Isolation and Molecular Characterization of *Bacillus thuringiensis* Harboring Putative *ps* Genes from Bangladesh

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Abstract

Parasporin, another type of δ- endotoxin from *Bacillus thuringiensis (Bt)*, renowned for its highly specific and preferential toxicity against different cancer cells, could be a potentially safe anticancer therapeutic. Considering the current cancer situation, isolation and molecular characterization of parasporin-producing *Bt* isolates were aimed. Hence, *Bt* isolates were obtained through a series of tests viz. heat treatment, acetate selection, starch hydrolysis, lecithinase activity, and microscopy for parasporal proteins. Then the biochemical properties, plasmid, parasporin gene, and δ- endotoxin protein profiles were checked. Primers were designed for *ps* gene detection and the molecular identification of the isolates was performed by 16S rRNA gene sequence analysis. From the tested samples (n=128), 91 *Bt* isolates were obtained and of them, 28 non-hemolytic *Bt* isolates were selected as potential parasporin producers. Plasmid profile analysis of these isolates revealed that non-hemolytic *Bt* isolates harbor low molecular weight plasmid (6-8 Kb) unlike the *Bt* with insecticidal properties. Through PCR detection, amplicons of the desired size for *ps2* and *ps3* genes were yielded by two isolates namely *Bt* DSc5 and *Bt* DSf3. The crude δ-endotoxin profiles and Proteinase-K digestion generated fragments of these isolates resembled previous reports. Finally, the 16S rRNA gene sequence analysis of these two isolates followed by blastn analysis confirmed them as *B. thuringiensis.* It is, therefore, anticipated that effective anticancer proteins could be obtained from indigenous *Bt* to make them useful in cancer therapy.

Significance | Novel type of anticancer agents from *Bacillus thuringiensis*

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Abbreviations: *Bt*, *Bacillus thuringiensis*; *ps*, parasporin; kDa, kiloDalton; bp, base pair; M, Molar; *Btk*, *Bacillus thuringiensis kurstaki*; g, Gram; fwd, Forward; rev, Reverse; UV, Ultraviolet; PCR, Polymerase chain reaction; dNTPs, Deoxynucleotide triphosphates; *Taq*, *Thermus aquaticus*.

Introduction

Cancer is among the leading causes of death in the contemporary world and the high cost of available treatments made it a matter of great concern for both developed and developing countries (WHO media center 2017). It is an urgent need to produce a low-cost anticancer drug with specific cytotoxicity to fight this deadly disease. *Bacillus thuringiensis* (*Bt*), a gram-positive bacterium with the ability to produce crystalline parasporal inclusions which are insecticidal and mostly comprised of δ-endotoxins, were also reported with preferential cancer cell killing ability (Beegle and Yamamoto 1992; A. Shishir et al. 2014; E Mizuki et al. 1999; Okassov et al. 2015; Nagamatsu et al. 2010; Okumura et al. 2010).

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Interestingly, the specific toxicity against the cancer cells was observed with non-insecticidal *Bt* strain-derived δ- endotoxins which were named as Parasporin (Ps) (Okumura et al. 2010; E Mizuki et al. 1998). Although it was reported that the non-insecticidal *Bt* strains were more widely distributed than insecticidal ones (Hastowo, Lay, and Ohba 1992; Martin and Travers 1989; Meadows et al. 1992; Ohba and Aizawa 1986; E Mizuki et al. 1999; Ohba, Mizuki, and Uemori 2009), the number of Ps proteins discovered so far is quite a few compared to

the insecticidal proteins (Aktar et al. 2019; Ferdous et al. 2018). Nevertheless, many *Bt* strains exerting anticancer activities have been isolated around the world in recent years, e.g., LDC-391 from India (K Poornima, Selvanayagam, and Shenbagarathai 2010; Kkani Poornima et al. 2012), 64-1-94 from Caribbean Islands (Gonzalez et al. 2011), and *Bt*18 from Malaysia (Nadarajah et al. 2008). Hence, more extensive investigations should be carried out continuously for the discovery of more and novel *ps* genes.

