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New Innovations in *Bacillus Thuringiensis* Research- A Review

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Abstract

Bacillus thuringiensis (*Bt*) "Wonder insecticide" is a well-known entomopathogenic bacterium used worldwide as an environmentally compatible biopesticide. This review lightens on new innovations of *Bt* research, categorizes into isolation of novel *Bt* strains from various environmental sources, novel approaches for molecular characterization of *Bt* and diverse application of *Bt*. These new findings will expands the world of *Bt* application in safe, specific, effective, economic and ecofriendly manner. It has also been observed that new innovative activities in the emerging fields of *Bt* technology are increasingly growing. Opportunities are immense and sky is the limit for researchers who are interested to take R&D in *Bt* technology.

Highlights

- New *Bt* innovations aid in isolation and characterization of more novel *cry* genes.
- *Bt* new innovations sheds light on alternative choices of insecticides for potential problems associated with insect resistance.
- New *Bt* innovations expands its applications in environmental decontamination and biosafety appraisal studies.

Keywords: *Bacillus thuringiensis*, entomopathogen, innovations, Novel Bt strains, wonder insecticide

The rapidly increasing world population has imposed new demands on the agricultural community (Zhu *et al.*, 2007) urging the development of highly efficient pest control strategies to minimize crop losses worldwide (Bravo *et al.*, 2007, Gatehouse 2008). The strict specificity of *Bacillus thuringiensis (Bt)* delta-endotoxins (Cry proteins) to certain insect species is considered as a major advantage for agricultural application because effects on non-target insects, including omnivorous predators (Torres and Ruberson 2008). Cry proteins are active against insects of the orders Lepidoptera, Coleoptera, Diptera, Hymenoptera as well as against nematodes (Bravo *et al.*, 2007, Gatehouse 2008).

For a sustainable use of *Bt*, it is imperative that there are (1) collections of *Bt* isolates, crystal proteins and strains of related species, (2) research on the persistence of crystal proteins and possible long-term effects on non target organisms and the environment, (3) development of improved resistance management strategies, and (4) genetic engineering of *Bt* genes into the plastid genomes of transgenic crops. The developments described above underscore the validity of genetic manipulation to improve efficacy/ cost-effectiveness, and to expand the markets for *Bt*-based bioinsecticides. This review attempts analyze and categorized new innovative research in the field of *Bt* based research.

Isolation of novel *Bt* strains from various environmental sources

Despite the isolation and characterization of a relatively large number of different insecticidal proteins to date, there is a need for identification, isolation and characterization of new insecticidal proteins. The reasons for this are manifold. Firstly, due to the specificity of insecticidal proteins towards particular groups of target pests (host insect spectra), there is a need to clone genes encoding proteins with different spectra of activity, so that for different crops and different geographic regions suitable proteins for combating insect pests are available. The spectra of activity of *Bt* Cry proteins, for example, is mostly limited. Identification of toxins with specificity towards different target insects remains desirable. Second, after prolonged use in one geographic region, insects are known to have the capacity to develop resistance towards chemical insecticides and microbial sprays (for example based on *Bt* spore-crystal mixtures), and are believed to have the capacity to develop resistance towards plants expressing insecticidal proteins. The development of resistance within insect populations could render existing insecticidal proteins ineffective, creating a need for novel genes and proteins. Third, for health and environmental reasons it is desirable to identify proteins with high, specific insecticidal potency and acute bioactivity towards target insect species (Arnaut *et al.,* 2011).

Bacillus thuringiensis (*Bt*) is a naturally occurring bacterium common in soils throughout the world and belongs to the group *Bacillus* cereus sensu lato (Huang *et al.,* 2001). *Bt* is from the family of *Bacillaceae* which encompasses two genus divisions, namely Clostridium and Bacillus. Hence, *Bt* is a ubiquitous bacteria with Gram-positive, spore-forming, rod-shaped in nature and is approximately 1 µm in width and 5 µm in length (Konecka *et al.,* 2006). Asokan *et al.,* 2013 used soil samples of Great Nicobar Islands to isolate new *Bt* strains, where no collection has been characterized previously. Lee *et al.,* 2012 isolated *B.thuringiensis* from forests soils in Korea. The results suggest that forest areas in Korea are a rich source of *B. thuringiensis* and need to be further explored to discover novel *B. thuringiensis* isolates. Konecka *et al.,* 2012 Isolated *B. thuringiensis* strains from soil and water. Baig and Mehnaz, 2010 isolated 31 *Bt* strains from Arabian Sea sedimentary rocks. PCR approach was used to analyze the presence of different crystal toxin encoding genes with six pairs of universal primers that could detect the *cry1, cry4, cry7, cry8, cry9,* and *cry10* genes. Strains containing *cry1* genes were the most abundant in our collection (49.5%). These bacterial strains survived in highly saline environment. This character may lead to carry novel insecticidal genes proving high toxicity against insects especially storage insects of sea food. Reports of *Bacillus* from marine sources rarely mentioned the presence of *B. thuringiensis* isolates Maeda *et al.,* 2000.

