Tropical Agricultural Research Vol. 19: 36 - 45 (2007)

A Novel Plasmid Encoded Endo-β-1, 4-Glucanase Activity from an *Acinetobacter* spp. Capable of Decomposing Rice Straw

D.M.N. Weerakoon and D.M. Sirisena[1]

Department of Botany
University of Kelaniya
Kelaniya, Sri Lanka

ABSTRACT. *An Acinetobacter spp., a gram negative cellulase positive bacterial strain previously isolated from decomposing rice straw was used for this study. The ability of this strain to hydrolyze carboxymethyl cellulose (CMC) indicated the production of an endo-β-1,4-glucanase (carboxymethyl cellulase). This is one type of cellulases responsible for hydrolysis of cellulosic compounds. This strain was also resistant to a high level of ampicillin, suggesting the possibility of having a plasmid in these cells. This possibility was examined by carrying out plasmid isolation and subsequent gel electrophoresis. It was revealed that the bacterial strain harbors a plasmid of 23 kb in size. Transfer of this plasmid into a non cellulolytic, ampicillin susceptible E. coli-JM109 cells by electroporation changed the phenotype of transformants to make them cellulase positive and ampicillin resistant. High level of endo-β-1,4-glucanase activity of transformed E. coli cells as determined by CMC hydrolysis with crude enzyme preparation is clear evidence for the presence of endo-β-1,4-glucanase gene in the plasmid. Thus, the Acinetobacter sp. associated with decomposing rice straw seems to be involved in cellulose hydrolysis as a result of the presence of this plasmid, which also determines ampicillin resistance of the cells. The presence of plasmids carrying genes for cellulase activity in cellulolytic bacteria has not been reported earlier.*

INTRODUCTION

Recently more emphasis has been placed on the use of agricultural waste as raw materials for various industrial and biotechnological applications. Cellulose is the most abundant component in agricultural residues that can be used as a renewable source of fuels and chemicals.

In Sri Lanka, rice is the principal crop that generates huge amount of residues including straw, husk and bran during harvesting and post harvesting process. Most of these agricultural waste is used as a source of fuel, cattle bed and feed in rice cultivating areas. Although use of rice straw for production of biofuels and other chemicals is not economically feasible yet in Sri Lanka, there is a potential of using it as an organic fertilizer. However, the major obstacle that prevents using rice straw in the field is its slow rate of decomposition.

---

[1] Department of Botany, Faculty of Science, University of Kelaniya, Kelaniya, Sri Lanka.

Since the major component of rice straw is cellulose, the rate of its decomposition is primarily determined by the action of cellulose degrading (cellulolytic) microorganisms. Bacteria and fungi are the prominent cellulolytic microorganisms. It is thought that degradation of cellulose is achieved by the synergistic action of multi enzyme systems produced by these organisms and they are confined to three major classes namely; endoglucanases, exoglucanases and β-glucosidase (Schwarz, 2001; Knowels *et al*., 1987).

Although both bacteria and fungi are involved in efficient decomposition of cellulosic substrates, studies on bacterial cellulase systems are less compared to those of fungi. Furthermore, information on bacteria involved in rice straw decomposition is limited. Therefore, identification, evaluation of the cellulolytic activity and understanding the genetic basis of the cellulase activity of bacteria associated with the decomposing rice straw would pave the path to innovate efficient cellulolytic bacterial strains that could rapidly degrade rice straw.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The *Acinetobacter* spp. which had been isolated and identified from decomposing rice straw (Weerakoon and Sirisena, 2004) and confirmed to have a high level of carboxymethyl cellulase (CMCase) activity was initially maintained on carboxymethyl cellulose (CMC) agar medium containing CMC 1.0 g, peptone 0.5 g, dipotassium hydrogen phosphate 0.2 g, magnesium sulphate hydrate 0.2 g, potassium carbonate anhydrous 4.0 g, calcium chloride 0.2 g, ferrous sulphate 0.2 g, sodium chloride 0.2 g, agar 15 g/L and 1 L of water

After detection of its antibiotic resistance, this bacterial strain was grown on LB broth (pH 7.2) containing ampicillin (100 µg/mL). Stock cultures were prepared using 15% glycerol and maintained at -80°C. *E. coli*-JM109 without CMCase activity or antibiotic resistance was grown in LB broth. Stock cultures were also prepared and maintained in 15% glycerol at -80°C.

