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In vitro antifungal potential of surfactin isolated from rhizospheric *Bacillus thuringiensis* Berliner 1915 against maize (*Zea mays* L.) fungal phytopathogen *Fusarium graminearum* Schwabe

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Abstract: *Fusarium graminearum* fungus cause significant loss in maize (*Zea mays* L.) and other cereal crops all over the world. The usage of chemical agents cause severe environmental problems. *Bacillus* species and other plant growth-promoting bacteria (PGPR) play key role in biopesticide development. A wide range of environmentally safe antimicrobial agents are already being manufactured. The current investigation was focused on exploring the antifungal activity of *Bacillus thuringiensis* lipopeptide surfactin against fungal phytopathogen *Fusarium graminearum*. *B. thuringensis* was isolated from the rhizosphere of maize crop and cultivated to produce lipopeptides. Surfactin was identified by high-performance liquid chromatography (HPLC) from the extract at 210 nm, retention time 3-5 minutes and the obtained peaks area was 3.990. The growth of *F. graminearum* was successfully inhibited by surfactin at different concentrations. Among these, 80 % concentration showed the highest zone of inhibition in comparison to 60 %, 40 % and 20 % concentrations ($p < 0.005$), respectively. The current study concludes *B. thuringensis* lipopeptide surfactin has a high potential to inhibit the growth of *F. graminearum*.

Key words: surfactin; *Bacillus;* biological control; HPLC; *Fusarium graminearum*

In vitro protiglivni potencial surfaktina, izoliranega iz bakterije *Bacillus thuringiensis* Berliner 1915 iz rizosfere koruze (*Zea mays* L.) proti patogeni glivi *Fusarium graminearum* Schwabe

Izvleček: Gliva *Fusarium graminearum* povzroča znantne izgube v pridelku koruze in drugih žit širom po svetu. Uporaba kemičnih sredstev za zatiranje povzroča resne okoljske probleme. Vrste iz rodu *Bacillus* in druge rast vzpodbujajoče bakterije (PGPR) igrajo ključno vlogo pri razvoju biopesticidov. Proizveden je bil že širok spekter okolju prijaznih antimikrobnih agensov. Raziskava se osredotoča na uporabo protiglivne aktivnosti lipopeptidnih surfaktinov iz bakterije *Bacillus thuringiensis* proti patogeni glivi *Fusarium graminearum*. Bakterija *B. thuringensis* je bila izolirana iz rizosfere posevka koruze in gojena za proizvodno lipopeptidov. Surfaktin je bil določen s tekočinsko kromatografijo visoke ločljivosti (HPLC) iz izvlečka pri 210 nm, retencijskim časom 3-5 minut, dobljeni višek je bil 3.990. Rast patogene glive je bila uspešno zavrta pri različnih koncentracijah surfaktina. 80 % koncentracija surfaktina je pokazala največjo sposobnost zaviranja v primerjavi s koncentracijami 60 %, 40 % in 20 % ($p < 0{,}005$). Na osnovi te raziskave lahko zaključimo, da ima lipopeptidni surfaktin iz bakterije *B. thuringensis* velik potencial za zaviranje rasti glive *F. graminearum*.

Ključne besede: surfaktin; *Bacillus;* biološka kontrola; HPLC; *Fusarium graminearum*

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1 INTRODUCTION

Globally *Bacillus thurenginisis* is considered to be the most predominant soil-dwelling bacterium found in the plants rhizosphere known for their antimicrobial properties. Aforementioned, *Bacillus* strains are known as plant growth promoting rhizobacteria (PGPR) that are associated with plants tolerance against biotic, and abiotic stresses caused by certain fungal phytopathogens (Saxena et al., 2019). In this context the worldwide major loss of maize and other cereal crops is due to fungal phytopathogens. The repertoire of fungal phytopathogens including *Acremonium alternatum* Link (Pal and Gardener, 2006), *Ustilago maydis* (DC.) Corda[(Kwon *et al.,* 2021), *Aspergillus niger* van Tieghem, *Aspergillus flavus* Link, *Puccinia sorghi* Schwein., *Fusarium* species (Rehman et al., 2021), *Helminthosporium*, *Alternaria*, *Rhizopu*s, *Penicillium*, *Drechslera* (Snetselaar and McCann, 2017), *Macrophomina phaseolina* (Tassi) Goid., and *Colletotrichum graminicola* D.J. Politis (Saleem et al., 2012), cause varieties of disease in maize.

