Title: A CHITINASE FROM BREVI BACILLUS LATEROSPORUS, ITS PRODUCTION AND USE THEREOF

Abstract: The present invention provides a new strain of BrevibaciUus laterosporus LAK 1210 (MTCC 5487) having an accession number MTCC 5487, as a dual producer of insecticidal proteins and inducible chitinolytic enzymes with a potential utility in agriculture and forest insect pest management, plant disease control and mosquito control programs. The multiple effects of this new insecticidal strain of BrevibaciUus laterosporus LAK 12 10, as a chitinase producer with the ability to degrade marine wastes finds many biotechnological applications in the areas of agriculture, forestry, medicine and environment. Furthermore, the object of the invention is also directed to a new chitinase with novel characteristics from a newer source of microorganism and its novel applications in biomass utilization and waste management. Methods and compositions of the invention utilize the vegetative cells, their derivatives (spores, cellular extracts, enzyme fractions) and the polypeptides to develop a natural pesticide for control of insects and phytopathogenic fungi. Another object of this invention is to expand the synergistic effect of chitinases on insecticidal activity for an improved pesticidal activity where a wider range of pests are impacted.
A CHITINASE FROM BREVIBACILLUS LATEROSPORUS, ITS PRODUCTION AND USE THEREOF

FIELD OF INVENTION

The field of invention is drawn to pesticidal strains producing novel enzymes with applications in plant protection, more specifically controlling insects and phytopathogenic fungi of economic significance to agriculture and forestry sectors. This invention relates to antifungal activity of chitinases to control phytopathogenic fungi and synergistic interaction of chitinases with the insecticidal proteins of *Brevibacillus laterosporus* to exhibit greater efficacy and enhanced toxicity to achieve a higher degree of insect control. The present invention can be applied to the rational design and development of new generation biopesticides based on *Brevibacillus laterosporus* for agricultural and forest pests.

BACKGROUND OF THE INVENTION

The plant diseases caused by insect pests and phytopathogenic fungi are a major constraint on forest and agricultural productivity. The use of conventional pesticides for plant protection is being undermined by problems related to insect resistance, pest resurgence and environmental concerns. Copping et al (2000) have reported that a variety of insect pests cannot be effectively controlled with available pesticide regimens (Biopesticides: a review of their action, applications and efficacy. Pest Manag Sci 56(8):651-676). In principle, biopesticides may effectively address most of the challenges related to the use of pesticides and as a result they received ample attention in current research programs on sustainable crop protection.

However, it has been reported, despite the flurry of research initiatives in comprehensive, ecologically based integrated pest management, the widespread commercial use of biopesticides has so far been limited by their lack of efficacy (Mensink BJWGH, Scheepmaker JWA (2007) How to evaluate the environmental safety of microbial plant protection products: A proposal. Biocontrol Sci Tech 17(1-2):3-20). Biocontrol research has prompted to look for new compounds, new strains and
novel methods of biological control of insect pests and phytopathogenic fungi of economic significance to agriculture and forestry sectors. As alternatives to synthetic pesticides, certain naturally-occurring agents have been isolated and developed as pesticides. These include natural strains, novel polypeptides and proteins. Consequently, there is a great interest and utility in finding natural strains and polypeptides with deleterious effect on insect pests and phytopathogenic fungi.

Fravel (2005) has reported that many efforts in the area of biological control are directed towards the use of new pesticidal strains or novel compounds derived from such organisms to develop biopesticides and biological controls for agricultural and forest pests (Fravel DR Commercialization and implementation of biocontrol. Ann Rev Phytopathol 43:337-359). There is a need for novel microbial strains with enhanced activity, efficacy and broad spectrum pesticidal/antifungal activity to meet the long term and sustainable needs of integrated pest/plant disease management. Only a limited number of wild strains were characterized and identified as effective biocontrol agents for insect control and controlling phytopathogenic fungi.

Bacterial agents are environmentally safe due to their host specificity, required in very low dosage, easy to prepare commercially in large-scale and are less costly. Since the 1950s, strains of *Bacillus thuringiensis* have been used as potent insecticides to control economically important agricultural insect pests. Formulations of *B. thuringiensis* var. *kurstaki* have been used for many years as commercial insecticides for lepidopteran pests. But the toxins of *B. sphaericus* and *B. thuringiensis* var israelensis in particular, do not persist long in nature and require frequent application which is a limiting factor for these organisms to be most successful and potent biolarvicide. The development of insect resistance to *B.t* necessitates a continuing search for new biocontrol agents having activity against a wide variety of insect pests and improved insecticidal activity. There have been few effective microbial insecticides since *Bacillus thuringiensis* (Bt) and there is a quest for novel strains as alternative to Bt control. The present invention based on a new, chitinolytic, pesticidal strain of Brevibacillus
*laterosporus* IAK 1210 producing a novel chitinase can be an answer to the resistance and other disadvantages of *B.t.*

The implication of role of chitinases as biocontrol agents has been investigated and the approach to chitinase for biocontrol of fungal and insect pathogens is based on the widespread presence of chitin as an integral part of the cell walls of fungi, insect cuticle and crustacean exoskeletons (Herrera-Estrella A and Chet I (1999) Chitinases for biological control. EXS: 87:171-84; Gooday GW (1999) Aggressive and defensive roles for chitinases. EXS:87:157-69). Chitin is an unbranched polysaccharide polymer consisting of N-acetyl-D-glucosamine units ("GluNAc") joined by beta-1,4 glycosidic linkages. Chitin is insoluble in water, dilute mineral acids and bases but can be broken down enzymatically by chitinase, the degradation products being soluble monomers or multimers of GluNAc. Chitinases are a class of hydrolytic enzymes which degrade chitin by endolytic or exolytic mechanisms. Chitinase is produced by certain naturally occurring bacteria, fungi, actinomycetes, nematodes, insects, crustaceans, plants and some vertebrates and there have been reports of the role of chitinase in the suppression of pathogens. (Huang CJ, Wang TK, Chung SC, Chen CY (2005) Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28-9. J Biochem Mol Biol 38(l):82-88). US patent 6329504 discloses an antifungal polypeptide and methods for controlling plant pathogenic fungi.

Sampson and Gooday (1998) first investigated the ability of chitinases to improve the insecticidal activity of *Bacillus thuringiensis* strains and subsequently reported the role of chitinases is to weaken the peritrophic membrane of the insect gut epithelium to facilitate the permeation of insecticidal toxins. Synergism between chitinases of different origin in combination with *Bacillus thuringiensis* (*B.t*) to increase the potency of the strain has been reported by several authors (Regev A, Keller M, Strizhov N, Sneh B, Prudovsky E, Chet I, Ginzberg I, KonczKalman Z, Koncz C, Schell J, Zilberstein A (1996) Synergistic activity of a *At* delta-endotoxin and a bacterial endochitinase against Spodoptera littoralis larvae. Appl Environ Microbiol 62(10):3581-3586).
Brevibacillus laterosporus, has long been known to include strains toxic to certain invertebrate organisms. Favret and Yousten (1985) found that over half of the 29 isolates they tested were toxic larvae of the mosquito, C. quinquefasciatus (Favret, ME and Yousten A. Insecticidal activity of Bacillus laterosporus. J. Invertebr. Pathol. 45:195-203). It has been demonstrated that the strains of Brevibacillus laterosporus has mosquitocidal activity similar to that observed in Bacillus thuringiensis (Orlova MV, Smirnova TA, Ganushkina LA, Yacubovich VY, Azizbekyan RR (1998) Insecticidal activity of Bacillus laterosporus. Appl Environ Microbiol 64(7):2723-2725). Though Brevibacillus laterosporus is reported to have a very wide spectrum of biological activity compared to the most popular insecticidal bacteria Bacillus thuringiensis and Bacillus sphaericus, its biological control potential has not been fully explored since the attempts to isolate this organism from different ecological niches was not successful since the distribution of strains of Brevibacillus laterosporus is limited compared to the strains of Bacillus thuringiensis and Bacillus sphaericus (Oliveira E J D, L Rabinovitch L, Monnerat RG, Passos LKJ and Zahner V (2004) Molecular characterization of Brevibacillus laterosporus and its potential use in biological control. Appl. Environ. Microbiol. 70(11): 6657-64).

