp. and surfactin by *Bacillus* spp. had been reported to induce defence responses in melon plants and reduce powdery mildew disease caused by *Podosphaera fusca*. Different *Bacillus* spp. and *Streptomyces* spp. are also known for their insecticidal potential against pests such as *Bactrocera oleae* (Gmelin), *Helicoverpa armigera* (Hubner), *Spodoptera litura* (Fabricius) and *S. littoralis*, damaging more than 150 species of host plants.

A single bacterial strain co-producing different types of biomolecules might be more suitable for field scale application due to its synergistic and broad spectrum antifungal activity rather than using combination of different biocontrol agents to have effective control of fungal infestation. Different strains of *B. subtilis* have emerged as potential candidates for developing bioformulations due to their ability to co-produce different CLPs belonging to surfactin, iturin A and fengycin families, active against broad range of fungal pathogens. The field application of commercial bio-formulations based on bacterial isolates *viz.* Serenade (*Bacillus subtilis*), Biosave (*Pseudomonas syringae*), Mycostop (*Streptomyces griseoviridis*), Bt (*B. thuringiensis*) had been
reported to control the spread of different pathogens/pests on economically important vegetables and cereal crops. However, their application is still not as prevalent as chemical pesticides due to their inconsistent performance under different agro-climatic zones. Thus, formulations based on indigenous microflora can provide long-term protection from pathogens, as they can establish in the rhizosphere of host plant and effectively colonize the roots for eliciting defence responses in host plant. Presently, another drawback of BCA based pest control strategies is their inability to provide complete protection against diverse pathogens. This limitation can be addressed by developing mixed formulation of BCA with chemical fungicides so as to develop integrated pest management strategies which may broaden the disease control spectrum with lower use of chemical fungicides.

Therefore, the present study was taken up with an aim to isolate and screen the indigenous rhizospheric bacterial diversity for potential antifungal biomolecules. The summary of results obtained from the experimental work carried out is presented below:

**Isolation and Screening of bacterial isolates for antifungal potential**

The rhizospheric soil and root samples were collected from agricultural fields in State of Punjab (India), cultivating wheat, rice, sugarcane, tomato, onion, maize, potato, cucumber and mustard, during different cropping seasons. A total of 201 morphologically distinct bacterial isolates were obtained, with 171 isolates obtained from rhizospheric soil samples and 30 endophytic isolates obtained from roots of different plants. All the isolates were further screened for their antifungal potential against 7 different phytopathogenic fungi *viz.* *Alternaria alternata, Colletotrichum* sp, *Fusarium oxysporum, Fusarium moniliforme, Helminthosporium* sp., *Magnaporthe grisea* and *Rhizoctonia oryzae* by dual culture plate assy. Out of 201 bacterial isolates, 20 isolates affording antifungal activity against all the 7 phytopathogenic fungi were selected for further studies.

The bacterial isolates were characterized by routine microscopic and biochemical protocols, and were tentatively identified by 16S rRNA oligonucleotide cataloguing. The isolate R2, R5, N4, M136, M56, S8, S9, R11, P5, P4, A22, RC3, RC16 and A2 belonged to different species of genus *Bacillus*. Isolate M32 and M34 were identified as *Brevibacterium* spp., whereas isolate A28, SB11, S41 and N16 were
identified as *Pseudomonas* sp., *Paenibacillus polymyxa*, *Gordonia lacunae* and *Pseudomonas extremaustralis* respectively.

**Production and extraction of antifungal biomolecules**

The cells of selected isolates (20) were grown in YME broth and the biomolecules were extracted from the CFS by acid/alkaline precipitation or solvent extraction. The precipitation of biomolecules in CFS of isolates R2, R5, R11, M32, M34, M56, M136, S8, S9, N4, A2, A22, P5, RC3 and RC16 was observed when pH was lowered to 2.0. The CFS of isolates N16, SB11, A28, S41 and P4 did not show any precipitation under both acid and alkaline condition and their CFS were extracted with different solvents to extract biomolecules. The yield of antifungal molecules by different extraction protocols ranged from a minimum of 0.21 g L$^{-1}$ in case of isolate M56 to a maximum of 0.89 g L$^{-1}$ for isolate R2. The stock solutions (1% w/v) of extracted biomolecules from respective bacterial isolate were prepared in methanol and used for further studies.