Maize (*Zea mays* L.) is the most important cereal crop in the world, covering 75 % of the food requirements all over the world (Hussain et al., 2013). In Pakistan, among the cereal crops, maize is the third most important crop, after wheat and rice. Among these, globally the most important and significant phytopathogen is *Fusarium graminearum*, which causes significant loss of grain crops (Rauwane et al., 2020). The wide range of diseases caused by this plant pathogen includes; fruit rots, *Fusarium* head blight (FHB), wilts, and root rots (Kant et al., 2011).

Chemical compounds have been used to manage these fungal phytopathogens for many decades. They have a potential to generate major environmental problems. Alternative and less environmentally detrimental measures are required to control these plant diseases. *Bacillus* species and other PGPR play a key role among biopesticides. They produce various antimicrobial compounds such as enzymes, lipopeptides, and antibiotics that stimulate plant development while inhibiting pathogenic microbes (Shafi et al., 2017). For *B. thurenginisis* cyclic peptides including, surfactin, mycobacillin, mycosubtilin, subtilin, bacilysin, fengycin, bacillomycin, and iturin are reported that exhibit both antibacterial, and antifungal properties (Khan et al., 2021; Ntushelo et al., 2019).

Surfactin is a lipopeptide composed of cyclic depsipeptides of *β-hydoxy* hepta with possible amino acid combinations of alanine, valine, leucine, or isoleucine at positions 2, 4, and 7 in the cyclic depsipeptide moiety and *β-hydoxy* fatty acid chain variants of C_{13} to C_{16} in the cyclic depsipeptide moiety and *β-hydoxy* fatty acid chain variant (Hue et al., 2001). According to the investigations surfactin has natural antifungal properties produced by *Bacillus* spp. that could inhibit the growth of certain fungal species including, *F. graminearum* (Khan et al., 2021), *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen (Kim et al., 2010), *Colletotrichum gloeosporiodes* (Penz.) Penz. & Sacc. (Snook et al., 2009), *Fusarium verticillioides* (Sacc.) Nirenberg (Dunlap et al., 2011), and *Fusarium moniliforme* (Sacc.) Nirenberg (Vitullo et al., 2012).

Therefore, the current study was designed to isolate and characterize *B. thuringiensis* lipopeptide from rhizospheric soil and also to assess its antifungal efficacy against the fungal phytopathogen *F. graminearum* of maize.

2 MATERIALS AND METHODS

2.1 BACTERIAL AND FUNGAL ISOLATION

A total of 20 maize rhizospheric soil samples were collected from various locations in Peshawar, Pakistan, for the isolation of *B. thuringiensis* (Figure 1). *B. thuringiensis* was identified using colony morphology, gram staining, and biochemical-tests such as citrate hydrolysis, catalase, indole production, nitrate reduction, Voges-Proskauer (VP), motility, H_2S production, and crystal formation (Amin et al., 2015). *F. graminearum* was isolated using a sample acquired from a diseased maize plant in Peshawar, Pakistan (Figure 1), and identified using colony morphology and microscopic analysis (Uddin et al., 2019; John et al., 2006).

