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Recovery of Biomass Energy on the Farm: Identification of Cellulolytic Bacteria in Agricultural Residues for On-Site Bioethanol Production

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Authors' contributions

This work was carried out in collaboration between all authors. Authors FMM, SB and MB contributed to the design of the study and wrote the manuscript. Author FL carried out the experiments and their analysis. All authors read and approved the final manuscript.

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ABSTRACT

Farms are significant sources of unused biomass, conversion of which into energy would contribute to decreasing the environmental footprint associated with farming activities. A promising alternative for energy conversion involves bioethanol production. Bioethanol can be fermented from simple sugars that in turn must be extracted from biomass. To this end cocktails of enzymes may be used to deconstruct lignocellulosic biomass, but their cost and efficiency are often prohibitive. One could circumvent these drawbacks by finding locally-established, well adapted bacteria that produce enzymes with relevant specificities. Here we identified such bacteria and compared their ability to hydrolyse cellulose from agricultural and industrial biomass residues. By collecting environmental samples at a local farm we identified 54 strains, of which 12 exhibited cellulolytic activity. Based on 16S rDNA sequence analyses, we found that these strains were relatives of *Bacillus aryabhattai*, *B.*

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cereus, *B. licheniformis*, *B. pseudomycoides* and *B. thuringiensis* species. This article reveals the first experimental evidence of cellulase activity from *B. aryabhattai*, confirming earlier predictions. The abilities of these strains to produce simple sugars from carboxymethyl cellulose, treated maize biomass, and papermaking primary sludge were investigated. *B. licheniformis* and *B. thuringiensis* related strains both showed high extracellular cellulase activity and sugar production when grown on treated maize. This study suggests that local microbial biodiversity should be considered when developing enzymatic strategies for exploitation of farm residues.

Keywords: Bioethanol; biomass; cellulase; cellulose; Bacillus aryabhattai; Bacillus licheniformis; Bacillus thuringiensis.

ABBREVIATIONS

CMC : Carboxymethyl Cellulose
Mm : Minimal Medium
PCR : Polymerase Chain Reaction Amplification

1. INTRODUCTION

Bioethanol is considered to be a sustainable substitute for petroleum-based fuels, mainly because it is produced from renewable biomass. The most straightforward bioethanol production strategy relates to "first generation bioethanol" which uses parts of plants with high concentrations of readily-hydrolyzed sugars such as starch (corn kernels) or sucrose (sugar cane). Second generation bioethanol is produced from any kind of plant residue, either from farming or from forestry activities. This avoids conflicts between bioenergy production and food production, a problem with first generation biofuels [1-3]. Plants are composed primarily of cellulose, hemicellulose and lignin, the proportions and distributions of which vary according to source. Hydrolysis of cellulose (or hemicellulose) into monosaccharides is a prerequisite for fermentation to ethanol. Cellulose comprises tightly packed regions (crystalline cellulose) and hydrated regions (amorphous cellulose), and is associated with lignin and hemicelluloses. Its complete hydrolysis to glucose requires several types of cellulase enzymes: endocellulase (EC 3.2.1.4) which has a higher affinity toward amorphous cellulose, exocellulase (EC 3.2.1.91) which can hydrolyse both cellulose types, and cellobiase (EC 3.2.1.21) which can hydrolyse the disaccharide cellobiose produced by exocellulases into glucose [4].

A few years ago, three local agri-businesses (located in Québec, Canada) initiated an ambitious project named Agrosphère. The objective of Agrosphère is to become an integrated biomass processing center, leading to cellulosic sugar production from local crop residues. Pioneering work from Barnabé's group has helped define the scientific challenges and opportunities associated with this project [5]. One interesting avenue to support the full exploitation of biomass from farms involves the on-site production of cellulase enzymes already adapted to biomass specific to Quebec soils and climate. This production could be achieved at a very low cost by robust local cellulose-degrading bacteria using substrates such as farm residues, organic waste or other biomass rejects. To this end we report here the identification and initial characterisation of cellulolytic bacteria established on a local farm. We found 12 strains related to *Bacillus* genus which secreted cellulase(s), and investigated the ability of the most promising strains to extract sugars from biomass samples.