Genes encoding Cry toxins are thought to reside on large plasmids with many *Bt* strains containing multiple *cry* genes. The protein PS1Aa1, established earlier as Cry31Aa1, is a polypeptide of 723 amino acid residues weighing 81 kD and the length of the corresponding gene is 2,169 bp. As reported by Mizuki et al. (Eiichi Mizuki et al. 2000) and Katayama et al. (Katayama et al. 2005), PS1Aa1 exhibits cancer cell-killing activity only when digested with proteases. Protease-treated PS1Aa1 (81 kDa) generates a heterodimer protein consisting of 15- and 56- kDa polypeptides upon digestion in the N-terminal region which is toxic to the cancer cells. According to Ito et al. (Ito et al. 2004) PS2Aa1 (former Cry46Aa1) is a polypeptide of 338 amino acid residues and a molecular weight of 37 kD, product of a 1,014 bp long gene. Proteolytic processing of the 37-kDa precursor protein yields a 30-kDa cytotoxin active on cancer cells. The protease digestion, in this case, occurs in both N and C-terminal regions. Again, the protein PS3Aa1 has a typical three-domain structure and consists of 825 amino acid residues with a deduced molecular weight of 93,689. The activation of PS3Aa1 is also dependent on proteolytic digestion of the 81-kDa precursor both at N- and C- terminal regions resulting in a 64-kDa toxic moiety. The fourth parasporin, PS4Aa1 (former Cry45Aa1), comprises 275 amino acid residues weighing 30 kD. The corresponding gene is 828 bp long and the three-domain structure is not associated with this protein (Supplementary Table 1).

The identification of subclasses of *ps* genes (1-6) was accomplished by *in vitro* cytotoxicity analysis of δ-endotoxins upon detection of those genes based on the available amino acid sequences (Okumura et al. 2010). The small *ps* gene library and a large number of cytotoxicity analyses are a few of the key hindrances to the detection of more and novel *ps* genes. Initially, pore-forming like Parasporin proteins was detected followed by the further discovery of aerolysin and epsilon-like toxins (Ferdous et al. 2018). Although the Cry toxins are mainly found in parasporal crystals, some proteins have been reported to be found within the coat of spores (Aronson et al. 1982). Hence, the possibility, that more PS proteins with different structures yet to be discovered, could be very high. It was also reported that peptide sequences with anti-cancer properties could be identified in the combination of bioinformatics and proteomic analysis. Hence, new approaches should be adopted involving metagenomic analysis, bioinformatics, and *in silico* proteomics studies before the cytotoxicity study for high throughput cost-effective screening process (Aktar et al. 2019). Nevertheless, screening for more potent strains following traditional isolation and characterization technique is still valuable where the limitation of sophisticated and advanced instruments is present.

The study was therefore aimed at isolation and characterization of indigenous non-hemolytic *B. thuringiensis* strains producing parasporin inclusion proteins. The endeavor was made based on the so far reported *ps* genes and their corresponding parasporin proteins.

Materials and Methods

Isolation and Characterization of *Bacillus thuringiensis*

Samples were collected from different locations of Bangladesh comprising soil, stored dust, leaves, and sand. Strains of *B. thuringiensis* were isolated through acetate selection, presence of parasporal crystal protein, and starch hydrolyzing ability (Travers, Martin, and Reichelderfer 1987; A. Shishir et al. 2014) and were preserved in 15% glycerol stock. In brief, a 1.0 g soil sample was added to 20 mL of LB (Luria-Bertani) broth supplemented with 0.25 M sodium acetate for 4 h at 30°C. In this process, sodium acetate at a concentration of 0.25 M and more, selectively inhibits the germination of *Bt* spores solely whereas the spores of other *Bacillus* species germinate. At this stage, the culture was heated at 80 °C for 5 min to kill the vegetative cells i.e. the spores of other *Bacillus* spp. that just germinated. Then, 1.0 mL of the heat-treated culture was serially diluted to 10^{-6} folds and inoculated on LB agar medium by spread plate method followed by further incubation.

