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ORIGINAL RESEARCH ARTICLE

Optimization and Purification of Chitinase Produced by *Bacillus subtilis* and Its Antifungal Activity against Plant Pathogens

S.Kavi Karunya*, D.Reetha, P.Saranraj and D. John Milton

Department of Microbiology,Annamalai University,Chidambaram – 608 002, India

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ABSTRACT

Microorganisms, which secret a complex of mycolytic enzymes are considered to be possible biological control agents of plant diseases. Since Chitinases are digestive enzymes that break down glycosidic bonds in chitin. In this present study, an industrial enzyme chitinase produced by *Bacillus subtilis* was optimized, purified and its antifungal activity was investigated against plant pathogens. The bacteria *Bacillus subtilis* was isolated from soil sample and it was identified by staining techniques, plating on selective media and biochemical characteristics. The *Bacillus subtilis* showed highest chitinolytic activity in Colloidal chitin agars that degrade chitin in 0.6 mm zone of clearance. The production of chitinase by *Bacillus subtilis* was optimized under different media, substrates, substrate concentrations, pH, temperature and incubation period. The maximum chitinase production was observed in Luria Bertaini Broth amended with 0.3% colloidal chitin at pH 7.0 and temperature 35˚C after four days of incubation. The enzyme was partially purified by Dialysis method. Protein concentration of 200µg/ml was estimated according to Lowry's method. The Chitinase has antifungal activity against plant pathogens *viz*, *Aspergillus niger*, *Aspergillus flavus* and *Penicillium chrysogenum.*

Key words: Chitinase, Chitin, *Bacillus subtilis*, Fungal plant pathogens and Antifungal activity.

1. INTRODUCTION

Enzyme technology is an interdisciplinary field, and enzymes are routinely used in many environmental-friendly industrial sectors. With the advancement in biotechnology especially in the area of genetics, protein engineering, developments in bioinformatics, and the availability of sequence data have opened a new era of enzyme applications in many industrial processes [1]. Chitinase, a group of enzymes capable of degrading chitin directly to low molecular weight product [2] and found in a broad range of organisms, including bacteria (*Bacillus, Aeromonas, Vibrio, Pseudomonas, Serratia, Enterobacter, Actinomycete*), fungi (*Trichoderma* and *Aspergillus*), and higher plants, insects, crustaceans, invertebrates and some vertebrates. The roles of these chitinases may be divided into several categories; for example, a major role of fungi, crustaceans and insects is modification of the organisms structural constituent chitin [3, 4, 5].

Chitin $(C_8H_{13}O_5N)n$, the (1-4)-β-linked homopolymer of N-acetyl-D-glucosamine, is produced in enormous amounts in the biosphere. It is the most abundant biopolymer next to cellulose with an annual production of 10^{10} to 10^{11} tons per annum. Shrimp and crab shell, are waste products from canning industries. They are the best sources of chitin. Cell walls of many microorganisms and plants are made of chitin cross-linked with other biopolymers. Chitin binds easily with other materials and can be cast into sheets or films. Chitin and most of its derivatives have a strong positive charge on them. This property has been exploited for many uses. Chitin and its derivatives are biodegradable and biocompatible to humans and most of the animals. For these reasons, there are lot of emphasis on studies on possible uses of Chitin and its derivatives in recent years [6].

The production of chitinase by plants has been suggested to be a part of their defense mechanism against fungal pathogens [7]. Chitinases have received increased attention due to their wide range of biotechnological applications, especially in the biocontrol of fungal phytopathogens [8]. Chitinase have been implicated in plant resistance against fungal pathogens because of their inducible nature and antifungal activities *in vitro*. Chitinase in fungi is thought to have autolytic,

***Corresponding Author:** S.Kavi Karunya**, Email:** kavi.vit@gmail.com, **Phone No:** +91- 9629144780

nutritional and morphogenetic roles. In viruses, chitinases are involved in pathogenesis [1]. Due to multiple applications of chitinases in biocontrol, waste management, medicine and biotechnology they become interesting enzymes for study [9]. Chitinase as a bio-control agent against insect pest, *Monochamus alternatus*, a pine sawyer beetle which is one of the important factors causing pine tree destruction in Japan. Chitinase (50µ liter, 0.3 to 3µM) were individually administrated orally into the digestive tube of the pine sawyer beetle and most of the beetles died in 20 hrs. thus chitinase have potential as a pest-control agent [10].