2. MATERIALS AND METHODS

2.1 Bacteria Screening

Bacteria were isolated from farm residues (pig manure, corn crop residues), grain storage unit and soils (soy plantation and corn plantation) on the site of Olivier Lépine farm in Saint-Alexis-de-Montcalm (Quebec, Canada). Samples of pig manure and corn crop residues (mixture of stover, cob and leave) were collected following standardized sampling and storage protocols of MDDEP (2008) for microbial analysis. Each isolate was preincubated at 30°C for an hour and then a small amount (10% w/v) was added to 100 ml of liquid minimal medium (Mm) composed of K_2HPO_4, 2 g/l; KH_2PO_4, 2 g/l; NH_4NO_3, 1.5 g/l; yeast extract, 0.25 g/l; peptone, 1 g/l; Wolfe's mineral solution, 5 ml/l and carboxymethyl cellulose, 5 g/l. Various growth conditions were tested. pH was varied from 6 to 8, and incubation temperatures of 37°C and 50°C were compared. Samples were aliquoted after 0, 1, 24 and 48 hours and spread on Mm (without yeast extract and with 15 g/l agar) plates and were incubated

under the same conditions (pH and temperature, with an appropriate dilution factor). Each different colony was subcultured ten times on Mm plates, incubated at their optimal growth temperature, and preserved in 15% glycerol at -80°C.

2.2 Bacteria Characterization

Various tests were performed to identify bacterial strains. Characterization of bacteria was performed as described by Meddeb-Mouelhi et al. [6].

2.3 Identification by Sequencing of 16S rRNA Genes

For DNA extraction, individual colonies from the various isolated strains were suspended in 20 µl of 20mM Tris-HCl buffer and subjected to two freeze thaw cycles (-80°C for 15 minutes, boiling water for 10 minutes). Cell fragments were eliminated by centrifugation and the supernatant was used for PCR.

The 16S rRNA genes were amplified using the following primers: 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1522R: 5'- AAGGAGGTGATCCANCCRCA-3'. PCR mixtures (50 µl) were prepared using 5 µl of buffer 10X, 1µl (10 mM) dNTP, 1µl (20 mM) of each primers, 0.5 µl Taq DNA polymerase, 41 µl of sterile water and 0.5 µl of supernatant. The thermal cycling started with a 2 min (95°C) denaturation cycle. An amplification cycle (incubation for 30 seconds at 98°C, 1 min at 55°C, 2 min at 72°C) was repeated 30 times, and followed by a final extension step of 5 min at 72°C. The amplified 16S rRNA genes were sequenced with an ABI Prism 3700 by the Biomolecular Analysis Platform (Université Laval, Québec).

For each isolate the forward and reverse sequences were combined to obtain a fragment of DNA of approximately 1.2 kbp. A BLASTn investigation (NCBI) was performed to find sequences similar to each isolate gene. Settings included nucleotide collection (nr/nt), an expected threshold of 10, word size of 28, match/mismatch scores of 1 and -2, respectively, and a linear gap cost. Using MEGA 5.1 and CLUSTAL W, multiple sequences were aligned and a phylogenetic tree was inferred using the neighbor-joining method [7].

2.4 Cellulase Activity

Ten (10) µl of bacteria supernatant (from culture grown in liquid Mm and 0,5% CMC at 37°C for 24 hours and then centrifuged to remove cells) was spotted on a 0.5% CMC agar plate, incubated for an hour, and then stained with 3 ml of Gram's Iodine solution for 5 min. Appearance of clear halos around the bacterial spots indicated the presence of cellulase activity [8]. Each strain that showed cellulase production was precultured in 10 ml of LB for 6 to 12 hours. Cultures were then adjusted to 1 OD_{600} in Mm, then added with CMC, to insure similar starting amounts of bacteria prior to cellulase expression and quantitation. Cultures (1 mL) were sampled at various intervals during growth at 37°C, centrifuged, and the supernatant was collected for cellulase assays. The quantity of reducing sugar released by cellulase at pH 7 was measured using the DNS method optimized by the National Renewable Energy Laboratory (NREL) [9]. The reducing sugars generated by enzymatic hydrolysis were measured using the absorption at 540 nm and comparison to a standard curve generated using glucose [10]. One international enzyme unit (IU) was defined as the amount of enzyme necessary to release 1 µmol of reducing sugar per min under our assay conditions. Enzyme activity was also tested using two alternative carbon sources, treated maize (extrusion) and primary paper sludge, as substrates. To this end, Mm medium was supplemented with 0.5 g/L of cellulose rich substrate (treated maize or primary paper sludge). In addition to reducing sugar production, growth was monitored using the vial count method with 0.1 ml cell broth (or appropriate dilution) spread on a TSA plate incubated at 37°C for 16 hours [11].

