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Optimization Studies for Cellulase Production by Bacteria Isolated from Solid Waste

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A B S T R A C T

Cellulose degrading bacteria plays a crucial role to convert cellulose into valuable products through cellulase enzyme. In this study, 21 bacteria were isolated from dump site of sewage pumping station, Khajod, Gujarat, India. Amongst, three bacteria *viz.*, SWCDB6 (1.32; 0.47; 0.36 and 0.46), SWCDB21 (1.27; 0.55; 0.36 and 0.48) and SWCDB5 (1.20; 0.52; 0.33 and 0.43) showed potent Cellulolytic Index (CI), CMCase (U/ml) activity, FPase (U/ml) activity and cell biomass (OD_{660}), respectively at 72 hrs at 30 ºC ± 0.2. Effect of varied pH, temperature, incubation period, carbon sources (starch and cellulose) and nitrogen source (ammonium sulphate and urea) studied to optimize the maximum cellulase production by these three isolates. Maximum CMCase, FPase and biomass production was recorded with pH 7.0 at 35 ºC ± 0.2 at 96 hrs along with addition of 0.5 % ammonium sulfate and starch in the medium for all three isolates. However, SWCDB21 was found to be potent cellulase producer and molecularly identified as *Staphylococcus* species. It can be further explored for agriculture waste degradation after thorough *in vivo* studies.

Introduction

Cellulose is the most abundant and important renewable natural raw material for industries like paper and pulp, animal feed, manure, and fuel. In order to convert the cellulose into valuable products, chemical and physical treatments followed in the industries created secondary pollutions (Agbor *et al.*, 2011). Thus, microbial cellulase is the best eco-friendly approach for biodegradation or bioconversion of cellulosic waste materials. Cellulase refers to a class of enzymes produced chiefly by fungi and bacteria (Immanuel *et al.*, 2006). Numerous bacteria *viz.*, *Cellulomonas*, *Pseudomonas*, *Bacillus* and *Micrococcus* have been reported to exhibit cellulolytic properties but

unfortunately very few are examined for their industrial applications (Lee and Koo, 2009). Further, extracellular enzymes like CMCase and FPase produced by the cellulose degraders are more sensitive to the various physiochemical condition of the environment. Soil of the waste dump site is represents the most suitable site to isolate the cellulose degrading bacteria. Therefore, present study aims to isolate and identify the novel cellulose degrading bacteria from waste dump site and to optimize the physiochemical parameters for maximum enzyme production.

Materials and Methods

Isolation and screening of cellulose degrading bacteria

Soil sample from the depth of 5 to 10 cm were collected from waste dump site of sewage pumping station, Khajod, Surat, Gujarat, India (21°05'45.2"N; 72°48'18.8"E). Isolation was done through standard microbiological method using serial dilutions and spread plate technique on sterile nutrient agar plate. Purified bacterial colony preliminary screened for cellulose degraders on Carboxymethyl cellulose (CMC) agar plate as per method of Missa *et al.*, (2016). Plates were flooded with 1 % congo red solution and colonies that showed discoloration of congo red were considered as cellulose degraders. Cellulolytic index (CI) was measured using the formula: Diameter of total zone – Diameter of bacterial colony/Diameter of bacteria colony. The isolates that showed significant cellulolytic index (CI) on CMC agar plate were further screened quantitatively using CMCase and FPase assays along with bacterial biomass (OD_{660}) according to method as described by Sherief *et al.,* (2010).

Effect of physiochemical conditions

Physiological condition was studied using varied incubation time, pH and temperature (Bhagat and Kokitkar, 2021). Each of selected bacteria were inoculated into the CMC broth and incubated at 30 ºC ± 0.2 for different incubation period (24, 48, 72 and 96 hrs). Similarly, each of the selected bacteria was cultured in CMC broth and subjected for varied temperatures (25 ºC, 35 ºC, 45 ºC and 55 ºC) and pH (5, 6, 7, 8, 9 and 10). Different nitrogen (ammonium sulphate and area) and carbon sources (starch and cellulose) with the concentration of 0.1 %. 0.5 % and 1.0 % as co-substrates were added in CMC broth. CMCase, FPase and biomass was analysed for each parameter along with control flasks.

