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PROCESS OPTIMIZATION OF EXTRACELLULAR CHITINASE PRODUCTION FROM *BACILLUS* SP. ISOLATED FROM FISH WASTE DUMPING SITE

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ABSTRACT

Microbial chitinases have wide range of applications in biotechnology, agricultural (biocontrol agent of phytopathogenic fungi) and bioremediation (bioremediation of seafood waste). Non- availability of chitinases with desired properties and low enzyme yields are a challenge in the commercial application of these enzymes. Chitinase producing bacteria were isolated from the soil samples collected from the chitin degrading sites. Isolate giving maximum chitinolytic activity was selected on the basis of chitin hydrolysis zone and chitinase activity in liquid assay. Various physicochemical parameters were standardized for maximal enzyme yield. An eight fold increase in the chitinase activity was achieved from the optimization studies done on the selected isolate for its hyper production. Maximum activity was obtained when media was supplemented with 1% chitin as carbon source and 0.5% casein as nitrogen source and incubated at 37ºC, 150 rpm for 72 hours. Cultural, physiological, and biochemical characteristics indicate that the bacterial isolate belongs to the Genus *Bacillus*.

KEYWORDS: Chitinase, optimization, phytopathogenic fungi, biocontrol agent.

INTRODUCTION

Chitin is a biopolymer of N-acetyl-D-glucosamine units, which are linked by β-(1→4) glycosidic bond. It is one of the most abundant naturally occurring polymer, present in the cell walls of fungi, yeast and lower plants.[16] It is also present in huge amounts as structural components in the exoskeletons of arthropods and marine invertebrates.[41] Chitin is known to be degraded by chemical, physical and enzymatic methods but the enzymatic degradation of chitin with chitanases is much more eco-friendly and cheaper alternative.[24]

The chitinases (EC 3.2.1.14) are glycosyl hydrolases which hydrolyse the β-(1→4) linkages in the chitin molecule and is produced by insects, higher plants, animals and wide variety of microorganisms.[26] Microorganism are the preferred source because of their rapid growth, limited space required for their cultivation, longer shelf life, the ease with they can be genetically manipulated to generate improved enzymes.[52,12,25,46] All types of microorganisms; virsues, fungi, bacteria are known to produce chitinases.[18,35]

Microbial chitinases are in demand because of their application in various fields like biotechnology, food, medicine, wastewater treatment and agriculture industry.[3,7,33] Moreover, chitinases are also commercially exploited as antifungal biocontrol agents.[28,18] Bacteria are preferred sources of enzymes because enzymes production does not involve the problem of high biomass as in case of fungi. Although bacteria of different genera viz. *Aeromonas, Serratia, Vibrio, Streptomyces* and *Bacillus* are known to produce chitinases.[14,53,35] But there are some limitations for the application of chitinases on the commercial scale such as low chitinase yields, high production time and low catalytic activity of the available enzymes hence low chitin degradation.[18,48] Therefore, the aim of the present study was to isolate extracellular chitininase producing bacteria and optimizing fermentation conditions for the hyperproduction of the chitinase so that enzyme can be explored for various applications in the different fields.

2 MATERIALS AND METHODS

2.1 Culture Media

Minimal Medium recommended by Hsu and Lockwood, 1975 (Designated as MMHL) containing 0.5% colloidal chitin, 0.05% $MgSO_4.7H_2O$, 0.03% KH_2PO_4, 0.07% K_2HPO_4, 0.0001% $MnCl_2$, 0.001% $FeSO_4.7H_2O$, 0.0001% $ZnSO_4$, pH 7.0 was used for growth and enzyme production. Colloidal chitin was prepared according to the modified method as described.[20]

2.2 Isolation and selection of the chitinolytic bacteria

Soil samples were collected from various dumping sites of fish markets of Chandigarh and its surrounding areas for the isolation of chitinase producing bacteria. 1g of the soil was suspended in MMHL medium containing 1% chitin and incubated at 37ºC for 96 h for the enrichment.

After enrichment, appropriate dilutions were made and spread plated on these MMHL plates and incubated for 72 h at 37°C. The isolates showing a clear zone around colonies were further screened for the extra cellular chitinase production in liquid medium and isolate producing highest yield of chitinase was selected for the further optimization studies.

