Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 10 [8] July 2021 : 153-159

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Characterization and production of groundnut-shell degrading cellulase of *Streptomyces mutabilis*

Shinde G.M., Jadhav A.G.*
Department of Microbiology, Government Institute of Science, Aurangabad MS India – 431004.
*Email: akgjadhav@gmail.com

ABSTRACT
Groundnut is a leguminous crop, grown for seed and oil worldwide. Groundnut shells are the residual agriculture waste remained after removal of seed from its husk, this has very low degradation rate under natural conditions.The residues have 65.5 % cellulose content. The use of GNS substrate for enzyme production by actinomycetes strains is less explored area. In present study a cellulase degrading isolate of actinobateria identified by biochemical and 16S rRNA sequence comparison as Streptomyces mutabilis was selected for cellulase production under submerged condition using Groundnut shell. This was also screened for extracellular enzymes such as amylase, pectinase and cellulase using a novel substrate, Ground nut shell (GNS). Along with purified substrates, crude materials were also efficiently degraded and hydrolysis of different substrates accomplished after 24 h of incubation, where CMC substrate was maximally hydrolyzed by the crude enzyme preparation. The results showed that medium containing 3% (w/v) GNS and 0.3% (w/v) ammonium sulphate resulted in the highest production of cellulase (0.051 U mL^{-1}), after 24 hours. The results revealed that S. mutabilis capable to utilize GNS and thus can be explored processes.
Keywords: *Streptomyces mutabilis, Ground nut shell, cellulase, agriculture waste, degradation.*

Received 29.06.2021 Revised 06.07.2021 Accepted 11.07.2021

INTRODUCTION

Agriculture wastes are leftover after use or removal of usable product, majority of it is not treated and widely available. The major components of these agricultural residues consist cellulose, hemicelluloses and lignin containing substrates and. These components are resistant to natural biodegradation if disposed in a natural environmental and degraded slowly. In some part of the globe these are burned imposing environmental pollution hazards [17]. According to the National policy for management of crop residues (NPMCR) [22] 501.73 Mt residues generate in India, amongst that most used as domestic and industrial uses but there is still 140.84Mt surplus residues out of that 92.81Mt were burned every year [8]. This suggest the need of methodology for management of waste and converting or using this in a value aided product formation like food, feed, paper and bioenergy industries [26, 6, 27,8, 9].

Groundnut is used for its seed and oil purpose throughout the world. Groundnut shell is a leftover after the removal of seed from its pod, an abundant agro-industrial waste which has a very slow degradation rate under natural conditions [28]. The residue have a 65.5 % cellulose content (dry weight) in its cell wall makes it an appropriate substrate for variety of process. Since it is a nontoxic, various studies showed that it is used for variety of purposes including production of biodiesel, Hydrogen, SCP and biosorbant in dye removal etc. and thus this waste can be a beneficial bio-waste [3, 1, 12].

The degradation of several such wastes is majorly influenced by its composition. The bioconversion of wheat straw is enhanced by its relatively low lignin content and higher amount of degradable hemicellulose. The activity essential for degradation of lignin [23, 4] along with the cellulose and hemicellulose degradation was reported in various actinomycetes [14]. For the degradation of cellulosic waste material to glucose, from various substrates, the saccharifying activity of *Streptomyces* strains may be of considerable interest. Present study highlighted hydrolysis of GNS and its component by *Streptomyces mutabilis* and suggested its utilization for production of hydrolytic enzymes.

MATERIAL AND METHODS

Isolation and screening for Cellulose degrading bacteria.

Soil sample collected from wooden waste (saw mill) site, Aurangabad (19°52'43.4"N 75°20'20.3"E19.878710, 75.338971) (MH, India). The sample inoculated in medium containing CMC 2.0

gm, KH_2PO_4 0.5 gm, $MgSO_4$ 0.25 gm, Gelatin 2.0 gmL^{-1}distilled water, pH adjusted to 7.0 and incubated at 30°Cfor 3 days. The growth obtained was inoculated in solid medium having same composition with additional Congo red (0.2 gm) pH 7 ± 0.2 [16] to confirm cellulose degrading ability. The colonies showing zone of clearance were screened for further study.

