JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2009. Vol. **3**(1), p. 67-74

Characterisation of Extracellular Cellulase (Endoglucanase) Activity of *Bacillus subtilis* and *Bacillus stearothermophilus* Isolated from Marine Sponges of Gopalpur Coast, Orissa (Bay of Bengal)

A. Vimla, C. Chandi Rath[1]* and A. Sree

Forest and Marine Product Division, Institute of Mineral and Material Technology(IMMT),Bhubaneswar, India.
Formerly: Regional Research Laboratories (CSIR), Bhubaneswar, India.
[1]P.G. Departmentof Botany, North Orissa University, Takatpur, Baripada - 757 003, India.

(Received: 18 July 2008; accepted: 03 October 2008)

In the present study 83 bacteria in toto were isolated from eleven sponges; one gorgonian and one antipatherian from the Bay of Bengal and screened for their extracellular cellulase activity. Thirteen isolates were positive for cellulase activity. Three isolates with higher zones of activity viz. *B. stearothermophilus* (RRL-11), *B. subtilis* (RRL-12) and *B. subtilis* (RRL-36) were selected for further characterization studies using two different media (Medium-1 and Medium-2). Optimum pH for enzyme activity was 7.5 except RRL-12 which showed a range of pH 6-8 at which the activity was stable (medium-1). Optimum temperature for enzyme activity was 40° C while RRL-11 and 12 exhibited a stable range of 50°–70° C and 45°-65° C respectively. Of the three isolates studied, RRL-36 showed an optimum NaCl concentration of 7% for maximum enzyme activity. From metal ions studies, Ca^{2+} was found to be the inhibitor ion.

Key words: Extracellular cellulase, Bacteria, Marine Sponges, Bay of Bengal.

Scientific investigations have been carried out on the saccharification of cellulose waste material by enzymes[26]. Potential application of cellulose in biotechnology has been reviewed[13]. Very few reports are available on endogluconase production from marine sources. Forty-one bacteria have been isolated[9] from several invertebrates, macroalgae, sea grass, and the surrounding water that exhibited different patterns of hydrolytic enzyme activities measured as the hydrolysis of either native biopolymers or fluorogenic substrates. There are reports on endogluconase production from shipworm (*Psiloteredo healdi*)[12], endogluconase from shipworm (*Teredenobeta turniral*)[1] and from marine rotifer *Brachinous plicatilis*[6]. Certain marine *Vibrio* sp., *Streptomyces* and fungi exhibited cellulolytic activity. Among marine invertebrates, sponges remain the most prolifically studied phylum in the search for novel pharmacologically active compounds[10]. Some

* To whom all correspondence should be addressed.

enzymes with interesting features have been isolated from sponges and the microbes associated with sponges[18,22]. In contrast to the literature on natural compounds, studies on enzymes with biotechnological potential from microbes associated with sponges are rare. Bacteria isolated from six marine sponges (*Spirastrella* sp., *Phyllospongia* sp., *Ircinia* sp., *Aaptos* sp., *Azorica* sp., and *Axinella* sp.) were identfied to produce amylase, carboxymethylcellulase, and proteases[24].

Therefore, the present work was aimed at investigating cellulolytic activity of bacterial isolates associated with marine sponges.

MATERIAL AND METHODS

The sedentary fauna were collected from Bay of Bengal at Gopalpur, Orissa coast from a depth of 30 meters by SCUBA drivers[28]. The bacteria were isolated from sponges using different media as described earlier[27]. Isolation and identification of bacterial isolates was carried out by following standard microbiological methods[28]. All the 83 bacterial isolates were screened for cellulose activity on cellulose test agar plates containing nutrient agar medium supplemented with 1% (w/v) carboxymethyl cellulose. A small portion of the cell paste of the isolate was scraped and deposited onto the carboxymethylcellulose agar plates. After incubation at 37ºC for 2 days, petriplates were washed with an aqueous solution of congo red (1% congo red in distilled water) for 10 min. The congo red solution was then poured off, plates were further flooded with 1 N NaCl for 5-10 min. The bacterial growth was stopped by flooding the CMC agar plates with 1 N HCl, which changed the dye color to blue-violet (pH 0.1). A clear zone of hydrolysis around cells of marine bacteria indicated cellulolytic activity. Bacterial isolates showing higher zones were taken for further characterization.

