High-performance liquid chromatography-based characterization of fengycin produced by *Bacillus amyloliquefaciens* against *Fusarium graminearum* and *Rhizoctonia solani*

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Abstract

A substantial loss of crop production worldwide is attributed to fungal phytopathogens. The most important economic pathogens among these fungal phytopathogens are *Fusarium graminearum* and *Rhizoctonia solani*, which cause a wide range of plant diseases. In the present study, *Bacillus amyloliquefaciens* secondary metabolite fengycin was identified by High-performance liquid chromatography (HPLC) and in vitro screened against the *Fusarium graminearum* and *Rhizoctonia solani.* Based on the HPLC result, fengycin was identified at 215nm wavelength, a retention time of 5-7 minutes, and a peak area of 3.914. The obtained results indicated that fengycin (1, 1/2, 1/4, and 1/8) concentrations have a significant effect ($p<0.005$) on the growth of *F. graminearum* and *R. solani*. The current study concluded that *B. amyloliquefaciens* secondary metabolites fengycin have a high potential to inhibit the growth of *F. graminearum* and *R. solani.*

Keywords: Bacillus amyloliquefaciens; biopesticides; fengycin; phytopathogens; Secondary metabolite.

1. Introduction

Bacillus species produce various volatile agents that encourage the Plant's defense mechanism. Due to its pivotal bio-control activity, most bacterial and fungal phytopathogens have been controlled via bio-agents which play a significant role in phytopathology, the antimicrobial substances produced by *Bacillus* spp. Includes; subtilin, bacilysin, mycobacillin, bacillomycin, mycosubtilin, iturin, fengycin, and surfactin, having both antifungal and antibacterial activity (Ntushelo *et al.,* 2019).

Bacillus is used as an ideal biocontrol bacterium because it produces various types of environment-friendly antimicrobial agents. *B. amyloliquefaciens*, has been recognized as a safe bacterial spp. With outstanding excellent in-plant colonization capacities (Cao *et al.,* 2011; Liu *et al.,* 2017) and have a greater ability to protect plants from pathogenic microorganisms (Ongena & Jacques, 2008). Studies revealed that *B. amyloliquefaciens* could directly suppress pathogenic entities by secondary metabolites production, including polyketides, nucleic acids, proteins produced by microbes, amino acids, and lipopeptides (Ongena & Jacques, 2008; Chen *et al.,* 2015; Yan *et al.,* 2018). Lipopeptides with lower- molecular weight are the most common antimicrobial agents (Koumoutsi *et al.,* 2004). Fengycin, surfactin, and Iturin are the 3 significant families of

cyclic peptides with unique chemical and physical characteristics (Khan *et al.,* 2021; Santoyo *et al.,* 2012; Caulier *et al.,* 2019).

Fengycin is a cyclic lipodecapeptide made of a β-hydroxy fatty acid and a side chain of 16-19 carbon atoms (Steller *et al.,* 2000). According to previous studies, fengycin is produced mainly by *B. amyloliquefaciens* and *B. subtilis* (Hanif *et al.,* 2019; Geetha *et al.,* 2010). These metabolites are reported to inhibit the growth of filamentous fungi and fungal enzymes such as aromatase and phospholipase A2 (Deleu *et al.,* 2005). Furthermore, studies revealed that fengycin is effective against various types of fungi, including *Magnaporthe grisea*, *Plasmodiophora brassicae, Botryosphaeria dothidea, C. gloeosporiodes, F. verticillioides, F. solani, F. solani f. sp. radicicola, F. oxysporum, F. oxysporum f. sp. spinaciae, F. verticillioides* and *F. graminearum* (Zhang & Sun., 2018; Li *et al.,* 2014; Fan *et al.,* 2017; Kim *et al.,* 2010; Li *et al.,* 2012). In addition, Fengycins inhibit *F. graminearum*'s production by disrupting cell membrane permeability, and inhibition depends upon the concentration of fengycin (Liu *et al.,* 2019).

