

OPTIMIZATION OF CHITINASE ENZYME FROM THE MARINE RHIZOSPHERE SOIL

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Abstract—Chitinase enzyme has diverse applications in the field of biotechnology, due to high attention especially in the field of agriculture for bio control of phytopathogenic fungi and harmful insects. In the present study, bacteria were isolated from marine rhizosphere soil. Bacterial isolates were screened for chitinolytic activity and isolates were selected for Chitinase production in broth media. Most powerful isolates were recognized as *Aeromonas hydrophila* and were selected for additional study, based on enzyme production. Studies were conducted on the effects of media composition and various fermentation conditions for optimization of chitinase production. At 37 °C and pH 8.0 after 24-48 h of incubation by *Aeromonas hydrophila*, maximum production of chitinase is obtained. Among the substrates, colloidal chitin was the best for the strain. Regarding carbon sources, maltose was the best for isolated strain; while meat was found as the best nitrogen source for *Aeromonas hydrophila*. Among surfactants, SDS was the best which shows major enzymatic activity.

INTRODUCTION

Chitin ((1,4)-2-acetamide-2-deoxy-beta-D-glucan) is a polysaccharide created by beta, 1-4 bonding of N-acetyl glucosamine residue. Chitin depends on molecular weight and chain length for their functional properties and physiological activities (Singh, 2010). Chitin is basic, almost all polysaccharides that are either acidic or neutral. Chitin is the main component of insect cuticles that protect intestines of insects. Intestines which are infected by pathogenic bacteria are required to pass this chitin-rich barrier first (Anuradha and Revathi, 2013). Chitin is present in insects, in tracheal tubes, on their upper skin and muscles along with protein. Chitin is hydrolyzed by two types of enzymes, namely Chitinase and beta-N-acetyl hexosaminidase (Mahadevan, 1997).

Bacterial Chitinase enzymes are involved in breakdown of micelles of fungal pathogens, in protection against parasites in fungi, protozoa and invertebrates. Chitinase enzyme is involved in the

plants defense mechanisms and vertebrates. Chitinase enzyme is produced by plants to defend themselves against fungal pathogens. For pathogenicity chitinase enzyme is produced by baculoviruses, which are used in bio control of pests (Bartholomew *et al.*, 1952).

Chitinase enzyme helps to produce chito oligosaccharides. Chito oligosaccharides are the degraded products of chitin, produced in the plant defense mechanism and has a significant use in the field of medicine having antioxidant and anti-inflammatory properties. The chemical chitin structure has similarity to the structure of cellulose. The C₂ position of chitin contain acetylamino group instead of hydroxyl group (Parameswaran, 2005). Cell wall of fungi consists of chitin that is present as an exoskeletons of insect arthropods, and molluscs in different form (Bryn *et al.*, 1973).

Chitinase enzyme break down chitin that catalyses the breakdown of β-1, 4 bonds in N-acetyl-β-D-glucosamine in chitin (Sandhya, 2005). Chitinase contribute's nitrogen and carbon in the

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environment. Chitinases are found in most organisms like fungi or other organisms. Chitinase is present in many bacteria such as *Streptomyces*, *Bacillus*, *Aeromonas*, *Vibrio*, *Pseudomonas* etc. and in fungi.

Chitinase is classified into endo Chitinase and exo Chitinase. Endo Chitinase acts on the internal site and does not produce N-Acetyl glucosamine, dimer di-cetylchitobiose and chitotetraose. Exo Chitinase does not produce the N-acetyl glucosamine and it is divided into chitobiosidases and 1-4- β -glucosaminidase. Chitobiosine catalyse the release of di acetylchitobios from non-reducing microfibril end of chitin (Priya, 2011). Based on the amino acid sequence chitinase is classified into three groups 18, 19 and 20 that consist of endo-chitinase from different organism include micro-organisms, insect and plants members of group 18 are sensitive to allosamidine but the members of group are insensitive. The amino acid sequence of these three groups doesn't have any similarities. It can also be classified on the basis of N-terminal sequence, isoelectric pH, signal peptide and inducers.

METHODOLOGY

Collection of sample: The soil sample was collected from fish harbour, Kollam.

Substrate Preparation: 1g soil sample was collected, weighed and kept in 99ml distilled water and incubate it overnight at room temperature for the growth of microorganisms

Preparation of colloidal chitin: Colloidal chitin was made from commercially available chitin powder based on the method by Arnold and Solomon (1986). 20 g of chitin powder was mixed with 400 ml of concentrated HCl, then kept overnight in cool room. Then the pH of the resulting suspension was neutralised by adding 10N NaOH. After keeping the suspension overnight in the refrigerator, it was centrifuged (5000 rpm for 30 min) and desalting was done that was used as substrates for chitinase production.