Determination of antibiotic resistance of cellulose utilizing bacteria

Bacterial cells obtained from a fresh culture of the *Acinetobacter* spp. were inoculated separately on L-agar plates supplemented with erythromycin, tetracycline or ampicillin in different concentrations (25, 50, 100 and 200 µg/mL). These plates were incubated at 37°C for 24 hrs and examined for growth of bacterial strain.

Isolation and purification of plasmid DNA from the strain with high cellulase activity and antibiotic resistance

The cellulase positive *Acinetobacter* spp. was found to have a high level of ampicillin resistance. Therefore, plasmid DNA isolation was carried out from this bacterial strain by alkaline lysis method (Sambrook *et al.*, 1989).

A single colony from *Acinetobacter* spp. was inoculated into 2 mL of LB broth containing ampicillin (100 µg/mL) and incubated overnight at 37°C with vigorous shaking. Then 1.5 mL of the culture was transferred into a microfuge tube and centrifuged at 12,000xg for 30 sec at 4°C. The dry pellet was suspended in 500 µL of ice cold solution of STET buffer (50 mM glucose, 10 mM Ethylenediamine Tetraacetic Acid (EDTA) (pH 8.0), 25 mM Tris/Hcl) and mixed by vigorous vortexing. After that freshly prepared 0.2 N NaOH and 1% SDS were added to the suspension and contents were mixed by inverting the tube rapidly five times. Then these tubes were stored on ice and 150 µL of ice cold potassium acetate solution was added to each tube and votexed gently in an inverted position for 10 s. These tubes were then stored on ice for 5 min. Supernatant of each tube was separated by centrifugation at 12,000 g for 5 min at 4°C. Phenol chloroform extraction was carried out in order to remove proteins from the samples. Double stranded DNA was precipitated with two volumes of ethanol at room temperature. After adding, ethanol mixed by vortexing and allowed the mixture stand for 2 min at room temperature. Nucleic acid pellet was rinsed with 70% ethanol and again centrifuged at 12,000xg for 5 min. Dry pellet of nucleic acid was redissolved in TE buffer (pH 8.0) containing DNase free pancreatic RNase.

Isolated plasmid DNA was purified using an ultrapure plasmid mini prep kit (Bangalore Genei) according to the manufacture's instructions. One milliliter of equilibration buffer was added to ion exchange column and allowed to empty by gravity flow. Plasmid DNA dissolved in the TE buffer was added into the column and allowed to bind. Then plasmid DNA was eluted by adding 0.80 mL of elution buffer to the column and was precipitated with 0.56 mL of isopropanol. Supernatant was removed by centrifugation at 10,000 g for 30 min at room temperature. Nucleic acid pellet was washed with 1.0 mL of 70% ethanol and centrifuged for 10 min. Air dried pellet was redissolved in TE buffer.

Visualization of plasmid DNA by agarose gel electrophoresis

Electrophoresis of isolated nucleic acid was performed on a 0.7% agarose gel at constant voltage of 75 V using Bio Rad Sub-Cell GT agarose gel electrophoresis system to confirm the presence of plasmid DNA in the nucleic acid sample isolated from *Acinetobacter* spp. The gel was stained with ethidium bromide and examined on a UV transilluminator.

Restriction analysis of plasmid DNA isolated from *Acinetobacter* sp.

Plasmid DNA isolated from *Acinetobacter* sp. was digested with restriction enzymes *Hind*III and *Pst*I separately. Digested samples were subjected to agarose gel electrophoresis to separate the DNA according to their sizes. The approximate size of DNA bands were determined by comparison with the Lambda *Hind*III digested molecular weight marker DNA.

