African Scientist Vol. 23, No. 2 June 30, 2022
Printed in Nigeria

1595-6881/2021 $10.00 + 0.00

http://www.niseb.org/afs

AFS2022010/23202

Preliminary Screening of Cellulase Producing Bacteria Isolated from the Gut of African Palm Weevil Larvae

O.M. Immanuel*, T.L. Omoko and B.U. Egumandi

Department of Biological Sciences, University of Africa, Toru-Orua, Sagbama, Nigeria

*Corresponding author; Email: immanuelomega@gmail.com Tel: +234 (0) 803 522 9190

(Received April 3, 2022; Accepted in revised form April 14, 2022)

ABSTRACT: Insects of the taxonomic order Coleoptera are recognized for considerable cellulolytic activity in their digestive tract. However, the cellulolytic activity of the gut microbiome of the African palm weevil (*Rhynchoporus phoenicis*), has not been reported. Thus, this study aimed at isolating cellulose degrading bacteria from the gut of the insect's larva and assessing their cellulolytic activities. The larva of the insect was dissected and inoculated in carboxymethylcellulose-Congo red (CMC-CR) agar medium and incubated at room temperature (28±2) for 14 days. Isolate with the highest cellulolytic index was identified as *Pseudomonas luteola.* The isolate was used in the fermentation study for the production of enzyme in Erlenmeyer flasks for 12 days. Glucose production and enzyme activities were measured every two days. A total of 25 pure colonies of bacteria were isolated from the sample, among which 9 were found to hydrolyze CMC. The isolate produced glucose ranging in concentration from 0.101 - 0.320 mg/ml. The enzyme activities of crude enzyme extracts ranged from 0.028 – 0.200 UI/ml. This study therefore highlights the prospect of bacteria from the gut of *R. phoenicis* larvae as important sources of cellulase production for potential industrial and environmental applications.

Keywords: African palm weevil, *Pseudomonas luteola*, cellulolytic activity, cellulase production

Introduction

Cellulose is the most abundant biopolymer produed on earth and the major constituent of agricultural and industrial wastes (Dashtban *et al*., 2010). Lignocellulosic biomasses are huge renewable, cost effect and raw materials that can be useful industrially if it can be converted to its monomeric units (glucose) which can in turn be used for the production of biofuels and other valuable products (Moe *et al.,* 2012; Zabed *et al*., 2016). The key to achieving this lies in finding suitable means of degrading this polymer by the use of cellulases with animals, bacteria, fungi and plants haven been identified as capable of producing these enzymes (Su *et al*., 2013).

The sugar chain complex structures of lignocellulosic materials make them sturdy for microorganisms to break down into simple sugar (Tillman *et al.,* 2006; Hill *et al*., 2006; Tan *et al.*, 2016). Nevertheless, several microbes degrade lignocellulosic material, and for this reason, there is a growing interest in prospecting for microorganisms that can produce cellulases sufficiently and efficiently such that they can be applied for the large-scale conversion of the abundant lignocellulosic biomasses into glucose (Sukumaran *et al*., 2005; Yang *et al*., 2011).

Insects, especially the highly adapted phytophagous, are known to produce their own cellulolytic enzymes and are regarded to be very promising candidate for the search of novel cellulases since they forage on lignocellulosic biomasses (Oppert *et al*., 2010; Su *et al*., 2013). The larva of African palm weevil (*Rhynchoporus phoenicisi*), a phytophagous insect of the taxonomic order Coleoptera, is cherished delicacy among the many communities in

Nigeria and other parts of Africa, especially in those places where palms (oil, Raphia and coconut) are cultivated (Ogbuagu *et al*., 2011; Womeni *et al*., 2012).
The gut of *R. phoenicis* larvae is expected to contain a group of cellulolytic microbes that secret these cellulolytic enzymes (endoglucanases, exoglucanases and β-glycosidase) in other for them to utilize the palm fibre as food. This study sought to screen for cellulase producing bacteria from the gut of *R. phoenicis* larva.