Muniady *et al.,* 2011 used chicken manure samples for quick isolation and characterization for the of novel *B. thuringiensis* strains. The main reason for obtaining samples from different locations Malaysia to broaden the probability of getting *Bt* strains. The designated protocol uses heat treatments, in which it selectively eliminates the entire nonspore forming bacteria and the vegetative cells. Thus only endospore forming bacteria which grow on Nutrient Agar plates were subjected to characterization.

Novel approaches for characterization of *Bt* strains

Ye *et al.,* 2012 designed a high-throughput system for the identification of novel crystal protein genes (*cry*) from *B. thuringiensis* strains. The system was developed with two goals: (i) to acquire the mixed plasmid-enriched genomic sequence of *B. thuringiensis* using next-generation sequencing biotechnology, and (ii) to identify *cry* genes with a computational pipeline (using BtToxin_scanner). The system was able to evaluate 21 *B. thuringiensis* strains in a fast and efficient way. A total of 113 candidate Cry sequences were extracted from the 21 strains, and 8 of them were identified. Among them, 3 potentially represent novel *cry* gene types (primary ranks) and 5 of them became *cry* holotypes, which were designated *cry8Ac1*, *cry7Ha1*, *cry21Ca1, cry32Fa1*, and *cry21Da1* by the *B. thuringiensis* Toxin Nomenclature Committee, were identified.. These results proved the efficiency of this system to mine *cry* genes. Indeed, the mining of novel sequences must be related to the previous strain selection. Still, it is important to note that the selection of the sequencing strategy affects the final prediction results, thus a choice has to be made between cost and efficiency.

Wang *et al.,* 2013 studied the high-throughput identification of promoters and screening of highly active promoter-59-UTR DNA region with different characteristics from *B. thuringiensis*. Identified 1203 active promoter candidates in *B. thuringiensis* through analysis of the genome-wide TSSs based on the transcriptome data. There were 11 types of s-factor and 34 types of transcription factor binding sites found in 723 and 1097 active promoter candidates,

respectively. Moreover, within the 1203 transcriptional units (TUs), most (52%) of the 59-UTRs were 10–50 nucleotides in length, 12.8% of the TUs had a long 59-UTR greater than 100 nucleotides in length, and 16.3% of the TUs were leaderless. We then selected 20 active promoter candidates combined with the corresponding 59-UTR DNA regions to screen the highly active promoter-59-UTR DNA region complexes with different characteristics. These results provide a substantial contribution to molecular biology research and biotechnological applications of *B. thuringiensis*, and our work has made the first step in developing a novel protein expression system in this regard.

The metabolic regulation of sporulation and parasporal crystal formation in *Bacillus thuringiensis* revealed by transcriptomics and proteomics with the objective to elucidate the metabolic regulation mechanisms of insecticidal crystal protein (ICP) synthesis (Wang *et al.,* 2013). For the first time, systematically reveals the metabolic regulation mechanisms involved in the supply of amino acids, carbon substances, and energy for *B. thuringiensis* spore and parasporal crystal formation at both the transcriptional and translational levels. 1) During sporulation, some operons and genes involved in amino acid transport and biosynthesis were either specifically induced or up-regulated in response to amino acid starvation, more importantly, abundant proteases with high activities could efficiently promote protein recycling to meet the requirements for amino acids. 2) *B. thuringiensis* has developed various strategies to provide carbon and energy substances for sporulation and parasporal crystal formation. When nutritional substances are rich, cells store intracellular (e.g. PHB) and extracellular (e.g. Acetoin) carbon substances that could be reused under nutrient-deficient conditions. Some low-quality carbon and energy sources that remained unused during the exponential growth phase could be fully utilized during sporulation. 3) The central carbohydrate metabolism pathways (particularly, the TCA cycle) were significantly modified during sporulation. 4) The oxidative phosphorylation-associated enzymes and cytochromes were remarkably up-regulated during sporulation. This study lays the foundation for metabolic engineering and industrial strain improvement of *B. thurigiensis*, and the construction of a heterologous gene expression system in *B. thurigiensis*.

