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An Efficient Method of Production of Colloidal Chitin for Enumeration of Chitinase Producing Bacteria

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Abstract

Chitin is a long-chain polymer of an N-acetyl glucosamine, a derivative of glucose, and is one of the most abundant natural polymer which has proved versatile for several medical, industrial and biotechnological purposes. Chitinase enzymes have received wide spread attention for its biotechnological applications mainly in the field of agriculture as a bio control agent against fungi and certain insects. An efficient way of obtaining colloidal chitin and the enumeration of chitinase producing bacteria with fungicidal properties were the main objectives of the current study. For this purpose two different species of fungi, *Alternaria sp.* and *Fusarium sp*. were isolated from infected vegetables. Chitinase can be used as a potential alternative to chemical fungicides. The current study elucidates an effective method of preparation of colloidal chitin to enumerate the chitanase producing bacteria.

Keywords: Colloidal chitin, Fungal cellwall, Chitinase

1. Introduction

Chitin is a long-chain polymer of anN-acetyl glucosamine, a derivative of glucose, and is found throughout the natural world

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and is the second most abundant natural polymer after cellulose. It is a characteristic component of the cell wall of many fungi, exoskeletons of arthropods such as crustaceans (e.g., crabs, lobsters and shrimps) and insects, the radulae of molluscs, and other soft tissues of fishes. In its pure, unmodified form, chitin is translucent, resilient, and quite tough. Combined with calcium carbonate, as in the shells of crustaceans and molluscs, chitin produces a stronger composite. Chitinases are hydrolytic enzymes that break down glycosidic bonds in chitin. As chitin is a component of the cell walls of fungi and exoskeleton elements of some animals (including worms and arthropods), Chitinases are generally found in organisms that either need to reshape their own chitin or dissolve and digest the chitin of fungi or animals [1].

One of the major issues threatening the existence of life on earth is population explosion and environmental pollution. For the production of more food, yield of crop plants should be more. Most of the plant diseases are controlled using large quantities of chemical fungicides. Fungicides are essential in agricultural production, but they pose a serious risk to humans who are exposed to them directly through various ways and indirectly through diet. Chemical fertilizers can cause several negative outcomes such as neurological problems, birth defects, foetal death, and neuro developmental disorder [2]. This paper discusses an efficient way of obtaining colloidal chitin from commercially obtained chitin and isolating and enumerating strains of Chitinase producing bacteria in chitin media. These strains were used to produce Chitinase enzyme whose fungicidal activity were investigated. This could be used as an efficient fungicide and a suitable alternative for chemical fungicides as it is environmental friendly.

2. Materials and Methods

2.1 Preparation of moist colloidal chitin

Chitin is not readily water soluble, chitin is often chemically modified to form colloidal chitin, with a small particle size that is more readily manipulated to obtain homogenous distribution in agar media, compared to use of physically modified, finely ground chitin that can be difficult to obtain. 20g of chitin powder was

measured and taken in a 1000ml beaker. It was then treated with 150ml of 12M HCl by the slow addition of HCl along the sides and was then stirred every 5min for 60min. The chitin – HCl mixture was filtered through eight layers of cheese cloth and the filtrate obtained was treated with 2 litres of ice cold distilled water to allow precipitation of colloidal chitin. After overnight incubation, the filtrate was then passed through three layers of Whatmann filter paper kept in a Buchner funnel seated in a vacuum filtration flask. Around three litres of tap water was passed through the colloidal chitin using this assembly to increase the pH and this is continued till pH 7 is attained. The colloidal chitin obtained was autoclaved and was stored 4°C until further use. [3]

2.2 Collection of Soil Samples and preparation of Chitin Media

The soil samples for the isolation of chitin utilizing bacteria was collected from Dharmaram, Christ University, Bangalore. 10g of soil was collected from the side of the pond and 1g of this soil was taken from this and was serially diluted by mixing it in 10ml water and then making it a 10^{-1} mixture by taking 1ml from it and pouring it in 9ml. Similarly, it was serially diluted up to 10^{-3} and this was used for spread plating in the chitin media for obtaining chitinase producing bacteria.

2.3 Preparation of Chitin Media

For the isolation of chitin utilizing bacteria, a solid agar medium of chitin was used which provided the bacteria with chitin as the primary carbon source. Since chitin is a complex polymer other carbon sources along with the chitin media was avoided so that the bacteria won't depend primarily on the other carbon source. Thus a media was prepared supplemented with chitin [4]. The media composed of moist Colloidal chitin, K_2HPO_4, KH_2PO_4, NaCl, Agar. This media was spread plated with serially diluted samples of soil from pond in Dharmaram, Christ University, Bangalore. The culture was observed after three days of incubation.

