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Full Length Research Paper

Chitin degrading potential of three aquatic actinomycetes and its optimization

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Eighty actinomycetes obtained from Krishna River in Satara district, India were screened for their chitinolytic activity on colloidal chitin agar. Fifty-two isolates showed clear zones of hydrolysis of chitin. Three isolates: *Streptomyces canus, Streptomyces pseudogriseolus* and *Micromonospora brevicatiana* were selected on the basis of zone of clearance to colony size ratio, for further studies. The period of optimum chitinase production was at 120 h for *S. canus* and 132 h for *S. pseudogriseolus* and *M. brevicatiana*. It was observed that *S. canus* and *M. brevicatiana* showed maximum activity from 40 to 60°C and *S. pseudogriseolus* showed maximum activity from 40 to 50°C. The optimum pH for chitinase production by all tested actinomycetes was at pH 8. *S. canus* and *M. brevicatiana* showed maximum activity at 1.2% chitin concentration and *S. pseudogriseolus* showed maximum activity at 1% substrate concentration.

Key words: Chitinase, actinomycetes.

INTRODUCTION

Chitin is the most abundant biopolymer next to cellulose. It is the β –1, 4 linked polymer of N-acetylglucosamine and is the structural component of fungi as well as the shell or cuticles of arthropods. Contribution of chitin to soil is in the form of animal biomass in the marine environment (Deshpande, 1986). Both soil and aquatic habitat act as major sources of chitin degraders. Due to multiple applications of chitinases in biocontrol, waste management, medicine and biotechnology, they have become interesting enzymes for study (Muzzarelli et al., 1985, 1997). In nature chitin is not found alone but forms a part of very complex system, such as chitin- protein complex, calcium carbonate in addition to protein and organic substances (Hackmen, 1954).

Microorganisms, lower animals, birds, fungi and plants are known to produce chitinases. Bacteria are well studied, amongst which *Serratia marcescens* is well known to produce chitinases. *Streptomyces* are also explored for chitinase production (Deshpande, 1986).

Actinomycetes are prominently known for their degradative abilities. Keeping these views in mind, Krishna river water from Satara district (M. S.) India was screened for chitinolytic actinomycetes. The period of optimum chitinase production and effect of factors like, pH, temperature, substrate concentration were observed.

MATERIALS AND METHODS

Eighty actinomycetal isolates obtained from Krishna River in Satara district (M. S.), India, were screened for chitinolytic activity.

Primary screening for chitin degraders

Primary screening of actinomycetes for chitinase activity was performed by spot inoculating the isolates on colloidal chitin agar supplemented with 5 to 7 mg in 100 ml^{-1} griseofulvin at neutral pH. Zone of chitin hydrolysis (clearance) and colony diameter were recorded after 7 days at 35°C. Selection Ratio (S/R) for each isolate was determined on the basis of zone of hydrolysis and colony diameter. Three efficient isolates (KS10, RE 14 and BH 1) were selected for further studies.

Selection ratio = Zone of Clearance (mm) / Colony diameter (mm).

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Table 1. Time course of chitinase production (Units/ml).

Time (h)	Isolate code No.		
	KS 10	RE 14	BH 1
12	-	-	-
24	-	-	-
36	-	-	-
48	-	-	-
60	-	-	-
72	-	-	-
84	0.62	-	0.57
96	1.2	0.54	1.83
108	2.5	1.5	2.7
120	3.1	2.8	3.6
132	3.1	3.4	4.2
144	3.1	3.4	4.2

KS 10 = *Streptomyces canus*; RE 14 = *Micromonospora brevicatiana*; BH 1 = *Streptomyces pseudogriseolus*.

Identification of chitinolytic bacteria

The identification of actinomycetes was carried out based on Williams et al. (1989). Morphological characters were studied by coverslip culture technique. Lactose, sucrose, xylose, mamnitol, maltose, ribose, rhamnose and arabinose utilistion test, arginine hydolysis, lipase, urease, catalase and H_2S production test and growth at 30°C and 50°C were used for the identification of Actinomycetes up to species level.

A working key was also used for generic identification of actionmycetes (McCarthy and Williams, 1990). Species level identification was carried out by using MICRO-IS software (Portyrata and Krichevsky, 1992). Probable identifications were made using (Williams et al., 1983).

