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OPTIMIZATION OF CULTURE CONDITIONS FOR PRODUCTION OF BACERIAL CHITINASE ISOLATED FROM MARINE CRUSTACEAN SHELLS

Anuradha Venkatraman[1], *Syed Ali Mohamed Yacoob*[*2], *Yogananth Nagarajan*[2], *P. Kalitha Parveen Peer Mohamed*[2]

Address(es): Mohamed Yacoob Syed Ali,
[1]PG& Research Department of Biochemistry, Mohamed Sathak College of Arts and Science, Sholinganallur, Chennai-600119, India.
[2]PG& Research Department of Biotechnology, Mohamed Sathak College of Arts and Science, Sholinganallur, Chennai-600119, India.

*Corresponding author: syedmicro555@gmail.com

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ABSTRACT

Six bacterial isolates identified from marine crustacean shell wastes' were studied under different growth parameters to optimize the production of chitinase. The enzyme activity was determined with different growth media and optimized under different conditions of pH, Temperature and substrate concentration. The enzyme activity was found to be maximum in nutrient broth with 0.3% colloidal chitin. The optimum pH and temperature were found to vary between the isolates. Under optimized condition, the chitinase activity was doubled than in the normal condition.

Keywords: Crustacean shells, bacterial chitinase, chitin, optimization

INTRODUCTION

Chitin is the most abundant biopolymer in the earth, next to cellulose. Annually 1- 100 billion metric tones of chitinous waste were recovered by marine invertebrates **Rattanakit *et al.*, (2002)**. Chitin is an insoluble polysaccharide composed of linear chains of α -1,4- N-acetylglucosamine (GlcNAc) residue that are highly cross-linked by hydrogen bonds. It is found in the outer skeleton of insects, fungi, yeasts, algae, crabs, shrimps, lobsters and in the internal structures of other invertebrates **(Flach *et al.*, 1992; Felse and Panda, 1999)**. The biodegradation of chitin requires the synergistic action of several hydrolytic enzymes for efficient and complete breakdown. The combined action of endochitinases (EC 3.2.1.14) and exochitinases [chitobiosidases and β-N-acetyl hexosaminidase (EC 3.2.1.82)] results in the degradation of chitin polymer into the soluble N-acetyl D-glucosamine **Gkargkas *et al.* (2004)**. The capacity to degrade chitin is widespread among taxonomic groups of prokaryotes including the gliding bacteria, vibrios, Photobacterium spp., enteric bacteria, actinomycetes, bacilli, clostridia and archaea. Bacteria employ several proteins, including chitin-binding proteins, to degrade chitin, but the hydrolysis by chitinase is the key step in the solubilization and mineralization of chitin. The capacity to degrade chitin would seem to be an important attribute of marine bacteria given the presumed high input of detrital chitin into the sea.
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 to expand their usefulness. Chitinases produced by different microorganisms have received increased attention due to their wide range of biotechnological applications, especially in the production of chitooligosaccharides and N-acetyl D-glucosamine, biocontrol of pathogen and pests, preparation of sphaeroplasts and protoplasts from yeast and fungal species, and bioconversion of chitin waste to single cell protein (**Felse and Panda, 1999**). Based on this information, the aim of the present study is to optimize the culture conditions for the production of bacterial chitinase isolated from marine shell waste.

MATERIAL AND METHODS

Sample Collection

Freshly removed shell waste samples from various marine sources like Prawn, Crab and Fish scales were collected from local market in six different parts of Chennai, Tamil Nadu State, India. The shells of fish, shrimps and crab were immersed in 75% sodium chloride (sterilized) in three separate sterile polythene bags.

