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Alteration of the whole cell protein profile in nifH containing sesame rhizospheric bacteria in response to Cry1Ac toxin

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Abstract
Rhizospheric soil was isolated from flowering stage of sesame plant. From the soil sample, a total of 22 strains of bacteria were isolated by the process of enrichment and isolation on nitrogen free medium. Out of these 22 strains, two were selected by their partial identification through 16S rRNA phylogeny. Growth characteristics were studied for two strains viz. tempetrature and pH requirement for growth. RM4 and RM5 showed 99% similarity with *Sphingobium sp.* The Bt-endotoxin was purified from *Bacillus thuringiensis* subsp. *Kurstaki*. Bioassay of isolated toxin was done on the larvae of *Helicoverpa armigera* to check their mortality and effectivity, and to calculate LC_{50} of endotoxin. The integrity of protein was checked on 10% SDS-PAGE. The protein profiles of bacteria were compared in absence and in presence of two different condition of Cry1Ac endotoxin. Different protein profile was observed for the different concentration of toxin for the same strain.

Keywords: Bacillus thuringiensis, Cry1Ac, LC_{50}, bioassay, SDS-PAGE, protein profile

Introduction
Bt is a naturally occurring ubiquitous soil bacterium that produces a toxin lethal to certain insects. Bt transgenic crops are plants that have been genetically modified to express the insecticidal proteins (e.g. Cry1Ab, Cry1Ac, and Cry13A) from subspecies of the bacterium Bt to kill the coleopteran pests that feeds on potato (Flores *et al*., 2005) [5]. Even today the release of Bt transgenic crop is still highly controversial in many countries due to the concern over their potential detrimental effects on ecology and human health (Liu *et al*., 2005) [12]. Although Bt is a soil borne bacterium that naturally releases Bt toxins into the soil, Bt toxin from crop sources are likely to result in the additional exposure of soil organisms to these toxins. Soil ecosystem is not only the reservoir pool of exotic genes and their expression products of Bt transgenic crop but also the centre of biosphere and terminal habitat of microorganisms. Thus, concern for soil ecosystem effects of Bt transgenic crop has been raised in recent years. Usually, the Bt-endotoxin could be introduced into the soil through root exudation or decomposition of the crop residues (Palm *et al*, 1996; Sims and Holden, 1996; Saxena *et al*., 1999; Saxena and Stotzky, 2000) [16, 23, 20, 21]. Once in the soil, Bt-toxin could be adsorbed on bound or clay particles, humic components, or organic mineral complexes and then be protected against degradation by soil microorganisms. In this way, it could accumulate to certain concentration that might affect the composition and activity of soil microbial communities (Tap & Stotzky, 1995) [27].
The effect of Bt endotoxin from *Bacillus thuriengiensis* on two native bacteria isolated from Sesame plant rhizospheric soil has been studied. The bacteria isolated were free living, in nature and detected positive for presence of nifH gene, a gene coding for nitrogenase enzyme essential for biological nitrogen fixation.

Materials and methods
Soil sampling
Soil sample was collected from sesame plant rhizosphere Department of Zoology, North Campus, University of Delhi (ridge area). Soil sampling was done aseptically in sterilized polythene bags. The soil samples were taken randomly from different plants at the depth of 0-10 cms from surface.

Composite mixture was made and plant roots, stones and other debris were removed by hand picking from the samples. Some soil was sieved with < 4mm size sieve and stored at 4 ˚C for pH, microbial activity and percentage (%) C, H, N and S analysis. Remaining soil samples were stored immediately at -20 ˚C before analysis. Soil pH, Organic carbon, Available phosphate (P2O5), Available potassium (K2O), Ammonical nitrogen (N) and Nitrate nitrogen (N) content of the soil was determined using HIMEDIA soil testing kit according to instruction manual. Soil dry matter content, Moisture content (%), Water holding capacity was determined according to procedure described by (Alef *et al*.,) For total C, H, N, S, analysis, the samples were oven dried at 60 ˚C for 8-10 hrs. The dried soil samples were then sieved through BSSM standard sieves numbers 200 and 300. Sieve 200 had mesh size 0.075 microns. Sieve 300 had mesh size 0.05 microns. These samples were analysed for C.H.N.S. at University Scientific Instrumentation Centre by the C.H.N.S.O analyser Elementar Vario ELIII.

Isolation of bacterial strain

5 grams of soil sample aseptically and inoculated into 100 mL of nitrogen free LGI medium. This was kept at 28˚C, 150 rpm for 15 days. After 15 days, the solid particles were allowed to stand for 1 hr and 10 mL of supernatant was used to inoculate fresh LGI medium. This was repeated for 4 weeks and every time with fresh LGI medium. An aliquot of 100 µL was taken from LGI medium. This aliquot was diluted to 100 times to 108 times by using autoclaved milliQ water. Different dilutions were spread on LGI agar plates by using sterilized glass spreader. Plates were incubated at 28˚C for 48 hrs. Only those plates with 100 dilutions were found to have distinct colonies. The discrete colonies of microbes showing luxurious growth were isolated depending upon their distinct morphological features such as shape, size, color etc.