Other applications of chitinases are bioconversions of chitin waste to single cell proteins and ethanol and fertilizers. Industrial applications of chitinases have been governed mainly by key factors such as cost production, shelf-life stabilities and improvement in enzyme properties by immobilization [11]. Chitinase producing marine bacteria play an important role in the degradation on chitin in the oceans [12]. Various species of *Bacillus* have been shown to secrete chitinase, including *Bacillus circulans, Bacillus licheniformis, Bacillus laterosporus, Bacillus amyloliquefaciens, Bacillus magaterium, Bacillus pabuli, Bacillus stearothermophilus, Bacillus subtilis, Bacillus thuringiensis* [13]. In this present study, an industrial enzyme chitinase produced by *Bacillus subtilis* was optimized, purified and its antifungal activity was investigated against plant pathogens.

2. MATERIALS AND METHODS

2.1 Sample collection

Soil sample was collected from the agricultural field in different area. The soil samples are taken from 2 to 3 cm depth with the help of sterile spatula and put in sterile plastic bags and it mouth was tied properly and brought to laboratory for further processing.

2.2. Isolation and identification of *Bacillus subtilis*

Pour plate technique was used for the isolation of *Bacillus subtilis*. Well grown bacterial colonies were picked and further purified by streaking. The isolated strains were maintained on Nutrient agar slants and stored at 4°C. Identification of the bacterial isolates was carried out by the routine bacteriological methods *i.e*., By the colony morphology, preliminary tests like Gram staining, endospore staining, motility, catalase and oxidase, plating on selective medias and performing biochemical tests.

2.3. Screening method

2.3.1 Preparation of colloidal chitin

Colloidal chitin was prepared from chitin flakes. The chitin flakes were ground to powder. Five gram of chitin powder was added slowly into 90 ml of concentrated HCl under vigorous stirring and the mixture was added to 500 ml of ice cold ethanol with rapid stirring and kept overnight at 25˚C and stored at -20˚C until use when needed, the precipitate was collected by centrifugation at 6000 rpm for 10 min at 4˚C and washed with sodium phosphate buffer until the colloidal chitin became neutral pH 7 and used for further applications.

2.3.2 Screening for chitin degradation

The bacterial isolates were grown in Nutrient broth containing 1% colloidal chitin and it was incubated at 100 rpm in a rotary shaker at room temperature for 2 days. After 2 days of incubation the culture were harvested, centrifuged at 10000 rpm for 15 min at 4˚C and supernatant was collected. Colloidal chitin agar (CCA) plates were prepared and a plain disc is dipped in the Culture filtrate of isolate was placed in the agar and incubated at 37˚C. After 12 hrs, the development of clear zone around the disc was observed.

2.4. Optimization of culture condition

2.4.1. Effect of Medium on chitinase production

Two different medium namely Nutrient broth and Luria Bertaini broth amended with 0.1% colloidal chitin was sterilized. The *Bacillus subtilis* culture was inoculated and incubated at 37˚C for 24 hours. The chitinase production was observed in spectrophotometer at 540 nm.

2.4.2. Effect of substrates on chitinase production

The Luria bertaini broth was prepared and *Bacillus subtilis* culture was inoculated in the broth which contains 0.2% of different substrate like crab shell waste, chitin flake, colloidal chitin. The series of tubes were incubated at 37˚C for 24 hours. The chitinase production was observed in spectrophotometer at 540 nm.

2.4.3. Effect of substrate concentration on chitinase production

The *Bacillus subtilis* was grown in Luria bertaini broth with different concentrations (0.2%, 0.3%, 0.4%, 0.5%) of colloidal chitin and incubated at 37˚C for 24 hours. The chitinase production was observed in spectrophotometer at 540 nm.