Transformation of *E. coli*-JM109 bacterial strain with plasmid DNA isolated from *Acinetobacter* sp. by electroporation

Preparation of competent cells of *E. coli*-JM109

E. coli-JM109 strain without any antibiotic resistant phenotype was used for transformation. An overnight cultural of *E. coli*-JM109 culture (20 mL) was inoculated into

1 L of LB and incubated at 37^oC until A_{600} = 0.5-0.8. This culture was chilled on ice for 15 min and then centrifuged at 4000 g for 15 min. Cell pellet was washed twice by suspending in ice cold sterilized H_2O and centrifugation as above.

The cell pellet was resuspended in 20 mL of ice cold sterilized 10% glycerol and centrifuged at 3000 g for 15 min at 4^oC. Finally the cells were suspended in 3 mL of ice cold sterilized 10% glycerol.

Transformation of competent cells with plasmid DNA isolated from *Acinetobacter* sp. by electroporation

Competent bacterial cells (40 µL) were mixed with 2 µL of plasmid DNA isolated from *Acinetobacter* spp. Then transformation was carried out with Electroporator (Eppendorf 2510). Time and voltage pulse parameters were set to 3 mes and 1800 V. As a control, a competent cell sample of *E. coli*-JM109 was treated in the same manner, but without adding plasmid DNA. Both electroporated sample and the control sample were mixed with 1 mL of L-broth and incubated at 37^oC for 1 hr to express the transferred genes. Then 100 µL of each sample was plated on L-agar containing ampicillin (100 µg/mL) and incubated over night at 37^oC.

Determination of cellulase activity of transformed bacterial strain

Cellulase activity of transformed bacterial strains was determined according to Teather and Wood (1982). Cells of *E. coli*-JM109 transformed with the plasmid from *Acinetobacter* spp., non transformed *E. coli*-JM109 and *Acinetobacter* spp. were separately inoculated into CMC broth and incubated for 3 days at 37^oC. These cultures were centrifuged at 5000 g for 5 min and supernatant of each culture was used as a crude enzyme preparation. One hundred of each crude enzyme preparation was then placed on the wells on CMC agar plates containing 1% CMC and incubated for 24 hrs at 37^oC. These plates were then flooded with aqueous congo red (0.01 mg/mL) for 15 min. The congo red solution was then poured off and plates were treated with 1 M NaCl for 15 min to visualize clear zones of hydrolysis around the wells.

RESULTS AND DISCUSSION

Acinetobacter spp. used in this study had been identified as a cellulase positive strain associated with decomposing rice straw. Since both CMC hydrolysis test and enzyme assay experiments had confirmed its high level of endo-β-1,4-glucanase activity, we decided to further characterize this strain. Results of antibiotic resistance tests clearly indicated that this strain is able to tolerate a high level of ampicillin (100 µg/mL), but not erythromycin or tetracycline. It was sensitive to even 25 µg/mL of these two antibiotics, erythromycin or tetracycline. The high level of ampicillin resistance is often determined by a plasmid encoded gene (Vega *et al.*, 1976). Therefore, plasmid isolation was carried out to examine this possibility.

Isolation and analysis of plasmid of the *Acinetobacter* sp.

Gel electrophoresis data clearly indicated the presence of a plasmid in the *Acinetobacter* spp. Plasmid DNA digested with *Hind*III generated eight fragments and plasmid DNA digested with *Pst*I produced a smear on the gel. According to the size of fragments obtained with *Hind*III digestion, this is a relatively large plasmid of about 23 Kb. (Plate 1)

