Journal Homepage: - www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI: 10.21474/IJAR01/4053
DOI URL: http://dx.doi.org/10.21474/IJAR01/4053

RESEARCH ARTICLE

CELLULOSE DEGRADING CAPABILITIES OF CELLULOLYTIC *BACILLUS* SP. ISOLATED FROM THE INTESTINAL TRACT OF THE BEETLE, *ORYCTES RHINOCEROS.*

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Manuscript Info

Manuscript History

Received: 23 February 2017
Final Accepted: 24 March 2017
Published: April 2017

Key words:-
Cellulase, *Oryctes rhinoceros*, Cellulolytic organism isolation, *Bacillus* sp, 16S rDNA typing

Abstract

A few cellulolytic bacteria were isolated from the gut of a phytophagous beetle, *Oryctes rhinoceros* that feeds on the unopened leaves of coconut plant. On an average of 3.06 x 10^5 cellulolytic bacterial colonies were isolated from the gut of the beetle. The cellulolytic bacteria were found to be facultative anaerobes. Among the isolates, the most efficient cellulolytic bacterial strain was selected on carboxymethylcellulose agar plate and was further identified and characterized through 16s rRNA typing. The bacterial strain was identified as *Bacillus* sp. based on 16s rRNA analyses. The 16s rRNA sequence of *Bacillus* sp. is deposited in gene bank NCBI data base. The cellulolytic capability of the isolated organism was studied at varying pH and temperature.

Introduction:-

Cellulase is a group of inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo,2001). The complete enzymatic hydrolysis of cellulosic materials needs different types of cellulases such as, endoglucanase (1, 4-β-d-glucan-4-glucanohydrolase), exocellobiohydrolase (1, 4-β-d-glucan glucohydrolase) and β-glucosidase (β-d-glucoside glucohydrolase) (Yi *et al*.,1999). The endoglucanase randomly hydrolyzes the β-1, 4 bonds in the cellulose molecule and the exocellobiohydrolases in most cases release a cellobiose unit showing a recurrent reaction from chain extremity. Lastly, the cellobiose is converted to glucose by β-glucosidase(Bhat and Bhat,1997). Cellulase has attracted much interest because of the diversity of their application. The major industrial applications of cellulase are in textile industry for 'bio-polishing' of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness (Paulo, 1998). They are also used in animal feeds for improving the nutritional quality and digestibility. In food processing industries such as fruit juices, baking and paper industry. A potential challenging area where cellulase would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals (Gong *et al*., 1999). Numerous cellulase enzyme producing organisms such as, bacteria and fungi have been reported (Immanuel, 2006). In general, bacterial cellulases are constitutively produced, whereas fungal cellulase is produced only in the presence of cellulose (Suto and Tomito, 2001).

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The present study was initiated to isolate new strains of bacteria with better adaptability and superior cellulolytic activity to improve bioethanol production from cellulosic materials so that the whole process of bioethanol production could be made economically viable *Orcytes rhinoceros* a phytophagous beetle feeds mainly on coconut tender leaves. The coconut leaves are commonly composed of cellulosic materials. The young unopened leaves have more contents of water and protein and have less carbohydrate when compared to matured leaves. The carbohydrate content is more than protein and water content. *O. rhinoceros* has the digestive enzymes to utilize these carbohydrate sources. These digestive enzymes to digest the cellulosic materials could be produced either by the digestive glands of the digestive tract or by the symbiotic bacteria living in the digestive tract. Here we isolated a few bacteria from the digestive tract of *O. rhinoceros* that are involved in utilizing cellulosic substrate. The cellulolytic capability of one of the isolated microorganisms was studied with respect to different pH and temperature.

Material and Methods:-

Collection of insect from field:-

The adult beetle *O. rhinoceros* were collected from the coconut plantation in and around Tiruchirappalli. Tiruchirappalli is situated at the centre of the Indian state of Tamil Nadu. It lies at an altitude of +78 M above sea level. Alive adult beetle were collected and taken to the laboratory for a complete microbial analysis. The beetle were dissected out under aseptic conditions and the alimentary canal was removed and grinded in physiological saline and was serially diluted and plated onto nutrient agar as well as carboxymethyl cellulose (CMC) containing agar plates and incubated at 37^{o}C for 24 to 48 h (Banjo *et al*.,2003).