The advantage of the strains of Brevibacillus laterosporus is even the acrystalliferous strains were reported to exhibit strong biocidal action against mosquito larvae and the insecticidal activity was also detected against larvae of the black fly (Simulium vittatum) and the yellow-fever mosquito (Aedes aegypti). Rivers et al (1991) studying essentially the same strain collection, detected activity against coleopteran (but not lepidopteran) larvae as well (Rivers, D. B., C. N. Vann, H. L. Zimmack, and D. H. Dean. Mosquitocidal activity of Bacillus laterosporus. J. Invertebr. Pathol., 58:444-447). U.S patent 80761 19 discloses an invention which relates to a method for biological control of insects belonging to the order Diptera (flies, mosquitos, horseflies and midges) and especially against the species Musca domestica. Brevibacillus laterosporus strain compositions containing the same and method for the biological control of dipterans.
A small number of research articles have been published about the enzymatic profile and effects of toxicity from *Brevibacillus laterosporus* strains. Only nematicidal activity has been reported for an extracellular protease from *Brevibacillus laterosporus* (Huang X, Tian B, Niu Q, Yang J, Zhang L, Zhang K (2005) An extracellular protease from *Brevibacillus laterosporus* G4 without parasporal crystals can serve as a pathogenic factor in infection of nematodes. Res Microbiol 156(5-6):719-727).

The literature on chitinase from *Brevibacillus laterosporus* and its biotechnological applications, in particular agricultural and environmental applications is scarce. It is an object of the present invention to provide a new, wild type (i.e naturally occurring) chitinolytic, insecticidal strain of *Brevibacillus laterosporus* LAK1210 which produces a novel chitinase. The chitinolytic enzymes from this new biocontrol strain synergises the insecticidal activity and enhances the entomotoxicity of the said strain making it effective against a wide spectrum of insects and plant pathogenic fungi.

**SUMMARY OF THE INVENTION**

The invention broadly pertains to a novel isolate of *Brevibacillus laterosporus*, *Brevibacillus laterosporus* LAK 1210 that exhibits strong chitinolytic and insecticidal activity. The new strain has been deposited at Microbial Type Culture Collection MTCC), an International repository at IMTECII, INDIA and can be accessioned as 5487. The subject of invention also concerns a natural strain that could be quickly developed into an effective, safe, natural biopesticide/ biocontrol formulation.

The present invention further relates to the discovery of a new chitinase and antifungal metabolites (polypeptides) that exhibit broad spectrum antifungal activity against phytopathogenic fungi. The novel polypeptides represent a new group of protein antifungals obtainable from the new strain, *Brevibacillus laterosporus* LAK1210. The present invention provides method of effecting or modulating phytopathogenic infection, in particular, fungi and insects, using cell suspensions of *Brevibacillus laterosporus* LAK 1210 or supernatant containing metabolites (antifungal chitinases) or
purified metabolites to all or part of a plant or a plant seed, under conditions effective to control insect pests and plant diseases of agronomic and horticultural importance.

In accordance with the present invention, synergistic effect of the chitinases potentiate the insecticidal action resulting in additive enhancement of the toxicity, efficacy and substantially broadens the spectrum of activity against the insects to be controlled and also allows lower application rates. In addition to the synergistic action on insecticidal proteins, the chitinases according to the invention also exhibit broad spectrum antifungal activity which may contribute significantly to the enhanced, persistent fungicidal action against a wide range of phytopathogenic fungi.

The invention has particular utility for a new chitinase from a new insecticidal strain as an agriculturally beneficial biocontrol agent in controlling/ inhibiting/modulating phytopathogenic infection caused by insects, phytopathogenic fungi, bacteria and nematodes. This invention is directed at antifungal combinations of chitinolytic enzymes and use thereof for topical or internal application to inhibit germination or replication of phytopathogenic fungi and synergistic action of chitinases to enhance the insecticidal effectiveness. More specifically, the invention provides methods and compositions for preventing and controlling fungal diseases and combating insects of economic significance to agriculture and forestry sectors.

So far, lepidopteran activity against economically important insects hasn't been reported for strains of *Brevibacillus laterosporus*. Advantageously, this novel isolate, *Brevibacillus laterosporus* LAK 1210 has lepidopteran activity as tested against diamond backmoth (*Plutella xylostella*), a devastating pest of crucifers.

An aspect of the present invention relates to a biologically pure culture of a new strain of *Brevibacillus laterosporus* designated as *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487, a variant or a mutant thereof.

Another aspect of the present invention relates to an isolated 16S rRNA gene sequence of the strain *Brevibacillus laterosporus* LAK 1210 having accession number MTCC
548, wherein the nucleotide sequence of gene is as set forth in SEQ ID NO: 1 having GenBank accession number HQ412764.

Yet another aspect of the present invention relates to a chitinolytic enzyme having molecular weight selected from a group consisting of 25 kDa, 55 kDa, 70 kDa, 75 kDa, 90 kDa, wherein the enzyme is active at a pH ranging from 3.0 to 11.0 and at a temperature ranging from 30°C to 90°C.

Further aspect of the present invention relates to a process for producing a supernatant comprising chitinases and insecticidal proteins, wherein the process comprises culturing cell culture of novel strain of *Brevibacillus laterosporus* designated as LAK 1210 having accession number MTCC 5487 in a culture medium comprising 0.1-1 % (w/v) colloidal chitin or 0.1-1% (w/v) marine chitinous waste to obtain cell culture, and obtaining the supernatant comprising chitinases and insecticidal proteins from the cell culture.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 shows phylogenetic relationship of *Brevibacillus laterosporus* Lak1210 to selected species from the genera *Bacillus, Paenibacillus* and *Brevibacillus*, based on 16S RNA genes. The tree was constructed with the Treebuilder tool provided by the Ribosomal Database Project (see method section for details). *Brevibacillus laterosporus* Lak 1210 is indicated with an arrow.

Fig. 2 shows a) chitinolytic activity of *Brevibacillus laterosporus* Lak. 1210. The plate contained 0.2 % colloidal chitin as the only carbon source. b) Chitin plate stained with 1% Congo Red after 7 days of incubation (30°C). Chitin degradation is shown as a clear halo around the colony. The figure also shows the morphology of a typical older Lak1210 colony, filamentous with fringed margins. c) Photomicrograph of the 72 h sporulating culture of *Brevibacillus laterosporus* LAK 1210.

Fig.3 shows a) SDS PAGE. Proteins precipitated from the supernatant of chitin-induced culture (lane 1) and chitin affinity purification (lane 2). Protein bands that were
subjected to characterization by trypsination and mass spectroscopy are numbered. M, protein size markers b) Activity staining. Proteins precipitated from the supernatant of chitin-induced culture (lane 1) and chitin affinity purification (lane 2).

Fig. 4 is characterization of the chitinase mixture using 4MU-NAG2 as substrate. Each point represents the average of three measurements. Standard deviations are not shown because they were below 2% of the value and thus hidden by the data points. (a) pH activity profile. The buffers used were: Citrate phosphate (pH 3.0 - 5.0), sodium acetate (pH 5.0 - 6.0), sodium phosphate (pH 6.0 - 8.0), Tris-HCl (pH 8.0 - 9.0), Carbonate-bicarbonate (pH 9.0 - 11.0). (b) Temperature activity profile (c) Thermostability profile-the enzymes were incubated for 30 minutes in 50 mM Tris-HCl pH 8 at the indicated temperatures, after which residual activity was determined. The measurements from the incubation at 25°C were used as the 100% value.