Materials and methods

Sample collection: African palm weevil (*R. phoenicis*) larvae were collected in the month of November, 2021 from Toru-Orua community, Sagbama Local Government Area, Bayelsa State in sterile plastic containers. The samples were transported to the Microbiology Laboratory at the Department of Biological Sciences, University of Africa. Toru-Orua.
Buffer preparation: Acetate buffer of pH 4 contains Sodium acetate anhydrous, 0.14g; acetic acid, 0.6005g in 0.1L distilled water. Acetate buffer pH 5 contains Sodium acetate anhydrous, 14.27g; acetic acid, 0.6005g in 0.1L distilled water. Phosphate buffer of pH 6 contains NaH_2PO_4. $2H_2O$, 1.56g; $NaHPO_4$, 0.22g in 0.1L of distilled water and pH 7 contains NaH_2PO_4. $2H_2O$, 1.56g; Na_2HPO_4, 2.257g in 0.1L of distilled water. The solutions were diluted to 500ml with distilled water which were used to prepare the media.
Culture media: Carboxymethyl Cellulose-Congo Red agar medium (CMC-CR agar) containing CMC, 2.5g; $MgSO_4$. $7H_2O$, 0.05g; NaCl, 0.5g; $(NH_4)_2SO_4$, 0.25g; KH_2PO_4, 0.34g (excluded in Phosphate buffer); Yeast extract, 0.25g; Agar powder, 3.75g; $FeSO_4$. $7H_2O$, 0.0025g, and Congo red, 0.25g dissolved in 250ml of each of the acetate buffer pH (4 and 5) and phosphate buffer (pH 6 and 7).
Isolation and screening of cellulolytic microbes: The African palm weevil larva was dissected and the gut (99 mg in mass) was collected and homogenized in 200ml Ringer solution (containing NaCl, 120 mg; Sodium lactate, 62 mg; KCl 6 mg; $CaCl_2$, 4 mg) using an electronic blender to make total suspension of the gut. The pH value of the solution was measured as 6.5. The suspension was transferred to a 1000 ml volumetric flask and filled up to 1000ml with distilled water.
Ten-fold serial dilution of the suspension was carried out. Aliquots **(**0.1 ml) of the dilutions were inoculated into CMC-CR agar plates prepared using acetate buffer (pH 4 and 5) and phosphate buffer (pH 6 and 7), using a sterile spread rod. The plates were incubated at room temperature (28±2^{o}C) for 14 days.
Cellulolytic activity of bacterial isolates: Clear zones of degraded CMC around the colonies was used to determine the cellulolytic activity of bacterial isolates by measuring the diameter of clear zones. The formula described by Ferbiyanto *et al*., (2019) was used to calculate the cellulolytic index.

$$\text{Cellulolytic Index } (CI) = \frac{diameter\ of\ zone\ (cm) - diameter\ of\ bacterial\ colony\ (cm)}{diameter\ of\ bacterial\ colony\ (cm)}$$

Identification of isolate: Isolate with the highest cellulolytic index was subcultured in nutrient agar to obtain a pure culture and then identified using API 20E strip.
Production of cellulases enzyme: CDB7 with the highest cellulolytic index on CMC-CR agar plate was selected for quantitative determination of cellulases activities in submerged fermentation as described by Arimurti *et al*. (2017). One loopful pure culture of CDB7 was suspended in 10 ml sterile distilled water and inoculated into 100 ml nutrient agar broth in a 250 ml conical flask and incubated overnight at room temperature on a magnetic stirrer set at 120 rpm. After growth overnight, 20 ml of the culture suspension with equal cell density (1.4 OD at 600 nm) was subcultured in two 250 ml conical flasks one containing 100 ml of 80 % w/v CMC solution and the other 100 ml of 20 % w/v CMC solution both prepared with phosphate buffer of pH 6. Both flasks were incubated at room temperature (28±2^{o}C) for 12 days on a magnetic stirrer set at 120 rpm.
At every 2 days interval, 10 ml of the fermented broth was centrifuged at 4000 rpm for 5 minutes. The supernatant of each broth was used as crude enzyme extract for determination of enzyme activity.
Determination of endoglucanase activity: Endoglucanase activity was measured according to the method described by Zhang *et al*. (2009). The enzyme activity was determined through estimation of produced reducing sugar in fermentation medium. The content of reducing sugar was evaluated colorimetrically using 3,5-dinitrosalicylic acid (DNS) method. Three samples were prepared in the Dinitrosalicyclic reagent method: Positive control sample using glucose (1.20mg/ml), negative control using distilled water and the actual sample (crude enzyme extract). The