Screening for extracellular enzymes

It was performed by inoculating pure culture in medium containing 0.2% of Starch, CMC and pectin for amylase, cellulase and pectinase respectively.

Characterization of Isolate

The morphological, cultural, biochemical and molecular characteristics were used for identification of isolated strain. The phylogenetic description was determined using 16S rDNA nucleotide sequencing at National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune (India). For the phylogenetic identification, the total genomic DNA of isolate was obtained as per Sambrook *et al*. [24]. Subsequently, the 16S rRNA gene was amplified from the total chromosomal DNA using universal specific primer. The 16S rRNA nucleotide sequence obtained was compared with database sequences by using BLASTnat NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequence of isolate has been submitted to the GenBank database and assigned with accession number.

Ground Nut Shell Substrate preparation

Ground nut shell (GNS) were collected from the farm, washed with distilled water, 1% $HgCl_2$ and dried at room temperature for 2 days. After drying it was grinded to formed powder using electric grinder. It was subjected for acid and alkali pretreatment [7] with some modification. Total 50ml of 1% sulphuric acid was added to 100 gm of substrate and heated for 20 min, after this allowed to cool, then 100 ml of 4% sodium hydroxide was added and heated for 30min then allow to cool the substrate at room temperature, pH was adjusted to 7.0.

Growth of Isolate in various substrates and detection of hydrolytic enzymes

The ability of the selected isolate to utilize and hydrolyze various substrates was examine by inoculating the culture in a medium with a specific carbon source, The medium composition: (g/L)Ammonium sulphate3, Potassium dihydrogen phosphate 2.4, Dipotassium hydrogen phosphate trihydrate 5.6, Magnesium sulphate-heptahydrate 1.0, Copper sulphate-pentahydrate 0.0064, Ferrous sulphate-heptahydrate 0.001, Manganese chloride heptahydrate 0.008, Zinc sulphate-heptahydrate 0.0015, and 0.2 % respective carbon substrate viz; pectin, xylan, CMC, starch, lignin, glucose and 3% of GNS was inoculated with 2% pure inoculum in Erlenmeyer flasks and incubated for 5 days at room temperature and observed for growth.

Crude cellulase enzyme production

The GNS substrate 3.0 gm was added to 100 ml of above mentioned medium composition in 250ml Erlenmeyer flask, autoclaved at 121°C for 20 min. This was inoculated with 5ml culture of *Streptomyces mutabilis* and incubated on rotary shaker at 120 rpm at room temperature for 5 days.

Extraction of crude enzyme

After incubation biomass and remaining substrate was removed by filtration with filter paper followed by centrifugation at 8000 RPM for 10 min in cooling centrifuge at 4°C. The supernatant was used as crude enzyme and store at 4°C. This was used to detect various hydrolytic enzyme activities such as Pectinase, Amylase and cellulase by DNSA method [19]. Standard graph of galacturonic acid used for pectinase and glucose was used for cellulase and amylase

Determination of cellulase activity

Cellulase activity was determined by 3,5-dinitrosalicylic acid (DNS) method through measuring of the amount of reducing sugars released from CMC. Crude cellulase, 0.5ml was mixed with 1ml of 1% of CMC prepared in 0.05M sodium citrate buffer and incubated at 50°C for 30 min. The reaction was stopped by adding DNSA reagent then the reaction mixture kept in boiling water bath for 10 min and cooled to room temperature. The absorbance was measured at 540nm. The amount of reducing sugar was measured based on glucose standard curve. One unit of enzyme activity was defined as the amount of enzyme that liberated 1µmol of glucose per min.

Effect of Temperature, pH and thermo stability of crude cellulase enzyme

The effect of Temperature and pH on crude cellulase enzyme was determined by testing the activity of the crude enzyme solution at different temperatures from 30 to 80°C and different pH values from 4.0 to 10.0. Thermal stability assay were performed: crude enzyme were kept at different higher temperature for 1h and CMCase enzyme assay were performed.

Statistical analysis

The reported results and enzyme units were the average value obtained from three independent experiments. Statistical analysis was performed using Microsoft Excel.