SCREENING: Different types of organisms were isolated from the soil by serial dilution method and they were screened for the production of Chitinase by plate assay method. To the sterile production media organisms were inoculated. After incubation period, isolated colonies were streak plated. From the pure culture the organism is identified by microscopic and biochemical tests.

(A) Primary Screening: Chitin degrading bacteria were isolated by serial dilution of soil sample and plated on 0.5% colloidal chitin agar (CCA) medium. After 24h of incubation at 37°C the isolates capable of degrading chitin on CCA were selected and streaked in a nutrient agar plate to obtain pure culture and from the pure culture it is sub cultured in nutrient broth slants and maintained (Bhattacharya, 2007).

(B) Secondary Screening: The isolated colonies were selected and confirmed by microscopic and biochemical test.

Identification of Chitinolytic Bacteria

(A) Microscopic Examination

Gram staining: It is the method of staining bacterial species into gram positive or gram negative. Gram staining differentiates the bacteria by its chemical and physiological characteristics of the cells. A heat-fixed smear of a bacterial culture is prepared and primary stain (crystal violet) is applied (Ruzzante, 1996). After 1 minute wash out the primary stain and add iodide, which binds to crystal violet and traps it in the cell. Rapid decolourization with ethanol or acetone. Counterstaining with secondary stain (safranin) and keep it for 30 seconds. Wash out the secondary stain and viewed under the microscope. Prepare a thin smear of organism in a glass slide and heat fix it. Pour crystal violet stain and wait for one minute. Then wash the stain with tap water and pour gram's iodine solution, after one minute wash the smear with alcohol for decolourization. Then add counter stain safranin to the smear, after 30 seconds and wash the smear and dried and view under microscope in oil immersion (Divatar *et al.*, 2016).

(B) Biochemical Tests: Biochemical tests were performed to confirm the organism.

Oxidase Test: It is used to determine whether the bacterium produces certain cytochrome C oxidase. A loop full of pure bacterial culture where aseptically transfer to the oxidase disk. The disk where impregnated with a reagent N-tetramethyl-p-phenylenediamine (DMPD). The oxidase-positive bacteria possess cytochrome oxidase by from an indophenol blue colour (E. Andronopoulou *et al.*, 2004). The organism is rubbed in a sterile filter paper disc impregnated with N, N-dimethyl-p-phenylenediamine oxalate, if the organism is oxidase positive it oxidized the dye to produce a violet colour to the disc (Frandsberg *et al.*, 1994).

Sugar fermentation test: The test is used to determine whether or not bacteria can ferment a specific carbohydrate. To each pair of test tubes glucose, lactose and sucrose are taken and nutrient broth with phenol red is added to each tube. Then place the Durham's tube and sterilize all the tubes. After sterilization three tubes were inoculated and serve as test, other three tubes without organism serves as control. Incubate overnight at 37 °C for 24 hrs (Juni, 1986). 0.075 gm sugars such as glucose, sucrose and lactose added to sterilized peptone broth and pour on tubes and inoculated. Place Durham's tube to view acid production.

Vogues Proskauer test: The test depends on the digestion of glucose to acetylmethylcarbinol. In this test inoculated nutrient broth kept in overnight incubation at 37°C. After incubation, to the broth VP1 and VP2 reagents are added and wait for 15 min under aerobic condition (Fay and Barry, 1974). Nutrient broth was prepared, sterilized and inoculated with organism. Then add VP1 and VP2 reagents, colour change may observe (Fay and Barry, 1974).

Optimization of chitinase enzyme

Effect of media and incubation time on Chitinase production: From existing different broth mediums, in the present study nutrient broth (g L⁻¹: yeast extract, 1.5; NaCl, 5; beef extract, 1.5; amended with 1% colloidal chitin) was used to determine the growth of bacteria and Chitinase production. The culture was inoculated (1%) and incubated at 37 °C for 24 h in a rotary shaker (120 rpm). After 24 h of incubation, the cultures were harvested, centrifuged at 5,000 rpm for 20 min and the supernatant used for Chitinase assay. Then rate of absorbance was measured for different sources like nitrogen sources, carbon sources, and detergents using calorimeter.

(B) Effect of temperature and pH on Chitinase production: The effect of temperature on enzyme production was determined by incubating inoculated medium at different temperatures (22, 37, 40, 50 and 55 °C) for optimized period of time. By varying the initial pH of the culture medium from 5 to 9 and at optimized temperature and incubation period, the effect of the initial pH value on the Chitinase production was studied. From those five pH values highest value was obtained using pH meter. Chitinase assay was analysed as per the standardized protocol.

