# "ISOLATION AND MOLECULAR IDENTIFICATION OF GUT MICROFLORA OF TERMITES WITH CELLULOLYTIC POTENTIAL"

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**Abstract**

Efficient biodegradation of cellulosic biomass needs a battery of enzymes targeting cellulose, hemicellulose, and lignin. Termites cause damage to both cellulose and non-cellulose containing substances like living trees, crop plants, due to its symbiotic association with different microorganisms. Hence in this study, gut microflora of termites with cellulolytic potential were screened and identified. Termites were collected from different locations of Mandsaur and Neemuch Districts of Madhya Pradesh Malwa region. The microflora of termites gut were isolated and cultured on CMC media for Cellulolytic strains identification. Total 56 bacteria were isolated from the gut of Termites, whereas 31 were found with cellulolytic activity through screening on carboxymethylcellulose (CMC) media. Among 31 cellulolytic active bacteria, five bacterial isolates showed more promising cellulolytic activity than others. The five cellulolytic strains were further analysed for the accurate molecular identification through the PCR amplification of 16S rRNA gene followed by Sanger sequencing. Based on 16s rRNA gene sequencing, we have identified Cellulolytic bacterial strain as *Bacillus* sp. (in: firmicutes) strain NTG 4, *Aneurinibacillus migulanus* strain MTG 15, *Bacillus subtilis* strain STG 8, *Streptomyces pseudovenezuelae* MTG 17, *Bacillus megaterium* strain MTG 19. The *B. megaterium* strain MTG 19 showed highest cellulolytic activity followed by *Bacillus subtilis* strain STG 8 in these five cellulolytic isolates.

**Key words:-** *Bacillus megaterium* strain MTG 19, Cellulolytic activity, biodegradation, 16s rRNA gene.

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## Introduction

It has been suggested that termites (Isoptera) can break down lignocellulosic materials since these insects produce cellulases and hemicellulases along with symbiotic association with different microorganisms in its own gut, which as a result of evolution and modification, makes the termites able to degrade the lignocelluloses, hemicelluloses in their diets. Symbiotic bacteria are essential to termite survival. The several symbiotic bacteria in termite guts are in charge of breaking down the lignocelluloses. Numerous bacteria in the termite have not yet been identified since it is challenging to grow them outside of the termite stomach. Additionally, several of the microbes are only found in termite stomachs. According to Konig H et al. (2006), the guts of lower termite species (Mastotermitidae, Hodotermitidae, and Kalotermitidae) are filled with many flagellates. Since symbiotic bacteria are necessary for termite digestion, they are referred to as mutualistic interactions. The digestive system of a termite consists of three parts: the foregut, midgut, and hindgut. Due to a number of intestinal processes, termite digestive enzymes can take longer to digest [Albuquerque et al., 2014]. *Acetonema longum* and *Clostridium mayombei* from *Macrotermes gilvus* (Hagen) are just two of the bacteria that have been collected and identified from termite guts [Nakashima K et al., 2002]. Four of the bacteria that the study team identified from the stomach of the *Odontotermes formosanus* are capable of digesting lignin and cellulose [Sun LQ et al., 2015].

The world's oldest economic sector, agriculture, depends more than any other sort of trade on rich soils and a stable climate [Fahad et al., 2015]. Today, wheat is one of the most important cereals grown around the world. Wheat farming is a major activity with significant economic development implications. The world faces a significant difficulty when it comes to disposing of the vast quantities of straw that are produced as agricultural waste after harvest. The majority of this waste typically 85%, is burned in place in the open air with the aim of preparing the fields for double-cropping or the upcoming agricultural cycle [Quintero et al., 2008]. The air quality is impacted by the wheat straw, which is burned on-site and releases significant amounts of particulate matter (PM), carbon monoxide (CO), and Methane ($CH_4$) every year. Due to termites' capacity to digest lignin and cellulose, which was previously discovered to be broken down by symbiotic bacteria, microbes can be isolated from their guts to fix this issue. Therefore, the objective of the current investigation was to identify the potential cellulolytic bacterial species from termite guts.

