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Isolation and profiling of proteins of *bacillus* spp. and their influence on *Meloidogyne incognita*

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Abstract
Indigenous endophytic five isolates of *Bacillus weihenstephanensis* (TSB4), *B.cereus* (CLB2D), *B. subtilis* (TSB5), *B. cereus* (TSB4D), *B. licheniformis* (TSB4) possessing antinemic/antifungal property were assayed for the presence of different proteins through SDS-PAGE analysis. The analysis showed the presence of four proteins with different molecular weight in all the isolates of *Bacillus* spp. Further 2D page analysis of the proteins of *B. weihenstephanensis* (TSB4) identified as the most effective isolate among the five isolates exhibited both up regulated and down regulated proteins with molecular weight ranging from 14 to 96 kda. The crude proteins of all the five indigenous isolates of *Bacillus* had a significant effect to inhibit egg hatching and to cause mortality of juveniles of *M. incognita* with positive correlation between their antinemic effect and concentration time as well as period of exposure to crude antibiotics. Of all the five isolates the *B. weihenstephanensis* (TSB4) crude antibiotics exhibited the highest ovicidal and larvicidal effect over root knot nematode and it was differing significantly from other isolates.

Keywords: Egg hatching, dialysis, juveniles mortality, *Meloidogyne incognita*, precipitation, protein, SDS-PAGE, 2D PAGE

Introduction
The exploration of the intracellular protein levels of bacterial species is of importance to understand the pathogenic mechanisms of diseases caused by harmful organisms. Among the bacterial bioagents, the *Bacillus* spp. are considered as promising bio-control agents, effective against phytonematodes and plant pathogens by several authors [1-4]. The inhibitory effect of *Bacillus* spp. are related to their crude antibiotics/proteins/toxigenic genes/antibiotic biosynthetic genes in general.
The main objective of this experiment,is to isolate antagonistic proteins from beneficial bacteria (*Bacillus* spp.) by Electrophoresis method. Polyacrylamide gel electrophoresis (PAGE) is one of the most widely used laboratory electrophoresis method to separate biological macromolecules such as proteins and nucleic acids, Sodium Dodecyl Sulfate (SDS) is used as bio-agent for separation ofbacterial proteins by Electrophoresis method. The binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass. As a result, negatively charged proteins will migrate towards the positive electrode and will be fractionated by approximate size during electrophoresis.This procedure is called SDS-PAGE.
2D-PAGE is a form of gel electrophoresis in which separation and identification of proteins in a sample are done by displacement in 2 dimensions oriented at right angles to one another. This technique is also used to compare two or more samples to find differences in their protein expressions.This method consists of two steps for separation of proteins according to their iso-electric point. In first dimension (1st step), it focuses iso-electric point followed by separation on the basis of molecular weight by acryl-amide gel electrophoresis in the second dimension (2nd step). Visualization of separated proteins is carried out by using different stains.
However, the available literature on protein profiling using molecular techniques and their influence on nematodes is very meager. Therefore, the present study was initiated to isolate and characterize proteins of the most promising five native isolates of endophytic *Bacillus* spp. [5] and to know their influence on root knot nematode, *M. incognita*.

Materials and Methods
Isolation of Proteins
To initiate the experiment, the nutrient broth of a desired quantity inoculated with the most

effective isolates of *B. weihenstephanensis* (TSB4), *B.cerus* (CLB2D), *B. subtilis* (TSB5), *B.cerus* (TSB4D) and *B. licheniformis* (TSB3) was incubated at 30 ºC for 5 days at 170 rpm and thesupernatant was collected by centrifugation at 10,000 rpm for 10 min. The supernatant was dissolved with ammonium sulphate 80% (w/v) and incubated at -40ºC for overnight. The protein pellet collected by centrifugation at 8000 rpm for 15 min was dissolved in sterile water.

A protein sample of 10µl from different isolates of *Bacillus* spp. were taken and mixed with 30 µl of sample buffer in a micro-centrifuge tube, boiled for 4 min at 100^0C. The ammonium sulphate precipitates were dissolved in 5 ml potassium phosphate buffer and dialyzed against thesame buffer using dialysis membrane with molecular cut off of 12,000 to 14,000 Dalton (Spectra/Por, SPECTRUM Laboratories, CA, USA.) and incubated at 4 ^{0}C for 30 min.