Screening for Cellulase producing Bacteria:-

The axenic culture of each colony appeared on nutrient agar plate was obtained by streaking on to fresh nutrient agar plate and was incubated at 37^{o}C for 24-48 h. Stock culture of each organism, was made and kept on agar slants at 4^{o}C and these were used for further analysis.

The isolated bacterial cultures suspended, serially diluted up to 10^{-5} and plated on CMC containing agar plates and was incubated at 37°C for 2 days. The plates were stained with Congo red, destained with 1 M NaCl solution and scored for a clear zone surrounding the colony for cellulolytic activity (Wood and Bhat,1988).

Production of Crude Cellulase:-

The production of cellulose enzyme by the isolated bacterial strains was assayed using liquid broth. A single colony from agar plate was inoculated into 50 ml-culture tube containing 5 ml of production medium with carboxymethylcellulose powder in nutrient broth and incubated at 180 rpm and 37°C for 24hr. This culture was then subculture into 500 ml capacity Erlenmeyer flask containing 95 ml of the same medium and incubated at 37°C for 48 h. Two ml aliquots were withdrawn at 2 h interval and were centrifuged at 5,000 rpm for 10 min at 4°C. The cell pellets were washed twice with 50 mM Tris–HCl buffer, pH 7.0 and resuspended in 2 ml of the same buffer. Cellulase activity was measured in both cell free supernatant and in the cells to detect the extracellular and the cells bound enzyme activity. Cells and insoluble materials were removed by centrifugation at 5,000 rpm for 10 min at 4°C and the cell-free supernatant was filtered through a 0.45-µm pore-size membrane filter and was used as the source of crude cellulase enzyme. The same procedure was followed also for the cells to measure the cellulase activity (Ibrahim and Diwany,2007).

Enzyme Assay:-

Cellulase activity was measured by the DNS (3,5-dinitrosalicylic acid) method (Miller,1959), through the determination of the amount of reducing sugars liberated from CMC solubilized in 50 mM Tris–HCl buffer, pH 7.0 (Bailey *et al*.,2003). This mixture was incubated for 20 min at 70°C. For crystalline cellulose substrates, incubation time was extended to 2h and the reaction was stopped by the addition of DNS solution. The treated samples were boiled for 10 min, cooled in water bath for color stabilization, and the optical density was measured at 550 nm. The cellulase enzyme activity was determined using a calibration curve for glucose. One unit of enzyme activity was equivalent to the amount of enzyme that released 1 µmol of glucose per minute. The substrate specificity of the crude enzyme was determined by performing the assay CMC as the substrates.

Protein Determination:-

Protein concentration of bacterial cell lysate was determined according to the method described by Bradford, 1976. One ml of Bradford reagent was added to 50 µl of sample and the extinction was measured after 5 min at 595 nm.

Different concentrations of bovine serum albumin (BSA) were used as a protein standard: 10, 20, 40, 60, 80 and 100 µg/ ml distilled water. One ml of Bradford reagent was added to 50 µl BSA standard and the extinction was measured after 5 min at 595 nm.

Effect of Temperature and pH on the Activity of Cellulase:-

The influence of temperature on the catalytic activity of cellulase was determined by measuring the enzyme activity at temperatures ranging from 30 °C to 60 °C under the standard assay conditions. The influence of pH on the enzyme activity was determined by measuring the enzyme activity at varying pH values ranging from 5 to 9 at 40 ºC using different buffers, 50 mM sodium acetate (pH 5.0 and 6.0), 50 mM sodium phosphate (pH 7.0, 7.5 and 8.0), 50 mM glycine-NaOH buffer (pH 8.5, 9.0), respectively[10]. Experiments were performed in triplicate and mean values calculated.