Isolation of Chitin Degrading Bacteria

Chitin degraders were isolated by serial dilutions of shell samples and plated on 0.5% colloidal chitin agar (CCA) medium according to the method of Vogan *et al.*,2001). After 48 h of incubation at room temperature, the isolates capable of degrading chitin with distinct zone of clearance on CCA were selected and sub cultured in NA slants and maintained. All the strains isolated were identified by FAME-GC analysis and 16s rDNA sequence. Their biochemical characteristics have been determined by routine analysis according to Bergey's manual of Systematic Bacteriology. The strains were identified as *Vibrio aestuarianus, Flavobacterium odoratus, Shewenella putrefaciens, Exiguobacterium strain, Bacillus subtilis* and *Bacillus atrophaeus* (**Anuradha and Revathi., 2013**).

Preparation of Colloidal chitin

Colloidal chitin was prepared from the chitin flakes (Sigma Chemicals Company, USA) by the method of **Mathivanan (1995)**. The chitin flakes were ground to powder and added slowly to 10 N HCl and kept overnight at 4° C with vigorous stirring. The suspension was added to cold 50% ethanol with rapid stirring and kept overnight at 25° C. The precipitate was collected by centrifugation at 10000 rpm for 20 min and washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). It was freeze dried to powder and stored at 4° C until further use.

Determination of Chitinase Production

1ml of inoculum (chitinolytic bacteria) was inoculated in 100ml yeast nitrogen basal media (liquid broth). The tubes were incubated at 100rpm in rotary shaker at room temperature. After 2 days, the culture was harvested. It was centrifuged at 10000rpm for 15 minutes. The supernatant was used for chitinase assay.

Preparation of reaction mixture and Chitinase Assay

The reaction mixture contained 1 ml of 0.1% colloidal chitin in sodium acetate buffer (0.05 M, pH 5.2) and 1 ml culture filtrate was incubated at 37ºC for 2 h in a water bath with constant shaking. Suitable substrate and enzyme blanks were included. Chitinase activity was assayed by the colorimetric method of (**Reissig et al., 1955**). The reaction was terminated by adding 0.1 ml of 0.08 M potassium tetraborate, pH 9.2 to 0.5 ml of reaction mixture and then

boiled in a water bath for 3 min. Then 3 ml of diluted p-dimethylaminobenzaldehyde (p-DMAB, Sigma Chemicals Company, USA) reagent was added and again incubated at 37ºC for 15 min. The released product in the reaction mixture was read at 585 nm in a spectrophotometer Hitachi, Japan). Chitinase activity was determined using N-acetylglucosamine (Sigma Chemicals Company, USA) as the standard.

One unit of chitinase activity was defined as the amount of enzyme, which produces 1 µ mole of N-acetylglucosamine in 1 ml of reaction mixture under standard assay condition **(Mathivanan *et al*., 1998).**

$$\frac{\text{Amount of NAG liberated}}{\text{Molecular weight of NAG}} \times \frac{1000}{\text{Incubation time}}$$

Optimization of Culture Conditions

Media Composition

Two different media namely NB and Luria Bertaini broth amended with 1% colloidal chitin were used to determine the growth and chitinase production. 1 mL inoculum of all the six bacterial strains with 0.5 OD was inoculated with 100 ml of each medium and incubated at 100 rpm in a rotary shaker at room temperature. After two days of incubation, the cultures were harvested, centrifuged at 10000 rpm for 15 min and the supernatant was used for chitinase assay.

Substrate concentration, pH and temperature

All the six bacterial strains were grown at different concentrations (0.1 - 0.5%) of colloidal chitin amended NB broth to determine the optimum concentration of substrate for chitinase production. In addition, the bacterium was grown at different pH (4 - 10) and temperature (20 - 40° C) in NB amended with 0.3% of colloidal chitin to determine the optimum pH and temperature for chitinase production. After two days of growth, the cultures were harvested, centrifuged and the supernatant was used for chitinase assay.

