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Chitinase enzyme in bacterial species and fungal growth inhibition against *A. niger* and *Penicillium*

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Abstract

Chitin is the most abundant polysaccharide in nature, second after cellulose as it found in the structure of crustaceans, insects and fungi being a significant component. Sea food waste disposal pose a great problem as chitin in nature degrade at a very gradual rate. Efforts are being made to use chitinase enzyme from different organisms to increase the rate and efficiency of chitin waste disposal. Moreover, chitinase and chitinase producing bacteria are widely being exploited to be used as bio-control agents to inhibit crop pathogens. On the basis of its mechanism of action, chitinase can either be endochitnase or exochitinase. Endo chitinases randomly hydrolyses internal glycosidic bonds in chitin. Exochitinase is exoglycosidase that can be divided into two subtypes based on the specificity of their action Chitinase is synthesized by a number of different microbes including bacteria, fungi and other organisms like mammals, insects etc. This paper gives information about the production of different chitinase enzymes from different bacterial gram-positive species. Antifungal activity for different isolates were tested against *A. niger* and *Penicillium*. The isolates showed varying degree of fungal growth inhibition against different fungi.

Introduction

Biopolymers are made up of repeating monomers of one or more than one type, produced by living organisms. Chitin is among the most abundant biopolymers in nature. It is second most widespread polymer after cellulose existing in the form of ordered polysaccharide micro-fibrils. Chitin has structural homology to cellulose with a hydroxyl group on C2 position replaced with acetyl amide functional group (- NHCOCH3) [1]. In nature chitin is both profuse and ubiquitous. The worldwide turnover of chitin is around 10 billion tons yearly [2]. In fungal cell walls, chitin is the major structural component as well as it is found in many organisms, including protozoa, nematodes and the arthropods (insects and crustaceans) making their exoskeleton, shells, and linings of gut. Chitin is the highly insoluble linear homopolysaccharide of ß-1, 4-linked N-acetyl D-glucosamine units. On the basis of difference in the orientation of micro-fibrils, chitin can be divided into three allomorphs α,β and γ chitin. In α-chitin antiparallel configuration of individual chains exists. In β-chitin monomer chains run in parallel direction whereas γ-chitin is combination of both α and β-chitins [3, 4] β chitin being most copious is found in exoskeletons of crab, lobster etc. β -chitin is extracted from squid pen whereas γ-chitin can be isolated from fungi and some yeast [5, 6]. Amino groups in chitin are deacetylated to produce polysaccharide chitosan, for biomedical applications. Both chitin and chitosan are bio-renewable and biocompatible with vast applications in various fields [7]. Metal ions react with chemical compounds known as chelating agents to form water soluble products. Both chitin and chitosan have high potential as chelators, they are heavy metal trappers that are known to trap and remove arsenic in drinking water. In waste water chitin and chitosan mixture has been found to effectively remove petroleum and petroleum products [8]. Oxidative molecules and free radicals are produced when food is broken down during cellular metabolism. These free radicals and oxidative species can lead to oxidative stress and can cause serious illnesses such as cancer, cardiovascular disorders, arthritis, and inflammation [9]. Antioxidants protects against the hazardous effects of these radicals. Chitin and its derivatives are among the various compounds to exhibit antioxidant ability. Non toxicity of chitin to cells makes it a suitable alternate for harmful synthetic antioxidants such as butylated hydroxytoluene [10, 11].

Chitinases as the hydrolytic enzymes

PChitinases are the hydrolytic enzymes responsible for cleav-

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ing glycosidic bonds in polymers (chitin) to produce soluble and insoluble oligosaccharides. Chitinase enzymes are present in chitin containing organisms like fungi, crustaceans, nematodes etc. But they can also be produced in organisms that do not produce chitin such as bacteria, viruses, plants and animals. In these organisms chitinase plays significant role in morphogenesis, recycling of nitrogen and carbon in the environment and moreover it safeguards the plants from chitin containing pests and insects. Based on the differences of catalytic domain chitinases are sub divided into three families of glycosyl hydrolases, family 18, 19 and 20. Family 18 is further sub grouped into three categories. Subgroup A consists of bacterial chitinases. Subgroup B consists of enzymes from plants whereas subgroup C consist of high molecular weight newly discovered enzymes. An organism can have multiple genes that encode chitinase enzyme. More than one type of chitinase enzyme produced by an organism can lead to efficient degradation of chitin because different types of enzymes work synergistically and provide contrasting affinities for broad range of substrates. For instance, *S. marcescens* secrete four types of chitinases namely, ChiA, ChiB, ChiC1 and ChiC2. These enzymes work synergistically to degrade chitin [12].