Isolation of pure strain

Each morphologically different colony was streaked on fresh LGI agar plates separately. Isolates were purified further by streaking on fresh LGI agar plates on every alternate day for 1 week. Each isolated strains was studied for their basic colony characteristics namely abundance of growth, pigmentation, size, form, margin of colony and elevation of colony on LB agar plates after 48 hrs of growth. The selected strains were distinguished as Gram's positive and Gram's negative strain by KOH method.

Isolation of genomic DNA

Genomic DNA was isolated from each strain according to the method described by Sambrook *et al*., (1989) [19] DNA quantity and quality were checked by agarose gel electrophoresis and by Nano Drop ND 100, spectrophotometer. For purity check ratio of absorbance at wavelength 260 nm and 280 nm was taken in account. If A260/280=1.8 ± 0.1 the DNA was considered pure. The isolated DNA was also visualized for integrity and for RNA and protein contamination on 0.8% agarose gel stained with ethidium bromide and visualized under UV-light.

Amplification of nifH gene from the isolated strain

The presence of 370 bp *nifH* gene encoding for enzyme nitrogenase was checked in isolates. For that, 370 bp *nifH* genes from all the soil isolates were amplified using primers PolF: 5'-TGC GAY CCS AAR GCB GAC TC-3' and PolR: 5'-ATS GCC ATC ATY TCR CCG GA-3' designed by Poly *et al*., (2001a). Amplification was observed on 2% agarose gel using UV visualization of Et-Br stained band. The PCR amplified products were loaded on 2% agarose gel stained with ethidium bromide and visualized under UV-light.

16S rRNA gene amplification from isolated strains

The 16S rRNA gene was amplified from each strain by colony PCR technique. The amplified PCR products were loaded on 1% agarose gel stained with ethidium bromide and visualized under UV-light. *PCR product was eluted* and sent to Macrogen Korea for sequencing using universal primers for 16S rRNA gene. The sequences obtained were analyzed using ABI sequence analysis software. The sequence obtained for each strain was subjected to nucleotide BLAST search program for identification of the soil isolates. Out of 30 isolated strains, strain RM1 and RM2 were selected on the basis of 16S rRNA gene sequence similarity for further studies. Partial 16S rRNA gene sequence for the strains RM4 (606bp) and RM5 (410bp) were submitted to NCBI Bankit to obtain the accession number and for future references.

2.1.6. Optimization of growth condition for RM4 and RM5

To optimize the growth conditions i.e. temperature and pH conditions for strains, both the strain, RM4 and RM5 were grown at different temperature and different pH. 5 ml of LB broth was inoculated with a single bacterial colony. It was allowed to grow till optical density reached 0.7 at λ595nm.

Optimization of temperature and pH condition

To optimize the temperature for bacteria, 2 ml of culture (OD of 0.7 at λ595nm) was inoculated into flasks containing 200 ml of LB broth each. Each inoculated flask was kept on shaker at three different conditions (20 ºC, 150 rpm), (30 ºC, 150 rpm), and (40 ºC, 150 rpm), for 48 hrs. 2 ml of sample was taken out in microcentrifuge tubes at interval of every 2 hrs starting from 0 hr. OD was monitored on UV-visible light at λ595nm. Experiment was performed in triplicate for each temperature.

LB broth with different pH: 5.3, 6.3, 7.3, 8.3, and 9.3 were prepared and autoclaved. To optimize the pH condition of bacteria, 2 ml of culture (OD at λ595nm) was inoculated into each flask containing 200 ml of LB broth. Each inoculated flask kept on shaker at 30 ºC, 150 rpm, for 48 hrs. 2 ml of sample was taken out in microcentrifuge tubes at interval of every 2 hrs starting from 0 hr. Optical density was monitored on UV-visible light at λ595nm. Experiment was performed in triplicate for each pH.

Purification of Cry1Ac toxin

The spore crystal mix was isolated from the bacteria *Bacillus thuringiensis* by the method described by Howard *et al*., (1979) [9]; Mittal *et al*., (2007) [15]. Cry1Ac toxin was isolated from the spore crystal mix of *Bacillus thuringiensis* by slight modification of the method described by Babu *et al*., (2002) [4]. Spore crystal mixture was mixed with NaOH (50 mM, pH 12.0) in the ratio of 1:12 (W/V) and incubated at 37 ºC in a shaker for 30 min. After incubation, the tube containing spore crystal mixture and alkali buffer was centrifuged at 10,000 rpm for 10 min at 4 ºC to remove the

spores and undissolved inclusions from the supernatant. The supernatant was filter sterilized and stored at -20 ºC for further analysis. The presence of viable spores in the purified toxin was detected by plating 25 µl of the purified toxin on the LB agar plate. The germination of the spores was checked after 24 and 48 hrs of incubation at 37 ºC. The concentration of the purified endotoxin was estimated by the process described by Lowry *et al*., (1951). Integrity of the isolated Cry1Ac toxin was checked by Sodium Dodecyl Sulphate–Polyacrylamide Gel electrophoresis (SDS-PAGE) according to Laemlli, (1970) [11]. The gel was stained in Coomassie brilliant blue G-250 solution prepared by dissolving 0.25 mg Coomassie Brilliant Blue G-250 in 100 ml of 25% methanol, 10% glacial acetic acid and 65% double distilled water. The staining was carried out for 2 hrs under mild shaking followed by destaining using methanol: glacial acetic acid: double distilled water in the ratio of 25:10:65 (V/V) for several hrs till the protein bands became clearly visible with no background colour. The gel was then visualized under densitometer.