2.5 Biomass Samples

Pretreated corn crop residues were obtained from Agrosphere company (Quebec, Canada) after applying a twin-screw extrusion process to fractionate hemicelluloses and lignin from cellulose. It contained 42% cellulose, 30% hemicellulose and 11% lignin (w/w). Primary paper sludge was obtained from Kruger tissue mill at Crabtree (Quebec, Canada) and the sample used in this study contained 28% of cellulose (w/w) and 11% of hemicellulose (w/w).

3. RESULTS AND DISCUSSION

Bacteria isolated from farm biomass samples were initially screened for growth on 0.5% CMC-Mm plates. The 54 strains obtained were subjected to a second screening using a Gram's iodine test to confirm production of extracellular

cellulase(s) (typical results shown in Fig. 1). Out of 54 strains only 12 showed significant zones of CMC degradation. These twelve bacterial strains were identified based on 16S rRNA gene sequencing, and a phylogenetic tree revealed that the strains were related to *Bacillus* species. Isolates were related to clades identified as *B. licheniformis* (strains 1D, 1A, 4D, S9A), *B. cereus* (4C, S9B), *B. thuringiensis* (8B, 4A, S4A, 9D), *B. aryabhattai* (S1A) and *B. pseudomycoides* (S1C) (Fig. 2). It is not too surprising to find strains with names that do not match the classification proposed, since the differences among *Bacillus* species are very limited, sometimes causing confusion as to which strain belongs to what group [12].

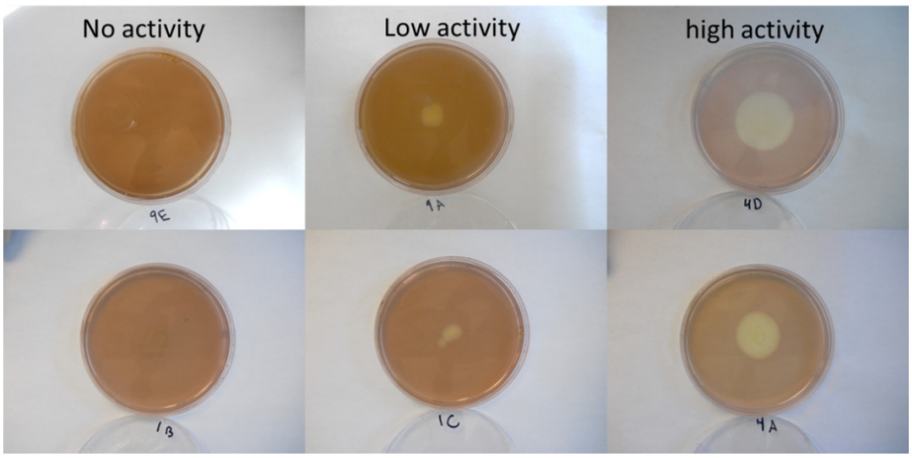

Fig. 1. Extracellular cellulase activity by six representative bacterial strains. Halos around colonies indicate cellulase activity. Each strain was inoculated by tip-touch at the center of petri dishes and incubated for 24 hours at 37˚C. Plates were stained with Gram's iodine for 5 min

