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Isolation and identification of cellulose degrading bacteria from gut of two herbivorous pest larvae

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Abstract

Insect gut microflora contributes towards the utilization of complex food resources in the gut of the host. The interaction of the host and the micro organisms can be symbiotic or transient. The present study explored the gut flora of two Lepidopteran pest larvae namely black cutworm (Agrotis ypsilon) and Colocasia esculenta leaf roller (Cnaphalocrocis sp.) to isolate potential cellulolytic bacteria from their gut. We could screen a total of 19 cellulolytic bacterial isolates from the gut of these two insect species. The bacterial isolates were aseptically isolated and screened on CMC (Carboxymethylcellulose)-agar medium by using CMC as a sole carbon source. Their cellulolytic potential was checked by using Congo-red overlay method and Gram's iodine method. The latter method was found to be more efficient with rapid and distinctly visible zone of hydrolysis. Basic identification of the bacterial genera was conventionally done by Bergey's Manual of Systematic Bacteriology based on their biochemical properties. Their cellulolytic efficiency was determined based on CMCase and FPase (Filter paper) assays. Estimation of reducing sugar was done as per DNSA (3, 5- Dinitrosalisylic acid) method (Miller, 1959).

Key words: *Agrotis ypsilon, Cnaphalocrocis sp*., Lepidoptera, Congo-red, Gram's Iodine, Carboxymethylcellulose, CMCase, FPase assay, DNSA.

Introduction

The cellulolytic capacity of microorganisms is of great importance from the stand point of industrial microbiology and in terms of microbial ecology. To determine the number of cellulolytic microorganisms present in any complex ecosystem pose great challenge (Teather and Wood, 1982). There are two possible ways for enumeration of cellulolytic bacteria namely direct and indirect method. The direct enumeration method seriously underestimates the number of cellulolytic bacteria present in the studied environment (Bryant and Burkey, 1953). Whereas, in case of indirect enumeration

method, by using a non-selective medium to isolate wide range of bacteria, followed by testing of each isolate for cellulolytic activity, severely limits the nature and number of studies undertaken (Smith, 1977; Mahasneh and Stewart, 1980). So an alternative method was proposed to use a soluble substrate (carboxymethyl cellulose) which gets precipitated with detergent to visualize a zone of hydrolysis on an agar plate (Hankin and Anagnostakis, 1977). The interaction of the direct dye Congo-red with intact β-D-glucans provides the basis for a rapid and sensitive assay system for bacterial strains possessing β-(1→4),(1→3)-D-glucanohydrolase, β-(1→4)-D-glucanohydrolase and β-(1→3)-D-glucanohydrolase activities (Teather and Wood, 1982). Congo-red overlay method is considered to be most popular staining technique for selective technique. But it has been reported that staining with Congo-red has less efficiency and it also deactivates the microbes. Hence, Gohel *et al*., 2014 proposed that Gram's iodine solution as an alternative stain for the determination of extracellular activity on CMC agar plate.

A large number of bacteria are capable of degrading cellulose, but only a few of them produce significant quantities of cell-free bioactive compounds capable of completely hydrolyzing crystalline cellulose *in-vitro* (Patagundi *et al.,* 2014). There is a rise in demand for stable, highly active and specific cellulase enzyme for industrial application (Cherry and Fidants*,* 2003). The degradation of cellulosic materials has been reported from numerous studies, but few reports are there regarding which organisms met the industrial requirement (Lee *et al.*, 2008). It also have been reported that bacteria with higher growth rate as compared to fungi, also considered having a better potential for cellulase production (Sethi *et al.,* 2013). So it is utmost necessary to keep on exploring new sources of cellulase enzymes. Insect gut micro-flora contributes towards the utilization of complex food resources in the gut of the host. Herbivorous insects harbour pool of microorganisms which aid in their digestion. Our present investigation was designed to isolate and screen cellulolytic bacteria from two Lepidopteran pest larvae gut.