reaction medium contained 0.5ml of 1% CMC, and 0.5ml of enzyme preparation for the actual sample. The mixture was kept for 20 minutes in water bath at 40˚C. Specifically, 3 mL of DNS reagent (10ml of 2N NaOH, 0.5g of 3,5-dinitrosalicyclic acid in 25ml distilled water, 15g of sodium potassium tartrate tetrahydrate and was diluted to a final volume of 50ml) was added to the reaction mixture and the resulting mixture was boiled at 100 °C for 5 minutes. The test tubes were cooled and absorbance was measured using a UV-Visible spectrophotometer (BKI-UV1000) at 540 nm. A standard glucose curve was used to calculate the amount of glucose released. Enzyme activity was determined using the formular:

$$\text{Enzyme activity (UI/ml)} = \frac{W \cdot 1000}{V.t.M}$$

where: W - the amount of released glucose equivalents,
M - the molecular weight of the glucose,
V - the volume of the measured sample,
t - the reaction time.

After determining the amount of reducing sugars, the CMCase activity is determined and expressed in units (UI). In all assays, one enzyme unit (UI) was defined as the amount of enzyme producing one µmol of reducing sugar measured as glucose per minute under standard assay conditions (Arimurti *et al*., 2017).

Results

Cellulose degrading bacteria: Table 1 shows the cellulolytic index of the cellulose degrading bacteria. The cellulolytic index ranges from 0.2 - 4. Isolate CDB7 had the highest cellulolytic index of 4; isolate CDB3 and CDB4 had the lowest cellulolytic index of 0.2.

Table 1: Cellulolytic index of isolated cellulolytic bacteria

Isolate code	Diameter of colony (cm)	Diameter of cellulolytic zone (cm)	Cellulolytic index
CDB1	0.90	1.50	0.67
CDB2	0.40	0.70	0.75
CDB3	1.00	1.20	0.20
CDB4	1.00	1.20	0.20
CDB5	1.00	1.40	0.40
CDB6	0.30	0.90	2.00
CDB7	0.20	1.00	4.00
CDB8	0.50	1.00	1.00
CDB9	1.10	1.70	0.55

Plate 1 shows API 20E test reactions for the isolate with the highest index (CDB7). Isolate CDB7 was identified as *Pseudomonas luteola*.

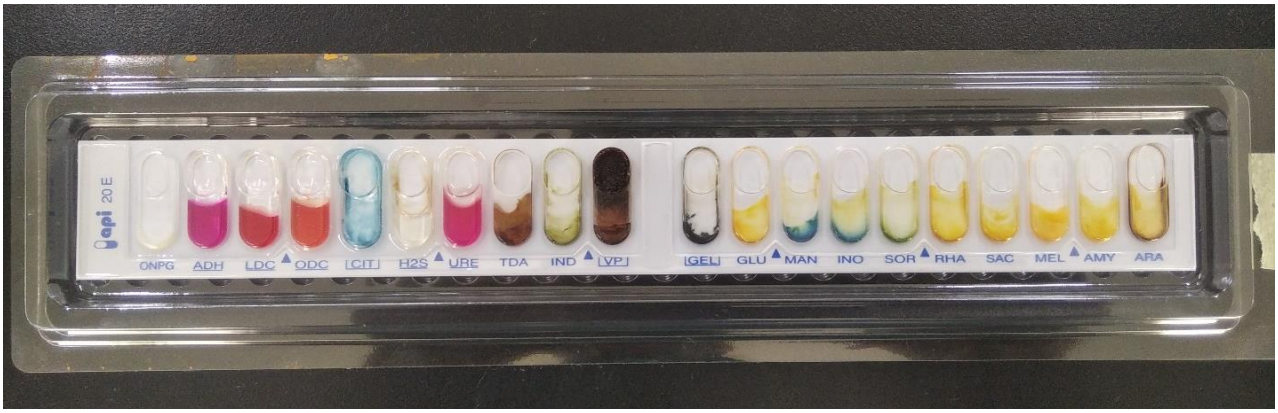

Plate 1: API 20E test reactions

β- endoglucanase Assay: Table 2 shows the absorbance of supernatant in different amount of CMC using DNS reagent method and read at A_{540}. The lowest absorbance was 0.059 (day 12) and the highest was 0.240 (day 2) in the 80% CMC medium, while in the 20% CMC medium, the lowest absorbance value was 0.086 (day 12) and the highest 0.273 (day 6).