Diverse Application of *Bt*

B. thuringiensis is a bacterium that has been found to produce many substances that are useful in biodegradation and bioremediation (Lin *et al.,* 2012; Dave *et al.,* 2012). Sukhumungoon *et al.,* 2013 used *B. thuringiensis* in biodegradation of ethidium bromide. *B. thuringiensis*, strain PSU9 demonstrated the ability to degrade EtBr shown by clear zone formation on EtBr-supplemented Tryptic soy agar and thin layer chromatography (TLC). In TLC experiment, the results suggested that the large portion of EtBr could be degraded within 18 h using bacterial culture as well as cell-free supernatant of *B. thuringiensis* PSU9. These results may suggest the promising solution using microorganism to solve the problem of EtBr waste in the laboratory for the decrease of pollutant in the environment.

Lin *et al.,* 2012 characterized the extracellular cellulose-degrading enzymes from *B. thuringiensis* strains. *Bt* strains produced novel cellulases which could liberate glucose from soluble cellulose, carboxymethyl cellulose (CMC), and insoluble crystalline cellulose. The maximal cellulase activities were obtained after 60 hrs incubation at 28ºC in a LB broth medium with 1% CMC. Maximum CMCase activities were got at 40ºC and pH 4.0, respectively, and more than 50% of its maximal activity was retained at 40-60ºC for 1 hr, while approximately 40% of its maximal activity was also retained after incubating at 70ºC for 1 hr. Most metal ions and reagents such as Ca_2+, Mg_2+, Cd_2+, Pb_2+, Zn_2+, Cu_2+, EDTA, and SDS inhibited the enzyme activities, but K+ and Mn_2+ activated the activities. The enzymes from *B. thuringiensis* strains could be applied in bioconversion of lignocellulosic biomass into fermentable sugars. These enzymes, which had not been reported previously, exhibited hydrolytic activities toward CMC, avicel and filter paper at pH 4.0 and 40ºC, and also showed broad temperature and pH stabilities. These results suggest that Bt strains might become a novel and interesting source of lignocellulose-degrading enzymes with important economic advantages, thus might be of potential applications in the industry.

Ramírez *et al.,* 2004 studied the antifungal Activity of *B. thuringiensis* Chitinase and Its Potential for the Biocontrol of Phytopathogenic Fungi in Soybean Seeds. The chitinolytic enzyme produced by *B. thuringiensis* var *israelensis* was used to investigate the inhibition both of *S. rolfsii* in soybean seeds and also in the growth of 11 phytopathogenic fungi, to extend its biotechnological application. Antifungal chitinase activity on phytopathogenic fungi was investigated in growing cultures and on soybean seeds infested with *Sclerotium rolfsii.* Fungal inhibition was found to be 100% for *S. rolfsii*; 55% to 82% for *A. terreus, A. flavus, Nigrospora* sp, *Rhizopus* sp, *A. niger*, *Fusarium*

sp, *A. candidus*,*Absidia* sp, and *Helminthosporium* sp; 45% for *Curvularia* sp; and 10% for *A. fumigatus* ($P < 0.05$). When soybean seeds were infected with *S. rolfsii*, germination was reduced from 93% to 25%; the addition of chitinase (0.8 U/mg protein) increased germination to 90%. These results showed that *B. thuringiensis* chitinase may be considered for the biocontrol of *Sclerotium rolfsii* in soybean seeds and has the potential for the biocontrol of other phytopathogenic fungi.

Jung *et al.*, 2008 studied the stability and antibacterial activity of bacteriocins produced by *B thuringiensis* and *B thuringiensis* ssp. *Kurstaki*. *B. thuringiensis* strain NEB17 and *B. thuringiensis* subsp. *kurstaki* BUPM4 produce the bacteriocins thuricin 17 (3,162 Da) and bacthuricin F4 (3,160.05 Da), respectively. These bacteriocins have functional similarities and show a similar spectrum of antimicrobial activities against indicator strains. the effects of sterilization methods on the recovery and biological activities of these bacteriocins were also studied. They were completely degraded by autoclaving and the two were similarly affected by the tested filter membranes. Polyvinylidene fluoride (PVDF), polyestersulfone (PES), and cellulose acetate (CA) are suitable for filter sterilization of these bacteriocins. The two bacteriocins were stable across a range of storage conditions. These data will facilitate their utilization in food preservation or agricultural applications.