Fig. 5 shows a) Antagonism against Fusarium equiseti b) Antagonism against Fusarium oxysporum f.sp lycopersici c) Antagonism against Fusarium moniliforme d) Antifungal assay showing antagonism against Rhizoctonia solani. Paper discs were treated with 100, 200 and 500 µl of the mixture of purified chitinases, in 10 mM sodium phosphate, pH 6.0; the samples are marked 1 to 3 respectively. C indicates a control disc impregnated with sodium phosphate buffer. The dotted line indicates a clear border of fungal growth for sample 3.

Fig. 6 shows Agar cup assays for scoring of the larvae of Plutella xylostella a) Control (Third and fourth instar larvae), b) Dead larvae (showing discolouration from green to brownish- black), c) Feeding assay experiment in a tissue culture tray.

Fig. 7 is the effect of chitinases on insecticidal activity. The graph shows survival of second and third instars larvae on cabbage leaves treated with sterile water or with a suspension of non-induced Brevibacillus laterosporus Lakl210 supplemented with 0-500 µl of supernatant from a chitin induced culture of Brevibacillus laterosporus Lakl210. Each point represents the average survival from three independent measurements (10 larvae per measurement).
SEQUENCE LISTING AND TABLES

SEQ ID NO: 1 shows 16 s rDNA sequence (1-1500 bp) having NCBI Genbank Accession No HQ_4 12764.

SEQ ID NO: 2 to 18 shows peptide sequence present in the chitinase(s) produced by a new strain of Brevibacillus laterosporus designated as Brevibacillus laterosporus LAK 1210 having Accession number MTCC 5487.

SEQ ID NO: 19 shows universal forward primer sequence

SEQ ID NO: 20 shows universal reverse primer sequence

Table 1 depicts peptide sequence present in the chitinase(s). The sequence information was derived for a limited number of the six proteins (usually only one), as listed in the second column marked "protein". Peptides are scored positive (1) on the basis of corresponding peptide masses. For example, the sequence ASVPTNYK was determined twice, while analyzing proteins 3 and 6, and belongs to a peptide with a molecular mass that was detected in the peptide maps of proteins 2, 3 & 6. These analyses are not absolute. Certain peptides may remain undetected in certain samples, whereas other peptides may be detected unexpectedly in certain samples due to cross-contamination (e.g. between proteins 2 & 3, which are very close to each other on the gel). X, unknown amino acid.

DETAILED DESCRIPTION OF THE INVENTION

The new strain of Brevibacillus laterosporus LAK 1210 has been deposited at Microbial Type Culture Collection, an International repository at IMTECH, INDIA with the accession number MTCC 5487 under the Budapest Treaty. This new strain encode novel polypeptides of agricultural interest, with biological activity against insects and phytopathogenic fungi and in particular embodiments, these polypeptides synergises and enhance the insecticidal activity relative to the activity of insecticidal proteins alone.
The embodiments involve the discovery of a rarely distributed, natural insecticidal strain producing a novel chitinase and the genes that encode the novel polypeptides. The embodiments further relate to the identification of fragments and variants of the naturally-occurring coding sequence that encode biologically active polypeptides with chitinolytic activity. The nucleotide sequences of the embodiments find direct use in methods for impacting pests, particularly insect pests such as pests of the order Lepidoptera. Accordingly, the embodiments provide new approaches for impacting insect pests that do not depend on the use of conventional insecticides.

The embodiments encompass a wild type (i.e. naturally occurring) bacterial strain which finds use in compositions and methods for impacting insect pests of the plant, such as, for example, lepidopteran pests and also protecting the plants from phytopathogenic fungi. Insect pests may be tested for insecticidal activity of compositions of the embodiments in early developmental stages, e.g., as second, third and fourth larval instars. The synergism of chitinolytic enzymes potentiates the insecticidal activity effecting changes in insect feeding, growth, and/or behaviour at any stage of development, including but not limited to killing the insect retarding growth, preventing reproductive capability, anti-feedant activity and the like.

In one of the embodiments of the present invention, there is provided a biologically pure culture of a new strain of *Brevibacillus laterosporus* designated as *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487, a variant or a mutant thereof.

In another embodiment of the present invention provides a biologically pure culture of a new strain of *Brevibacillus laterosporus* designated as *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487, a variant or a mutant thereof, wherein the strain produces chitinases and insecticidal proteins.

An embodiment of the present invention provides a biologically pure culture of a new strain of *Brevibacillus laterosporus* designated as *Brevibacillus laterosporus* LAK 1210
having Accession number MTCC 5487, a variant or a mutant thereof, wherein the strain exhibits antifungal activity against phytopathogenic fungi.

Another embodiment of the present invention provides a biologically pure culture of a new strain of *Brevibacillus laterosporus* designated as *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487, a variant or a mutant thereof, wherein the strain exhibits an inhibitory effect on insects.

Yet another embodiment of the present invention provides an isolated 16S rRNA gene sequence of the strain *Brevibacillus laterosporus* LAK 1210 having accession number MTCC 5487, wherein the nucleotide sequence of gene is as set forth in SEQ ID NO: 1 having GenBank accession number HQ412764.

Compositions and methods of the embodiments comprise chitinolytic enzymes encoded by a naturally-occurring nucleic acid of the embodiments. More specifically, the embodiments provide novel polypeptides having antifungal activity comprising the amino acid sequences set forth in (Table 1).

In another embodiment, the present invention provides a process for producing a supernatant comprising chitinases and insecticidal proteins, wherein the process comprises culturing liquid culture of novel strain of *Brevibacillus laterosporus* designated as LAK 1210 having accession number MTCC 5487 in a culture medium comprising 0.1-1 % (w/v) colloidal chitin or 0.1-1% (w/v) marine chitinous waste to obtain cell culture, and obtaining the supernatant comprising chitinases and insecticidal proteins from the culture broth.

Further embodiment of the present invention related to marine chitinous wastes, wherein the example of the marine chitinous wastes includes but is not limited to shrimp shell powder, crab shells, squid pen powder.

In another embodiment, the present invention provides a process for producing a supernatant comprising chitinases and insecticidal proteins, wherein the process comprises culturing liquid culture of novel strain of *Brevibacillus laterosporus*
designated as LAK 1210 having accession number MTCC 5487 in a culture medium comprising 0.1-1 % (w/v) colloidal chitin or 0.1-1% (w/v) marine chitinous waste to obtain cell culture, obtaining the supernatant comprising chitinases and insecticidal proteins from the cell culture, and purifying chitinases from the supernatant using purification method comprising ammonium sulphate precipitation followed by affinity or ion exchange chromatography.

Yet another embodiment of the present invention provides a chitinolytic enzyme having molecular weight selected from a group consisting of 25 kDa, 55 kDa, 70 kDa, 75 kDa, 90 kDa.

Another embodiment of the present invention relates to chitinolytic enzymes which are active at a pH range of 3.0-11.0, with two pH optima (pH 6.0 and 8.0). The enzymes are alkaline active under alkaline conditions (at a pH above 7.0 and in a pH range of pH 9.0-11.0) but may also be active under neutral and/or acid conditions. The chitinolytic enzymes of the present invention are particularly effective in controlling insects, because they are active under alkaline conditions. As a result, these enzymes can be ingested by insects and then attack the insects by degrading their chitin-containing, alkaline digestive tracts.

Further embodiment of the present invention provides a chitinolytic enzyme having molecular weight selected from a group consisting of 25 kDa, 55 kDa, 70 kDa, 75 kDa, 90 kDa, wherein the enzyme is active at a temperature ranging from 30°C to 90°C, wherein the enzyme has a temperature optimum of 70°C.