2.3 Chitinase production in liquid medium

The 20ml broth of MMHL medium (pH 7.0) was inoculated with 1% inoculum of overnight grown cells. Incubation was done at 150 rpm at 37°C for 96 h. Culture was centrifuged at 10000rpm for 10 min at 4°C. Chitinase activity was assayed in cell-free supernatant.

Chitinase Assay

Chitinase activity was measured in terms of the amount of N-acetyl-D-glucosamine sugars released from chitin by the chitinase action as described[43] using DMAB as a coloring reagent. Enzyme units were expressed in international units (IU) as micromoles of N-acetyl glucosamine released by 1ml of enzyme in 1 minute under the assay conditions.

2.4 Characterization of bacterial isolates

The identification of selected bacterial isolate was carried out by the morphological and biochemical tests performed according to the methods described in Bergey's Manual of Systematic Bacteriology.

2.5 Optimization of chitinase production by one variable at a time (OVAT)

Optimization of the chitinase production was done by varying different physico-chemical factor one at a time keeping the other factors constant.

2.5.1 Effect of Incubation time on enzyme production and growth

The effect of incubation time on enzyme production was studied by withdrawing the samples from the culture at different time intervals upto 120 hr and doing the assay using cell free supernatant. Growth curve was studied by determining the log CFU at different time intervals by dilution method.

2.5.2 Effect of pH

The effect of pH on enzyme production was studied with different pH ranging from 5.0-9.0 and doing the assay using cell free supernatant.

2.5.3 Effect of incubation temperature

The effect of incubation temperature on enzyme production was studied at different temperature ranging from 25-45°C.

2.5.4 Effect of inoculum size

The effect of inoculum size was studied at different inoculum concentration ranging from 0.1 - 2%.

2.5.5 Effect of carbon source

The effect of different carbon sources *viz.* starch, glucose, fructose, chitin, galactose and sucrose was studied at 0.5% concentration.

2.5.6 Effect of nitrogen Source

The effect of different nitrogen sources *viz.* casein, peptone, yeast extract, NH_4SO_4, beef extract, NH_4NO_3, gelatin and urea was studied at 0.5%.

2.5.7 Chitin concentration

The effect of chitin was studied at different concentration ranging from 0.1-1.5%.

2.5.8 Casein concentration

The effect of casein was studied using different concentration ranging from 0.1-1.0%.

3 RESULTS AND DISCUSSION

3.1 Isolation and selection of chitinolytic bacteria

Enriched soil samples were plated on minimal medium plates supplemented with 0.5% colloidal chitin. Five bacterial isolates showing the zone of colloidal chitin hydrolysis having diverse colony morphology were selected and designated as CH-1, CH-2, CH-3, CH-4 and CH-5.

Out of the five, Isolate no.1 and 3 did not show any chitinase activity in liquid assay which can be because of the intracellular enzyme in these isolates, Isolate no. 2, 4, 5 showed the extracellular chitinase activity. Bacteria have been reported to produce chitinases intracellularly[34] and extracellularly.[32,39]

Out of Isolate no. 2, 4, 5 Isolate no. 2 showed the highest activity of 0.31 IU (Table 1, Fig. 1). Chitinolytic activities in the same range have been reported by most of the bacteria like *Bacillus* sp. (0.09IU)[38], *Serratia marcescens* DSM 30121(0.5IU)[37], *Bacillus sp.* (1.27IU)[31] etc. Isolate no. 2 was selected for further studies.

Table 1: Assessment of extracellular chitinase activity.

Isolate No.	Enzyme activity (IU)
CH-1	ND
CH-2	**0.31**
CH-3	ND
CH-4	0.09
CH-5	0.07

ND: Not Detected.

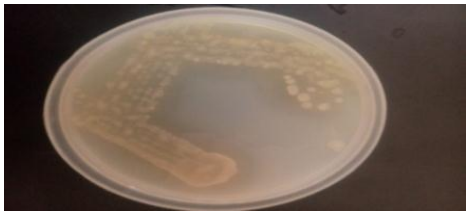

Figure 1: Chitinolytic colonies of isolate CH-2.