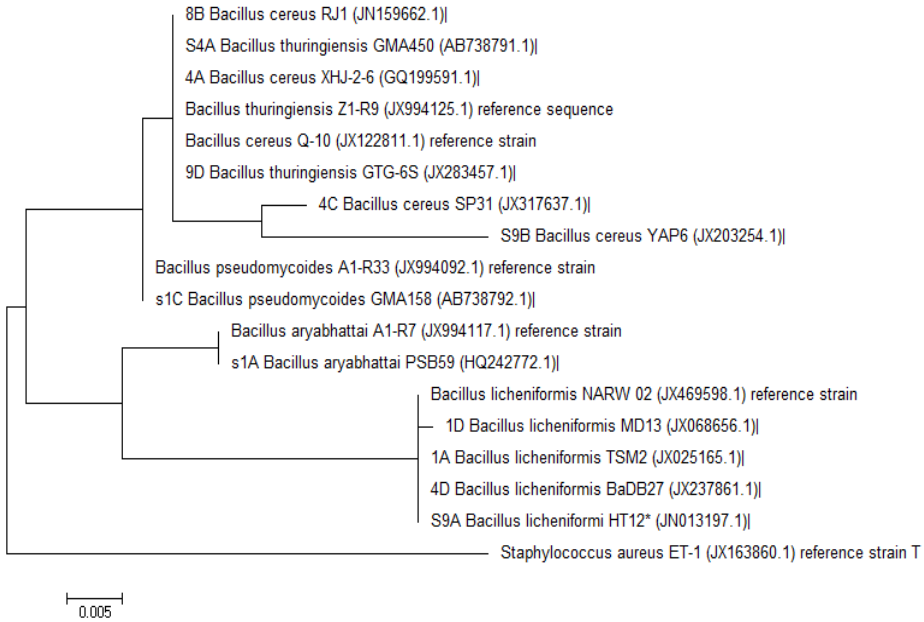

Fig. 2. Neighbor-joining inferred tree based on 16S rRNA gene sequences showing the evolutionary relationship of isolates within previously-characterized species. *Staphylococcus aureus* was used as the 'outgroup'. Reference strains are indicated. GenBank accession numbers for 16S rDNA sequences are shown in parentheses. Bar length represent the number of base substitutions per site

Strains distributed among the five clades represented on the phylogenetic tree were further investigated for their ability to produce reducing sugars from CMC (i.e. cellulase or CMCase activity). The relationship between growth and production of cellulase activity for one representative of each clade (4D, 4C, 9D, S1A and S1C) is shown in Fig. 3. Growth of all strains appears to plateau after 48 hours. Regarding cellulase activity, the 5 strains analyzed behaved differently. The best early producers were *B. licheniformis* and *B. thuringiensis*. Strains related to *B. aryabhattai* showed a continuous increase in the total cellulase activity (up to 72 hours). We did not investigate the source of such differences in enzyme expression kinetics, but the secretion (or lack of) of such enzymes may delay activity detection. Since the *B. thuringiensis* 9D and *B. licheniformis* 4D strains produced higher activities during the first 12 to 24 hours, we selected them for further investigation.