The separating gel solution as given below was poured between the glass plates with a pipette and left1/4 of the space free for loading the stacking gel. Covered the top of the resolving gel carefully with 50% isopropanol and kept for 30 min to resolve gel polymerization. Discarded theisopropanol when a clear line appear between the gel surface and the top of the solution oncompletion of the process of polymerization. Then the stacking gel solution as detailed below poured carefully with a pipette to avoid formation of bubbles and comb was inserted and allowed the gel to polymerize for 30 min. There after the combs were removed carefully to put the gel in the tank of electrophoresis (bottom and top reservoir) with fresh 1X Tris-glycine-SDS Buffer (0.05 M Tris HCl 1.2g; 0.192M glycine 28.8g,0.1% SDS 2g; sterile water 2l) and made sure that the gel wells were covered with the bufferfollowed by loading of protein ladder / marker and probes and set appropriate voltage of 80. The running of electrophoresis was stopped when the dye reached the bottom of the gel anddisassemble the gel sandwich to proceed for gel staining.

Table 1: Separating gel

Sterile water	5.03 ml
30% acrylamide	6 ml
1.5M Tris HCl Ph	3.75 ml
10% Sodium dodecyl sulphate	150 µl
10% Ammonium per sulphate	75 µl
Tetra methyl ethylene diamine	20 µl

Table 2: Stacking gel

Sterile water	9 ml
30% acrylamide	1.98 ml
0.5M Tris HCl pH (7.00)	3.78 ml
10% Ammonium per sulphate	75 µl
Tetra methyl ethylene diamine	20 µl

The gel was stained with staining solution made with Coomassie Brilliant Blue (R250) 0.2 g, methanol 40 ml, acetic acid 10 ml and water 50 ml for 6 h. Again the gel was destained with destaining solution prepared with methanol 40 ml, acetic acid 10 ml and water 50 ml. Finally the banding pattern of proteins was observed in light illuminator.

Bacterial proteins profiling by 2D-PAGE analysis

Sample preparation

In this study the *B. weihenstephanensis* strain TSB4 identified as most effective isolate was used for protein extraction. The strain was grown in 50 ml nutrient agar broth at 30 ^{0}C in 250 ml flask on a rotary shaker at 175 rpm continuously a week for better growth of bacterial cells. The bacterial cells were removed by centrifugation at 8000 rpm for 20 min at 4^0C followed by filtration through a 0±2 nm nitrocellulose filter (Millipore) or through a membrane (pore size = 0.2 µm). The proteins were then precipitated twice using the deoxycholatetetrachloroacetic acid method. The pellet was washed twice with ethanol: ether (1:1) dried and stored at – 80 ^{0}C until use. The proteins content of the pellet was determined as per Bradford method [6].

Two dimensional electrophoresis

The protein pellets were resuspended in sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.6% Pharmalytes (Amersham Pharmacia Biotech, Uppsala, Sweden), 2 mMtricarboxyethylphosphine (TCEP) (Pierce, Chester, UK), 40 mMTris and subsequently diluted in IPG rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.6% Pharmalytes, 2 mM TCEP). IPG strips (17cm length) in the linear pH range 4–7 (Bio-Rad, Hercules, CA, USA) were chosen because preliminary experiments revealed no proteins below pH 4 and few proteins above pH 7. The IPG strips were rehydrated for 16 h with 400 µl of rehydration buffer and loaded 20 µg protein per IPG strip for spot quantification and 100 µg protein per IPG strip for proteins identification. The proteins were separated by IEF on an Amersham Pharmacia Multiphor II horizontal electrophoresis system for 32 000 Vh. The IPG strips were then equilibrated for 15 min in 50 mMTris pH 8.8, 6 M urea, 30% glycerol, 1% SDS and 1% DTT and then for 20 min in 50mM Tris pH 8.8, 6 M urea, 30% glycerol, 1% SDS, 1.6% iodoacetamide. The second dimension was run on a 10% to 15% gradient SDS-PAGE, on a 16 x 20 x 0.1 cm Bio-Rad Protean II xi vertical slab gel. The gels were silver stained for spot detection and quantification or stained with Coomassie Blue G250 for the identification of proteins.