## Materials and methods

**Termite collection and identification:** Around 30 to 40 worker termites were collected from termite mounds, using cellulose traps according to the method of El-Sebay (1991) from different locations of Mandsaur and Neemuch Districts of Madhya Pradesh Malwa region. The sample were taken to lab, washed to get rid of dirt and were identified based on their morphological characteristics. Termites were identified based on its morphological characteristic at the laboratory of Faculty of Life Sciences, Mandsaur University, Mandsaur, M.P.

**Figure1:** Figure showing the termites, which were collected for the present study.

The collected Termites were surface sterilized using 70% ethanol for 30 seconds. The rectum of termites was stabbed by an inoculation needle, and then streaking was done on nutrient agar plate medium.

**Isolation of termite gut bacteria:** Termites were sterilized with 70% ethanol and then washed with sterile distilled water. After this, their heads were cut and gut was removed and macerated in sterilized autoclaved sand. Serial dilution was done in the saline water up to $10^{-6}$ and 500 µl of different dilute sample were spreaded on nutrient

agar (NA) plates. Plates were incubated at 28± 2°C in BOD incubator for 24 hr and observed for the bacterial growth. Single colony from different sample plates have been picked and streaked on NA plates for pure culture.

Sub-culturing was performed as per the requirement in NA medium. To select the cellulolytic bacteria and determine the ability of the bacteria to break down cellulose, 1 mL of the 24 hour grown bacterial culture was spread on carboxymethylcellulose (CMC) agar plates medium composed of K2HPO4 (0.2 g/L), KH2PO4 (0.2 g/L), MgSO4 (0.2 g/L), NaCl (0.2 g/L), NaNO3 (1 g/L), CaCO3 (0.01 g/L), yeast extract (0.5 g/L), CMC (10 g/L), Agar (15 g/L), pH 7.0 and incubated for 48 h at 37°C. Re-streaking on CMC agar plates purified every bacterial isolate. For more research and characterization, the cellulase-producing isolates that showed the most promising results were chosen [Gupta et al.,2012].

**Screening for cellulolytic bacteria:** The pure colonies from the isolates were grown in medium modified by Sharma et al., (2015), containing (g/L) 0.2 K2HPO4, 0.01, 9 MgSO4, 1 NaCl, 0.5 NaNO3, 0.05% yeast extract and 10 g CMC Inoculated cultures were incubated for 72 h at 37°C. After incubation, 1 ml of culture was centrifuged at 5000 rpm for 10 min to obtain bacterial pellets. After this, 6 mm wells were punched in 1.5% agar containing media plated supplemented with 1% CMC. The isolated bacterial pellet was then resuspended in medium and 100 µL from this suspension was filled in wells and incubated at 37 °C for 24 h. The incubated plates were observed for clear zone formation after submerging the plates in 0.1% Gram's Iodine (Kasana et al., 2008).

**Cellulolytic activity assay:** Cellulolytic activity was measured as a diameter of clear zone after the CMC plate was poured Gram's Iodine.

$$\text{Cellulolytic index (CI)} = \frac{\text{Diameter of activity zone} - \text{Diameter of Bacterial colony}}{\text{Diameter of Bacterial colony}}$$

Cellulolytic index (CI) was calculated using formula as follows (Ferbiyanto et al., 2016):

**Molecular characterization of selected isolates**: Genome isolation of the selected bacterial isolates were done using phenol chloroform method. The five isolates were amplified by PCR using the universal primers (reverse primer: 5′-GAGAGTTTGATCCTGGCTGGCTCAG-3′ and forward primer: 5′-AAGGAGGTGATC CAGCC G CA-3′ ) for the 16S rDNA gene analysis for accurate molecular identification (Cheng et al.,2010). The PCR was carried out using the thermal cycle 2720 (Applied Biosystem, USA) in a total volume of 25 µL, which also contained 7.5 µL of water nuclease-free, 1 µL of each primer, 3 µL of template DNA (50 ng/ µL), 12.5 µL of Gene Dire X's PCR master mix, and 1 µL of each primer. The 16S rDNA gene was amplified under the following conditions: denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min and final extension at 72°C for 10 min.

**Sequence analysis of 16S rDNA gene:** The PCR products were purified using EXOSAP Clean up in accordance with manufacturer's instructions. Out sourcing was done for sequencing of PCR products (Eurofins Pvt Ltd, bangalore)

**Computational analysis**:
For the purpose of the multiple sequence alignment and phylogenetic dendrogram, the Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used. The Phylogenetic trees were produced using the unweighted pair group method with arithmetic mean (UPGMA), according to Kumar et al. (2004).

