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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2020.1345.1350

Short Communication
Pseudomonas stutzeri CM1, Novel Thermotolerant Cellulase-Producing Bacteria Isolated from Forest Soil

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Abstract

Background and Objective: Cellulase is an important enzyme that useful for agricultural residue hydrolysis such as plant stover, molasse, rice straw. Thermotolerant cellulases are required to apply in textile, food, detergent, biofuels and pharmaceutical applications. This research aimed to isolate the thermotolerant cellulase-producing bacteria from forest soil and to determine cellulase activity from isolated bacteria. **Materials and Methods:** Soil samples were collected from the Roi Et Rajabhat University forest. One gram of soil sample was mixed with Luria-Bertani (LB) broth medium and incubated at 37°C with shaking at 150 rpm for 24 h. The cultured broth was streaked on LB agar plate and incubated at 37°C for 24 h. Cellulase-producing bacteria were isolated using Carboxymethylcellulose (CMC) agar plate. Four bacterial isolates which presented a clear zone on CMC agar plate after flooded with iodine solution, named CM1, CM2, CM3 and CM4. Cellulase activity of 4 isolated bacteria was determined against various pH (pH 4-8) and temperature (50-100°C). **Results:** The results indicated that CM1 isolate showed the highest cellulase activity at 0.074 unit mL^{-1} at 80°C and pH5. All isolates were identified using 16S rRNA gene sequencing. The results indicated that CM1, CM3 and CM4 were identified as *Pseudomonas stutzeri*. while isolate CM2 was *Bacillus subtilis*. **Conclusion:** This is the first report presenting the thermotolerant cellulase produced by *Pseudomonas stutzeri*. The thermotolerant cellulase produced from *Pseudomonas stutzeri* in this study will be useful in many industrial processes using cellulase at high temperatures.

Key words: Cellulose-degrading bacteria, *Bacillus subtilis*, *Pseudomonas stutzeri*, forest soil, thermotolerant cellulase

Citation: Rattanasuk, S., A. Songsaeng and T. Sriwarom, 2020. *Pseudomonas stutzeri* CM1, novel thermotolerant cellulase-producing bacteria isolated from forest soil. Pak. J. Biol. Sci., 23: 1345-1350.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cellulose, a polysaccharide of D-glucose units linked by β-1,4 bonds, is the most abundant carbohydrate on earth[1,2]. It is an important main component of plant biomass and considered as the most important natural renewable resource for bioconversion to value-added bioproducts[3,4]. The use of cellulose obtained from industrial waste and agricultural waste as inexpensive carbon sources have been raised and importantly interesting economic.

Cellulase is an enzyme that hydrolyzes the main chain of cellulose into glucose molecule[2]. Cellulase in microorganisms is divided into 3 hydrolytic enzymes including endoglucanases (β-1,4-D-glucan-4-glucanohydrolase, EC 3.2.1.4, carboxymethyl cellulase, EC), exoglucanases (β-1,4-D-glucan-4-glucohydrolase, EC3.2.1.91, cellobiohydrolase, CBH) and cellobiases (β-D-glucoside glucohydrolase, EC 3.2.1.21, β-1,4-D-glucosidase)[5,6]. Cellulases are inducible enzymes that are synthesized by a large number of microorganisms during their growth including bacteria and fungi such as *Bacillus cereus* Razmin, *Enterobacter aerogenes* Razmin, *Chryseobacterium kwangyangense* Strain Cb.[7], *Acinetobacter junii*, *Bacillus subtilis*, *Cellulomonas biazotea*, *Pseudomonas cellulose*, *Acetivibrio cellulolyticus*, *Butyrivibrio fibrisolvens*, *Clostridium thermocellum*[5], *Paenibacillus* sp., *Aeromonas* sp., *Bacillus* sp.[2], *Aspergillus niger*, *Rhizopus oryzae*, *Penicillium notatum*, *Mucor racemosus*, *Aureobasidium* sp., *Trichoderma citrinoviride*, *Fusarium salani*[6], *Ruminococcus*, *Alteromonas* and *Acetivibrio* etc[8]. Cellulase production from bacteria has been considered more advantageous than that from fungi since the bacteria provide higher growth rates compared to those of fungi[9]. The applications of cellulase are in the textile industry for bio-polishing, fabric softness, brightness textile, food, detergent, fermentation of biomass into biofuels and pharmaceutical applications[8,10].