Influence of *Bacillus* proteins

Against eggs of *M.incognita*

Five ml of each concentrations of proteins mixed with distilled water were transferred to sterilized Petri dishes having 5 cm dia. Then uniform sized egg mass of *M.incognita* obtained from pure culturing of the root knot nematode were transferred @ one / Petri dish to evaluate the effect of precipitated proteins of different isolates of *Bacillus* spp. on inhibition of hatchability of eggs of *M.incognita*. All the protein concentrations of isolates of *Bacillus* spp. were replicated thrice in completely randomized design (CRD) and compared with distilled water as untreated controls.

Against juveniles of *M. incognita*

As above the different concentrations of proteins of various isolates of *Bacillus* spp. were taken in a sterilized Petri dish (5 cm dia) @ 5ml / Petri dish. The freshly hatched out second stage juveniles of *M. incognita* were transferred @100 J_2 / Petri dish to study the effect of precipitated proteins of isolates of *Bacillus* spp. on the juveniles of *M. incognita* under laboratory conditions (28±2 ^{0}C). Suitable replications were maintained in CRD. Observations were recorded on mortality of juveniles of *M. incognita*at 24h interval for three days and the per cent mortality of juveniles was worked out after confirming their irreversible reaction of *M. incognita* on exposure to the proteins of test isolates of *Bacillus* spp.

Results and Discussion

Confirmation of presence of proteins with the endophytic

***Bacillus* spp. using SDS-PAGE**

The effective isolates of *Bacillus* spp.*viz*. *B. weihenstephanensis* (TSB4), *B.cereus* (CLB2D), *B.subtilis* (TSB5), *B.cereus* (TSB4D) and *B.licheniformis* (TSB3) were tested for isolation of protein to study the nematicidal property through SDS – PAGE. The result showed the presence of four different proteins having different molecular weight of 25, 43, 66 and 95 Kda in the *B. weihenstephanensis* (TSB4) (Plate 1). The molecular weight of 14, 22, 35 and 95 Kda proteins was comparatively lesser with the next most effective species of *B. cereus* (CLB2D). However the trend was not maintained with the molecular weight of proteins present in the remaining endophytes of *B. subtilis* (TSB5) (22, 35, 43 and 95 Kda), *B. cereus* (TSB4D) (22, 25, 43 and 66 Kda). *B. licheniformis* (TSB3) (25, 43, 66 and 95 Kda)

Hence it is suggested that the different proteins of *Bacillus* spp. needs to be studied individually for their antimicrobial property against root knot nematode in order to arrive conclusive result.

Proteins profiling of *B. weihenstephanensis* (TSB4) through 2D - PAGE

The protein profiling of *B. weihenstephanensis* (TSB4) observed through 2D-PAGE, analysis. It showed the prominent banding pattern of different proteins present in the bacterium. The 2D-PAGE analysis of proteins of *B. weihenstephanensis* (TSB4) exhibited both up regulated and down regulated proteins with molecular weight ranging from 14 to 94 kda (Plate 2).

In this connection it is suggested that indepth study in future with MALDI -TOF will be useful to understand the proteomics of *B. weihenstephanensis* (TSB4) and its influence over *M.incognita*.

Influence of proteins of different isolates of *Bacillus* spp. against *M. incognita*

The results of *in vitro* experiment furnished in Table 3 and 4 revealed the effectiveness of proteins of *Bacillus* spp. to maximize the per cent inhibition of egg hatching and mortality of juveniles of *M. incognita.*

Effect of proteins of different isolates of *Bacillus* spp.

With regard to proteins of the above five isolates, the *B. weihenstephanensis* (TSB4) was found to be most effective to achieve the highest per cent inhibition in egg hatching (99) and mortality of juveniles of *M.incognita* (100) at cent per cent concentration after 72 h exposure (Table 3 and 4). Its nematicidal effect of the proteins was followed by *B.cereus* (CLB2D), *B.subtilis* (TSB5), *B.cereus* (TSB4D) and *B.licheniformis* (TSB3) and differed significantly from each other (Table 3 and 4).