Materials and methods

Isolation of bacteria

The insect larvae were collected freshly from field in and around the North-Eastern Hill University (NEHU) campus. The larvae were killed by putting in deep freeze for half an hour and were surface sterilized with 5.25% sodium hypochlorite solution prior to dissection. The dissection was done aseptically under laminar air flow hood. The microbes present in the guts were enumerated by serial dilution of macerated gut content in distilled water onto CMC agar plate as inoculum. The media composition (g L^{-1}) for isolation of cellulolytic bacteria contains: 10g peptone, 10g CMC, 0.2g $MgSO_4$, 0.5g $NaCl_2$, 0.1g $CaCl_2$, 15g Agar at pH 6.5. CMC was used as a sole carbon source. The inoculated plates are incubated at 32^0C for 24-48 hours (Dantur *et al*., 2015).

Screening of bacteria

The incubated CMC agar plates were flooded with 1% Congo-red solution and

allowed to stand for 15 minutes. The dye was poured off and the plates were washed with 1M $NaCl_2$ thoroughly several times. Bacterial isolates producing cellulase enzymes produced clear zone of hydrolysis around the colony. We flooded the duplicate CMC-agar plate with Gram's Iodine solution to check the cellulolytic potential of the isolates. The bacterial colonies having clear zones were selected for further analysis. Further bacterial stains were purified by repeated streaking. The pure cultures were preserved at 4^0C till further investigation.

Screening for cellulase enzymes

Development of inoculum

The selected bacterial isolates were inoculated in broth medium (gL^{-1}) containing: 1.5g KH_2PO_4, 2.5g $Na_2HPO_4.7H_2O$, 0.3g $MgSO_4.7H_2O$, 0.5g $NaCl_2$, 0.1g $CaCl_2$, 0.005g $FeSO_4.H_2O$, 0.0016g $MnSO_4$, 10g CMC at pH 6.4. The broth cultures were incubated in shaker incubator at 37^0 C up to 120 hours at 150 rpm. At intervals of every 24 hours the bacterial cultures were collected in 15 ml centrifuge tube and centrifuged at 12000 rpm for 10 minutes in cooling centrifuge to collect supernatant. The supernatant obtained after centrifugation served as crude enzyme source.

Estimation of cellulase enzymes

Estimation of cellulase enzyme activity was assayed using 3, 5 – Dinitrosalicylic acid (DNS) reagent (Miller, 1959) by estimation of reducing sugars released from CMC (CMCase assay) and Whatman no. 1 filter paper (FPase assay). For CMCase assay 2% Carboxymethylcellulose (w/v) (2g CMC in 0.5 M sodium citrate buffer pH 5.5) and for FPase assay 50 mg Whatman no. 1 filter paper (1.0 cm × 6.0 cm) strips saturated in 0.5 M citrate buffer pH 5.5 were used. To the test tubes 2% CMC (w/v) with 250 µl of crude enzymes were added and incubated at 50^0 C for 30 min. For FPase assay filter paper strips and 500 µl of crude enzyme was added to test tubes and incubated for 60 min at 50^0 C. To the incubated mixture 3ml DNS reagent is added to stop reaction and heated in boiling water for 5 min to develop colour. The test tubes are allowed to cool down and 1 ml of Rochelle salt are added to each tube when they are still warm. Reducing sugar liberated during the reactions was measured as absorbance at 540 nm. One unit (IU) of enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugar equivalent to glucose (mg min^{-1}) mg^{-1} protein during the reaction (Ghose, 1987)

Identification of bacterial isolates

Bacterial isolates were identified by using standard identification tests as per Bergey's Manual of Determinative Bacteriology (Buchnan and Gibbons, 1974).

Optimization of culture condition

Optimization of temperature- To determine the effective temperature for cellulase production by the bacterial strains, isolates were incubated at different temperature viz.