Soil samples from the natural forests of the university are good sources of cellulose bacteria because the soil samples are not contaminated with chemicals. It is also an area covered with cellulose from leaves and branches. This research aimed to isolate and to characterize cellulose-degrading bacteria from soil collected from Roi Et Rajabhat University.

MATERIALS AND METHODS

Study area: All the experiments were performed during October, 2018-April, 2019 in the Microbiology Laboratory, Major of General Science, Department of Science and Technology, Faculty of Liberal Arts and Science, Roi Et Rajabhat University, Roi Et, Thailand.

Soil samples collection: Soil samples were collected from the Roi Et Rajabhat University forest and were used as the cellulase-producing bacterial source. Collected soil samples were put in the sterile bottles and transferred to the microbiology laboratory, Faculty of Liberal Arts and Science, Roi Et Rajabhat University.

Isolation and screening for cellulose-degrading bacteria: One gram of each soil sample was added into 50 mL of sterilized Luria-Bertani (LB) broth medium and was incubated at 37°C with shaking at 150 rpm for 24 h. The cellulose-degrading bacteria were isolated using LB agar containing 1% carboxymethyl cellulose (CMC). The plates were incubated at 37°C in the bacterial incubator for 24 h. Detection of cellulolytic bacteria solubilization was observed by a clear zone formation after flooded with iodine solution. The colonies that presented the clear zone were collected for further experiments.

Preparation of crude enzyme: The single colony of bacterial isolates that showed the clear zone was cultured using 50 mL of sterilized LB broth medium and incubated overnight at 37°C with shaking at 150 rpm. The bacterial cell concentration was adjusted at OD600 nm of 0.1 and bacterial final concentration at 1% was continued cultured with LB broth containing 1% CMC at 37°C, 150 rpm for 24 h. The cultures were then centrifuged for 5 min at 10000 rpm and the clear supernatant was used as a source of crude cellulase solution.

Cellulase activity assay: An aliquot of 500 µL of crude cellulase was mixed with 500 µL 1% CMC (pH 4) and incubated at 50°C for 5 min. The cellulase reaction was terminated by adding DNS-reagent. The reducing sugar liberated in the enzyme reaction was measured as glucose reducing equivalents by the Nelson[11] and Somogi[12] method. One unit of enzyme is defined as the amount of enzyme which liberates reducing sugars equivalent to 1 µmol glucose standard per minute under the experimental conditions described above[13].

Cellulase thermal and pH stability: An aliquot of 500 µL of crude cellulase was mixed with 500 µL 1% CMC (pH 4-8) and incubated at 50, 60, 70, 80, 90 and 100°C for 60 min. Cellulase activity was stopped by adding DNS reagent. The amount of reducing sugars was determined by measuring at 540 nm.

Bacterial morphological characterization: Bacterial cell morphology was observed under a light microscope using a Gram staining technique which used to differentiate between Gram-positive and Gram-negative bacteria.

Bacterial identification: Bacterial isolates were cultured overnight at 37°C using LB broth. The bacterial cell was lysed by boiling at 100°C for 5 min. The bacterial lysate was centrifuged at 4,000 rpm for 5 min and 5 µL of the supernatant was used as a DNA template in a Polymerase Chain Reaction (PCR). The primers used to amplify 16S rDNA genes were fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-AAGGAGGTGATCCAGCC-3')[13]. PCR reaction was done using an initial denaturation at 94°C for 2 min and followed by 35 cycles with denaturation at of 94°C (45 sec), annealing at 55°C (45 sec) and extension at 72°C (2 min) and a final extension at 72°C for 10 min. The purified 16S rDNA product was sent to the Macrogen Company (Korea) for DNA sequencing. The resulting DNA sequences were compared with the non-redundant nucleotide database from GenBank using the BLAST program.

Data analysis: In this study, it was used experimental design followed by descriptive analysis. All graphs and data were done using Excel version 2016.