Susceptibility test of Cry1Ac toxin to *Helicoverpa armigera*

Bioassay

Adult males and females of *Helicoverpa armigera* were allowed to mate in a chamber and eggs layed were collected on muslin clothes in a small air tight container whose lid was light screwed for exchange of gases. Larvae came out after 1-2 days. They were cultured on a semi synthetic diet, which contained chickpea as its main component (Singh and Rambold, 1992). The colony was maintained in a culture room with a mean temperature of 27 ºC, 60% RH and with a photoperiod of 14:10 (L:D) The stock culture in sixth to ninth generation was used for the bioassay. Five concentrations of Bt-endotoxin were prepared (0, 0.0312, 0.3720, 0.0625, 0.9120) µg/ml along with control. Each treatment was replicated for three times and at least ten larvae formed one experimental unit. Mortality of larvae was scored every 24 hrs for 7 days. Each bioassay was repeated 2-3 times. In each experiment, mortality pattern for five concentrations were used to calculate median lethal concentration (LC_{50}), All the experiments were carried out in a room with a photoperiod of 12:12 (L: D) and at an average temperature of 27 ºC, 60% RH. Mortality was recorded for the dose of toxin tested and corrected mortality was calculated in case where mortality was recorded in the control using Abott's formula.

$$\text{Corrected Mortality} = \frac{P - P^o}{100 - P^o} X100$$

Where,

P = Mortality in experimental unit.

P^o = Mortality in control unit.

Corrected percent mortality was converted to their respective probit value. A graph with probit values (Y-axis) against $\log_{10}$ concentration (X-axis) was plotted and a best straight line was drawn through plotted points, then this line was used to estimate the $\log_{10}$ concentration associated with a probit value of 5 (the probit of 50%). LC_{50} value was calculated by converting this $\log_{10}$ value to antilog.

Effect of Bt-endotoxin on the protein profile of the selected strains

Nine flasks with 200 ml of LB broth each were prepared (three flasks for each concentration). Two concentrations i.e. 1.6 and 3.2 µg/ml was used for both the strain for further experiment. The cells were harvested by centrifugation at 10000 g at 4 ºC for 15 mins, washed twice in cold milliQ water and resuspended in 1 mL of milliQ water. 30 µL of this cell suspension was diluted in 50 µL of 50 mM/L) Tris buffer (pH 7.5). To this suspension, 20 µL of 4X SDS-loading dye (200 mM/L, Tris–HCl, pH 6.8, 10% (w⁄v), glycerol, 2% (w⁄v), SDS, 5% (v⁄v), β-mercaptoethanol and 0.05% bromophenol blue) were added, and the samples were denatured by heating at 95ºC for 5 mins in a water bath (Laemmli, 1970) [11]. After denaturation, samples were centrifuged at 10000g for 10 mins. Protein concentration of the whole cell protein extract was determined by Bradford's method. SDS-PAGE for the whole cell protein extract was carried out according to the method described earlier. The gel was stained in Coomassie brilliant blue G-250 for several hrs till the protein bands become clearly visible with no background colour. The gel was then visualized and quantification of bands was done by densitometry.

Result and discussion

Soil biota consists of a large range of microorganisms which interact among each other and also with the plants and provide them nutrition along with other benefits (Welbaum *et al*., 2004) [29]. Manipulation in the population of plant growth promoting rhizobacteria (PGPR'S) has tremendous potential to enhance crop yield, improve soil fertility and sustain soil health. A number of factors including plant type, age, soil type, agricultural practices and composition of microbial communities, root exudates and chemical compounds applied to the soil may influence their activity and diversity (Martinez-Toled *et al*., 1991; Rajakumar *et al*., 1995) [14, 18].

Microbes are natural components of soil and water environment. They usually, occupy a volume of less than 0.1% of the soil but are responsible for numerous transformations that cycle elements and energy in nature. Microbial density may be as high as 109 per gram of soil, with a biomass upto several tone per hectare. The structural analysis of microorganism in soil presents a far new complex problem than their functional analysis. Microbial communities in soil are extremely diverse and it is assumed that with conventional microbiological cultivation methods only about 1% of the indigenous species are recovered. Soil physicochemical properties are important factors that govern the type and properties of the micro-flora and micro-fauna found in a particular soil. The physio-chemical properties of experimental soil are shown in table1. Out of the different plates inoculated with different dilutions of enrichment culture, only the plate having 100 and 102 were showing countable numbers of colonies after the 7 days of incubation. A total of 22 colonies were isolated by the enrichment technique from the LGI plates. Microorganism use naturally occurring and synthetic chemicals for their growth. They use these molecules as a source of energy, Carbon (C), Nitrogen (N), Phosphorous (P), Sulfur (S) or other elements, which are required for their growth. LGI medium used in this study had sucrose as a carbon source. As the strains were isolated by enrichment method in the LGI medium, it can be expected that the strains growing in

this medium were able to utilize sucrose as a carbon source very well, apart from other carbon sources.

