African Journal of Microbiology Research Vol. 4(24) pp. 2822-2827, 18 December, 2010
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2010 Academic Journals

Full Length Research Paper

Purification and characterization of chitinase from *Micrococcus* sp.AG84 isolated from marine environment

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Accepted 7 December, 2010

Chitinase producing bacterial strain *Micrococcus* sp. AG84 isolated from marine sediments grew maximally in shake flask and produced chitinase at 35°C, pH 8.0. Chitinase activity was found to be maximum at 45°C, pH 8.0, and the enzyme was 100% stable even at 60°C and pH 11.0. Added with Fe^{2+}, Ca^{2+} and Ni^{2+} chitinase activity increased but it was inhibited by EDTA. The molecular weight of purified chitinase is 33 kDa.

Key words: *Micrococcus* sp. AG84, chitinase, thermostable, alkaline, marine.

INTRODUCTION

Chitin is a polysaccharide consisting of 1,4-linked N-acetyl- D-glucosamine moieties, the second most abundant biopolymer and also a constant source of renewable raw materials on earth. Along with its deacetylated derivative chitosan, chitin has recently gained much biotechnological significance, not only because of their favorable pharmaceutical features, such as antimicrobial, anticholesterol, and antitumor activities, but also because of its potential for wastewater treatment (Flach et al., 1992), drug delivery (Kadowaki et al., 1997), wound healing and as a dietary fiber (Dixon, 1995). Hence, identification of chitin modifying enzymes and elucidation of their activities could facilitate the efficient production of specific chitin products.

Chitinases are a group of enzymes which hydrolyses the β-1, 4-linkages in chitin to low-molecular-weight products and have been shown to be produced by a number of microorganisms. Generally, chitinase-producing strains will use chitin or colloidal chitin as a carbon source and comprise of chitinases (EC 3.2.1.14) and N-acetylglucosaminidase (EC 3.2.1.52). Chitinases can be classified into two major categories. Endochitinases (EC 3.2.1.14) cleaves chitin randomly at internal sites, generating low molecular mass multimers of GlcNAc, such as chitotriose, chitotetraose and diacetylchitobiose. Exochitinases can be divided into two subcategories: chitobiosidases (EC 3.2.1.29), which catalyze the progressive release of diacetylchitobiose starting at the non-reducing end of chitin microfibril, and β-(1,4) N-acetyl glucosaminidases (EC 3.2.1.30), which cleaves the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc (Sahai and Manocha, 1993).

Chitinase is widely distributed in bacteria, actinomycetes and plants which play an important role in the degradation of chitin in the oceans (Park et al., 2000). Chitinases from marine bacteria have been isolated and their properties reported, play a role in nutrition and parasitism. In addition to the above potential applications, chitinases can be used for the production of chitooligosaccharides, which have been found to function as antibacterial agents, elicitors of lysozyme inducers and immune-enhancers (Wen et al., 2002). Chitinases can also be used in agriculture to control plant pathogens (Dahiya et al., 2005). However, the preparation of chitin involves demineralization and deproteinization of shellfish waste with the use of strong acids or bases (Synowiecki and Al-Khateeb, 2000; Wang et al., 2008). The utilization of shellfish waste not only solves environmental problems but also decreases the production costs of microbial chitinases. The production of inexpensive chitinolytic enzymes is an important element in the process

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(Wang et al., 2008; Liu et al., 2005).

In the present study, we describe the isolation of a chitinases producing bacterium, *Micrococcus* sp. AG84 from marine environment, purification and characterization of chitinase.

MATERIALS AND METHODS

Isolation and identification of chitinase producing bacteria

Chitinase producing bacteria was isolated from marine sediment collected at parangipettai (Latitude, 11°46' Longitude, 79°46'), east coast of India, using an enrichment procedure. The enriched culture broth was serially diluted and spreaded on chitin agar plates containing (g/l) 1.0% swollen chitin, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.01% $MgSO_4.7H_2O$ (pH 9.0) and incubated at 37°C for 48 h. Isolated colonies were screened for further chitinase production. The colonies with highest clear zone was considered as potential strain and subjected to morphological and biochemical identification. The chitinase producing bacterial strain was identified according to Bergey's Manual of Determinative Bacteriology (Buchnan and Gibbons, 1974).