2.4 Collection of samples for the isolation of fungal culture

The pure cultures of fungi for investigating the fungicidal activities of chitinase were isolated from fungal infected beans which were handpicked from vegetable shops. They were wrapped in wet

newspaper and was covered in a plastic cover and was incubated for 2 – 3 days for further infection. These infected beans were used for imprinting in MRBA media and in PDA media for obtaining fungal cultures. The fungal cultures obtained after an incubation of 3 days were stained using Lacto phenol cotton blue and was observed under the microscope for fungal spores and mycelium morphology.

2.5 Preparation of Chitinase production broth

The media for the production of chitinase from chitinolytic bacteria consisted of Colloidal chitin, K_2HPO_4, KH_2PO_4 and NaCl. This broth was autoclaved and was inoculated with chitin utilizing bacteria. This mixture was incubated for 3 days after which, the broth was centrifuged. The supernatant obtained was precipitated using ammonium sulphate. The precipitate obtained after centrifugation was dissolved in sodium phosphate buffer and stored. [5]

3. Results and Discussion

3.1 Isolation of Chitin Utilizing bacteria from the soil

The chitin media plates after the incubation for 3 days gave several colonies. The bacteria formed pale white colonies, which were smooth in appearance and showed clearing zones around the colonies [Fig. 1 & Fig. 2]. This is due to the fact that in these bacteria the enzyme chitinase is extracellular or secretory. Bacteria is one of the best chitin utilizers in soil. In soil systems, chitin hydrolysis rates has shown to be proportional to bacterial abundance, but depending on temperature, pH, or the successional stage of the degradation process, even fungi may be quantitatively important agents of chitin degradation. Chitin, naturally found in nature may vary in the degree of deacetylation and therefore form distinctions such as chitosan, which is the completely deacetylated form of the polymer. The process by which chitin is cleaved and degraded are called chitinoclastic process. Growth on chitin is not necessarily accompanied by the direct dissolution of its polymeric structure. Alternatively, chitin can be deacetylated to chitosan or possibly even cellulose-like forms, if it is further subjected to deamination [6]. These colonies which showed clearing zones were sub cultured

and preserved. There is clearly a need for an updated account of the diverse mechanisms involved in chitinolysis and the ecological consequences of this process for bacteria. A focus on bacteria rather than all other organisms involved in chitin degradation is warranted since bacterial chitin degradation takes place in all major ecosystems. Also, their metabolism and growth have such a central role in most ecosystem-scale biogeochemical cycles. In light of recent developments in molecular methods, a particular emphasis should be on the molecular level understanding of the process of chitinolysis.

3.2 Isolation and Purification of fungal colonies

The colony no. 1 observed was found to be *Alternaria sp*. based on the spore morphology. [Fig. 3] Alternaria is a genus of ascomycete fungi. *Alternaria sp*. are known as major plant pathogens. There are 299 species in the genus; they are ubiquitous in the environment and are a natural part of fungal flora almost everywhere. The spores are airborne and found in the soil and water, as well as indoors and on objects. The club-shaped spores are single or form long chains [Fig. 5]. They can grow thick colonies which are usually green, black, or grey. At least 20% of agricultural spoilage is caused by *Alternaria sp*.; most severe losses may reach up to 80% of yield, though. *Alternaria sp.* fungus is usually transmitted through seeds (seed borne). The fungi colonize the seed coat during the seed development stage and when the seed germinates, they become active. The fungal disease causes damping-off and stunted seedling. Infection usually develops slowly. The leaf spots vary in sizes from very tiny spots up to 5 cm in diameter. The spots begin as small yellow or brown spots that slowly enlarge to about 5 cm in diameter, dark colored spots with concentric rings. When all the spots join together, the leaves will turn yellowish or blackish and drop off. Disease-free seed and crop rotation is usually practiced to prevent Alternaria infection [7].

The second colony obtained was found to be Fusarium sp. [Fig. 4] Based on fungal spore morphology [Fig. 6]. Fusarium is a large genus of filamentous fungi, part of a group often referred to as hyphomycetes, widely distributed in soil and associated with

plants. Some species produce mycotoxins in cereal crops that can affect human and animal health if they enter the food chain [8].

Fusarium wilt is a common vascular wilt fungal disease, exhibiting symptoms similar to Verticillium wilt. The pathogen that causes Fusarium wilt is *Fusarium oxysporum* which generally produces symptoms such as wilting, chlorosis, necrosis, premature leaf drop, browning of the vascular system, stunting, and damping-off. The most important of these is vascular wilt. Fusarium wilt starts out looking like vein clearing on the younger leaves and drooping of the older lower leaves, followed by stunting of the plant, yellowing of the lower leaves, defoliation, marginal necrosis and death of the plant [9].