**Antifungal activity of bacterial extracts against different phytopathogenic fungi**

The antifungal potential of extracted biomolecules was evaluated against seven (7) different fungal pathogens by agar well diffusion and TLC based agar overlay assay. It was evident from the agar well diffusion assay studies that the APB of isolate R2 supported a clear inhibition zone of 18, 25, 25 and 26 mm against *F. moniliforme*, *Helminthosporium* sp., *Colletotrichum* sp. and *A. alternata* respectively and a hazy inhibition zone of 27 mm against *R. oryzae*, which were higher among all other isolates. On the other hand, the SEB of isolate N16 and APB of isolate M32 supported clear inhibition zone of 17 and 21 mm against *F. oxysporum* and *M. grisea* respectively, which were higher than observed for biomolecules of other isolates.

Further, the crude extracts of isolates supporting clear zones of inhibition against fungal pathogens in agar well diffusion assay were used to perform TLC based agar overlay assay. The biomolecules of different isolates supported varied inhibition patterns of growth for different fungal pathogens in comparison to control plates, thus indicating to diversity of biomolecules produced by different bacterial isolates. On the basis of these observations and higher antifungal activity among the screened isolates,
R2 (*Bacillus vallismortis*), M32 (*Brevibacterium* spp.) and N16 (*Pseudomonas extremaustralis*) were selected for further studies.

**Optimization studies for improving the production of antifungal biomolecules**

The effect of different physico-chemical parameters and nutrient supplements on the yield of biomolecules of isolate R2, M32 and N16 respectively was evaluated. The antifungal potential of the APB/SEB of isolates R2, M32 and N16, produced under different set of conditions against pathogen *A. alternata, M. grisea* and *F. oxysporum* respectively was determined by agar well diffusion assay.

The effect of incubation period, pH, temperature and incubation under static/shaking conditions on yield and antifungal activity of biomolecules of isolate R2, M32 and N16 was evaluated. The cells of isolate R2, M32 and N16 grown in YME broth (pH 7.0) incubated at 30°C supported maximum yield and antifungal activity of biomolecules after an incubation of 48 h. The maximum yield and antifungal activity of biomolecules of isolate R2, M32 and N16 was observed when the flasks were incubated at 100, 150 and 200 rpm respectively.

The effect of different types of growth media *viz.* PM, YME, LB, TSB, NB, KB and MSM broth on yield and antifungal activity of biomolecules of isolate R2, M32 and N16 was studied. The cells of isolates R2 and M32 grown in PM broth and cells of isolate N16 grown in KB broth supported maximum yield and antifungal activity of biomolecules. Therefore, the respective broths were used for further studies for these isolates.

The effect of addition of different sources of carbon *viz.* glucose, glycerol, fructose, lactose, sucrose and starch to respective growth medium on yield and antifungal activity of biomolecules of isolate R2, M32 and N16 was evaluated. It was observed that among all the carbon sources, 2.5% (w/v) and 2% (w/v) supplement of glucose to PM broth supported maximum yield and antifungal activity of biomolecules of isolates R2 and M32 respectively, and 2% (v/v) glycerol supplement to KB broth supported best results for isolate N16. These supplements to respective media were used for further studies.
The effect of addition of different organic nitrogen sources *viz.* YE, BE, ME, peptone and tryptone to growth medium on yield and antifungal activity of biomolecules of isolate R2, M32 and N16 was also studied. The 2% (w/v) supplement of YE for isolate R2 and 2% (w/v) supplement of peptone for isolates M32 and N16 supported maximum yield and antifungal activity of biomolecules.

The effect of addition of different inorganic nitrogen sources *viz.* NaNO₃, NH₄Cl, NH₄H₂PO₄, (NH₄)₂SO₄ and NH₄NO₃ to growth medium on yield and antifungal activity of biomolecules of isolate R2, M32 and N16 was evaluated. The 0.5% (w/v) NaNO₃ supplement to PM broth supported maximum yield and antifungal activity of biomolecules of isolates R2 and M32, whereas no effect of different inorganic nitrogen supplements on yield and antifungal activity of biomolecules of isolate N16 was observed.