Molecular identification of bacteria

The 16S-rDNA sequencing of potential cellulose degrader, SWCDB21 was performed for its proper identification. Bacterial genomic DNA was extracted and purified following the protocol as described by Nakada *et al.,* (2010). The 16S rDNA genes were amplified using primers 27F (AGAGTTTGGATCMTGGCTCAG) and 1492R (CGGTTACCTTGTTACGACTT). Sequencing services were taken from Eurofins Genomics India Pvt. Ltd., Bengaluru, India.

Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The consensus 16S rDNA sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and the optimal tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and evolutionary analyses were conducted in MEGA11 (Tamura *et al.*, 2021).

Statistical analysis

Primary screening, secondary screening and all physiochemical parameters were analyzed in triplicates. To study level of significance, the complete randomized design with factorial concept was used. The critical difference (CD) among the variance was calculated at $P \leq 0.05$ (Panse and

Sukhatme, 1967). The results are expressed as mean with standard deviation (mean±SD) or standard error (mean±SE).

Results and Discussion

Isolation and screening of cellulase producers

A total of 21 bacterial isolates, coded as SWCDB1 to SWCDB21 were isolated from the soil dumping site of sewage pumping station on nutrient agar media. Amongst, 15 isolates showed cellulolytic index (CI) in the range of 0.14 to 1.32 at 72 hrs at 30 ºC ± 0.2 on CMC agar media (Fig.1). Cellulolytic index > 1.0 was observed with the bacteria SWCDB21 (1.32) followed by SWCDB5 (1.27), SWCDB6 (1.20), SWCDB17 (1.10), SWCDB13 (1.09) and SWCDB3 (1.07). Thus, these six bacteria were studied for quantitative analysis of enzyme activities or secondary screening. Earlier, Hatami *et al.*, (2008) screened cellulolytic bacteria from farming and forest soil and reported CI value between 1.38 to 2.33 and 0.15 to 0.37, respectively. Gaur and Tiwari (2015) also selected bacteria that have > 1.0 CI on CMC agar plates.

Bacterial isolates SWCDB21 showed maximum CMCase (0.55 U/ml) and FPase (0.36 U/ml) followed by SWCDB5 and SWCDB6 (Table 1). Bacterial biomass was increased with incubation time (Table 1). Drastic change in the pH of the medium was not observed. Saini *et al.*, (2017) reported that an isolate NAB37 produced highest CMCase (0.948 U/ml) and FPase (0.125 U/ml) activity among the 371 bacteria isolated from soil.

Optimization of physiochemical condition

The selected three bacterial isolates *viz*., SWCDB21, SWCDB5 and SWCDB6 were optimized for its physiological conditions like incubation period, pH and temperature with respect to their CMCase, FPase and cell biomass activity. The optimum incubation period was found to be 96 hrs. The isolate SWCDB21 showed maximum activity of CMCase (0.55 U/ml), FPase (0.36 U/ml) and biomass (0.48 $_{OD660}$) followed by SWCDB5 and SWCDB6 (Fig. 2a). Previously, Lugani *et al.*, (2015) also studied effect of incubation period on cellulase production by *Bacillus* sp. Y3 and found that maximum FPase and CMCase at 96 hrs. The optimum temperature for maximum enzyme activities and cell biomass was recorded at 37 ºC. Bacterial isolates SWCDB21 (CMCase-0.60 U/ml; FPase- 0.40 U/ml and biomass$_{OD660}$-0.58) showed maximum activities followed by SWCDB6 and SWCDB5 (Fig. 2b). Further, moderate activities were noted at 25 ºC; while diminished at high temperatures (45 and 55 ºC). Similarly, Premalatha *et al.*, (2015) found 30 ºC as the optimized temperature for highest cellulase production by *Enhydrobacter* sp. Khatiawada *et al.*, (2016) also reported that *Serratia* sp. produced 0.20 U/ml of cellulase at 35°C. The effect of varied pH revealed that pH 7 and 8 was optimum for bacterial growth along with enzyme activities (Fig. 2c). SWCDB21 recorded remarkable enzyme activities *viz*., CMCase of 0.50 U/ml, FPase of 0.32 U/ml and cell biomass of $_{OD660}$ 0.52 than the other isolates. These results supported with the work of Dar *et al.*, (2018) who isolated *Klebsiella* sp. MD21 and reported maximum cellulase activities at pH 7.0.

