JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2015. Vol. **9**(2), p. 1033-1038

Isolation and Screening of Cellulose Degrading Microorganisms and Evaluation of its Cellulolytic Activity

Viraj Krishna Mishra[1], Vishwa Rawat, Satish Rana and J. Dubey

Department of Biotechnology Engineering,
Ambala College of Engineering and Applied Research, Devsthali, Haryana, India.

(Received: 11 March 2015; accepted: 03 May 2015)

Cellulose degrading bacteria and fungi were isolated from humous soil, pond water, plants and guts of cellulose feeding invertebrates (locusts and termites) on PDA and NA media. Growth of isolates was also observed on paper with minimal media. Cellulolytic activity was measured by measuring the clear zone around the colonies and calculating the hydrolytic value of the isolates on Congo Red Agar Medium. Highest Hydrolytic Capacity (HC) value was shown by fungal isolate FW .FPCase (Filter paper cellulose) activity ranged from 0.080-0.104IU/mL for bacteria and 0.108-354IU/mL for fungi. CMCase activity ranged from 0.243 to 0.370 IU/mL for bacterial isolates and 0.308-0.528IU/mL for fungal isolates.

Key words: Hydrolytic capacity (HC), FPCase activity ,CMCase activity, Cellulose.

Microbial conversion of cellulosic biomass into simple reducing sugars is a promising strategy for low cost bio-mass processing. Many fungi and some bacteria (gliding bacteria, *Clostridia* and *Actinomycetes*) produce extracellular cellulases that hydrolyse cellulose[1], paper can be used as raw material to produce ethanol as it is made up of cellulose which can be degraded into glucose and fermented to produce ethanol. Several insect cellulases have been purifed and characterized[2, 3]. Termites are said to dissimilate a significant proportion of cellulose (74-99%) and hemicelluloses (65-87%). Termites play an important role in the turnover and mineralization of complex biopolymers, cellulose and hemicelluloses containing materials[4]. Fuels produced by non food materials are called second generation bio-fuels.

* To whom all correspondence should be addressed.
E-mail: viraj.krishna@gmail.com

Ethanol as a fuel has a potential to decrease dependency on fossil fuel and to reduce air pollution. After burning of ethanol, the released CO_2 is recycled back via photosynthetic fixation of carbon dioxide. Ethanol production processes uses energy from renewable sources; no carbon dioxide is added to the atmosphere, making ethanol an environment friendly energy resource. Toxicity of ethanol is lower than that of petroleum sources, contains 35% oxygen that helps in complete combustion of fuels and thus reduces particulate emissions that pose health hazards to living organisms. And, cellulosic ethanol technologies advance the use of organic content of the municipal solid waste as a transportation fuel feedstock and simultaneously reduce externalities associated with waste disposal[5]. For these enormous advantages, the production of ethanol from various raw materials is being considered. Developing countries have therefore begun to explore bio-fuels policies of their own, work on bio-fuels like butanol and ethanol has tremendous international recognition[6].

MATERIALS AND METHODS

Collection of Samples

Samples were collected from humous soil, pond water, plant material (leaves, root and peel of fruits) and cellulose feeding termites for the isolation of cellulose degrading bacteria and fungi. Plant parts were washed with hydrogen peroxide and bromine water [7]. Guts of collected insects were crushed in 0.9% saline water in aseptic condition[8].

Isolation of cellulose degrading micro-organisms

Serial diluted samples were grown on PDA and NA for isolation of pure bacterial and fungal colonies. Growth was observed on Congo Red Agar medium (KH_2PO_4 0.5g ;$MgSO_4$ 0.25g,cellulose 2g,agar 15g,Congo-Red 0.2 g, and gelatine 2g; distilled water 1L at pH 6.8-7.2) to confirm the cellulose degrading ability of the isolates. Congo Red is an indicator and is absorbed by the bonds between polysaccharide chains and the amino groups of the dye, thus provides a basis for rapid and sensitive screening test for cellulose degrading micro-organisms|[8]. Isolates were also grown on basal salt media with following composition:(KH_2PO_4 0.5g ;$MgSO_4$ 0.25g ; Agar 15; gelatin 2g in a liter and at pH 6.8-7.2 containing paper discs of area 48cm^2 added to each Petri Plate. Bacterial cultures were incubated for 2 days at 32°C and fungal cultures were incubated at 28°C for 3 days. Colonies showing growth on paper and showing clear zones were taken as cellulose degrading colonies, and were used for further studies. Hydrolytic Capacity (HC) of the isolates was calculated by taking the ratio of diameter of clear zone and colony[8].