Table 2: Absorbance of crude enzyme extract in different amount of CMC with the same inoculum using DNS reagent method and read at A_{540}

Amount of CMC (%)	Fermentation duration (Days)					
	2	4	6	8	10	12
80	0.240	0.152	0.189	0.125	0.102	0.059
20	0.221	0.120	0.273	0.108	0.100	0.086

The amount of glucose produced in 80% CMC medium as deduced from the glucose calibration curve ranged from 0.101 – 0.320mg/ml while the amount produced in 20% CMC medium, ranged from 0.133 – 0.360mg/ml (Fig. 1).

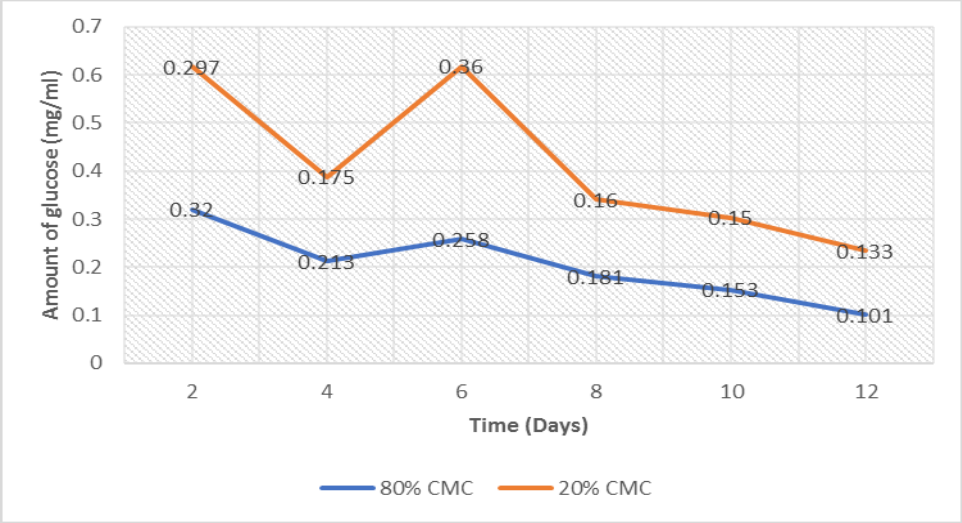

Fig. 1 Amount of glucose produced

The highest cellulolytic activity in 80% CMC medium was 0.178UI/ml on day 2 and the least was 0.056UI/ml on day 12, while 0.200UI/ml was the highest cellulolytic activity on day 6 and 0.074UI/ml the lowest in the 20% CMC medium on day 12 (Fig. 2).

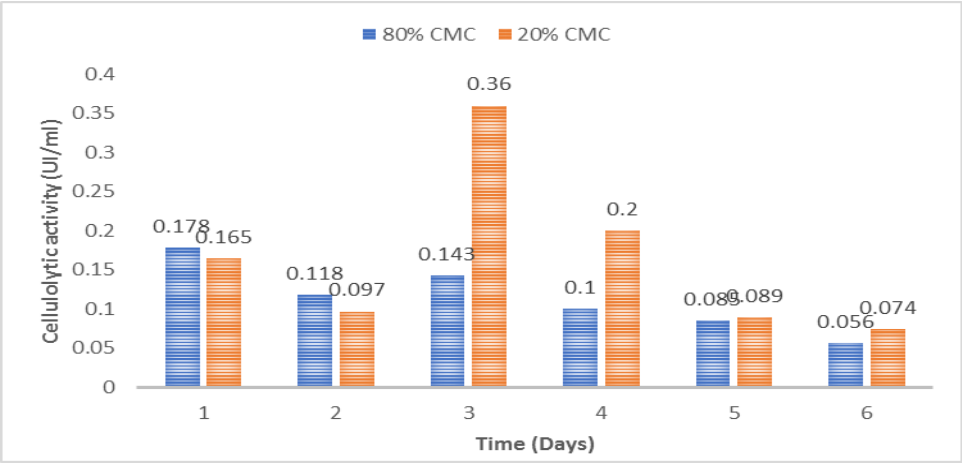

Fig. 2: Cellulolytic activity

Discussion

This work investigated the cultivable CMC-degrading bacteria present in the gut of palm weevil (*Rhynchophorus phoenicis*) larvae. A total of 9 isolates were found to hydrolyse CMC. The isolates gave cellulolytic index values ranging from 0.2-4, which was found to be higher than previously reported cellulolytic index range of 0.75 – 2.5 by Kondakindi *et al*., (2017) but slightly lower than cellulolytic index ranging from 0.54-4.93 reported by Erick *et al*. (2021).