Worldwide, major loss of crop production is due to fungal phytopathogens (Ntushelo *et al.,* 2019). Among these fungal phytopathogens, the most significant and economic pathogens are *F. graminearum* and *R. solani,* which cause an extensive range of plant diseases (Kant *et al.,* 2011; Ajayi & Bradleyb, 2018). To control these fungal phytopathogens, chemical fertilizers, pesticides, and fungicides cause severe environmental issues (Khan *et al.,* 2021; Shafi *et al.,* 2017; Din *et al.,* 2019). Therefore, solving these issues requires an alternative approach for these fungal phytopathogens in a controlled environment.

Therefore, the study aimed to characterize *B. amyloliquefaciens* secondary metabolites, further analyzed through the high-performance liquid chromatography (HPLC) technique and screened against phytopathogens *F. graminearum* and *R. solani.*

2. Material and Methods

2.1 Study design

100 samples were collected from the rhizosphere of cereal crops to isolate *B. amyloliquefaciens* from places in Peshawar, Pakistan. To isolate *F. graminearum* and *R. solani*, the samples were collected from the diseased cereal crops in Peshawar, Pakistan.

2.2 Characterization of *B. amyloliquefaciens*

Soil samples were then processed through the serial dilution method. After the serial dilution with distilled water, the samples were streaked on nutrient agar media and incubated at 28°C to 30°C for 2-3 days. After incubation, the suspected colonies were examined through Gram's staining method. Gram-positive, rod-shaped, and spore-forming bacteria were selected for further Identification. *B. amyloliquefaciens* were identified through biochemical tests including citrate hydrolysis, catalase, indole production, nitrate reduction, Voges-Proskauer (VP), motility, H_2S production, and crystal formation (Amin *et al.,* 2015).

2.3 Production of secondary metabolites from *B. amyloliquefaciens*

B. amyloliquefaciens colonies were inoculated into a shaking flask containing a nutrient broth medium. After inoculation, the flasks were incubated at 30°C with a constant 200rpm at a shaking incubator for 16hours. Subsequently, 1ml of culture was transferred into an Erlenmeyer flask containing 99ml of Tryptic soy Broth (TSB) media and incubated at 30°C at a constant 200rpm on a shaker incubator overnight. The optical density (OD) of the growth curve of *B. amyloliquefaciens* was measured at 600nm using a spectrophotometer. Afterwards, the culture was removed and centrifuged for 30 minutes at 5590rpm. Finally, the supernatant was filtered at a sterile 0.22µm filter (Mater *et al.,* 2009).

2.4 High-performance liquid chromatography (HPLC) analysis

The supernatant was adjusted with concentrated HCL to pH 2 and centrifuged for 10 minutes at 894rpm and 20°C. The residue was dissolved in pH 8, methanol and water (50:50, v/v) solution and again filtered through a 0.22 µm membrane filter. The sample was treated three times with 20ml chloroform for purification. The lower layer was collected, and chloroform was evaporated through heat. The residue was dissolved in methanol. The secondary metabolites from the extract were identified by injecting 50µm of the extract in Shimadzu 20A UV-Vis HPLC at a wavelength range of 200–250nm. The isocratic HPLC method and 4.6×150mm C-18 normal phase column were used (Mater *et al.,* 2009). Acetonitrile was used as a mobile phase for the HPLC experiment. The obtained peak was compared to previously published data, and secondary metabolites were identified (Meena *et al.,* 2014).

2.5 Characterization of *F. graminearum* and *R. solani*

The infected part of the Plant was sliced by around 5mm, and 5% silver chloride was sterilized and washed with purified water 3 times. After sterilization, potato dextrose agar (PDA) was placed on the specimens and incubated for 3-4 days at 28°C (Uddin *et al.,* 2019). After incubation, the *F. graminearum* and *R. solani* were identified based on their colony colour, dense mycelia, and morphological characteristics on the microscope (John *et al.,* 2006).