RESULTS AND DISCUSSION

Isolation and screening for cellulose-degrading bacteria: Four bacterial isolates named CM1, CM2, CM3 and CM4 were isolated from soil samples collected from the Roi Et Rajabhat University forest. These isolated strains presented the clear zone on LB agar containing CMC after iodine staining. Many sources were used to isolated cellulose-degrading bacteria including waste dumping sites[5], sugar industry waste[2], termites[7], mangrove sediment[14], humus soil[1], soil[4], pulp wastewater, rotten wood[6], sediment from fish ponds[15], cow dung[3], hindgut of wood-feeding termite[16], saw dust[17], molasses[18], brown garden snail (*Cornu aspersum*)[19], decaying banana pseudostem and *Strelitzia alba*[20]. Cellulolytic activity of CM4 showed the highest cellulase activity at 0.0081 unit mL^{-1} followed by CM1, CM3 and CM2 at 0.004835, 0.00229 and 0.003562 unit mL^{-1}, respectively (Fig. 1).

Cellulase thermal and pH stability: Crude cellulase was determined for the enzyme stability by incubating with various temperature and pH for 60 min. The results indicated that at pH 5 and 80°C, CM1 isolate showed the highest cellulase activity at 0.074 unit/ml. At pH 4 and 80°C, CM2, CM3 and CM4 isolates showed the highest cellulase activity at 0.056282, 0.070806 and 0.068991, respectively (Fig. 2). The effects of temperature and pH on the stability of the cellulase were elucidated in many previously reported. The highest cellulase production from *Paenibacillus* sp. showed the optimal at pH 7.0 and 40°C temperature on 24 h[2]. The highest cellulase activity from *Cellulomonas* sp. ASN2 was found at 60°C and pH 7.5[21]. *Scophthalmus maximus* was presented the highest cellulase activity obtained at 50°C and pH 5[22]. The optimum pH and temperature of a purified cellulase of *Cellulomonas uda* NCIM 2353 was 7 and 50°C, respectively[23]. The optimum activity of *Aspergillus flavus* was at pH 10 with 60°C[24] and *Scytalidium thermophilum* SKESMBKU02 showed high cellulolytic activity at 45°C and pH 5.0-6.0[25]. The optimum condition of cellulase from different organisms was not similar. This research presented the thermotolerant cellulase that showed the highest cellulase activity at 80°C which higher than many previously reported. The greater stability of the cellulose can be useful for many applications[25].

Bacterial morphological characterization and identification: The result from a microscopic observation demonstrated that CM1, CM3 and CM4 isolates were Gram-negative bacilli but CM2 isolate was Gram-positive bacilli (Fig. 3). The result