Chitinase assay

Chitinolytic activity in the cell medium was determined by using swollen chitin as a substrate and by measuring the release of reducing sugars with dinitrosalicylic acid method (Miller, 1959). One mililitre of reaction mixture contained 0.3 ml of substrate (1% w/v aqueous suspension of the swollen chitin), 0.6 ml of 0.05 M citrate-phosphate buffer (pH 6.0) and 0.1 ml of the enzyme sample (culture supernatant). Reaction mixture was incubated in a shaker water bath (100 rpm) at 35°C for 60 min. Reaction was stopped by adding 1 ml of dinitrosalicylic acid followed by boiling in a water bath for 10 min. The reaction mixture was diluted obtain to reach a total volume of 100 ml with distilled water. The solution was then filtered by using Whatman filter paper (Grade 613). The A_{540} of the filtrate was measured on systronics 106 colorimeter. A standard curve for N-acetyl glucosamine was carried out in parallel to measure reducing sugar released. A unit of enzyme activity was defined as the amount of enzyme required to release 0.5 micromole of NAG h^{-1} at 37°C.

Estimation of protein

Protein content of all enzyme preparations was measured by Biuret method.

Criteria used for species level identification

The following criteria were considered for the identification of actinomycetes isolates:

Microscopic examination (Kawato and Shinobu, 1951).
Chemical composition of cell wall (Toru Hasegawa et al., 1983).
Whole cell sugar composition (Toru Hasegawa et al., 1983).
Biochemical characters (Williams et al., 1989).
Nutritional characters (Shirling and Gottlieb, 1966).

Study of effect of chitin concentration on chitinase activity

The crude extract of enzymes was incubated with different (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 1.8%) concentrations of chitin at 30°C for 1 h in a shaker incubator. The enzymes activity was measured. The experiment was performed in triplicate.

Effect of pH and temperature on chitinase activity

Crude extract of enzymes with substrate was subjected to different pH levels; 2, 4, 6, 8 and 10. Level of pH, in the reaction mixture, ranging from 2 - 10 were adjusted by using four different buffers (0.05 M); glycine HCl (pH range 2.2 - 3.4), acetate buffer (pH range 3.6 - 5.6), phosphate buffer (pH range 6.0 - 8.0) and glycine NaOH (pH range 8.6 - 11.2). The mixture was incubated at 35°C for 1 h on shaker incubator.

The crude extract of enzymes with substrate was incubated at temperatures ranging from 20 to 70°C for 1 h in a shaker incubator. The enzyme activity at the end of the incubation period was measured. The experiment was performed in triplicate.

RESULTS

Of the 80 actinomycete isolates screened for chitinolytic activity, fifty two isolates tested showed clear zones of hydrolysis of chitin. Isolates showing chitinase activity were observed for their Selection Ratio, out of which three isolates KS10, RE14 and BH1 were found to have maximum ratios (S/R) of 4.5, 4.4 and 5.2 mm, respectively. The three isolate identified were actinomycetes (KS10) *Streptomyces canus,* (RE14) *Micromonospora brevicatiana* and (BH1) *Streptomyces pseudogriseolus.*

The time of production of detectable levels of chitinase by *S. canus, M. brevicatiana*, *S. pseudogriseolus* was investigated. *S. canus* and *S. pseudogriseolus* produce the enzyme at 84 h whereas *M. brevicatiana* produced the enzyme at 96 h. There was a gradual increase in chitinase production up to 144 h (Table 1) after which chitinase activity decreased. The pH of production medium was 8, it declined to 6.5 within the first 60 h after which it increased up to 8.5 during 144 h of chitinase activity.

Growth of the isolates was measured in terms of protein after every 12 h of incubation. It was found that the protein content increased from 5.02 to 14 mg ml^{-1} 108 h, after which the protein content decreased until 144 h of incubation.