(C) Effect of nitrogen, carbon and detergent sources on Chitinase production: The effects of

various carbon, nitrogen and detergent sources (1%) were used as additional supplement in media for maximum enzyme production. The supplemented media were inoculated with 1% inoculums and fermented at an optimized condition.

Five conical flasks were prepared with first nitrogen source, yeast (0.00003) and other compositions such as colloidal chitin (0.4), MgSO₄·7H₂O (0.05), K₂HPO₄ (0.07), FeSO₄·7H₂O (0.001), MnCl₂ (0.0001), NaCl (0.00003), distilled water (100 ml). At the same way, the other four conical flasks were prepared using the other four nitrogen sources with the above mentioned compositions at the same amount given above. Prepared five conical flasks were incubated for 24 hrs in a rotator shaker. After that incubation period nitrogen source compositions were taken for centrifugation at 5000 rpm for 20minutes. After the completion of centrifugation processes, supernatant of each nitrogen source compositions were taken in each cuvette and absorbance is measured using calorimeter. The same procedures which has done on nitrogen source was carried out in the case of carbon source and detergents for measuring the absorbance. And the compositions were, colloidal chitin (0.4), MgSO₄·7H₂O (0.05), K₂HPO₄ (0.07), FeSO₄·7H₂O (0.001), MnCl₂ (0.0001), NaCl (0.00003), distilled water (100 ml) in case of both carbon sources and detergents (Singh *et al.*, 2008).

RESULTS

Upon performance of serial dilution and spread plate method, different bacterial colonies were observed on the nutrient agar plates. Subculture of these colonies from pure cultures on 1% chitin agar plate, showed four colonies with promising growth. These strains were selected for further studies and



Fig. 1. Bacterial colony showing clear view on colloidal chitin agar [Master plate].



Fig. 2. Bacterial growth on nutrient broth.

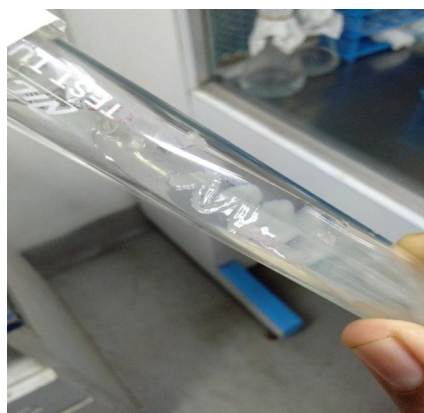


Fig. 3. *Aeromonas hydrophila* slant.

their colony morphology was observed. All the colonies were found to be gram negative rods with different arrangement of colonies, when exposed to gram staining,

Identification test

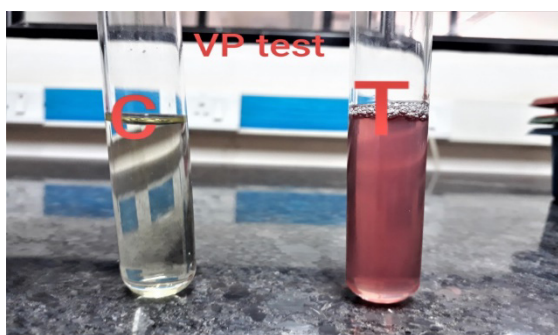


Fig. 4. Vogues proskaeur test.

Optimization of enzyme

(A) **Effect of pH on Chitinase production by *Aeromonas hydrophila*:** To evaluate the effect of pH of media on the Chitinase production, bacterial cultures were grown at different pH (5-9). pH 8 for

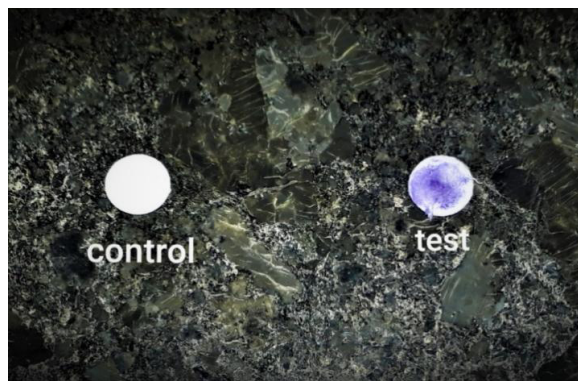


Fig. 5. Oxidase test.