The present study results are also in accordance with the work carried out by Islam and Roy (2018) wherein *Paenibacillus* species showed good growth and cellulase activity of 0.89 U/ml at pH 7. Further, all three bacteria showed the significant differences and maximum enzyme activity with cell biomass at 37ºC with pH 7 at 96 hrs of incubation period.

Different nitrogen and carbon source as co-substrates in CMC broth at 0.1 %, 0.5 % and 1.0 % was carried out in order to accelerate the microbial process. The maximum activity was observed with 0.5 % of ammonium sulphate as compared to other concentration. SWCDB21 showed higher activities than the other isolates (Fig. 3a). Biomass and enzyme activities were drastically decreased in the presence of urea. However, moderate enzyme activities and biomass was recorded with 0.1 % of urea with the isolate SWCDB6 (Fig. 3b).

Table.1 Quantitative analysis of cellulase production

Isolates	CMCase (U/ml)				FPase (U/ml)				Cell biomass (OD_{660})			
	24	48	72	96	24	48	72	96	24	48	72	96
SWCDB3	0.13	0.19	0.23	0.26	0.06	0.10	0.14	0.19	0.39	0.43	0.47	0.43
SWCDB5	0.33	0.37	0.46	0.52	0.20	0.20	0.26	0.33	0.41	0.43	0.46	0.49
SWCDB6	0.33	0.40	0.44	0.47	0.19	0.21	0.26	0.36	0.43	0.46	0.46	0.48
SWCDB13	0.15	0.20	0.24	0.32	0.06	0.09	0.16	0.20	0.38	0.40	0.43	0.41
SWCDB17	0.12	0.16	0.20	0.23	0.07	0.13	0.16	0.21	0.39	0.42	0.49	0.46
SWCDB21	0.31	0.33	0.44	0.55	0.21	0.29	0.31	0.36	0.44	0.48	0.48	0.54
S.Em. ±	0.005	0.007	0.012	0.011	0.004	0.005	0.007	0.006	0.008	0.009	0.013	0.011

Fig.1 Cellulolytic Index (CI) of the isolates

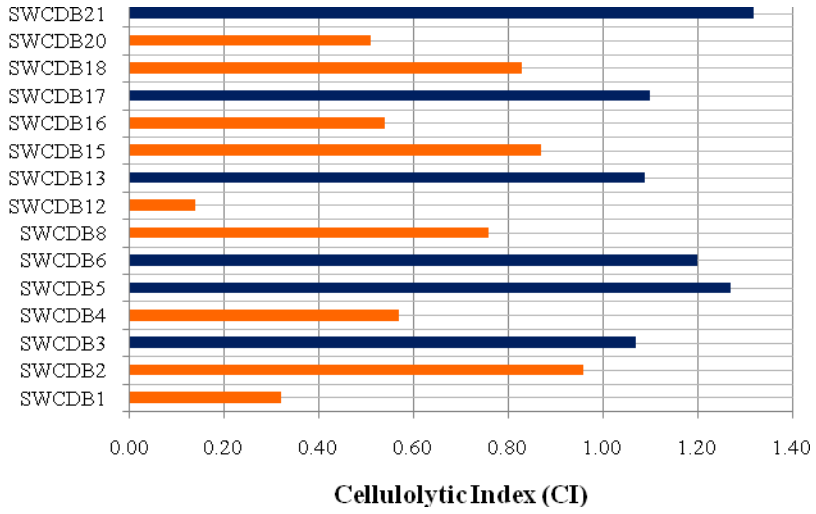
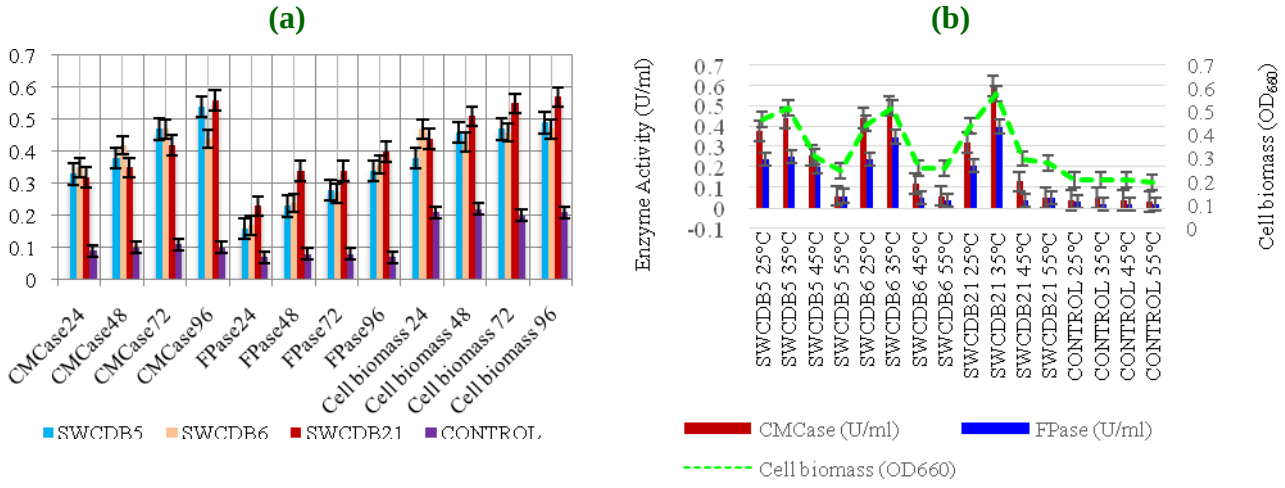