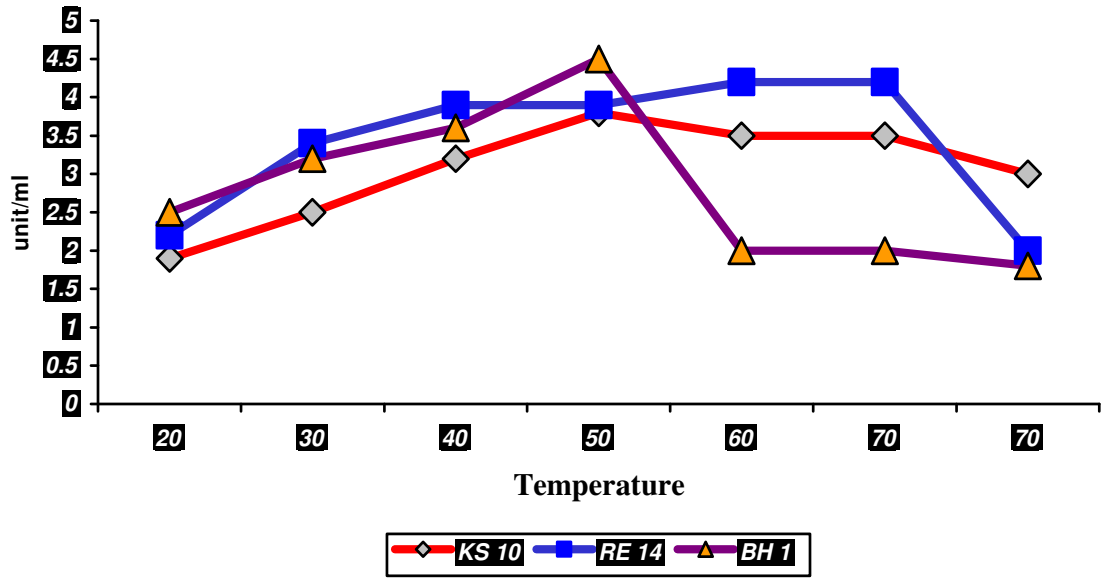

Figure 1. Effect of temperature on chitinase activity. KS 10 = *Streptomyces canus*; RE 14 = *Micromonospora brevicatiana*; BH 1 = *Streptomyces pseudogriseolus.*

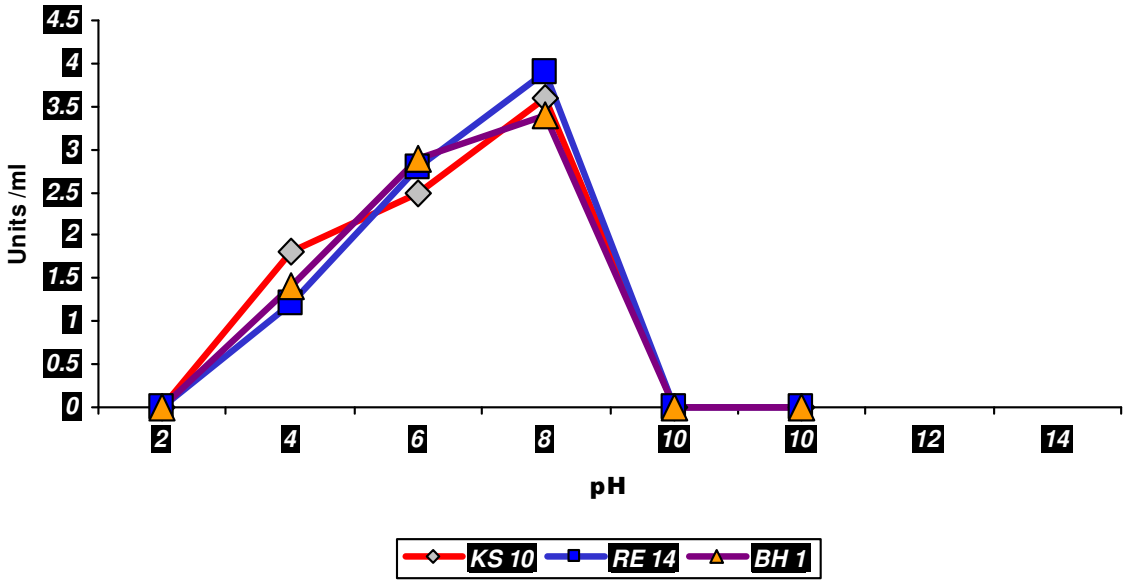

Figure 2. Effect of pH on chitinase activity. KS 10 = *Streptomyces canus*; RE 14 = *Micromonospora brevicatiana*; BH 1 = *Streptomyces pseudogriseolus.*

Effect of temperature and pH on chitinase activity

It was observed that the enzyme produced by *S. canus* and *M. brevicatiana* showed maximum activity from 40 to 60°C, *S. pseudogriseolus* showed maximum activity from 40 to 50°C. Thereafter the activity sharply declined (Figure 1). When chitinase was when assayed at different pH for activity, optimum activity was at pH 8. No activity was observed at pH 2 and 10 and lowest recorded activity was observed at pH 4 (Figure 2).