Effect of different period of exposure/concentrations of proteins

Further it is observed that the nematicidal property of the proteins of the above bacteria was increasing with increase in the concentrations / period of exposure with all the five tested isolates of *Bacillus* spp. and it was 73.74 to 95 and 91.44 to 99 per cent at the lowest (25%) and highest (100%) concentrations respectively in inhibiting the egg hatching of *M.incognita*. The trend was similar in respect of juveniles of *M.incognita* also with the per cent mortality of juveniles of *M.incognita* as 56.00 to 92.66 and 65 to 100 respectively at the lowest and highest concentrations (Table 1 and 2).

The effect of dialyzed proteins of isolates of *Bacillus* spp. against *M. incognita*

Ammonium sulphate protein precipitation is a method used to purify proteinsby altering their solubility. This technique is useful to remove large amounts of contaminated proteins quickly before assaying the effect of proteins against a test organism.

In the above study, the proteins of all the five isolates *viz. B. weihenstephanensis* (TSB4), *B. cereus* (CLB2D), *B.subtilis* (TSB5), *B.cereus* (TSB4D), *B. licheniformis* (TSB3) had a significant effect to maximize the per cent inhibition of egg hatchingfrom 89.11 to 99 and mortality of juveniles of *M. incognita* with the range of 53.44 to100. Further, the nematicidal property of the proteins of the above bacteria was increased with increase with an in the concentrations / period of exposure irrespective of endophytestested.

Among the five endophytes, the *B. weihenstephanensis* (TSB4) ranking first found to register the highest per cent inhibition in egg hatching (99%) and mortality of juveniles (100%) of *M.incognita* at the highest concentration and longest period of exposure showing direct relationship between their ovicidal / larvicidal effect and proteins per cent concentration/ period of exposure (Table 1 and 2).

Although no information pertaining to the protein effect of the above five tested

endophytes is available to support the present findings, the literature available with different endophyte of *B.pumilus* showed the effectiveness of proteins of the bacterium against bacterial pathogens *viz*. *E.coli* and *S. aureus*; fungal pathogens *viz. Aspergillus niger and A. flavus.* Similarly the proteins of *B. subtilis* reported to be inhibitory to many fungal pathogens *viz. Fusarium oxysporom* f. sp. *cubense, Corynespora cassicola, Rizoctonia solani, Botrytis cinarea, Colletotrichum gloceopoioides* [7-9].

Therefore, it is believed that there is a chance for the possession of antinemic property against root knot nematode by the proteins of endophytes tested in the present study.

Hence the present study clearly confirming the presence of four different proteins which could be related to its promising biocidal effect over root knot nematode.

However no information is available absolutely to match the present finding of presence of protein with *B.weihenstephanensis* (TSB4) through the technique of SDS – PAGE.

The findings of earlier workers coinciding with the present results are discussed below [8] proved the inhibitory activity of *B. subtilis*strain B29 on mycelia growth of *Fusarium oxysporum*, *Rhizoctonia solani*, *Fusarium moniliforme* and *Sclerotinia sclerotiorum*. In continuation of the study the authors purified the antifungal protein fraction of the *B.subtilis*having the molecular mass of 42.3 KDa by SDS - PAGE and designated the same as B29I.An attempt of purifying antifungal fraction of proteins through SDS – PAGE was studied before which is used in this present experimental study [7]. The authors observed a single band with the bacterium, *B.subtilis* B25 and identified further as amino acid sequence of 14 peptides segments by NanoLC–ESI / MS. Finally the authors concluded that it was a hypothetical protein (gi154685475), with a relative molecular mass of 38708.67 Da and isoelectric point of 5.63 which believed to exhibit good inhibitory effect over many fungal pathogens.

Reconfirmation of presence of proteins in *B. weihenstephanensis* (TSB4)

A study was performed using 2D-PAGE to analyze the

proteome of *B. weihenstephanensis* (TSB4) extracellular proteins. In this study around one hundred spotswith molecular weight of 20 to 95 KDa were visualized in the gel of *B. weihenstephanensis* (TSB4) extracellular proteins. The results confirmed the presence of proteins in the bacterium which is predicted to be responsible for its antimicrobial property.