2.6 Screening of secondary metabolites

The secondary metabolite was screened against *F. graminearum* and *R. solani*. Four wells of 5mm were made by using a sterile cork borer on potato dextrose agar (PDA). Various concentrations (1, 1/2, 1/4, and 1/8) of secondary metabolites were taken, and the zone of inhibition was measured in millimetres (mm) (Mater *et al.,* 2009).

2.7 Statistical Analysis

The data collected was analyzed and organized using the Statistical Package for Social Sciences (SPSS) version 23.0 software using a single ANOVA test.

3. Results

3.1 Characterization and cultivation of *B. amyloliquefaciens*

Among the 100 soil samples, *B. amyloliquefaciens was* isolated or found in 08 samples, which were confirmed based on colony morphology, gram staining, and biochemical tests (Figure 1). *B. amyloliquefaciens* was cultivated to produce secondary metabolites, and the growth curve's optical density (OD) was determined (Figure 2).

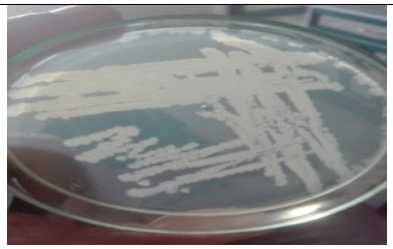
Bacillus amyloliquefaciens							
 Circular, rough, opaque, fuzzy white or slightly yellow colonies							
Gram Staining	**Shape**	**Motility**	**Catalase**	**Indole production**	**Citrate utilization**	**H2S production**	**Crystals formation**
Gram Positive	Rod shaped	Positive	Positive	Negative	Positive	Negative	Negative

Fig. 1. Morphological and Biochemical characteristics of *B. amyloliquefaciens.*

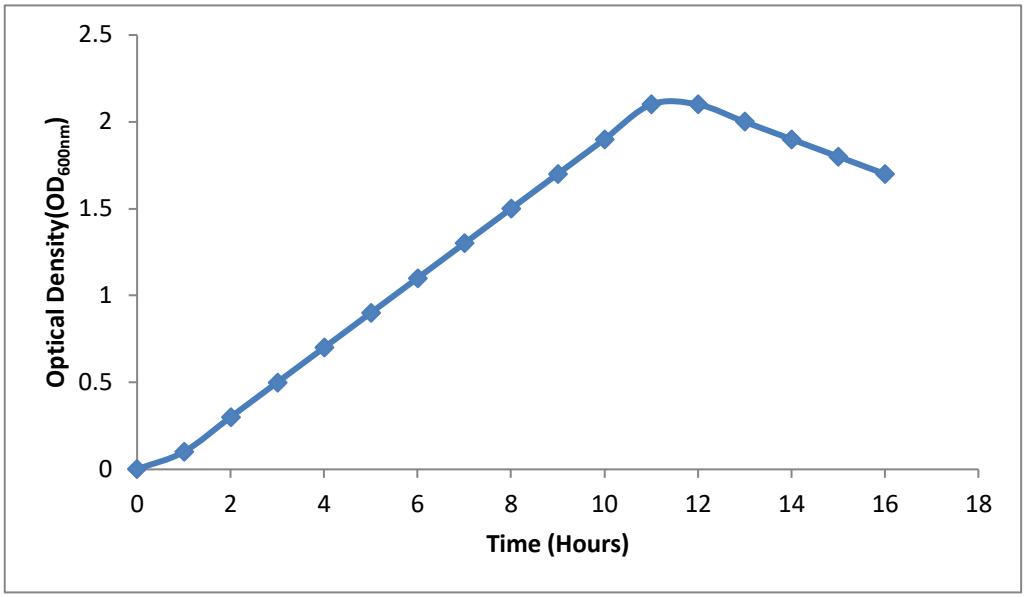


Fig. 2. Optical density (OD) of the growth curve of *B. amyloliquefaciens* at 600 nm wavelength

3.2 HPLC analysis of *B. amyloliquefaciens* secondary metabolites.

B. amyloliquefaciens purified secondary metabolites were analyzed using acetonitrile as the mobile phase. At 215nm and a retention time of 5-7minutes, the obtained peaks are similar to those obtained previously in literature data on fengycin (Figure 3). So, the secondary metabolite was confirmed as fengycin of *B. amyloliquefaciens* (Table 1).