25^0 C, 37^0 C, 45^0 C and 60^0 C.

Optimization of pH- The most suitable pH for the cellulase production was determined by adjusting the pH of the medium to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0.

Optimization of incubation period- Some microorganisms produce maximally during their exponential phase, whereas others in their stationary growth phase. The incubation period ranged from 24 hours to 120 hours, the assay was performed at an interval of 24 hours.

Optimization of salinity- Optimum salinity is required to maintain osmolarity of cell cytoplasm. Therefore, percentage of salinity ranging from 1-6 was investigated.

Morphological and biochemical characterization

The isolates showing highest cellulolytic activity were further subjected to morphological characterization, oxidase test, catalase test, MR VP test, citrate test, TSI test, Indole test, Urea hydrolysis test, Nitrate reduction test, Starch hydrolysis test, DNAse test, Carbohydrate fermentation tests. The results of morphological characterization and biochemical characterization are summarized in the Table 1 and Table 2 respectively.

Results and discussion

A total of 19 bacterial isolates were screened from two Lepidopteran insects, 12 isolates from taro leaf roller (*Cnaphalocrocis sp*.) larva gut and 7 isolates from gut of *Agrotis ypsilon* larva. All the isolates showed positive cellulolytic activity on CMC agar plate when stained with both Congo-red and Gram's iodine solution. But later when cellulase activity was examined only two isolates (CWI 6 and TCI 11) one each from two insect larvae showed considerable cellulase activity (both CMCase and FPase). The result revealed that both isolates showed higher FPase activity than CMCase activity. Previous study reports very low CMCase activity in Lepidopteran gut fluid (generally < 0.06 U/mg proteins). The origin of cellulolytic enzymes probably reflects the phylogenetic relationship and feeding strategies of different insects (Su *et al.,* 2013). Morphological and physiological characteristics intensely depend on environmental conditions, hence the accurate identification of isolates turned out to be very difficult (Bakri *et al.,* 2010). The molecular techniques are more significant for the characterization of the new isolates, allowing grouping the strains.

Table1. Morphological characterization of bacterial isolates

Colony Morphology	TCI 11	CWI 6
Size	5 mm	1.3 cm
Colour	Creamy white	Creamy white
Elevation	Flat	Flat
Margin	Irregular	Irregular
Opacity	Opaque	opaque
Gram's staining	+ve (rod)	-ve (rod)
Motility	+ve	-ve

Furthermore, complex studies (morphological, biochemical and molecular) are essential, when the identification of new isolate is the purpose of the investigation (Rathnan *et al.,* 2013). Enzyme production is closely controlled in microorganisms and for improving its productivity, these controls can be improved. Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculums size, pH value, temperature, and presence of inducers, aeration, growth time, and so forth (Immanuel *et al.,* 2006).

(a) **(b)** **(c)** **(d)**

Figure 1. (a) *Agrotis ypsilon* larva, (b) taro leaf roller (*Cnaphalocrocis sp*.) larva, (c) CMC agar plate flooded with Congo-red dye showing zone of hydrolysis, (d) CMC agar plate flooded with Gram's iodine dye showing zone of hydrolysis

During the present investigation it was found that the zone of hydrolysis was more prominent and readily visible when the CMC agar plate was stained with Gram's iodine (GI) than conventional staining method with Congo-red (CR) (Figure 1). The diameter of zones of hydrolysis was found to be 2.1 cm and 1.5 cm respectively for Gram's iodine and Congo-red staining method showed by CWI 6 bacterial isolate of a colony with diameter of 1.3 cm. For TCI 11, a colony with a diameter of 5 mm gave zone of hydrolysis of 1.2 cm and 7 mm for GI and CR respectively. So, the cellulolytic index was found to be higher with Gram's iodine than Congo-red staining technique (Table 3). Clear zone appeared around growing bacterial colonies indicating cellulose hydrolysis (Irfan *et al.,* 2012).