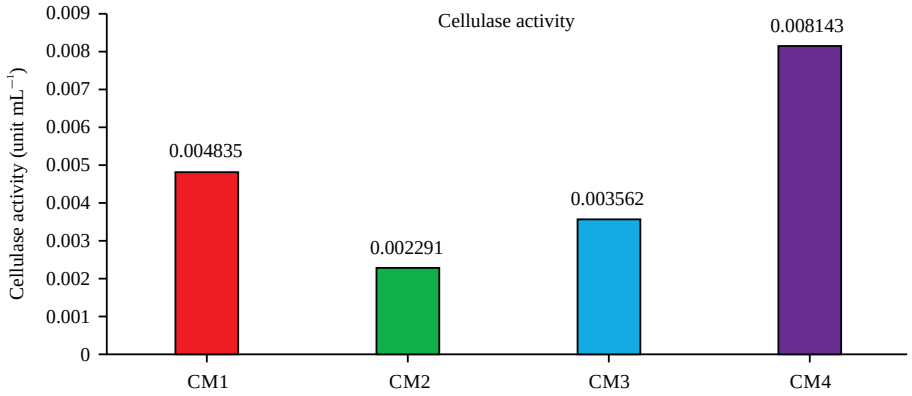

Fig. 1: Cellulase activity of isolated bacteria

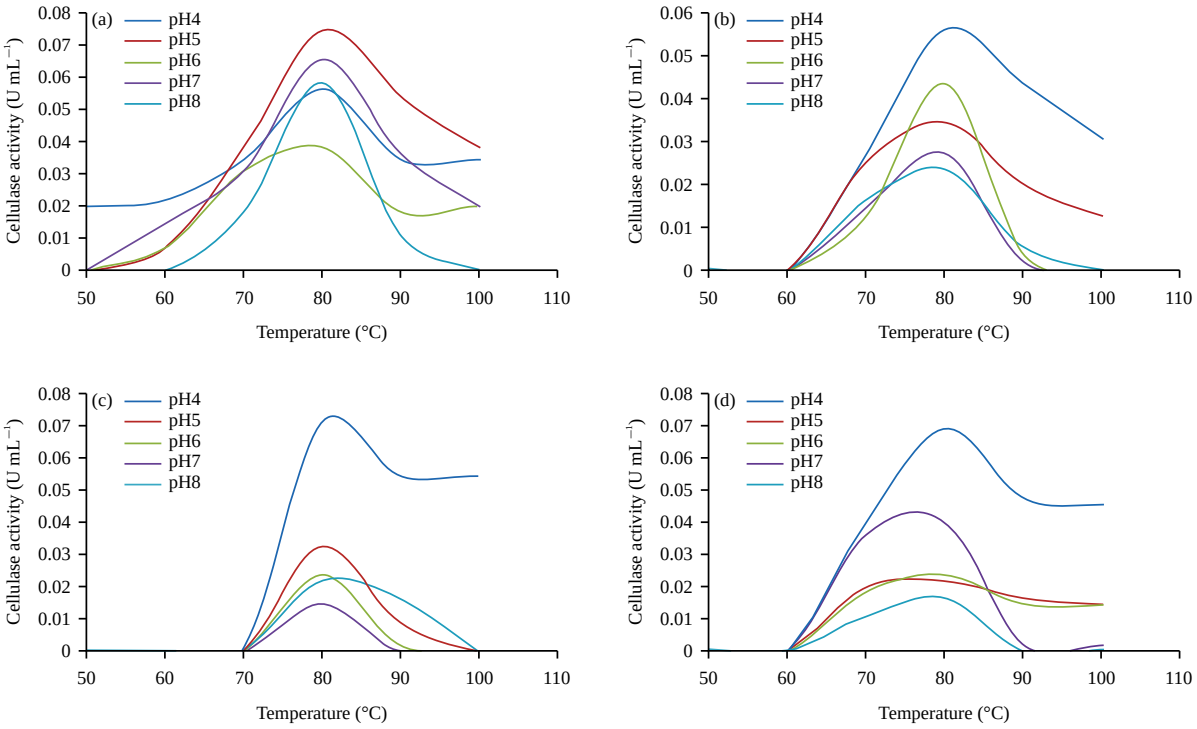

Fig. 2(a-d): Effect of temperature and pH on cellulase activity by (a) *Pseudomonas stutzeri* CM1 isolate, (b) *Pseudomonas stutzeri* CM2, (c) *Bacillus* sp. and (d) *Pseudomonas stutzeri* CM4

