Production of Chitinase from Chromobacterium violaceum Using Agro Industrial Residues under Solid State Fermentation

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ABSTRACT: Chitinases have huge potential applications and biological value in industries, they are used in generating single cell proteins, sweeteners, insecticides, antifungal drugs, anti cancer agents, biopesticides, food processing agents, degrading agents for sea waste etc. Chitinases are used for the conversion of chitin a polysaccharide into monomers. Chitin was produced enormously in the biosphere annually from fungal cell walls, exoskeletons of insects and crustaceans. Chitinases producing microorganisms Bacilluslicheniforms, Chromobacterium violaceum, Rhizobium radiobacter, Streptomyces griseous were obtained from MTCC. Solid state fermentation was done on Naturally available substrates like Fish scale waste, Prawn shell waste, Wheat brawn, Potato peel, Grape leaf to check the enhanced production of Chitinase. Prawn shell waste was found to be the best natural substrate for high activity of Chitinase (464.98 U/g). The overall maximum yield of Chitinase, Chitinolytic activity (531.66 U/gds) was achieved with the optimized process parameters of incubation time (60th hour), temperature (34ºC), inoculum age (3rd day), inoculums volume (20% v/w), pH (7.0) moisture content (60% v/w), 1.5% w/w starch as Carbon source supplement and 1% w/w urea as Nitrogen source supplement. Both physico-chemical and nutritional parameters had played a significant role in the production of the Chitinase enzyme.

KEYWORDS: Chitinase, Chitin, Chromobacterium violaceum, Chitinolytic activity, Solid state fermentation.

I. INTRODUCTION

Chitin is the insoluble polysaccharide which consists of β-1, 4-linked to N-acetyl-β-glucosamine. Chitin was broken down to its monomer NAG by chitinases, which this process called chitinolysis. Chitin has high resistance to organic solvent and requires strong mineral acids for solubilization. Chitin and its derivatives are biodegradable and biocompatible to human and most of the animal [1].

Chitinase is monomeric protein that forms in single strand of polypeptide with molecular weight range in between 21 kDa to 70 kDa. Chitinase also known as poly 1,4 - (N-acetyl-β-D-glucosaminide) glycanohydrolase, (EC 3.2.2.14). Chitinase have been classified into families 18 and 19 of the glycosyl hydrolase superfamily, and chitobiases, to family 20. These families are grouping based on primary structure comparisons and it is well established that enzymes that belong to the same family share several common properties in terms of folding of the catalytic domain, substrate specificity, and stereochemistry of the reaction as well as catalytic mechanism. Chitinase is capable to cleave the bond between C1 and C4 of two consecutive N-acetyl-β-glucosamines of a chitin polymer into low molecular weight product [2]. Broadly, chitinase are classified as the endochitinases, 2 N-acetyl-glucosaminidase and exochitinases. The exochitinases are defined as the progressive action starting from the non-reducing end of the chitin with release of successive diacetyl chitobiose units. N-acetyl-glucosaminidase plays a role in the activities are defined as a random cleavage at the internal point in the chitin chain [1]. Chitinases was produced by wide range of microorganisms such as fungi, bacteria, actinomycetes, streptomycyes, crustaceans, and insect as well as...
organisms that do not contain chitin such as viruses, higher plants, animals and humans [3]. Recently, chitinases have been receiving attention because of their possible application for biological control of chitin-containing organisms and also for the exploitation of natural chitinous materials [4]. Chitinases are used extensively in biological control for the protoplasts due to its ability to degrade fungal cell wall and it makes an attractive alternative as an environmentally safe biocontrol agent. Furthermore, the ability of chitinase to hydrolyze chitin makes it very useful for production of value added products such as sweeteners, growth factors and single cell protein [1]. Purification and characterization of chitinase producing bacteria is important due to the microbial chitinase’s application in agricultural and pollution control. Purification process is mean by separation of interest protein from mixture of protein while maintaining its biological function. This can be conduct by controlling the pH, temperature, and ionic strength of the interest enzyme. All purification method is based on molecular size, electrostatic properties, and relative solubility in salts until purification homogeneity achieved.

**II. MATERIALS AND METHODS**

**Microorganism used and inoculum preparation:** Chitinase producing *Bacillus licheniformes* (MTCC 429), *Chromobacterium violaceum* (MTCC 2656), *Rhizobium radiobates* (MTCC 8917), *Streptomyces gresius* (MTCC 4734) obtained from MTCC, Chandigarh, were used in the present study. The strains were grown and maintained on yeast manitol, Nutrient agar and streptomycyes medium, respectively. After cultivating for 2-3 days at 30°C, cultures were preserved at 4°C for short term storage and were sub cultured every 4 weeks. The production of chitinase enzyme requires the preparation of inoculum. Culture was scraped and washed from the slant culture with 10ml sterile water; and 2ml of the inoculum was added to each 250ml flasks containing production medium.