Cellulolytic potential of the cellulose hydrolyzing bacterial isolates was determined by calculation of their Hydrolysis Capacity (HC) (Lloyd and Tarun, 2016). Cellulolytic index was calculated by using formula as follows (Ferbiyanto *et al.*, 2015):

$$\text{Cellulolytic index} = \frac{\text{Diameter of zone} - \text{Diameter of bacterial colony}}{\text{Diameter of bacterial colony}}$$

Bacterial isolate CWI 6 from *Agrotis ypsilon* insect gut showed maximum activity in its lag phase at 120 hour culture.

Table 2. Biochemical Characterization of the bacterial isolates

Biochemical properties	TCI 11	CWI 6
Catalase	+	+
Oxidase	-	-
MR-VP	+ +	+ +
Indole	+	-
Citrate	+	-
TSI	Fermentation(-),gas (-), H_2S (-)	Fermentation(+),gas (-), H_2S (-)
DNAse	+	-
Nitrate reduction	+	+
Carbohydrate utilization		
(a) Manitol	+	+ (gas)
(b) Innositol	+ (gas)	+
(c) Sorbitol	+ (gas)	+
(d) Maltose	+	+
(e) Galactose	+	+ (gas)
(f) Lactose	+	+ (gas)
(g) Sucrose	+ (gas)	+

