

ENTOMOPATHOGENIC FUNGI: BIOPROCESSING TOOL FOR MODERN SCIENCE

Abstract

The biological control measures to enhance the quality and quantity of the agricultural productivity have attracted the researchers and scholars of the entire globe. The untapped potential of biological tools has lowered the dependency on the chemical based products for safeguarding the damage of agri-products. Exploiting certain living organisms, microorganisms like fungi, bacteria and others have led the rise of feasible and reasonable pest management techniques. Fungi (species infecting arthropods) have shown promising safeguarding tool over past few decades. Fungal genera such as *Hirustella*, *Metarhizium*, *Beauveria* etc are examples of such entomopathogenic fungi.

Besides there pathogenic acitivity some of the fungal genera have shown a complementary behaviour which has been observed to be useful in plant growth. Recent studies have shown that few secondary metabolites produced have found to be promoting plant growth. The article presents an state of art collection of the various research works in the taxonomic, molecular aspect, application of such fungal genera in recent trends of biotechnological and strain improvement techniques.

Keywords: Entomopathogenic Fungi, Taxonomy, Molecular Approaches, Applications, Methods.

Authors

Dr. M.M. Ali
PhD(Mycotoxicologist)
T.N.B. College
TMB University
Bhagalpur, Bihar, India.

Ashish Ranjan
M.Sc (Biotechnology)
T.N.B. College
TMB University
Bhagalpur, Bihar, India.

Rachna Kumari
M.Sc (Zoology)
College of Commerce, Arts and Sciences
Patliputra University
Patna, Bihar, India.

I. INTRODUCTION

The net productivity of the agriculture produces was needed to be enhanced due to the limited farming areas and gradual increase in the number of consumers. There seemed to be two probable ways to enhance the net productivity, firstly, by increasing the agriculture production from farmlands or secondly, by safeguarding the gross production from damage caused by insect/ pests. One of the techniques followed to increase the gross agriculture production was the implementation of chemical pesticides in farmland to check the loss caused by insects/ pests. The tremendous use of such chemical weedicides/ pesticides adversely affected the quality of food crops, ecofriendly and beneficial organisms, health concerns of end consumers and environmental degradation (Kumar and Kalita, 2017). Further, it has also been reported that continuous use of such chemicals lead to the evolution of certain tolerant varieties of pests/ insects (Mantzoukas and Eliopoulos, 2020). These undesirable consequences led the scholars around the world to develop biological control practices.

Among the broad arrays of biological techniques under the Integrated Pest Management Program, pest control method by entomopathogenic microorganism proved to be the most effective alternative (Mahar *et al.*, 2008; Ruiu, 2015; Shaver *et al.*, 2018; Fanning *et al.*, 2018). Such fungi are eukaryotic, genetically varied and heterotrophic in nature. These fungi regenerate via asexual and sexual spores (Mora *et al.*, 2017). “Entomopathogenic” as terminology refers to those fungi/ organism which are competent enough to attack insects/ pests and further utilizing those insects/ pests as host to complete their life cycle (Delgado and Murcia, 2011). The nature of relationship between entomopathogenic fungi and its host insects can be mutualistic, facultative or obligate parasitic or commensalism with immense ability of sporulation (Charnley and Collins, 2007; Mora *et al.*, 2017). Approximately, 750-1000 species of such fungi (under 100 genus) are known to exist that can cause infection to insects and pests (Leger and Wang, 2011). It has been estimated that around 80% diseases in insects are due to infection by fungi ultimately leading to fatal condition (Batista, 1989). The most leading edge of entomopathogenic fungi over any other biocontrol agent is that it does not need to be inoculated in the gut of insects/ inner body part, however, just a simple touch or entangling of spore on any part (even on exterior surface) of insect body can lead to pathogenesis (Mantzoukas and Eliopoulos, 2020). These fungi penetrates the host’s cuticle, secreting enzymes and growth of germination tube from spores take place inside the host insect (Sandhu *et al.*, 2012). However, any other prospective biocontrol agents need to be ingested by the insects in order to cause pathogenesis.

The entomopathogenic fungi belong to several taxonomic groups under the kingdom fungi. In accordance with the system of classification and the Index Fungorum, the entomopathogenic fungi has further been divided into six phylum which are further grouped into twelve classes of fungi kingdom (Abdelgany, 2015). The fungi that are entomopathogenic are reported from Ascomycota, Basidiomycota, Chytridiomycota, Deuteromycota, Oomycota and Zygomycota groups. Among them most of the entomopathogenic fungi have been reported from class Entomophorales (Zygomycota) or Hyphomycetes (Deuteromycota) (Maina *et al.*, 2018). The host specificity and pathogenesis of these entomopathogenic fungi varies to a great extent within genera and species. In spite having variation in the specificity, a common mode of life cycle of entomopathogenic fungus involves steps such as conidial attachment, germination of spore tube, excretion of enzymes,

penetration through the host insect's cuticle, vegetative part of life cycle growth inside the host insect, protrusions of fungal body outside the host and conidiogenesis. During initial interface between the fungus and host insect the mechanical force is exhibited to invade the cuticle, certain metabolic acids and enzymes are secreted which result in pathogenesis by fungus. Post invasion into host, certain fungal toxins are secreted which causes distortion and breakage of cells of host for the hyphae to penetrate. These neuromuscular toxins cause sluggishness, mild paralytic attack etc. to the host as initial symptoms (Leger *et al.*, 1987). Certain toxins depolarize the membrane coating muscles of insect by rapidly activating calcium ions. Lastly, with the favourable temperature and humid conditions, the hyphae crosses the tegument of the host. These hyphae forms conidiophores which are infection causing entities.