On the LGI agar plate, different types of colonies were found to be having different types of morphological characteristics such as abundance of growth, pigmentation, size, margin of colony and elevation of colony (Table 2). The strain RM4 had good abundance of growth, medium size of colony with smooth margin and convex elevation of colony. The strain RM5 had good abundance of growth, medium size of colony with smooth margin and convex elevation of colony. Both the strains showed pigmentation. Earlier LGI medium was described as a selective substrate for the isolation of Azotobacter being successfully employed to screen soil isolates for the presumptive recognition of micro-organisms belonging to the genus of interest. But now it has been reported that utilization of LGI medium is differential more than selective, allowing free living diazotrophes to be preliminary recognized on the basis of morphology, consistency, and pigmentation of the colonies. Moreover, the presence of pH indicator (Bromothymol blue) permitted the individualization of the Azotobacter acidifying strains (Aquilanti *et al*., 2004) [3]. Out of the 22 strains isolated, three strains, RM1, RM2 and RM23 were detected as Gram's positive. All other strains were detected as Gram's negative (Table 3). Genomic DNA, isolated from all the strains was shows different yields. 1.5 kb 16S rRNA gene was successfully amplified from all the strains by PCR technique (Fig 1). 360 bp nifH gene was successfully amplified from RM4 and RM5 using genomic DNA as template (Fig 2). According to the16S rRNA gene sequence similarity, the strains were compared with their closest gene bank match and the strain RM4 was showing 99% similarity with the *Sphingomonas sp*. and RM5 was showing 99% similarity with the *Novosphingobium sp*. Strain RM4 and RM5 were selected out of 22 strains as they were showing 99% similarity with their closest gene bank match. Rest other strains were showing 100% similarity with their closest gene bank match. The optimum temperature and the optimum pH for the strain RM4 and RM5 were obtained as 30ºC and 7.3 (Fig 3). The Cry1Ac toxin purified was free of viable spores as no germination of spores was seen when plated on LB agar plates. A calibration curve was made using different concentrations of standard BSA stock solution. The value of slope obtained was 0.351. The value of unknown protein sample was determined by dividing OD of unknown sample by value of slope of calibration curve. The concentration of Cry1Ac toxin extracted from ECE53 was 0.868 µg/µl and concentration of Cry1Ac toxin from 4D1 spore crystal mix was 0.376 µg/µL. Only single band of size approximately 135 kDa was visible in the lane loaded with crystal protein purified from spore crystal mix of *Bacillus thuringiensis* (4D1), as shown in (Fig.8). With the 5 different Bt-toxin concentrations on the neonate larvae of Helicoverpa *armigera*, maximum i.e. 100% motality was recorded for the toxin concentration of 0.9120 µg/mL. Untreated replicates were not showing mortality even after 7 days (Table 3.13). From the dose response relationship graph the LC50 value for Bt-endotoxin was calculated as 0.647 µg/mL (Fig.6). After 12 hrs of incubation considerable growth was seen in all the six flasks, crude protein extracted was estimated by Bradford's method the value of slope was 0.036. The value of unknown protein sample was determined using slope of calibration curve. The yield of protein for different treatment showed that for strain RM4 the control, with no toxin incubation gave maximum yield of 5.31 ± 0.15 mg/mL, which goes down to 0.69 ± 0.02 mg/mL, and showed slight increase 0.79 ± 0.03 mg/mL for toxin concentration 3.2 µg/mL. For strain RM5 the control, with no toxin incubation gave maximum yield of 5.05 ± 0.14 mg/mL, which goes down to 0.76 ± 0.00 mg/mL, corresponding to 1.6 µg/mL of Bt-endotoxin and showed increase upto 2.81 ± 0.06 mg/mL for toxin concentration of 3.2 µg/mL (Table.5). The SDS–PAGE of whole cell proteins of strains RM4 and RM5, with and without Cry1Ac toxin incubation generated protein profiles containing 40–50 bands each, with molecular weights ranging from 11-230 kDa (Fig.9). A quantitative as well as qualitative difference was observed in protein bands having molecular weight in the region of 28–36 kDa, and also in the 17-28 kDa. The bands present in the lower region (<11 kDa) of the SDS–PAGE gels in all samples did not resolve well. A differential expression of protein bands were seen corresponding to different doses of Bt- endotoxin. For quantification of protein bands by densitometry showed that for the strain RM4, band size ranging from 28-36 kDa (2) was showing increase in intensity with an increase in the concentration of Cry1Ac toxin used which decreased further with increase in toxin concentration. For the strain RM5, this band was almost negligible for control but its intensity increased with the increased dose of toxin concentration. A band in the range 17-24 kDa (4) was also showing difference in intensity. For the strain RM5 although, this band was present in the control, its intensity went up with the increased concentration of toxin and decreased with further increase in toxin concentration. For the strain RM4 the result was quite different as band intensity increased with increase in toxin dose. Also, a band of approximately 36 kDa (1) was present in the control for both the strains, but got dissappeared in the presence of toxin. Another band of 28 kDa (3) was also showing difference in intensity with different toxin dose. For the strain RM4, band intensity increased slightly with initial increase in toxin dose which decreased significantly with increase in toxin concentration further (Fig 10). For the strain RM5 the result was same. The bands differing in intensity needs to be identified further by mass spectrometry.