Effect of substrate concentration on chitinase activity

Colloidal chitin was used to determine the effect of substrate concentration on the chitinase activity. It was observed that *S. canus* showed maximum activity at 1.2% chitin concentration. (4.8 unit ml^{-1}) and the activity remained constant up to a concentration of 1.6%. *Micromonospora. brevicatiana* showed maximum activity at substrate concentration 1.2% and the activity decreased thereafter. *S. pseudogriseolus* showed activity up to 1%

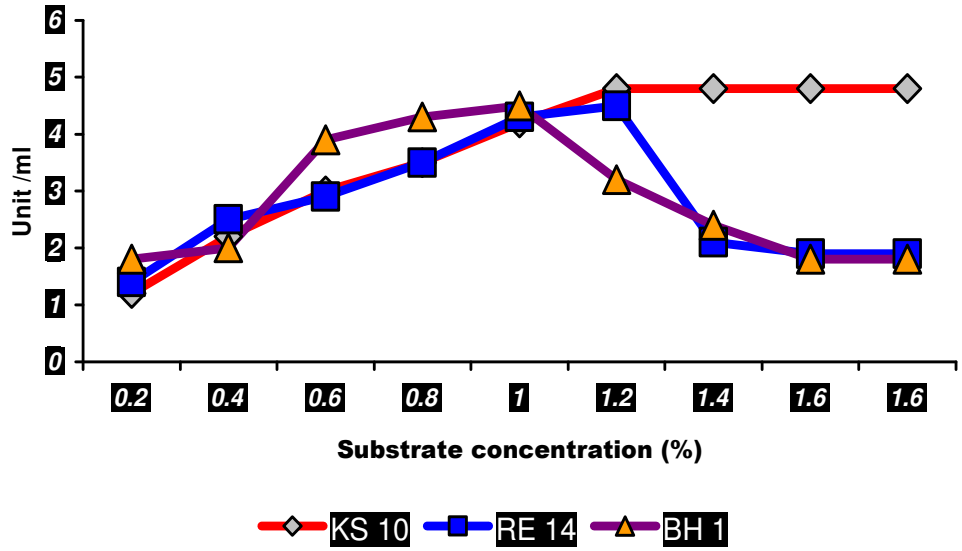

Figure 3. Effect of substrate on chitinase activity. KS 10 = *Streptomyces canus*; RE 14 = *Micromonospora brevicatiana*; BH 1 = *Streptomyces pseudogriseolus.*

substrate concentration and declined thereafter (Figure 3).

DISCUSSION

In this study on chitinase production, extra cellular chitinase activity was determined. There was no chitinase, until 46 h of incubation, after which the amount increased from 84 to 120 h. The chitinase activity remained until 144 h. Similar observations had been made by Young and Bell (1985) and Neugebour, (1991) during production of chitinase from *S. marcescens* and *S. lividans*, respectively. The growth of the culture was slow at the beginning and was exponential after 84 h. Enzyme production increased was in exponential phase and more amounts were detected in the stationary growth phase. Maximum enzyme activity was detected in the decline growth phase.

Chitinases are fairly stable over broad pH range. The pH stability of chitinase varies from organism to organism. Chitinase from *Streptomyces* are found to be stable over a pH range of 4.0 to 10.0. Chitinases of *S. marcescens* and *Serratia liquifaciens* have pH optima between 5.0 to 6.0 (Brurberg et al., 1996). *Streptomyces. erythraceus* chitinases has an optimum pH 5.0 (Hara et al., 1989). According to our study chitinases from *S. canus*, *M. brevicatiana* and *S. pseudogriseolus* have optimum activity at pH 8.

The temperature optima for chitinases range from 40 - 60°C depending on source. Optimum temp for *S. lividans* is 50°C. *S. erythraceus* chitinase has optimum activity at 60 - 70°C but the enzyme was not stable above 60°C (Hara et al., 1989). According to our study, *S. canus* and *M. brevicatiana* showed maximum activity at 40 - 60°C. Substrate concentration of *S. pseudogriseolus* showed maximum activity at 40 - 50°C. 1.2 to 1.6% gave optimum chitinases activity forom all isolates.

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