Culture of bacterial isolate

For cellulase production the bacterial isolates were cultured in two different liquid media at pH 7. In the first medium (Medium-1) nutrient broth along with 0.1% CMC was prepared in 50% aged seawater. The second medium (Medium-2) contained 0.5% peptone, 0.5% $MgSO_4.7H_2O$, 0.1% KH_2PO_4 and 0.1 % CMC in 50% aged sea water. 200ml of each liquid media was inoculated with 1ml of bacterial culture. The cultures were incubated (at 37°C) on a rotary shaker at 200 rpm for 48hrs.

Preparation of enzyme

The bacterial culture was centrifuged at 12,000rpm in a cooling centrifuge (Remi R24 model) at 4°C for 30 min and the supernatant was collected. The supernatant was aseptically, passed through a 0.22µm (Sartorius) Millipore filter paper. The filtered supernatant was taken as enzyme solution for assay.

Assay method

Cellulase activity was determined by using CMC as the substrate[21]. The glucose liberated in the endoglucanase activity was measured by dinitrosalicylic acid method[23] .One IU of enzyme activity was defined as the amount of enzyme that liberates 1 ìmole of glucose per minute under the experimental conditions. The specific activity was calculated as IU/ mg protein. For the assay, 4ml of CMC (1% CMC in a 50 mmol/1 acetate buffer pH-5.2) was pipetted out in a test tube followed by 1ml of buffer and 2ml of enzyme solution. The test tubes were kept on water bath at 30°C for 2 hrs. 1ml of the reaction mixture was pipetted out into a test tube after 30 min of incubation. Then, 1ml of DNS reagent was added to it. The mixture was heated in a boiling water bath for 5 min. To it 1ml of Rochelle's salt was added when the mixture was hot. Tubes were cooled under tap water. The optical density was measured at 575 nm. Amount of reducing sugar released in the reaction mixture was calculated using standards prepared from glucose.

The effect of time on enzyme activity was evaluated by incubating the assay mixture at different time intervals (30, 60, 90 and 120 min) at 37°C. Enzyme activity was measured after each incubation period.

To study the enzyme activity at different pHs, the enzyme preparation was incubated with 1% CMC in 0.1M citrate buffer (pH 3.0-6.0), 0.2M phosphate buffer (pH 6.5-8.0) and 0.2M glycine-NaOH buffer (pH 8.5-10.5). The assay was carried out as described above.

The effect of temperature on enzyme activity was evaluated by incubating the enzyme preparation at different temperature (40°-80°C)

at increment of 5°C for 30 min. All further, assays were performed in the buffer showing optimum activity

The enzyme solution was pre-incubated with individual metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, K^{+} and Cd^{2+}) at concentrations ranging from 1-10 mmol with 2mmol increments. Separate blanks with individual metal ions were prepared. **The residual activity was measured**

Protein content of enzyme samples was measured with the Folin phenol reagent[19].

RESULTS AND DISCUSSION

A total of 83 bacteria were isolated from eleven sponges; one gorgonian, one unidentified antipatherian and sediment along with 25 other isolates, which lost their viability during cultivation[28]. Based on the screening studies of 83 bacterial isolates, 13 (15.66%) isolates were found to be positive for extracellular endoglucanase activity. The producer isolates belonged to genera *Acinetobacte*r, *Bacillus*, *Citrobacter*, *Micrococcus*, *Pseudomonas* and *Vibrio*.

Based on the screening results on CMCase plates, three isolates with higher zones of activity *viz*., *Bacillus stearothermophilus* (RRL-11), *Bacillus subtilis* (RRL-12) from the sponge *Acanthella ramosa* and *Bacillus subtilis* (RRL-36) from *Raspailia* sp were selected for further studies and characterization of cellulases.

Enzyme production was more in culture media containing 0.1% CMC and nutrient broth (medium-1) for *B. subtilis* (RRL-12) and *B. subtilis* (RRL-36) while media containing 0.1% CMC along with 0.5% $MgSO_4.7H_2O$, 0.5% KH_2PO_4 and peptone (medium-2) showed more enzyme production for *B. stearothermophilus* (RRL-11). All the three isolates showed extracellular endoglucanase activity. Extracellular cellulase producing bacteria were reported by many authors. Extracellular cellulase was reported from *Pseudomonas fluorescens*[29]. An extracellular alkalophilic cellulase was reported from a *Bacillus* sp (N-1139) [11]. An extracellular cellulase from a thermophilic fungus *Thermoascus aurantiacus* was reported[20].