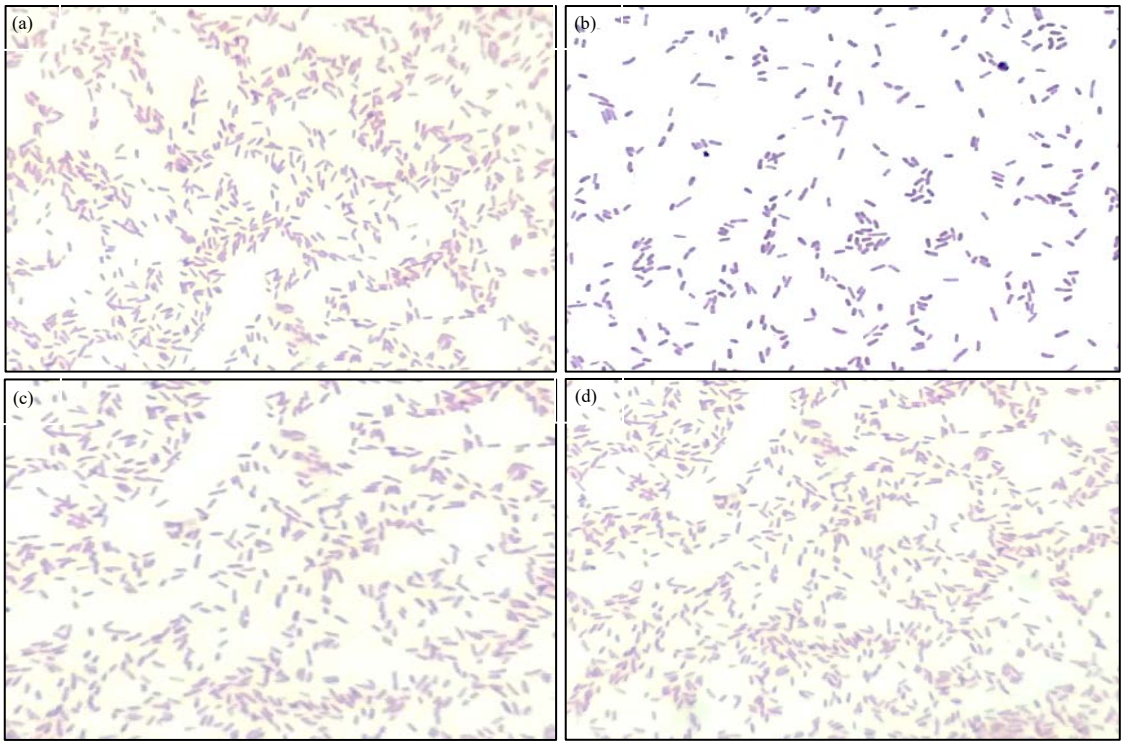

Fig. 3(a-d): Gram staining of (a) CM1, (b) CM2, (c) CM3 and (d) CM4 isolates

Table 1: Identification of isolated cellulase-producing bacteria

Isolates	Closet strain in GenBank (accession number)	Identity (%)	Identification
CM1	*Pseudomonas stutzeri* (NR_103934)	99.17	*Pseudomonas stutzeri*
CM2	*Bacillus subtilis* (NR_112116)	97.59	*Bacillus subtilis*
CM3	*Pseudomonas stutzeri* (NR_103934)	98.91	*Pseudomonas stutzeri*
CM4	*Pseudomonas stutzeri* (NR_103934)	99.11	*Pseudomonas stutzeri*

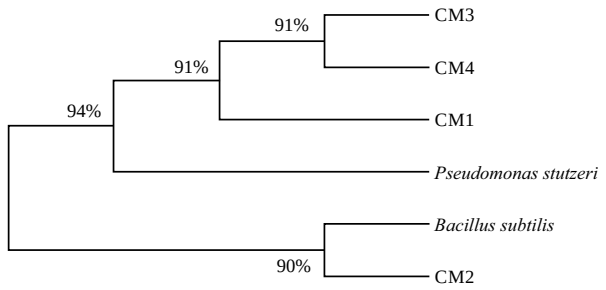

Fig. 4: Phylogenetic tree of isolated cellulose degrading bacteria

from DNA sequencing revealed that CM1, CM3 and CM4 isolates were *Pseudomonas stutzeri* and CM2 isolate was *Bacillus subtilis* (Table 1). The phylogenetic tree was constructed and presented that CM1 CM2 and CM4 are related to *Pseudomonas stutzeri* and CM2 is related with *Bacillus subtilis* (Fig. 4)[26]. The result from this research was similar to Islam and Roy[18], Sakpetch *et al.*[27], Listyaningrum *et al.*[28] that isolated cellulase-producing *Bacillus*. The results of this research were similar to Goel *et al.*[29] who reported *Pseudomonas* sp. and Berlemont *et al.*[30] who presented that *Pseudomonas stutzeri* was isolated from soil. The finding of this study presented the diversity of thermotolerant cellulase producing-bacteria isolated from soil, Thailand. It will be useful in many industrial applications such as textile, fermentation and bioenergy production, etc. The enzyme purification and factor affecting enzyme activity will be conducted.