RESULT AND DISCUSSION

Isolation and screening for Cellulolytic activity

An isolate showed clear zone of hydrolysis on CMC agar medium containing Congo red was selected for further studies. It showed typical actinobacterial characteristics. The colonies were white in color, irregular in shape with Gm +Ve. The partial 16S rRNA sequence was obtained, submitted to GenBank (Accession no. MW575257) and compared with the database sequences using nucleotide blast program (https://blast.ncbi.nml.nih.gov/Blast.cgi.). This showed significant similarity with *Streptomyces mutabilis* (99.14%). This was further compared with those of the type strains of *Streptomyces* species. On the basis of BLAST, phylogenetic analysis and tree constructed of neighbor joining type with boostrap value 1000 showed that strain is most closely related to *Streptomyces mutabilis* and *AJ781374 Streptomyces violaceorubidus*. The biochemical and morphological characteristics along with the evolutionary tree based on 16S rRNA confirms the isolate identification.

TableI: Biochemical characteristics of the isolate

Character	Result
Gm character	Gm + ve
Catalase	+
Nitrate reduction	+
CAMP test	+
Urea	+
Arabinose	-
Manitol	-
Glucose	+
Maltose	-
Sucrose	-
Trehalose	-
Sucrose	-
Xylose	+

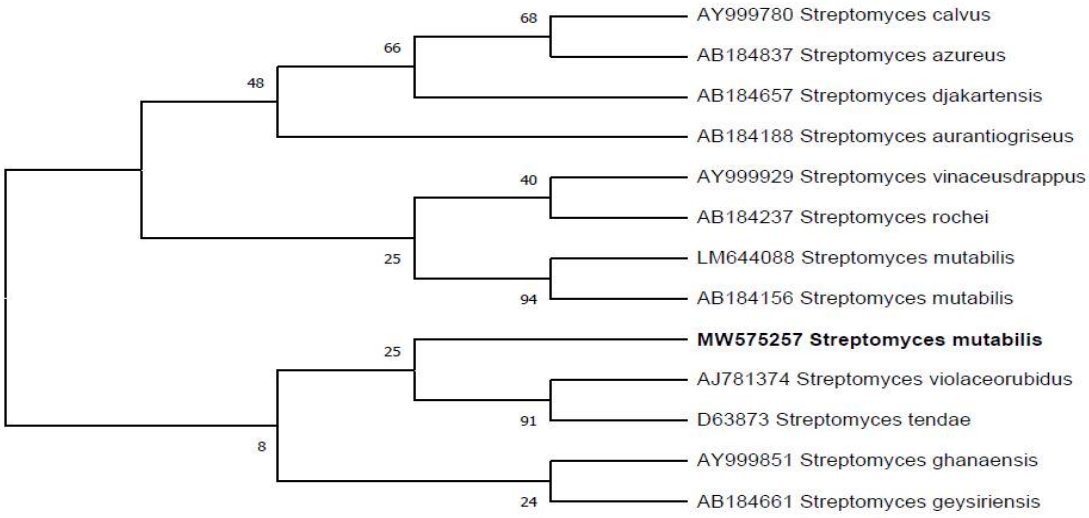

Fig. 1: Phylogenetic tree of the isolate S GAA1 based on 16S rRNA sequences plotted using MEGAX (Kumar *et al.* 2018). The trees were constructed by using the neighbour-joining method. GenBank accession numbers are given in parentheses; the sequence of this study represented in bold type.

Growth in various substrates

The isolate was identified as *S. mutabilis* showed growth in various substrates. The shell contains lipids (1.2%), minerals (4.5%), protein (7.3%), carbohydrates (21.2%) and cellulose (65.7%) (Vyas *et al.* 2016).The isolate showed growth in mineral medium with GNS and other carbon sources after 24h of incubation except for xylan (Table II). There are reports of using it as a substrate for enzyme production (Salihu *et al.* 2014), fungi and bacteria (Aspergillus and Bacillus) have been explored for production of hydrolytic enzymes.