3.3 Production of chitinase enzyme

After the incubation of 3 days the bacterial broth was subjected to centrifugation and the supernatant was mixed with ammonium sulphate for the precipitation for chitinase protein. No precipitate was obtained during this procedure. This was assumed to be because of the unfavourable temperature of the shaker incubator as the preferred temperature was suggested to be 30°C [10]. The possibility of the bacteria being non-viable also persists as the culture was stored in deviating low temperatures in the fridge. The colloidal chitin used for the isolation of bacteria was different from that of the one used for the production of chitinase enzyme. This might also be the reason for the lack of chitinase in the supernatant as pH of the second batch of colloidal chitin was slightly lower than that of the first batch. Also metal ions play a very important role in the production of chitinase and for the various characteristics of the enzyme. The media was not supplemented with any salts and didn't have crucial metals such as Manganese and Cobalt which helps in chitinase stability.

Chemical Fungicides are toxic, they are also potentially hazardous to humans, animals, other organisms, and the environment. Therefore, people who use pesticides or regularly come in contact with them must understand the relative toxicity, potential health effects, and preventative measures to reduce exposure to the products they use. The symptoms of pesticide poisoning can range from a mild skin irritation to coma or even death.

4. Conclusion

This work was primarily aimed at the identification of a suitable, simple and cost efficient method for the preparation of colloidal chitin and an attempt to prepare chitinase enzyme from chitin utilizing bacteria isolated and enumerated from the soil. Fungal cultures were isolated and purified from infected beans for checking the extent of anti-fungal activity of chitinase. In biological control of fungal phytopathogens, application of agents containing various metabolites of microorganisms, including CHIs, appears to be the most efficient, since they show stronger fungicidal activity than purified chitinolytic enzymes. The use of agents constituting a consortium of chitinolytic microorganisms seems to bring better results in fighting fungal phytopathogens. The current study identified optimum conditions for the production of colloidal chitin for testing for bacterial chitinase activity. This could be used to check the chitin degradation efficiency of chitinases produced by different bacterial species and strains. Further work is planned on quantifying the enzyme activity of these bacterial chitinases on plant pathogenic fungal cellwall degradation. If optimized, these could serve as potential weapons against plant pathogenic fungi posing a threat to economically important crop plants.

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Appendix

Figure 1 Chitin utilizing bacterial colony – A

Figure 2 Chitin utilizing bacterial colony – B

Figure 3 *Alternaria spp*.

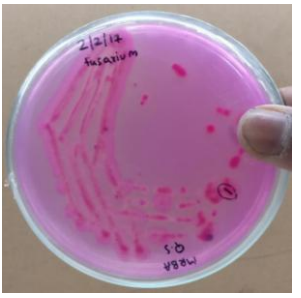

Figure 4 *Fusarium spp*.

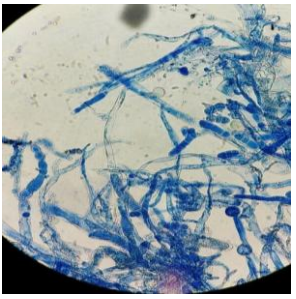

Figure 5 Spore Morphology of *Alternaria sp*.

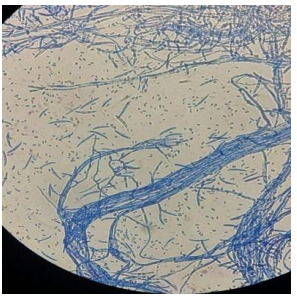

Figure 6 Spore morphology of *Fusarium sp*.

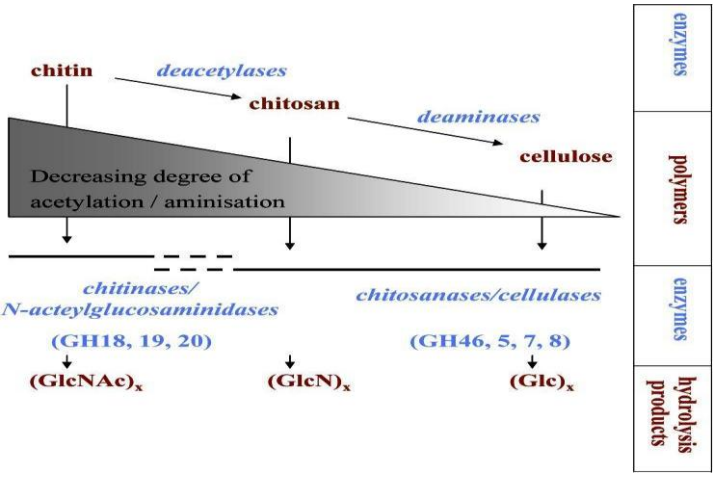

Figure7 Graph showing chitinolytic activity