Table 1: Physical and chemical properties of experimental soils

Soil characteristics	Values
pH	9.0
Total C (%)	2.95 ± 0.22
Total H (%)	2.02 ± 0.16
Total N (%)	2.77 ± 0.28
Total S (%)	0.12 ± 0.01
Organic carbon (%)	0.505-0.750
Available phosphate (P_2O_5)	0 kg/hectare
Available potassium (K_2O)	Above 392 kg/hectare
Ammonical nitrogen (N)	15 kg/hectare as N
Nitrate nitrogen (N)	50 kg/hectare as N
Dry matter content (%)	87.93 ± 0.13
Dry matter content (%)	12.06 ± 0.13
Water holding capacity (%)	51.84 ± 1.7

Table 2: Characteristics of the soil isolates on LB agar after 24 hrs of growth.

Strains	Abundance of growth	Pigmentation	Size	Margin of colony	Elevation of colony
RM1	Good	N	Large	Smooth	Convex
RM2	Good	N	Large	Smooth	Flat
RM4	Good	Y	Medium	Smooth	Convex
RM5	Good	Y	Medium	Smooth	Convex
RM5	Good	Y	Medium	Smooth	Convex
RM7	Good	Y	Medium	Smooth	Convex
RM8	Good	Y	Medium	Smooth	Convex
RM9	Good	Y	Medium	Smooth	Convex
RM10	Good	Y	Medium	Smooth	Convex
RM11	Good	Y	Medium	Smooth	Convex
RM12	Good	Y	Medium	Smooth	Convex
RM14	Good	Y	Medium	Smooth	Convex
RM15	Good	Y	Medium	Smooth	Convex
RM16	Good	Y	Medium	Smooth	Convex
RM17	Good	Y	Medium	Smooth	Convex
RM18	Good	Y	Medium	Smooth	Convex
RM19	Good	Y	Medium	Smooth	Convex
RM20	Good	Y	Medium	Smooth	Convex
RM21	Good	Y	Medium	Smooth	Convex
RM22	Good	Y	Medium	Smooth	Convex

*Y-yes **N-no

Table 3: Differentiation of soil isolates as Gram's positive and Gram's negative strain.

Strains	KOH test result	Gram's +/ Gram's -
RM1	-ve	Gram's +ve
RM2	-ve	Gram's +ve
RM4	+ve	Gram's -ve
RM5	+ve	Gram's -ve
RM5	+ve	Gram's -ve
RM7	+ve	Gram's -ve
RM8	+ve	Gram's -ve
RM9	+ve	Gram's -ve
RM10	+ve	Gram's -ve
RM11	+ve	Gram's -ve
RM12	+ve	Gram's -ve
RM14	+ve	Gram's -ve
RM15	+ve	Gram's -ve
RM16	+ve	Gram's -ve
RM17	+ve	Gram's -ve
RM18	+ve	Gram's -ve
RM19	+ve	Gram's -ve
RM20	+ve	Gram's -ve
RM21	+ve	Gram's -ve
RM22	+ve	Gram's -ve

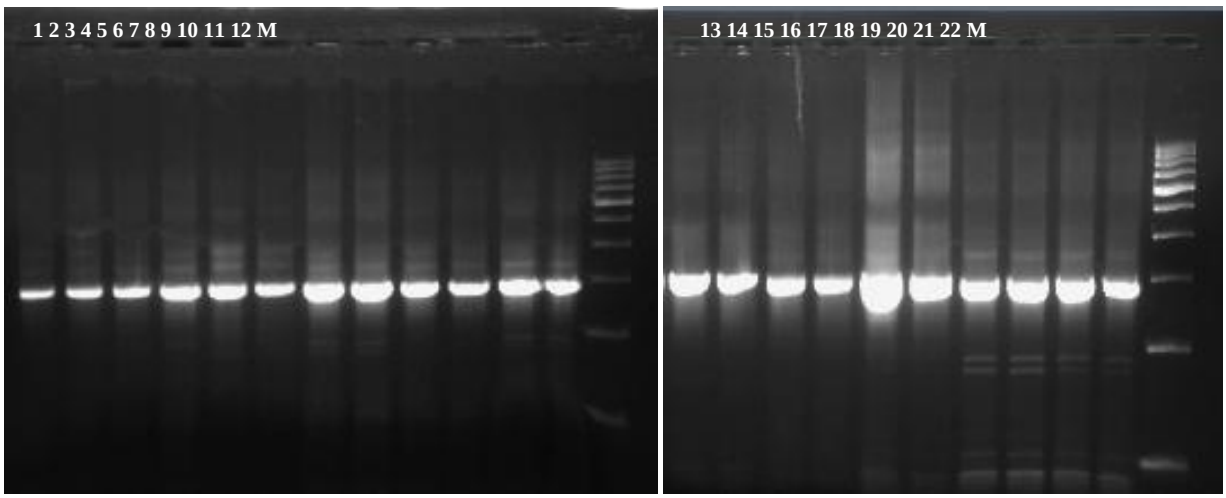


Fig 1: 0.8% agarose gel stained with ethidium bromide showing 16S rRNA gene amplification from samples 1, 2, 3, 5and 6 isolates (Numbers above the wells indicate the name of the strains). Lane M shows 500 bp DNA size marker.