3.2 Morphological and Biochemical characteristics of Isolate CH-2

Isolate CH-2 was Gram positive, small sized rod, present singly, or in pairs and was motile. On the MMHL plate bacteria showed whitish to milky, smooth colonies. Organism could grow at pH 6.0-8.0 and temperature ranging 30-45°C. Detailed biochemical characteristics of Isolate CH-2 have been enlisted in Table 2. Morphological, physiological and biochemical characteristics indicated bacterial Isolate CH-2 to be a species of Genus *Bacillus* and designated as *Bacillus* sp. CH-2.

Table 2: Biochemical characteristics of Bacterial Isolate CH- 2.

TEST	RESULT
Catalase, Oxidase	Positive, Negative respectively
Indole	Negative
Citrate utilization	Negative
Nitrate reduction	Positive
Urease production	Negative
D-Glucose, mannitol, xylose, sucrose	Positive, positive, negative, positive respectively
Starch hydrolysis	Positive
Methyl red	Negative
V-P reaction	Negative
H/L	Motile
Triple Iron Sugar , H_2S Production	A/A, Negative respectively

3.3 Optimization of chitinase production by one variable at a time (OVAT)

For industrially important enzymes, their hyper-production is an issue of central importance for commercial application. Microbial enzyme/s production is influenced by the physico-chemical factors e.g. temperature, agitation, pH, culture/production medium constituents.[15,29,13,9,21,22] Optimization of these factors for improving the yield of enzyme is crucial to make the process economical.[4] The chitinase production with isolate CH-2 was optimized with respect to previous parameters:

3.3.1 Effect of incubation time on enzyme production and growth

Kinetics of growth and chitinase production was investigated. The organism showed a typical bacterial growth curve, no extracellular chitinolytic activity was observed during the early log phase, enzyme production started in mid log phase reaching maximum (0.28IU) in the stationary phase at 72hours. (Fig. 2).

Most of the organisms have been reported to produce chitinases in the stationary phase. *Bacillus* sp. HSA, 3-1a and *Streptomyces hygroscopicus* VMCH2 produced chitinase after in late stationary phase after 72 h and 96h respectively.[40,42]

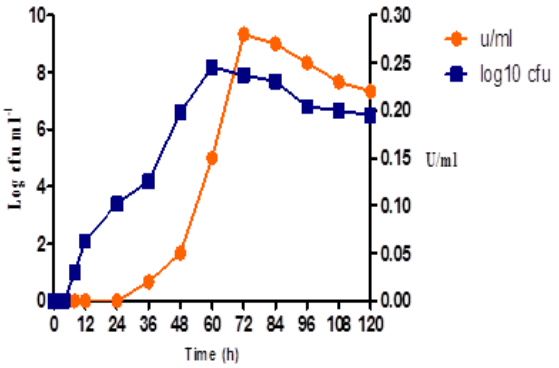

Figure 2: Growth curve of *Bacillus* sp.CH-2 and enzyme activity upto 120h.

3.3.2 Effect of pH

Organisms showed minimal growth at lower and higher pH value hence the less enzyme production was observed. Organism showed good growth in the pH range of 6 to 8 and optimal enzyme production (0.5IU) was observed at pH 6.5. (Fig. 3). Most of the organisms are known to produces chitinases in the neutral range e.g. *Serratia marcesence*[8], *Streptomyces sp.*[50] *Paenibacillus sp.* D1[45] showed maximum enzyme production at pH7. However alkalophilic organisms such as *Bacillus sp*. BG-11[6], *Beauveria bassiana* (49) *Actinomycete*, *Nocardiopsis prasina* OPC-131[51] are known to produce chitinases at higher pH.

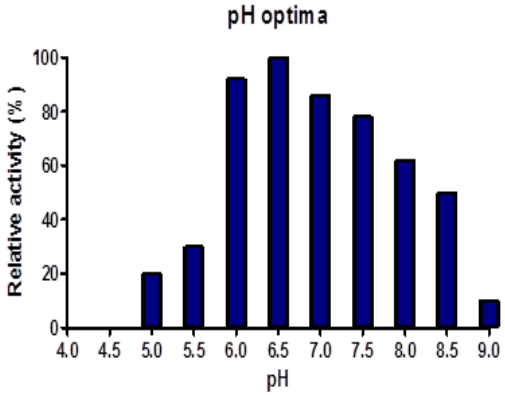

Figure 3: Production of chitinase from *Bacillus* sp. CH-2 at various pH.