Two novel parasporin (PS) genes were cloned from *B. thuringiensis* B0462 strain (Kuroda *et al.*, 2013). One was 100 % identical even in nucleotide sequence level with that of parasporin-1Aa (PS1Aa1) from *B. thuringiensis* A1190 strain. The other (PS1Ac2) showed significant homology (99 % identity) to that of PS1Ac1 from *B. thuringiensis* 87-29 strain. The 15 kDa (S113–R250) and 60 kDa (I251–S777) fragments consisting of an active form of PS1Ac2 were expressed as His-tag fusion. Upon purification under denaturing condition and refolding, the recombinant polypeptides were applied to cancer cells to analyze their cytotoxicities. 3-(4,5-Dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide assay revealed that either of 15 or 60 kDa polypeptide exhibited no cytotoxicity to HeLa cells, but they became cytotoxic upon mixed together. These results suggested that PS1Ac2 was responsible for the cytotoxicity of *B. thuringiensis* B0462 strain, and that the formation of hetero-dimer of 15 and 60 kDa polypeptide was required for their cytotoxicity.

Despite significant achievements have been made in isolation of new *B.thuringiensis* strains, the patents and patentable technologies were very limited confined to certain geographical regions. Emphasis must be given to the patents and patentable technologies and this will enable the

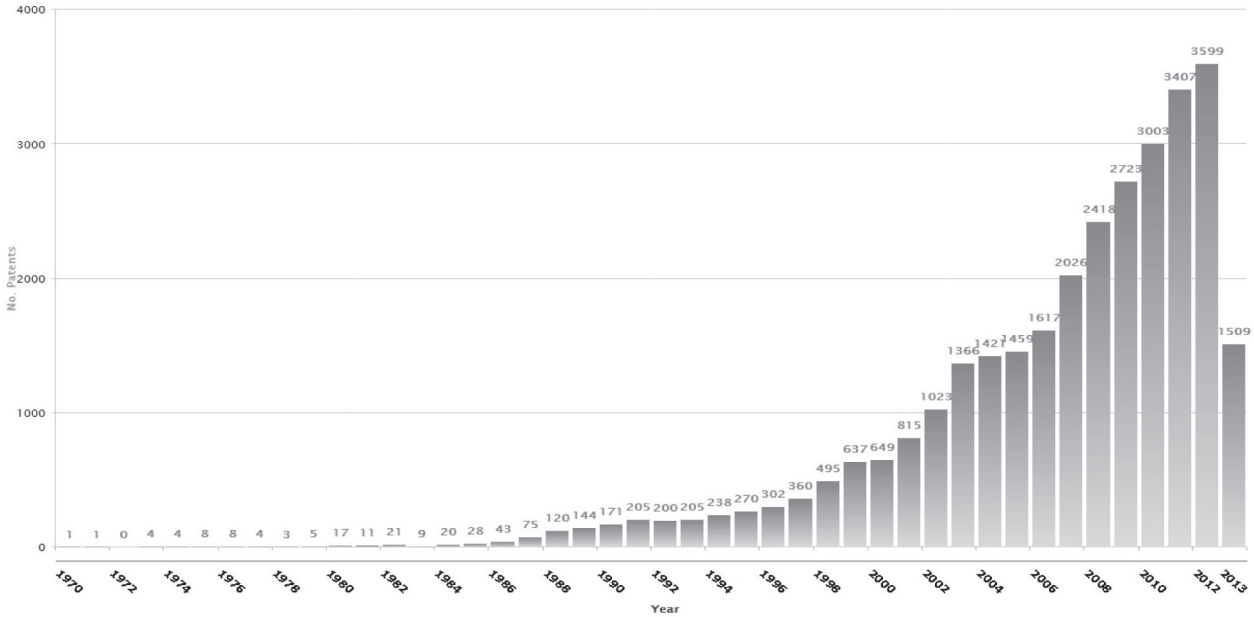

Fig. 1: Number of *B. thuringiensis* patents granted from 1970 to 2013. Patent analysis was done using AcclaimIP a web application includes patents from USPT, USAPP, WIPO, EP, JP.