**Results and Discussion**
During present study cellulose-degrading bacterial species have been isolated and screened for the cellulose activity potential from the termites gut. Termites were collected from different locations of Mandsaur and Neemuch Districts of Madhya Pradesh Malwa region.

**Isolation and screening of cellulase producing Bacterial Isolates**
The rectum of termites was stabbed by an inoculation needle, then streaked on nutrient agar medium for the growth of gut micro flora of termites as given in figure 2. The microflora of termites gut were isolated and cultured in CMC media for Cellulolytic strains. In total, 56 bacteria were isolated from the gut of Termites, whereas 31 were found with cellulolytic activity through screening on carboxymethylcellulose (CMC) media but Five bacterial isolates showed more cellulolytic activity than others (figure 3).

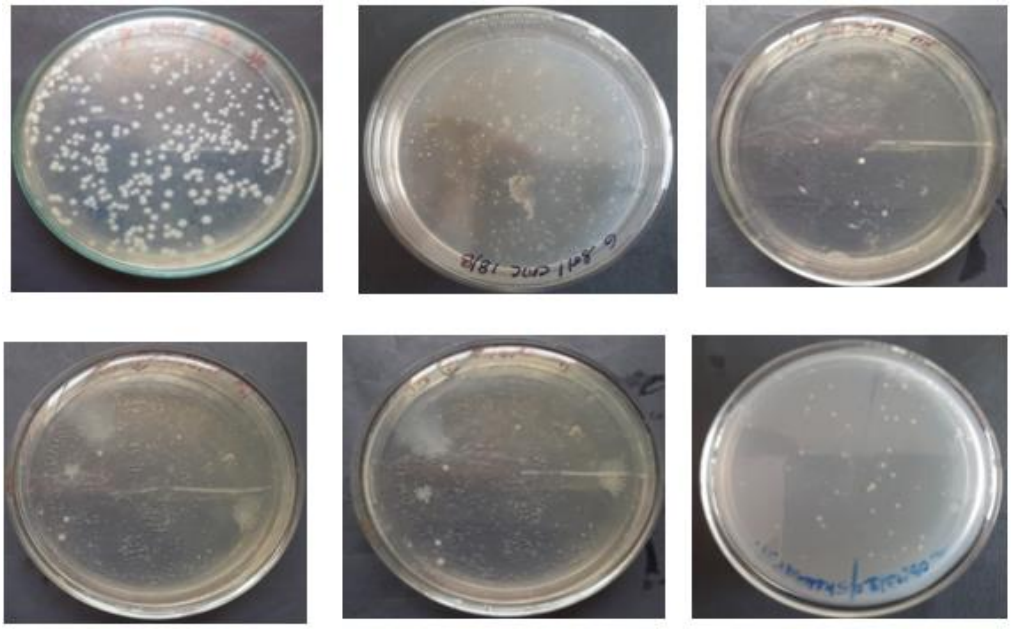

**Figure 2:** Present figure showing the growth of gut micro flora (bacterial sp.) of termites on nutrient agar media plate.

**Cellulolytic activity of bacterial isolates**

Cellulose is the main building blocks of plants and have major fraction of organic carbon in soil. Microorganisms, which live in soil, are accountable for recycling of this organic carbon to the Environment (Wang et al., 2008). Degradation of cellulosic materials is a complex process and requires participation of microbial cellulolytic enzymes. Several microorganisms have been discovered for decades which have capacity to convert cellulose in to simple sugars (Perez et al.,2002) but need for newly isolated cellulose degrading microorganisms still continues (Nirajane et al., 2007). In this study, we have succeeded in isolating and identifying five strains of symbiotic aerobic cellulolytic bacteria from the gut of the termites indicating that they play a role in cellulose digestion. Cellulolytic activity of bacterial isolates was based on clear zone of degraded CMC area around the colony (Figure 3). Based on cellulolytic index and growth, isolate MTG 19 and STG 8 were found potential isolates as shown in table 1. The results showed the highest cellulolytic activity in MTG 19 (CI=3.57) followed by STG 8 (CI=2.64) while reported low in MTG 17. Similar trend were reported by Sreena et al. (2015) in different bacterial species.