The effect of time and media on endoglucanase activity is shown in Fig. 1. Maximum enzyme activity was seen at 30 min incubation for the two isolates RRL-36 and RRL-12. But a degree of variability was observed in case of the isolate RRL-11. A slight increase in enzymatic activity was reported in medium-1 up to 90 min and the activity remained constant up to 90 min. in medium-2. A decrease in enzyme activity was reported on further incubation.

The optimum pH for enzyme activity of RRL-36, RRL-12 and RRL-11 in both the media is shown in Figure 2. Maximum enzyme activity of *B. subtilis* (RRL-36) was obtained at pH 8 in medium-1 and at pH 7.5 in medium-2. The enzyme activity of RRL-36 was greatly reduced beyond pH 8. The optimum value of both RRL-12 and RRL-11was observed to be pH 7.5. Only RRL- 12 showed a range of pH 6-8 at which the activity was stable (medium-1). The optimum pH is comparatively higher than that of *Euphausia suberba* isolated from Antarctic krill[5], *Alcaligenes* sp.[2] and *Streptomyces* sp. isolated from the digestive tract of *Barnea birmanica philippi*[3].

In our experiments *B. subtilis* (RRL-36) from the sponge, *Raspailia* sp showed highest enzyme activity at alkaline pH (8.0). Similar results were reported from *Bacillus* sp isolated from *Axinella* sp showing optimum endoglucanase activity at pH 8[25]. CMCase activity of cell suspension of *Cytophaga* sp, showing a maximum activity at pH 8 was reported[4].

In our results optimum CMCase activity of most of the isolates was found at pH 7.5 and more than 50% of the activity was retained at pH 8, which is higher than that reported[16], where an extracellular cellulase producer *Streptomyces* sp showed optimum enzyme activity at pH 7 and temperature of 40°C.

An alkaline *Bacillus* sp N-4 that produced multi CMCase which was active over broad pH range (pH 5-10) was reported[15]. An extracellular alkalophilic *Bacillus* sp (N-1139), which was stable over the pH 6-11 and most active at pH 9 with stable activity at 40°C was reported[11]. Alkaline cellulase from alkalophilic organisms *Bacillus* sp. B38-2 and *Streptomyces* sp. S36-2 that showed optimum pH and temperature of crude enzyme activity in the range from pH 6-7 at 55°C for *Streptomyces* sp and at pH 7 and 60°C for *Bacillus* sp. was

reported[7]. This shows that cellulases of present investigation are comparable to the cellulases studied from different alkalophilic organisms.

The temperature and media effect on the enzyme activity of the three isolates is shown in Figure 3. The optimum temperature for maximum endoglucanase activity of all isolates was found to be 40°C. However, the two isolates RRL-11 &12 exhibited stable enzymatic activity within a range from 50-70°C and 45-60°C respectively.

The effect of NaCl on endoglucanase activity of RRL-36, RRL-11 and RRL-12 is shown in Table .1.

The optimum NaCl concentration for maximum enzyme activity was found to be 7% and 6% for RRL-36 in medium-1 and medium-2 respectfully. The optimum NaCl concentration of maximum enzyme activity for RRL-12 was observed to be 5% and 3% and 2% and 4% for RRL-11 in medium-1 and medium-2 respectfully. Similar results which showed NaCl as an important factor for endoglucanase synthesis by marine organisms were reported (3, 8). An optimum endoglucanase activity at 3% NaCl concentration was observed by *Bacillus* sp[25], which is comparatively less to that of our findings. The effect of metal ions and media on the endoglucanase activity of RRL-36, RRL-11 and RRL-12 is shown in Table. 2, 3 and 4.