Entomopathogenic fungi are extensively distributed mostly in the terrestrial habitats. Variability in the existence of species has been reported with climatic conditions and geographical distributions (Augustyniuk-Kram and Kram, 2012). These fungi thrive in soil as well as on the superficial ground level environment. These pathogenic fungi have also been found in the litter of leaves in forest areas with high amount in temperate forests than in agricultural areas (Aung *et al.*, 2008). The existence of these infectious fungi in farmlands and forest areas render additional benefit to the crops/ plants in the vicinity. The ecological aspect of their existence not only provides protection to the crops from insects or mites but also benefits the standing crops with their proficiency in the rhizosphere (Behie *et al.*, 2012). The metabolism that provide systemic resistance to the plants/ crops also enhance the plants competence for the adverse biotic conditions. Some of the adverse biotic conditions can be growth retardation, improper nutritional uptake, salinity etc. During the interaction various beneficial secondary metabolites are secreted by the fungi.

The secondary metabolites secreted from these entomopathogenic fungi have capacity to control some of the insects, however, the target insect for development of commercial mycoinsecticides is important. Similarly, the development of biologically controlling mechanism dependent on entomopathogenic fungi requires the thorough research on communication between fungus and crops. Exploration of certain important informations which include physiological mechanism of fungi-plant interaction and its variation with plant species. Further, the success of execution of entomopathogenic fungi as biocontrol agent need a clear picture of various factors (abiotic and biotic) that can accelerate the pathogenicity of fungi towards host insect.

Molecular techniques (PCR based) have been used for characterization and exploring the species and phylogenies of entomopathogenic fungi (Glare *et al.*, 2008). The PCR product once digested by restriction enzymes yielded DNA fragments of various sizes. Further, RFLPs are used characterize the DNA of donor fungi. For ecological studies RAPD are in use along with other methods to isolate certain species from soil (Bidochka *et al.*, 2001). Other molecular techniques such as AFLPs, ISSR, SSRs, ITSs have been extensively used to study the fungal systematic (Bowman *et al.*, 1992; Driver *et al.*, 2000). Augmenting molecular techniques with the recombinant DNA will equip fungus with virulent genes which will enhance the potential of entomopathogenic fungi for intense pathogenesis towards insects. These alterations should be under trial initially to assure no adverse affect on environment/ ecology.

The entomopathogenic fungi fit into the position of highly advantageous microbial biocontrol for harmful insects/ pests. These fungi are very less or non-toxic to the non-target organisms/ crops. Furnished with capabilities like short regeneration/ resting cycle coupled with high reproductive capacity, target oriented host specific activity along with potential to survive outside the host (as spores) makes them the most potent biocontrol measures. Owing to such high efficiency to control insects, mycopesticides covers 27% of the total market of biopesticides globally (Kabaluk et al., 2010; Patrick and Kaskey, 2012). Hence, it has been experienced that the global market of biopesticides are on increasing trend.

II. TAXONOMY

1. Phylum Oomycota: The group comprises of 6 genus of entomopathogenic fungi which includes: Lagenidium, Leptolegnia, Pythium, Crypticola, Couchia, Aphanomyces. Structurally, coenocytic hyphae is made of cellulose. As per the records collected, these group of fungi infect insects of order Diptera (Araujo and Hughes, 2016). Mostly parasitic with few species reported as saprophytic in nature. Such pathogenic insect are found as parasites of the hosts in the habitats of freshwater, including ponds as well as rivers (Alexopoulos, 1996) and treeholes or leaf axiles (Saunders et al. 1988 and Frances et al. 1989).

- **Distinguishing Features:** Reproduction is performed by biflagellate zoospore. Tinsel shaped and longer flagellum is pointed forward. Antagonistically, whiplash flagellum which is comparatively shorter is pointed backward (Barr, 1992; Dick, 2001). At times they reproduce by formation of oospore which is thick-walled in nature. Their mitochondrion is observed with tubular cristae in contrast to fungi containing mitochondria with plate like falt cristae (Araujo and Hughes, 2016). Alexopoulos et al., 1996 has reported about its cellulosic cell wall.
- **Genus: *Leginidium*:** consists of facultative parasites. An observation has been recorded about the floating pattern of zoospores headed for surface of water (Golkar et al., 1993). Observations from above showed the swimming behaviour of spore is due shape of cell as well as physical center of gravity instead of sensory stimulus.

2. Phylum Chytridiomycota: The group consists of 4 genera of insect pathogen fungi: Myiophagus, Coelomycidium, Myrmicinosporidium, Coelomomyces. As most fungi, it has chitinous cell wall and hyphae are coenocytic.

- **Distinguishing Features:** Chytridiomycota exceptionally possesses locomotor cells once in their life cycle. Zoospores have one whiplash flagellum pointed backward, supporting its aquatic life cycle (Barr and Désaulniers, 1988). Physiological responses are through chemotactic stimulus helping them to track its host, an essential phenomenon for its pathogenicity in aquatic habitat (Sparrow, 1960).
- **Genus: *Coelomomyces*:** These species include human disease causing vectors such as mosquitoes culex, aedes etc. as well as Simulin. Martin, 1978, reported that genera includes species infecting eggs larvae as well as Lucarotti and Klein, 1988 reported similar in adults. In species such as *Coelomomyces psophorae*, Whisler et al., 1975 reported that copepod is a requisite for life cycle.