Screening of cellulose degrading micro-organisms

Determination of Celluloytic Activity

Glucose production as a result of cellulose degradation was estimated too with the help of DNSA method[19]. Supernatant of the isolates with 50 mg paper grown on basal media was collected and stored at 4°C as enzyme preparation. Enzyme assay was done according to the methods of (IUPAC) International Union of Pure and Applied Chemistry[9]. Endoglucanase (CMCase) activity is determined by measuring amount of sugar produced from amorphous cellulose and (Filter paper cellulose) FPCase activity was determined by measuring the amount of sugar produced from filter paper. For measuring endoglucanase activity,0.05mM sodium citrate buffer (pH 4.8) 0.5mL and 0.5mL of 2% cellulose solution were incubated with 0.5mL of supernatant for 50°C for 60minutes.For FPC activity,0.5mL of supernatant was incubated with 0.05mM of sodium citrate buffer (pH4.8) 1.0 mL containing filter paper strip (50mg) for 50°C for 60minutes.After incubation,reaction was terminated by adding 3.0 mL of DNS reagent to 1 mL of reaction mixture. Reducing sugars obtained were measured spectrophotometrically using DNS reagent with standard glucose curve[10]. The enzymatic activity was defined in international units IU.

RESULTS AND DISCUSSION

Isolation and screening of Cellulose degrading micro-organisms

Total 29 isolates were isolated from different natural samples i.e. from humous soil, pond water, plant material (leaves, root and peel of fruits) and cellulose feeding insects like locusts and termites. Out of which 15 were bacterial and 14 were fungal isolates. These isolates showed positive activity on Congo Red Agar Media byproducing clear zone around the colony (Figure 1,2).

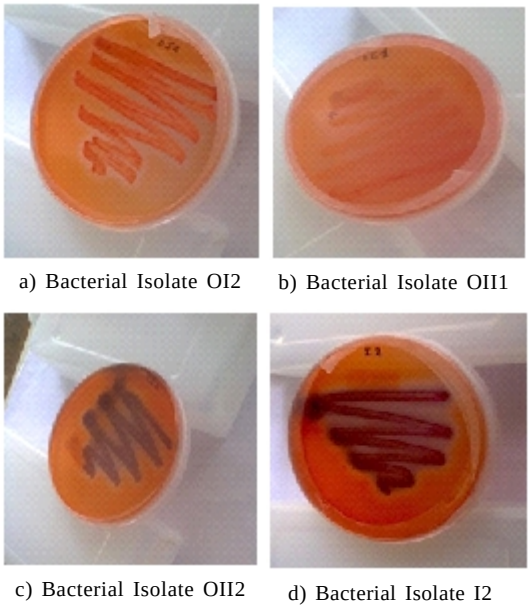
Hydrolytic capacity of the isolates was calculated and it was found that it ranged from 1.2 to 9 mm for all isolates(Table 1 and 2), highest value was given by FPP6 (9.0), FW (7.5) in fungi and by BOI1 ;BOII1, both 7.2 in bacteria. The range of HC value obtained is similar to that of range reported by [12], [8], [13]. Hydrolytic capacities were measured by taking the ratio of diameter of clear zone to that of the diameter of the colony. After confirming that the isolates were able to grow on cellulosic media, they were checked for their conversion of cellulose into glucose. Enzymatic activities of the isolates were calculated too. On the basis of growth on minimal media with paper, hydrolyzing capacity, amount of glucose produced and their resulting enzymatic activities, three fungal isolates i.e FPP1,FPP3,FW and four bacterial isolates OI2,OII1,OII2 and I2 were chosen. FPP1 and FPP3 were estimated to be of genus *Penicillium* and FW was estimated to be of genus *Aspergillus* by fungal staining and cultural

characteristics and for bacteria OI2 resembled *Clostridium*, OII1 resembled *Bacillus*, OII2 showed properties of *Acitobacter* sp and I2 resembled *Rhizobium* in many ways but it can't be exactly the same genus for the same strain.