The enzyme activity of RRL-36 in medium-1 was activated by Mg^{2+}, Mn^{2+}, K^{+} and Cd^{2+} (10mmol/l) and inhibited by Cu^{2+} and Ca^{2+} at 25mmol/l. Inhibition of enzyme activity of *Alcaligenes* sp. due to Cu^{2+} and Mn^{2+} has been

Table 1. Effect of NaCl concentration on enzyme activity

% of NaCl	Isolates (IU/mg protein)					
	RRL-36		RRL-11		RRL-12	
	Medium-1	Medium-2	Medium-1	Medium-2	Medium-1	Medium-2
1	0.264	0.238	0.130	0.366	0.336	0.468
2	0.264	0.252	0.171	0.432	0.351	0.522
3	0.279	0.421	0.126	0.450	0.360	0.570
4	0.291	0.431	0.102	0.459	0.378	0.357
5	0.333	0.470	0.100	0.447	0.456	0.354
6	0.334	0.503	0.092	0.372	0.414	0.297
7	0.390	0.438	0.090	0.348	0.291	0.291
8	0.375	0.471	0.089	0.249	0.207	0.276
9	0.360	0.441	0.084	0.225	0.180	0.219
10	0.318	0.344	0.063	0.225	0.177	0.213
11	0.315	0.312	0.063	0.222	0.138	0/204
12	0.285	0.229	0.055	0.207	0.138	0/204

Table 2. Effect of metal ions on the enzyme activity of RRL-36

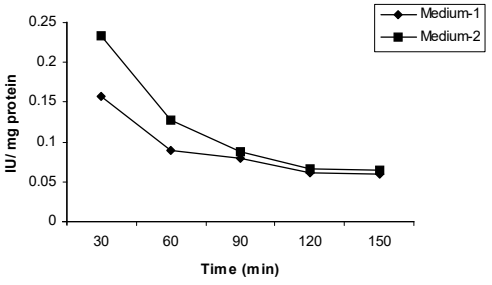
Metal ions	Percentage activity vs Concentration of Medium									
	Medium-1 (in mmol/l)					Medium-2(in mmol/l)				
	1	3	5	7	10	1	3	5	7	10
Mg^{2+}	102	85	93	104	106	125	115	104	104	104
Mn^{2+}	139	158	164	183	239	167	134	112	108	108
Ca^{2+}	125	118	118	110	81	150	114	115	117	115
Cu^{2+}	270	214	172	95	85	164	208	211	276	225
K^{+}	72	77	85	89	120	119	118	123	128	149
Cd^{2+}	112	195	102	122	133	209	138	154	159	172

Table 3. Effect of metal ions on the enzyme activity of RRL-12

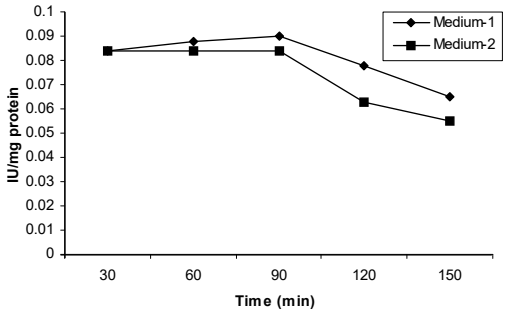
Metal ions	Percentage activity vs Concentration of Medium									
	Medium-1 (in mmol/l)					Medium-2(in mmol/l)				
	1	3	5	7	10	1	3	5	7	10
Mg^{2+}	97	100	88	85	107	67	55	53	54	53
Mn^{2+}	119	103	81	73	75	103	74	73	63	57
Ca^{2+}	42	42	48	50	65	43	33	29	23	22
Cu^{2+}	60	65	72	80	83	53	56	60	62	79
K^{+}	51	51	53	56	63	26	38	46	46	46
Cd^{2+}	34	35	39	48	53	29	31	33	67	70

observed (2). Enzyme activity of RRL-36 in medium-2 was inhibited by Ca^{2+}, Cd^{2+}, Mg^{2+} and Mn^{2+} at 10mmol/l and was activated by Cu^{2+} and K^{+}.

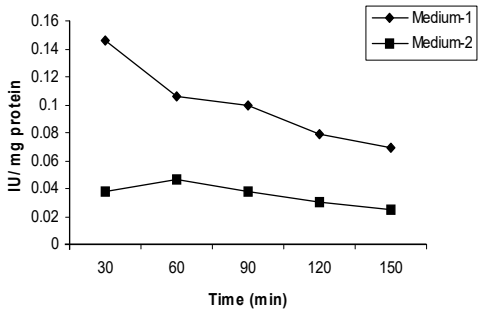
Enzyme activity of RRL-12 was activated by Mg^{2+} at 10mmol/l and inhibited by Cu^{2+} Cd^{2+}, K^{+} $Ca^{+2}$and Mn^{2+} in media-1 whereas activity was inhibited by all the cations in medium-2. The enzyme activity of RRL-11 in medium-1 and 2 was inhibited by all the cations *viz*., Cu^{2+}, Mg^{2+}, Mn^{2}, Cd^{2+}, K^{+} and Ca^{2+}. No change in activity was observed with increase or decrease in metal concentration. Inhibition of endoglucanase activity by *Bacillu*s sp and *Alcaligenes* sp by Co^{2+}, Cu^{2+} and Mn^{2+}at 25 mmol/l whereas it was activated by Mg^{2+} and Ca^{2+} at higher concentration



