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Prospecting the gut fluid of giant African land snail, *Achatina fulica* for cellulose degrading bacteria

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ABSTRACT

The rapidly increasing human population poses a serious risk of fast depletion of natural resources like fossil fuels. Therefore, production of ecofriendly renewable alternatives like biofuels from waste materials has sparked more curiosity from the industrialists as well as academicians. Similarly, production of bioenergy from agricultural wastes is gaining interest worldwide. In view of the above objective, cellulose degrading bacterium G9-KDP isolated from the crop fluid of giant African land snail, *Achatina fulica* was identified as *Bacillus tequilensis* based on 16S rRNA gene sequencing. The *B. tequilensis* G9 produced cellulase with the highest activity of 956.9 IU/ml extract on 8th day of incubation when grown in BMS medium induced by sugarcane bagasse. The optimal temperature and pH of the organism were 60 °C and 6.6, respectively. It was observed that the activity of crude enzyme was affected to various extents by different metal ions. The activity was inhibited by Co^{2+} and Ca^{2+} at 5 mM concentrations but enhanced by K^{+} and Mn^{2+} ions. However, EDTA at 1% concentration increased the activity by 94% while SDS reduced it by 57% at the same concentration. Scanning electron microscopy of the filter paper used as substrate revealed significant structural modifications after the treatment with bacteria. The degradation of the various cellulosic compounds into simpler sugars by this isolate brands its potential applications in industrial biotechnology.

1. Introduction

Lignocellulosic (LC) plant biomass is an important renewable carbon resource for the bio-refinery industry, and thus is considered a sustainable and environment friendly alternative to the current petroleum platform (Kamm and Kamm, 2004). The LC biomass is the chief source of natural polysaccharide i.e., cellulose. Lignocellulosic wastes (LCW) can be converted into a worthwhile and effective asset by utilizing microorganisms which can digest the cellulose and result in the production of value added products like biofuels (Lynd et al., 1991), enzymes, and antibiotics (Sun and Cheng, 2003). Unfortunately, most of these wastes are incinerated after harvesting the crop throughout the world (Maki et al., 2012). The bioconversion of LCW into useful sugars has gained more interest during the past decade. Since, the natural way of recycling this waste into useful products seems the most promising and appropriate solution, therefore several governmental and non-governmental organizations have recently come to the front to tackle this problem.

The degradation of cellulosic biomass involves a suit of enzymes and cofactors called cellulosome. The cellulosome usually consists of three main cellulases viz., exo-β-1, 4-glucanases (EC 3.2.1.91), endo-β-1, 4-glucanases (EC 3.2.1.4), and β-1, 4-glucosidase (EC 3.2.1.21) (Wilson and Irwin, 1999). It is the synergy/compatibility of these three enzymes that makes the bioconversion of cellulose polymer to glucose easy and sustainable (Zhang et al., 2017a). The key industrial applications of cellulases include biofuel generation (Da Silva et al., 2005), laundry (Bhat, 2000), organic acids (Piškur et al., 2009) and production of animal feed (Dienes et al., 2004). Being inducible enzymes, secretion of cellulases could be significantly affected by the substrate used for fermentation (Rastogi et al., 2010). Accordingly, low-cost microalgae (Zhang et al., 2017b) and easily accessible agro-wastes, such as grass straw, wheat husk, sugar cane bagasse, etc. could prove desired cellulosic materials for the production of various sugars.

Cellulases are widely spread in nature, predominantly produced by microorganisms, including molds, fungi (Zhang et al., 2017c), bacteria and some animals like molluscs, termites etc (Tokuda and Watanabe, 2007; Gascón-Garrido et al., 2013). Microorganisms that are capable to degrade cellulosic compounds are of great importance from biological and ecological point of view. However, most of the fungal cellulases, e.g., *Trichoderma* cellulase, have shown several drawbacks like, low

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specific activities, reduced yield, and end-product inhibition (Del Menezzi et al., 2008). Therefore, isolation and characterization of potential microorganisms that can thrive under extreme conditions and hydrolyze agricultural cellulose are of vital importance for industrialization (Gabani et al., 2012). Bacteria are generally considered robust and versatile cellulase producers because of their high growth rate, stability at adverse conditions and presence of multi enzyme complexes. Bacteria are generally preferred over fungi because of their higher recombinant production of enzymes (Maki et al., 2012). However, bacterial cellulases are not studied well as compared to fungal enzymes (Mansour and Salem, 2015). Thus, attempts were made to characterize the crude cellulase of bacterial species.

The gastrointestinal (GI) tract of animals such as *Achatina fulica* (Bowdich) (Dar et al., 2015; Pawar et al., 2015; Cardoso et al., 2012) harbors a diverse array of bacterial flora that ameliorates the digestion of cellulosic food they feed on. But the diversity and functionality of cellulolytic bacteria from the crop fluid (gut region) of *A. fulica* has not been as thoroughly investigated as termite and compost, despite its economic importance as a pest of over 500 plant species. Therefore, efforts were taken to investigate the cellulolytic bacteria residing in crop fluid of *A. fulica*. Further, various sources of lignocellulosic wastes including wheat husk, grass straw, sugarcane bagasse etc. were used to evaluate the cellulolytic potential of the most efficient bacterial strain.