Enzyme production in shake flasks

Chitinase production was done by culturing the bacteria into the production medium which contains 1.0% swollen chitin, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4 and 0.01% $MgSO_4.7H_2O$ (pH 9.0). Chitinase production was performed in shake flasks, in a 500 ml conical flask containing 100 ml of production medium. The culture conditions were as follows: pH 9.0, temperature 37°C, and 1.0% chitin and 150 rpm. Culture medium was seeded with 1% inoculum (1.85x106 CFU ml^{-1}) and maintained at above culture conditions for 48 h. Bacterial growth and enzyme production was determined from the samples collected at 6 h intervals. Growth was estimated by measuring optical density of the culture broth collected at different time intervals at 600 nm.

Purification of chitinase enzyme

The culture from the shake flask was harvested after 72 h and the cells were removed by centrifugation at 6000 x g for 30 min at 4°C. The cell free supernatant was used as crude enzyme. The crude chitinase was precipitated with ammonium sulphate at 60% saturation and allowed to stand overnight at 4°C. The precipitates were collected by centrifugation and dialyzed against 50 mM Tris-HCl buffer, pH 9.0 for 24 h. Dialyzed enzyme solution was loaded onto a DEAE-cellulose column (2.0 X 25 cm) equilibrated with Tris-HCl buffer, pH 9.0. The enzyme was eluted with linear gradient of NaCl (0-1 M in 100 mM Tris-HCl buffer) at a flow rate of 25 ml/h. The eluted fractions were assayed for enzyme activity. This partially purified enzyme solution was used to investigate the effects of temperature, pH and various metal ions on enzyme activity and stability.

Enzyme assay

Chitinase activity was measured with colloidal chitin as a substrate. Enzyme solution (0.5 ml) was added to 1.0 ml of substrate solution, which contained a 1.3% suspension of colloidal chitin in 50 mM Tris-HCl buffer (pH 9.0). The mixture was incubated at 37°C for 30 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by the Dinitrosalicylic acid (DNS) method (Miller, 1959) with N-acetylglucosamine as a reference compound. One unit of chitinase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugars per min.

Protein estimation

The amount of protein in crude as well as purified enzyme was determined according to the method of Lowry et al. (1951) with Bovine serum albumin (HiMedia, Mumbai) as standard.

Effect of temperature for enzyme activity and thermostability

Optimum temperature for enzyme activity was determined by incubating the reaction mixture at different temperature levels. Thermostability of the enzyme was performed in eppendorf tubes (1.5 ml) containing 0.2 ml of purified enzyme in 50 mM in Tris-HCl buffer (pH-9.0) was incubated at 35, 40, 50, 60, 70, 75, 80, 85, 90 and 95°C for 1 h, aliquots were withdrawn every 15 min and the residual activity was determined by standard assay condition with colloidal chitin as a substrate.

Effect of pH on enzyme activity and stability

The effect of pH on enzyme activity was carried out with five different buffer systems (50 mM): Citric acid-Na_2HPO_4 (pH 5 to 6), phosphate buffer (pH 6 to 8), boric acid-NaOH buffer (pH 8 to 9.5), phosphate-NaOH buffer (pH 9.5 to 12) and sodium hydroxide solution buffer (12-13) and the residual activity was determined by the standard assay. The stability of the enzyme was evaluated by incubating enzyme solution at different pH for 1 h and the residual activity was assayed.

Effect of metals on enzyme activity

The effect of metals on enzyme activity was determined by incubating the enzyme with different metals (5 mM concentration) at room temperature for 1 h. The activity of enzyme without any metal was considered as control and the activity was taken as 100%.

Molecular weight determination

SDS-PAGE was performed to estimate the molecular weight of the purified protein using 5% stacking gel and 12% resolving gel according to the method of Laemmli (1970). Molecular weight was estimated by comparing the relative mobility of proteins of different molecular sizes, using a standard molecular weight marker (205, 97.4, 66, 43 and 29 kDa) (Genei; Bangalore, India).