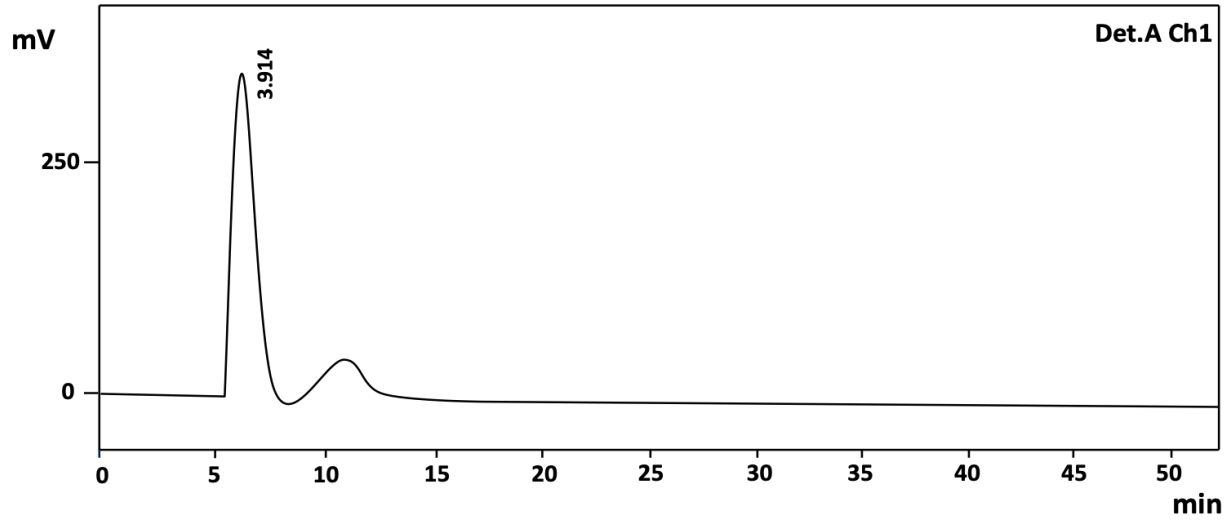


Fig. 3. HPLC Chromatogram of *B. amyloliquefaciens* secondary metabolites fengycin obtained at 215nm and retention time between 5-7 minutes.

Table 1. HPLC analysis results of secondary metabolites produced by *B. amyloliquefaciens*

***Bacillus* Strains**	**Wavelength (nm)**	**Retention time (minutes)**	**Peak area (Volts-minute)**	**Secondary metabolites**
Bacillus amyloliquefaciens	215	5-7	3.914	Fengycin

3.3 Characterization of fungal species from infected Plant

F. graminearum was isolated from infected maize plants and identified based on their colony morphology, white to the pinkish and microscopic examination of hyaline septate hyphae, two to multi-celled and sickle-shaped. *R. solani* was also isolated from infected maize plants and identified based on their white to brown colony morphology and on microscopic examination of the long tube and septa or partition inside (Figure 4).

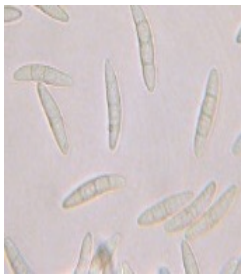
S.No.	Colony Morphology	Microscopic examination	Fungal species
1.	White to pinkish 	Hyaline septate hyphae, two to multi-celled and sickle-shaped 	***Fusarium graminearum***
2.	White to brown 	Long tube and septa or partition inside 	***Rhizoctonia solani***

Fig. 4. *F. graminearum* and *R. solani* results of colony morphology and microscopic examination.

