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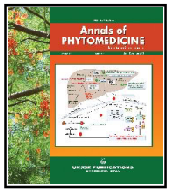

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Insights into the cellulose degradation for the production of fermentable sugars by *Alkalispirochaeta odontotermitis* JC202^T

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Abstract

The primary component of plant biomass cellulose is the most prevalent polymer on earth. It is biodegraded by a variety of microorganisms which produce a set of unique enzymes such as glycosyl hydrolases and glycosyl transferases that work in conjunction to complete the degradation process. Moreover, cello oligomers produced as a result of degradation of cellulose are having several industrial and pharmaceutical applications. Symbiotic gut bacteria are a good source of metabolic and degradative enzymes compared to their hosts. Symbiotic Spirochaetes are one such group of bacteria producing these enzymes but because of their difficulty in cultivation and their complex nutritional requirements for growth, have been poorly described and their metabolic potential is not well studied. *Alkalispirochaeta odontotermitis* JC202^T, an obligate anaerobic bacteria isolated from the hind gut of *Odontotermes obesus* was found to be an active cellulose hydrolyzing bacteria. The present study mainly focuses on the identification of cello oligomers generated as a result of cellulose degradation and identification of genes encoding the proteins responsible for its degradation using genome sequence. This study indicated the presence of a putative amino acid sequence in the draft genome of this strain, showing a similarity (44% amino acid identity) to the extracellular glycosyl hydrolases of Bacillus species. The current study will demonstrate how choosing poorly characterized organisms may result in the identification of potential biomolecules, enzymes and reveal information on unidentified metabolic pathways.

1. Introduction

Oligosaccharides are produced mainly through organic synthesis reactions catalysed by specific enzymes like glycosyl transferases or by selective hydrolysis by the accomplishment of particular enzymes, hydrolases (Zhang and Kong, 2003; Monsan and Paul, 1995; Yang and Kong, 2005). The potential for these sugars to be used in the fields of food, animal feed, medicine, cosmetics, the paper industry, immune modulators and prebiotics has sparked fresh study into understand their production and learn about their bio-technological potential (Alcalde *et al*., 1999). In addition, derivatives of oligomers owing to their complicated structures and diverse biological activities are gaining considerable interest in drug discovery as cancer preventive agents (Giese *et al*., 2010). Studies showed that bacteria aid in facilitating cellulose digestion in insects. *Actinobacteria* connected with termites assist in nutrient attainment from a diversity of polysaccharides, together with cellulose and hemicelluloses (Lewin *et al*., 2016; Shepherdson *et al*., 2023). *Proteobacteria* associated with insects are also involve in carbohydrate degradation (Zing *et al*., 2020), and can be involved in provisioning other nutrients such as amino acids (Moran and Baumann, 2000). Most termites' gut microbiota contains a large group of bacteria called spirochaetes, which has been linked to cellulose degradation and may constitute to half of all bacteria in wood-eating termites (Tokuda *et al*., 2018).

Cellulose degradation is considered as a complex process involving the activity of various glycoside hydrolases (GH) families in combination. The super family of GH is a significant group of enzymes responsible for the degradation of cellulose. They comprise enzymes having different folds and catalyzing many biochemical reactions (Horn *et al*., 2012). While various GH functions are shared by numerous families, some GH families induce a particular reaction. These families include at least three described types of proteins active on 1,4 glycosidic bonds. They are: (i) endocellulases active on internal 1, 4 glycosidic bonds, (ii) exocellulases degrading the polymer by its extremities, and (iii) glucosidases producing glucose from cellobiose.