Enzymatic activity of the isolated strains

Twelve bacterial and ten fungal isolates were chosen for the estimation of enzymatic activity. FPCase activity was highest for fungal isolate FPP1 with 0.354 IU/mL and highest endoglucanase (CMCase) activity was shown by fungal isolate FW with 0.528 IU/mL. The FPCase activity ranged from 0.080-0.104 IU/mL for bacteria and from 0.100-0.354 IU/mL for fungi and CMCase activity ranged from 0.243-0.373 IU/mL for bacteria and CMCase activity ranged from 0.281- 0.528IU/mL for fungi (Figure 2,3).

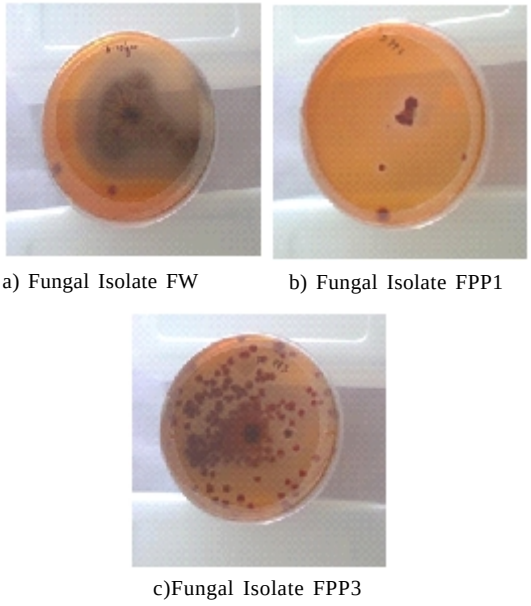
Results similar to these were reported for *Actinobacter anitratus* and *Branhamella* sp. grown separately on Carboxy methyl cellulose and glucose. Maximum enzyme activity of *A.anitratus* culture supernatant were 0.48 and 0.24U/mL for CMC and glucose,respectively. For *Branhamella sp.*, the maximum enzyme activities of the culture supernatant were 2.56 and 0.34 U/mL for CMC and glucose respectively [8].



a) Bacterial Isolate OI2 b) Bacterial Isolate OII1

c) Bacterial Isolate OII2 d) Bacterial Isolate I2

Fig. 1. Bacterial isolates showing clear zone on cellulose congo red agar medium



a) Fungal Isolate FW b) Fungal Isolate FPP1

c)Fungal Isolate FPP3

Fig. 2. Fungal isolates showing clear zone on cellulose congo red agar

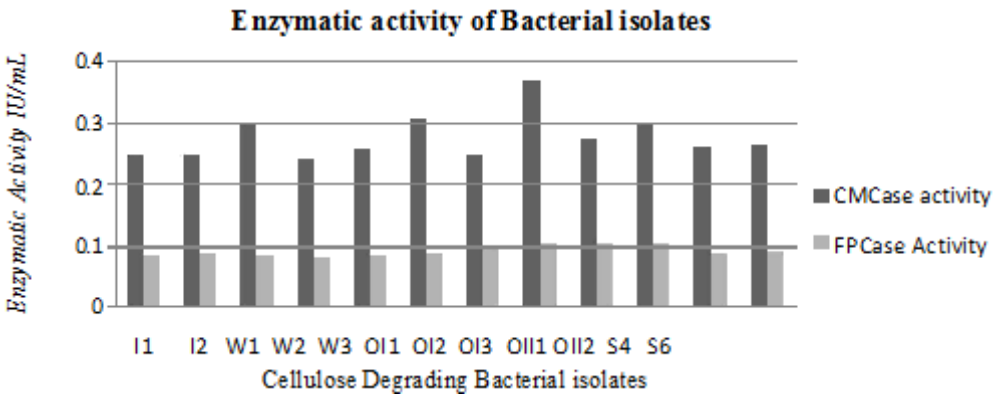


Fig. 3. Enzymatic activity of Bacterial isolates

Actinomycetes isolated from different sediment samples from the Bhitarkanika Mangrove Forest showed extracellular cellulase activities ranged from 0.266±0.001 to 0.734±0.001 IU/mL for FPC and 0.501±0.014 to 1.381±0.024 IU/mL for CMCase or endoglucanase assay[14]. *Pseudomonas* sp. and and *Bacillus sp showed* specific enzyme activity in the crude sample round 6.0U/mg and 8.4 U/mg and of partially purified sample was found to be 6.97 U/mg and 9.3 U/mg respectively[15].