Fig.2 Effect of (a) incubation period (b) temperature and (c) pH on CMCase, FPase and biomass production



(c)

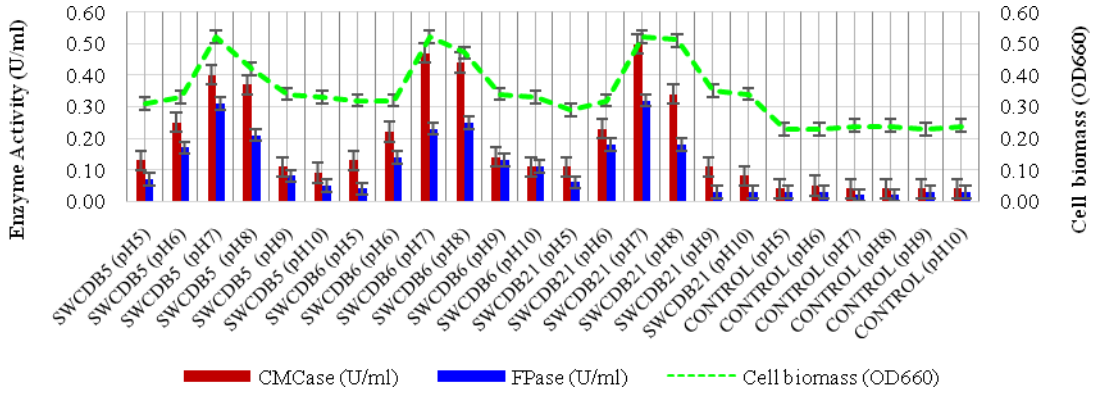
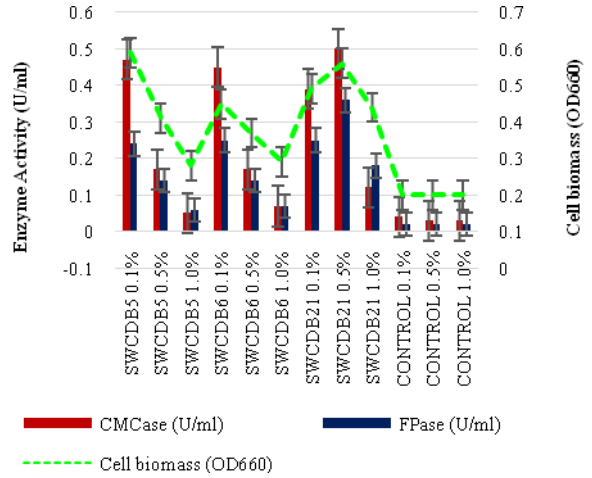
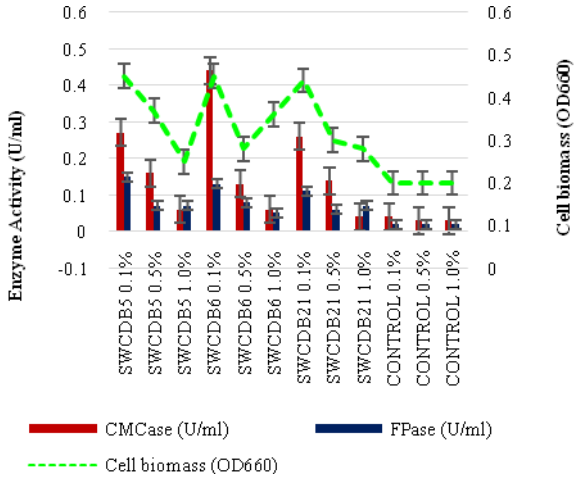