- 3. Phylum Zygomycota:** The group consists of 10 genera: *Strongwellsea*, *Entomophaga*, *Tarichium*, *Erynia*, *Eryniopsis*, *Massospora*, *Furia*, *Entomophthora*, *Pandora*, *Zoophthora* (Koiri et al., 2017). Mycelium lacks regular septations, asexual reproduction usually by sporangiospores (formed within multispored sporangia) and absence of cells with flagella (Alexopoulos et al., 1996).
- **Distinguishing Features:** mycelium is haploid with multinucleate hyphae (Goettel et al., 2005). The resting spore (ie, zygospore) is produced which is thick-walled within zygosporangium. These zygosporangium are fusion product of two hyphae or gametangia (Alexopoulos et al., 1996). As reported by Horn and Lichtwardt, 1981, some fungi of this species exclusively make the guts of different arthropods their habitat.
 - **Class: Zygomycetes** – are saprobes or haustorial or nonhaustorial parasites of animals, plants, or fungi.
 - **Class: Trichomycetes** – are found as symbionts in the gut or at times around the anal region of arthropods (in insects and their larvae). They remain attached to their hosts with a cellular or noncellular holdfast (Lichtwardt 1986).
- 4. Phylum Ascomycota:** The group consists of five genera of entomopathogenic fungi: *Beauveria*, *Cordycepioideus*, *Metarhizium*, *Nomurae*, *Lecanicillium* (Koiri et al., 2017). They produce septate hyphae and are filamentous.
- **Distinguishing Features:** Formation and development of ascospores (sexual spores) in sac-like ascus (Araujo and Hughes, 2016) is its characteristic feature. Large number of entomopathogenic fungi as ascomycetes develop the spores inside flask shaped/ subglobose ostiolate ascoma structure namely perithecia. The said structure contains numerous asci (Evans et al., 2011). Reproduction occurs by ascospores, and conidia have been observed.
 - **Genus: Cordyceps:** They form single or multiple erect stromata on host, with perithecia restricted to an apical/ subapical fertile segment or with scattered on stromatic face. The perithecia is flask-shaped fully immersed in stroma. Asci is elongated with condense apical cap pierced by a fine pore with 8 filiform, multiseptate ascospores. These ascospores usually fragment to form single celled part-spores.
- 5. Phylum: Basidiomycota:** The phyla consists of three genera of insect pathogenic fungi: *Fibularhizoctonia*, *Uredinella* and *Septobasidium* (Araujo and Hughes, 2016). Hibbett et al., 2007 have reported to exhibit their dikaryotic phase.
- **Distinguishing Features:** formation and development basidiospores (sexual spores) outside characteristic reproductory cells namely basidia. Such spores are discharged under pressure by certain specialized arrangement (Pringle et. al., 2005). A unique as well as important feature is clamp connections. These structures develop during nuclear segregation on the axial end of budding hyphae, forming dikaryotic condition as reported by Alexopoulos et al., 1996. The study can be exploited for the identification of fossil discovered in this phyla (Krings et. al., 2011).

- **Genus: *Uredinella*:** The presence of binucleate uredospore stands as distinguishing morphological among the different genera of fungus under this phylum (Couch, 1937). The fungal pathogen is rightly said to be transition of rusts and Septobasidium (Aime and McTaggart, 2021).
- 6. Phylum: Deuteromycota:** The phylum of Deuteromycota (Fungi Imperfecti) includes class of diverse fungi that are grouped on the basis of their asexual characteristics rather than their sexual (Boucias and Pendland, 1998).
- **Distinguishing Features:** distinct and developed septate mycelium with conidiophores, however, unicellular thallus in few. Certain fungi imperfect lack conidia and is only found to form sclerotia.
 - **Genus: *Nomuraea*:** Conidiophores are synnematosus/ mononematous bearing whorls of branches with bunch of phialidic conodigenous cells (Reblova et al., 2020). Conidia is single celled, smooth wall in divergent chains. Potential dimorphic fungi that cause epizootic fatality of insects/ pests (Samanta, 2015).

III. MOLECULAR APPROACHES

An entomopathogenic fungus penetrates the cuticle region of insects by releasing proteinases. Insect on counterpart also release anti-fungal protein/ peptides which is regulated by acetylation/ deacetylation of histone proteins. The defense mechanism of insect is nullified by the immediate response of pathogenic fungi by chymotrypsin and metalloproteins to degrade host proteins. This correlation was studied by LC/MS and RT-PCR analysis (Mukherjee and Vilcinskis, 2018). For detailed understanding of insect-entomopathogenic fungi interaction, dual RNA seq technique is helpful (Westermann et al., 2012). The RNA transcripts formed as result of coding and non-coding of genome during the interaction and mapping them against referencing genomes has found to be useful in collating the desired informations. Study between interaction of *B. bassiana* and black moth was carried successfully (Chu et al., 2016). Infected host showed the expression of certain genes post several hours of infection and further informations collected from the study of genes expressed helped in understanding the interaction between insect and entomopathogenic fungi at molecular level.