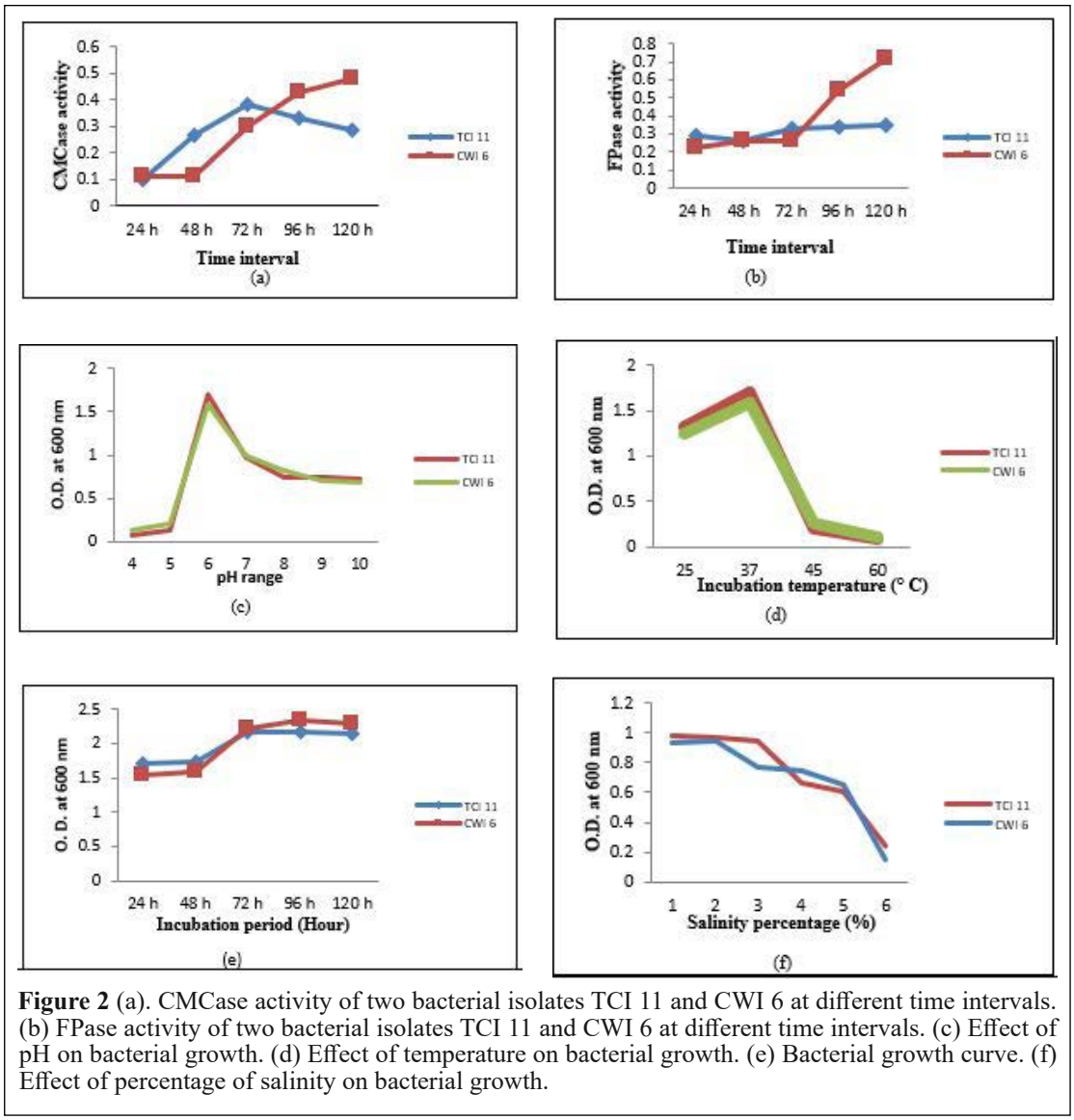

Figure 2 (a). CMCase activity of two bacterial isolates TCI 11 and CWI 6 at different time intervals. (b) FPase activity of two bacterial isolates TCI 11 and CWI 6 at different time intervals. (c) Effect of pH on bacterial growth. (d) Effect of temperature on bacterial growth. (e) Bacterial growth curve. (f) Effect of percentage of salinity on bacterial growth.

Whereas, TCI 11 from taro leaf roller larva gut showed highest CMCase activity at 72 hours incubation period and there was a sharp decline in its activity. But it showed maximum FPase activity at 120 hours incubation (Figure 2a and b). The optimal growth condition of the bacterial isolates revealed that both of them are mesophilic, neutrophilic, and can withstand up to 2% of salt concentration (Figure 2c, d and f).

Table 3. Cellulase assay and cellulolytic index

Isolates	CMCase assay	FPase assay	Cellulolytic index (GI)	Cellulolytic index (CR)
TCI 11	0.233 IU	0.279 IU	1.4	0.4
CWI 6	0.378 IU	0.508 IU	0.615	0.153

Conclusion

With the help of biochemical tests (summarized in Table 2) we could conclude that bacterial isolate CWI 6 belongs to genus *Klebsiella* and TCI 11 belongs to genus *Bacillus.* The isolate CWI6 belonging to phylum Proteobacteria and TCI 11 belongs to phylum Firmicutes. Both families are reported by Huang *et al., 2012* to harbour a pool of cellulolytic bacteria. In contrast to the previous reports which claims that the CMCase activity in lepidopteran gut fluid is less than 0.06 U/ mg protein we could get better cellulase activity in case of both isolates from two different insect larvae gut.

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References

Bakri, Y., Masson, M. and Thonart, P. 2010. Isolation and identification of two new fungal strains for xylanase production. *Applied Biochemistry and Biotechnol*ology, 162:1626-1634.

Bryant, M. P. and Burkey, L. A. 1953. Numbers and some predominant groups of bacteria in the rumen of cows fed different rations. *Journal of Dairy Science,* 36:218-224.

Cherry, J. R. and Fidants, A. L. 2003. Directed evolution of industrial enzymes: an update, *Current Opinion in Biotechnology*, 14(4): 438- 443.

Ferbiyanto, A., Rusmana, I. and Raffiudin, R. 2015. Characterization and identification of cellulolytic bacteria from gut of worker *Macrotremes gilvus. Hayati Journal of Biosciences*, 22(4):197-200.