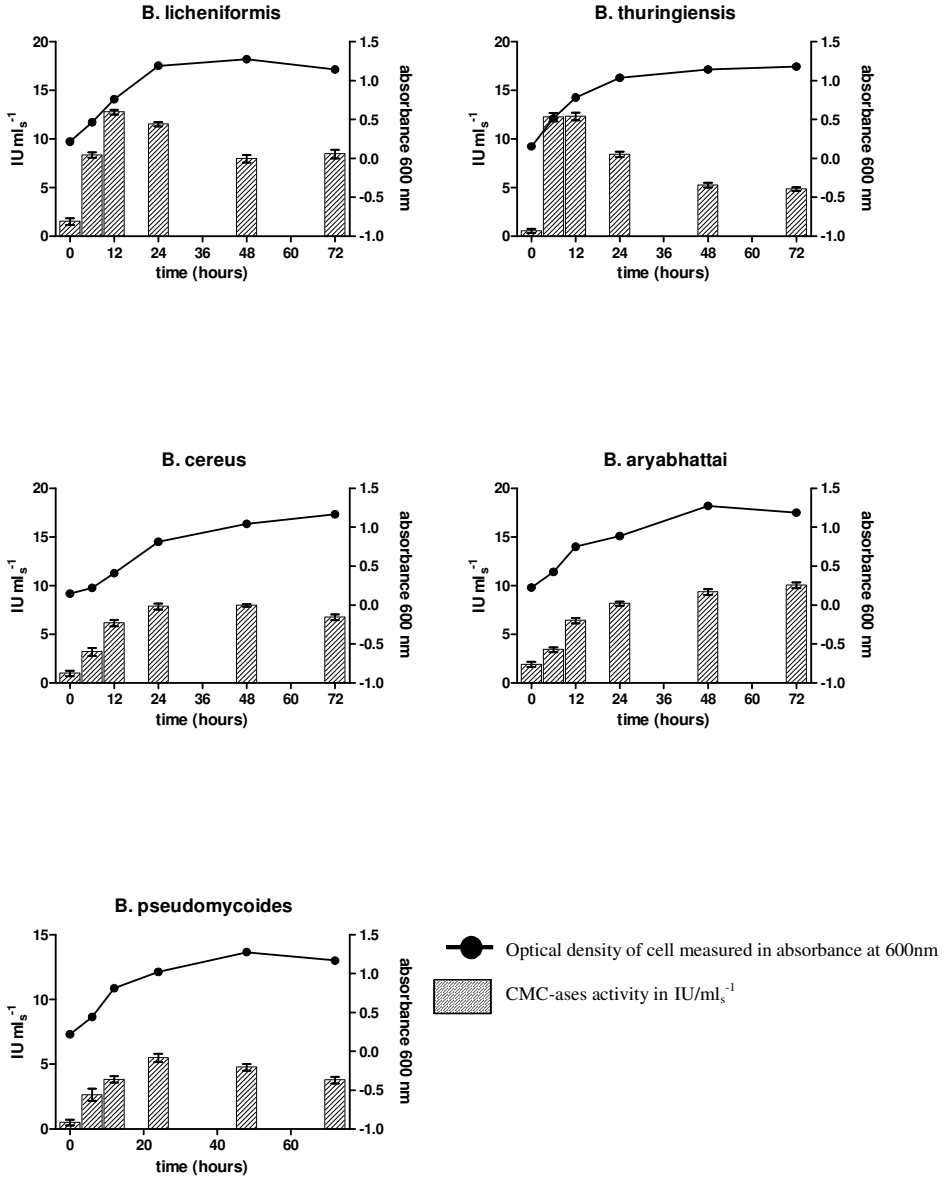

Fig. 3. Growth and production of reducing sugars for selected strains in the presence of CMC. The axis on the left refers to CMCase activity (U/ml of supernatant) determined by reducing sugar release detection, while cell density (OD_{600}) is shown on the right axis

The ultimate goal of this project is to identify bacteria for biomass degradation, so the abilities of the two *Bacillus* strains to hydrolyze different biomass substrates were investigated. Fig. 4 shows their abilities to produce reducing sugars when using biomass found at the farm (maize residues) or biomass that was collected from an industrial biotope (sludge from a paper mill) as carbon sources. Biomass concentrations were set at 5g (dry weight) per liter of Mm medium, and a control experiment measured in the presence of 0.5% CMC is shown for comparison. The preference of *B. thuringiensis and B. licheniformis* strains toward maize was revealed by comparing both growth and enzyme production measurements to results obtained using the other carbon sources. In the presence of maize residues, both strains displayed more cellulase activity than in the presence of either CMC or paper mill sludge. *B. thuringiensis*