The isolate with the highest cellulolytic index was identified as *Pseudomonas luteola*. *Pseudomonas luteola* had been reported to have capability to degrade cellulose. Maharsiwi *et al.,* (2020) isolated and identified cellulolytic strains of *Pseudomonas luteola* from the sponges. Ohkuma *et al.,* (2003) isolated and identified cellulolytic *Pseudomonas luteola* from termite.

Pseudomonas luteola was used to produce cellulolytic enzymes, measured by the amount of glucose produced. After fermentation, the highest glucose concentration (0.320mg/ml) was produced on the second day in 80 CMC broth and 0.360mg/ml on the sixth day in 20% CMC broth of incubation in the medium containing 80% CMC. The values decreased to 0.101mg/mi and 0.133mg/ml after 12 days. These values are within range of values (0.07 – 1.03mg/ml) reported by Kasing *et al*. (2000).

The endoglucanase activity measured in the 80% CMC medium varied from 0.056 UI/ml-0.178, with the highest activity recorded on the second day. Recorded enzyme activity declined after the second day to the twelfth day. In the 20% CMC medium, the maximum endoglucanase activity (0.36UI/ml) was observed on the sixth day followed by the second day 0.200UI/ml and the least activity (0.074 UI/ml) was observed on the twelfth day. Observed endoglucanase activities were higher than other unoptimized study such as 0.02UI/ml and 0.058UI/ml by *Brevibacillus sp*. DUSELG12 and *Geobacillus sp*. DUSELR7 as reported by Rastogi *et al*., (2009); 0.0701UI/ml by Bacillus cereus reported by Erick *et al*., (2021) and 0.07 UI/ml from *Bacillus subtilis* AS3 reported by Deka *et al*. (2011). The results obtained in this study are, however, lower than endoglucanase activity of 2.02UI/ml and 2.08UI/ml under optimized conditions as reported by Shilpa and Pethe (2017) and Liang *et al*., (2014) respectively. Cellulases production can be increased through optimization of various factors such as pH value, growth time, temperature, and introduction of inducers in fermentation medium (Sethi *et al*., 2013). Therefore, the fact that the present study did not optimize the culture conditions might have affected the low production of enzymes relative to optimized studies.

Conclusion

This preliminary investigation indicates that the microflora of the gut of palm weevil larvae include bacteria capable of hydrolyzing cellulose into reducing sugars. The high cellulolytic activities reported for the isolates encourages further research in optimization using *Pseudomonas luteola* for possible application in large-scale cellulase production.

Acknowledgements

The authors wish to thank the Bayelsa State Education Development Trust Fund (EDTF) for supporting the Bayelsa suya initiative to the University of Africa, Toru-Orua.

We also wish to thank Professor Mansi El-Mansi and Professor Francis Sikoki for their encouragement and stimulating discussions.

References

Arimurti S, Nurani Y, Ardyati T, Suharjono S: Screening and identification of indigenous cellulolytic bacteria from Indonesian coffee pulp and investigation of its caffeine tolerance ability. Malays J Microbiol 13(2): 109-116. 2017.

Dashtban M, Maki M, Leung KT, Mao C, Qin W: Cellulase activities in biomass conversion: Measurement methods and comparison. Critical Rev Biotechnol 30(4): 302-309. 2010.

Deka D, Bhargavi P, Sharma A, Goyal D, Jawed, M, Goyal A: Enhancement of cellulase activity from a new strain of Bacillus subtilis by medium optimization and analysis with various cellulosic substrates. Enzyme Res 151656. 2011.

Erick MS, Aneth DM, Cyprian BM, Sylvester LL: Cellulase-Producing Bacteria Isolated from Mufindi Paper Mill Industrial Effluent, Iringa Tanzania. Tanz J Sci. 47(1): 204-213. 2021.