Since anaerobic bacteria have reported on so few studies, cellulose breakdown by these organisms is currently garnering a lot more interest. The cellulolytic approach used by aerobic and anaerobic groups differs significantly. With just a few notable exceptions, anaerobes generally break down cellulose using sophisticated cellulose or glycosyl hydrolase systems. Many anaerobic species that consume cellulose localize their complex cellulases right on the cell surface rather than releasing detectable amounts of extracellular cellulose (Lynd *et al.*, 2002). The breakdown of polymers used as raw materials for the generation of ethanol or other fuels and chemicals by fermentation is currently the subject of intense attention. The primary

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goal in this field is to convert polysaccharides (such as cellulose, starch, *etc*.) into fermentable monosaccharides without the use of chemicals or enzymes. Exometabolome analysis may also provide important data by enabling us to assess the degree of degradation through the identification of degraded products that are present in the extracellular media. An integrated approach of exometabolome and genome will enable us to identify novel bacterial gene clusters (BGS) responsible for the biosynthesis of expressed metabolites. Recently, for the first time tambromycin comprising of nonstandard amino acid monomeric units showing antiproliferative activity against cancerous B and T cell lines was discovered by correlating metabolite data with genome (Goeing *et al*., 2016).

Till now, attempts were made only for the description of *Spirochaetes* and no reports were found on their bioprospecting potential. In the present study, for the first time attempts were made to study cellulose degradation pattern focusing on fermentable sugars produced by them and analysis of genome was carried out for the identification of enzymes responsible for its degradation.

2. Materials and Methods

2.1 Cultures used

A. odontotermitis, *Alkalispirochaeta cellulosivorans* JC227^T, *Alkalispirochaeta alkalica* DSM8900^T, *Alkalispirochaeta americana* DSM14872^T, *Alkalispirochaeta sphaeroplastigenens* JC133^T are used for the present study. To screen for the production of oligosaccharides by these strains, they were allowed to grow on carboxy methyl cellulose (CMC) (5 g l^{-1}) as the only carbon source in alkaline medium. The media used is specific spirochete media composed of : Na_2CO_3 (2 g l^{-1}); $NaHCO_3$ (4.5 g l^{-1}); NaCl (48 g l^{-1}); NH_4Cl (0.5 g l^{-1}); KH_2PO_4 (0.3 g l^{-1}); $MgCl_2$. $6H_2O$ (0.2 g l^{-1}); resazurin (0.001 g l^{-1}); yeast extract (0.5 g l^{-1}); sucrose (5 g l^{-1}); SL7 (trace elements solution; 1 ml) [SL7 contained (unless indicated, mg l^{-1}) HCl (25 %, v/v) (1 ml); $ZnCl_2$ (70); $MnCl_2$. $4H_2O$ (100); H_3BO_3 (60); $CoCl_2$. $6H_2O$ (200) $NiCl_2$. $6H_2O$ (20); Na_2MoO_4 . $2H_2O$ (40); $CuCl_2$. $2H_2O$ (20)] and 10 ml vitamin solution (Wolin *et al*., 1963). Autoclaved Na_2S. $9H_2O$ (0.5 g l^{-1}) was added separately after sterilization. The final pH of the medium was adjusted to 11-12.5 (Sravanthi *et al*., 2016).

2.2 Extraction of metabolites

A. odontotermitis was grown on CMC under strict anaerobic conditions and after 3 days of inoculation, cells were harvested by centrifugation (10,000 × g, 10 min, R_T) and 1000 ml of supernatant was collected and concentrated to 5 ml under vacuum in rotary flash evaporator (Heidolph, Germany) at 39^{0}C. This concentrated supernatant was used for the study of production of oligomers (Figure 1).

Figure 1: *A. odontotermitis* grown in serum vial bottle.

2.3 Thin layer chromatography

To identify the oligomers produced as a result of cellulose degradation, one-dimensional chromatography was used in concurrence with thin layer chromatography (TLC) and silica gel TLC (Kieselgel 60 F254; Merck). 1-butanol, methanol, 25% ammonia solution, and water were used in the ratio of 41.6: 33.3: 16.6: 8.3, respectively, to develop chromatograms. Spraying agent was made by dissolving 1 g of diphenylamine in 100 ml of acetone along with 1 ml of aniline. Prior to usage, this combination is combined with 85% orthophosphoric acid (10:1 v/v) and utilized as a visualizing agent for the detection of sugars (Anderson *et al*., 2000).