Determination of Incubational change in Chitinase activity under Optimised Conditions

Chitinolytic bacterial strains were grown in an optimized growth conditions with culture media of 0.3% colloidal chitin. The pH was maintained at 8.0 and incubated in rotary shaker at 37°C for 10 days. Every 2 days intervals the culture was filtered and the supernatant was used for assay. The production of chitinase in the culture filtrate was assayed.

RESULTS AND DISCUSSION

Totally, Six potent chitin degrading bacterial strains were identified after screening and studied for optimized production of chitinase. Chitinolytic bacterial strains were grown in an optimized growth conditions in LB and NB media with 0.3% colloidal chitin. The pH was maintained at 8.0 and incubated in rotary shaker at 37°C for 10 days. Every 2 days intervals the culture was filtered and the supernatant was used for assay. The production of chitinase in the culture filtrate was assayed. In this experiment the chitinase production was high in nutrient broth compared with Luria Bertani broth. *Shewenella putrefaciens*, *Exiguobacterium* strain and *Bacillus atrophaeus* showed maximum chitinase activity than other three strains (Table 1).

Table 1 Determination of growth and chitinase production in Luria Bertani and nutrient broth

Strains	Activity in Luria Bertani broth (IU/l)	Activity in Nutrient broth (IU/l)
Vibrio aestuarianus	11.30	16.95
Flavobacterium odoratus	22.60	22.60
Shewenella putrefaciens	22.60	33.90
Exiguobacterium strain	28.25	33.90
Bacillus subtilis	22.60	22.60
Bacillus atrophaeus	28.25	33.90

Effect of Substrate concentration for chitinase production

The effect of substrate concentration of the medium on chitinase production was studied by using various concentration of colloidal chitin (0.10% to 0.50%). Chitinase production was increased when increasing the substrate concentration (Table 2). Maximum chitinase production was recorded at 0.30%. At 0.40 and 0.50% the chitinase production level did not increase. *Shewenella putrefaciens* showed maximum production when compared with other strains. 0.3% of colloidal chitin is reported as optimum concentration for maximum chitinase production by *B.licheniformis MB*-2 (**Toharisman *et al*., 2002**). The same concentration of colloidal chitin has already been reported as optimum for chitinase production in *Fusarium chlamydosporum* **(Mathivanan et al., 1998**). However, addition of colloidal chitin at 0.5% and above induced the maximum chitinase production in *Bacillus sp. NCTV2* (**Wen *et al*., 2002**), *Alternaria alternata* (**Sharaf, 2005**) and *Trichoderma harzianum* (**Sandhya *et al*., 2005**).

Table 2 Chitinase activity at different substrate concentration

Strains	Chitinase activity (IU/l) in varying substrate concentration				
	0.10%	0.20%	0.30%	0.40%	0.50%
Vibrio aestuarianus	5.65	16.95	22.60	22.60	22.60
Flavobacterium odoratus	11.30	16.95	22.60	22.60	22.60
Shewenella putrefaciens	11.30	16.95	33.90	33.90	33.90
Exiguobacterium strain	11.30	16.95	28.25	28.25	28.25
Bacillus subtilis	5.65	11.30	22.60	22.60	22.60
Bacillus atrophaeus	5.65	11.30	16.95	16.95	16.95

Effect of pH for Chitinase Production

The effect of pH of the medium on chitinase production was studied by adjusting the medium pH from 4 to 10 by using 0.1N NaOH and HCl. Chitinase production with different pH and the enzyme activity was shown (Table 3). The pH of the culture medium is playing an important role in chitinase production. Chitinases are fairly stable over broad pH range. The pH stability of chitinase varies from organism to organism. Majority of bacteria reported to produce maximum level of chitinase at neutral or slightly acidic pH and whereas fungi mostly secret it in acidic conditions. Maximum enzyme activity was observed at the pH level of 8. At this pH, chitinase produced by *Shewenella putrefaciens* and *Exiguobacterium strain* was maximum than other strains. Similar optimum pH of 8 has been reported for chitinase production in *B. pabuli K1* (**Frandberg et al., 1994**).