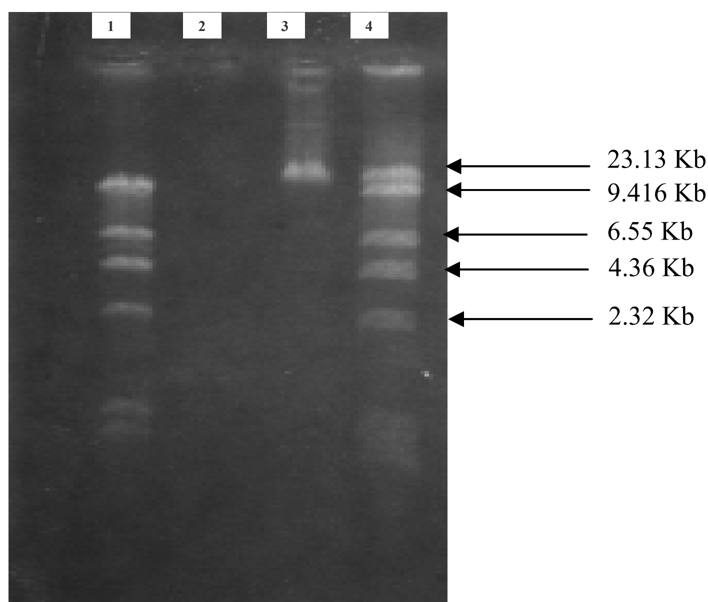


Plate 1. Analysis of plasmid DNA isolated from *Acinetobacter* spp. by agarose gel electrophoresis.

Note: Lane 1, plasmid DNA digested with *Hind*III; Lane 2, plasmid DNA digested with *Pst*I; Lane 3, none digested sample of plasmid DNA; Lane 4, molecular weight marker DNA (Lambda DNA - *Hind*III digested).

Although several bacterial species with high cellulase activity have been isolated from different sources, and characterized at molecular level (Gutierrez-Nava *et al*., 2003; Eckert *et al*., 2002; Kim *et al*., 2000; Cazemier *et al.,* 1999; Han *et al*., 1995) presence of plasmids within these strains has not been reported earlier. Therefore, this is an important genetic characteristic of the *Acinetobacter* spp. associated with decomposing rice straw.

Electroporation of *E. coli*-JM109 bacterial strain with plasmid isolated from *Acinetobacter* sp.

To examine whether the endo-β-1-4-glucanase (CMCase) activity and ampicillin resistance of this particular *Acinetobacte*r spp. are plasmid encoded, it was transferred into *E. coli*-JM109 without CMCase activity, or antibiotic resistance. *E. coli*-JM109 cells transformed with plasmid isolated from the *Acinetobacte*r spp. produced colonies on LB medium supplemented with ampicillin (100 µg/mL) while non-transformed *E. coli*-JM109 did not show any growth in this selective medium (Plate 2). These results indicated that *E. coli*-JM109 cells were successfully transformed with the plasmid. The growth of

transformants in the presence of ampicillin (100 µg/mL) shows that ampicillin resistance of the *Acinetobacte*r spp. is a plasmid encoded characteristic, as it was presumed.

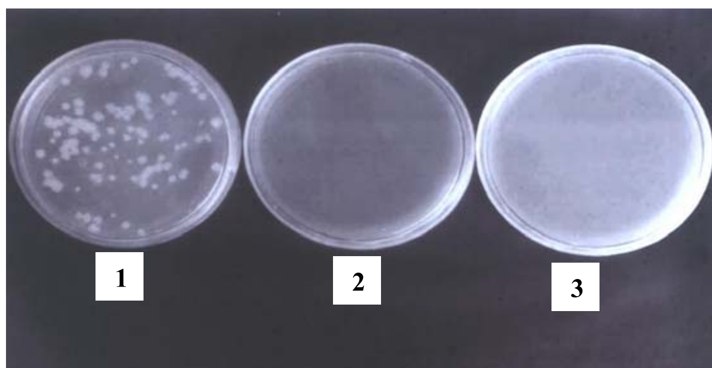


Plate 2. Growth of transformed and non transformed bacterial colonies after 24 hrs incubation on the LB- plates containing ampicillin.

Note: (1) transformed *E.coli*-JM109; (2) non transformed *E.coli*-JM109; (3) Non transformed *E .coli*-JM109 on LB plates without antibiotics.