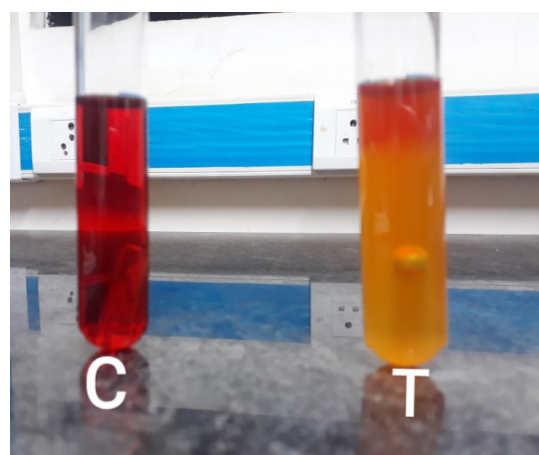


Fig. 6. Sugar fermentation test.

A. hydrophila (0.37 for zeroth day and 0.97 after 24 hrs) supported the maximum Figure 6 Effect of pH.

Chitinase production we can conclude that pH of media not only helps in the production of Chitinase

Effect of nitrogen sources on Chitinase production:

The addition of yeast extract, peptone, meat, beef and maltose in *A. hydrophila* each sources had its own significant effect on Chitinase production among the various tested nitrogen sources, meat extract in *A. hydrophila* shows the most favourable and highest rate of absorbance which means the bacterial growth is more in meat extract.

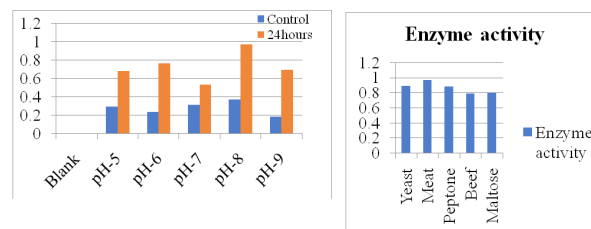


Fig. 7. Effect of Nitrogen Source

Effect of carbon sources on Chitinase production:

Different carbon sources (1%) were tested for maximum Chitinase production namely lactose, sucrose, fructose, glucose, and maltose were tested for maximum Chitinase production. Among the carbon sources, maltose supported maximum Chitinase production for *A. hydrophila* followed by glucose and sucrose.

Effect of detergents on Chitinase production:

A total of five detergents namely glycerol, SDS, Tween 80, Tween 20 and Triton X-100 were tested for maximum Chitinase production. Among these various tested detergents SDS in *A. hydrophila* shows favourable and highest rate of absorbance or better bacterial growth.

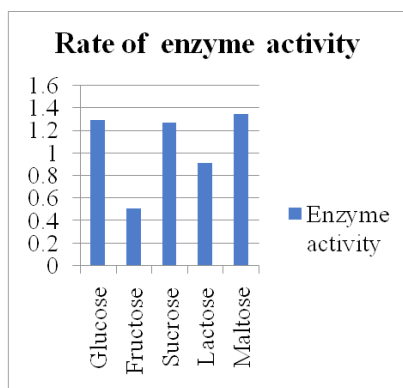


Fig. 8. Effect of Carbon source

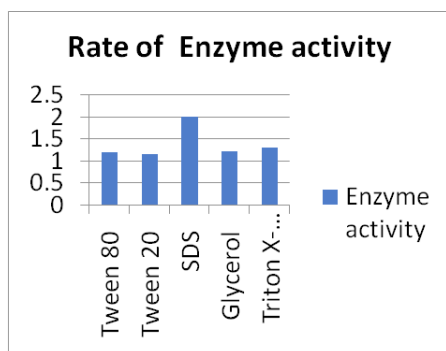


Fig. 9. Effect of Surfactant

DISCUSSION

Chitinase is the enzyme that cleaves bond between the chitin molecules. They are classified into exo Chitinase and endo Chitinase, also based on the amino acid sequence they classified into three types (Goodday, 1998). Now day Chitinase enzymes have potential applications in medicinal, bio pesticides, cosmetics, waste management etc. studies on

Pseudomonas sp. *Aeromonas schubertii* and *Bacillus* sp. have stated parallel studies. Maximum Chitinase activity was detected to occur after 24 hrs of incubation In the *A. hydrophila* HS4 and persisted invariant up to 48 h, while *A. punctata* HS6, after 48h of incubation, maximum enzyme was produced. The main reasons for decrease in chitinase production include- production of toxic chemicals in medium or lack of nutrients in cultiure medium, which results in inactivation of secretary machinery of the enzymes or breakup of enzyme by proteases, are the main reasons for the decrease in chitinase production.