Mechanism of action and hydrolysis

On the basis of its mechanism of action, chitinase can either be endochitnase or exochitinase. Endo chitinases randomly hydrolyses internal glycosidic bonds in chitin generating chitobiose, chitotriose etc. that are low molecular weight oligomers of glucosamine. Exochitinase is exoglycosidase that can be divided into two subtypes based on the specificity of their action I.e., chitobiosidases, breaks down chitin into dimers (catalyze the hydrolysis of chitobiose at terminal ends releasing N- acteylchitobiose) and N-acetylglucosamines, produce monomers from dimers (specifically catalyze the release of N-acetylglucosamine units by cleaving oligomers, resulted by the activity of endochitnase.

Annually, bulk of chitin approximately 10 billion tons is released into the environment as waste from food industries especially as waste of shrimps, crabs and fishes etc. Chitin in this form is impure and is tightly bound with carotenoids, proteins and calcium carbonate [13]. To extract pure chitin from the shellfish waste in undergoes through an ordered series of process which includes demineralization which is acid treatment mostly with HCl to remove minerals, deproteinization includes alkaline treatment with NaOH to isolate protein content and discoloration that uses of bleaching agents that result in colorless products [14]. Regardless of the fact that chitin is released in abundance to the environment; it does not accumulate due the presence of numerous natural and synthetic mechanisms that degrade insoluble chitin into soluble chitin oligomers and GlcNAc [15]. The hydrolytic products of chitin act as mediators of chitin degrading enzymes [16]. Chitin can be hydrolyzed through a number of different processes categorized into physical, chemical and enzymatic methods [17].

Depolymerizations of chitin by acid or enzymes leads to the production of chitin oligomers that constitute up to ten GlcNAc residues [18]. Chitin oligomers are water soluble with a number of additional biological and chemical properties that makes them more adaptable for industrial use than chitin itself. The most commonly employed acid for hydrolysis of long chain polymers of chitin is concentrated HCl. The key mediators in this process are the concentration of acid, temperature and time period of incubation. Chitin oligomers can also be produced by chitosan by treating it first with concentrated HCl resulting in chitosan oligomers and then N-acetylation these oligomers partially by acetic anhydride to convert them into chitin oligomers [19]. Different physical adjunct treatments are known that assist depolymerization of chitin by chemical and enzymatic methods. Ultra-sonication has been found to be effective for depolymerization as it cleaves the bonds that are most susceptible and lower the MW of the polymer thus preserving its chemical nature [20]. For producing chitin oligomers, irradiation in microwave has been also proved to be beneficial. HCl is added to chitin prior subjecting to conventional microwave for irradiation [21]. Other physical technique is gamma irradiation that requires no chemical or enzymatic additives. Chitin degradation is mediated by specific enzymes that catalyses' the removal of acetyl groups from chitin. These enzymes are termed as chitinases that belong to glycosylhyrolases; enzymes responsible for the hydrolysis of glycosidic bonds in carbohydrates. Complete polymerization of insoluble chitin polysaccharide consists of three steps that are: Formation of oligomers by cleaving polymers, Production of dimers b splitting oligomers, Formation of monomers by cleaving dimers [22]. Lysozymes have also shown hydrolytic action against chitin [23].