RESULTS

Microorganism

The colony of the strain was smooth, circular and dirty white. The strain was Gram positive Cocci (Figure 1) and it grows between the temperature range of 25 to 45°C. The strain was negative for indole, methyl red, Vogus Proskuer, catalase test and it is positive for oxidase and nitrate reduction test. The strain utilized carbohydrates

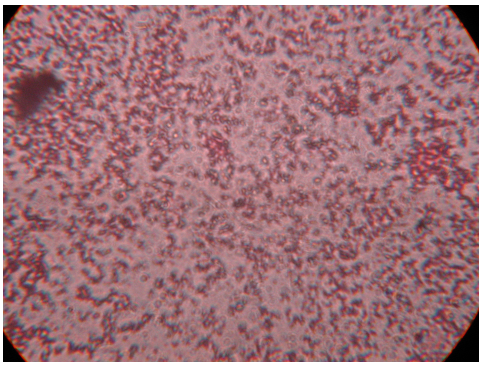

Figure 1. Microscopical view of chitinase producing bacterium *Micrococcus* sp. AG84.

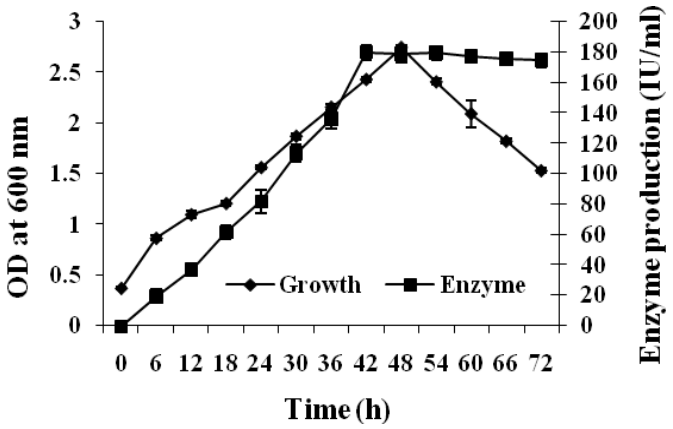

Figure 2. Bacterial growth vs. enzyme production. Results represent the means of triplicates and bars indicate standard deviation. Absence of bars indicates that errors were smaller than symbols.

Table 1. Morphological and biochemical characteristics of *Micrococcus* sp. AG84.

S. no	Biochemical reactions	Results
1	Gram staining	+
2	Shape	Cocci
3	Oxidase	+
4	Catalase	+
5	Nitrate reduction	+
6	Mannitol	+
7	Sucrose	+
8	Dextrose	+
9	Galactose	+
10	Arabinose	+
11	Dulicitol	-
12	Cellobiose	+
13	Maltose	+
14	Lactose	-
15	Sorbitol	-
16	Growth at temperature	37ºC

such as mannitol, sucrose, dextrose, galactose, arabinose, cellobiose and it did not utilize lactose, sorbitol and dulicitol. From these results of physiological and biochemical characteristics, the strain was identified as *Micrococcus* sp. AG84 (Table 1). Enzyme production was started from the logarithmic phase itself and maximum production occurred at (175 IU/ml) 42 h. But bacterial growth, maximum growth occurred at the 48th h of the culture (Figure 2).

Enzyme purification and characterization were performed at room temperature (28±2°C) and the summary of purification steps were presented in Table 2. Purification of chitinase with ammonium sulphate followed by DEAE-cellulose column chromatography yielded 43.72 and 19.95% recovery with 6.71 and 10.33-fold respectively. The molecular weight of the protein was estimated as 33 kDa (Figure 3). The enzyme was active at temperatures between 30 to 50°C and optimum being at 40°C. The enzyme was 100% stable even up to 60°C and also it exhibited 83% activity at 70°C. At 90°C and above, it completely lost its activity (Figure 4).

The chitinase from *Micrococcus* sp. AG84 was active between wide ranges of pH between 7 to 13 and optimum being at pH 8.0. Regarding stability, the enzyme was 100% stable between pH 9 to 11 and it retained more than 80% of its activity at pH 12.0 and 75% at pH 13.0 (Figure 5). The effect of various metal ions on the activity of the purified enzyme is shown in Table 3. The metals like Fe^{2+}, Ca^{2+}, Ni^{2+} and Mn^{2+} enhanced the enzyme activity whereas CO^{2+}, Cu^{2+} and Hg^{2+} inhibited the enzyme activity and all the other metals were neither enhanced nor inhibited. The metal chelator EDTA also slightly inhibits the enzyme activity (60%) (Table 3).