Bt isolates were grown on T_3-agar (in 1.0 L: Tryptone 3.0 g, tryptose 2.0 g, yeast extract 1.5 g, $MnCl_2$ 0.005 g, Phosphate buffer 50 mM, agar 15.0 g; pH: 6.8) for 4 days at 30 °C and the parasporal crystal protein was observed under a light microscope following coomassie brilliant blue staining method described by Rampersad (Rampersad, Khan, and Ammons 2002). Non-hemolytic Bt isolates as determined by hemolytic ability on sheep blood agar were subject to different biochemical tests including Oxidase, Catalase, Methyl red, Voges-Proskauer, Nitrate reduction, Indole, Urease, Citrate utilization, Starch hydrolysis, Casein hydrolysis and Motility ("Bergey's Manual of Determinative Bacteriology" 1958) along with fermentation of monosaccharide and disaccharide (Murad et al. 2010).

Plasmid DNA of the selected non-hemolytic strains and *Btk* HD-73, a reference strain of pesticidal *Bt* was prepared with overnight grown bacterial culture by following alkaline lysis method (A. Shishir et al. 2014) and visualized upon agarose gel electrophoresis in Gel documentation system (Alpha imager mini, USA) (Trevors 1984).

Genomic DNA isolation and 16S rRNA gene sequencing

Genomic DNA was isolated by following the method described by Sambrook and Russel 2001 and 16S rRNA gene was amplified by PCR with universal primers for *Bacillus* spp.: fwd (20F); 5'-GAGTTTGATCCTGGCTCAG-3' (position 9-27), and rev (1500R); 5'-GTTACCTTGTTACGACTT-3' (position 1492-1509) (M. A. Shishir et al. 2015). The amplicons were analyzed by electrophoresis in 1% (w/v) agarose gel (Promega, USA) in 1× TBE buffer and visualized against UV light. The purified PCR products were sequenced through ABI 3130 Automated Genetic Analyzer (Hitachi, Japan) and submitted to the NCBI database.

Analysis of Inclusion Protein

For protein profile analysis, *Bt* isolates were grown for 4 days on T_3- agar medium, and the crude parasporal inclusion mixture was prepared by following the partial purification method (Saitoh et al. 1998). Sporulated culture was scrapped off from the medium and suspended in 1.0 ml of sterile distilled water by vortex machine. The suspension was then centrifuged at 10000×g for 20 min at 4°C, and the pellet was washed with 1.0 M NaCl once. Then washing with sterile distilled water was performed thrice and the parasporal inclusions were solubilized in 50 mM- Na_2CO_3, (pH 10.0). Insoluble materials were separated by centrifugation at 10000×g for 10 min at 4°C, and the resulting supernatant was referred to as solubilized parasporal inclusions. The protein concentration of the supernatant was then estimated by the Bradford method (Bradford 1976). The non-denatured crystal protein inclusion mixture was then treated with different concentrations of proteinase-K (Ishii and Ohba 1994) and the effect of the proteolysis was observed through SDS-PAGE. Electrophoresis was performed in a 10% separating gel initiated with a 4% stacking gel and the gel was then kept in the staining solution (0.02% Coomassie Brilliant Blue- G250 in 2% (w/v) phosphoric acid, 5% aluminum sulfate, and 10% ethanol) (Kang et al. 2002) for 2 hours. The molecular weight of the proteins was determined with the help of Alphaview SA software by comparison with molecular standards obtained from Sigma.