The present invention further provides relates to chitinolytic enzymes with high thermostability, with an optimum temperature of 70°C for the mixture of chitinases. The temperature activity curve coincidences with the thermostability curve, indicating that enzyme activity at high temperatures is impaired by the stability of one or several of the proteins in the mixture. The high thermostability of the chitinases is remarkable considering that the thermostable chitinases from a mesophilic bacterium adapted to a mangrove forest biome with moderate temperatures is a rare find and there are very few
reports on thermostable enzymes from mesophiles. Thermostability is an important parameter for industrial use of enzymes and the high thermostability of the chitinases from *Brevibacillus laterosporus* Lakl210 could thus be an advantage in further utilization of the strain in biomass utilization, biofuel research and in other industrial processes where the harsh environment demands higher pH and temperature.

In another embodiment, the present invention provides a composition comprising cells of the *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487 or a metabolite obtained from the *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487 and a carrier.

Yet another embodiment of the present invention provides a composition the supernatant obtained from the culture of the *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487 or a metabolite obtained from the *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487 and a carrier.

Still another embodiment of the present invention provides a composition comprising the purified chitinases obtained from the supernatant obtained from the cell culture of the *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487 or a metabolite obtained from the *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487.

In an embodiment of the present invention, there is provided a synergistic composition comprising the chitinases obtained from the supernatant obtained from the cell culture of the *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487 or a metabolite obtained from the *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487 and insecticidal proteins selected from the group consisting of i) culture supernatant of *Brevibacillus laterosporus* LAK 1210 having accession number MTCC 5487 ii) cells of strain *Brevibacillus laterosporus* LAK 1210 having accession number MTCC 5487 and iii) one or more derivatives of *Brevibacillus laterosporus* LAK 1210 having accession number MTCC 5487, wherein the derivatives are selected.
from a group consisting of spores, inclusions, enzymatic fractions and cellular extracts; and a carrier.

The compositions as disclosed in the present invention can be formulated in the form of wettable powders, dust, pellets, granules, seed treatment products, emulsions, sprayable solutions, aqueous solutions, oil or water based dispersions, ULV formulations or microencapsulations.

The compositions as disclosed in the present invention can be a biopesticide, biofungicide, insecticide or nematicide.

Further embodiment of the present invention provides a method for protecting or treating or modulating phytopathogenic infection in plant or a part thereof, wherein the method comprises applying synergistically effective amounts of the composition as disclosed in the present invention to the plant or the part thereof, natural or artificial soil or planting media.

In a specific embodiment, the combinations are compositions, particularly compositions for use in treating or modulating phytopathogenic infection, more specifically phytopathogenic fungi and insects. The above mentioned combinations may also be formulated into compositions with an agriculturally acceptable shelf life. As defined herein, "phytopathogenic infection" means infection of plants by pathogenic fungi, bacteria, insects and nematodes.

Preferred combinations can be formulated with an acceptable carrier into a biopesticide/biocontrol agent. Furthermore, these combinations of this invention have an advantage of being formulated as an adjuvant, a colloid, a wettable powder, an emulsifiable concentrate, an aerosol or spray, a dusting powder, a dispersible granule or pellet, an impregnated granule, a suspension, a solution, an emulsion and also microencapsulations. The compositions can be formulated by conventional methods such as those described in, for example, Winnacker-Kuchler (1986), "Chemische Technologie" [Chemical Technology], Vol. 7, C. Mauser Verlag Munich, 4th Ed. 1986; van Valkenburg, "Pesticide formulations", Marcel Dekker N. Y., 2nd Edition 1972-73;

Necessary formulation aids include such as carriers, inert materials, surfactants, solvents, and other additives. Such formulated compositions may be prepared by concentration of a culture of cells comprising the polypeptides, desiccation, extraction, filtration, centrifugation, sedimentation, homogenization and lyophilization.

The invention is additionally directed to a method of treating or modulating the phytopathogenic infection, in particular phytopathogenic fungi and insects. These formulations can be applied to the environment hosting a target insect pest/pathogenic fungus, e.g., soil, water or a plant or a part thereof wherein seeds, plantules, plants, foliage of plants of the plant are treated with an effective amount of the formulation.

The phytopathogenic infection as described in the present invention is a plant disease caused by at least one fungus selected from the group consisting of *Fusarium*, *Rhizoclonia*, *Pythium*, *Phylophthora*, *Cercospora*, *Puccinia*, *Venturia*, *Alternaria*, *Uncinula*, *Ustilago*, *Colletotrichum*, *Erysiphe*, *Bolrytis*, *Sclerotium* and *Monilinia* which comprises contacting such fungus with purified antifungal chitinases. effective to obtain said inhibiting. The method of the present invention involving application of a chitinolytic enzyme can be carried out through a variety of procedures when all or part of the plant is treated, including seeds, roots, stems and leaves etc.

The examples of the *Fusarium* species include *Fusarium oxysporum* f. sp. *lycopersici*, *Fusarium moniliforme* or *Fusarium equiseti*.

The active compounds and compositions of the embodiments display activity against insect pests, which may include economically important agronomic, forest, greenhouse, nursery, ornamentals, food and fiber, public and animal health, domestic and commercial structure, household, and stored product pests. Insect pests include insects selected from the orders Diptera, Lepidoptera, Coleoptera, Hymenoptera, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Isoptera, more specifically insects from Diptera, Coleoptera and Lepidoptera.
Further, the phytopathogenic infection as described in the present invention may be caused by at least one insect belonging to Lepidoptera, Diptera, Coleoptera, Homoptera or Hymenoptera.

The novel strain of *Brevibacillus laterosporus* LAK 1210 exhibits an "improved pesticidal activity" which refers to synergistic action of chitinolytic enzymes of the embodiments that has enhanced insecticidal activity where a wider range of insects is impacted relative to the range of insects that is affected by activity of its corresponding non-chitinolytic strains of *Brevibacillus laterosporus* strains and may be other wild-type non-chitinolytic insecticidal strains.

The formulations of the embodiments can be used in combination with other Bt toxins or other insecticidal proteins to increase insect target range for insect resistance management and control. Furthermore, the formulations can also be used in combination with other biocontrol agents or chemical fungicides for integrated pest management.

In another embodiment of the present invention there is provided an improved method for protecting or treating or modulating phytopathogenic infection in plant or a part thereof, wherein the method comprises applying the composition as disclosed in the present invention concurrently with the Bacillus-based insecticides, wherein the method enhances the insecticidal effectiveness of the Bacillus-based insecticides for insect control.

The example of *Bacillus* species includes *Bacillus thuringiensis* or *Bacillus sphaericus*.

**EXAMPLES**

Following are the illustrative and non-limiting examples, including the best mode, for practicing the present invention.

**Example 1: Screening of insecticidal bacteria for novel chitinolytic enzymes**

About 300 strains of microorganisms from marshy, mangrove soil from Andhra Pradesh, India were screened for their ability to clear chitin-containing agar plates
buffered at pH 10. From this screening studies, a strain of *Brevibacillus laterosporus* was isolated that secreted chitinolytic enzymes which were active at pH 9.1, when the strain was grown on nutrient agar plates (pH 9.0) or liquid culture medium (pH 9.0) containing 0.2% colloidal chitin.