2.4.4. Effect of pH on chitinase production

The *Bacillus subtilis* was inoculated in Luria bertaini broth at different pH ranges from pH 4 to pH 10, and incubated at 37˚C for 24 hours. The

chitinase production was observed in spectrophotometer at 540 nm.

2.4.5. Effect of temperature on chitinase production

The *Bacillus subtilis* was inoculated in Luria bertaini broth at different temperature ranges from 25˚C to 45˚C, and incubated for 24 hours. The chitinase production was observed in spectrophotometer at 540 nm.

2.4.6. Effect of incubation time on chitinase production

The bacterial culture was grown in Luria bertaini broth (LBB) with different incubation period ranges from 1 to 7 days. The chitinase production was observed in spectrophotometer at 540 nm.

2.5. Partial purification by Dialysis

The culture supernatant fluid was subjected to precipitation with ammonium sulphate to 80 % saturation and stirred for 2 hours. Then the precipitate was allowed to stand for overnight and then collect by centrifugation at 10000 rpm at 4˚C for 30 min. The precipitated pellets were dissolved in phosphate buffer and centrifuged and dialyzed against the same buffer which was then partially purified.

2.6. Estimation of Protein content

The protein concentration was estimated by the following the method of Lowry *et al*. (1951)[14] using Bovine serum albumin as the standard.

2.7. Determination of Antifungal activity of chitinase

2.7.1. Test microorganisms

Three plant pathogenic fungi, *viz*., *Aspergillus flavus, Aspergillus niger* and *Penicillium chrysogenum* obtained from Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Chidambaram. The cultures were sub-cultured and maintained on Sabouraud's dextrose agar slants and stored in refrigerator at 4°C.

2.7.2. Inoculum preparation

Fungal inoculum was prepared by inoculating a loopful of test organisms in 5 ml of Sabouraud's dextrose broth and incubated at room temperature for 3 days.

2.7.3. Antifungal activity

The antifungal activity was assayed *in vitro* by inhibiting the growth of fungus on Sabouraud's dextrose (SDA) agar medium. SDA agar medium were prepared and sterilized and poured into the Petri plates. After solidification, the fungal colonies were spread along the plates and Chitinase discs were placed on the SDA agar medium and incubated at room temperature. After three days of incubation, the diameter of the zone of inhibition was measured and expressed in mm.

3. RESULTS AND DISCUSSION

Chitin, a structural component of fungal cell wall and plants. They are commercially obtained from shrimp and crab shell waste from the fishing industry. In Recent advances, understanding the structure and properties of chitin and its derivatives has opened a lot of new avenues for its applications. The properties of chitin are improved in particular application and it can be easily brought about by chemical modifications. The applicability of chitin in many areas and its easy manipulation has resulted in a considerable amount of research being done on the possible applications of chitinase [6].

The bacteria used in this study, *Bacillus subtilis* was isolated from agricultural soil sample in Vellore, isolated colonies of the bacteria are got after serial dilution followed by Spread plate technique. Morphological characteristic of bacterial isolate was studied by Gram staining technique, Motility (Hanging drop method) and Spore staining. The bacterium was isolated as Gram-positive, rod shaped, motile and spore forming organism which grow in the range of 37˚C to 60˚C and hydrolyzed starch, gelatin and chitin. The isolate was biochemically characterized and found to be positive for Voges Proskauer test, Citrate utilization test, Catalase test, Urease, Nitrate reduction test, Glucose, Sucrose, Maltose, Mannitol and the negative test for Indole test, Methyl red test, Oxidase test, H_2S production and Lactose fermentation. Based on morphological, microscopical and biochemical characterization, the bacterium was identified as *Bacillus subtilis*. The bacterial isolate obtained from agricultural field was hydrolyzed the colloidal chitin and produced a prominent and maximum clear zone in Colloidal Chitin agar plates.