Demonstration of the presence of the plasmid in *E. coli*-JM109 transformants

Since the ampicillin resistance phenotype of transformants indicated the transfer of the plasmid into *E. coli*-JM109, as a confirmation step, plasmid DNA was isolated again from the transformed *E. coli*-JM109 and subsequently gel electrophoresis was carried out. As evident from Plate 3. Plasmid DNA isolated from the *Acinetobacter* spp. and transformed *E. coli*-JM109 migrated at the same rate and produced bands at the same level. This clearly confirmed the presence of the same plasmid in transformed *E. coli*-JM109 cells.

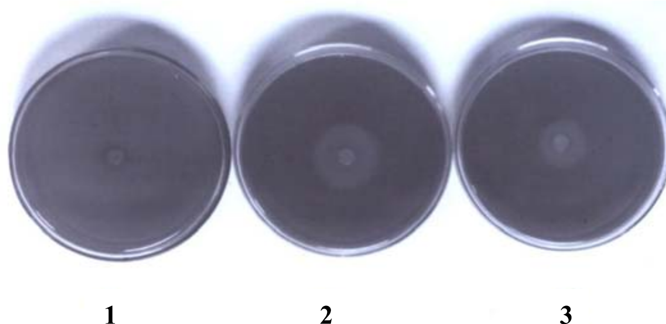
Plate 3. Agarose gel electrophoresis of plasmid DNA isolated from *Acinetobacter* spp. and transformed *E. coli*-JM109 cells.

Note: Lane (1), plasmid DNA from the *Acinetobacter* spp; Lane (2), plasmid DNA from the transformed *E. col*i-JM109.

Determination of CMCase (endo-β-1-4-glucanase) activity of transformed bacterial strain

After the demonstration of transfer of the plasmid with ampicillin resistance marker into *E. coli* strain, we were interested to examine whether the CMCase positive phenotype of the *Acinetobacter* spp. was associated with this plasmid. Therefore, initially both transformed and non transformed *E. coli* cells were inoculated into a medium containing CMC as the sole source of carbon. Only the transformed *E. coli*-JM109 cells were able to grow in this medium indicating the change of genotype of *E. coli*-JM109 so that it can hydrolyse CMC as a result of acquiring the plasmid.

The positive colonies for endoglucanase activity were randomly selected and further assessed for their ability to hydrolyze CMC. Plate 4 and Figure 1 show the results of the CMC assay with crude enzyme preparation of the transformed *E. coli*-JM109. Only the transformed *E .coli*-JM109 and original *Acinetobacter* spp. produced clearing zones on CMC plates incubated with crude enzyme preparations derived from the three-day old bacterial cultures. No clearing zones were observed with non transformed *E. col*i-JM109.



1 2 3

Plate 4. **Congo red stained CMC agar plates containing 1% (w/v) CMC, after incubation for 24 hrs at 37°C.**

Note: The central well of each plate was inoculated with 100 µL of crude enzyme preparation from (1) *E. coli*-JM109 (non transformed bacteria); (2) transformed *E. coli* -JM109; (3) original *Acinetobacter* spp.

Figure 1 shows the diameter of clearing zones produced by the crude enzyme preparations from original *Acinetobacter* spp. and transformed *E. coli*-JM109 bacterial strain on CMC plates after visualization as described previously by congo red method. The crude enzyme preparation from *Acinetobacter* spp. produced the largest clearing zone around the well on CMC plates.

The congo red method used to visualize the hydrolysis zone of CMC enables numerical evaluation for the endo-β-1,4,glucanase activity of the transformed *E. coli*-JM109, non transformed *E. coli*-JM109 and *Acinetobacter* spp. Diameter of the clear hydrolysis zones produced by crude enzyme preparations of these bacterial strains were used to compare their endo-β-1,4-glucanase activity.