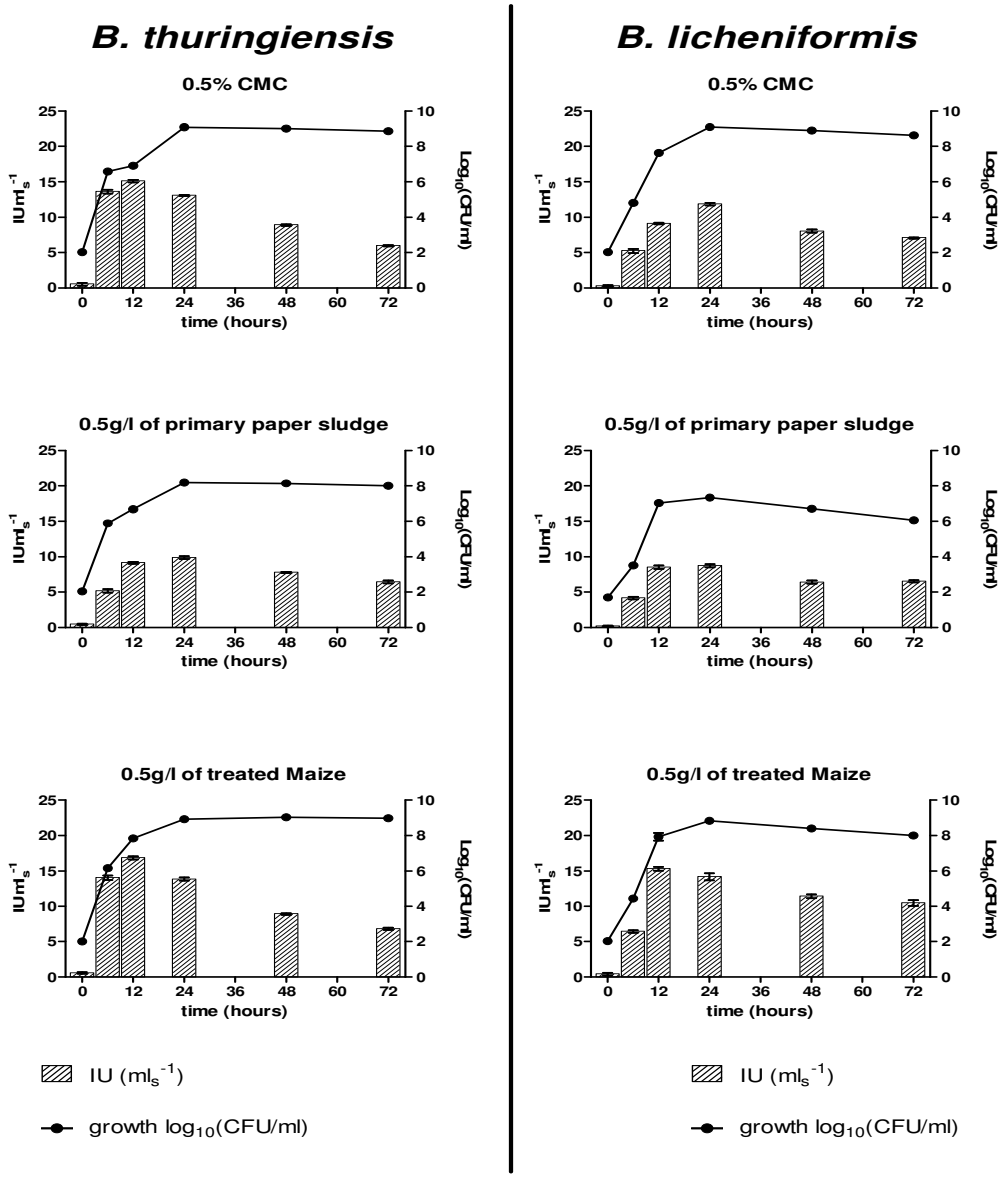

Fig. 4. Bacterial growth and cellulase activity measured in the presence of maize residues and paper mill sludge. Data recorded for bacteria grown with CMC are shown for comparison. Growth was calculated as CFU/ml on LB agar plates, and not as optical density because biomass was highly turbid

achieved the highest CMCase activity after 12 hours, followed by a decrease over the next 60 hours. *B. licheniformis* performed similarly, but retained higher activity after 72 hours. Regarding growth, strain 9D from *B. thuringiensis* performed well in all three media, but for 4D strain *B. licheniformis*, growth was decreased when paper mill sludge was used. The maize residue used here contains glucose that is mostly present as marginally soluble crystalline cellulose [13]. The ability to thrive on such a growth substrate strongly suggests that both *Bacillus* produce potent exocellulase(s), and not only endocellulase(s), although one has to keep in mind that other substances (hemicellulose) might contribute to supporting growth. Clearly, the bacterial strains found on site are adapted to local substrates and show diversity in their behavior regarding growth and enzyme production peak.

Comparison of the strains' ability to thrive on biomass samples is not devoid of pitfalls. First, using CMC should favor strains secreting endocellulases that can hydrolyse this modified cellulose substrate. Second, the biomass samples used are very different in terms of cellulose accessibility. Paper mill sludge is expected to contain a significant amount of free cellulose fibrils. By contrast, maize residue cellulose fibrils, mainly crystalline, are tightly associated with other biomass components, including lignin and hemicelluloses. Extrusion of maize has been performed in order to increase the amount of cellulose accessible to cellulase hydrolysis during the bacterial growth [14]. Since maize residue is mainly insoluble crystalline cellulose [13], *Bacillus* strains likely produce a potent exocellulase in addition to endocellulase(s), in order to extract sugars from it. Finally, another source of variation comes from the chemistry associated with paper mill sludge. By using a low (0.5% g/L) concentration for the source of carbon we tried to limit the impact of potential enzyme or growth inhibitors [15].

The locally-adapted and available bacteria in this study that could produce cellulase enzymes when grown on cheap locally-available biomass residues were identified as *Bacillus* species. The presence of *Bacillus* strains in soil samples is not surprising, and has been observed previously by a number of researchers (for a review, see Hurst et al. 2010 [16]). The identification here of a strain related to *B. aryabhattai* is notable. This species was first found in 2009 by Shivaji et al. [17] at high altitude, and since then has been found in a variety of environments, including forest soil and sugar cane fields. The ability of *B. aryabhattai* to digest cellulose was inferred from its genome sequence by Wen et al. 2015 [18] and the identification of a cellulase gene by this group. To our knowledge, we present here the first experimental evidence of extracellular cellulase production for this species (Fig. 3).

4. CONCLUSION

Bacillus strains producing extracellular cellulase(s) when grown on maize residues were identified at a local farm. These strains will be instrumental for the development of the integrated biomass processing initiative known as Agrosphère.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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