The overall increase of 4.25, 3.58 and 1.89 folds in the yield of biomolecules of isolates R2, M32 and N16 respectively was achieved after optimization studies. The inhibition zones afforded by biomolecules extracted by cells grown under optimised conditions also improved as compared to those of cells grown under un-optimized conditions.

The renewable and non-conventional carbon sources were explored for their potential to support production of biomolecules by the isolates so as to lower the cost of production. The carbon source in PM and KB broth was replaced with by-products of agro- and food industries *viz.* cane molasses and waste/spent frying oils (sunflower oil, soyabean oil and rice bran oil). It was observed that addition of soyabean oil (2.5% v/v) to growth medium for isolate R2 and sunflower oil (2% v/v) to growth medium of isolates M32 and N16 supported maximum yield and antifungal activity of biomolecules. Although, the yield of biomolecules was significantly lower than the conventional carbon supplements, however the same could be improved by undertaking optimization studies to make it commercially competitive with chemical fungicides.

**Potential of cells and biomolecules of bacterial isolates to control fungal infestation on different plants**

The potential of cells and biomolecules of isolate R2 was evaluated to control infestation of wheat, rice and tomato by *A. alternata, M. grisea* and *F. oxysporum*.
respectively. The ability of cells and biomolecules of isolate M32 and N16 was evaluated to control infestation of rice and tomato plants by *M. grisea* and *F. oxysporum* respectively. The plants/plant parts infected with spore suspension (10^4 spores mL^{-1}) of respective fungal pathogen were treated with either cell suspension (10^8 CFU mL^{-1}) of respective bacterial isolate or different concentrations of biomolecules.

The treatment of leaves of wheat infected with *A. alternata*, with cells and APB of isolate R2 resulted in 77.34% and 93.2% reduction in infection respectively as compared to infected leaves not treated with BCA. The rice leaves infected with *M. grisea* and treated with cells and APB of R2 supported 71.32% and 94.35% reduction in infection, and cells and APB of isolate M32 supported 54.83% and 80.95% reduction in infection respectively. The tomato leaves infected with *F. oxysporum* and treated with cells and APB of isolate R2 supported 77.0% and 95.2% reduction in fungal infection, on the other hand the treatment of *F. oxysporum* infected tomato leaves with cells and SEB of isolate N16 supported 64.0% and 88.4% reduction in infection respectively.

The treatment of wheat grains infected with *A. alternata*, with cells, 25 mg and 50 mg APB of R2 resulted in 65.0%, 74.44% and 82.21% reduction in disease incidence respectively as compared to untreated infected grains. Similarly, the rice grains infected with *M. grisea* and treated with cells and APB (25 mg) of R2 resulted in 67.1% and 90.81% reduction in disease incidence and in case of infected rice grains treated with cells and APB (25 mg) of isolate M32 a reduction in disease incidence by 46.05% and 78.95% respectively was observed. The infected grains treated with BCA germinated into healthy plants, whereas those not treated with BCA did not germinate into healthy plants and showed significant reduction in their overall growth.

The tomatoes infected with *F. oxysporum* and treated with cells and APB (10 mg mL^{-1}) of R2 showed reduction in infection by 67.05% and 100% respectively, and a significantly lower reduction in incidence of infection i.e. 33.47% and 58.7% was observed in infected tomatoes treated with cells and SEB of isolate N16.

In light of the higher antifungal potential of cells of R2 and their APB preparations against all the fungal pathogens compared to that of isolate M32 and N16, further studies were taken up with isolate R2 only.

**Antifungal properties of APB of *B. vallismortis* R2**
The effect of different concentrations of APB of R2 on growth of *A. alternata*, *M. grisea* and *F. oxysporum* in liquid medium was evaluated by microtiter plate assay. It was observed that the growth of all fungal pathogens was inhibited with increase in concentration of APB from 5 to 500 µg mL⁻¹ supplement to YME broth, ranging from 1.6% to 72.9%, 0.53% to 63.41% and 0.45% to 57.39% respectively. The effect of APB on the hyphal morphology and cellular components was observed under light and scanning electron microscopes. The untreated hyphae were healthy and showed characteristic smooth, regular, and hyaline hyphae. The severity of morphological deformities *viz.* stunted, distorted, swollen, collapsed, ruptured and empty hyphae, extensive vacuolization and granulated cytoplasm in fungal pathogens increased with increase in concentration of biomolecules in the growth medium.