2.2 LIPOPEPTIDE EXTRACTION AND IDENTIFICATION

In a shaking flask containing nutrient broth medium (Oxoid™), all morphological and biochemical based confirmed isolated colonies of *B. thuringiensis* were injected. The flask was incubated for 16 hours at 30 °C with shaking incubator at 200 rpm. Afterward the culture was transferred to an Erlenmeyer flask containing 99 ml of Tryptic Soy Broth (TSB) medium (Oxoid™) and incubated overnight at 30 °C with shaking incubator at 200 rpm. The optical density (OD) of the *B. thuringiensis* growth curve was measured at 600 nm using a spectrophotometer (Shimadzu, UV-1800). After the decline phase of *B. thuringiensis* growth, the culture was removed and centrifuged at 6000 rpm for 30 minutes. The supernatant was filtered using a sterile 0.22 µm filter (Mater et al., 2009). The extract was then centrifuged for 10 minutes at 1000 rpm and 20 °C. The deposit was dissolved in a solution

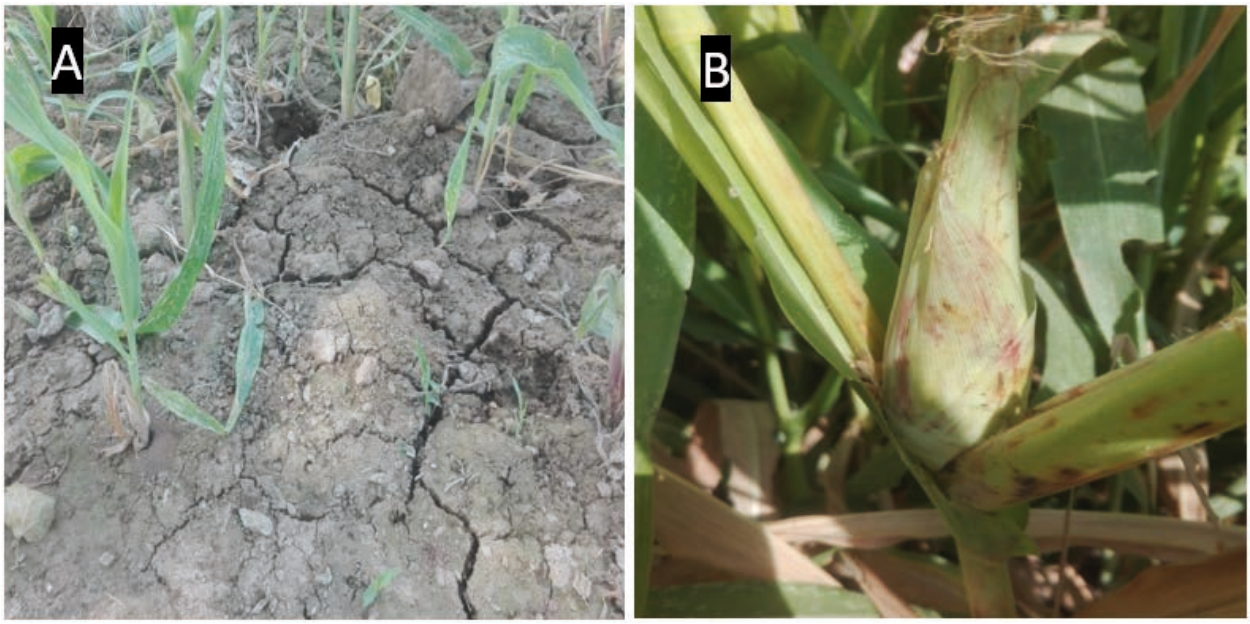

Figure 1: (A) Sampling site of maize rhizospheric soil for the isolation of *B. thuringiensis,* **(B)** Diseased maize for the isolation of *F. graminearum*

of methanol (Analytical grade, VWR Chemicals BDH®) and water (50:50, v/v) and filtered again using a 0.22 µm filter membrane. For purification, the sample was treated three times with 20 ml chloroform (VWR Chemicals BDH®). The bottom layer was collected and chloroform was evaporated at 50 °C temperature by using a hotplate stirrer. Methanol was used to dissolve the residue. Surfactin from the extract were identified by introducing 50 µl of the extract into a Shimadzu 20A UV-Vis HPLC at a wavelength range of 200–250 nm. The isocratic HPLC method was employed, along with a 4.6 × 150 mm C-18 normal phase column (Mater et al., 2009). For the identification of surfactin by HPLC experiment, acetonitrile was utilized as a mobile phase. Surfactin were discovered after comparing the observed peak to previously published data (Meena et al., 2014).