**Chemicals used:** For Colloidal chitin: Concentrated HCl and Chitin, For Acetate Buffer: Acetic acid and sodium acetate, For citrate phosphate buffer: citrate phosphate and citric acid, For DNS Reagent: 3,5-dinitrosalicylic acid, Sodium potassium tartarate and Sodium hydroxide

**Preparation of Colloidal Chitin:** 10.0 g Colloidal chitin was prepared according to the modified method of Shimahara and Takiguchi [5]. Ten grams of chitin flakes from crab shells (Sigma–Aldrich, C9213) were mixed with 200 ml concentrated HCl on ice with vigorous stirring for 3–4 h and continued incubation on ice overnight. Then, the mixture was filtered through cheesecloth and dropped slowly into 600 ml of 50% ice-cold ethanol with rapid stirring on ice. Then, the colloidal chitin was collected by centrifugation at 8000rpm, for 30 min at 4°C and washed several times with tap water until the pH was neutral (pH 7.0). Alternatively the filtrate was re-filtered with suction through Whatman No. 1 filter paper and washed with water until the washing solution was neutral. The colloidal chitin was kept at 4°C until used. [6].

**Substrate used in Solid State Fermentation:** In the present study, five substrates which are Wheat Brawn, Prawn shell Waste, Fish Scale, potato peel and grape leaf were used in the production of Chitinase. Fish scale waste, prawn waste, wheat bran, was collected at local fish market (MVP colony, Visakhapatnam) and dried and graded with mixer. 5 g of individual powder taken in 250 ml Erlenmeyer flask and mixed with distilled water to a solid liquid ratio of 5:4(w/v). The thoroughly mixed substrate was autoclaved for 20 min at 121°C, 15 lb and cooled to room temperature. Under aseptic conditions, the sterilized solid state medium was inoculated with 2 ml (23 x 10^5 c.f.u/ml.) spore inoculum. The contents were mixed thoroughly and the flaks were placed in an incubator at 34°C for 60 h intervals [7].

**Enzyme extraction:** After incubation, the fermented solid was mixed with 25 ml (5 X volume, based on initial dry weight of the substrate) distilled water and mixed thoroughly on a rotary shaker 150 rpm for 30 min. The entire contents were squeezed through a whatmann no 1 filter paper. After extracting twice the extracts were pooled, centrifuged at 4°C for 20 min at 10,000 rpm and the clear supernatant was used as crude enzyme for assay [8].

**Analytical Procedures:** Chitinase activity was measured by incubating 1 ml of enzyme solution with 1% 0.3ml of colloidal chitin with addition of 0.3ml 1% acetate buffer (pH 7.0) at 37°C for 1 h. The catalytic reaction was terminated and analyzed on adding dinitrosalicylic acid reagent (0.5 mL). The mixture was boiled for 15 min, chilled
and centrifuged at 5000rpm for 5 min to remove insoluble chitin. The resulting adduct of reducing sugars were measured spectrophotometrically at 540 nm[7].

III. EXPERIMENTAL RESULTS

Selection of suitable substrate and strain: In SSF, the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation. In the present study, five substrates, viz. Wheat Bran, Prawn Waste, Fish Scale, potato peel, grape leaf were screened for growth and production of Chitinase using various strains viz Bacillus licheniformes (MTCC 429), Chromobacterium violaceum (MTCC 2656), Rhizobium radiobates (MTCC 8917), Streptomyces gresius (MTCC 4734). All the substrates and strains supported growth and production of Chitinase, while fish scales and prawn waste was proved superior to other substrates and Chitinase activity of 315.28 U/g and 464U/g were obtained using Chromobacterium violaceum (MTCC 2656) [16]. The solid state fermentation (SSF) process was observed to be less sensitive to contamination than submerged fermentation [9]. Solid substrate fermentation holds tremendous potential for the production of microbial enzymes [10]. Solid substrate fermentation can be of special interest in those processes in which the crude fermented product may be used directly as an enzyme source [11]. Different types of substrates which contain chitin have been tried for the production of chitinase which included fungal cell wall, crab and shrimp shells, wheat bran, prawn waste [12];[13]; [14];[8];[15]. As prawn waste supported maximum enzyme activity with Chromobacterium violaceum this combination was chosen for indepth study.

The physicochemical parameters like incubation time, temperature, inoculum age, inoculum volume, pH and moisture content were optimized. The nutritional supplementation study was carried out using various nitrogen sources to enrich the production medium to enhance the enzyme yield. The results of study presented and discussed in the light of existing literature.

Fig.1 shows the photos of various substrates used in the experiment,(a) fish scales, (b) wheat bran, (c) prawn waste, (d) grape leaves powder, (e) potato peel.
Fig. 2 shows graphical representation of the screening of potential substrate and organism for Chitinase production. In the screening of five substrates, prawn shell waste was found to be the best natural substrate for high activity of Chitinase (464.98 U/g) and was selected.