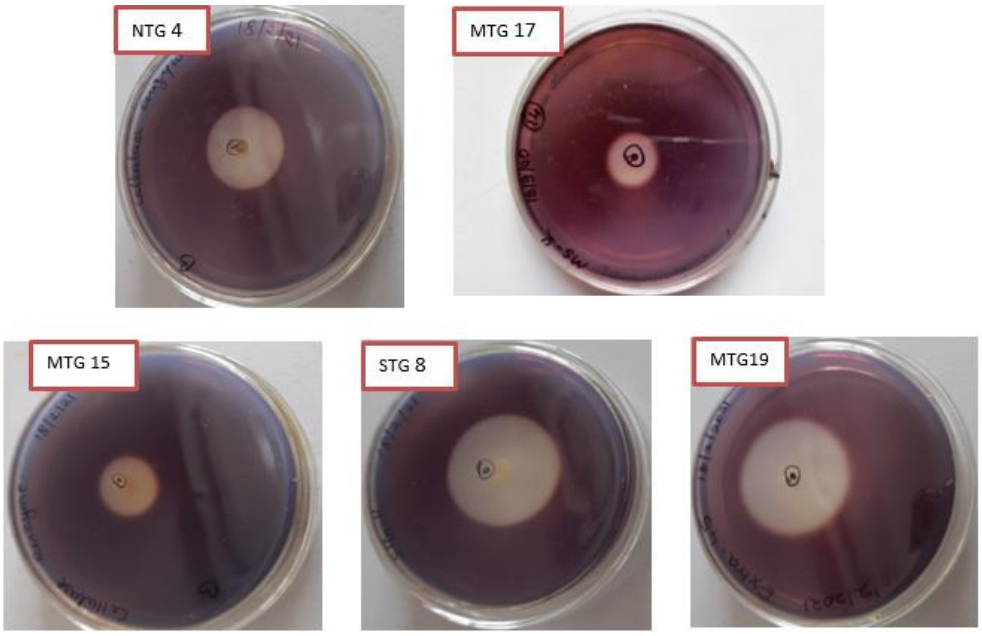


**Figure 3.**The ability of the isolates to degrade CMC by spotte diffusion assay method.

**Table 1 :** Cellulolytic index of isolated cellulolytic bacteria isolates from the micro flora of Termites gut.

| S.No. | Isolate code | Diameter of colony (mm) | Diameter of cellulolytic zone (mm) | Cellulolytic index |
|---|---|---|---|---|
| 1 | NTG 4 | 2 | 4.5 | 1.25 |
| 2 | MTG 15 | 1.5 | 3 | 1 |
| 3 | STG 8 | 2.2 | 8 | 2.64 |
| 4 | MTG 17 | 3 | 5 | 0.67 |
| 5 | MTG 19 | 2.3 | 10.5 | 3.57 |

**16S rRNA gene sequencing analysis**

The environmental microbial communities' analysis has largely relied on a PCR-dependent amplification of genes entailing species identity as 16S rDNA (Rosselli et al. 2016). The selected cellulolytic strain were further analysed for the accurate molecular identification through the PCR amplification of 16S rRNA gene followed by Sanger sequencing (Table 2). Based on biochemical tests and 16s rRNA gene sequencing we have identified Cellulolytic bacterial strain as *Bacillus* sp. (in: firmicutes) strain NTG 4, *Aneurinibacillus migulanus* strain MTG 15, *Bacillus subtilis* strain STG 8, *Streptomyces pseudovenezuelae* MTG 17, *Bacillus megaterium* strain MTG 19. Among all *B. megaterium* strain MTG 19 showed highest cellulolytic activity. Butera et al. (2016) have also used 16S rRNA gene sequence to identify isolated cellulolytic bacteria from the gut of *Reticulitermes lucifugus*, demonstrated its role in cellulose digestion.