Table II: Growth of S. m in various substrates

Sr. No.	Substrate	Growth		
		24 h	48h	72h
1	CMC	+	+++	++++
2	Pectin	+	+++	++++
3	Xylan*	-	-	-
4	Starch	+	++	++
5	Lignin	-	+	+
6	Glucose	+	+	++
7	GNS	+	+	++

*Growth occurred after 10 days incubation

The growth in medium with GNS and other CMC, pectin and starch as a sole carbon source were further analyzed for extracellular enzymes, cellulase, pectinase and amylase (Table III). This suggests that the GNS is supporting growth of the isolate along with a good stimulant for extracellular enzymes.

Table III: Extracellular crude enzyme activity S. *mutabilis* strain in different substrates after 24hrs at 30°C

Sr. No.	Carbon source	Cellulase (U/ml)	Pectinase (U/ml)	Amylase (U/ml)
1	CMC	0.040	ND	ND
2	Pectin	ND	0.102	ND
3	starch	ND	ND	0.021
4	GNS	0.046	0.045	0.015
5	GNS with N2 source	0.051	0.038	0.014

ND- Not Detected

The interest in the use of low cost, unutilized or underutilized left over agriculture residues for enzyme production by microorganisms have been increased significantly. These have been explored for various enzyme productions including cellulase production [2]. There are studies already been described for enzyme production like xylanase and protease production by actinobacteria using different residues [11, 20]. The enzyme, cellulase was detected at different concentration of the GNS (1% to 5%), the growth was similar in almost all quantities but the slightly higher amount of enzyme was detected at 3% GNS. Along with the GNS, different nitrogen sources were used, medium with ammonium sulphate showed higher cellulase activity.

Present study focused on cellulase production by *Streptomyces strains* isolated from wood waste (saw mill) soils using GNS which is a waste, low-cost residue from agriculture, as the main substrate. The isolate, *Streptomyces mutabilis* grown in 0.2% of CMC, pectin and starch and produce around 0.040 U mL^{-1} CMCase, 0.102 U mL^{-1} pectinase and 0.021 U mL^{-1} amylase, whereas 0.041 U mL^{-1} CMCase, 0.045U mL^{-1} pectinase and 0.015 U mL^{-1} amylase using 3% of pretreated GNS, in the similar studies with same conditions an activity of 0.22 U mL-1 was obtained using wheat bran by *Streptomyces drozdowiczii* [15].