Ferbiyanto A, Rusmana I, Raffiudin R. Characterization and identification of cellulolytic bacteria from gut of worker Macrotermes gilvus. HAYATI J. Biosci 22(4): 197-200. 2019.

Hill J, Nelson E, Tilman D, Polasky S, Tiffany D: Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Natl Acad Sci U.S.A. 103: 11206–11210. 2006.

Kasing A, Bor CJ, Mohd AS: Screening and isolation of a cellulolytic and amylolytic Bacillus from sago pith waste. J Gen Appl Microbiol. 46: 263–267. 2000.

Kondakindi VR, Tatiparti V, Pabbati R, Maddela NR: Characterization of some efficient cellulase producing bacteria isolated from pulp and paper mill effluent contaminated soil. Braz Arch Biol Technol. 60: 4. 2017.

Liang Y, Zhang Z, Wu M, Wu Y, Feng J: Isolation, screening, and identification of cellulolytic bacteria from natural reserves in the subtropical region of China and optimization of cellulase production by *Paenibacillus terrae* ME27-1. BioMed Res Int. 1-13. 2014.

Maharsiwi W, Astuti RI, Meryandini A, Wahyudi AT: Screening and characterization of sponge-associated bacteria from Seribu Island, Indonesia producing cellulase and laccase enzymes. Biodiversitas. 21(3): 975-981. 2020.

Moe ST, Janga K.K, Hertzberg T, Hägg MB, Øyaas K, Dyrset N: Saccharification of lignocellulosic biomass for biofuel and biorefinery applications–a renaissance for the concentrated acid hydrolysis? Energy. Procedia 20: 50-58. 2012.

Ogbuagu MN, Ohondu I, Nwigwe C: Fatty Acid and Amino Acid Profiles of the Larva of Raffia Palm Weevil: *Rhynchophorus phoenicis*. Pac J Sci Technol 12(2): 392- 400. 2011.

Okhuma M: Termite symbiotic systems: efficient bio-cycling of lignocellulose. Appl Microbiol Biotechnol 61: 1-9. 2003.

Oppert C, Kingeman WE, Willis JD, Oppert B, Jurat-Fuentes JL: Prospecting for cellulolytic activity in insect digestive fluids. Comparative Biochemistry and Physiology. Part B, Biochem Mol Biol 155(2): 145-154. 2010.

Tan H, Corbin KR, Fincher GB: Emerging technologies for the production of renewable liquid transport fuels from biomass sources enriched in plant cell walls. Front Plant Sci. 7:1854. 2016.

Tilman D, Hill J, Lehman C: Carbon-negative biofuels from low input high-diversity grassland biomass. Science. 314: 1598–1600. 2006.

Rastogi G, Muppid GL, Gurram RN, Adhikari A, Bischoff KM, Hughes SR: Isolation and characterization of cellulose-degrading bacteria from the deep subsurface of the Homestake gold mine, Lead, South Dakota, USA. J Ind Microbiol Biotechnol 36, 585–598. 2009.

Sethi S, Datta A, Gupta BL, Gupta S. Optimization of cellulase production from bacteria isolated from soil. Int Sch Res Notices 1: 1-7.2013.

Shilpa L, Pethe AS: Isolation and screening of cellulolytic bacteria from soil and optimization of cellulose production. Int J Life Sci 5(2): 277-282. 2017.

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

Sukumaran RK, Singhania RR, Pandey A: Microbial cellulases-production, applications and challenges. J Sci Ind Res. 64: 832-844. 2005.

Womeni MH, Tiencheu B, Linder M, Nabayo CME, Tenyang N Mbiapo TF, Villeneuve P, Fanni J, Parmentier M: Nutritional value and effect of cooking, drying and storage process on some functional properties of *Rhynchophorus phoenicis*. Int J Life Sci Pharma Res 2: 203-219. 2012.

Yang B, Dai Z, Ding SY, Wyman CE: Enzymatic hydrolysis of cellulosic biomass. Biofuels. 2(4): 421-449. 2011.

Zabed H, Sahu JN, Boyce AN, Faruq G: Fuel ethanol production from lignocellulosic biomass: an overview on feedstocks and technological approaches. Renew Sust Energ Rev 66: 751-774. 2016.

Zhang YH, Hong J, Ye X: Cellulase assays. In: Mielenz J (Eds) *Biofuels*. Methods Mol Biol (Methods and Protocols) 581: 213-231. 2009.