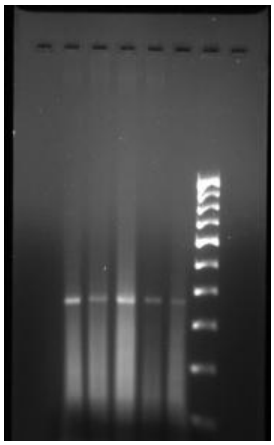
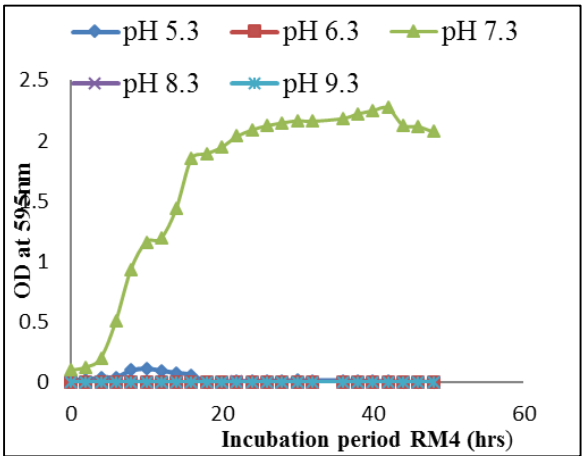
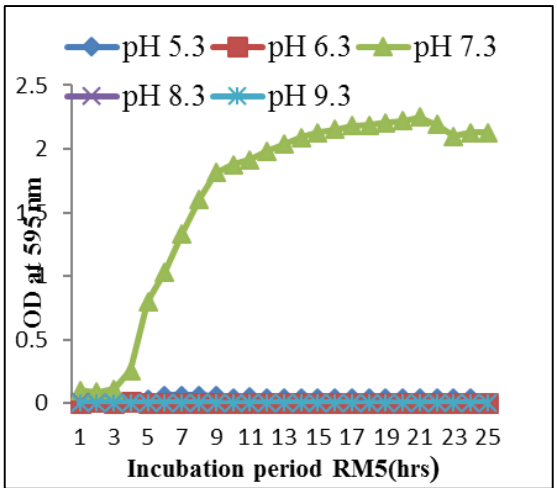


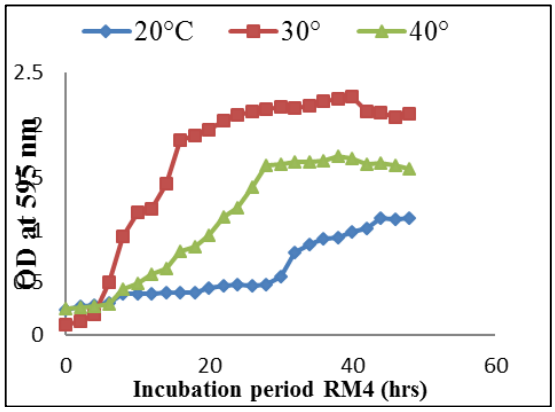
Fig 2: 1% agarose gel stained with ethidium bromide showing 360 bp *nifH* gene amplified from the strain RM4 and RM5. Lane M contains 100 bp DNA size marker. Lane A. br. contain positive control.