Ghose, T. K. 1987. Measurement of Cellulase Activities. *Pure & Applied Chemistry,* 59: 257-268.

Gohel, H. R., Contractor, C. N., Ghosh, S. K. and Braganza, V. J. 2014. A comparative study of various staining techniques for determination of extra cellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates. *International Journal of Current Microbioiology and Applied Science*, 3(5): 261-266.

Hankin, L. and Anagnostakis. 1977. Solid medium containing carboxymethylcellulose

to detect C_x cellulase activity of microorganisms. *Journal of General Microbiology,* 98:109-115.

Huang , S., Sheng, P. and Zhang, H. 2012. Isolation and Identification of Cellulolytic Bacteria from the Gut of *Holotrichia parallela* Larvae (Coleoptera: Scarabaeidae). *International Journal of Molecular Sciences,* 13:2563-2577.

Immanuel, G. R., Dhanusha, P. P. and Palavesam, A. 2006. Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *International Journal of Environmental Science and Technology,* 3(1):25-34.

Irfan, M., Safdar, A., Syed, Q. and Nadeem, M. 2012. Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production and activity. *Turkish Journal of Biochemistry,* 37 (3): 287 -293.

Dantur, I. K., Enrique, R., Welin, B. and Castagnaro, A. P. 2015. Isolation of cellulolytic bacteria from the intestine of *Diatraea saccharalis* larvae and evaluation of their capacity to degrade sugarcane biomass. *AMB Express,* 5:15.

Lee, K. S., Kim, N., Choo, Y. M., Hong, S. J., Seol, K. Y., Je, Y. H., Sohn, H. D., Jin, B. R. 2008. Molecular cloning and characterization of a glycosyl hydrolase family 9 cellulase distributed throughout the digestive tract of the cricket *Teleogryllus emma*. *Comparative Biochemistry and Physiology- Part B: Biochemistry and Molecular Biology,* 150:368–376.

Lloyad J. and Tarun. 2016. Molecular characterization of cellulose hydrolyzing bacteria isolates from the gut of Philippine milk termite (*Coptotermes gestroi*). *Asia Pacific Journal of Research,* I:XXXVII:136-143.

Mahasneh, A. M. and Stewart, D. J. 1980. A medium for detecting B-(1-3) glucanase activity in bacteria. *Journal of Appied Bacteriology,* 48:457-458.

Miller, G. L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, 31(3):426-428.

Patagundi, B. I., Shivasharan, C.T. and Kaliwal, B. B. 2014. Isolation and Characterization of Cellulase producing bacteria from Soil. *International Journal of Current Microbiology and Applied Science,* 3(5): 59-69.

Rathnan, R. K., John, D. and Balasaravanan, T. 2013. Isolation, screening, identification and optimized production of extracellular Cellulase from Bacillus subtilis using cellulosic waste as carbon source. *Journal of Microbiology, Biotechnology and Food Sciences,* 2 (6): 2383-2386.

Sethi, S., Datta, A., Gupta, B. L. and Gupta, S. 2013. Optimization of Cellulase

Production from Bacteria Isolated from Soil. *Hindawi Publishing Corporation*, *ISRN Biotechnology*. Article ID 985685.

Smith, R. E. 1977. Rapid tube test for detecting fungal cellulose production. *Applied and Environmental Microbiology,* 33:980-981.

Su, L. J., Zhang, H. F., Yin, X. M., Chen, M., Wang, F. Q., Xie, H., Zhang, G. Z. and Song, A. D. 2013. Evaluation of cellulolytic activity in insect digestive fluids. *Genetics and Molecular Research,* 12 (3): 2432-2441.

Teather, R. M. and Wood, P. J. 1982. Use of Congo Red Polysaccharide Interactions in Enumeration and Characterization of Cellulolytic Bacteria from the Bovine Rumen. *Applied and Environmental Microbiology,* 43(4):777-780.