The effect of APB of R2 on integrity/permeability of cell membrane of *A. alternata*, *M. grisea* and *F. oxysporum* was determined by comparing the electric conductivity of supernatant of respective fungal cultures grown in absence and presence of APB of R2. The increased electric conductivity of the supernatant of growth medium supplemented with APB as compared to control indicated to a compromised cell membrane, resulting in leakage of intracellular ions into the growth medium.

The damage caused by APB of R2 to the fungal cell membrane was further confirmed by increase in uptake of a fluorescent dye PI by cells grown in presence of different concentration of APB. This dye can pass through only damaged membranes and fluoresces after interaction with cytoplasmic material of the cell. It was observed that the intensity of fluorescence in the fungal hyphae increased with exposure to increasing concentration (25 to 400 µg mL⁻¹) of APB, indicating an increase in membrane permeability of fungal hyphae. However, the fluorescence decreased in fungal hyphae grown in presence of 500 µg mL⁻¹ of APB, which may be attributed to leakage of cellular material due to extensive damage to the cell membrane structure.

The antifungal activity of APB of R2 was stable after exposure to temperature range from 4-100°C as no significant reduction in zone of inhibition against respective fungal pathogen was observed. The APB retained 76.62%, 89.74% and 85.43% antifungal activity against *A. alternata*, *M. grisea* and *F. oxysporum* after autoclaving at
121°C, 15 lbs for 20 min, which indicated to the thermo-stable nature of biomolecules of isolate R2.

**Chemical Mutagenesis**

The cells of isolate R2 were mutagenized by growing them in presence of ethyl methanesulfonate (EMS). The mutant designated F5, lacking antifungal activity against *A. alternata* was selected. The mutant showed significantly lower yield of 0.80 g L\(^{-1}\) APB as compared to 3.79 g L\(^{-1}\) APB of the wild type R2. The APB of mutant did not support the characteristic antifungal activity in comparison to APB of R2 as evident from agar well diffusion assay and TLC based bioautograph against *A. alternata*. Similarly, no effect of APB of mutant F5 on morphology of fungal hyphae was evident by light micrographs. Further, the APB preparation of mutant F5 failed to control infection of *F. oxysporum* on tomatoes, whereas 93.25% reduction in infection was observed after treatment of infected tomatoes with APB of R2.

**Purification, Identification and antifungal activity of biomolecules produced by isolate R2**

The biomolecules of the crude APB preparation of R2 having antifungal activity were purified by column chromatography. The fractions *viz.* Sp-3, 4 and 7 having biomolecules with characteristic antifungal activities and Rf values as compared to profiles in crude APB were obtained. The liquid chromatography-mass spectrometry of purified fractions (Sp-3, 4 and 7) indicated the presence of CLPs putatively belonging to surfactin (Sp-3), iturin (Sp-4) and fengycin (Sp-7) family. The individual fraction supported antifungal activity against *A. alternata* in agar well diffusion assay and an increase in antifungal activity was observed with mixture of purified fractions. The biomolecules of fraction Sp-4 and Sp-7 supported significantly higher reduction in fungal growth and induced adverse effects on morphology of fungal hyphae as compared to biomolecules in fraction Sp-3. Similarly, the exposure of hyphae of *A. alternata* to biomolecules of fraction Sp-4 and Sp-7 resulted in more damage to cell membrane as evident from higher fluorescence of PI as compared to hyphae exposed to fraction Sp-3.

The production of CLPs by isolate R2 was further confirmed by amplification of *ituD*, *fenD* and *sfp* genes coding for antifungal biomolecules iturin A, fengycin and
surfactin respectively, using specific primers. The amplicons of expected sizes of all the 3 genes (*ituD*-1203 bp, *fenD*-293 bp and *sfp*-675 bp) were obtained.