DISCUSSION

Microbial production of chitinase had great attention of both industrial and scientific environments, not only because of its wide spectrum of applications but also for the lacuna of an effective production method (Ahmadi et al., 2008). In the present study, an extracellular chitinase producing bacteria isolated from marine environment, identified as *Micrococcus* sp. AG84 was screened for chitinase production. However, many more bacteria have been studied for chitinase production such as *Vibrio* sp. (Park et al. 2000), *Enterobacter* sp. (Dahiya et al., 2005), *Aeromonas* sp. (Ahmadi et al., 2008), *Alcaligens xylosoxydans* (Vaidya et al., 2003).

The extracellular chitinase produced by *Micrococcus* sp. AG84 was purified by two step purification method such as ammonium sulphate precipitation, DEAE

Table 2. Summary of the purification of chitinase.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture filtrate	1812	16315.2	9.003	1.0	100
$(NH_4)_2SO_4$ Precipitate	118	7134	60.45	6.71	43.72
DEAE-Cellulose	35	3256	93.02	10.33	19.95

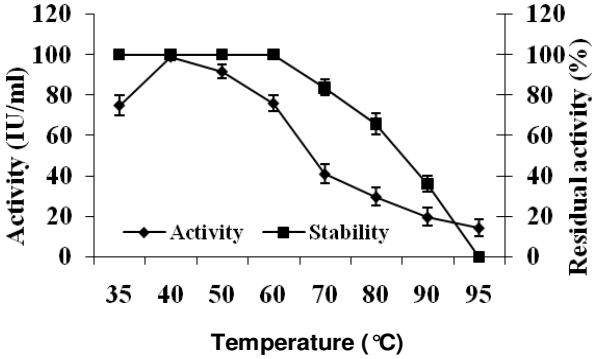

Figure 3. Effect of temperature on enzyme activity and stability. Temperature stability of the purified enzyme was tested by pre-incubating at different temperatures for 1 h. The residual activity was measured by standard assay conditions. The pre-incubated samples at 4°C were used as control. The values are mean ± SD, n=3. Absence of bars indicates that errors were smaller than symbols.

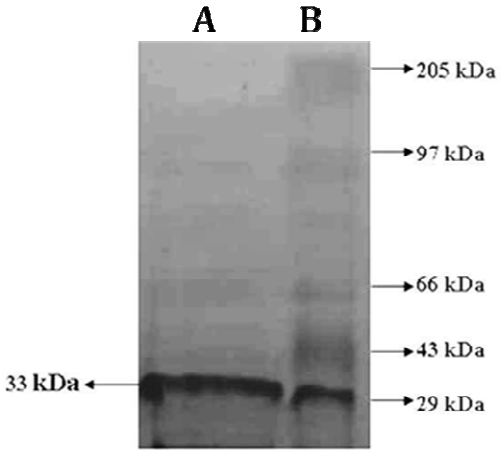

Figure 5. SDS-PAGE analysis of purified chitinase. **Lane 1**, Purified chitinase; myosin (205 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa). **Lane** 2, Molecular markers.

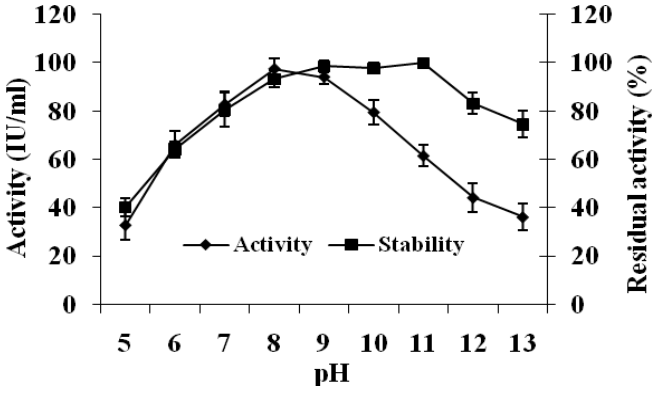

Figure 4. Effect of pH on enzyme activity and stability. The enzyme activity was assayed at 37°C for 10 min and the stability was measured after 1-h pre-incubation of the enzyme at 4°C in various buffers. The values are presented in percentages of the maximum activity under standard assay conditions. The values are mean±SD, n=3. Absence of bars indicates that errors were smaller than symbols.

Table 3. Effect of metal ions on chitinase activity.