Example 2: Identification of the Strain

*Brevibacillus laterosporus* Lakl210 was isolated from mangrove, marshy soil in Palasa located in Srikakulam district of Andhra Pradesh, India, as part of an effort to isolate novel pesticidal strains with chitinolytic potential. The active strain was identified as a member of *Brevibacillus laterosporus*, based on morphological and physiological characteristics, and 16 s r RNA sequence (SEQ ID NO: 1). The strain was accessioned into the Microbial Type Culture Collection, MTCC 5487. The 16S rRNA was amplified from genomic DNA using the universal primers p27f (SEQ ID NO: 19 - 5-AGA GTT TGA TCM TGG CTC AG-3) and pl525r (SEQ ID NO: 20 - 5-AAG GAG GTG WTC CAR CC-3). Genomic DNA was extracted using DNAzol (Invitrogen, California USA). The genomic DNA was subjected to PCR in a 50 µl reaction containing 1 µl DNA (0.2 ng), 1 µl each of forward and reverse primers (25 µM), 2 µl of each of the dNTPs, 2 µl MgCl2, 2.5 µl 10X PCR-Buffer II (without MgCl2), and 0.2 µl Taq-DNA Polymerase (2.5 U). The PCR conditions were as follows: initial activation of the Taq-DNA Polymerase for 30s at 94°C, followed by 30 cycles comprising of a denaturation step, for at 94°C, an annealing step for 1 min at 58°C, and an extension step for 50 sec at 72°C, followed by a final elongation step for 5 min at 72°C. PCR products were excised from a 1% agarose gel after electrophoresis, purified with the QIA quick Gel Extraction kit (QIAGEN, Inc.) and sequenced directly using a Biotech Diagnostic Big Dye sequencing kit (Biotech Diagnostics, Laguna Niguel, CA,USA) and an ABI 377 sequencer (Applied Biosystems, Foster City, CA,USA). For the phylogenetic analysis, representative sequences (45 in total) from the genera *Bacillus*, *Paenibacillus* and *Brevibacillus* were obtained from the Ribosomal Database Project (Release 10) (Cole et al. 2009). The selected sequences were aligned with Lakl210 and assigned to
phylogenetic groups using the Treebuilder tool provided by the Ribosomal Database Project. The 16S rRNA sequence of *Brevibacillus laterosporus* Lakl 210 (1500 bp) did not exhibit 100% identity with any 16S rRNA sequences in Genbank or the RDP database (Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucl Acid Res 37 (Database issue):D141-145). The closest neighbours are *Brevibacillus laterosporus* BPM3 and *Brevibacillus laterosporus* S62-9, both with more than 99% identity. The strain exhibited 96.5-97.0% identity with the type strains of *Brevibacillus ginsengisoli*, *Brevibacillus invocatus*, *Brevibacillus reuszeri*, *Brevibacillusformosus* and *Brevibacillus agri* (Figure 1).

Phenotypic studies showed that the isolated bacterium was aerobic and Gram-positive. Cells appear as small rods, 0.7-1.0 μm wide by 3.0-5.0 μm long, occurring singly or in short chains. We observed formation of ellipsoidal spores in swollen sporangia, laterally positioned with a canoe-shaped parasporal body (Figure 2 c). Colonies of *Brevibacillus laterosporus* Lakl210 grown on nutrient agar were opaque, granular, jagged and 1.0-2.0 mm in diameter after incubation for 2 days at 30°C. The pH and temperature range for growth were 7.0-11.0 and 25-37°C, and the optimum growth pH and temperature were 7.0-8.0 and 30-32°C, respectively.

**Example 3: Culturing of Brevibacillus laterosporus LAK1210 (MTCC5487) and production of chitinolytic enzymes**

The bacterial cultures were maintained on nutrient agar slants and plates [Lab-Lamco powder, 10.0 g, Bacto peptone (Difco), 10.0 g, sodium chloride, 5.0 g and Bacto agar (Saveen Werner AB, Limhamn, Sweden), 2.0 g per liter of water], at 30°C. For liquid cultures, the medium (pH 7.0) was composed as follows (g per liter): K$_2$HPO$_4$, 3.0 g, (NH$_4$)$_2$SO$_4$, 3.0 g, MgSO$_4$, 7H$_2$O, 0.03 g, peptone 2.0 g, yeast extract, 1.0 g, CaCl$_2$ 2H$_2$O, 18 mg, FeSO$_4$, 7H$_2$O, 0.75 mg, MnSO$_4$, 7H$_2$O, 50 mg, CuSO$_4$, 5H$_2$O, 7.5 mg, ZnSO$_4$, 7H$_2$O, 7.5 g and optionally, 0.5% (w/v) colloidal chitin. Alternately, 1%
sundried shrimp shell powder or other marine chitinous wastes (crab shell powder, squid pen powder) were also used as a source of carbon and nitrogen. The cultures were grown in flasks with constant shaking at 30°C at 180 rpm for 7 days.

**Example 4: Detection of chitinolytic activity**

For qualitative detection of chitinolytic activity, cells were grown on colloidal chitin agar plates (Sampson MN, Gooday GW (1998) Involvement of chitinases of Bacillus thuringiensis during pathogenesis in insects. Microbiology 144:2189-2194) containing a semi-minimal medium. The medium consisted of a 1:1 mixture of minimal medium containing 0.1% ammonium sulphate, 0.03% MgSO$_4$·7H$_2$O, 0.6% K$_2$HPO$_4$, 1% K$_2$HP0$_4$, pH 7.0) and nutrient broth (Oxoid, UK), supplemented with colloidal chitin (0.2%) and solidified with 1.5% agar. After incubating the plates at 30°C for 5 to 7 days, they were flushed with 1% Congo red solution for 10 minutes, followed by washing with 1M NaCl. The zones of chitin clearing became visible as translucent haloes around the colonies (Figure 2 a). The older colony shows a filamentous morphology with fringed margins. Chitinase production is evident from the clear halo around the colony which became visible after staining the plate with Congo Red (Figure 2 b).

The ability of *Brevibacillus laterosporus* to degrade chitin is not well documented. The literature available on enzymatic profile and chitinases produced by *Brevibacillus laterosporus* is scarce and the present invention relates to the inducible chitinolytic enzymes produced by newly discovered, hyperchitinolytic strain of *Brevibacillus laterosporus* LAK 1210 has been characterized.

**Example 5: Isolation and purification by chitin affinity chromatography**

Culture supernatant obtained by centrifugation (4°C, 15,000 × g, 20 min), were filtered through a 0.45 μm membrane (Filtropur S 0.45 PAT, Sarstedt, Nurmbrecht, Germany). Thereafter, the supernatant was saturated to 80% with ammonium sulphate and incubated overnight at 4°C. The precipitate was collected by centrifugation (4°C, 15,000 × g, 20 min), dissolved in 20 mM sodium phosphate buffer pH 6.0 (10 ml per 1
liter of culture supernatant) and dialyzed against the same buffer. 15 ml of the dialyzed solution was adjusted to 1M ammonium sulphate and 50 mM Tris-HCl, pH 8.0 and applied on a 2.5x5 cm column packed with chitin affinity beads (New England Biolabs, Hertfordshire, UK) at a flow rate of 2.5 ml/min. Following a wash with the starting buffer (1M ammonium sulphate, 50 mM Tris-HCl pH 8.0) to remove unbound protein, adsorbed proteins were eluted with 20 mM acetic acid. Fractions displaying chitinase activity towards (4MU-NAG) (see below) were pooled to around 10 ml in total and concentrated approximately 10 times using Vivaspin-20 ultrafiltration membrane concentrators (10 kDa cut off, Vivascience AG, Hannover, Germany). Enzyme purity was checked on SDS-PAGE gels stained with the Pierce silver staining kit (Thermo Fisher Scientific, USA).

**Example 6: Enzyme assays for chitinolytic activity**

Chitinolytic activity was measured by using the fluorogenic chitin derivatives, 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4MU-NAG), 4-methylumbelliferyl-P-D-N,N"-diacetylchitobioside (4MU-NAG₂) and 4-methylumbelliferyl-P-d-N,N',N"-triacetylchitotriose (4MU-NAG₃) (Sigma Chemicals, St.Louis, USA).