Identification of *ps* genes

Four specific sets of primers were designed using Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) software aligning *ps* gene sequences (PS1-PS4) (Okumura et al. 2010). FASTA sequence of *ps* gene sequences was retrieved from NCBI database (Supplementary Table 2) and four specific primer set was designed (Supplementary Table 3). PCR for specific *ps* genes was carried out in a thermal cycler (MJ mini, Bio-Rad) using both genomic and plasmid DNA in total 50 µl reaction mixture including 5 µl of 10× buffer, 1.0 µl 10 mM dNTPs, 2.0 µl $MgSO_4$, 1.0 µM of each primer, 20-50 ng DNA template, 0.25 µl Platinum *Taq* DNA polymerase (Promega, USA) and 2 µl DMSO. Amplification was achieved by 35 cycles (96 °C for the 50s, 47 °C (*ps1*, *ps2*, and *ps3*) and 50 °C (*ps4*) for 45s, 72 °C for 2 min) with an initial denaturation step at 96 °C for 10 min and a final extension step at 72 °C for 10 min and the amplicons were analyzed by electrophoresis in 1.5% agarose gel along with 100 bp marker (Invitrogen, USA).

Plasmid Profiling

Plasmid DNA was prepared by following the alkaline lysis method (A. Shishir et al. 2014). Pellet of a 5.0 ml overnight culture grown in LB broth at 30 °C and 120 rpm was lysed with 0.85 ml of lysis solution containing TE buffer (50 mM Tris, 20 mM EDTA; pH 8.5) and lysozyme (2 mg/ml) (Wako, Japan). Then 0.05 ml of 20% SDS solution and 5U proteinase-K (Nacalai tesque, inc. Japan) were added. Subsequently, 0.03 ml of 3.0 N NaOH was added and mixed gently for 3 min followed by neutralization with 0.06 ml of 2 M Tris-HCl (pH 7.0). Afterward, 0.1 ml of 5.0 M NaCl was added to the suspension and placed on ice for 15 min and centrifuged with 12,000 × g at 4 °C for 15 min (Tomy, MX-305, High-Speed Refrigerated Micro Centrifuge, Japan). The supernatant was then transferred into a fresh microfuge tube and a double volume of ice-cold ethanol was added and incubated at -20 °C for 15 minutes. Afterward, the mixture was centrifuged at 12,000 × g for 15 min and the pellet was air-dried and re-suspended in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA) and preserved at -20 °C after visualization through agarose gel electrophoresis (Promega, USA) (Trevors 1984).

Results

Biochemical properties of the *Bt* Isolates

Bacillus isolates evidenced with the parasporal crystal proteins after 4 days of incubation on T_3- agar were presumed as *Bacillus thuringiensis* (Fig. 1). Thus 104 isolates were observed with blue stained objects as revealed under a light microscope.

Isolates with lecithinase activity and starch hydrolysis ability were checked for hemolysis property which yielded a total of 28 isolates (Fig. 2). The biochemical characterization revealed that the traits were variable among the isolates. However, they were mostly positive for starch hydrolysis, Voges-Proskauer, casein hydrolysis, oxidase, catalase, utilizing citrate as sole carbon source, urease, indole production, etc. and were mostly non-motile (Supplementary Table 4). Moreover, they were able to ferment monosaccharides (glucose) and unable to ferment disaccharides such as sucrose and lactose (Supplementary Table 5).

Molecular and Genetic profiles

The tested *Bt* isolates were found to harbor plasmid DNA. The molecular weight of these plasmids was relatively lower, ranging from