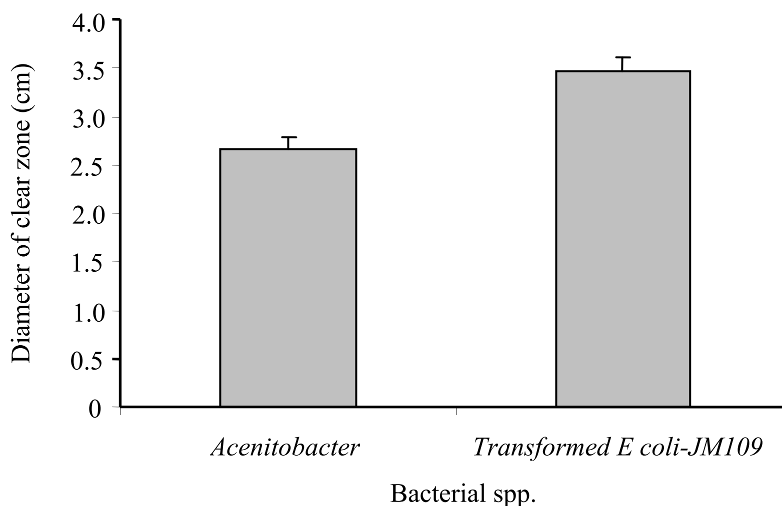


Figure 1. Evaluation of the endoglucanase activity of transformed *E. coli*-JM109.
Note: c – Average value obtained in four independent trials.

Thus, the results of CMC hydrolysis tests show that the *E. coli* cells transformed with the plasmid from *Acinetobacter* spp. gain the ability to hydrolyse CMC. Therefore, endo-β-1-4-glucanase activity of the transformants is clearly determined by a plasmid encoded gene. In previously reported studies with different cellulolytic bacteria only chromosomal endo-β-1-4-glucanase genes have been identified (Gutierrez-Nava *et al*., 2003; Bauer *et al*., 1999; Her *et al*., 1999**;** Lee *et al.,*1987**)**. However, transformed *E. coli*-JM109 bacterial strain has shown a higher endoglucanase activity than the plasmid containing *Acinetobacter* spp. Therefore, new cellular environment in *E. coli*-JM109 bacterial strain appears to have some positive effect on the expression of the endoglucanase gene. However, this difference in enzyme activity in two strains is not statistically significant.

In the *Acinetobacter* spp. used in this study endo-β-1,4,glucanase gene is located in a plasmid with ampicillin resistance maker, Therefore its identification and characterization would be relatively easy. For future genetic manipulation of bacteria associated with rice straw to enhance their cellulase activity, this plasmid encoded gene would be a potential candidate.

Many endoglucanase genes have been isolated and cloned into different vectors to evaluate their enzyme activity on CMC. Endoglucanase gene of 3.2 Kb in size designated *CelA* gene of *Clostridium thermocellum* has been cloned and successfully expressed in *E. coli* DHI (Schwarz *et al*., 1986). Similarly a 1.9 Kb fragments of *Pseudomonas* spp. YD-15 was essential for the expression of endo-β-1,4, glucanase activity, while a 3.5 Kb fragment isolated from *Cellulomonas flavigena* has allowed identification of three open reading frames of endo-β-1,4,glucanase genes (Gutierrez-Nava *et al*., 2003; Her *et al*., 1999). Therefore, it is possible to contain one or several endoglucanase genes in the 23 Kb plasmid of *Acinetobacter* spp. isolated in this study.

CONCLUSION

Acinetobacter spp., a cellulolytic bacterial strain associated with decomposing rice straw, showed high level of endoglucanase activity as indicated by the results of CMC hydrolysis test and saccharification of CMC, both of which are clearly correlated. Antibiotic resistance is a feature of this cellulolytic bacterial strain and plasmid of *Acinetobacter* spp. can be transformed to non cellulolytic *E. coli*-JM109 by electroporation. The endoglucanase activity of transferred *E. coli*-JM109 clearly indicated that the plasmid of *Acinetobacter* spp. is carrying a gene(s) responsible for endoglucanase activity.