Fig.3 Effect of (a) ammonium sulfate (b) urea (c) starch and (d) cellulose

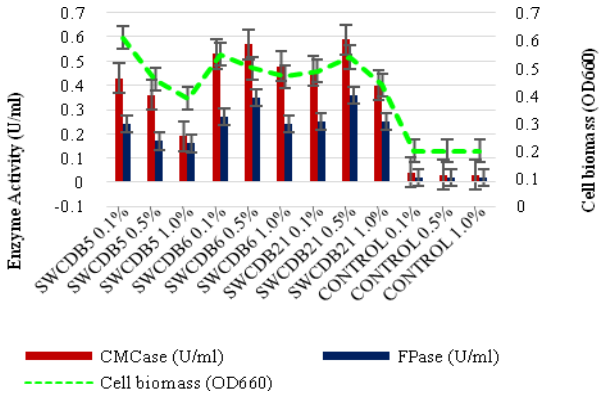
(a)



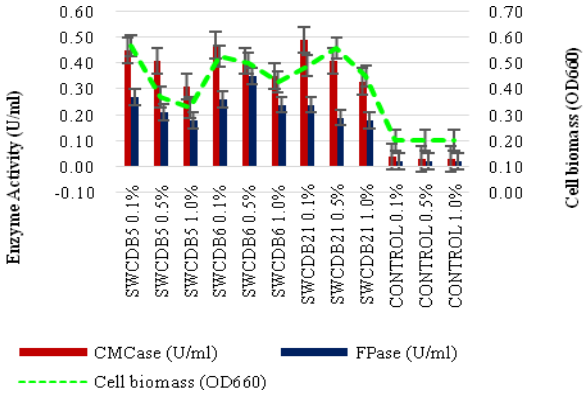
(b)



(c)



(d)



Earlier, Sethi *et al.*, (2013) reported higher cellulase activity by *Pseudomonas fluorescens*, *Bacillus subtilIs*, *E. coli*, and *Serratia marcescens* with 0.5 % of ammonium sulfate in the medium; while inhibition of enzyme activity in the presence of 0.5 % urea. Similar kind of results obtained by Vyas *et al.*, (2016), wherein CMCase and FPase activities of *Bacillus subltilis* M1was decreased in the presence of urea.

Among the varied concentration of carbon sources studied, 0.5 % starch was found to be optimum CMCase, FPase and biomass production for the bacterial isolates SWCDB21 and SWCDB6; while 0.1 % starch was for SWCDB5 (Fig. 3c). Moderate activities were noted with 0.1 % cellulose with all the isolates (Fig. 3d). However, starch was found to be a better as co-substrate as compared to cellulose. Similarly, *Ochrobactrum anthropi* (YZ1) and *Bacillus anthracis* isolated by the Duza and Mastan (2015) reported cellulase activity of 2.69 U/ml and 2.81 U/ml; 4.13 U/ml and 4.38 U/ml with 0.5 % and 1.0 % starch, respectively. Thus, ammonium sulfate and starch at 0.5 % in the CMC medium more optimum for the bacterial biomass and enzyme activities.