Similarly it is observed that more than one hundred spots in a gel of another *Bacillus* endophyte of *B.subtilis* extracellular proteins through 2D–Page [10]. The observations of the authors are in agreement with the findings of the present study. Therefore, the information generated through the present study have been added to the related protein database of *Bacillus* spp.

In advancement of the similar study the confirmation of the presence of different proteins were identified as collagenase, phospholipases, haemolysins, proteases and enterotoxins [11]. Therefore it is suggested to confirm the different proteins present in *B. weihenstephanensis* (TSB4) in future to use as nematicidal proteins.

Table 3: Influence of crude proteins of different isolates of *Bacillus* spp. against eggs of *M. incognita*

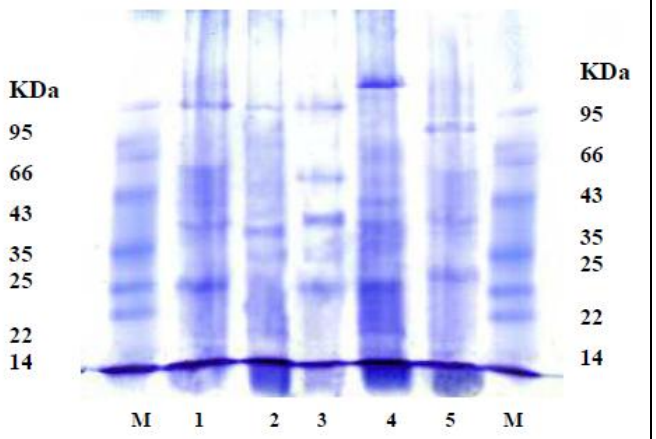
***Bacillus* spp. with accession number**	**Per cent inhibition in egg hatching at different concentrations (%) and time of exposure (h)**								
	25			**50**			**100%**		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**
B.weihenstephanensis(TSB4) KJ734726	98.12 (76.59)	96.00 (78.37)	95.00 (78.50)	99.38 (86.59)	97.41 (80.76)	96.89 (79.84)	99.38 (88.83)	94.26 (85.39)	99.00 (85.89)
B. cereus (CLB2D) KJ734726	92.49 (74.17)	91.11 (72.67)	93.33 (75.04)	97.41 (79.92)	97.11 (82.76)	93.88 (80.21)	98.44 (82.40)	98.15 (80.76)	97.50 (81.87)
B.subtilis(TSB 5) KJ734724	91.11 (66.37)	88.15 (70.01)	83.76 (72.66)	93.44 (72.16)	92.22 (73.82)	90.62 (73.97)	96.88 (81.30)	94.82 (76.88)	94.66 (76.66)
B. cereus (TSB4D) KJ734723	90.22 (65.38)	87.77 (73.33)	82.50 (71.59)	92.00 (71.57)	90.00 (71.00)	88.88 (73.60)	95.12 (82.12)	93.93 (78.35)	93.78 (78.17)
B.licheniformis(TSB3) KJ778904	89.11 (63.56)	81.11 (71.78)	73.74 (70.81)	90.00 (66.74)	88.88 (73.54)	83.12 (73.57)	94.66 (77.97)	92.24 (80.21)	91.44 (79.47)
Ammonium sulphate (35%)	57.23 (47.00)	59.46 (73.60)	60.71 (50.67)	65.76 (74.00)	68.45 (74.60)	69.71 (52.48)	66.13 (47.02)	69.50 (74.60)	69.71 (74.75)
Untreated control	53.33 (42.53)	90.00 (80.22)	150.00 (150.00)	53.33 (42.53)	90.00 (80.22)	150.00 (150.00)	53.33 (42.53)	90.00 (80.22)	150.00 (150.00)
SEd	4.13	4.71	6.71	3.37	4.58	6.70	4.29	4.46	6.74
CD(P=0.05)	8.99	10.26	14.63	7.36	9.99	14.59	9.35	9.72	14.70

Note: The figures in parentheses are arcsine transformed values.