Analyses of 16S rRNA Gene Sequences:-

Identification of isolate using 16S- rRNA Typing:-

Among the isolated celluloytic bacterial strains one strain was found to hydrolyse cellulose very efficiently. This strain was identified by 16s rRNA typing (Synergy, Chennai). The 16S-rRNA gene sequence of the isolate was aligned with reference to 16S-rDNA sequences of the European Microbiological Laboratory (EMBL), GenBank (gb, Germany) and the data base of Japan (DBJ) using the BLAST algorithm (Altschul *et al*.,1997) available in NCBI (National Centre for Biotechnology information) in internet.

Table 1:- Characteristics and number of colonies of the bacteria isolated from the digestive tract of *O. rhinoceros* (n=3±SD)

Organism	**Source**	**Oxygen tolerance**	**No. of colonies on Nutrient agar**	**No. of colonies on Cellulose agar**
O. rhinoceros	Entire digestive tract	Facultative anaerobe	4.201 ± 0.011 x 10^5	3.06 ± 0.28 x 10^5
		Obligate anaerobe	-	-

Fig. 1-16S rDNA Gene Sequence of isolated *Bacillus* sp.

1 cagtgtcccg ttacagtaac gaatgctcta gcgcgtgatg cctggctcag taagtcctat
61 caaggttaac ccgtattgga tcccctaaca gtttgatcca cggctcacgt aagactggac
121 atagctcccg gaaaccgggg ctcataccgg ataacatttt gaaccgcatg gttcgaaatt
181 gaaaggcggc ttccgctgtc acttatggat ggacccgcga ctcattaact agttggtgag
241 gtaacggctc accatttcat cgatgcctat ccgacctgag agggtaatct gcccactggg
301 actttgaaaa agcccatact ccatccggga cccaactgta tggtaaactt tgtactgtaa
361 ggttaaaggg gttccccccc cttaatgctt gaaggtaacg ccttaagccc tccccctgg
421 ggaattcggg cgcaaggctt aaaattaaag gaattgacgg gggcccccc caagggggga
481 acctggggtt taatttggaa caaagggaaa aaccttaccc ggttctgacc ttctttggca
541 accccaaaaa aaggggtttt cctttgggaa caaaatgacc gggggggcca ggttgttgtt
601 aacttgggtt ctgaaaaggt gggttaaatt ccgcaacgaa ggcaaccctt tatttttgtt
661 gccatccatt aattgggccc tttaagggtg acgccggggg caaaccggag gaagggggggg
721 aaggagttaa attctccttc ccctttttgc cttgggtacc ccactggtac caatggcggt
781 tcaaaaaagt gccaggaccc gaggggggaag taattttaaa aaaccgtttt ccatttggaa
841 tgttgggtgc cacttgccta cctggaaacg gattccccgg tattggggct taacaagttt
901 gctccgattc cgttacgtgg caacaaggta acccctattg gatcctctag agtttgatac
961 ctggctcagg aagtcgtaac aaggtaacca gtattggatc ctctaga

Fig. 2:- Effect of pH on the activity of cellulase synthesized by *Bacillus* sp.