In due course of evolution, the decreased virulence of fungi and increased defense mechanism of host insect and the stress caused due to susceptibility towards environmental changes in fields might have resulted into low virulence of entomopathogenic fungi towards its host insect (Gressel, 2007; Lovett and St. Leger, 2015). In such scenario molecular biology provided a suitable technique fortifying the fungi through genetic modifications and making them suitable biocontrol agent. With the acceptance of genetically modified organisms in agricultural sector, novel bioinsecticides through gene manipulation are being generated from entomopathogenic group of fungus. The technique of genetic engineering or gene manipulation has been aimed to enhance the virulence behaviour of such fungus with less infection time to kill insect. Further, the ability to combat the stress conditions of these fungi is also being improved. The improvised virulent strains are expected to be infectious to wider range of insects with comparatively lesser lethal dose of its conidia. Decrease in lethal

conidial dose will render hypervirulence behaviour of fungi even at lower quantity. These genetically engineered fungi are being devised to be more potent to cause infection following molecular strategies such as hyper expression of its infectious gene resulting into higher dose of pathogenic proteins, utilising proteins/peptides of target insect by insect killing fungi, incorporating genes from predators of insect or other insect pathogens in entomopathogenic fungi, artificially designed genes/ proteins for entomopathogenic fungi (Zhao et al., 2016).

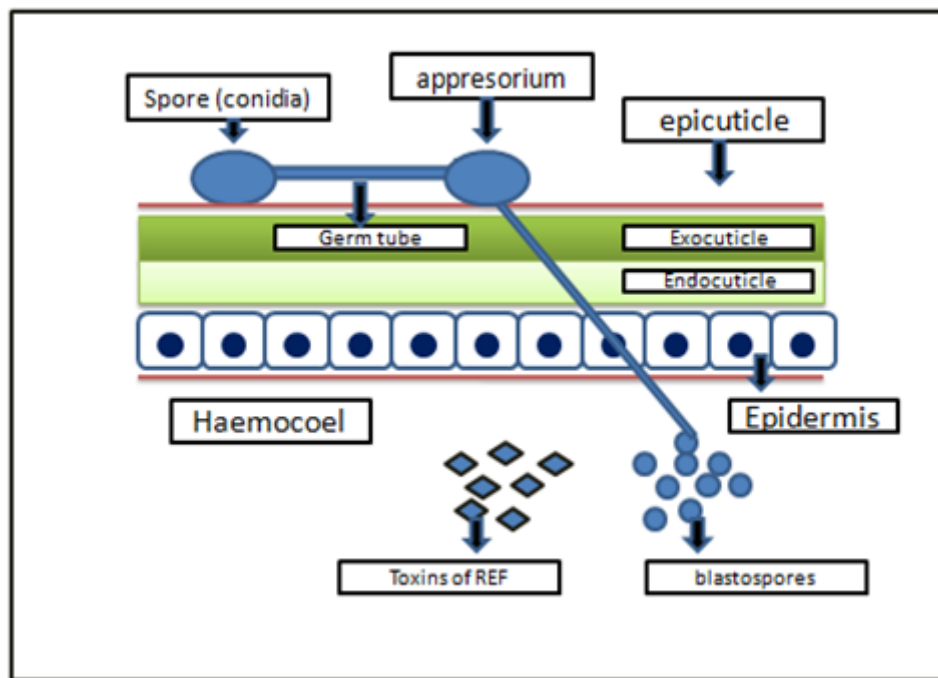


Figure 1: Schematic Representation of Action of Rec. Ento. Fungi

Molecular biology approaches to modify the genetic content of entomopathogenic fungi are being formulated to establish them as significant biocontrol agent of insects. Strategically, one of the scientific method is being developed by recombination of the virulent genes entomopathogenic fungus. The technique has resulted into certain promising results as the genes related to expression of pathogenicity in entomopathogenic fungi are highly controlled and regulated. Over expression of hydrolytic enzymes (protease and chitinase) have boosted the penetration of fungal hyphae into insect cuticle as compared to natural ones thereby increasing pathogenicity of fungi (Fang *et al.*, 2009). The transfer of proteases Pr1A gene from *M. anisopliae* into *B. bassiana* (Gongora *et al.*, 2004) reported to enhanced virulence. The recombinant for hyper-production of proteases and chitinases were formed by intergeneric fusion of protoplasts. Over expression of chitinase CHIT 1 of *B. bassiana* and ATM 1 (acid trehalase) gene in *M. acridium* increased the virulence along with lesser infection dose of conidia (Fang *et al.*, 2005; Penget *et al.*, 2015). Transforming *M. acridium* with Mest1 (esterase gene) of *M. robertsii* has widen the host range of former fungi as it is now infecting caterpillars apart from locusts (Wang *et al.*, 2011). These experiments develop an understanding that once characterization of entire continuum of genes depicting pathogenicity is done coupled with microbial mechanism of host specificity, the chances of improvising the virulence of these fungi can be increased by many folds. Conversely, another aspect that is trending to increase the combating skill of insect pathogenic fungi is expression of specific host insect protein in pathogenic fungi. The

approach is tactically based on the mechanism of pathogenic fungi damaging physiology (osmotic balance, digestion, sterol homeostasis, immunity and neural system) of host insects leading to increased chances of infection. The gene coding MSDH protein that regulates the salt water balance of insect *M. sexta* was incorporated in pathogenic fungi *B. bassiana* resulted in enhanced insecticidal activity (Fan *et al.*, 2012; Karaborklu *et al.*, 2018). Mr-NPC2a gene (codes for protein responsible as sterol transporter in insects) has been known to disrupt the physiology of insect by helping fungi to compete with insect for sterols which limit development of insect hemolymph and thereby increasing the capability of fungi to cause infection (Zhao *et al.*, 2014). This approach can be used as two-sided advantages: a. expression of common protein molecule in pathogenic fungi can cause infection to wide range of insect species and b. expression of targeted insect/ host proteins. The technique demands thorough research on the insect-pathogenic fungi mechanism and the physiological processes of insects which are adversely affected.