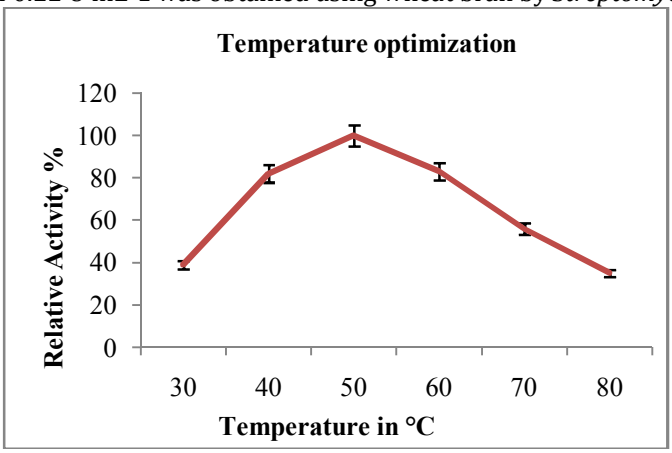

Fig.2: Temperature optimization of crude cellulase

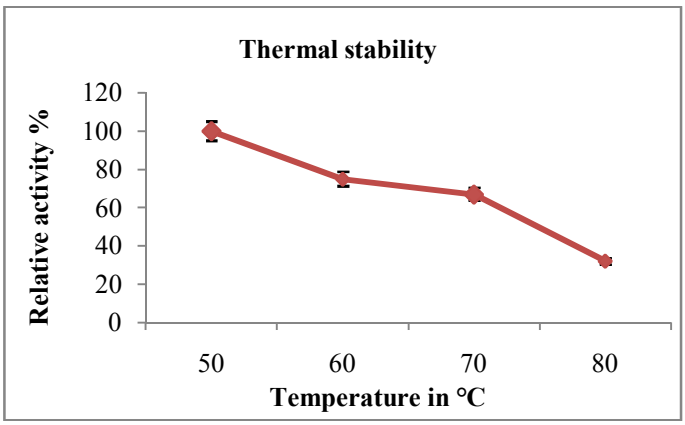

Fig 3: Temperature stability of crude cellulase enzyme

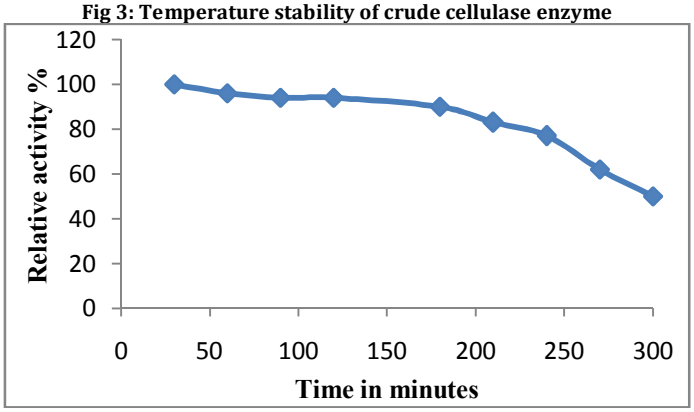

Fig.4: Activity of enzyme at 50°C and varying time interval

The supernatant from *Streptomyces sp*. grown on GNS in the best conditions was significantly active at 50°C and maximal activities (more than 80% relative activities) were observed within the range of 40–60°C whereas a lesser activity levels of i.e. 56% and 35% were detected at 70°C and 80°C respectively (Fig. 2). The crude enzyme retain about 90% relative activity up to 3h and 50% of relative activity at 5h (fig.4), this shows that enzyme is stable for long duration at 50°C. Results of this study are in accordance to those reported for various *Streptomyces sp.*[13, 15]. Similar result were also found in Nascimento *et al.* [21], the CMCase produced by *S. malaysiensis* AMT-3 was thermophilic, with residual activity of around 100% at temperatures between 40° and 60°C.

Thermal stability experiments are shown in Fig.3, crude enzyme was able to retain near about 100% residual activity at 50°C, 75% and 67% residual activity retain at 60°C and 70°C respectively, the similar result was found in Dalal *et al*. [9]. Cellulase produced by *Streptomyces mutabilis* strain (MW575257) shows stability at higher temperature also.

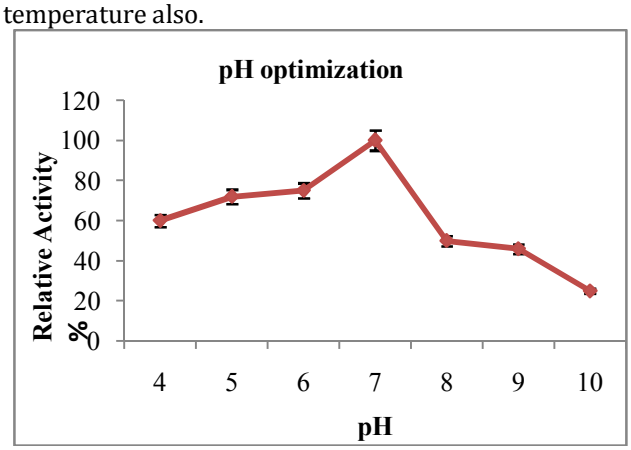

Fig. 5: pH optimization of crude cellulase

The pH activity of the crude enzyme obtained under the growth conditions in GNS substrate (Fig. 5) shows that around 70% CMCase activity was maintained over the pH range 5.0–7.0, with optimal activity at pH 7.0. CMCase activity in the pH range (4.0–8.0) was also detected for different species of Streptomyces [15, 21]. Although commercial cellulase is superior at pH values below 4 and above 8

(above 70% of relative activity) for *S. viridobrunneus* SCPE-09 in the pH range 4.0–7.0, with optimal activity around 5.0 [10].

However, while comparing results described by various studies, a significant point should be considered that substrate and conditions used for cellulase activity and enzyme production are not similar. Various studies reported significant difference in enzyme activity after growing various species of Streptomyces in different medium [10, 20, 25].