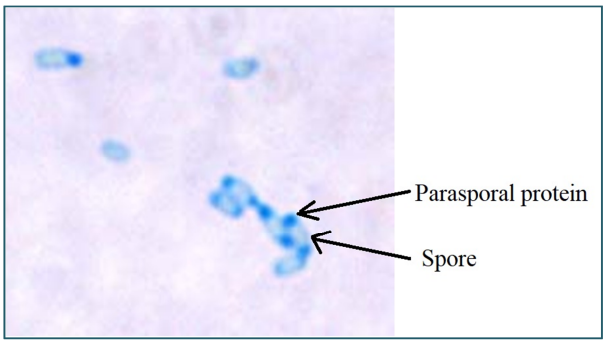
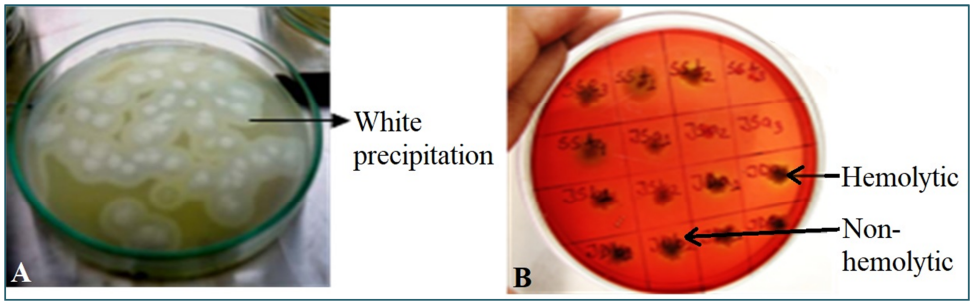


Figure 1| **Crystal proteins (blue) beside the spores were observed under light microscope in *Bacillus thuringiensis isolates.***

Figure 2| **A) Lecithinase activity was checked for the Bt isolates in Egg yolk agar medium. Isolates with lecithinase activity produced white precipitate around their colonies.**
B) Hemolytic and non-hemolytic Bt isolates were differentiated on sheep blood agar medium based on the clear zone of erythrocytes lysis.



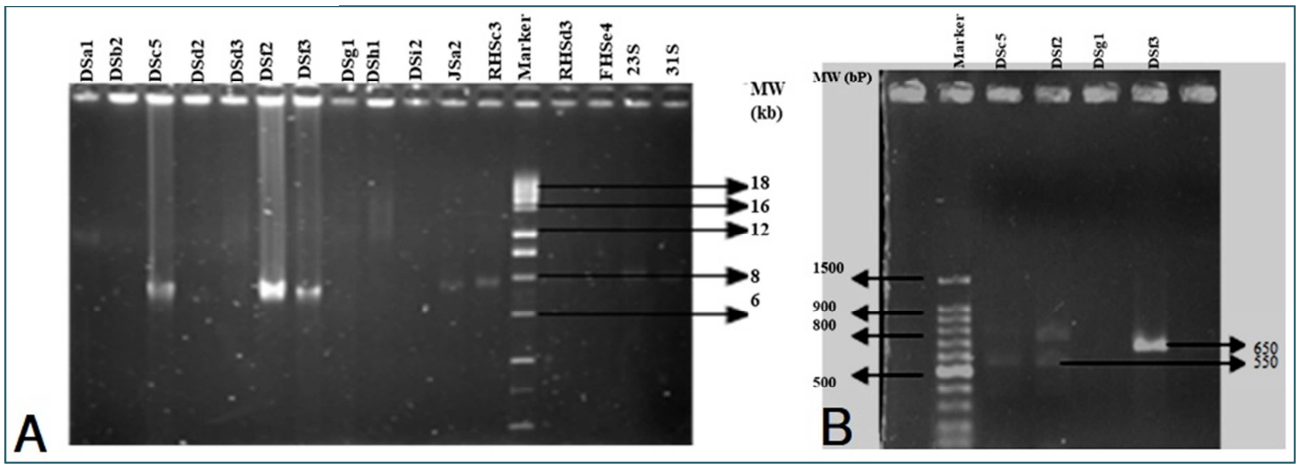


Figure 3| **A) Plasmid profile of the *B. thuringiensis* isolates. Name of the isolates are mentioned over the lanes. B) Agarose gel electrophoresis of PCR products of parasporin genes of *B. thuringiensis* DSf3 contains both *ps2* and *ps3*.**