In the meanwhile, researchers have been also trying different prospect to increase the pathogenicity of these fungi by incorporation of genes of predators (of the target insect) or pathogens of insect (other than fungi). The predators utilising its toxins kill insects. These toxins include chemical compounds which are diverse in their mechanism (neurotoxins, haemotoxins, enzyme regulators etc.) and once reach inside the body of insects cause consequences. The schematic approach of inserting these toxins inside the insect body using entomopathogenic fungi as vector is under trial. AaIT1 gene (from the scorpion/ arachnids) was incorporated in *M. anisopliae* (strain ARSEF549) which encoded the protein that blocked sodium ion channel. The death rate caused by the engineered fungi of insect *M. sexta* was recorded to be same, however, approx 22% less amount of conidia required (Fang *et al.*, 2014). Incorporation of Bt gene (bacterial gene) in *B. bassiana* proved to be lethal even when ingested and also through cuticle (Qin *et al.*, 2010). BmKit, another scorpion toxin when transformed in *Lecanicillium lecanii* had additional advantage of almost 26% reduction in survival time of infected insect along with 7 fold less conidial dose (Xie *et al.*, 2015). In one of the study, pathogenic fungi *M. acridum* when transformed with four toxins, AaIT1, κ -HXTX-Hv1c, hybrid of self-synergizing protein and ω -HXTX-Hv1 showed less time to kill insects at lower amount of conidial dose compared to toxins used singly (Fang *et al.*, 2014). The toxigenic genes from insect pathogens as virus, bacteria etc. can also be transformed into entomopathogenic fungi to enhance its mode of infection. Bt crystal protein from *Bacillus thuringiensis*, Bt vegetable insecticidal proteins (Vips) have been tried with remarkable responses. The transformed *B. bassiana* by Vip3A were as virulent as wild type, however, the course of action were specific to target insect and more environment friendly. The transformed fungi killed insects by both ways i.e. via conidial contact and conidial ingestion by insects.

With the application of protein engineering, scientists have been trying to formulate synthetic, multifunctional protein functionally amalgamation of different genes. Apparently, the methodology includes mixing the competent protein domains from various genes catered from insect killing fungi and also from another living organisms, synthesizing pathogenic proteins. Hybrid protein of CDEP1 and Bbchit1 genes expressed from *B. bassiana* showed quicker penetration into the cuticle compared to wild strain or over expression recombinant with anyone of fused gene (Fan *et al.*, 2010). Similarly, synthetically designed CDEP1-BmChBD protein enhanced production of peptides that were helpful in binding of pathogenic fungi to cuticle of insects (Zhao *et al.*, 2016). These studies open up a new vista for researchers in the field of molecular biology with immense possibility.

The pathogenicity of fungi is limited by high temperature, osmotic stress, UV radiation, low water and oxidative stress. The photolesions in DNA caused by modification of nitrogen base Thiamine under the influence of Ultraviolet irradiation, checks the survival of entomopathogenic fungi. Incorporation and expression of photolyase UV tolerant gene of *Halobacterium* has enhanced the survival of *B. bassiana* (McCready and Marcello, 2003) and *M. robertsii* under UV light maintaining pathogenicity as wild strains (Fang and St. Leger, 2012). Attempts to transform fungi with melanin synthesizing genes have been tried. *B. bassiana* was transformed with tyrosinase gene from *Aspergillus fumigatus*, which activated the synthesis and production of pigment on conidial surface of fungi rendering it tolerant towards UV (Shang *et al.*, 2012). Further, tolerance capacity of pathogenic fungus towards UV radiation, has been also enhanced by introduction of archaeal photoreactivation and pathways of synthesis of pigment from nonentomopathogenic fungi (Fernandes *et al.*, 2015). The elevated level of oxidative stress can be counteracted by incorporation of *trxA* gene from *E. coli* into recombinant entomopathogenic fungi (Ying and Feng, 2011). Hyper expression of superoxide dismutase protein in *B. bassiana* has been observed to improve the capacity of fungi to combat oxidative stress and UV tolerant (Xie *et al.*, 2010). Expression of bacterial thioredoxin and heat tolerant proteins as HSP25 successfully raised the thermal tolerant and osmotic stress level of entomopathogenic fungi (Ying and Feng, 2011; St Leger *et al.*, 2014). Molecular studies and genomic analyses of entomopathogenic fungi are being helpful in revealing the evolutionary and protein group features related with fungal adaptation to insect hosts.