From the Korean traditional food, a chitinase producing *Bacillus* sp. was isolated and was identified on the basis of phylogenetic ananysis of the 16S rDNA sequence, gyrA gene and phenotypic analysis. The amino acid sequence of the chitosanase from *Bacillus* sp. exhibits 88 and 30% similarity to those from *Bacillus subtilis* and *Pseudomonas* sp. The chitosanase was purified by glutathione S-transferase fusion purification system and its molecular weight will be 27 kDa. The pH and temperature optima of the enzyme were 7.5 and 50˚C, respectively [15].

Optimization of cultural condition for the production of chitinase was carried out using two different media such as Nutrient broth and Luria bertaini broth with 0.3% colloidal chitin as substrate, among these media, Luria bertaini broth supports highest chitinase production of 28 units/ml as compared to Nutrient broth (**Fig 1**). The *Bacillus subtilis* was grown at different substrates like crab shell waste, chitin flake, and colloidal chitin. Among these substrates, colloidal chitin showed highest chitinase production when compared to crab shell waste and chitin flake (**Fig 2**). The *Bacillus subtilis* was grown at different concentrations (0.2 to 0.5 %) of colloidal chitin amended with Luria bertaini broth to determine the optimum concentration of substrate for chitinase production. Among this, 0.3 % recorded highest chitinase production (**Fig 3**).

The effect of pH, temperature and incubation period of chitinase production was optimized using Luria bertaini broth. The production of chitinase was evaluated on pH 4 to pH 10. Among this pH 7 and pH 8 shows maximum chitinase production when compared to pH 4, pH 5, pH 6, pH 9and pH 10 respectively (**Fig 4**). The chitinase production was studied using the different temperature ranges from 25˚C, 30˚C, 35˚C, 40˚C and 45˚C. Among the various temperatures, 35˚C is suitable for maximum chitinase production where as the temperature 45˚C the chitinase production observed slightly (**Fig 5**). The chitinase production was evaluated in different incubation time period of 1 to 7 days. Among which the days 4th and 5th were observed the highest chitinase production (**Fig 6**). The amount of protein present in the culture precipitate was estimated and the culture was partially purified by Dialysis method. The concentration of protein was estimated by Lowry's method using bovine serum albumin as substrate and the protein concentration was 200 µg/ml

The antifungal activity against phytopathogenic fungi is exhibited from the five bacterial strains SGE2, SGE4, SSL3, MG1 and MG3, isolated from soil and sediment samples. Analysis of the 16S rRNA genes and the substrate spectra revealed that the isolates belong to the genera *Bacillus* or *Streptomyces*. The closest relatives were *Bacillus chitinolyticus* (SGE2, SGE4 and SSL3), *Bacillus ehimensis* (MG1), and *Streptomyces griseus* (MG3). The chitinase present in the culture supernatants of the five isolates revealed optimal activity between 45˚C and 50˚C and at pH values of 4 (SSL3), 5 (SGE2 and MG1), 6 (SGE4), and 5-7 (MG3). The crude chitinase preparations of all strains possessed antifungal activity [16].

In this present study, the antifungal activity of Chitinase was tested against the three different plant pathogenic fungi *viz*., *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum.* Among the three fungal pathogens tested, the chitinase showed maximum inhibitory activity against *Aspergillus niger* (7mm in dm). Next to *Aspergillus niger*, the chitinase showed maximum zone of inhibition against *Aspergillus flavus* (5mm in dm). The fungi *Penicillium chrysogenum* (4mm in dm) recorded least inhibitory activity (**Fig-7**).

A chitinolytic bacterium isolated from lily plant in Taiwan. This bacterium i.e., *Bacillus cereus* exhibited biocontrol potential on Botrytis leaf blight of lily as demonstrated by a detached leaf assay and dual culture assay. Two chitinases (ChiCW and ChiCh) were excreted from *Bacillus cereus*. The ChiCw-encoding gene was cloned and moderately expressed in *Escherichia coli*. Near homogenous ChiCW was obtained from the periplasmic fraction of *Escherichia coli* cells harboring ChiCw by a purification procedure. An *in vitro* assay showed that the purified ChiCW had inhibitory activity on conidial germination of *Botrytis elliptica*, a major fungal pathogen of lily leaf blight [3].