In a standard assay, 5 µl enzyme solution was added to a reaction mixture containing 50 µg/ml 4 MU-NAG₂ and 0.1 mg/ml bovine serum albumin (BSA) in 50 mM Tris-HCl buffer (pH 8.0) (in a total volume of 50 µl). The reactions were incubated at 50°C for 10 min without agitation in glass tubes in a water bath and terminated by adding 1.95 ml of 0.2 M Na₂CO₃. The amount of 4-methylumbelliferone (4MU) released was measured fluorometrically using a TKO 100 minifluorometer (Hoefer scientific instruments, CA, USA). Protein concentrations were determined using the Bradford protein microassay kit according to the manufacturers protocol (Bio-Rad, Hercules, CA, USA) using BSA as standard. The effect of the chitinase inhibitor allosamidin was checked in 50 µl reactions containing 5 µM allosamidin, 50 µg/ml 4MU-NAG₂, 0.1 mg/ml BSA and 5 µl of enzyme solution in 20 mM sodium phosphate buffer (pH 7.0).
Example 7: Detection of chitinases by SDS-PAGE and activity staining

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10 % NuPAGE® Bis-Tris precast gels in a XCell SureLock™ Mini-Cell (Invitrogen, California, USA). Protein samples were mixed 1:1 with loading buffer (15% sucrose, 2.5% SDS, 125 mM TrisHCl (pH 6.7), 0.01% Bromo-phenol Blue) and heated at 70°C for 10 min. SDS-PAGE analysis of the chitinolytic fractions obtained from chitin-affinity chromatography of ammonium sulfate precipitates from 96 h chitin-induced cultures revealed three major and several less prominent bands. In total six bands, three major bands representing estimated masses of 75 (2), 70 (3), and 55 (4) kDa, and three minor bands representing estimated masses of and 90 (1), 45 (5) and 25 (6) kDa were subjected to further analysis (Figure 3a). Zymographic analysis with 4MU-(GlcNAc)₂ after in situ protein renaturation showed clear positive signals for all the four bands. The chitinases were characterized as a mixture and the two chitinases of the molecular weight, 75 and 70 kDa were characterized separately. Fluorometric zymography performed by agar-overlaying containing 4- methyl umbelliferyl substrates revealed chitinase activity (Figure 3b).

Example 8: Purification of chitinolytic enzymes

Cultures were grown at 30°C for 7 days with a constant shaking (180 rpm). The biomass was removed from the broth by centrifugation at 6000 X g for 30 min. 4°C. The supernatant was filtered through mira cloth, then adjusted to 80% saturation with ammonium sulfate to isolate total protein. The precipitate was collected by centrifugation (4°C, 15,000 x g, 20 min), dissolved in 20 mM sodium phosphate buffer pH 6.0 and dialyzed against the same buffer. 15 ml of the dialyzed solution was adjusted to 1M ammonium sulphate and 50 mM Tris-HCl, pH 8.0 and applied on a 2.5x5 cm column packed with chitin affinity beads (New England Biolabs, Hertfordshire, UK) at a flow rate of 2.5 ml/min. Following a wash with the starting buffer (1M ammonium sulphate, 50 mM Tris-HCl pH 8.0) to remove unbound protein, adsorbed proteins were eluted with 20 mM acetic acid. Fractions displaying chitinase...
activity towards (4MU-NAG₂) (see below) were pooled to around 10 ml in total and concentrated approximately 10 times using Vivaspin-20 ultrafiltration membrane concentrators (10 kDa cut off, Vivascience AG, Hannover, Germany). Enzyme purity was checked on SDS-PAGE gels stained with the Pierce silver staining kit (Thermo Fisher Scientific, USA).

Example 9: Identification of proteins by mass spectroscopy analysis

Protein bands were excised from silver or coomassie stained gels, transferred to individual 0.2 ml PCR tubes and dehydrated twice by washing with 100 % acetonitrile. For reduction and alkylation, the gel pieces were submersed in 50 µl of a freshly made DTT solution (10 mM dithiothreitol in 100 mM ammonium bicarbonate) and incubated at 56°C for 30 min. After removal of the DTT solution, 50 µl IAA solution (55 mM 2-iodoacetamide, 100 mM ammonium bicarbonate) was added, followed by 30 minutes incubation in the dark. Then the gel pieces were washed once with 100 mM ammonium bicarbonate and once with acetonitrile. After air drying, the gel pieces were covered with 30-50 µl (depending on the size of the gel piece) Trypsin solution (10 ng/µl sequence grade trypsin (Promega, Madison, WI) in 25 mM ammonium bicarbonate containing 10 % acetonitrile). After incubation on ice for 90 minutes, 25 mM ammonium bicarbonate containing 10 % acetonitrile (no trypsin) was added as needed to completely hydrate the gels (typically 30 - 50 µl), followed by incubation overnight at 37°C.

After a brief centrifugation and removal of the superantant, the gel pieces were extracted by sonication for 10 minutes (in a Vibracell 505, Sonics, Newtown, USA) in 50 µl 1 % formic acid in dH₂O, followed by three extractions with 25 µl of 5% formic acid in 50% acetonitrile (each extraction step included 10 minutes of sonication). The four extracts were pooled, and after vacuum-drying of the solution, the peptides were dissolved in 25 µl of 0.1% trifluoroacetic acid. To facilitate de novo sequencing, peptides were derivatized with Lys-tag (Ktag) made in-house according to the protocol of (Peters et al. 2001). The identity of the six bands was investigated by tryptic
digestion followed by MALDI-TO FMS/MS analysis of the resulting peptides. This approach yielded peptide maps for all six proteins as well as 18 peptide sequences belonging to proteins 1, 2, 3 and 6. Despite multiple attempts no peptide sequences were identified for proteins 4 and 5. The amino acid sequence of the chitinases is shown as (Table 1) and strongly suggest that bands 1 and 2 belong to the same protein, and thus, that the 75 kDa protein 2 is a degradation product of the 90 kDa protein 1. Further, the results indicate that bands 3, 4, 5 and 6 are derived from the same protein. Family 18 chitinases tend to be multidomain proteins, often with flexible linkers between the domains. Such proteins are prone to in vivo proteolysis and protease-induced multiplicity of chitinases has indeed been reported in several cases (Neiendam Nielsen M, Sorensen J (1999) Chitinolytic activity of Pseudomonasfluorescens isolates from barley and sugar beet rhizosphere. FEMS Microbiol Ecol 30(3):217-227; Suzuki K, Taiyoji M, Sugawara N, Nikaidou N, Henrissat B, Watanabe T (1999) The third chitinase gene (chIC) of Serratia marcescens 2170 and the relationship of its product to other bacterial chitinases. Biochem J 343:587-596). Note that the size of the catalytic domains of family 18 chitinases varies from about 30 kDa in typical endochitinases such as ChiC from Serratia marcescens (Synstad B, Vaaje-Kolstad G, Cederkvist FH, Saua SF, Horn SJ, Eijsink VG, Sorlie M (2008) Expression and characterization of endochitinase C from Serratia marcescens BJL200 and its purification by a one-step general chitinase purification method. Biosci Biotechnol Biochem 72(3):715-723) to about 45 kDa in processive exochitinases such as ChiA and ChiB from S. marcescens (van Aalten DM, Synstad B, Brurberg MB, Hough E, Riise BW, Eijsink VG, Wierenga RK (2000) Structure of a two-domain chitotriosidase from Serratia marcescens at 1.9-A resolution. Proc Natl Acad Sci USA 97(11):5842-5847).

215(3):403-410) in Genbank did show significant similarities between some of the peptides found in proteins 1, 2 and 3 and chitinase A1 from Paenibacillus sp. oral taxon 786 (ZP_04850994.1), chitinase A-BL3 from Bacillus licheniformis (AC124006.1) and chitodextrinase from Vibrio angustum S14 (ZP_01234708.1).