Nonetheless, the results presented here suggest that *Streptomyces mutabilis* is a good producer of CMCase and produces copious amount of enzyme when grown on GNS a lignocellulosic wastes as carbon source. Furthermore, use of *S. mutabilis* for bioconversion can be very beneficial due the growth promoting ability and thus in converting agricultural residue to fertilizers. This way the waste which is habitually abandoned in the field and only a small portion is used as fertilizer or animal feed [3] can be a more useful.

CONCLUSION

The isolate *Streptomyces mutabilis* of this study showed growth and produce CMCase along with other lignocellulosic degrading enzymes using GNS as a carbon source. The maximum CMCase activity detected was of significance, as it obtained only with the raw substrate. These results were obtained by optimizing one factor at a time. The optimum of pH and temperature of crude extract were of 7.0 and 50°C, respectively, and retained 100% of activity after 1 h at 50°C.Considering the wide availability and low cost of the medium for enzymatic activity, the results indicate possible use for the substrate and enzyme in biotechnology processes.

ACKNOWLEDGEMENT

GMS gratefully acknowledged to Council of Scientific and Industrial Research (CSIR) New Delhi for Junior Research Fellowship.

REFERENCES

1. Adhikari, B., Dhungana, S., Muhammad, W. A., Adhikari, A., II-Doo Kim, Shin, D. H., (2019). Antioxidant activities, polyphenol, flavonoid, and amino acid contents in peanutshell. J. Saudi Soc. Agric. Sci. 18 (4), 437-442
2. Adsul, M. (2004). Polysaccharides from bagasse: applications in cellulase and xylanase production. Carbohydrate Polymers 57, 67–72 .
3. Andrade, W. (2011) Sisal fiber in Brazil. Brazilian fiber publishing Web. http://www. braziliansisal.com.
4. Ball, A. S., Betts, W. B., & McCarthy, A. J. (1989). Degradation of lignin-related compounds by Actinomycetes. *Applied and Environmental Microbiology*, *55*(6), 1642–1644.
5. Balsan, G., Astolfi, V., Benazzi, T., Meireles, M. A. A., Maugeri, F., Di Luccio, M., Dal Prá, V., Mossi, A. J., Treichel, H., & Mazutti, M. A. (2012). Characterization of a commercial cellulase for hydrolysis of agro-industrial substrates. *Bioprocess and Biosystems Engineering*, *35*(7), 1229–1237.
6. Bhatt S. M., and Shilpa (2014). Bioethanol production from economical agro waste (Groundnut Shell) in SSF. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, *5*(3), 1210–1218.
7. Bholay, A. D., Borkhataria B. V., Jadhav P. U., Palekar K. S., Dhalkari M. V., Nalawade P. M. (2012). Bacterial lignin peroxidase: A tool for biobleaching and biodegradation of industrial effluents. *Universal Journal of Environmental Research and Technology 2*(1), 58–64.
8. Bhuvaneshwari S., Hettiarachchi, H., & Meegoda, J. N. (2019). Crop residue burning in India: Policy challenges and potential solutions. *International Journal of Environmental Research and Public Health*, *16, 832*.
9. Dalal, S., Sharma, A., & Gupta, M. N. (2007). A multipurpose immobilized biocatalyst with pectinase, xylanase and cellulase activities. *Chemistry Central Journal*, 1(16), 1–5.
10. Da Vinha, F. N. M., Gravina-Oliveira, M. P., Franco, M. N., MacRae, A., Da Silva Bon, E. P., Nascimento, R. P., & Coelho, R. R. R. (2011). Cellulase production by Streptomyces viridobrunneus SCPE-09 using lignocellulosic biomass as inducer substrate. *Applied Biochemistry and Biotechnology*, *164*(3), 256–267.
11. De Azeredo, L. A. I., De Lima, M. B., Coelho, R. R. R., & Freire, D. M. G. (2006). Thermophilic protease production by Streptomyces sp. 594 in submerged and solid-state fermentations using feather meal. *Journal of Applied Microbiology*, *100*(4), 641–647.
12. Duc, P. A., Dharanipriya, P., Velmurugan, B. K. & Shanmugavadivu, M. (2019).Groundnut shell a beneficial bio-waste. *Biocatalysis and Agricultural Biotechnology*, *20*, 101206.
13. George, S. P., Ahmad, A. & Rao, M. B. (2001). Studies on carboxymethylcellulase produced by an alkalo thermophilic actinomycete. *Bioresource Technology*, *77*(2), 171–175.
14. Godden, B., Legon, T., Helvenstein, P. & Penninckx, M. (1989). Regulation of the production of hemicellulolytic and cellulolytic enzymes by a *Streptomyces sp*. growing on lignocellulose. *Journal of General Microbiology*, *135*(2), 285–292.
15. Grigorevski De Lima, A. L., Pires Do Nascimento, R., Da Silva Bon, E. P. & Coelho, R. R. R. (2005). Streptomyces drozdowiczii cellulase production using agro-industrial by-products and its potential use in the detergent and textile industries. *Enzyme and Microbial Technology*, *37*(2), 272–277.