B. subtilis (RRL-36)

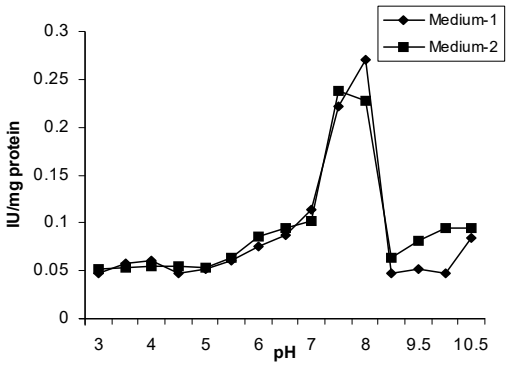


B. subtilis (RRL-12)

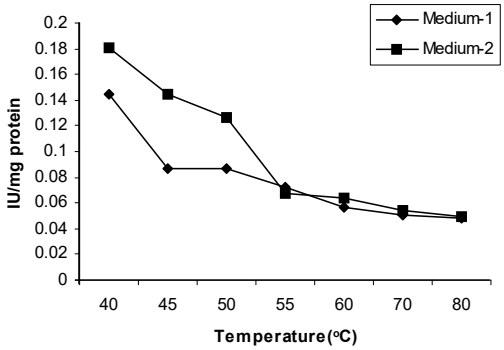


B. stearothermophilus (RRL-11)

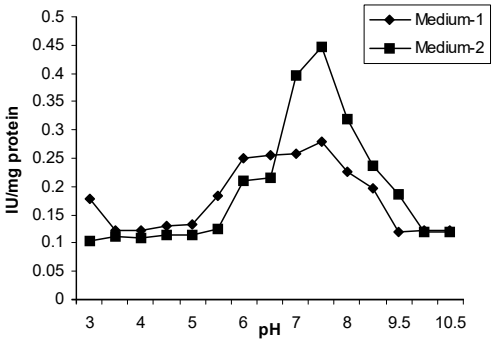
Fig. 1. Effect of time and media on endoglucanase activity



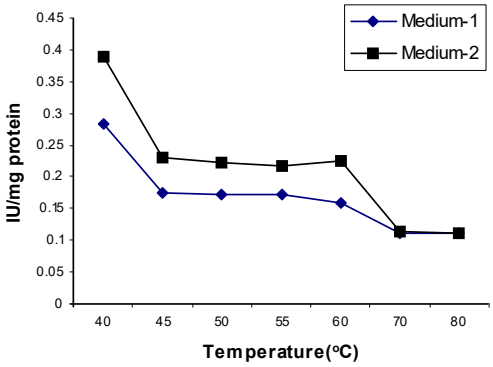
B. subtilis (RRL-36)



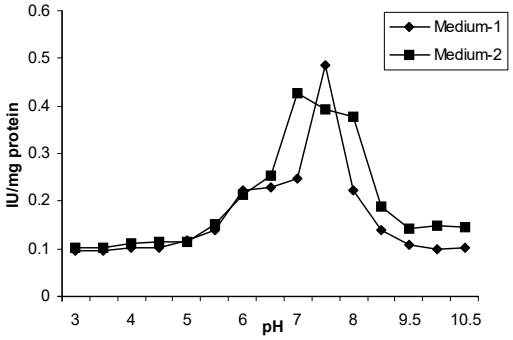
B. subtilis (RRL-36)