Example 10: Novel characteristics of chitinolytic enzymes from Brevibacillus laterosporus LAK1210

The pH optimum for the chitinase activity was measured using 4MU-NAG$_2$ under standard assay conditions, using the following buffers (all at 50 mM): citrate phosphate (pH 3.0-5.0), sodium acetate (pH 5.0-6.0) sodium phosphate (pH 6.0-8.0), Tris-HCl (pH 8.0-9.0), and carbonate - bicarbonate buffer (pH 9.0-1 1.0).

The temperature optimum for chitinase activity was determined by carrying out standard activity assays in 50 mM Tris-HCl buffer, pH 8.0 at temperatures ranging from 25°C to 100°C. Thermal stability of the enzyme was determined by preincubating the enzyme samples without the substrate in 50 mM Tris-HCl, pH 8.0, at temperatures ranging from 25°C to 100°C for 30 min, after which the residual enzyme activity of 10-fold diluted samples was measured under standard assay conditions. The pH-activity profile revealed two pH optima, one at pH 6.0 and the other at pH 8.0 (Fig 4a). The optimum temperature for chitinolytic activity for the mixture of chitinases was found to be 70°C (Fig 4b). The temperature activity curve coincidences with the thermostability curve (Fig 4c), indicating that enzyme activity at high temperatures is impaired by the stability of one or several of the proteins in the mixture. The high thermostability of the chitinases is remarkable considering that they originate from bacterium adapted to a mangrove forest biome with moderate temperatures. Thermostability is an important parameter for industrial use of enzymes and the high thermo'stability of the chitinases from Lakl210 could thus be an advantage in further utilization of the strain.

Chitinolytic enzymes which are alkaline active are of interest because the alkaline chitinases function well in the alkaline environment of insect gut to enable the insecticidal proteins to permeate through easily. U.S Pat 391 1110 discloses an invention
on chitinases that facilitate the action of insecticidal proteins, when used in conjunction with the entomopathogenic organisms.

Example 11: Antifungal Assays and antagonistic activity of *Brevibacillus laterosporus* LAK 1210 against *Fusarium* species and other phytopathogenic fungi

The antifungal activity of the purified mixture of chitinolytic enzymes from *Brevibacillus laterosporus* LAK 1210 by was assessed tested using *Fusarium equiseti* as indicator strain, on the basis of inhibition of hyphal extension using the paper disc diffusion method (Roberts WK, Selitrennikoff CP (1988) Plant and bacterial chitinases differ in antifungal activity. J Gen Microbiol 134:169-176. Mycelial discs from the test fungus (5 mm diameter) were laced in the centre of petridish containing Potato Dextrose Agar (PDA) and incubated for 3-4 days until the colony reached a diameter of about 3 cm. Sterile filter paper discs (5 mm in diameter) impregnated with varying amounts of chitinase solutions in 20 mM sodium phosphate buffer (pH 6.0) were then placed on the agar surface at a distance of 0.5 cm from the front edge of The growing fungal colony. Control discs were saturated with sodium phosphate buffer (pH 6.0). The plates were then further incubated at 28°C and the fungal growth inhibition was visually assessed by the presence of a zone of inhibition near the chitinase-treated discs.

Figure 5a shows that the enzymes indeed inhibited the fungus in a dose-dependent manner. The control disc is overgrown, disc 1 (with 100 μl chitinase solution) and disc 2 (with 200 μl chitinase solution) seem less overgrown, whereas disc 3 (500 μl) shows a clear zone of inhibition demarcated as an arc (Fig 5d). *Brevibacillus laterosporus* LAK 1210 exhibits antagonistic activity against other *Fusarium* species also, *Fusarium oxysporium* f.sp.lycopersici (Fig 5b), *Fusarium moniliforme* (Fig 5c) and the other phytopathogenic fungi, such as *Rhizoctonia solani* (Fig 5d).

Example 12: Bioefficacy of larval bioassays and synergistic action of chitinases on insecticidal activity

Bioassays were conducted to evaluate the effects of the chitinolytic enzymes set forth in (Table 1) on insecticidal activity, on a lepidopteran insect pest. To determine the effect
of chitinolytic enzymes on the growth and development of Lepidopteran insects, larval *Plutella xylostella* (diamond backmoth) were reared on a high wheat germ-based diet as per the method of Webb et al. (1988) Laboratory rearing of the imported Cabbageworm, *NY Food Life Sci. Bull.*, 122. Each bioassay included 3 treatments replicated three times. All larvae were weighed when controls reached the ultimate star and then monitored daily for developmental changes.

Feeding assays were conducted by incorporating the culture broth containing the chitinolytic enzymes (0, 0.25%, 0.5%, and 1% of chitinolytic enzyme mixture (Table 1) and the insecticidal proteins into lepidopteran-specific artificial diet placed in a 10 well- tissue culture tray (Figure 6c). The protein is suspended in Tris-HCl buffer at a pH of 8.0. One third instar larva was placed in each well to feed on the artificial diet for 5 days. Two negative controls included, one with the feeding diet topically treated with non-chitinolytic culture broth containing only the insecticidal proteins and the other to which the above buffer has been applied. Results were expressed as positive for larvae for observations like discoloration, stunting and mortality.

In another set of experiments, agar cup assays were performed to study the effect of the chitinases on the insecticidal activity of *Brevibacillus laterosporus* Lakl210 against larvae of diamond backmoth. Larvae of *Plutella xylostella* (diamond backmoth) were obtained from cultures raised on *Brassica oleracea* var. *capitata*. Agar cup assays were performed excised cabbage leaf discs were treated with 5 ml of uninduced cell suspension (1 mg/ml) mixed with varied amounts (0, 100, 250 and 500 µl) of the supernatant containing from a culture of Lak 1210 grown in standard growth medium supplemented with 1% (w/v) colloidal chitin. Second and third instar larvae of *Plutella xylostella* were placed in agar cups containing excised cabbage leaf discs (3.5 cm) which had been spread with the four different test suspensions. In each of the four treatments, 10 larvae were used in three replicates (in total 30 larvae), and mortality was recorded every 24 h for 7 days. Cabbage leaf discs sprayed with sterile distilled water were used as a negative control. The agar cups were kept at 21°C, > 40% R. H. and
continuous light. After the treatment with different test suspensions, the larvae were scored for developmental changes. The larvae became more sluggish with less wriggling movements and the larvae changed colour from light green to black when they died (Fig 6a and b) larvae that had pupated were scored as alive. The mortality rate for each treatment was calculated for each day after treatment. Using days as the unit, the time to death of 50% of the larvae, or LT50, was estimated based on the logistic model: \[ P(a \text{ larva is alive } t \text{ days after treatment } i) = \varepsilon x p(\mu + \beta t + \gamma t)[1 + \beta x p(\mu + \beta i + y_i)]. \]

Fig. 7 shows that supematants from chitin-induced cultures increased the lethal effect of \textit{Brevibacillus laterosporus} Lakl210 on the larvae in a dose-dependent manner. Upon addition of 500 µl supernatant, 100% mortality was observed on day 5, compared to day 7 without this addition. All treatments with \textit{Brevibacillus laterosporus} Lakl210 caused death of 100% of the larvae within 7 days. Fig 7 shows that the LT50 value decreased with increasing amounts of chitinolytic enzymes.

The bioassay experiments were done with second and third instar larvae because initial experiments showed that second and third instars were more susceptible compared to the fourth and fifth instars. The first larval instars have high natural mortality rates. The fifth instar larvae feed very little or have completely ceased to feed before pupating. A similar study has been carried out with \textit{Bacillus thuringiensis} ssp \textit{kurstaki} HD-1 against diamondback moth (Wiwat G, Thaithanun S, Pantuwatana S, Bhumiratana A (2000) Toxicity of chitinase-producing \textit{Bacillus thuringiensis} ssp \textit{kurstaki} HD-1 (G) toward \textit{Platella xylostella}. J Invertebr Pathol 76(4):270-277).