**Table 2 :** Molecular identification of the bacterial isolates, based on 16S rRNA gene sequencing.

| S. NO. | Isolates Code | Species | Identification method |
|---|---|---|---|
| 1. | NTG 4 | *Bacillus sp. (in: firmicutes)* | 16 S gene sanger sequencing, validate by https://blast.ncbi.nlm.nih.gov/Blast |
| 2. | MTG 15 | *Aneurinibacillus migulanus* | 16 S gene sanger sequencing, validate by https://blast.ncbi.nlm.nih.gov/Blast |
| 3. | STG 8 | *Bacillus subtilis strain* | 16 S gene sanger sequencing, validate by https://blast.ncbi.nlm.nih.gov/Blast |
| 4. | MTG17 | *Streptomyces pseudovenezuelae* | 16 S gene sanger sequencing, validate by https://blast.ncbi.nlm.nih.gov/Blast |
| 5. | MTG 19 | *Bacillus megaterium* | 16 S gene sanger sequencing, validate by https://blast.ncbi.nlm.nih.gov/Blast |

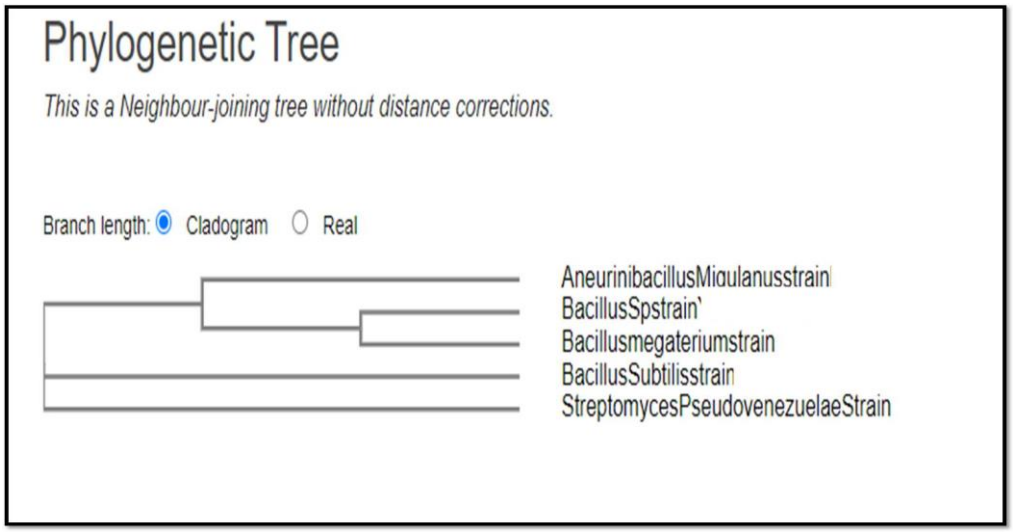


**Figure 4.**Neighbor-joining tree based on 16S rRNA gene sequences showing phylogenetic relationships among strains *Bacillus sp. (in: firmicutes)* strain *Aneurinibacillus migulanus* strain , *Bacillus subtilis* strain , *Streptomyces pseudovenezuelae* strain and *Bacillus megaterium* strain

**Phylogenetic analysis of 16S rRNA gene sequences**

Phylogenetic analysis was performed using DNA sequences of 16S rRNA gene of bacteria. The 16S rRNA gene sequences were identified using BLAST-N search program in national center for biotechnology information (http;//www.ncbi.nlm.nih.gov). The 16S rRNA

gene sequences were aligned and Phylogenetic tree was constructed using online Clustal Omega software. This phylogenetic showed the genetic homogeneity between *Bacillus subtilis* and *Streptomyces pseudovenezuelae* while *Bacillussp. (in: firmicutes)* and *Bacillus megaterium* making different sub cluster. The genetic homogeneity between species seems positively correlate with their cellulolytic activity potential.

**Conclusion:**

According to this study, our isolation screening and identification methods were quick and efficient for allowing us to identify several good cellulase producing bacteria from a wide variety of samples. Moreover, we were able to distinguish the isolates displaying the greatest cellulase activity for future study. In present study we have identified very promising cellulase activity carrying isolates such as *B. megaterium* strain MGT 19 and *B. subtilis* strain STG 8.