Metals (5 mM)	Residual activity (%)
$MnSO_4$	103
$FeSO_4$	126
$CoCl_2$	70
$MgSO_4$	98
$CuSO_4$	48
$CaCl_2$	146
EDTA	60
$HgCl_2$	42
$NiCl_2$	123

cellulose chromatography, and chitinase purified 6.71 fold with 19.95% recovery (Table 1). But only a 5.1 fold purification of chitinase was reported from *Serratia marcescens* NK1 (Nawani and Kapadnis, 2001) with ammonium sulphate precipitation and gel filtration chromatography. The molecular weight of the chitinase from *Micrococcus* sp. AG84 was determined as 33 kDa by SDS-PAGE. The molecular weight of other chitinase from *Bacillus circulans* was 45 kDa (Wiwat et al. 1999) and 57 kDa from *S. marcescens* (Nawani and Kapadnis, 2001). The molecular weights of microbial chitinases range from 20,000 to 120,000 with little consistency. The

Table 4. Biochemical properties of some of the previously reported chitinases.

Strains	References	Optimum temperature (°C)	Optimum pH	Thermostability (°C)	pH stability	Molecular weight (kDa)
Enterobacter sp.	Dahiya et al. (2005)	45	5.5	40	4.5-8.0	60
Vibrio sp.	Park et al. (2000)	45	6.0	45	6.0	98
Trichoderma atroviride	Hiraga et al. (1997)	40	5.0	60	5.0	42
Arthrobacter sp.NHB-10	Okazaki et al. (1999)	40	5.0	45	5.5	-
Pseudomonas aeruginosa	Wang and Chang (1997)	50	8.0	50	6-9	30-32
Vibrio alginolyticus-H-8	Ohishi et al. (1996)	50-55	6.5	40	7.0	-
Micrococcus sp.AG84	Present study	45	8.0	70	11.0	33

molecular weight of bacterial chitinases are mostly around 60,000 to 110,000, while those of actinomycetes are mostly 30,000 or lower, fungi are higher than 30,000. The molecular weight of plant chitinases are mostly around 30,000. The enzyme was highly active in the temperature range of 30 to 50°C but optimum at 40°C and pH 8.0. The results on temperature optimum were similar to the previous chitinase from *Aeromonas hydrophila* (Hiraga et al., 1997), *Pseudomonas aeruginosa* (Wang and Chang, 1997). Most of the bacterial chitinases were active at acidic pH (Dahiya et al., 2005; Murao et al., 1992; Ohtakara et al., 1979).

Conversely, the chitinase from *Micrococcus* sp.AG84 was not like all the other bacterial chitinases, it is active at pH 8.0. Regarding stability, the enzyme was 100% stable even at 60°C and pH 11.0 but most of the chitinases from *Arthrobacter* sp. NHBN-10 (Okazaki et al., 1999), *Vibrio alginolyticus* (Ohishi et al., 1996) were only stable between the temperature 40 to 50°C and pH 7.0 to 9.0 (Table 3). Hence this chitinase was comparatively better for industrial uses. Metals ions interfered in the activity of chitinase of the *Micrococcus* sp. AG84 at 5 mM concentration. The activity of the enzyme was stimulated by the addition of metals like Fe^{2+}, Mn^{2+}, Ca^{2+}, and Ni^{2+} but EDTA, Hg^{2+}, Co^{2+} and Cu^{2+} inhibited the enzyme activity. Other chitinases from *A. hydrophila* H-2330 (Okazaki et al., 1999), *Alteromonas* sp. O-7 (Tsujibo, 1992), *E. americana* (Inglis and Peberdy, 1997), *P. aeruginosa* K-187 (Wang and Chang, 1997), and *Fusarium chlamydosporum* (Mathivanan et al., 1998) were also inhibited by Fe^{2+}, Fe^{3+} and Cu^{2+}. Chitinase from *Alteromonas* sp. strain O-7 (Tsujibo et al., 1992) was activated by Na^{+} and Ca^{2+}.

The stimulation of chitinase by Fe^{2+} and Cu^{2+} could be related to the residues of aspartic and glutarmic acid in chitinases. It has been shown that these amino acids in the active sites of chitinases bind to certain divalent cations, thereby possibly stimulating chitinases (Milewski et al., 1992). In the present study, the chitinase which was active at alkaline pH and stable up to 60°C, was isolated from marine bacteria *Micrococcus* sp. AG84 (Table 4). From these promising results, this chitinase can be applied to use in the synthesis of chitin derivatives.

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