3.4 Screening of *B. amyloliquefaciens* secondary metabolites against fungal species

B. amyloliquefaciens secondary metabolites fengycin were screened at (1, 1/2, 1/4, and 1/8) concentrations against the *F. graminearum* and *R. solani.* The zones of inhibition were calculated (Figure 5 & Table 2).

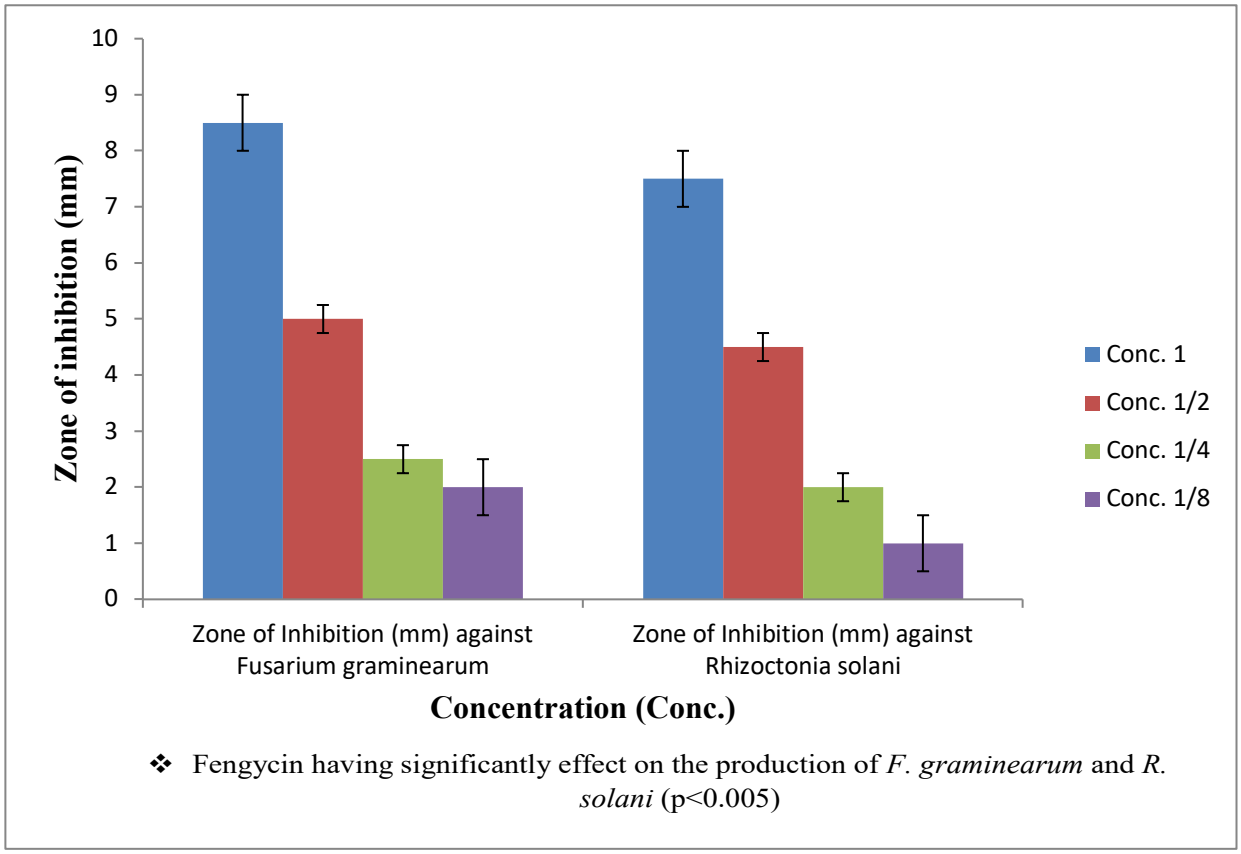


Fig. 5. *B. amyloliquefaciens* secondary metabolites fengycin zone of inhibition against *F. graminearum* and *R. solani*