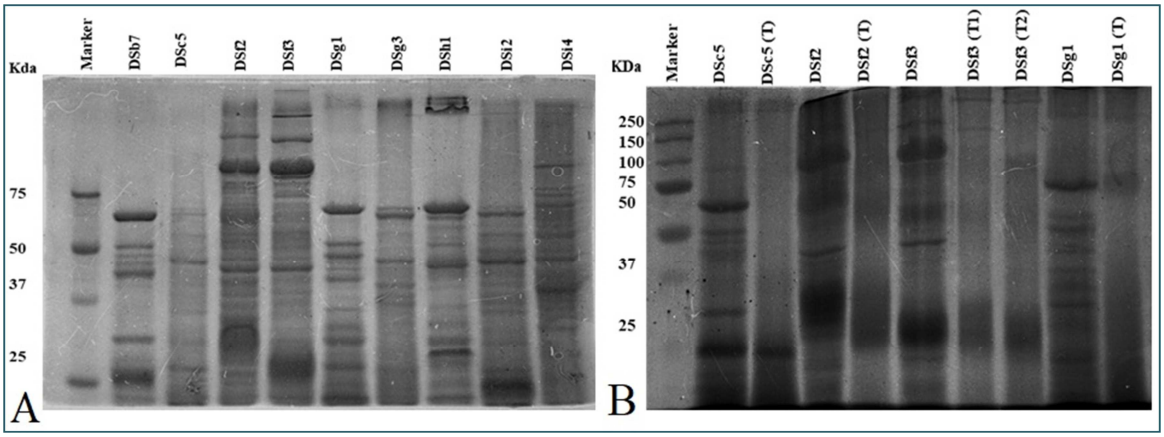


Figure 4| **(A) Cry protein profiles of the *B. thuringiensis* isolates observed through SDS-PAGE analysis. Isolates DSf2, DSf3 and DSf4 were observed with 88 kDa δ- endotoxin. Prominent protein band of 65 kDa was found in isolates DSb7, DSg1 and DSh1. (B) Digestion patterns of PS1 and PS3 were observed with isolates DSc5, DSf2, DSf3 and DSg1 in their 88 kDa and 34 kDa proteins upon Proteinase-K treatment.**

(6- 8kb) (Fig. 3A) hence considered as a cryptic plasmid. It is therefore likely that these plasmids might not harbor the *ps* genes. The PCR detection of *ps* genes in the *Bt* isolates revealed that only one isolate, DSc5 amplified *ps2* gene and one isolate DSf3 amplified *ps3* gene (Fig. 3B). None of the isolates were positive for *ps1* and *ps4* genes. Blastn results for the sequences of 16S rRNA gene amplicons (~1500bp) of the isolates assisted in their identification at the species level. The subsequent comparison revealed them (DSf3- GenBank KF741358 and DSc5- GenBank KF741360) as different species of *Bacillus*, maximum identities with *B. thuringiensis* and *B. subtilis* respectively.

Protein Profile

Among the isolates, *Bt* isolate DSf3 was found to express a protein band of 88 kDa which upon proteolytic digestion produced about 65 kDa and 27 kDa fragments. This is very similar to Ps1 protein. In another case, *Bt* isolate DSc5 was found to express a protein of about 34 kDa and in the case of proteinase-k treatment, resembled the digestion pattern of PS3 protein with fragment at 27 kDa (Fig. 4a & 4b). Most of the isolates produced a band at 27 kDa upon proteolytic digestion (Supplementary Table 6).