2.3 ANTIFUNGAL ACTIVITY OF LIPOPEPTIDE EXTRACT

To test the antifungal activity of surfactin, four 5 mm wells were created on potato dextrose agar (PDA) (Oxoid™) using a sterilized cork borer. The methanol was used as a control and also used to create concentrations of the lipopeptide extract of 20 %, 40 %, 60 %, and 80 %, respectively. The wells were filled with 200 *µl* of methanol (control), 20 %, 40 %, 60 %, and 80 % concentrations of lipopeptide extract, respectively. A colony of active growing *F. graminearum* was placed in the middle of media plates using sterile forceps and incubated at 30 °C for 3-7 days. Five repetitive antifungal analysis of the extracted lipopeptde was done by the same method described above. The inhibitory zones were measured and recorded (Mater et al., 2009). The obtained mean zone of inhibitions was analyzed using a one-way ANOVA test using the Statistical Packages for Social Sciences (SPSS) version 23.0 software and Microsoft Excel.

3 RESULTS AND DISCUSSION

3.1 BACTERIAL ISOLATE

In 20 rhizospheric soil samples *B. thuringiensis* 12 isolates were confirmed by various criteria such as, colony morphology, gram staining, and biochemical assays (Table 1). Previous results revealed that *Bacillus* species are primarily found in rhizospheric soil and that their metabolites have antibiotic characteristics as they can inhibit or restrict the development of other microorganisms (Amin et al., 2015).

3.2 FUNGAL ISOLATE

In context to this study, *F. graminearum* was isolated from infected maize plants and identified using colony morphology (white to pinkish), and microscopic assessment (Hyaline septate hyphae, two to multi-celled and

Table 1: Morphological and Biochemical characteristics of *B. thuringiensis*

Tests	Results
Colony Morphology	Circular, rough, opaque, fuzzy white or slightly yellow
Gram Staining	Gram Positive
Shape	Rod shaped
Motility	Positive
Catalase	Positive
Indole production	Negative
Citrate utilization	Positive
H2S production	Negative
Crystals formation	Positive
Identified Strains	B. thuringiensis

sickle-shaped) in the current investigation. *Fusarium* head blight (FHB) disease is caused by *F. graminearum* in maize. This fungus exhibit certain sign of early bleaching during infection which could reduce grain production and quality (Ntushelo et al., 2019).

3.3 LIPOPEPTIDE IDENTIFICATION

According to the current study findings, *B. thuringiensis* was grown to produce lipopeptides, and the optical density (OD) of the growth curve was measured (Figure 2). Lipopeptides isolated from *B. thuringiensis* were analyzed by HPLC using acetonitrile as the mobile phase. At 210 nm and retention period 3-5 minutes, the observed peak area was 3.990 (Figure 3), which is similar to the peaks found earlier in surfactin literature data (Mubarak et al., 2015). Previous studies are in agreement with our findings. According to the Deepak and Jayapradha (2015), they identified lipopeptide surfactin by HPLC which are produced by *B. thuringiensis*. In another study, the lipopeptde fengycin produced by *B. thuringiensis* was identified by HPLC techniques (Kim et al., 2004).

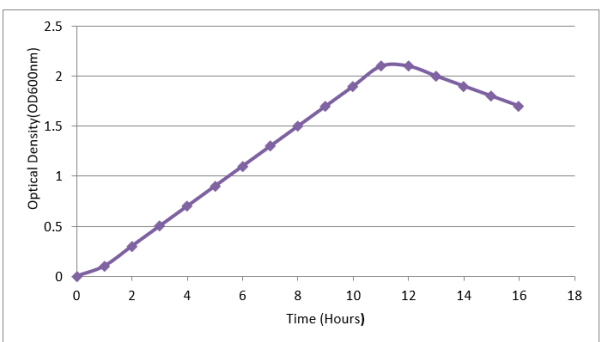

Figure 2: Optical density (OD) of the growth curve of *B. thuringiensis* at 600 nm wavelength