ACKNOWLEDGEMENTS

We acknowledge the financial assistance provided by the University of Kelaniya, Sri Lanka for this study.

REFERENCES

Bauer, M.W., Driskill, L.E., Callen, W., Snead, M.A., Mathur, E.J. and Kelly, R.M. (1999). An endoglucanase, EglA, from the hyperthermophilic Archaeon *Pyrococcus furiosus*, hydrolyzes β- 1,4 bonds in mixed linkage (1 - 3), (1 - 4)- β-D-glucans and cellulose. J. of Bacteriology 181 (1): 284 - 290.

Cazemier, A.E., Verdoes, J.C., Op den Camp, H.J.M., Hackstien, J.H.P. and van Ooyen A.J.J. (1999). A β-1,4-endoglucanase-encoding gene from *Cellulomonas pachnodae.* App. Microbio. and Biotech*.* 52: 232 - 239.

Eckert, K., Zielinski, F., Leggio, L.L. and Schneider, E. (2002). Gene cloning, sequencing and characterization of family 9 endoglucanase (CelA) with an unusual pattern of activity from the thermoacidophile *Alicyclobacillus acidocaldarius* ATCC 27009. App. Microbio. and Biotechnol. 60: 428 - 436.

Gutierrez-Nava, A., Herrera-Herrera, A., Mayorga-Reyes, L., Salgado, L. M. and Ponce-Noyola, T. (2003). Expression and characterization of the celcfIB gene from *Cellulomonas flavigena* encoding an endo-beta-1,4-glucanase. Current Microbio. 47 (5): 359 - 363.

Han, S.J., Yoo, Y.J. and Kang, H.S. (1995). Characterization of a bifunctional cellulase and its structural gene. The American Society for Biochemistry and Molecular Biology. 270 (43): 26012 - 26019.

Her, S., Lee, H.S., Choi, S.J., Choi, S.W., Choi, H.J., Yoon, S.S. and Oh, D.H. (1999). Cloning and sequencing of ß-1,4-endoglucanase gene (CelA) from *Pseudomonas* sp. YD-15. Letters in Applied Microbiology. 29: pp. 389 - 395.

Kim, J.O., Park, S.R., Lim, W.J., Ryu, S.K. and Kim, M.K. (2000). Cloning and characterization of thermostable endoglucanase (Cel8Y) from the hyperthermophilic *Aquifex aeolicus* VF5. Biochemical and Biophysical Rese. Communi. 279 (2): 420 - 426.

Knowles, J., Lehtovaara, P. and Teeri, T. (1987). Cellulase families and their genes. TIBTECH. 5: 255 - 261.

Lee, S.F., Forsberg, C.W. and Rattray, J.B. (1987). Purification and characterization of two endoxylanases from *Costridium acetobutylicum* ATCC 824. App. and Env. Microbio. 53: 644 - 649.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). In Molecular cloning- A laboratory manual, 2nd ed., Cold spring harbor laboratory press.

Schwarz, W.H., Grabnitz, F. and Staudenbauer, W.L. (1986). Properties of a *Clostridium thermocellum* endoglucanase produced in *Escherichia coli.* Applied and Envi. Microbio. 51 (6): 1293 - 1299.

Schwarz, W.H. (2001). The cellulosome and cellulose degradation by anaerobic bacteria. App. Microbial Biotech. 56: 634 - 649.

Teather, R.M. and Wood, P.J. (1982). Use of congo red- polysaccharide interaction in enumeration and characterization of cellulolytic bacteria from the bovine rumen. App. and Envi. Microbio. 43: 777 - 780.

Vega, R., Sadoff, H.L. and Patterson, M.J. (1976). Mechanisms of ampicillin resistance in *Haemophilus influenzae* Type B[1]. Antimicrobial Agents and Chemotherapy 9 (1): 164 - 168.

Weerakoon, D.M.N. and Sirisena, D.M. (2004). Endoglucanase activity of bacteria from rice straw, *In*: Proceedings of the 24th annual sessions of the Institution of Biology, Sri Lanka, Pp.18.