Discussion

The expense of cancer treatment is very high due to the development and maintenance of advanced medical infrastructure as well as skilled personnel. And the success of such therapy is achieved upon the preferential killing of cancer cells with minimum side effects to the body and mind (Ferdous et al. 2018). Parasporins, the anticancer proteins from *B. thuringiensis* with toxicity against specific cancer cells and low production cost, could be highly potential in minimizing the problems of conventional therapies. Therefore, the research aiming at isolation and characterization of non-hemolytic and parasporin-producing *Bt* was carried out. *Bt* isolation was performed in combination with heat treatment, acetate selection, microscopy for parasporal crystal protein, starch hydrolysis, and lecithinase activity so that *B. thuringiensis* could be differentiated from *B. sphaericus* (A. Shishir et al. 2014; M. A. Shishir et al. 2012). In this process, 91 presumptive *Bt* isolates were obtained and after the hemolysis test,
28 non-hemolytic *Bt* isolates were selected for further analysis. The biochemical properties of the isolates were mostly in conformation with those of the reference strain *Btk* HD-73 with few exceptions. Variation for few isolates was observed in the case of oxidase, catalase, urease, citrate utilization tests whereas the starch and casein hydrolysis ability were not common for all like indole production. Isolates also demonstrated variable results for VP and motility tests. These could be due to several factors such as slow growth kinetics, variation of origin of source, etc. since for environmental isolates such variations are common (Prado et al. 2010; González-Figueredo, Alejandro Flores-Estrella, and A. Rojas-Rejón 2019). The fermentation ability of non-hemolytic *Bt* isolates with monosaccharides and disaccharides was in agreement with previous other reports i.e. ability and inability to ferment monosaccharides and disaccharides respectively (Kkani Poornima et al. 2012).

B. thuringiensis harbor different types of *cry* genes in their plasmids besides the chromosomal DNA very often and these plasmids are transferrable both vertically and horizontally between different species of *Bacillus*. Moreover, recombination among the *cry* genes of different backgrounds occurs in *Bt* strains (Ishii and Ohba 1994; Kkani Poornima et al. 2012; González, Dulmage, and Carlton 1981; Kronstad and Whiteley 1986; Jensen and Gerdes 1995). Hence the plasmid profile is always an important concern in the molecular characterization of *Bt*. In this study, the tested non-hemolytic *Bt* was observed with plasmids of lower molecular weight, unlike the pesticidal *Bacillus thuringiensis kurstaki* strain HD-73. Variation in the plasmid profiling usually occurs due to the low copy number of large plasmids, extraction of which is sensitive to several artifacts. Hence, the exploration for *ps* genes (*ps1- ps4*) was carried out with the total DNA of each bacterial strain. Amplicons of the desired size were found only in *Bt* isolates DSc5 and DSf3 for *ps2* and *ps3* genes respectively. These genes would be sequenced for further confirmation.

Before the sequencing, another indicative experiment was performed with the presumptive Ps proteins. Since the protein profiles of the isolates revealed that the molecular weight of the proteins was very similar to that of Ps proteins, they were digested with Proteinase-k for fragment analysis. The SDS-PAGE analysis of the Proteinase-k digested proteins revealed that protein fragment patterns were similar to that of Ps2 and Ps3 proteins in the case of *Bt* isolates, DSc5 and DSf3 respectively. Then, 16s rRNA gene sequence analysis was used to identify these isolates at the species level and they were confirmed as *Bacillus thuringiensis*. Although the blastn hits included other species of *Bacillus* also, the presence of parasporal endotoxin was the key to conclude these two isolates as *B. thuringiensis*. Further research involving cloning and expression of the identified *ps* genes would facilitate their cytotoxicity study against different cancer cell lines.

Conclusion

Since the cancer situation of the world is worsening day by day and both the developing and undeveloped countries are at high risk, research for more effective as well as economic therapeutics which is easier to administer with minimum side effects should be carried out worldwide. These preliminary results upon further systematic studies could be translated into life-saving therapeutic agents.

Author Contributions

MMH, SNK, and NA conceived the idea of the research. NA performed the experiments. NA and MAS prepared and analyzed the data and prepared the manuscript. MMH, SNK, NA, and MAS reviewed the manuscript. All authors approved the manuscript.

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Competing Financial Interests

The authors disclose no potential conflicts of interest.

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