These findings demonstrate the synergistic effect of chitinases on the pesticidal activity of \textit{Brevibacillus laterosporus} Lakl210 and show that chitinases may enhance the strain's pesticidal potential. These observations concerning the possible role of chitinases are consistent with earlier reports on the effects of chitinases on the pathogenicity of pesticidal strains (Yuan ZM, Liu M, Cai QX, Liu HZ, Zhang BH, Yan JP (2002) Chitinolytic activities in \textit{Bacillus thuringiensis} and their synergistic effects on

**TECHNICAL ADVANTAGES**

*Brevibacillus laterosporus* LAK 1210 (MTCC 5487) is a novel pesticidal strain that meets all the requirements of enzyme prospecting in terms of the organism and the enzyme characteristics of an industrial enzyme.

The chitinolytic enzymes of the present invention are particularly effective in controlling insects insecticidal proteins are active under alkaline conditions. Chitinase from *Brevibacillus laterosporus* LAK 1210 (MTCC 5487) exhibits higher activity at alkaline pH (pH 8.0), alkaline active in the range of pH 9.0-11.0.

Synergistic action of the novel chitinase potentiates the insecticidal activity making the strain *Brevibacillus laterosporus* LAK 1210 (MTCC 5487) highly efficacious for biocontrol and a possible, viable alternative to Bt and Bt control.

*Brevibacillus laterosporus* LAK 1210 (MTCC 5487) is a new, natural strain that could be quickly developed into a safe biopesticide/biocontrol formulation.

The present invention that uses a new strain of *Brevibacillus laterosporus* LAK 1210 (MTCC 5487) provides significant economic benefits to agriculture and forestry sectors and environment.

The strain efficiently degrades the shell waste and could provide an effective solution to waste management and could also be commercially exploited to convert chitinous waste to value added products like glucosamine and N-acetylglucosamine.
Brevibacillus laterosporus LAK 1210 demonstrates lepidopteran activity of economically and agriculturally important lepidopteran insect pests, which so far have not been successfully reported for strains of Brevibacillus laterosporus.

The chitinase is alkaline active, thermoactive and thermostable, exhibiting activity over a wide range of pH (3.0 - 11.0) with two pH optima (pH 6.0 and 8.0) and an optimum temperature of 70°C and these unique characteristics of the novel chitinase from the new strain of Brevibacillus laterosporus LAK 1210 (MTCC 5487) can be exploited in industrial processes with harsh environment, in particular biomass utilization in a biofuel industry.

SEQ ID NO: 1

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Table 1: peptide sequence present in the chitinase(s) produced by a new strain of *Brevibacillus laterosporus* designated as *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487

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What is claimed is:

1. A biologically pure culture of a new strain of *Brevibacillus laterosporus* designated as *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487, a variant or a mutant thereof.

2. The *Brevibacillus laterosporus* LAK 1210 as claimed in claim 1, wherein the strain produces chitinases and insecticidal proteins.

3. The *Brevibacillus laterosporus* LAK 1210 as claimed in claim, wherein the strain exhibits antifungal activity against phytopathogenic fungi.

4. The *Brevibacillus laterosporus* LAK 1210 as claimed in claim 1, wherein the strain exhibits an inhibitory effect on insects.

5. An isolated 16S rRNA gene sequence of the strain *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487, wherein the nucleotide sequence of the gene is as set forth in SEQ ID NO: 1 having GenBank accession number HQ412764.

6. A chitinolytic enzyme having molecular weight selected from a group consisting of 25 kDa, 55 kDa, 70 kDa, 75 kDa, 90 kDa, wherein the enzyme is active at a pH ranging from 3.0 to 11.0 and at a temperature ranging from 30°C to 90°C.

7. The chitinolytic enzyme as claimed in claim 6, wherein the enzyme has two pH optima of pH 6.0 and 8.0.

8. The chitinolytic enzyme as claimed in claim 6, wherein the enzyme has a temperature optimum of 70°C.

9. A process for producing a supernatant comprising chitinases and insecticidal proteins, wherein the process comprises culturing liquid culture of novel strain of *Brevibacillus laterosporus* designated as LAK 1210 having accession number MTCC 5487 in a culture medium comprising 0.1-1 % (w/v) colloidal chitin or 0.1-
1% (w/v) marine chitinous waste to obtain cell culture, and obtaining the supernatant comprising chitinases and insecticidal proteins from the cell cultures.

10. The process as claimed in claim 9, wherein the marine chitinous wastes is selected from a group consisting of shrimp shell powder, crab shells, squid pen powder.

11. A method of purification of chitinases from the supernatant obtained by the process as claimed claim 9, wherein the purification method comprises ammonium sulphate precipitation followed by affinity or ion exchange chromatography.

12. A composition comprising cells of the *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487 or a metabolite obtained from the *Brevibacillus laterosporus* LAK 1210 having Accession number MTCC 5487 and a carrier.

13. A composition comprising the supernatant obtained by the process as claimed in claim 9 and a carrier.

14. A composition comprising the purified chitinases obtained by the method as claimed in claim 11 and a carrier.

15. A synergistic composition comprising the chitinases obtained by the method as claimed in claim 11 and insecticidal proteins selected from the group consisting of i) culture supernatant of *Brevibacillus laterosporus* LAK 1210 having accession number MTCC 5487 ii) cells of strain *Brevibacillus laterosporus* LAK 1210 having accession number MTCC 5487 and iii) one or more derivatives of *Brevibacillus laterosporus* LAK 1210 having accession number MTCC 5487, wherein the derivatives are selected from a group consisting of spores, inclusions, enzymatic fractions and cellular extracts; and a carrier.

16. The composition as claimed in claims 12 to 15, wherein the composition is formulated in the form of wettable powders, dust, pellets, granules, seed treatment products, emulsions, sprayable solutions, aqueous solutions, oil or water based dispersions. ULV formulations or microencapsulations.
17. The composition as claimed in claim 16, wherein the composition is a biopesticide, biofungicide, insecticide or nematicide.

18. A method for protecting or treating or modulating phytopathogenic infection in plant or a part thereof, wherein the method comprises applying synergistically effective amounts of the composition as claimed in claim 17 to said plant or the part thereof, natural or artificial soil or planting media.

19. The method as claimed in claim 18, wherein the phytopathogenic infection is a plant disease caused by at least one fungus selected from the group consisting of *Fusarium, Rhizoctonia, Pythium, Phytophthora, Cercospora, Puccinia, Venturia, Alternaria, Uncinula, Ustilago, Colletotrichum, Erysiphe, Botrytis, Sclerotium and Monilinia*.

20. The method as claimed in claim 19, wherein said *Fusarium* is *Fusarium oxysporum f. sp. lycopersici, Fusarium moniliforme* or *Fusarium equiseti*.

21. The method as claimed in claim 18, wherein the phytopathogenic infection is caused by at least one insect belonging to Lepidoptera.

22. The method as claimed in claim 18, wherein the phytopathogenic infection is caused by at least one insect belonging to Diptera, Coleoptera, Homoptera or Hymenoptera.

23. An improved method for protecting or treating or modulating phytopathogenic infection in plant or a part thereof, wherein the method comprises applying the composition as claimed in claim 17 concurrently with the Bacillus-based insecticides, wherein the method enhances the insecticidal effectiveness of the Bacillus-based insecticides for insect control.

24. The method of claim 23, wherein the said Bacillus is *Bacillus thuringiensis* or *Bacillus sphaericus*.
Figure 4