Fig 1: Optimization of cultural condition

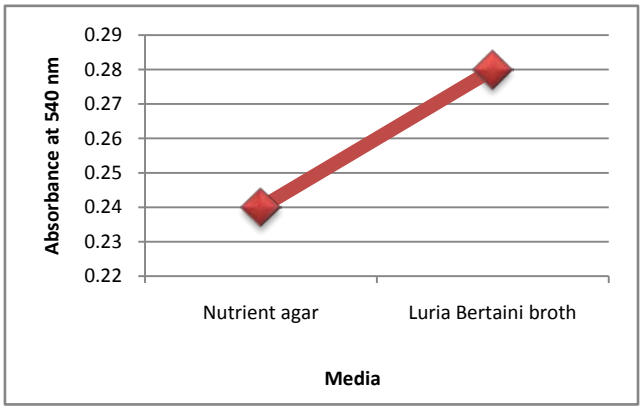


Fig 2: Optimization of substrates

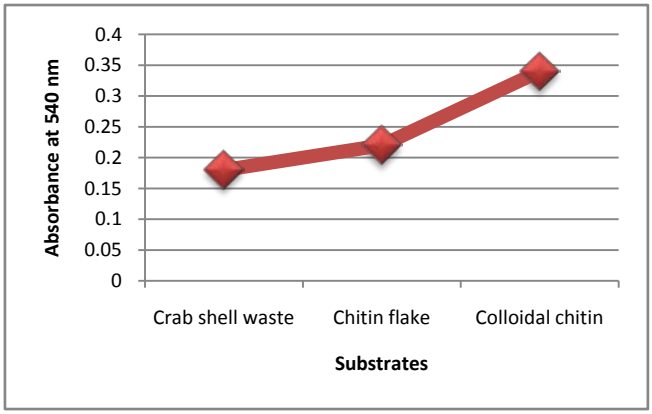


Fig 3: Optimization of substrate concentration

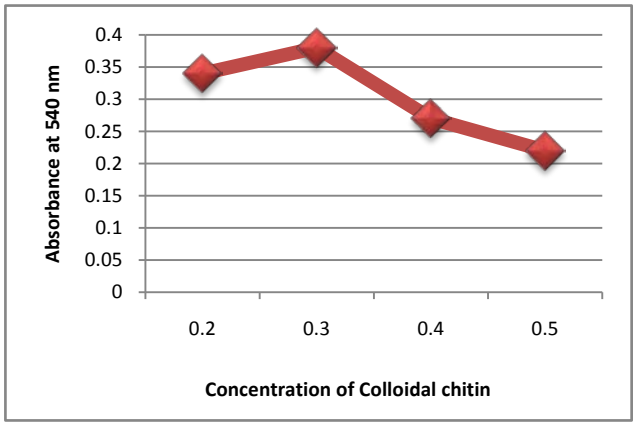


Fig 4: Optimization of pH

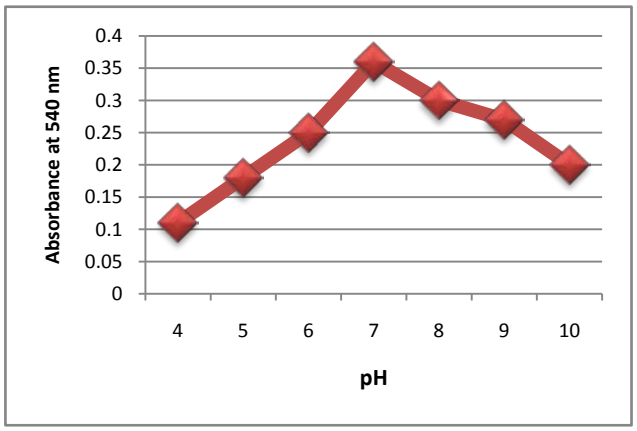


Fig 5: Optimization of temperature

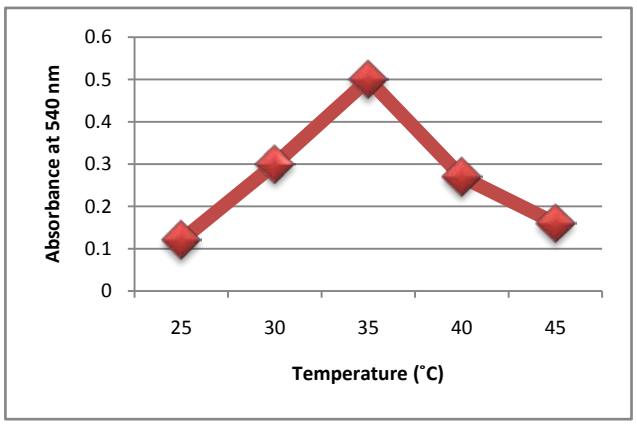


Fig 6: Optimization of incubation period

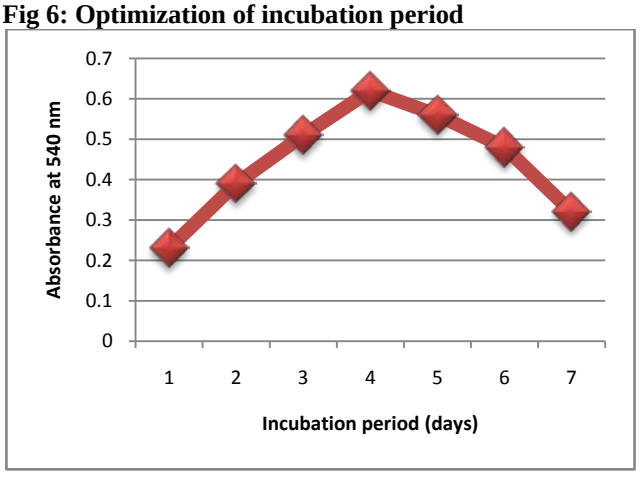
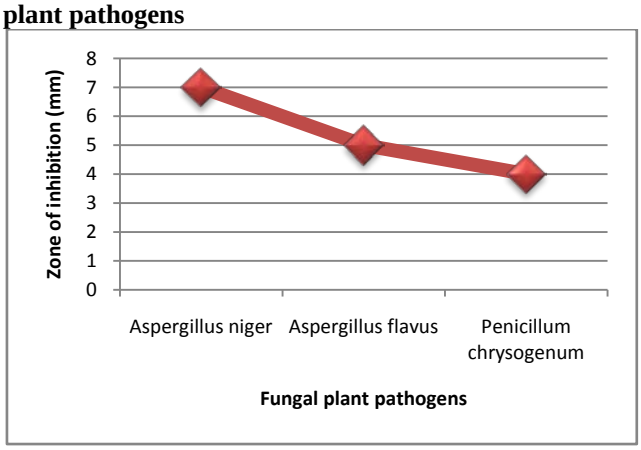


Fig 7: Antifungal activity of Chitinase against Fungal plant pathogens



4. CONCLUSION

Although chitinase have been isolated and characterized from a wide variety of sources, it is still important to screen for new sources for production of chitinase with more economical values and enhanced properties at expand their usefulness. Microorganisms, which secret a complex of mycolytic enzymes are considered to be possible biological control agents of plant diseases. Since Chitinases are digestive enzymes that break down glycosidic bonds in chitin. Because chitin composes the cell walls of fungi and exoskeletal elements of some animals (including worms and arthropods), chitinases are generally found in organisms that either need to reshape their own chitin or to dissolve and digest the chitin of fungi or animals. The substrate of chitinase is chitin, which is a common component of fungal cell walls. These enzymes are strong inhibitors of many important plant pathogens and these chitinases are able to lyse the hard chitin cell wall of the mature hyphae, conidia, chlamydospores and sclerotia.

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