2.4 HPLC analysis

High performance liquid chromatography (HPLC) was performed on model number waters 2695 and the column temperature was maintained at 37^{0}C. Mobile phase flow rate was 1.0 ml/min with 80% acetonitrile. The detection of oligomers was carried out using a refractive index (RI) detector. For each sample, the HPLC injection volume was 10 µl at a run time of 10 min. After each sample run, MilliQ water is used to rinse the needle and column.

2.5 Identification of genes coding for the degradation of cellulose

Under the accession numbers JRAS00000000 and SRR1562012, the genome of the strain *A. odontotermitis* was sequenced and uploaded to DDBJ/EMBL/GenBank (Tushar *et al*., 2015). Protein blast search in NCBI was used to identify genes.

3. Results

Of all the strains (*A. odontotermitis*, *Alkalispirochaeta cellulosivorans* JC227^T, *Alkalispirochaeta alkalica* DSM8900^T, *Alkalispirochaeta americana* DSM14872^T, *Alkalispirochaeta sphaeroplastigenens* JC133^T) screened for their growth on CMC, *A. odontotermitis* was found to be actively growing strain on CMC and is used for further analysis. By centrifuging the cells at 10,000 g for 10 min, extracellular metabolites were extracted from the fresh culture at various time periods and further analyzed for cellulose degradation activity.

3.1 Evaluation of CMC degraded products

CMC degraded products produced by *A. odontotermitis* were examined using thin layer chromatography. In culture supernatants collected at every time points, cellobiose was found to be the major degraded product (Figure 2). Culture supernatants taken at various times over 72 h time period were used for HPLC analysis. After promptly filtering through filter sheets with a 0.45 m pore size, cell-free supernatants were analyzed through HPLC. In culture supernatants obtained at 0 h, 24 h and 72 h indicated single peak attributable to cellobiose with retention times of 5.321 min, 5.170 min and 4.884 min, respectively. The peak area however, gradually and significantly grown from 0 h to 72 h demonstrating that strain *A. odontotermitis* is capable of converting CMC into the fermentable sugar cellobiose (Figures 3,4 and 5). By co-eluting the culture supernatant with D-glucose, it was determined that there was no peak in the supernatants that corresponded to the standard D-glucose peak. This demonstrates unequivocally that strain *A. odontotermitis* does not convert cellobiose to D-glucose; rather, it is possible that cellobiose uptake receptors absorbed this cellobiose into the cell.

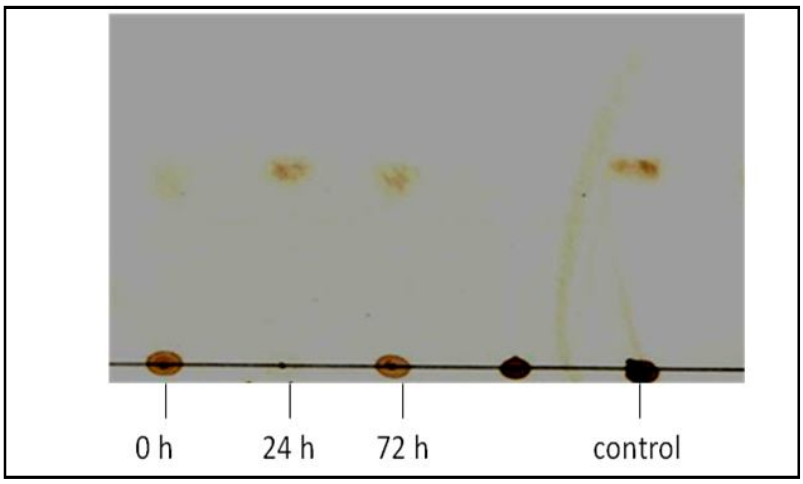

Figure 2: TLC plate of culture supernatant of *A. odontotermitis* grown of CMC.