Table 1: *Source of chitinase producing bacteria.*

Sr. No	Microorganism	Source of isolation
1	*Bacillus licheniformis*	Rhizosphere soil samples
2	*Bacillus thuringiensis*	Soil including chitin wastes
3	*Bacillus amyloliquefaciens*	Beaches
4	*Bacillus subtilis*	Soil sample of agricultural field
5	*Aeromonas hydrophila*	Soil of rice rhizosphere
6	*Serratia marcescens*	Fresh vegetables

Chitinase producing *Bacillus* spp

Bacillus thuringiensis

Bacillus thuringiensis is well known for its role as bio-control agent for insects. Its insecticidal properties were recognized before the bacteria itself was identified. Its insecticidal property is owed to the crystals present in the bacteria. *Bacillus thuringiensis* is a gram positive, endospore producing soil inhabitant microbe [24]. A number of *Bacillus thuringiensis* strains are reported with chitinase activity. These strains have been actively found to retard the growth of insect larvae and fungal hosts. Moreover, this bacterium is also exploited for producing insect and pest resistant genetically modified crops

such as cotton. Toxin genes from this bacterium are transferred to plants where they are expressed and so confer resistance to certain insect pests [25].

Bacillus licheniformis

Bio-control of insects and pests is an emerging agricultural technique to preserve environment from the hazardous effects of chemical insecticides and pesticides. Microorganisms commonly bacteria and fungi are used as biological agents to limit the use of harmful chemicals on crops. *Bacillus licheniformis* is among the most suitable bacterial species used for controlling pests and pathogenic fungal growth on crop plants as it secretes various chitin degrading enzymes. It is a gram positive, spore producing bacterium that is commonly isolated from rhizosphere of plants and birds' feathers. It has been delineated in a study that *Bacillus licheniformis* can have a great reducing effect on the growth of fungal pathogens i.e*., Phaeoacremonium aleophilum, Botryosphaeria* spp that effects grapevines [26].

Bacillus amyloliquefaciens

Bacillus amyloliquefaciens is a gram positive rhizobacterium that inhabits plant rhizosphere and is involved in plant growth and development by synthesizing an important plant hormone (Indole acetic acid) and increasing solubilization of potassium and phosphorus. It protects the plants from various Phytopathogens that can be fungi, bacteria, or fungus like organisms by competing for space, nutrients and releasing toxins. *Amyloliquefaciens* is commonly used as biopesticide and biofertilizer [27]. In another study it has been reported that *Amyloliquefaciens* shows a significant potential in protecting maize crops by retarding growth of common maize fungal pathogens i.e., *Rhizopus, Penicillium, and Fusarium* [28]. *Bacillus amyloliquefaciens* is among the bacteria with great chitinolytic activity.

Bacillus subtilis

Rhizosphere bacterial species can serve as eminent agents for biological control of soil borne pathogens of plants. A number of numerous bacterial species have been known to suppress fungal growth and one of them is *B. subtilis,* endospore producing, gram-positive rod-shaped bacteria that can endure extreme environmental conditions such as temperature, pH, osmotic pressure etc. This bacterium colonizes plant roots and plays a prominent role in the plant growth and kills plant pathogenic fungi by disrupting chitin containing mycelia. In a study*, B. subtilis* have shown to reduce the growth of *Colletotrichum gloeosporioides*, a fungus that is known to cause a disease called anthracnose of grass, ornamentals, vegetables, legumes and fruits [29].

Bacillus coagulans

Each year a several million tons of crops are lost both before and post-harvest significantly due to phytopathogens. *Fusarium* and *Botrytis* are the common cause of different diseases of crop plants including gray mold of grape wines and *Fusarium* rot different plant species i.e., tomatoes, wheat barley and oranges etc. To eliminate crop loss due to fungi many chemical fungicides are employed but the excessive use of these fungicides has led to increased resistance of plants to these chemicals. Moreover, the excessive use of these chemicals has detrimental effects on both the plants and the environment that opened the way to find an alternative to these chemical fungicides. *Bacillus coagulans*, a gram-positive bacterium, is used as a bioprotective agent that can preserve plant crops from fungal attack. The bacterium has ability to secrete antifungal lipopolypeptides and polysaccharides such as chitinase that suppress fungal growth [30].