In a study of Chitinase enzyme production and optimization, *Bacillus* sp. CH-2 which produced extracellular Chitinase was isolated from chitinous material dumping sites. A significant increase in enzyme yield was achieved by optimizing various physio chemical parameters of fermentation. Chitinase can be commercially explored from *Bacillus* sp.CH-2 for many applications such as bioremediation of sea food waste, antifungal biocontrol agent etc (Hamid *et al.*, 2013)

In a study conducted by Department of Industrial Biotechnology of Bharat University, at substrate level *Serratia marcescens* is capable of producing enzyme Chitinase and degrade chitin but to utilize it at commercial scale still a lot more is required to be studied and use it for the large scale degradation of chitin waste is generated in the sea-food industries (Hao *et al.*, 2012). Fermentation methodology to be adopted for maximum production of chitinase and enzyme assay kinetics at various stages is yet to be studied. Even though we have multiple important applications of chitin, we trust that the most capable applications are in the field of nanotechnology, which involves drug, gene delivery and scaffold for tissue engineering, and as chito-oligosaccharides in medicine and agriculture (Goodday, 1998).

In our study we isolate Chitinase producing strain from rizhosphere soil of fish harbour. The soil contain numerous of bacteria that can either be chitinolytic or non chitinolytic. From the soil Chitinase producing bacterial strain is isolated by serial dilution and pure culture of this strain is made by sub culture. The isolate strain used for further biochemical tests (like oxidase, vogues proskeur and VP) to identify the bacteria. Their hyper-production is an problem of central importance for commercial application, for industrially significant enzymes. Physico-chemical factors such as, temperature, agitation, pH, culture/production medium

constituents, Influence the production of Microbial enzyme/s. To make the production process economical, improving yield of enzyme is important and above factors should be optimized. The Chitinase production was optimized with respect to previous parameters (Islam and Datta, 2015). By changing different physico-chemical factors one at a time keeping the other factors constant, Optimization of the Chitinase production was done. This paper present product optimization of Chitinase by *Aeromonas hydrophila*. In the first step of the medium ingredients optimization for maximum enzyme production by *Aeromonas hydrophila*, the optimum nitrogen, carbon, and detergent sources were chosen for a one time procedure. Monosaccharides inhibited Chitinase biosynthesis and its synthesis in minor levels with low activity, based on study on the effect of carbon sources. Maltose is the carbon source which shows highest activity. The efficacy of nitrogen sources on enzyme production demonstrated that meat extract presumably is the most optimal nitrogen source for enzyme production (Monreal, 1969). The study on the effect of detergent sources showed that SDS is one among the five detergent sources which shows greater absorbance and thus bacterial activity.

The distinctive parameters i.e, Temperature and pH determine whether an enzyme is proper for biotechnological applications. The present result is based on previous studies where Chitinase production by other microbes was enhanced (Sambrook, 1986).

CONCLUSION

The naturally occurring and the second most abundant organic polymer is Chitin. The monomers of chitin have a wide range of application in industry. To obtain the monomers Chitinase is necessary. This makes the importance of Chitinase enzyme production in commercially. Chitinase mainly are of two types' exo Chitinase and endo Chitinase. Chitinase enzyme can produce by some bacteria and fungi. In our study we use the rhizosphere soil of fish harbour to obtain Chitinase producing bacteria.

The organism was collected from the rhizosphere soil of fish market, serial dilution is done and the plates were incubated after incubation several techniques have been developed to isolate and identify the Chitinase producing organism. From the pure culture microscopic and several

biochemical tests has been done to identify the Chitinase producing organism. In microscopic examination gram staining is done after that some biochemical tests i.e., oxidase test, sugar fermentation test and vogues proskeur test are done. From the tests the Chitinase producing strain from the rhizosphere soil is identified as *Aeromonas hydrophila*. Optimization of enzymatic activity was also done using various parameters. Carbon source, nitrogen source, and surfactants were used to find the highest enzymatic activities and graph of all those were plotted.

From the results present here in shows that the organism isolated from rhizosphere soil of fish market contain many organisms. In colloidal chitin agar plate only Chitinase producing can grow. The organism grown in colloidal chitin was isolated and different biochemical tests are done. From the tests we confirmed that organism, *Aeromonas hydrophila* can produce Chitinase enzyme. The maximum Chitinase production was obtained at 37 °C and pH 8.0 after 24–48 h of incubation by *Aeromonas hydrophila*. Regarding carbon sources, maltose was the best for isolated strain; while meat was found as the best nitrogen source for *Aeromonas hydrophila*. Among surfactants, SDS was the best which shows major enzymatic activity. This enzyme may also be useful in the management of sea food waste industries.

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