Table 4: Accession numbers for the strains RM4 and RM5

Sr. No	Strains	Bankit	Accession No
1	RM4	1391285	HQ267227
2	RM5	1391308	HQ267228







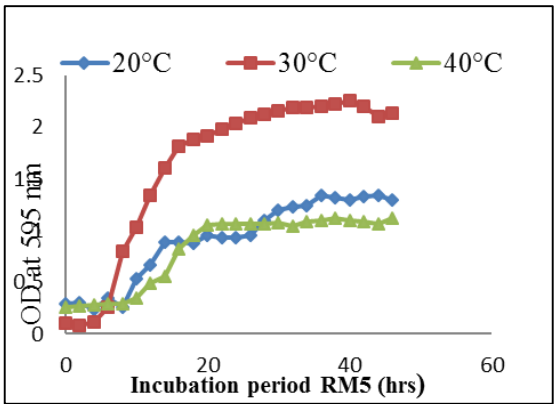
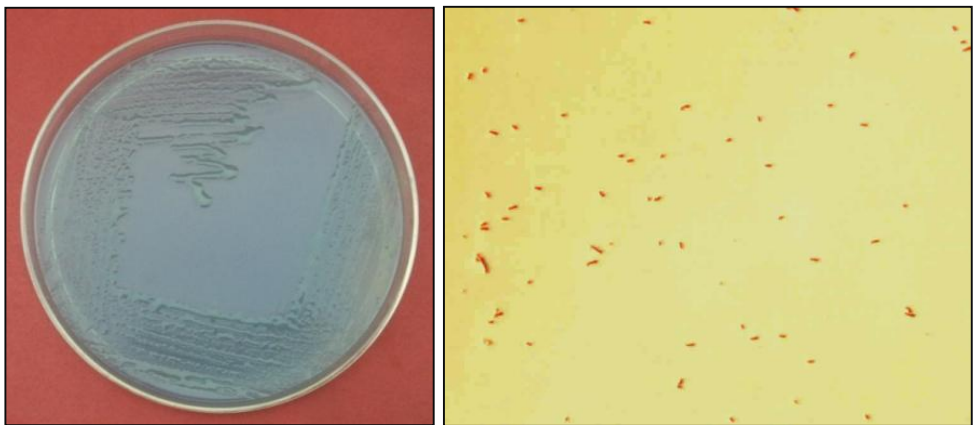
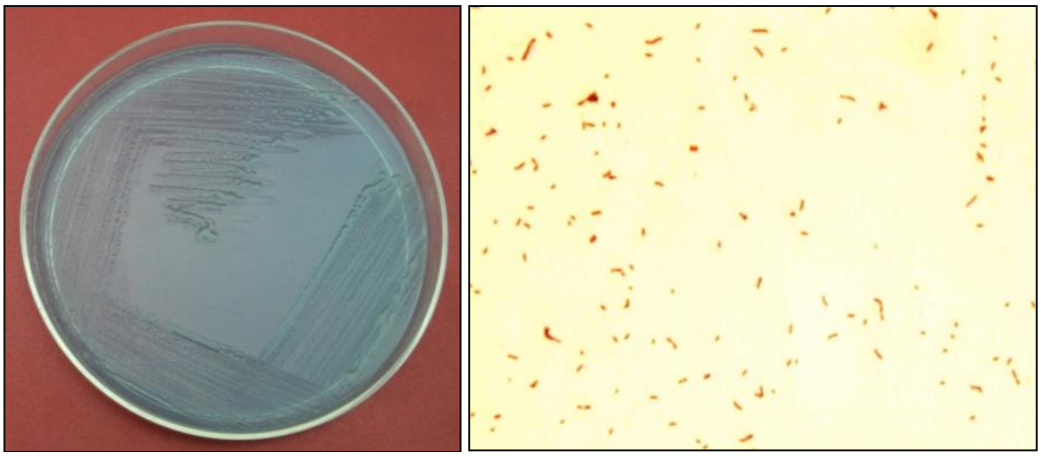


Fig 3: Growth curve of RM4 and RM5 at different temperature and pH.



a **b**



c **d**

Fig 4: (a) Morphology of the strain RM4 growing on LGI agar after 96 hrs. (b) Gram's staining of the strain RM5 (c) Morphology of the strain RM5 growing on LGI agar after 96 hrs. (d) Gram's staining of the strain RM5.

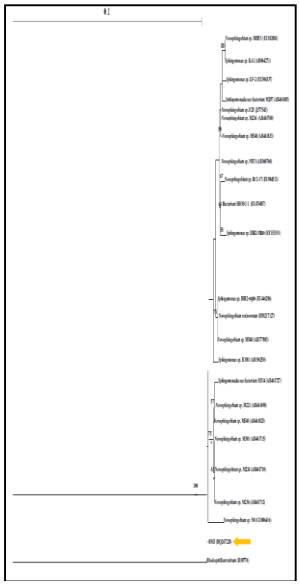


Fig 5: Phylogenetic tree RM4

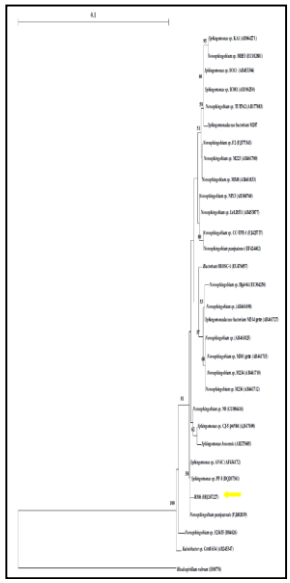


Fig 6: Phylogenetic tree RM5

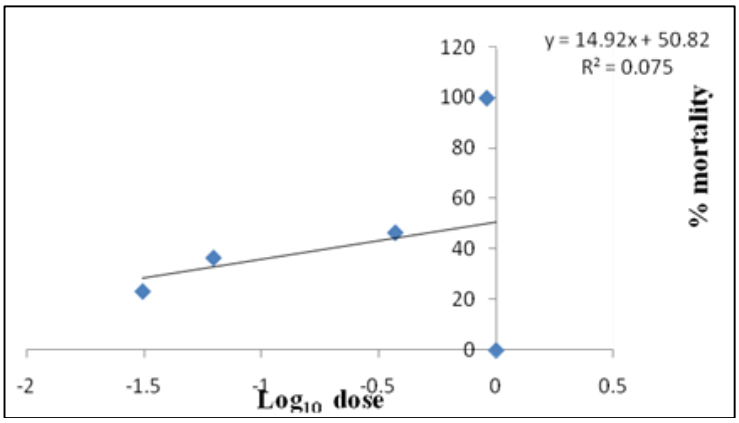


Fig 7: Dose response relationship of Helicoverpa armigera for Bt-endotoxin.