3.3.3 Effect of temperature

Since the organism is a mesophile very less growth hence low amount of enzyme production was observed at higher temperature of 45°C. Optimal enzyme production (0.52 IU) was observed at 37°C (Fig. 4). Similar results have been shown with other *Bacillus* strains like *Bacillus subtilis*[32] *Bacilus laterosporous*[44] showed maximum enzyme production at 35°C.

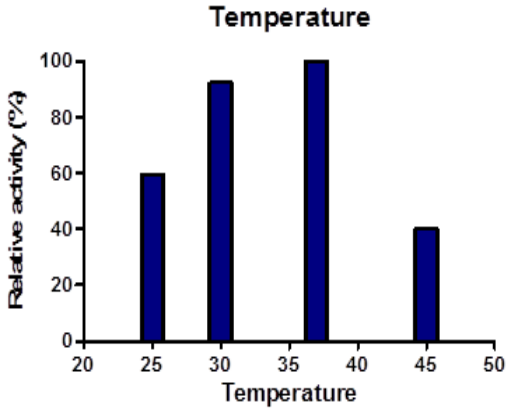

Figure 4 Chitinase production from *Bacillus* sp. CH-2 at different temperature range.

3.3.4 Effect of inoculum size

Small inoculums size leads to higher surface area to volume ratio as well as improved distribution of dissolved oxygen leads to high enzyme production, however if the inoculum size is too small deficient number of bacteria will produced and leads to low enzyme production. Therefore it is important to optimize the inoculums size for higher enzyme production. With 24h starter culture 1% of the inoculums size gave maximum enzyme production (0.53IU) (Fig. 5).

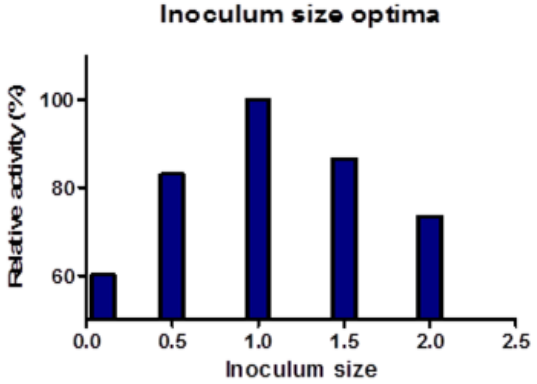

Figure 5 Production of chitinase from *Bacillus* sp. CH-2 at different temperature range.

3.3.5 Effect of carbon sources

It was observed that some quantity of chitinase was produced while using different carbon sources. However maximum enzyme yield (0.54IU) was achieved with chitin (Fig. 6). In similar results with *Aeromonas hydrophila* HS4[35] chitinase could be produced with various carbon sources but maximum production was achieved with chitin. *Streptomyces sp.*[47] produced maximum chitinase production using sucrose as a carbon source along with chitin.[17,42] have shown that no chitinase could be produced using carbon source other than chitin in case of *Beauveriabassiana* isolates and *Streptomyces hygroscopicus* respectively.

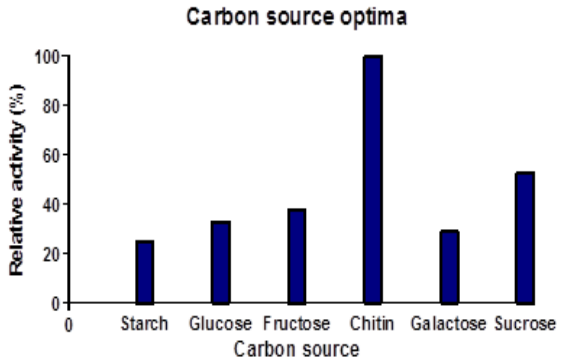

Figure 6 Production of chitinase from *Bacillus* sp. CH-2 by using different carbon sources.

3.3.6 Chitin concentration

As maximum enzyme production was achieved with chitin, varied concentrations of chitin were investigated for enzyme production and maximum enzyme production (1.0 IU) was achieved with 1% chitin (Fig.7).