CONCLUSION

Thermotolerant cellulase producing bacteria was isolated from forest soil collected from Roi Et Rajabhat University. Cellulase activity of isolated bacteria was affected by temperature and pH of incubation. *Pseudomonas stutzeri* CM1 presented the highest cellulase activity at 80°C and pH 5 (0.074 unit mL^{-1}). This is the first report that presented about thermotolerant cellulase produced from *Pseudomonas stutzeri.*

SIGNIFICANCE STATEMENT

This study discovers the thermotolerant cellulase producing-*Pseudomonas stutzeri* that can be beneficial for the textile industry for bio-polishing, fabric softness, brightness textiles, food, detergent, fermentation of biomass into biofuels and pharmaceutical applications. This study will help the researcher to uncover the critical areas of the screening method of thermotolerant enzyme producing-bacteria that many researchers were not able to explore. Thus, a new application using thermotolerant cellulase produced from *Pseudomonas stutzeri* may be arrived at.

ACKNOWLEDGMENT

This research was supported by Roi Et Rajabhat University Grant No. 639219.

REFERENCES

1. Karthi, V., A.R. Kumar, R. Ramesh, A. Stephen and S. Kumaresan, 2019. Isolation, identification and optimization of cellulase producing bacteria from forests of western ghats, india. J. Emerging Technol. Innovative Res., 6: 240-247.
2. Islam, F. and N. Roy 2019. Isolation and characterization of cellulase-producing bacteria from sugar industry waste. Am. J. BioSci., 7: 16-24.
3. Croos, A., S. Rajendran and K. Ranganathan, 2019. Isolation of a cellulase producing Bacillus cereus from cow dung and determination of the kinetic properties of the crude enzyme. J. Natl. Sci. Found. Sri Lanka, 10.4038/ jnsfsr.v47i2.9168.
4. Yotchaisarn, M., W. Saengha, T. Karirat, S. Deeseenthum, K. Maneewan and V. Luang-In, 2018. Identification of cellulase-producing bacteria from soil in Nasinuan forest, Kantarawichai district, Mahasarakham Province. National Res. counc. Thailand, 10.14457/MSU.res.2018.31.
5. Rashid, B., Z.A. Baba, M.A. Malik, A.H. Mir and F. Akhter *et al.*, 2019. Characterization of cellulolytic bacteria from waste dumping sites of Kashmir Himalaya. Int. J. Curr. Microbiol. App. Sci, 8: 2033-2048.