Genes from other insect pathogens		
<i>Vip3A</i>	Vegetative insecticidal protein	<i>Bacillus thuringiensis</i>
Improve tolerance to abiotic stresses		
<i>Try</i>	Tyrosinase	<i>Aspergillus fumigatus</i>
<i>BbSOD1</i>	Superoxide dismutase	<i>Beauveria bassiana</i>
<i>DHN-melanin synthesis pathway</i>	Three genes	<i>Alternaria alternate</i>
<i>MrPhr1</i>	CPD photolyase	<i>Metarhizium robertsii</i>
<i>HsPHR2</i>	CPD photolyase	<i>Halobacterium salinarum</i>
<i>trxA</i>	Thioredoxin	<i>Escherichia coli</i>
<i>HSP25</i>	Heat shock protein 25	<i>Metarhizium robertsii</i>

(Data source: Google links)

IV. APPLICATIONS OF BIOTECHNOLOGY

As biocontrol agents, entomopathogenic fungi have established their capability to infect insects, pests and arthropods. The host-fungus mechanism during pathogenesis has shown that these fungi also counteract the environmental stress simultaneously. Combating host's defense mechanism and environmental stress simultaneously lead to the natural production of various enzymes (chitinase, lipases, proteases, amylases etc.) by the pathogen. These enzymes possess properties such as high specificity of in action, ease in extraction, abundant availability, stability and safety for human. Owing to such properties, the industrial demands of enzymes are growing. Gradually, these entomopathogenic fungi are gaining wide reputation in field of research and commercial production of its biomass and metabolites. Various studies are being carried out by employing biotechnological tools to evaluate different strains of entomopathogenic fungi. The capacity to secrete useful enzymes in

considerable quantity, related proteomics and metabolites has made such fungi gained popularity among researchers.

Phylogenies and Classification: The advanced knowledge about the phylogenies, species as well as strains of these insect pathogenic fungi have been achieved with the help of PCR based techniques. Different techniques have been followed for the genetic characterization of the fungi. *M. anisopliae* and *B. bassiana* have been characterized by using AFLP method (Muro *et al.*, 2005; Inglis *et al.*, 2008). Similarly, *Metarhizium* species have been characterized with the help of RFLP technique (Bidochka *et al.*, 2001).

V. METHODS TO GENETICALLY TRANSFORM EF

The natural pathogenesis and defense mechanism of entomopathogenic fungi are being improvised by various biotechnological tools. They include:

1. Protoplast Fusion: Compatible protoplasts are rendered in close proximity to adhere to each other. The adhesion leads to fusion (in the presence of fusion inducer) of plasma membrane at certain localized points which gradually allows complete amalgamation of both the cytoplasm finally leading to formation of recombinant cell with desired characters. Entomopathogenic fungi of excessive virulent strains have been fused with *Aspergillus* and *Trichoderma* (Stasz *et al.*, 1988). Protoplast fusion of entomopathogenic fungus to yield hypervirulent fungal strain is being tried through various techniques as:

- Mechanical fusion
- Chemofusion
- Electrofusion

The production of protease by entomopathogenic fungi has further been tried to enhance by formation of hypervirulent strain through inter-genus fusion of *B. bassiana* and *M. anisopliae*. Post isolation of protoplasts from fungi, they are incubated with 20% PEG (Polyethylene glycol) along with CaCl_2 (0.01M) and glycine (0.05M) for half an hour. The activity of Pr1 and Pr2 genes (protease secreting) in fused fungi was found to enhance by two fold compared to mother fungi (Sirisha *et al.*, 2010). *B. bassiana* was hypovirulent to beetles like *Ostrinia nubilalis* and *Leptinotarsa decimlineata*. *B. sulfurescens* is non pathogenic to those beetles too. However, on protoplasmic fusion of *B. bassiana* and *B. sulfurescens*, the hybrid caused pathogenicity and thereby reducing the population of *O. nubilalis* (nearly 39 per cent) and *L. decimlineata* (nearly 73 per cent) (Couteaudier *et al.*, 1997). In another study carried by Inglis, 1997, it was observed that certain entomopathogenic fungi *Metarhizium anisopliae*, *Paecilomyces fumosoroseus* and *P. lilacinus* when transformed with β -tubulin gene from *Neurospora crassa* using protoplast fusion rendered pathogenic fungus tolerant towards fungicides used in field and subsequently increasing their pathogenicity.

2. Electroporation: Sodium channel blocker gene AaIT1 from scorpion was introduced in *M. anisopliae* (through electroporation technique) making the pathogenic fungi about 22 times more toxic towards tobacco hornworm *M. sexta* compared to its wild variant.

- 3. Biolistic Transformation:** The technique is implemented to the pathogenic fungi where protoplasmic fusion have been observed to be limited and in thick walled fungus. Entomopathogenic fungi specific to locusts in field as *M. acridium* is checked by herbicides. Transformation of *M. acridium* employing biolistic technique with Bar gene of *Streptomyces hygroscopicus* provided resistant to glufosinate ammonium (herbicide) (Utermark and Karlovsky, 2008).
- 4. Vector Mediated Transformations:** The multiple copies of protease gene Pr1 (chitin degrading) was cloned into *M. anisopliae* developing it into recombinant entomopathogenic fungi. This particular fungi upon transformation showed 25% lesser time to infect as well as kill *Manduca sexta* (St. Leger et al., 1996). Similarly, *B. bassiana* has single copy of Bbchit1 gene (an endogenous chitinase gene) naturally present in its wild variant. Transformation of *B. bassiana* using *Agrobacterium tumefaciens* to develop gene cassette of Bbchit1 gene by multiple cloning was performed. This resulted into decrease in the time of pathogen entry (19.8%) by rapid chitin degradation as compared to wild type. A recent trial with the hybrid gene Bbchit: CDEP1 using *E. coli* vector pAN52-1 has been used to transform wild strain of *B. bassiana*. The hybrid gene contained chitin degrading capacity of two pathogenic fungi: *B. bassiana* and *M. anisopliae*. Synergistic action of the genes increased the chitin degrading activity of the recombinant fungi to that of its wild strain of donor fungi (Fang et al., 2009). In another recent trial, employing vector pBarEx, the trehalase gene ATM1 of *M. acridium* was hyper expressed which resulted in 8.3 fold reduction in dose of fungi to infect locusts. Trehalose sugar was used by pathogenic fungi for their growth while causing infection to insects.