16. Gupta, P., Samant, K. & Sahu, A. (2012). Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. *International Journal of Microbiology*. doi:10.1155/2012/578925
17. Gutang, C. G., Dacua, M. and Deiparine, P.S. (2012). Peanut (Arachis hypogaea) Shells and Carton Bulletin board. http://www.studymode.com/essays/Peanut-Shells- 1128937.htm
18. Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35:1547-1549.
19. Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar.*Analytical Chemistry*, *31*(3), 426–428.
20. Nascimento, R. P., Claudia, Æ. M., Souza, R. F., Branquinha, Æ. M. H., Bon, E. P. S. & Rosalie, Æ. N. P. Æ. (2005). Production and partial characterization of extracellular proteinases from *Streptomyces malaysiensis*, isolated from a Brazilian cerrado soil. *Arch Microbiol* 184: 194–198.
21. Nascimento, R. P., Junior N. A., Pereira N., Bon E. P. S. & Coelho R. R. R. (2009). Brewer's spent grain and corn steep liquor as substrates for cellulolytic enzymes production by Streptomyces malaysiensis. *Letters in Applied Microbiology*, *48*(5), 529–535.
22. NPMCR. Available online: http://agricoop.nic.in/sites/default/files/NPMCR_1.pdf
23. Salihu, A., Sallau, A. B., Adamu, A., Kudu, F. A., Tajo, M. M., Bala, T. F. & Yashim, W. D. (2014). Utilization of Groundnut Husk as a Solid Substrate for Cellulase Production by *Aspergillus niger* Using Response Surface Methodology. *Waste and Biomass Valorization*, *5*(4), 585–593.
24. Sambrook, J., Fritsch, E.F. & Maniatis, T., (1989). Molecular Cloning, a Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, New York
25. Tuncer, M., Kuru, A., Isikli, M., Sahin, N. & Çelenk, F. G. (2004). Optimization of extracellular endoxylanase, endoglucanase and peroxidase production by Streptomyces sp. F2621 isolated in Turkey. *Journal of Applied Microbiology*, *97*(4), 783–791.
26. Vyas, A., Putatunda, C., Singh, J. & Vyas, D. (2016). Cellulase production by *Bacillus subtilis* M1 using pretreated groundnut shell based liquid state fermentation. *Biotropia*, *23*(1), 28–34.
27. Wilson, K., Yang, H., Seo, C. W. & Marshall, W. E. (2006). Select metal adsorption by activated carbon made from peanut shells. *Bioresource Technology*, *97*(18), 2266–2270.
28. Zheng, W., Phoungthong, K., Lü, F., Shao, L. M. & He, P. J. (2013).Evaluation of a classification method for biodegradable solid wastes using anaerobic degradation parameters. Waste Management, 33(12), 2632–2640.

CITATION OF THIS ARTICLE

Shinde G.M., Jadhav A.G. Characterization and production of groundnut-shell degrading cellulase of S*treptomyces mutabilis*. Bull. Env. Pharmacol. Life Sci., Vol10[8] July 2021 : 153-159