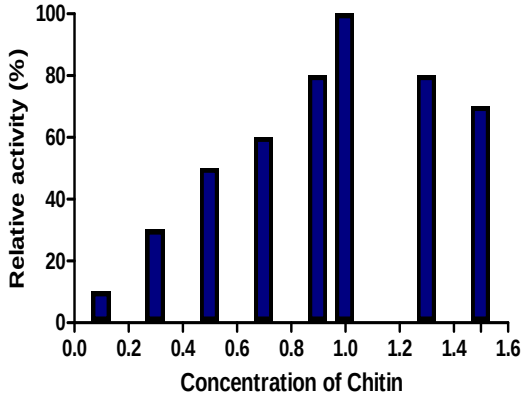

Figure 7 Production of chitinase from *Bacillus* sp.CH-2 at different concentration of chitin.

3.3.7 Effect of nitrogen sources

Like carbon sources, nitrogen source/s also significantly affects the enzyme production.[35] In the minimal medium chitin served both carbon as well as nitrogen source. In addition to chitin effect of other (organic as well as inorganic nitrogen sources) was studied. Enzyme production increased with most of the organic nitrogen sources and maximum enzyme yield (2.2 IU) was achieved with 0.5% Casein. Whereas no effect on enzyme production was observed with inorganic nitrogen sources (Fig. 8)[30] has also shown increased chitinase yield on addition of organic nitrogen source with *Bacillus* sp. but maximum enzyme production was with peptone than casein. Similarly in case of *Bacillus licheniformis*, *Pantoeadispersa* and *Axylosoxydans* maximum enzyme yield could be produced using peptone as nitrogen source.[2,23]

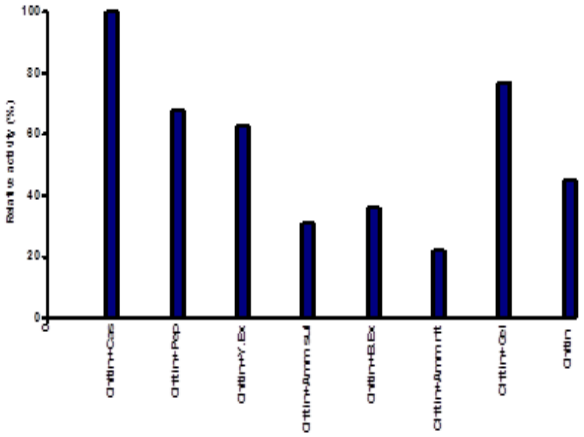

Figure 8 Production of chitinase from *Bacillus* sp. CH-2 by using different nitrogen source.

3.3.8 Casein concentration

As a considerable increase in enzyme yield was achieved with casein, various concentrations of casein were investigated for enzyme yield and maximum enzyme production (2.5IU) was achieved with 0.7% casein (Fig.9).

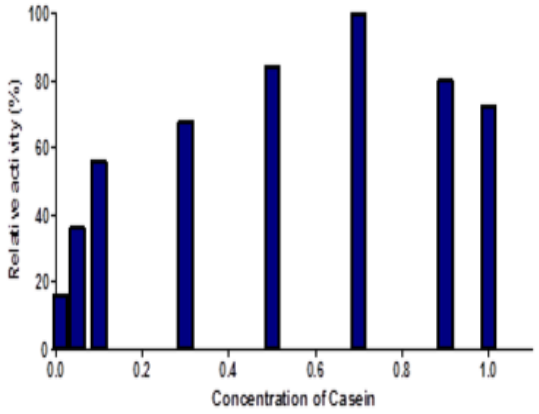

Figure 9 Production of chitinase from *Bacillus* sp. CH-2 at different casein concentration.

After optimization by one factor at a time 8 fold higher yield of chitinase than the unoptimized condition was achieved.

4 CONCLUSION

A number of attempts have been made to isolate the chitinolytic microorganisms for the degradation of chitin. But the search is still on to find organisms giving high yields of enzyme.

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 varios physico chemical parameters of fermentation. Chitinase from *Bacillus* sp.CH-2 can be commercially explored for various applications such as antifungal biocontrol agent bioremediation of sea food waste etc.

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