Table 2. *B. amyloliquefaciens* secondary metabolites fengycin zone of inhibition against *F. graminearum* and *R. solani*

***Bacillus* Strain**	**Secondary metabolites**	**Concentration**	**Zone of Inhibition (mm) against *Fusarium graminearum***	**Zone of Inhibition (mm) against *Rhizoctonia solani***
Bacillus amyloliquefaciens	Fengycin	1	8.5	7.5
		1 /2	5	4.5
		1 /4	2.5	2
		1 /8	2	1

❖ Fengycin has a significant effect on the production of *F. graminearum* and *R. solani* (p<0.005)

4. Discussion

4.1 Biocontrol of *B. amyloliquefaciens*

For several decades, chemical fertilizers, pesticides, and fungicides have been used to defend crops from the attacks of rodents and fungal plant diseases. The use of certain additives and agents has contributed to severe environmental problems. In biocontrol agents, production, and their environment-friendly behaviour, Plant growth-promoting rhizobacteria (PGPR) provides an appeal to replace these chemicals and fertilizers with biological agents. PGPR species found in soil, in the field of biopesticides, play an essential role since they produce several antimicrobial agents, including lipopeptides, antibiotics, and enzymes that stimulate plant growth and prevent pathogenic microorganisms (Lahmyed *et al.,* 2021; Dashti *et al*., 2021; Shafi *et al.,* 2017; Din *et al*., 2019). Among these PGPR, *Bacillus* spp. Develops metabolites with antibiotic properties capable of limiting or suppressing the development of other microorganisms (Amin *et al.,* 2015). Subtilin, bacilysin, mycobacillin, bacillomycin, mycosubtilin, iturin, fengycin, and surfactin are the antimicrobial substances developed by *Bacillus* spp. with both antifungal and antibacterial activity (Ntushelo *et al.,* 2019). *B. amyloliquefaciens* is known for producing antimicrobial substances and displayed antimicrobial activities against various fungal phytopathogens, including *F. proliferatum*, *F. asiaticum*, *F. verticilloides,* and *F. graminearum*, and some other diseases causing phytopathogens like *R. solani*, *C. coccodes*, and *B. cinerea*. According to previous reports, various *B. amyloliquefaciens* strains were more effective against *P. oryzae*, *R. necatrix*, *R. solani*, and *F. graminearum* (Yoshida *et al*., 2001; Gong *et al.,* 2015 & Yu *et al*., 2002). In the current study, *B. amyloliquefaciens* was isolated from soil, cultivated for the production of secondary metabolites and their secondary metabolites, such as fengycin produced by *B. amyloliquefaciens,* were characterized by HPLC, and their screening was performed against phytopathogens *F. graminearum* and *R. solani.* According to previous studies, *Bacillus* spp. Containing *B. amyloliquefaciens* were cultivated, and their extracts were identified through HPLC as fengycin, iturin, surfactin, bacillomycin, and cyclo (L-Pro-D-Tyr) (Hanif *et al.,* 2019; Jamal *et al.,* 2019; Gu *et al.,* 2017; Meena *et al.,* 2014). The findings mentioned above of various studies agree with the present study. The current study claimed that secondary metabolites produced by *B. amyloliquefaciens* have a high potential to inhibit the growth of *F. graminearum* and *R. solani.*

4.2 Biocontrol mechanisms of *B. amyloliquefaciens*

Due to cyclic lipopeptides, fengycin *B. amyloliquefaciens* broadly show antimicrobial activities (Ongena & Jacques, 2008). Both *B. subtilis* and *B. amyloliquefaciens* produce these lipopeptides (Ongena & Jacques, 2008; Wang *et al.,* 2007; Yoshida *et al.,* 2001); other bacterial spp. *B. licheniformis* and *B. megaterium* could also display antimicrobial activities attributed to fengycin and iturin (Kong *et al.,* 2010; Dey *et al.,* 2015). Additionally, a strain of *B. amyloliquefaciens* DA12 produced some volatiles inhibiting both fungi and bacteria. In the current study, we could not explore the inhibition mechanism. Still, the previously reported research claimed that the 2-hepatanone could be toxic for antifungal phytopathogens, including *R. solani* and some *Fusarium*

species. In addition, these compounds stop fungi colonization and spore germination (Lee *et al.,* 2017).