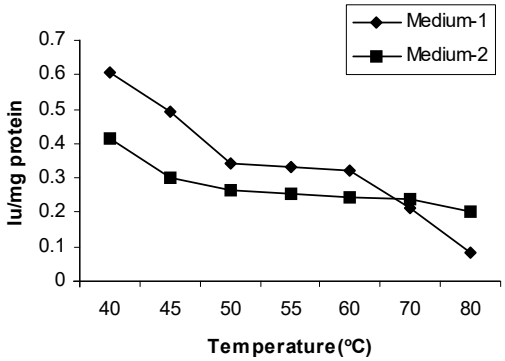
B. subtilis (RRL-12)



B. subtilis (RRL-12)



B. stearothermophilus (RRL-11)



B. stearothermophilus (RRL-11)

Fig. 2. Effect of pH and media on endoglucanase activity

Fig. 3. Effect of temperature and media on endoglucanase activity

(25mmol/l) was reported[25]. Another study reported an increase in the activity of endoglucanase of *Bacillus* sp (N-1139) by addition of Na^+ or K^+, complete inhibition due to Hg^{2+} or Cd^{2+} and inhibition to a lesser extent by Mn^{2+}, Cu^{2+} and Co^{2+},[11].

Studies have revealed that the bacteria closely associated with marine invertebrates play a major role in digestion of macromolecules like chitin[17] and cellulose [12]. The potential of marine microbial enzymes for the biodegradation of cellulosic material has not yet been realized. Commercially available cellulases display optimum activity at pH range 4-6[14]. Hence there is a need for enzymes having broad pH range activity, and high salt tolerant activity for application from industrial point of view.

Cellulolytic bacteria from marine microorganisms are not explored to a large extent and especially those from symbiotic microbes of sedentary fauna. There is scanty information available on cellulolytic enzymes of bacteria associated with sponges. Endoglucanase production from the bacterial associates of marine sponge *Axinella* sp. had been reported for the first time[25]. In the present study endoglucanase activity isolated from 4 sponges viz., *A. ramosa*, *A. agariciformis*, *Ircinia* sp and *Raspailia* sp has been reported. Two isolates from a gorgonian *H. flabellum* and one from antipatharian also showed endoglucanase activity, which becomes the first report of its kind.

ACKNOWLEDGEMENTS

The authors thank the Director, Regional Research Laboratory, Bhubaneswar; the Director, Central Institute Of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar and the Vice Chancellor, O.U.A.T, Bhubaneswar for providing research facilities, the Department of Oceanography, CSIR, India for financial support and the Indian Navy for diving assistance to collect marine organisms.

REFERENCES

1. Ahuja, S.K., Ferreira, G.M. and Moreira, A.R., Production of endoglucanase by the shipworm bacterium *Teridinobacter turnirae. J. Ind. Microbiol. Biotechnol*. 2004; **31**(1): 41-7.
2. Araki, T., Sugimoto, T. and Morishita, T., Purification and characterization of carboxymethylcellulase from *Alcaligenes* sp. CM-746. *J. Gen. App.Microbiol*. 1996; **42**: 439.
3. Balasubramanian, T., Lakshmanaperumalsamy, P., Chandramohan, D**.** and Natarajan, R., Cellulolytic activity of *Streptomycetes* isolated from the digestive tract of marine Borer. *Indian J. Mar. Sci*. 1979; **8**: 111.
4. Chang, W.T.H. and Thayer, D.W.**,** The cellulase system of *Cytophaga* species. *Can. J. Microbiol.* 1977; **23**: 1285-1292.
5. Chen, C.S. and Chen, H.Y., Purification and properties of carboxymethyl cellulose from Antarctic krill *Euphausia superba*. *J. Chin. Biochem. Soc*. 1983; **12**: 61-70.
6. Chun, C.Z., Hur, S.B. and Kim, Y.T., Purification and characterization of an endoglucanase from the marine rotifer, *Brachionus plicatilis*. *Biochem*. and *Mol.Biol. Int.* 1997; **43**(2): 241-9.
7. Dasilva, R., Yim, D.K., Asquieri, E.R. and Park, Y.K., Production of microbial alkaline cellulase and studies of their characteristics. *Rev. Microbiol*. 1993; **24**: 269-274.
8. Dhevendaran, K., Maya, K. and Natarajan, P., Studies on microbial enzymes and cellulolytic activity in Aeromonas of retting ground. *Proc. Natl. Acad. Sci. India Sect* B. 1992; **62**: 547.
9. Elena P. Ivanova, I. Yu. Bakunina, Olga I. Nedashkovskaya, Nataliya M. Gorshkova, Yulia V. Alexeeva, Elena A. Zelepuga, T.N. Zvaygintseva, Dan V. Nicolau, V.V. Mikhailov., Ecophysiological Variabilities in Ectohydrolytic Enzyme Activities of Some *Pseudoalteromonas* sps., *P. citrea*, *P. issachenkonii*, and *P. nigrifaciens. Curr. Microbiol*. 2004; **46**: 6-10
10. Faulkner DJ., Marine natural products. *Nat. Prod. Rep*. 2000; **17**: 7-55
11. Fukumori, F., Kudo, T and Horikoshi, K**.**, Purification and properties of cellulase from alkalophilic *Bacillus* sp. no. 1139. *J. Gen. Microbiol*. 1985; **131**: 3339-3345.
12. Greene, R.V., Griffin, H.L and Freer, S.N., Purification and characterization of extracellular endoglucanase from marine shipworm bacterium. *Arch. Biochem. Biophys.* 1988; **15**: 267(1): 334-41.
13. Ghosh, T.A and Pathak, A.N., Cellulase-1: source, technology. *Process Biochem*.1973; **8**: 35-38.
14. Horikoshi, K., Alkaliphiles from an industrial point of view. FEMS *Microbiol. Rev*. 1996;