3.4 ANTIFUNGAL ACTIVITY OF LIPOPEPTIDE

B. thuringiensis lipopeptide surfactin against the development of *F. graminearum* was investigated in this work. The surfactin lipopeptide efficiently suppressed the growth of *F. graminearum* (Figure 4). According to earlier research, isolated *Bacillus* spp. from the rhizosphere, particularly *B. subtilis*, reduced the growth of *F. graminearum*. *Bacillus* spp. is also effective in the prevention of *Fusarium* head blight (FHB) and root rot; they stimulate plant development and inhibit the mycelial growth of fungal infections through antagonistic action (Herba et al., 2020; Madhi et al., 2020; Dukare et al., 2020). In this study, lipopeptide surfactin from *B. thuringiensis* was tested against *F. graminearum* at 20 %, 40 %, 60 %, and 80 % concentrations (Figure 4). The zone of inhibition was the greatest at the 80 % concentration, followed by the 60 %, 40 %, and 20 % concentrations ($p < 0.005$), respectively. These findings are in agreement with previous report, in which the surfactin action against *F. oxysporum* (Deepak and Jayapradha, 2015) was screened. According to a recent study, microorganisms were isolated from plant anthers and wheat kernels to test their antagonistic activity against *F. graminearum*, the causative agent of *Fusarium* head blight (FHB). *B. subtilis* has a strong antifungal impact on *F. graminearum* mycelium, sporulation, and DON formation, with inhibition values of 87.9 %, 95.6 %, and 100 %, respectively (Zhao et al., 2014).

4 CONCLUSION

Lipopeptides obtained from *Bacillus* species have less negative environmental effects as compared to chemical compounds. The current study concluded that *B. thuringiensis* isolated from the rhizosphere of maize crop may produce lipopeptide surfactin, which has a high potential to inhibit the growth of *F. graminearum*. The study is also emphasizing surfactin as potential biological control agent with widespread usage. We are also encouraging other researchers to take advantage of newly invented techniques to explore mechanism of action of various *Bacillus* strains against phytopathogens.

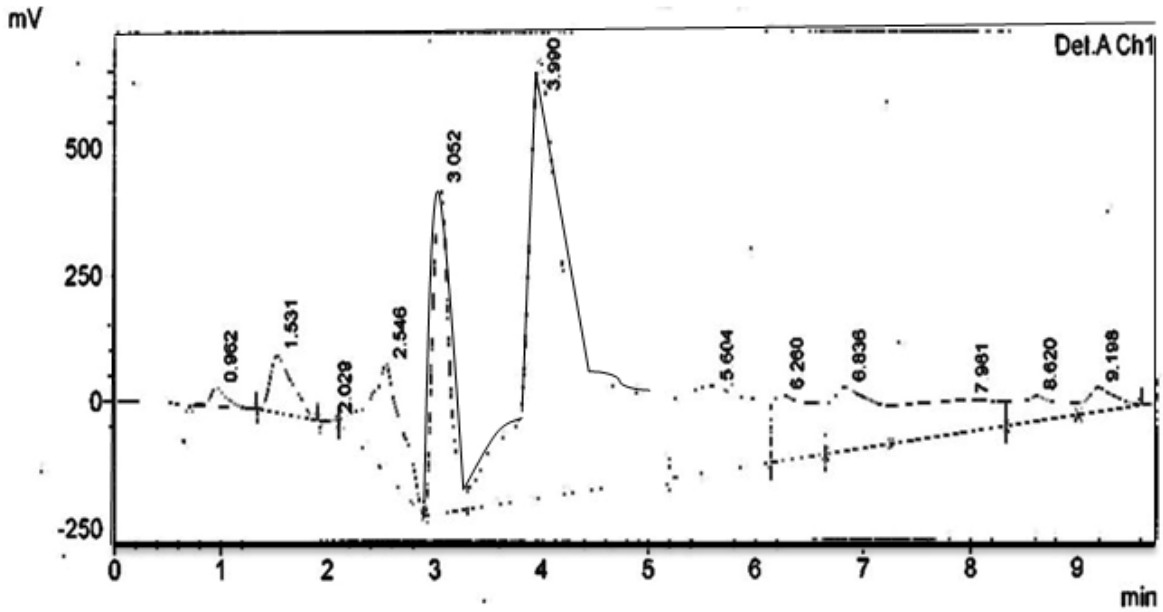

Figure 3: HPLC Chromatogram of *B. thuringiensis* lipopeptide surfactin obtained at 210nm, retention time between 3-5 minutes and peak area 3.990