4.3 Role of fengycin against Phytopathogens

Some *Bacillus* species, including *B. subsites*, and *B. amyololiquefaciens,* play a vital role against fungal plant pathogens. The impressive antibiotic ability is mainly owing to their genetic potential and non-ribosomal lipopeptides due to the biocontrol activity of *Bacillus* spp. Most bacterial and fungal phytopathogens have been controlled and effective against plant pathology (Ntushelo *et al*., 2019). Iturin, Surfactin, and fengycin are secondary metabolites produced by *Bacillus* spp. Comprises antifungal activity against various phytopathogens. According to previous studies, iturin, surfactin, and fengycin produced by *B. amyloliquefaciens, B. subtilis,* and *B. thuringiensis* were identified through thin layer chromatography (TLC) and HPLC having antifungal potential against *Aspergillus nigar, F. oxysporum,* and *F. graminearum* (Meena *et al*., 2014; Hanif *et al.,* 2019) *F. culmorum, F. solani* (Harba *et al.,* 2020), *R. solani, F. solani* (Madhi *et al.,* 2020; Fadhal *et al.,* 2019; Margani *et al.,* 2018), *F. graminearum* (Hanif *et al.,* 2019; Jamal *et al.,* 2019), *Helminthosporium oryzae, Curvularia lunata,* and *F. semitectum* (Saechow *et al.,* 2018). The current study's findings also claimed and correlated to the previous studies. In the present study, *B. amyloliquefaciens* secondary metabolites fengycin was analyzed through HPLC and successfully inhibited the growth of *F. graminearum* and *R. solani* at various concentrations. *F. graminearum* produces mycotoxin such as deoxynivalenol (DON), trichothecenes nivalenol (NIV), its derivatives 3-and 15- acetyldeoxynivalenol (3-ADON, 15-ADON), which cause diseases in plants, animals, and humans. *R. solani* indicators on varied hosts comprise crown rot, root rot, seed rot, hypocotyl rot, limb rot, stem rot, stem canker, pod rot, pre- & post-emergence damping-off, black scurf, and seedling blight (Ajayi & Bradleyb, 2018). According to previous studies, *B. amyloliquefaciens* inhibit mycotoxin biosynthesis of *F. graminearum* due to fengycin (Hanif *et al.,* 2019). The current finding is in agreement with previously reported studies, which claimed that *B. amyloliquefaciens* metabolites fengycin inhibits *F. graminearum* propagation and inhibits biosynthesis of their mycotoxins, besides these; also prevents the mycelial development of *R. solani.*

Furthermore, we analyzed the extracted secondary metabolites against both phytopathogens at various concentrations, but 100% (1) of secondary metabolites were highly effective against *F. graminearum* and *R. solani,* then 1 /2, 1 /4 and 1 /8 concentrations. Fengycin at various concentrations significantly affects the production of *F. graminearum* and *R. solani* ($p<0.005$). Our findings correlate with the previously reported study by Jamal *et al.,* the various concentrations of *Bacillus* spp. Secondary metabolites were a significant ($p<0.005$) effect on the phytopathogens growth (Jamal *et al.,* 2019).

5. Conclusions

Bacillus species is an excellent candidate to use as a biocontrol agent against fungal phytopathogens because they produce various antimicrobial substances and their environmentally

friendly behaviour. In addition, *Bacillus* will eliminate many of the existing commonly used control agents, such as fungicides, and cultural activities that harm health and the ecosystem in controlling phytopathogens. The current study concluded that *B. amyloliquefaciens* secondary metabolites fengycin have a high potential to inhibit the growth of *F. graminearum* and *R. solani.*

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