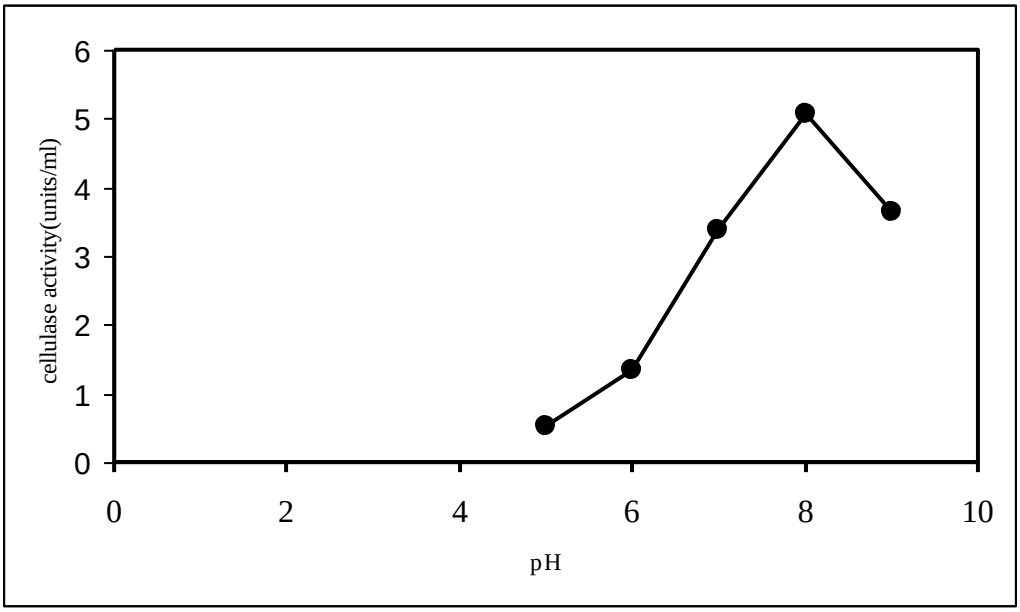
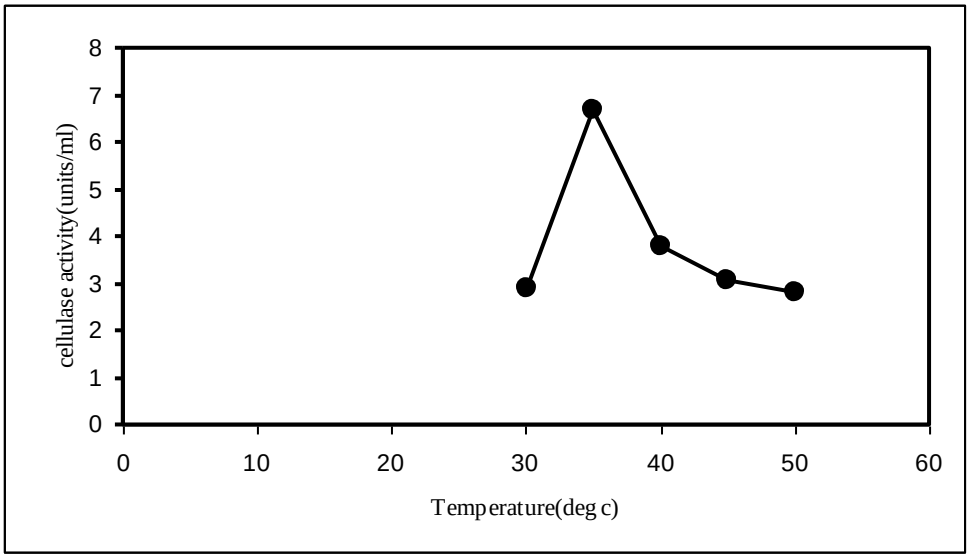


Fig. 3:- Effect of temperature on the activity of cellulase synthesized by *Bacillus* sp.



Results and Discussion:-

The total count of facultative anaerobic bacteria in intestinal samples collected from the beetle *O. rhinoceros* was 4. 2x 10^5. Out of these bacteria 3.1 x 10^5 cellulase producing bacteria were able to be isolated with direct plating method (Table 1). Four mechanisms have been proposed to account for cellulose digestion in insects: (i) exploitation of the cellulolytic capacity of protozoan symbionts residing in the hindgut; (ii) exploitation of the cellulolytic capacity of bacteria residing in the hindgut; (iii) reliance upon fungal cellulase, originating in the food, that remain active in the gut following ingestion; and (iv) secretion by the insect of a complete cellulase system.

The second mechanism, the exploitation of cellulolytic bacterial symbionts in the gut, has been assumed to explain the ability of many higher termites (Termitidae) to digest cellulose, but convincing evidence in support of this assumption is still lacking (Breznak,1982). Hindgut bacteria have been shown contribute cellulose digestion in the American cockroach, *Periplaneta americana* (Blattidae) (Bignell, 1981) and in the rhinoceros beetle, *Oryctes*

nasicornis (Scarabaeidae) (Bayon,1981) and seem likely to participate in the larvae of crane flies (Tipulidae) (Sinsabaugh *et al*.,1985; Griffith and Cheshire,1987).