Similarly, endoglucanase activity for culture filterate of *Penicillium pinophilum* MS 20 has specific activity of 69U/mg on CMC[16]. *Chaetomium thermophile* were optimized for maximal production of endoglucanase (EG): pH 5.0,temperature 50°C, incubation period 120 h, substrate 1% CMC. The enzyme was produced and isolated from theculture filtrate through centrifugation. The crude enzyme extract had 0.064 IU/mL endoglucanase activity[17].

Certain bacterial isolates from the guts of various plant eating invertibrates showed FPCase and endoglucanase activity in the range of 0.012-0.196IU/mL and 0.1622-0.400IU/mL respectively[8].

In other studies related to cellulose degrading microbes it was observed that *Trichoderma viride* Fd18 showed the highest specific activity of 1.30 U $mg^{H\,1}$ protein for xylanase, while the highest cellulase activity of 1.23 U mg^{H1} was shown by *Trichoderma* sp. F4[18].

Table 1. Maximum clearing zone and hydrolytic capacity (HC) value of Bacterial isolates on Congo Red agar media

Source	Isolate number	Maximum clearing zone(mm)	Average HC value	Maximum HC value
Soil	S1	20	2.0	2.6
	S3	10	1	1.6
	S4	10	1	1.8
Water	W1	20	2.5	3.3
	W2	12	3	3.6
	W3	30	6	6.7
Insects	I1	15	1.8	2.4
	I2	22	4.4	4.7
Plants	L1	30	1	1.2
	L2	30	1.2	1.4
	OI1	50	5.5	7.2
	OI2	12	2.6	3.8
	OI3	40	5.2	6.8
	OII1	30	6.1	7.2
	OII2	30	2.8	3.9

Table 2. Maximum clearing zone and hydrolytic capacity (HC) value of Fungal isolates on Congo Red agar media

Source	Isolate number	Maximum clearing zone(mm)	Average HC value	Maximum HC value
Soil	FS1	90	6.0	7.2
	FS2	90	6.0	7.0
	FS3	70	2.3	3.0
Insects	FI	55	5.5	6.7
Water	FW	65	6.5	7.5
Plants	FL	75	5.2	6.8
	FO1	30	6.0	7.2
	FO2	60	3.5	4.2
	FPP1	30	2.4	5.2
	FPP2	30	2.1	4.8
	FPP3	20	2.5	3.6
	FPP4	20	1.8	2.4
	FPP5	20	1.3	2.2
	FPP6	8	8	9

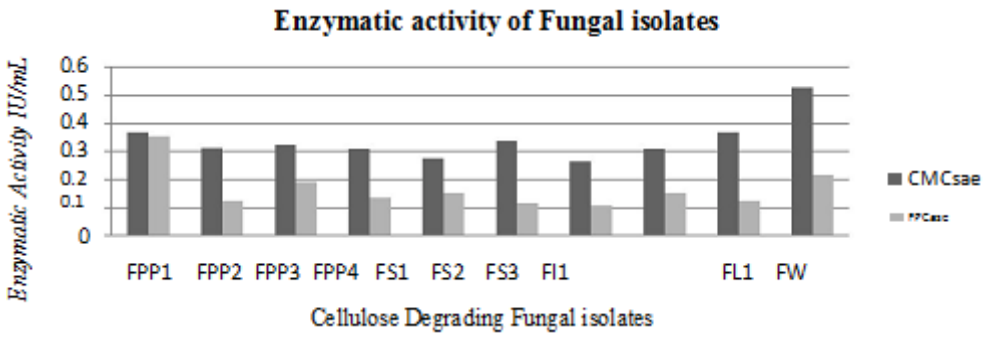


Fig. 4. Enzymatic activity of fungal isolates

In present study,fungi and bacteria were isolated from different sources. Some of them were able to degrade cellulose which was confimed by checking the clear zones formed around their colonies on Congo Red minimal media, they were grown on minimal medium with paper as the source of carbon. All of the isolates showed good enzymatic activities, which were relatively less than the activities reported by researchers on other micro organisms but here the substrate was paper and cellulose powder instead of CMC and glucose powder on which generally all the activities are calculated. This can be the factor for comparatively low levels of enzymatic activity. Proteins were produced were in favourable amounts too, 0.170-1.07 mg/mL. For ethanol, highest percentage was produced in the supernatant of FW i.e 10.25. Studies show that above 10% concentrations of ethanol are toxic for microbes.