Strain Improvement of EF: The genetic resource for mechanism and biochemical secretions causing pathogenesis when engineered by the tools and techniques of molecular biology will strategically help us to improvise the strain of entomopathogenic fungi. Increase in virulence, widening of infecting host range, overcome environmental stress, less dose of conidial requirement to cause infection, comparatively faster rate of killing insects are some of the traits of pathogenic fungi under manipulation. ESTs and cDNA microarray technique was used to investigate gene expression (Wang et al., 2005).

VI. PRODUCTION AND FORMULATION OF MYCOINSECTICIDES

- 1. Secondary Metabolites:** The insect pathogenic fungi are known to produce secondary metabolites which have found wide applications in industries (Khachatourians and Uribe, 2004). Gu et al., 2007 have reported the importance of secondary metabolites of *Cordyceps militaris* (an entomopathogenic fungus) in herbal biotechnology. Further, working on *C. militaris*, Ahn et al., 2000 and Zhou et al., 2002 reported pharmaceutical effects of secondary metabolites such as antimicrobial, anticancer, immunosuppressive properties etc. This substantiated the necessity of the genomic study of entomopathogenic fungi particularly those related with secretion of secondary metabolites. Biotechnological tools as Secondary Metabolite Analysis SHell (SMASH) and Secondary Metabolite Unique Regions Finder (SMURF) were employed (Wizaza et al., 2018). Other entomopathogens as *M. anisopliae*, *B. bassiana*, *M. robertsii* were also targeted as the subject of this study.

Genomic data was collected and cluster of genes responsible for the secretion of secondary metabolites were detected. The pathogenic fungi showed great variation in gene clusters of secondary metabolites thereby confirming diversity among same genus of fungi. The secondary metabolites were separated using chromatographic plates and its chemical structures were determined by mass spectrophotometry and techniques of molecular biology, which divides them in groups as peptides, dipeptides, peptide hybrids, polyketides, depsipeptides, terpenoids, amino acid derivatives (Donzelli and Krasnoff 2016; Wang *et al.* 2018). Biosynthetic pathway of these metabolites and its analysis can further help in medicinal, pharmacological benefits and subsequently industrial production can be promoted.

Entomopathogenic fungal genome	Number of gene clusters associated with secondary metabolites			Number of predicted genes in gene clusters associated with secondary metabolites		
	antiSMASH	SMURF	Overlapping ^a	antiSMASH	SMURF	Overlapping ^a
<i>Cordyceps militaris</i> strain CM01	72	24	22	1,160	238	197
<i>Cordyceps confragosa</i> strain RCEF 1005	107	28	28	1,984	277	244
<i>Cordyceps brongniartii</i> strain RCEF 3172	84	33	33	1,326	293	275
<i>Beauveria bassiana</i> strain ARSEF 2860	102	30	30	1,669	313	273
<i>Isaria fumosorosea</i> strain ARSEF 2679	81	24	24	1,283	213	202
<i>Moelleriella libera</i> strain RCEF 2490	72	14	12	1,158	71	64
<i>Metarhiziumanisopliae</i> strain ARSEF 549	119	51	47	2,214	509	490
<i>Metarhizium robertsii</i> strain ARSEF 23	122	53	51	2,235	489	484
<i>Metarhizium rileyi</i> strain RCEF 4871	69	26	26	1,084	271	220

^aOverlapping of number of gene clusters associated with secondary metabolites between antiSMASH and SMURF

^aOverlapping of predicted genes in gene clusters associated with secondary metabolites between antiSMASH and SMURF

(Data source: Google links)

- 2. Biocatalyst:** During pathogenesis entomopathogenic fungi shows tremendous changes in their physical and chemical physiology attributed by their communication with environment and interaction with host's physiology and defense system (Keyhani, 2018). Hence, the enzyme mechanism plays a major role in invading insect and combating with its various secretions (Dhawan and Joshi, 2017). This feature of pathogenic fungi to secrete broad spectrum of enzymes are being tried by various commercial production centers to produce biocatalysts (Mondal *et al.*, 2016; Amoboyne *et al.*, 2020). Primary enzymes for which fungi are industrially exploited are chitinase, lipases and proteases.

 - Chitinases:** Establishing its position as second most abundant natural polymer and owing to its global occurrence and diverse application, it has been a keen interest area of research. Its application as fungicides, biosensing, bioremediation, dye removal, drug delivery and others has made it one of industrial mining sector (Rameshthangam *et al.*, 2020).

β (1,4) bond of chitin is hydrolyzed into N-acetyl β -D-glucosamine monomer by chitinase enzyme. *Beauveria bassiana* produces extracellular chitinase using colloidal chitin as source of carbon during solid state and submerged fermentation techniques. However, improved enzyme productions are being tried by cloning chitinase gene in hosts such as *Pichia pastoris* (Fan *et al.*, 2007) and *E. coli* (Fang *et al.*, 2005). In one of the trial by Fan *et al.*, 2007, hybrid chitinase with enhanced activity was achieved amalgamating chitinase of *B. bassiana* with chitin binding domains and engineered to express them in *P. pastoris*. The recent trend in this direction has led to the isolation of heat tolerant *B. bassiana* strain which produces thermostable chitinase gene (Alali *et al.*, 2019). The industrial production of heat stable ascertains new dimension in beneficial biocatalyst even at higher temperature.