Bacillus megaterium

B. megaterium is a large sized, gram positive, mostly aerobic endospore producing bacteria that inhabits a wide range of habitats including sea water, soil, fish, rice paddies etc*. B. Megaterium* has served as an industrial microorganism for over 50 years. It has been employed in baking industry for modification of starch, in diagnostics for glucose blood tests, for producing antifungal toxins and penicillin [31]. *Aspergillus* and *Penicillium* are the main cause of rice grains contamination during storage that leads to great economic loss and poor-quality rice. *B. Megaterium* has shown significant reduction in growth of *Aspergillus* and *Penicillium* [32].

Table 2: *Common diseases of crops by fungal pathogens and bacillus species.*

Sr. No	Crop	Fungal disease	Causative agent	Bacterial species used for bio-control
1	Wheat	Head blight of wheat	*Fusarium graminearum*	*B. subtilis*
2	Banana	Black leaf Streak of banana	*Mycosphaerella fijiensis*	*B. subtilis*
3	Soybean	Damping- off of soybean	*R. solani*	*B. amyloliquefaciens*
4	Rice	Rice blast	*Magnaporthe grisea*	*B. amyloliquefaciens*
5	Rice	Rice blast	*M. grisea*	*Bacillus Licheniformis*
6	Grapes	*Esca* disease and diseases of grapevine	*Phaeoacremonium aleophilum*	*Bacillus Licheniformis*
7	Tomatoes	*Fusarium* rot of tomatoes	*Fusarium oxysporum radices*	*Bacillus coagulans*

Table 3: *Antifungal inhibition result.*

Sr. No	Isolate	*Aspergillus Niger*
1	**MDL3 (1)**	+ve
2	**MDL3 (2)**	-ve
3	**GMR4 (W1)**	+ve
4	**GMR4 (W2)**	-ve
5	**GMR4 (Y1)**	-ve
6	**GMR4(Y2)**	+ve
7	**MCR2(Y1)**	+ve
8	**MCR2**	-ve
9	**MSR1-2**	+ve
10	**MBM13**	+ve
11	**MCR2 (Y2)**	++ve
12	**MBM24**	++ve
13	**MSR1-1**	-ve
14	**MBM24-1**	-ve
15	**MBM24-2**	-ve

Antifungal assay on chitin producing fungal species

Antifungal assay performed on *Penicillium Aspergillus Niger* to check the fungal growth inhibition ability of isolates. Isolates showed different fungal growth inhibition abilities with different fungal strains. For the isolation of chitinolytic bacteria, the soil samples were collected from different areas in Pakistan. The samples were collected in sterile falcon tubes aseptically. The collected samples were labelled appropriately with the date and place of collection and transported to the lab in sterile plastic bags. The collected soil samples are processed to determine initial microbial flora and microbial count in each of the collected sample.

Conclusion

Soil borne pathogens like fungi pose immense threat to various crop plants. Each year a large proportion of crop yield is lost due to fungal attack on crops like rust and smut disease of wheat and corn. To protect these crops from being diseased by fungi many synthetic chemical fungicides are used. But as the result of excessive use of these harmful chemicals the fertility of soil is compromised moreover, the plants are also harmed. To avoid the excessive use of the synthetic pesticides and fungicides the alternative approach is the biological control of these pathogens [33]. The bacterial species isolated from soil that may be upper soil or rhizosphere, shows high ability to produce chitinase and hence potential to be used as BCAs to manage and prevent fungal crop diseases and crop loss. The property of the isolates to use chitin as a sole carbon source can be used in nature to manage and decompose chitinous waste such as shrimp or crab waste, lowering the cost of their disposal. Moreover, colloidal chitin serves as a cheap carbon source making the industrial application and manipulation of chitin degrading bacteria economical. By the virtue of in vitro analysis, it was found that the inhibitory effect of fungal growth of these isolates is attributed to the production of chitinase. Both the pure and crude enzyme can be produced on industrial scale and can used globally as bioweapon against different phytopathogens and human fungal pathogens. These chitin degrading bacteria can not only be employed for agricultural purposes but also in medicinal industry for the production of antifungal creams and ointments. Other than its property to be used as antifungal agent, chitinase enzyme can also be used to control insects and pests such as mosquitos that has developed resistance to widely used chemical sprays and other formulations.

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