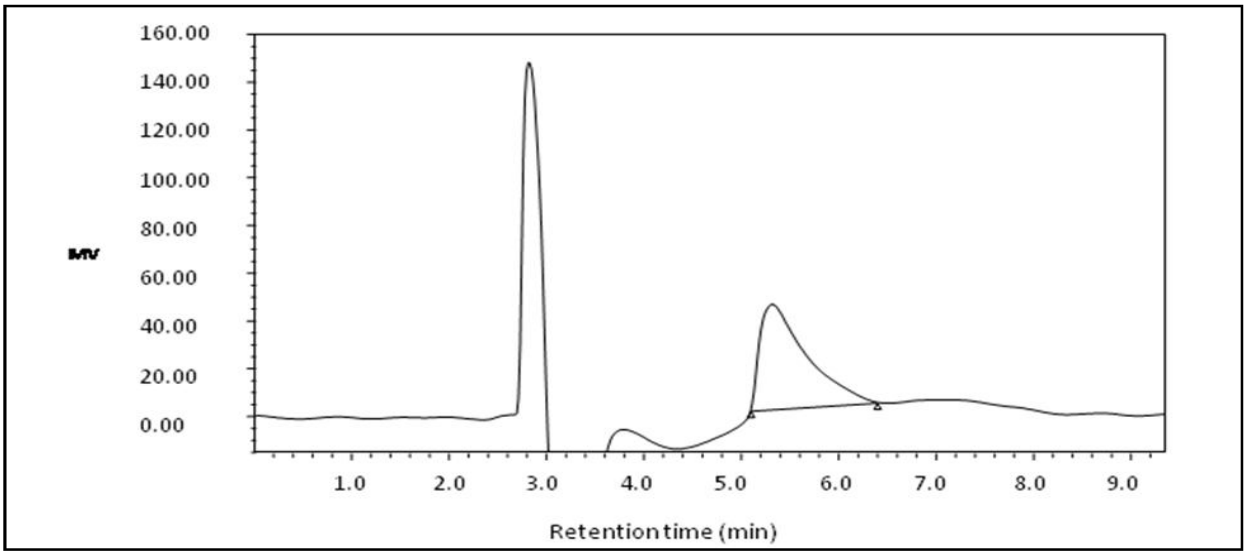

Figure 3: HPLC chromatogram of culture supernatant of *A. odontotermitis* grown on CMC at 0 h time interval.