**Pot studies for evaluation of antifungal potential of cells of isolate R2**

The field scale application of cells of R2 to suppress the growth of pathogen *A. alternata* and *M. grisea* on wheat and rice plant respectively was evaluated by carrying out pot studies. The soil drench method, involving 10 mL drench of suspension of spores of pathogen (10⁴ spores mL⁻¹) and cells of R2 (10⁸ CFU mL⁻¹) respectively, was used for exposure of plant to both pathogen and BCA. It was observed that exposure of roots to cells of R2 followed by infection with respective fungal pathogen, resulted in 65.5% and 35.89% reduction in disease index for wheat and rice plants as compared to their respective controls.

The ability of cells of R2 to colonize the rhizoplane was evaluated by isolating a rifampicin resistant variant of isolate R2 designated R2⁰ Rif⁺. It was observed that the cells of variant R2⁰ Rif⁺ inoculated initially at a concentration of 1 × 10⁸ CFU/mL, survived in the wheat rhizosphere even after 15 days of inoculation with a viable cell count of 1.9 × 10⁶ CFU/mL.

Further, the ability of cells of isolate R2 to elicit systemic response in tomato plants was evaluated. In this case the plants were treated with soil drench method as described above, however the pathogen was applied by foliage spraying of spore suspension. The histochemical analysis of leaves from tomato plants treated with cells of R2 and infected with *F. oxysporum* showed higher accumulation of H₂O₂ and cell wall strengthening due to deposition of lignin to counter fungal infection as compared to leaves of untreated infected plants.

**Studies on development of formulations of biological and chemical antifungal agents as tools for integrated pest management**

The possibility of use of BCA along with chemical pesticide to control fungal infestation was explored so as to develop environmental friendly pest management options, which might lead to lower dependence on chemical fungicides in future.

The compatibility of cells and APB of R2 with kemistin (commonly used fungicide to control *F. oxysporum* infection on tomato plants and fruits) was evaluated.
It was observed that after an initial lag of 24 h, the R2 cells were able to sustain growth in presence of 50 µg mL\(^{-1}\) kemistin. The addition of kemistin to APB preparation did not affect their activity and an increase in antifungal activity against \(F.\ oxysporum\) was observed in mixture, indicating to the possible synergistic interactions among chemical and biological molecules.

The application of 10 µg kemistin/fruit supported 100% reduction in infection on tomatoes, whereas 100 µg APB of R2/fruit was required to achieve 100% control of infestation. However, a mixture of 3 µg/fruit each of kemistin and APB achieved 100% reduction in infection of tomatoes by \(F.\ oxysporum\).

Similarly, the treatment of infected tomato plant with mixture of kemistin (25 µg/pot) and cells of R2 (5×10\(^8\) CFU/pot) showed reduction in infection by 47.64% in comparison to reduction of 52.0% and 43.28% achieved after treatment with kemistin (50 µg/pot) and cells of R2 (1×10\(^9\) CFU/pot) respectively. The infected plants which were not treated with chemical/BCA showed significant reduction in their overall growth in terms of shoot length, root length and dry weight as compared to treated plants.

These observations indicated that in presence of both cells and APB of R2, comparable results were obtained with lower concentration of kemistin as compared to treatments where higher levels of kemistin were used.

**Insecticidal potential of R2 against \(Spodoptera\ litura\)**

The insecticidal potential of isolate R2 was evaluated against polyphagous pest \(Spodoptera\ litura\) (Fabricius), known to have a wide host range and cause economic losses to number of crops. The effect of cells and APB of isolate R2 was evaluated against \(S.\ litura\). The feeding of larvae with castor leaves treated with different concentrations of cells of isolate R2 ranging from 7.0×10\(^7\) to 1.8×10\(^9\) CFU mL\(^{-1}\) and artificial diet supplemented with APB preparation from 50 to 250 µg mL\(^{-1}\) resulted in significant increase in larval mortality of \(S.\ litura\) as compared to control. Further, the diet amended with BCA significantly extended the development period of \(S.\ litura\) and reduced adult emergence. The toxicity of isolate R2 was evident from the emergence of morphologically deformed adults with crumpled and under developed wings. The nutritional physiology of larvae fed on APB supplemented diet was also adversely
affected as the relative growth and consumption rate as well as efficiency of conversion of ingested and digested food significantly reduced.