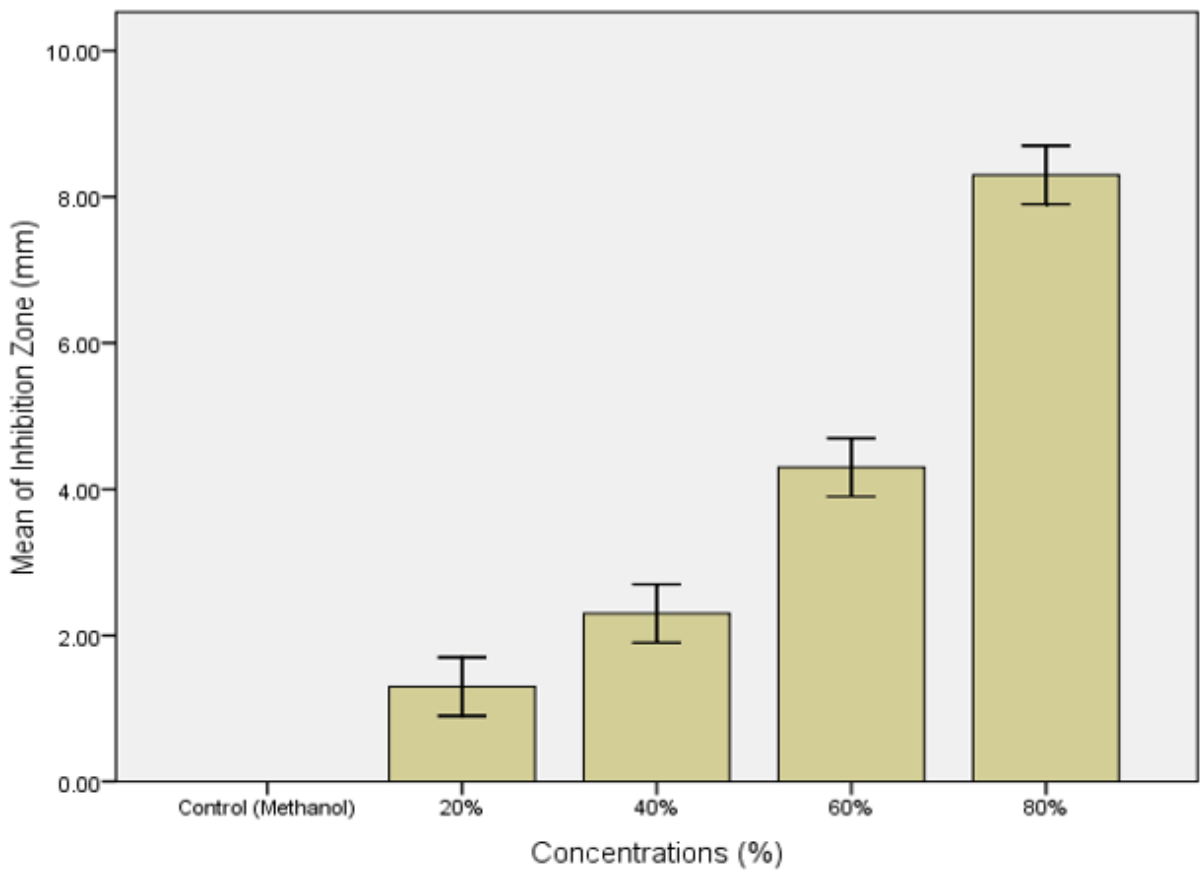

Figure 4: *B. thuringiensis* lipopeptide surfactin zone of inhibition (mean) against *F. graminearum* at various concentrations ($p < 0.005$)

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