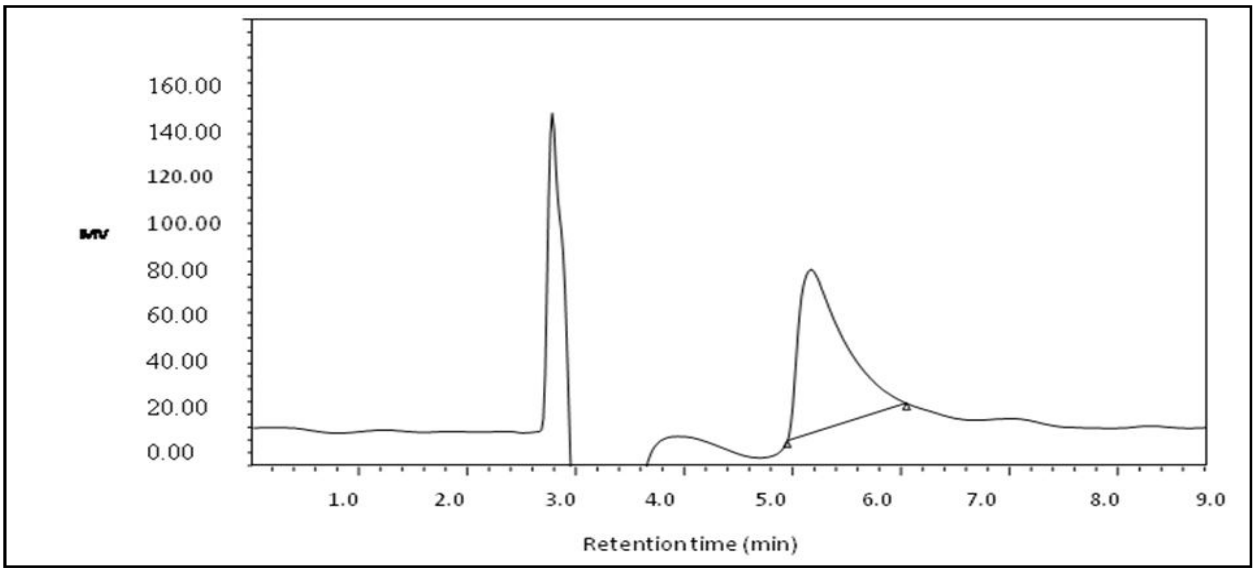

Figure 4: HPLC chromatogram of culture supernatant of *A. odontotermitis* grown on CMC at 24 h time interval.

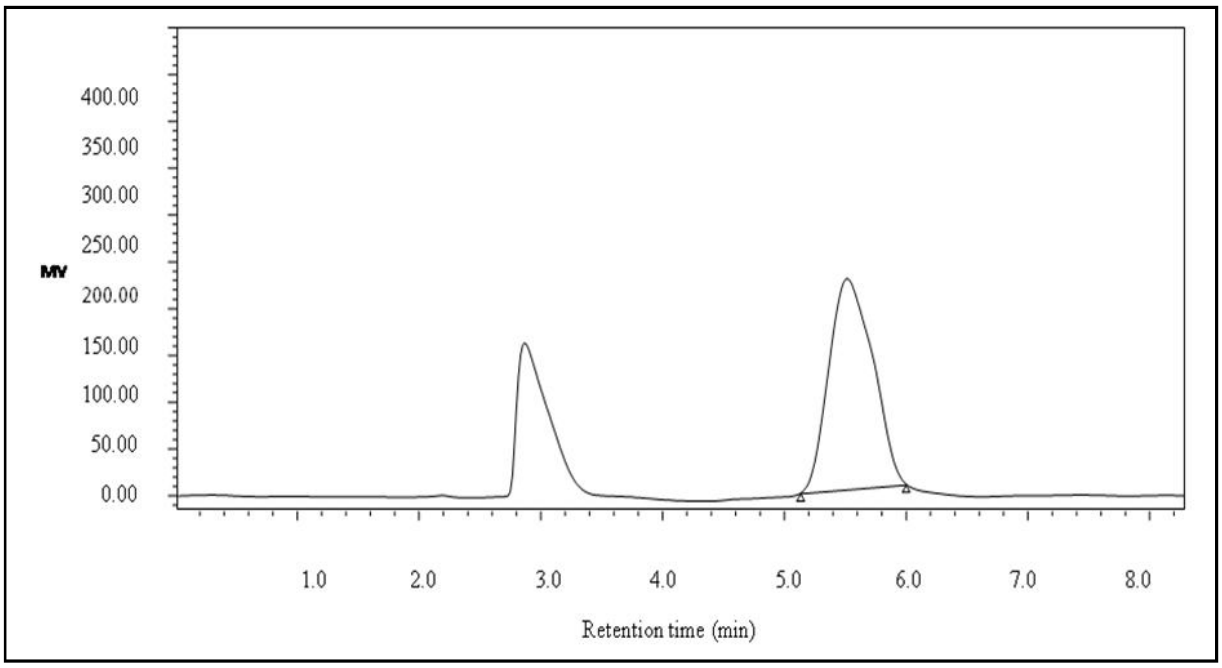

Figure 5: HPLC chromatogram of culture supernatant of *A. odontotermitis* grown on CMC at 72 h time interval.