Table 3 Chitinase activity at different pH concentration

Strains	Chitinase activity (IU/l) in varying pH				
	4	5	6	8	10
Vibrio aestuarianus	5.65	16.95	28.25	33.90	22.60
Flavobacterium odoratus	16.95	22.90	28.25	39.55	16.95
Shewenella putrefaciens	11.30	16.95	28.25	33.90	28.25
Exiguobacterium strain	5.95	16.95	22.60	39.55	28.25
Bacillus subtilis	16.95	22.60	28.25	33.90	16.95
Bacillus atrophaeus	5.95	11.30	16.95	28.25	11.30

Effect of Temperature for Chitinase production

Temperature effect on chitinase production was studied by incubating the strains in different temperature. The temperature optima for chitinases range from 40 - 60°C depending on source. Chitinase production was reached maximum at 37˚C (Table 4). *Flavobacterium odoratus* and *Exiguobacterium* strain showed maximum chitinase production at this temperature. Temperature below and higher then 37˚C affects the growth of the bacterial strains as well as the chitinase production. Similarly in previous studies maximum enzyme production of chitinase was observed from 30 to 40°C (**Gupta *et al.*, 1995; Golmes *et al.*, 2001**).

Table 4 Chitinase activity at different temperature

Strains	Chitinase activity (IU/l) in varying Temperature				
	0ºC	20ºC	37ºC	50ºC	60ºC
Vibrio aestuarianus	6.95	22.60	28.25	16.95	5.95
Flavobacterium odoratus	11.30	28.25	33.90	22.60	16.95
Shewenella putrefaciens	16.95	22.60	28.25	16.95	16.95
Exiguobacterium strain	11.30	22.60	28.25	22.60	5.95
Bacillus subtilis	16.95	28.25	33.90	28.25	16.95
Bacillus atrophaeus	5.95	16.95	28.25	22.60	11.30

Determination of activity of chitinase for 10 days incubation period

Table 5 Determination of chitinase production in day 2 interval upto 10 Days

Varying strains	Activity in day 2 (IU/l)	Activity in day 4 (IU/l)	Activity in day 6(IU\l)	Activity in day 8 (IU/l)	Activity in day 10 (IU/l)
Vibrio aestuarianus	28.25	33.90	50.85	62.16	52.57
Flavobacterium odoratus	22.60	39.55	56.55	63.51	58.64
Shewenella putrefaciens	33.90	33.90	39.55	50.85	46.24
Exiguobacterium strain	22.60	28.25	39.55	45.20	42.14
Bacillus subtilis	33.90	45.20	62.16	73.51	61.83
Bacillus atrophaeus	33.90	39.55	56.51	62.58	57.85

The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on growth rate and enzyme production. The incubation time varies with enzyme productions. After optimization of all the process parameters, the time course of maximal enzyme production was studied (Table 5). Our result elucidated that the incubation period influences the enzyme production, wherein, the chitinase activity increased steadily and reached maximum at 96 h of incubation among all the strains studied. Further increase in the incubation period led to a reduction in chitinase production. This might be due to the depletion of nutrients in the medium.

CONCLUSION

Due to the potential applications of chitinase, it is very important to study the organisms that can produce the enzyme. Microorganisms are generally preferred than to plant and animals as sources of industrial enzyme because their production cost is low and enzyme contents of microbes are more predictable and controllable. The ability of a microbial community to degrade chitin is important for recycling of nitrogen in the soil. Chitinolytic microbes occur widely in nature and prevent the polysaccharide deposited from dead animals and fungi from accumulating in land and marine sediments. The present study has revealed that the potential of any microbial culture could be increased for the production of different metabolites by adapting suitable cultural conditions. Since the enzyme activity was enhanced by optimizing the growth conditions, large scale Production of chitinase using bioreactors under optimized condition will extend the scope for industrial application of Chitinase from these sources.

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