Molecular identification of the isolate

The isolate SWCDB21 was found potential among the three isolates. Thus, it was characterized through 16S rDNA sequence. Morphologically SWCDB21 was gram positive cocci and produced tiny, small, circular, glistening and yellow pigmented colonies. Further, 16S rDNA based identification of the strain SWCDB21 was done. The 16S rDNA gene of SMCDB21 was successfully amplified through PCR employing universal primers for identification of species. Homology analysis indicated that sequence of the 16S gene of SWCDB21 showed somewhat similar sequence alignment, 86.69% homology with the genus *Staphylococcus* sp. strain JDMASP1 16S rRNA partial sequence (KX817938.1). Flimban *et al.*, (2019) reported that *Staphylococcus* was the most prominent group of bacteria that possess cellulolytic activity and identified six *Staphyloccus* species with significant cellulase activities using rice waste as a substrate. Cellulolytic bacteria are prominent to convert the agricultural waste into eco-friendly product. Diversified samples like soil, organic waste, and animal manure were used to isolate cellulose degrading bacteria. Municipal corporation waste possesses mixture of varied material and thus is the best source to isolate cellulolytic bacteria. Thus, an attempt made to isolate cellulolytic bacteria from waste dump site. Preliminary, 71.4 % of the bacteria showed cellulolytic index (CI) during primary screening that indicated that the soil of waste was rich in cellulose degrading bacteria. Six bacteria with > 1.0 CI were selected in order to select potential cellulose degraders. Cellulolytic activity is based on production of cellulase enzyme production and was studied in terms of secondary screening. Three bacteria *viz.*, SWCDB5, SWCDB6 and SWCDB21 out of six strains were identified with significant CMCase, FPase and biomass activity during secondary screening. CMCase and FPase are inducible enzymes that affected by physiochemical factors surrounding the environment.

Thus, effect of varied pH, incubation time and temperatures; addition of different concentration of ammonium sulfate and urea as nitrogen source; starch and cellulose as carbon sources were studied to optimize the physiochemical condition for higher CMCase and FPase production for the isolates SWCDB5, SWCDB6 and SWCDB21. It was noted that addition of 0.5 % ammonium sulfate and starch with pH 7.0 for 96 hrs at 37º C temperature favours maximum CMCase, FPase production and biomass of all three isolates as compare to other physiochemical parameters. Further, SWCDB21 bacterial isolate was found more potential to produce enzymes as compare to SWCDB5 and SWCDB6. Therefore, it was molecularly characterized and identified as *Staphylococcus* genus. Previously, research reports noted that *Staphylococcus* group have inbuilt ability to produce higher cellulase activity. Indeed, a detail *in vitro* and *vivo* study required to prospect the use of this strain in the field or industry.

Cellulose degrading bacterial strains possesses the enzymatic tool box that can be explored to degrade agricultural waste. Thus, an attempt was made to isolate and screen cellulose degrading bacteria and optimized physiochemical condition for maximum CMCase, FPase and biomass production. The present study introduced the cellulose degrading strains with optimized physiochemical parameters for higher CMCase and FPase production along with cell biomass. Moreover, comprehensive studies will be required to explore these bacterial strains for bio-conversion of agricultural residues into agricultural into valuable product for today's sustainable agriculture.

References

Agbor,V. B., Cicek, N., R., Berlin A. and Levin, D. B. 2011. Biomass pretreatment: Fundamental toward application. Biotechnol. Adv. 29 : 675-685. https://doi.org/10.1016/j.biotechadv.2011.05.005

Bhagat, S. A. and Kokitkar, S. S. 2021. Isolation and identification of bacteria with cellulose-degrading potential from soil and optimization of cellulase production. J. Appl. Biol. Biotech. 9(06):154-161. https://doi.org/10.7324/JABB.2021.96020

Dar, M., Shaikh, A., Pawar, K. and Pandit, R. 2018. Exploring the gut of *Helicoverpa armigera* for cellulose degrading bacteria and evaluation of a potential strain for lignocellulosic biomass deconstruction. Process Biochem. 73. https://doi.org/10.1016/j.procbio.2018.08.001

Duza, M. B. and Mastan, S. A. 2015. Optimization studies on cellulase production from *Bacillus anthracis* and *Ochrobactrum anthropi* (yz1) isolated from soil. Int. J. Appl. Sci. Biotechnol., 3(2): 272-284. https://doi.org/10.3126/ijasbt.v3i2.12616

Flimban, S., Oh, S. E., Joo, J. H. *et al.*, 2019. Characterization and identification of cellulose-degrading bacteria isolated from a microbial fuel cell reactor. Biotechnol Bioproc. 24: 622–631. https://doi.org/10.1007/s12257-019-0089-3

Gaur, R. and Tiwari, S. 2015. Isolation, production, purification and characterization of an organic solvent thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. BMC Biotech., 15: 19. https://doi.org/10.1186/s12896-015-0129-9

Hatami, S., Alikhani, H.A., Besharati, H., Salehrastin, N., Afrousheh, M. and Jahromi, Y. Z. 2008. Investigation on aerobic cellulolytic bacteria in some of north forest and farming soils. American-Eurasian J. Agric. & Environ. Sci. 3(5): 713 – 716.