3.2 Genome sequence analysis of genes encoding GH in *A. odontotermitis*

Draft genome of strain *A. odontotermitis* (3.82 Mb) contains 2,299 functionally identified genes. Of those identified genes, 1,361 (37.18%) were found to be hypothetical (Tushar *et al*., 2015). *A. odontotermitis* genome revealed the presence of genes encoding for potential cellulose using glycoside hydrolases (GH). Using NCBI protein blast search, it was discovered that genes encoding for cellulose degradation-related proteins belonged to the GH65 super family. This hypothetical protein was found to be JY97_17025; GI: 699052460 and consists of 763 amino acids. Additionally, BLAST analysis showed homology to *Bacillus* spp. GH genes, with which it shared 44% of the amino acid sequences. The deduced protein sequence also shared a 40-44% identity with the GH proteins of several other animals. Using CELLO2GO, a web server for protein sub cellular localization prediction using functional gene ontology annotation, the extracellular abundance of this enzyme was analysed (Yu *et al*., 2014).

4. Discussion

Several phyla of bacteria have diverse GH genes for degrading cellulose. Of 79% of the analysed genomic sequences contained putative genes belonging to GH1 and GH3 super families (Berlemont *et al*., 2013). Only a few *Actinobacteria*, *Fibrobacteres*, *Firmicutes*, *Proteobacteria* and *Spirochaetes* genomes were found to have genes related with GH9. Interestingly, genome data of *A. odontotermitis* has shown the presence of putative genes encoding for glycosyl hydrolases belonging to the super family, GH65. These enzymes have a wide range of industrial uses, including the chemo enzymatic and recombinant biosynthesis of new sugar biomolecules as well as the biosynthesis of glycol conjugates (Amin *et al.,* 2021). Finding the genes that encode the enzymes involved for metabolic processes using the metabolic foot printing or profiling of small-molecule metabolites from culture supernatants is widely used method (Mielko *et al*., 2019). In the current investigation, an analogous attempt was undertaken to connect the strain *A. odontotermitis* genome sequence with the degradation products found in extracellular metabolites. We have observed the concentration of cellobiose in the culture supernatant and also analyzed the putative genes responsible for its degradation *via* genome as it represents the downstream products of gene expression. HPLC analysis of extracellular metabolites taken from the fresh culture of *A. odontotermitis* at various time intervals revealed a single peak corresponding to cellobiose, demonstrating this strain is converting CMC into the fermentable sugar, cellobiose. *A. odontotermitis* draft genome annotation led to the discovery of a potential gene that may code for glycosyl hydrolases from the GH65 super family. Blastp analysis of this protein found that *Bacillus* species share 44% of its amino acid sequences, suggesting that it may be a new GH. However, further critical analysis must be performed to confirm this hypothesis.Till now, there were no reports on the production of fermentable sugars from polysaccharides by spirochaetes. Hence, this analysis will help in understanding the cellulose degradation pattern of this organism and further critical investigation will lead to the discovery of several unfold molecules, enzymes and metabolic pathways.

5. Conclusion

Identification of novel glycosyl hydrolases is receiving much more attention these days because they are essential for producing sugar monomeric units which are then converted into organic acids, ethanol, and other value-added products, as well as for use in biorefineries, textile processing, detergent formulations, and other industries. These enzymes serve as the cornerstone for the worldwide recycling of carbon and aid in the processing of carbon in various ways. In addition, they also have a number of applications in pharmacological and biotechnological fields. Present study indicated that spirochaetes

are capable of degrading polysaccharides and they are rich in enzymes responsible for polymer degradation which is evident from genome analysis. Thus, exploring unexplored organisms for the production of oligomers and identification of enzymes responsible for bio degradation will help in the discovery of novel enzymes and metabolites. There are several spirochaetes whose biotechnological potential was not at all received attention due to difficulty in handling and maintenance of these organisms. Hence, similar type of analysis could yield fruitful results if the unexplored organisms are explored and analysed for their applications.

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Conflict of interest

The author declares no conflicts of interest relevant to this article.

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