The present study, showed that *O. rhinoceros* carries an appreciable microbial flora which included *Bacillus* sp and *Pseudomonas aeruginosa* . This finding is similar to the previous report of Banjo *et al* 2006.

Identification of the Cellulase Producing Bacteria:-
The bacterial strain *Bacillus* sp showed clear zone on CMC agar after staining with Congo red. The phylogenetic analysis of this strain using its 16S rDNA sequence (Fig 1) data showed that this strain had highest homology (82 %) with *Bacillus* sp. CECRI (EF 532899.1). The 16s rRNA sequence was submitted to NCBI (Accession no- EU 629346). The bacterial strain was identified as *Bacillus* sp.

Effect of Temperature on the Enzyme Activity:-
The effect of temperature on the activity of crude cellulase was determined at various temperatures ranging from 30 °C to 50 °C at pH 7.0. The enzyme showed a good cellulolytic activity between 30 °C to 50°C with maximum activity at 35 °C (Fig 2).

Effect of pH on the Cellulase Activity:-
The effect of the pH on the crude cellulase activity of *Bacillus* sp was examined at various pH ranging from pH 5.0 to pH 9 as shown in (Fig 3). The enzyme has a broad range of pH activity (pH 5-9) with optimal pH at 8 which is close to the optimum pH value of most cellulase of *Bacillus* (Fukumori *et al*., 1985).

In the midgut, the pH usually ranges from 6.0 to 8.0 (Day and Waterhouse,1953; House,1974). Coleoptera larvae that feed on leaves of trees, which typically contain large quantities of tannins, had an average midgut pH of 8.67, while those that feed mostly upon herps and forbs had on average midgut pH of 8.29 (Berenbaum,1980). Most cellulases of microbial origin have pH optima in the acidic or neutral range, which is not good because of the alkalinity of detergent compositions. Alkaline cellulase found so far has been very few. For instance, Horikoshi *et al* 1984 partially purified two alkaline CMCase components from alkalophilic *Bacillus* sp. which resembles *B. pasteurii* (Fukumori *et al*., 1985) characterized an inducible alkaline CMCase from alkalophilic *Bacillus* sp. no.1139, which was similar to *B. firmus*. Recently, it was reported an alkaline cellulase from "neutrophilic" *Bacillus* sp, which was similar to *B. pumilus*. In the present study the isolation of alkalophilic *Bacillus* sp. which secretes a constitutive alkaline cellulase that fulfills the essential requirements for house in laundry detergents is reported. This isolate could also be classified as *B. circulans* or *B. pumilus*, although it differs in the pH optimum for growth and also in several taxonomic properties.

O. rhinoceros, a Coleopteran insect feeds on coconut leaves as a sole diet. The coconut leaves are said to have higher carbohydrate content. The isolated bacteria was found to be symbiotic, they were cultured thrice at different period. The isolated bacteria were also capable of degrading cellulose substrate such as cellulose. The isolated bacteria were considered to be symbiotic that helps in digestion of cellulose from coconut leaves. Coleopteran larvae have a gut pH ranging from pH 6.0-8.0. So the isolated and characterized bacterial strains were grown on alkaline pH to match that of the gut. These isolated bacterial strains were also seems to survive and efficiently utilize the cellulosic substrates at alkaline pH. Thus proves that the bacterial strains were symbiotic and helps in the digestion of cellulose substrate in their diet. The cellulolytic bacteria increase with larval stages also indicates that these bacterial isolates with the capacity of cellulose degradation helps in the digestion of cellulose, contributing to the growth of *O. rhinoceros.* This shows that the bacterial strain were indeed helpful in development of *O. rhinoceros.*

Acknowledgements:-

These authors gratefully acknowledge Srimad Andavan Arts and Science College (Autonomous) for financial support.

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