18: 259-270.

15. Horikoshi, K. and Akiba, T., Alkalophilic Microorganisms: A new microbial world. Springer-Verlag. Heidelberg, Tokyo, 1982.
16. Ishaque, M. and Kluepfel, K., Cellulase complex of a mesophilic streptomyces strain. *Can. J. Microbiol.* 1979; **26**: 183-189.
17. Lear, D.W. Jr., Occurrence and significance of chitinoclastic bacteria in pelagic waters and zooplankton. In: Oppenheimer C.H. (ed). Marine microbiology. Charles C Thomas, Publisher, Springfield, Ill. 1963; 594-610.
18. Lee YK, Lee J-H, Lee HK, Microbial symbiosis in marine sponges. *J. Microbiol.* 2001; **39**: 254-264
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the Folin phenol reagent. *J. of Biol. Chem*. 1951; **193**: 265-275.
20. Machuca, A. and Duran, N., Phenol Oxidases production and wood degradation by thermophilic fungus *Thermoascus aurantiacus*. *Appl. Biochem* and *Biotechnol*. 1993; **43**: 1993.
21. Mandels, M., Hontz, L. and Land Nystron, J., Enzymatic hydrolysis of waste cellulose. *Biotechnol. Bioeng*. 1974; **16**: 1471-1493.
22. Manz W, Arp G, Schumann-Kindel G, Szewzyk U, Reitner J., Wide field deconvolution epifluorescence microscopy combined with fluorescence in situ hybridization reveals the spatial arrangement of bacteria in sponge tissue. *J. Microbiol. Methods* 2000; **40**: 125-134
23. Miller, G.L., Use of dinitrosalicylic acid for determination of reducing sugars. *Anal. Chem.* 1959; **31**: 426-428.
24. Mohapatra BR, Bapuji M, Sree A., Production of industrial enzymes (amylase, carboxymethylcellulase and protease) by bacteria isolated from marine sedentary organisms. Acta Biotechnol. 2003; **23**: 75-84
25. Mohapatra, B.R., Perpeties of carboxymethylcellulase (endo-1,4-â-glucanase) from Bacillus sp. isolated from the marine sponge (*Axinella* sp.). *Ind. J. of Mar. Sci*. 1997; **26**: 292-296.
26. Ryu, D.D.Y. and Mandels, M.K., Cellulase biosynthesis and applications. Enzyme and *Microbial Tech.* 1980; **2**: 91-102.
27. Vimala, A., Rath, C.C. and Sree, A., Extracellular deoxyribonuclease of bacteria isolated from marine sponges. Microbes in our lives. 2005; 111-121.
28. Vimala, A., Studies on enzymes and bioactive substances from the bacterial associates of marine sponges. *Ph.D thesis*, Utkal University, Bhubaneswar, India 2004.
29. Yamane, K., Yoshikawa. T., Suzuki, H. and Nishizawa, K., Localization of cellulase components in *Pseudomonas fluorescens* var. cellulose. *J. Biochem*. 1971; **69**: 771-780.