Table 5: Estimation of whole cell protein by Bradford's method.

Strains	Concentration Of toxin µg/mL	Yield mg/mL	Average	S.D.	S.E.
RM4	0	5.19	5.05	0.197	0.14
	0	4.92			
	1.6	0.66	0.69	0.042	0.02
	1.6	0.72			
	3.2	0.75	0.79	0.056	0.03
	3.2	0.83			

RM5	0	5.47	5.31	0.219	0.15
	0	5.16			
	1.6	0.77	0.76	0.014	0.00
	1.6	0.75			
	3.2	2.88	2.81	0.091	0.06
	3.2	2.75			

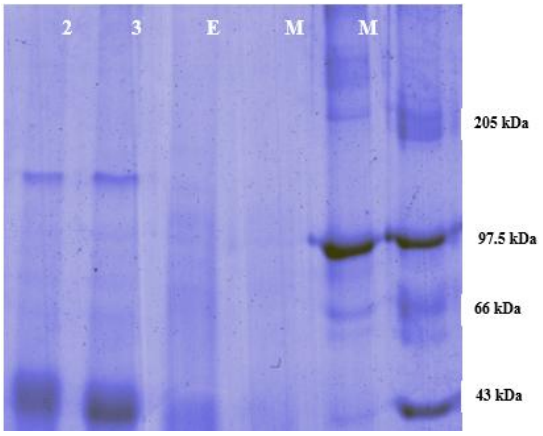


Fig 8: 10% SDS-PAGE showing extraction of 135 kDa Cry1Ac endotoxin from spore crystal mix of strain 4D1 (*Bacillus thuringiensis* subsp. *Kurstaki* HD1) Lane 1, 2 contain 135 kDa toxin band,. Lane E is empty. Lane M contain protein marker with molecular weight ranging from 29-205 kDa.

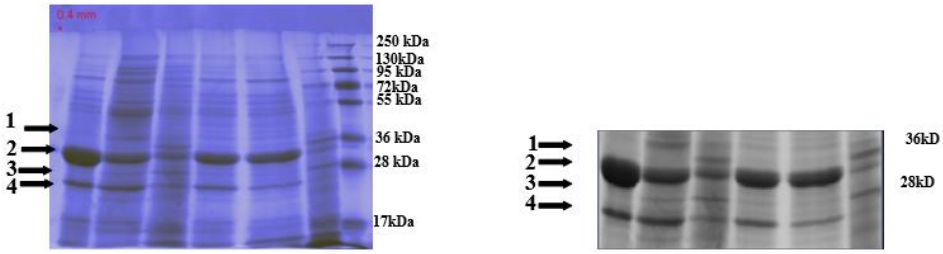


Fig 9: 12% SDS-PAGE showing whole cell protein profiling of bacterial strain RM4 and RM5 incubated in the absence and in the presence of different concentrations of Cry1Ac endotoxin. Lane 1- Strain RM5 incubated with 3.2µg/ml.
Lane2- Strain RM5 incubated with 1.6µg/ml. Lane3- Strain RM5 without toxin
Lane4- Strain RM4 incubated with 3.2µg/ml Lane5- Strain RM4 incubated with 1.6µg/m. Lane6- Strain RM4 without toxin. LaneM- Marker with molecular weight ranging from 11-250 kDa

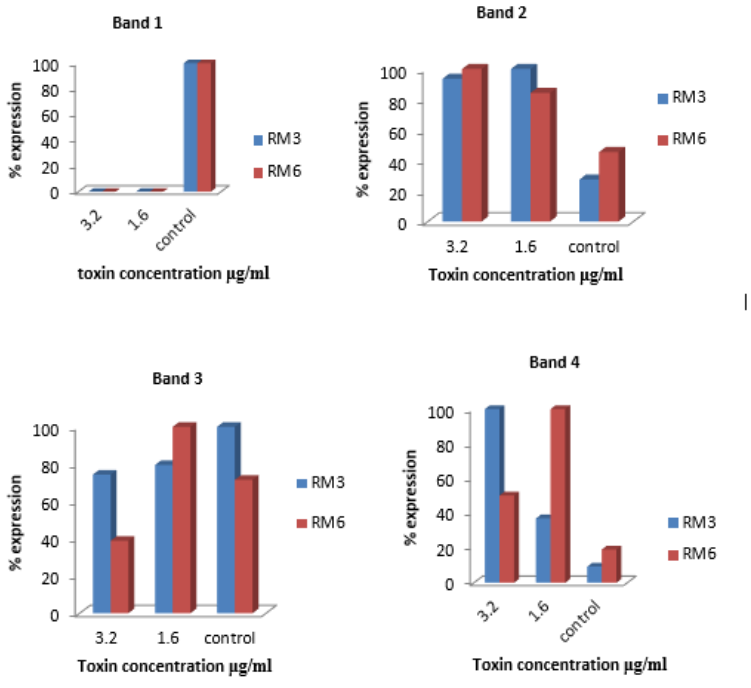


Fig 10: Comparative % expression of proteins bands in RM4 and RM5 at different *Bt*-endotoxin concentration.

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