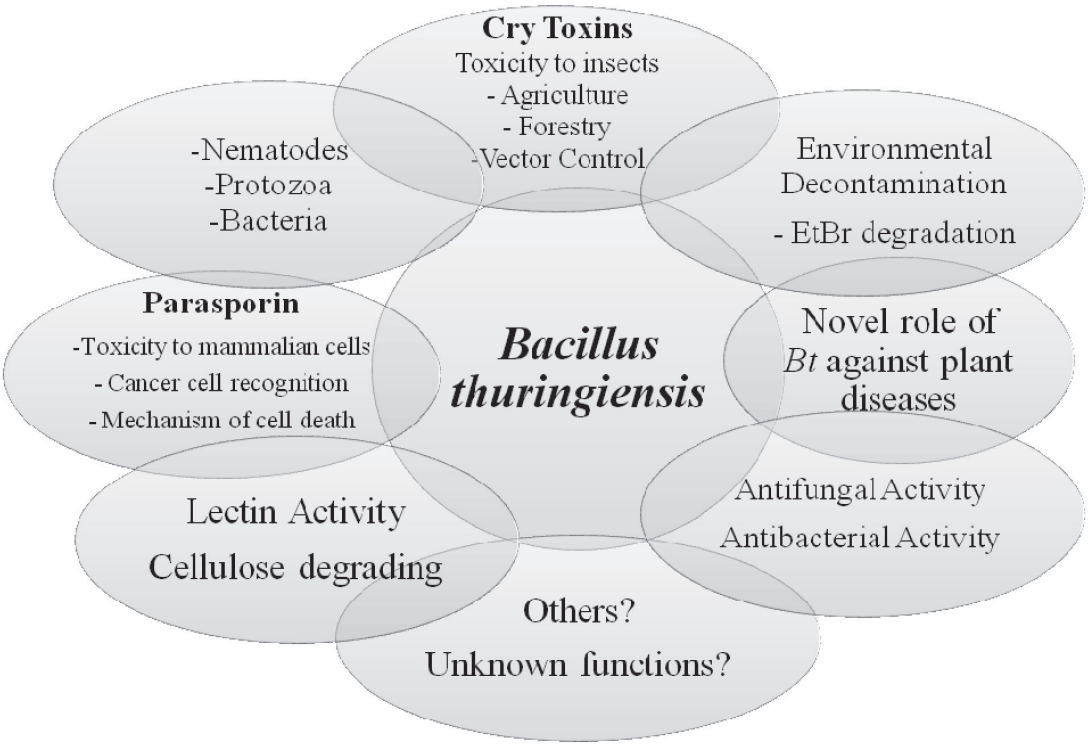

Fig. 2: A bird's eye view of *B. thuringiensis* new innovations and its applications in various fields in near future.

B.thuringiensis technology for the effective control of economically important insect pests for sustainable crop productivity in an ecofriendly manner. Patent documents related to bacterium *B.thuringiensis* are rich source of technical knowledge having commercial application and, thus, patent analysis is considered as a useful vehicle for R&D management. Many aspects of *B.thuringiensis* technology fall under the scope of intellectual property and can be protected with patents, although many of these aspects (processing and formulation in the case of *Bt* sprays, gene transfer and expression in the case of *Bt* crops) are not specific to *B.thuringiensis* and can have wider ramifications. The number of patents granted to *B.thuringiensis* depicted in the figure 1, indicating the patent applications increased in a stead state over the years. Many companies and academic institutions are working on *Bt* technology have sought patent protection, and in 1996 when the first *Bt* crops were commercialized, these were divided more or less equally between the 'old guard' companies which developed *Bt* topical products and the 'new wave' of companies expressing *B.thuringiensis* genes in plants.

Debashis *et al.,* 2013 studied the comparison of nutrients in transgenic and non transgenic cabbage (*Brassica oleracea*. L) for bio-safety evaluation. The nutritional content of non transgenic and *Bt*-transgenic variety of cabbage was compared. The potassium, calcium, magnesium, sulphur and proteins were taken as criteria to judge whether there is any adverse effect due to insertion of an alien gene. The transgenicity study was confirmed by PCR analysis. They found that there was no significant difference of nutrient contents like calcium, magnesium and sulphur content in transgenic cabbage plant in between the lines and variety and the phosphorus content were also within the known limits. Such studies are necessary for bio-safety appraisal of transgenic crop is a must before they can be adopted for commercial cultivation in turn which will encourage the *Bt* transgenic technology.

Conclusion

Summary of new innovations of *Bt* research depicted in Figure 2. Although the majority of the toxins were cloned continuously, the search for novel toxins against insects is still important for increasing the toxicity and host range. This may lead to alternative choices of insecticide for potential problems associated with insect resistance. In general, these new innovations aid in isolation and molecular

characterization of more novel *cry* genes would benefit further development of the Cry protein as a competitive biological insecticide. One of those genes could be genuinely novel. This insight has led, and will lead, by experimentation, to a better understanding of the basis of specificity and the practical application of improved toxins in agriculture. These the new innovations in *Bt* research are a starting point for future research determining potential usefulness *Bt* , which contributes to molecular biology research and the biotechnological application of *B. thuringiensis*.

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