6. Effiong, T., M. Abdulsalami, N. Egbe and V. Bakare, 2019. Screening of fungi isolates from soil, pulp waste water and rotten wood for cellulase producing potentials. J. Appl. Sci. Environ. Manage., 23: 1051-1055.
7. Azizi-Shotorkhoft, A., T. Mohammadabadi, H. Motamedi, M. Chaji, H. Fazaeli, 2016. Isolation and identification of termite gut symbiotic bacteria with lignocellulose-degrading potential, and their effects on the nutritive value for ruminants of some by-products. Animal Feed Sci. Technol., 221: 234-242.
8. Shanmugapriya, K., P.S. Saravana, M.M. Krishnapriya, A. Mythili and S. Joseph, 2012. Isolation, screening and partial purification of cellulase from cellulase producing bacteria. Int. J. Adv. Biotechnol. Res., 3: 509-514.
9. Sethi, S., A. Datta, B.L. Gupta and S. Gupta, 2013. Optimization of cellulase production from bacteria isolated from soil. ISRN Biotechnol., Vol. 2013. 10.5402/2013/985685.
10. Hussain, A.A., M.S. Abdel-Salam, H.H. Abo-Ghalia, W.K. Hegazy and S.S. Hafez, 2017. Optimization and molecular identification of novel cellulose degrading bacteria isolated from Egyptian environment. J. Genetic Eng. Biotechnol., 15: 77-85.
11. Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., 153: 375-380.
12. Smogyi, M., 1952. Notes on sugar determination. J. Biol. Chem., 195: 19-23.
13. Rattanasuk, S. and M. Ketudat-Cairns, 2009. *Chryseobacterium indologenes*, novel mannanase-producing bacteria. Songklanakarin J. Sci. Technol., 31: 395-399.
14. Kurniawan, A., S.P. Sari and E. Asriani, 2019. Molecular identification of cellulolytic bacteria from mangrove sediment at tin minning region in west bangka. Int. J. Appl. Biol., 3: 7-14.
15. Dat, T., V. Tam, T. Dung, L. Bui, H. Anh and P. Oanh, 2019. Isolation and screening of cellulose and organic matter degrading bacteria from aquaculture ponds for improving water quality in aquaculture. IOP Conference Series: Earth and Environmental Science, 2019 IOP Publishing, pp: 012002-012002.
16. Afzal, M., M.Z. Qureshi, S. Khan, M.I. Khan and H. Ikram *et al*., 2019. Production, purification and optimization of cellulase by *Bacillus licheniformis* hi-08 isolated from the hindgut of wood-feeding termite. Int. J. Agric. Biol., 21: 125-134.
17. Akinleye, H.A., F.C. Samuel-Osamoka and H.M. Moniloye, 2018. Optimization of cellulose production by bacteria isolated from saw dust. Nig. J. Biotechnol., 35: 16-24.
18. Islam, F. and N. Roy, 2018. Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses. BMC Res. Notes, Vol. 11, No. 1. 10.1186/s13104-018-3558-4.
19. Ndlovu, T.M., and J.P.H. Van Wyk, 2019. Isolation of cellulase enzyme from brown garden snail (*Cornu aspersum*) for the saccharification of waste paper materials MethodsX, 6: 1030-1035.
20. Legodi, L.M., D. La Grange, E.L.J. Van Rensburg and I. Ncube, 2019. Isolation of cellulose degrading fungi from decaying banana pseudostem and *Strelitzia alba*. Enzyme Res., 10.1155/2019/1390890.
21. Irfan, M., A. Safdar, Q. Syed and M. Nadeem, 2012. Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production and activity. Turk. J. Biochem., 37: 287-293.
22. Pachauri, P., S. More, V. Aranganathan, S. Sullia and S. Deshmukh, 2018. Kinetic study and characterization of cellulase enzyme from isolated *Aspergillus niger subsp. awamori* for cellulosic biofuels. J. Sci. Ind. Res., 77: 55-60.
23. Swathy, R., K. Rambabu, F. Banat, S.H. Ho, D.T. Chu and P.L. Show, 2019. Production and optimization of high grade cellulase from waste date seeds by *Cellulomonas uda* NCIM 2353 for biohydrogen production. Int. J. Hydrogen Energy, 10.1016/j.ijhydene.2019.06.171.
24. Bano, A., X. Chen, S. Prasongsuk, A. Akbar and P. Lotrakul *et al*., 2019. Purification and characterization of cellulase from obligate halophilic *Aspergillus flavus* (TISTR 3637) and its prospects for bioethanol production. Appl. Biochem. Biotechnol., 189: 1327-1337.
25. Santoshkumar, S., E. Sujatha and P. Shivakrishna, 2015. Production and optimization of exo and endocellulases from thermophilic fungi scytalidium thermophilum SKESMBKU02. Walailak J. Sci. Technol., 13: 9-22.
26. Kumar, S., G. Stecher, M. Li, C. Knyaz and K. Tamura, 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol., 35: 1547-1549.
27. Sakpetch, P., H. Aran and A. Chandumpai, 2017. Isolation and screening of potential lignocellulolytic microorganisms from rubber bark and other agricultural residues. Walailak J. Sci. Technol., 14: 953-967.
28. Listyaningrum, N., A. Sutrisno and A. Wardani, 2018. Characterization of thermostable cellulase produced by bacillus strains isolated from solid waste of carrageenan. Conference Series: Earth and Environmental Science, March 1, 2018, IOP Publishing pp: 012043-012043.
29. Goel, N., R. Patra and S. Verma, 2019. Purification and characterization of cellulase from *Pseudomonas* sp. Isolated from waste dumping site soil. J. Appl. Biotechnol. Bioeng., 6: 118-124.
30. Berlemont, R., M. Delsaute, D. Pipers, S. D'amico, G. Feller, M. Galleni and P. Power, 2009. Insights into bacterial cellulose biosynthesis by functional metagenomics on Antarctic soil samples. ISME J., 3: 1070-1081.