CONCLUSIONS

Simple strains cultured from environment have the very potential to degrade cellulose and help in the process of ethanol making. Further, the strains can be optimized for pH and temperature for glucose production. Instead of paper and cellulose they can be made to grow on CMC and glucose for better enzyme activities. Not only individual strains but also the consortia of micro-organisms can be beneficial in ethanol production. Despite the progress achieved, more effort is needed for cellulolytic enzymes and/or micro organisms to have significant industrial impact on bioethanol production.

ACKNOWLEDGEMENTS

Authors are thankful to director and management of Ambala College of Engineering and Applied Research for providing necessary support and facility to complete the research work.

REFERENCES

1. Prescott, Harley, and Klein (2008). Prescott, Harley, and Klein's Microbiology. 7 th ed. McGraw-Hill Publishers, America.36.
2. Gupta R. B., Demirbas A. (2010). Gasoline, Diesel and Ethanol Biofuels from Grasses and Plants. Cambridge University Press, UK.
3. Delalibera I. Jr., Handelsman J., and Raffa1 K. F. (2005). Contrasts in Cellulolytic Activities of Gut Microorganisms Between the Wood Borer, *Saperda vestita* (Coleoptera: Cerambycidae), and the Bark Beetles, *Ips pini* and *Dendroctonus frontalis* (Coleoptera: Curculionidae). *Environ. Entomol.* 34(3): 541-547.
3. Genta, F. A., Terra W. R., and Ferreira. C. (2003). Actionpattern, speciÞcity, lytic activities, and physiological role of the digestive beta-glucanases isolated from *Periplaneta americana. Insect Biochem. Mol. Biol*. 33: 1085-1097.
4. Upadhyaya S. K., Manandhar A., Mainali H., Pokhrel A. R.., Rijal A., Pradhan B., Koirala B.(2012). Isolation and Characterization of Cellulolytic Bacteria from Gut of Termite. *Rentech Symposium Compendium* 1(4):14-18.
5. Otulugbu K. (2012). Production of ethanol from ellulose (sawdust). Master's Thesis , University of Lund, Lund , Sweeden.
6. Dong F. (2007). Food security and biofuels Development: The case of China. Briefing paper 07-BP 52, Centre for Agriculture and Rural Development lowa State university.
7. Chawla H. S (2002). Introduction to plant biotechnology. 2 nd ed. Oxford and IBH Publishing Co.Pvt Ltd.,New Delhi.
8. Gupta P., Samant K. and Sahu A. (2011). Isolation of cellulose degrading bacteria and determination of their cellulolytic potential. *International journal of microbiology*. 2012:1-5.
9. Ghose T. K. (1987). Measurement of cellulase activity. *Pure and Applied Chemistry*. 59:.257-268.
10. Adney B. and Baker J. (1996). Measurement of Cellulase Activities, Laboratory Analytical Procedure (LAP), National Renewable Energy Laboratory, Technical report, NREL/TP-510-42628.
11. Crowell E. A. and Ough C. S. (2010). A Modified Procedure for Alcohol Determination by Dichromate Oxidation. *American journal of enology and viticulture* .30(1):61-63.
12. Lu W. J., Wang H. T., Yang S. J., Wang Z. C., and Nie Y. F. (2006). Isolation and characterization of mesophilic cellulose-degrading bacteria from flower stalks-vegetable waste co-composting system. *Journal of General and Applied Microbiology*. 51(6):353–360.
13. 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(3): 2563–2577.

14. Mohanta Y. K. (2014).Isolation of Cellulose-Degrading *Actinomycetes* and Evaluation of their Cellulolytic Potential. *Bioengineering and Bioscience* 2(1): 1-5, 2014
15. Shaikh N. M.,Patel A. A., Mehta S.A. and Patel N.D. (2013). Isolation and Screening of Cellulolytic Bacteria Inhabiting Different Environment and Optimization of Cellulase Production. *Universal Journal of Environmental Research and Technology*.3:39-49.
16. Dipali P., Laxman R. S. and Rao M. (2012). Purification and biochemical characterisation of endoglucanase from *Penicillium pinophilum* MS 20. *Indian Journal of Biochemistry*. 49:189-194.
17. Naim S. and Jamil A.(2007) Production of endoglucanase from a thermophillic fungi. *Pakistan journal of Agriculture science.* 44(1).
18. Jàafaru M..I. (2013).Screening of fungi isolated from environment samples for Xylanase and cellulose production.*ISRN Microbiol*. 2013:2013
19. Miller G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*. 31(3):426-428.