Immanuel, G., Dhanusha, R., Prema, P. *et al.*, 2006. Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. Int. J. Environ. Sci. Technol. 3: 25–34. https://doi.org/10.1007/BF03325904

Islam, F. and Roy, N. 2018. Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses. BMC Res. Notes., 11: 440-445. https://doi.org/10.1186/s13104-018-3558-4

Khatiawada, P., Jahed, A., Mehadi, H. S., Kamrul, I. and Abul, K. A. (2016). Isolation, screening and characterization of cellulase producing bacterial isolates from municipal solid wastes and rice straw wastes. J. Bioprocess. Biotechnol., 6(4): 1-6. https://doi.org/10.4172/2155-9821.1000280

Lee, S. M. and Koo, Y. M. 2009. Pilot-scale production of cellulose using *Trichoderma reesei* Rut C-30 in fed-batch mode. J. Microbiol. Biotechnol.1(1): 229-233.

Lugani, Y., Singla, R. and Singh, B. S. 2015. Optimization of cellulase production from newly isolated *Bacillus sp.* Y3. J. Bioprocess. *Biotech.*, 5(11): 264-269. https://doi.org/10.4172/2155-9821.1000264

Missa, H., Ari, S. and Ratna, S. 2016. Diversity and phylogenetic relationship of cellulolytic bacteria from the feces of Bali cattle in south

central Timor, east Nusa Tenggara, Indonesia. Biodiversitas. 17(2): 614-619. https://doi.org/10.13057/biodiv/d170232

Nakada, N., Hongo, S., Ohki, T., Maeda, A. and Takeda, M. 2002. Molecular characterization of NDRG4/Bdm1 protein isoforms that are differentially regulated during rat brain development. Dev, Brain Res. 135: 45-53. https://doi.org/10.1016/s0165-3806(02)00303-6

Panse, V. G. and Sukhatme, P. V. 1967. Statistical Methods for Agricultural Workers, 2nd Edition, Indian Council of Agricultural Research, New Delhi.

Premalatha, N., Gopal, O. N., Jose, P. A, Anandham, R. and Kwon, S. W. 2015. Optimization of cellulase production by *Enhydrobacter sp.* ACCA2 and its application in biomass saccharification. Front Microbiol., 6: 1046. https://doi.org/10.3389/fmicb.2015.01046

Saini, J. K, Saini, R. and Tewari, L. 2017. Lignocellulosic agriculture wastes as biomass feed stocks for second-generation bioethanol production: concepts and recent developments. Biotech. 5:337-353. https://doi.org/10.1007/s13205-014-0246-5

Saitou, N. and Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Sethi, S., Datta, A., Gupta, B. L. and Gupta, S. 2013. Optimization of cellulase production from bacteria isolated from soil. Int. Sch. Res. Notices Biotechnol., 7. https://doi.org/10.5402/2013/985685

Sherief, A. A., El-Tanash, A. B. and Atia, N. 2010. Cellulase production by *Aspergillus fumigatusi* grown on mixed substrate of rice straw and wheat bran. Res. J. Micro., 5: 199–211. https://doi.org/10.3923/jm.2010.199.211

Tamura, K., Nei, M., and Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA. 101:11030-11035. https://doi.org/10.1073/pnas.0404206101

Tamura, K., Stecher, G., and Kumar, S. 2021. MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution* https://doi.org/10.1093/molbev/msab120

Vyas, A. K., Putatunda, C., Singh, J. and Vyas, D. 2016. Cellulase production by *Bacillus subtilis* M1 using pretreated groundnut shell based liquid state fermentation. Se. Asian J. Trop. Biol., 23(1): 542-549. https://doi.org/10.11598/btb.2016.23.1.472

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