**References**


1. Ali, H. R., Hemeda, N. F., & Abdelaliem, Y. F. (2019).Symbiotic cellulolytic bacteria from the gut of the subterranean termite *Psammotermes hypostoma* Desneux and their role in cellulose digestion. *AMB Express*, *9*, 1-9.
2. Cheng, K., Lu, F. P., Li, M., Liu, L. L., & Liang, X. M. (2010).Purification and biochemical characterization of a serine alkaline protease TC4 from a new isolated *Bacillus alcalophilus* TCCC11004 in detergent formulations. *African Journal of Biotechnology*, *9*(31), 4942-4953.
3. de Albuquerque Lima, T., Pontual, E. V., Dornelles, L. P., Amorim, P. K., Sa, R. A., Coelho, L. C. B. B., ... &Paiva, P. M. G. (2014). Digestive enzymes from workers and soldiers of termite *Nasutitermes corniger*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *176*, 1-8.
4. El-Sebay, Y. (1991).A modified El-Sebay trap for subterranean termite. *Proc. 4th Arab Cong Plant Protection*, 245-247.
5. Fahad, S., Nie, L., Chen, Y., Wu, C., Xiong, D., Saud, S., ...& Huang, J. (2015). Crop plant hormones and environmental stress. *Sustainable Agriculture Reviews: Volume 15*, 371-400.
6. Ferbiyanto, A., Rusmana, I., &Raffiudin, R. (2015).Characterization and identification of cellulolytic bacteria from gut of worker *Macrotermes gilvus*. *Hayati journal of biosciences*, *22*(4), 197-200.
7. Gupta, P., Samant, K., &Sahu, A. (2012).Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. *International journal of microbiology*, *2012*.
8. Hartke, T. R., & Baer, B. (2011). The mating biology of termites: a comparative review. *Animal Behaviour*, *82*(5), 927-936.
9. Hongoh, Y. (2011). Toward the functional analysis of uncultivable, symbiotic microorganisms in the termite gut. *Cellular and Molecular Life Sciences*, *68*, 1311-1325.
10. Kasana, R. C., Salwan, R., Dhar, H., Dutt, S., & Gulati, A. (2008). A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Current microbiology*, *57*, 503-507.
11. König, H., Li, L., & Fröhlich, J. (2013).The cellulolytic system of the termite gut. *Applied microbiology and biotechnology*, *97*, 7943-7962.
12. Lenz, M., Kard, B., Creffield, J. W., Evans, T. A., Brown, K. S., Freytag, E. D., ... & De Santi, M. P. (2013). Ability of field populations of *Coptotermes* spp., *Reticulitermes flavipes*, and *Mastotermes darwiniensis* (Isoptera: Rhinotermitidae; Mastotermitidae) to damage plastic cable sheathings. *Journal of Economic Entomology*, *106*(3), 1395-1403.
13. Nakashima, K., Watanabe, H., & Azuma, J. I. (2002).Cellulase genes from the parabasalian symbiont *Pseudotrichonympha grassii* in the hindgut of the wood-feeding termite *Coptotermes formosanus*. *Cellular and Molecular Life Sciences CMLS*, *59*, 1554-1560.
14. Niranjane, A. P., Madhou, P., & Stevenson, T. W. (2007). The effect of carbohydrate carbon sources on the production of cellulase by *Phlebia gigantea*. *Enzyme and Microbial Technology*, *40*(6), 1464-1468.
15. Pérez, J., Munoz-Dorado, J., De la Rubia, T. D. L. R., & Martinez, J. (2002). Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *International microbiology*, *5*, 53-63.
16. Quintero Núñez, M., & Moncada Aguilar, A. (2008).Contaminación y control de las quemasagrícolasen Imperial, California, y Mexicali, Baja California. *Región y sociedad*, *20*(43), 3-24.
17. Rosselli, R., Romoli, O., Vitulo, N., Vezzi, A., Campanaro, S., de Pascale, F., ...&Squartini, A. (2016). Direct 16S rRNA-seq from bacterial communities: a PCR-independent

approach to simultaneously assess microbial diversity and functional activity potential of each taxon. *Scientific reports*, *6*(1), 32165.

18. Sun, L., Hse, C. Y., Shupe, T., Sun, M., Wang, X., & Zhao, K. (2015). Isolation and characterization of an endophytic fungal strain with potent antimicrobial and termiticidal activities from Port-Orford-Cedar. *Journal of economic entomology*, *108*(3), 962-968.
19. Wang, C. M., Shyu, C. L., Ho, S. P., &Chiou, S. H. (2008). Characterization of a novel thermophilic, cellulose-degrading bacterium *Paenibacillus* sp. strain B39. *Letters in applied microbiology*, *47*(1), 46-53.