- **Amylases:** The amylase produced by entomopathogenic fungi (Bb SG 8702) have been observed to display stability in the pH range of 3 to 8 although produced under acidic fermentation environment at the temperature of 40°C. Stability of these enzymes within such range of pH makes them promising biocatalysts for various industrial products (Feng and Ying, 2002). They are widely applicable in starch based food processing, biofuel production, paper manufacturing, fabric industries etc. (Bhatt *et al.*, 2020).
- **Lipases:** These enzymes have been found to play critical role in cuticular breakdown of insects (Keyhani, 2018). Lipases derived from pathogenic fungi (such as Bb 906.7 strain) are active in wide range of pH (acidic, neutral, alkaline). Further, few of them have exhibited mesophilic and thermophilic characteristics (Zibae *et al.*, 2011; Vici *et al.*, 2015). These characteristics make lipases potential biocatalyst for various industrial applications. Further, its potential has been enhanced by production of heterologous lipase protein in *P. pastoris* by engineered gene of Bb CFF74 strain.
- **Cellulase and other Enzymes:** The enzyme cellulase produced by entomopathogenic fungi such as strain Bb B14532 has demonstrated its maximal activity at 80°C (pH 6.5). Hence, this thermo stability property of enzyme renders it as potential biocatalyst for various industrial applications.

Asparaginases are produced by Bb SS18/41 act as biosensors and diminish acrylamide in fried or baked foods (Chand *et al.*, 2020). Catalase have found their varied applications in bioremediation, textile manufacture, dairy processing (Kaushal *et al.*, 2018). Chitosanase, which causes hydrolytic breakdown of chitosan plays an important role as bioconversion of various marine crustaceans into biomaterials besides biocontrol agent (Thadathil and Velappan, 2014). Beta-glucosidase is an important enzyme during cellulase degradation of host insect also acts as biocatalyst in production of biofuels, ethanol production (Ahmed *et al.*, 2017). Keratinase, a proteolytic enzyme finds its wide application as biocatalyst in cosmetics, fertilizers and animal feed industry (Hassan *et al.*, 2020). Superoxide dismutase detoxifies superoxide radicals and thus has been tried to treatment of various diseases including arthritis and diabetes (Younus, 2018). With the application of modern biotechnological tools and techniques, novel biocatalysts are being developed.

VII. INTEGRATED PEST MANAGEMENT STRATEGIES

From past few decades, uses of natural insecticides such as entomopathogenic fungi have gained attention and are employed as biological control (Sahayaraj, 2014). Depending on standing crops and environment, entomopathogenic fungi are being tried as pesticides either alone or in combination with chemicals. The application of pathogenic fungi as biocontrol against European cockchafer beetle is effective for almost 9 years and controls beetle growth on pastureland, orchards and other timbers of Europe (Keller et al., 1997). After almost two decades in California, the combination of azadirachtin with *B. bassiana* effectively diminished the honeysuckle aphid and rice root aphid by nearly 62% and remarkable reduction was observed in case of *Lygus hesperus* plant bug (Dara, 2015; Dara, 2016).

Production of mycoinsecticides

1. **Inoculum:** For the *in vitro* production of mycoinsecticides, the inoculum should have the capacity to retain its virulence behaviour during production, storage and in field. Single spore isolates are successfully being tried (as inoculum) compared to serial transfer of culture for *in vitro* culture and are subsequently stored in liquid nitrogen (Brownbridge et al. 2001; Charnley and Collins, 2007).
2. **Method:** Submerged liquid fermentation (SmF) technique involves deep tank size equipment for large scale production. The technique is comparatively faster and controllable. Surface cultivation on the other hand involves solid substrate, however, aeration, pH, temperature parameters are comparatively difficult to control (Feng *et al.*, 2000). The technique was further improvised in one of the study, wherein, packed-bed bioreactor with straw and rice were employed to produce *B. bassiana*. The production culture yielded 49×10^8 conidia/ gram without any basal support and $1.1-1.2 \times 10^{10}$ conidia/ gram on polypropylene foam (Kang *et al.*, 2005). At the commercialised center of Laverlam International, an automated forced-aeration solid state bioreactor was developed and the production gained in case of *B. bassiana* was 3×10^{13} / kg of initial mixture in one litre of volume of bioreactor (Wraight *et al.*, 2001).
3. **Harvesting:** From liquid culture, fungal conidia are harvested by centrifugation followed by rapid drying under controlled conditions. On the other hand, conidial cultures grown on solid substrate are washed off or dried under controlled moisture content followed and then powdered (Charnley and Collins, 2007).
4. **Formulation of Mycoinsecticides:** The obtained fungus post harvesting, needs to be stabilised, stored and retain its virulence characteristics during field operations (Wraight et al. 2001). *P. fumoroseus* blastospores when formulated with whole milk in starch-oil and stored at -20°C without losing its virulence nature for an year (Jackson et al. 2006). The conidia of *M. anisopliae* harvested in groundnut oil and then diluted with an antioxidant or with Shellsol K, deodorised kerosene (Edelex) retained viability of 60% for more than 2.5 years when stored at 17°C (Charnley and Collins, 2007). Phyllosilicate powders has been tried from since long as wettable powder for formulation and owing to its inert nature and cost-effectiveness have been widely used to retain conidial viability over extended period of time (Ward, 1984). Inclusion of nutrient in the conidial formulation allows saprophytic growth of spores and sporulation with increased viability.

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