Fig. 3 shows the graphical representation of the effect of incubation time on chitinolytic activity and for the optimal incubation time for Chitinase production, samples were periodically taken 12-hour interval. The below figure/results indicate that from day 1 to 3 there was very less production of chitinase. The production increased on 60th hour, increased gradually and attained high titers and gradually decreased on 72nd hour, hence 60th hour is optimal for high activity of chitinase.
Fig. 4 shows the graphical representation of the effect of different incubation temperatures, different incubation temperatures, viz. 26\(^\circ\)C, 30\(^\circ\)C, 34\(^\circ\)C, 38\(^\circ\)C and 42\(^\circ\)C, were tried to cultivate the culture for Chitinase production. It was another critical parameter that has to be controlled which varies from organism to organism [9]. The chitinase yields have increased most at 34\(^\circ\)C temperature. The organisms exhibited a better growth as well as highest enzyme production with *Chromobacterium violaceum*. A decrease in the yield of Chitinase was observed when the incubation temperature was higher or lower than the observed optimum incubation temperature 34\(^\circ\)C.

![Graph showing Chitinase activity at different incubation temperatures](image)

**Chitinase activity (U/g)**

**Incubation temperature (°C)**

26 30 34 38 60

Fig. 5 shows the graphical representation of the effect of moisture content i.e various moisture content levels (30, 40, 50, 60, 70 and 80\%) to study their effect on activity were selected. The chitinase yield for *Chromobacterium violaceum* was observed with 30 \%v/w moisture content,, further increase of moisture content from 30 to 60 \%v/w effected significantly in the chitinase content and the maximum yields for *Chromobacterium violaceum* were obtained at 60\% v/w moisture content. The chitinase yield dropped when the level of the inoculum increased further.

![Graph showing Chitinase activity at different moisture contents](image)

**Chitinase activity (U/g)**

**Moisture content (%v/w)**

30 40 50 60 70 80
Fig. 6 shows the graphical representation of the effect of inoculum age on the production of Chitinase, the solid substrate was inoculated with inoculum of different ages (1-5 days). Third day inoculum was found to be optimum for the maximum Chitinase production with *Chromobacterium violaceum*.

![Graph showing Chitinase activity vs. inoculum age (hours)](image1)

Fig. 7 shows the effect of various levels of inoculums on the chitinolytic activity. Various inoculum levels were tried (5, 10, 15, 20, 30, and 35%) to study their effect on Chitinase production. The chitinase yield for *Chromobacterium violaceum* was observed from 5% v/w inoculum level; further increase of inoculum level from 10 to 20% v/w effected significantly in the chitinase content and the maximum yield for *Chromobacterium violaceum* was obtained at 20% v/w. The chitinase yield dropped when the level of the inoculum increased further from 20 to 35% v/w. Hence the optimum inoculum level was 20% v/w.

![Graph showing Chitinase activity vs. inoculum level (% v/w)](image2)
Fig. 8 shows the Effect of pH on chitinolytic activity. The chitinase productivity increased with increase in pH from 5.0 to 9.0. Maximum yield of chitinase with *Chromobacterium violaceum* was attained at pH 7. A further increase in pH resulted in decreased Chitinase production.

Fig. 9(a) shows the effect of carbon sources on chitinase production. The effect of carbon enrichment on the production of Chitinase was examined by adding starch, glucose, galactose, maltose, fructose, arabinose, xylose, sucrose, mannose carbon sources (1% w/w) to the fermentation medium. Of the 9 carbon sources tested, starch has positive influence with maximum Chitinase activity with *Chromobacterium violaceum*.
Fig. 9(b) shows the effect of selected starch concentration on chitinase production. Significant observation was made when the medium was enriched with different concentrations of starch %w/w, increase in the production of chitinase is observed with 1.5% w/w addition of starch.

Fig. 10(a) shows the effect of supplementary nitrogen sources on chitinase production. The effect of nitrogen enrichment on the production of Chitinase was examined by the addition of different organic (peptone, yeast extract, malt extract) and inorganic (potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate, and urea) nitrogen sources (1% w/w) to the fermentation medium. Of the 8 nitrogen sources tested, urea has positive influence with maximum Chitinase activity with Chromobacterium violaceum.
Fig. 10(b) shows the effect of selected urea concentration on chitinase production. Significant observation was made when the medium was enriched with different concentrations of starch % w/w, increase in the production of chitinase is observed with 1% w/w addition of urea.

**IV. CONCLUSION**

The present study suggests that solid waste from prawn waste can be used as suitable substrate for chitinase production using *Chromobacterium violaceum*. Maximum chitinase activity of 531.66 U/g with *Chromobacterium violaceum* MTCC 2656, it can be comparable with earlier reports. Exploitation of prawn waste, not only solve the severe problem in disposal of solid waste but also used for value added important chitinase which has immense commercial value and is economical.

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