Table 4: Influence of crude proteins of different isolates of *Bacillus* spp. against juveniles of *M.incognita*

***Bacillus* spp. with accession number**	**Juveniles mortality (%) with different concentrations (%) and time of exposure (h)**								
	25			**50**			**100%**		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**
B.weihenstephanensis(TSB4) KJ734726	87.33 (69.21)	90.00 (71.62)	92.66 (74.29)	94.33 (76.27)	95.33 (77.83)	96.33 (76.83)	99.00 (84.98)	99.00 (84.98)	100.00 (88.83)
B. cereus (CLB2D) KJ734726	81.00 (64.17)	83.33 (65.40)	88.33 (70.07)	84.00 (66.42)	87.00 (68.90)	89.00 (70.98)	86.00 (63.03)	89.66 (71.27)	94.66 (76.83)
B.subtilis(TSB 5) KJ734724	76.00 (59.34)	77.66 (61.80)	79.00 (62.72)	73.00 (67.69)	75.00 (59.78)	78.00 (62.02)	69.66 (56.37)	72.00 (58.05)	74.00 (59.34)
B. cereus (TSB4D) KJ734723	62.33 (52.13)	65.00 (53.73)	67.66 (55.34)	65.00 (53.73)	69.33 (56.37)	71.00 (57.40)	67.66 (55.34)	69.00 (56.16)	72.00 (58.05)
B.licheniformis(TSB3) KJ778904	54.33 (47.67)	55.66 (48.25)	56.00 (48.63)	55.00 (47.86)	57.00 (49.02)	58.00 (49.60)	59.66 (49.40)	60.33 (50.18)	65.00 (50.77)
Ammonium sulphate	35.00 (24.66)	39.33 (27.43)	41.55 (30.56)	38.66 (27.15)	40.77 (28.39)	44.00 (35.67)	42.33 (31.30)	45.33 (36.89)	46.66 (38.58)
Untreated control	0.00 (1.16)	0.33 (2.68)	1.00 (5.00)	0.00 (1.16)	0.33 (2.68)	1.00 (5.00)	0.00 (1.16)	0.33 (2.68)	1.00 (5.00)
SEd	0.99	1.20	1.38	0.58	1.59	1.74	1.25	1.58	1.55
CD(P=0.05)	2.17	2.62	3.02	1.27	3.48	3.79	2.73	3.45	3.38

Note: The figures in parentheses are arcsine transformed values.



Sl.No.	*Bacillus* spp	Isolates
1.	*B.weihenstephanensis*	TSB4
2.	*B.cereus*	CLB2D
3.	*B.subtilis*	TSB5
4.	*B.cereus*	TSB4D
5.	*B.licheniformis*	TSB3

Plate 1: Confirmation of presence of proteins in five different isolates of *Bacillus* spp. using SDS – PAGE.

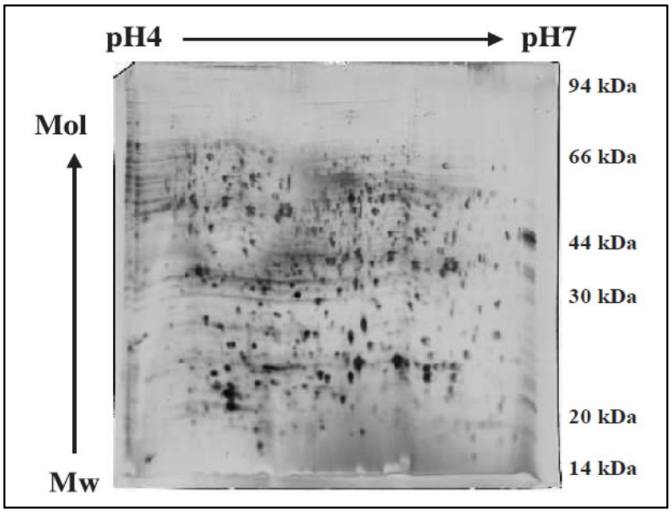


Plate 2: Protein profiling of *B.weihenstephanensis*(TSB4) using 2D-PAGE

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