2. Materials and methods

2.1. Cellulosic materials

Carboxymethyl cellulose (CMC), filter paper and Xylan from Birchwood were procured from Sigma Aldrich, (St. Louis, Mo, USA). All chemicals and reagents used were of analytical or molecular biology grade. Wheat husk, grass straw and sugar cane bagasse were collected locally from crop fields of Kolhapur, Maharashtra, India. Before using, all the LC agro-wastes were chopped into pieces of about 5–7 cm in length, soaked overnight in 0.1% NaOH solution, washed thoroughly with distilled water until neutrality, then air dried and autoclaved at 121 °C for 15 min before use.

2.2. Snail dissection, screening and selection of cellulolytic bacteria

The dissection was carried out as described previously (Dar et al., 2015). Fluid from the crop's region of GI tracts of snails (n = 3) were pooled together by using a 5 ml disposable syringe and then serially diluted in Phosphate Buffered Saline (PBS, pH 7.0) up to 10^{-7}dilutions. Hundred microliters (100 µl) of each dilution was spread on 0.5% CMC (w/v) agar plates and incubated at 37 °C for 24–48 h. Based on visual inspection of colony morphologies such as color, shape, elevation, margin, 2-3 colonies with similar morphology were picked and transferred to agar medium with Carboxymethyl cellulose source as well as Luria Bertani (LB) Agar medium.

After proper growth on LB media, the isolates were screened for CMC degradation by employing plate based technique. Briefly, bacterial isolates were patched and grown (patch size not more than 0.5 mm) on Berg Minimal Salt medium (BMS) agar plates which comprised of $NaNO_3$, 2g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.02 g; $MnSO_4.7H_2O$, 0.02 g; $FeSO_4.7H_2O$, 0.02 g; $CaCl_2.2H_2O$, 5 g per liter, supplemented with 0.5% CMC (w/v) and 1.8% agar (w/v). After an incubation for 24-48 h at 37 °C, the plates were flooded with 10 ml Congo red stain (0.1%, w/v) for 20 min followed by destaining with 1N NaCl for 10-15 min and then observed for the appearance of zone of clearance around the colonies. Cellulose degrading potential of the positive isolates was also semi quantitatively confirmed by calculating Hydrolysis Capacity (HC), the ratio of the diameter of zone of clearance to the diameter of colony (Hendricks et al., 1995). The isolate showing CMCase activity was preserved and selected for further studies.

2.3. Biochemical characterization

The screened bacterium was grown overnight on LB agar medium at 37 °C to examine the morphological and cultural characteristics, including cell shape, colony appearance, cell motility etc. Catalase test, Methyl Red (MR) and Voges-Proskauer (VP) reactions, indole production, hydrolysis of starch and citrate utilization, H_2S production and acid formation from carbohydrates and litmus milk reactions were determined as described by Barrow and Feltham (1993).

2.4. Identification and phylogenetic study

The genomic DNA from the overnight grown isolate was extracted by using Favor Prep Soil DNA Isolation Kit (Favorgen Biotech Corp, Pvt Ltd, India). The full length 16S rRNA gene was PCR amplified and sequenced using primer pairs, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Eden et al., 1991). The nucleotide sequence of the isolate and closely related strains from GenBank were aligned by using Clustal X program (Thompson et al., 1997) and were edited manually using DAMBE (Xia and Xie, 2001) to obtain an unambiguous sequence alignment. A Neighbor-Joining (NJ) (Saitou and Nei, 1987) phylogenetic tree was built using the MEGA 5.2 package (Tamura et al., 2011). The confidence values of branches of the phylogenetic trees were determined using bootstrap analyses (Felsenstein, 1985) based on 500 replicates.

For dot plot analysis, 16S rRNA gene sequence of the selected isolate was aligned with same gene sequences of *B. tequelensis* (most close NCBI relative) in EMBOSS program (Rice et al., 2000). Since window size and threshold value play a vital role in scatter plot, a threshold of 70 was used while keeping the windows size to 40 to get the best possible analysis.

2.5. Growth curve determination

The growth pattern of the bacterial isolate was studied over the period of 8 days. The inoculum was prepared by growing the bacterium overnight at 37 °C and 160 rpm on rotary shaker. One milliliter of inoculum was transferred to 100 ml of BMS media supplemented with 0.5% CMC. The media was then incubated at 160 rpm and 37 °C to check the exponential growth period of the bacterium. For every 8 h, 1 ml of culture media was sampled which was further processed for standard plate count. The optical density of the sampled culture was then monitored at 600 nm in a spectrophotometer. To confirm the growth curve determination, each sample was also serially diluted upto 10^{-5} in 50 mM PBS, later spread on 1% CMC agar plates. The plates were incubated at 37 °C for 24-48 h to get the standard plate count.

2.6. Effect of pH and temperature on enzyme production by the strain

To ascertain the effect of temperature and pH on different enzyme production by the promising isolate, 1 ml of overnight grown culture was inoculated to 100 ml of BMS CMC media with different pH (3.0–9.0). The cultures were then incubated at 37 °C on a rotary shaker at 160 rpm for 14 days. For the study of effect of temperature, the inoculated media with optimum pH was incubated at different temperatures ranging from 10 to 60 °C, keeping agitation speed constant over a period of 14 days. The sampling was done at 2 days interval by taking small aliquots of 5 ml, centrifuged at 10,000 rpm, for 20 min at 4 °C to separate the supernatant which was then used as enzyme extract. The different enzyme assays such as endoglucanase, exoglucanase, cellobiase and xylanase were carried out in triplicates as described below.

2.7. Filter paper degradation and scanning electron microscopy

For filter paper test, 1 mL overnight grown culture was inoculated in