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Characterization of Bacteria Community Isolated from Wood Decay

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Authors' contributions

This work was carried out in collaboration between all authors. Author FM performed sampling, microbiology work and wrote the first draft of this manuscript. Author JKM carried out enzymatic assays and contributed to manuscript editing. Author MB contributed to data analysis and edited the manuscript final version. All authors read and approved the final manuscript.

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ABSTRACT

In order to discover enzymes having potential for wood fibre modification, bacteria (fourteen strains designated MMB1 to MMB14) were isolated from a decomposing stump from a resinous tree. Phylogenetic analysis and biochemical characterization indicated that these isolates were related to *Microbacterium*, *Chryseobacterium*, *Lysinibacillus,* and *Bacillus* gene; although most demonstrated phenotypic differences compared to previously characterized relatives. Only the *Bacillus* strains showed cellulolytic activity (as CMCase detected with Congo red) and only *Bacillus subtilis* strains (MMB10 to MMB14) displayed cellulolytic and secreted xylanase activity. Phenotypic characterization of two strains (MMB8 and MMB9) related to a previously characterized isolate (*Bacillus* sp. JU2), supported their reassignment to the genus *Lysinibacillus*. The *Microbacterium* strain MMB1 produced a green pigment when grown in the presence of light. Some microbes from the consortium were devoid of wood polymer modifying enzymes, and may be dependent on other organisms for their survival in this biotope.

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1. INTRODUCTION

Enzymes are powerful biocatalysts now recognized as key elements of "green manufacturing" (global sales of $3.3 billion in 2010 expected to reach $4.4 billion by 2015 with a growth rate of 6% over a 5-year period [1]. The best-selling industrial enzymes are hydrolytic enzymes, such as lipases for detergents, amylases for biofuel production from maize, and cellulases and hemicellulases which are becoming important enzymes for modification of wood polymers for either biofuel production or papermaking [2]. Commercial enzyme manufacturers (Novozymes, Genencor) do not provide a sufficient variety of different enzymes to meet the needs of many industrial sectors [3,4]. It appears that production costs and market size prevail and as a consequence, most enzymes are derived from only a handful of microorganisms (*Aspergillus* and *Trichoderma*) to name a few [5]. Consequently, most commercial enzymes are adapted to the needs and growth conditions of this select group of microorganisms, and often are sub-optimal for potentially rewarding applications. Equally important is that high volume commercial enzymes are typically available to competitors in any sector as well, which precludes exclusivity.

In order to identify novel (possibly exclusive) enzymes with ideal properties for a given application, one can generate sequence diversity using accelerated evolution approaches and produce the novel enzymes in recombinant hosts [6]. One can also search the environment, and screen novel microbes which in turn produce enzymes that have different properties [7,8]. Sampling relevant environments is a prerequisite to finding microorganisms and enzymes adapted to specific conditions. For instance, thermophiles represent an obvious source of thermostable enzymes, and extracellular enzymes from thermophiles generally show high thermostability [9]. Similarly, microbes that grow on wood produce extracellular enzymes that break down the woody cell wall and more specifically, its polymers. Fibres can be found in many types of samples, including wood chips, straw, fruit peels, and pulp and paper sludge to name a few. Such substrates may demand different sets of enzymes or enzymes having different properties. In addition, depending on temperature, tree species, humidity and surrounding flora, one should find differences in microbial communities, and in their associated enzyme properties.

Pioneering work on the identification of microorganisms involved in the digestion of decaying wood dates back to 1950's [10]. More recently, such microbes have been of special interest for their ability to secrete industrially relevant enzymes [11,12]. Although a number of dominant species have been found in these environments (especially *Bacillus*), strains found in one study often differ from those identified in other reports [13,14]. Moreover, analysis of 16S and 23S rRNA genes have revealed high heterogeneity in the genus *Bacillus*, both genetic (G + C percent from 32 to 69) and phenotypic (respiratory type, metabolism of sugars or wall composition), demonstrating that closely related microbes can display different enzymatic profiles. Finally, the proteome of such microbes may evolve as the environment and the microbes themselves evolve. On this basis, screening environment samples such as decaying wood for microbial diversity is a nearly infinite source of potentially different enzymes.

Here we have analysed the microbial biodiversity contained in a decaying stump of a resinous tree grown in Trois-Rivières, Québec, Canada. Unlike previous studies on wood or pulp and paper sludge microbial populations, we report a thorough analyses of morphology, biochemistry and hydrolytic activities having potential interest for several applications. This analysis provides a complete description of these microbes, emphasizing differences compared to previously identified relatives.

2. MATERIALS AND METHODS

2.1 Isolation of Hydrolytic Bacteria

Bacterial strains producing hydrolytic enzymes were isolated from decomposing wood chips taken from the tree. Wood chips (1g) were mixed in 100 ml of sterile distilled water, then 0.1 ml was removed and spread on the surface of nutrient agar medium (0.3% beef extract, 0.5% peptone, 0.5% NaCl, and 1.5% agar at pH 7.0). Plates were incubated at 20 or 37ºC for 48 hours. Morphologically different colony types were picked and re-streaked to isolate pure cultures. Isolates were then grown overnight in 10 ml LB medium (pH 7.0) with agitation (230

rpm) and stored as a frozen suspension in 10% (v/v) glycerol at -80ºC. *Escherichia coli* (ATCC 25922) and *Salmonella enteritidis* were used as negative controls.

2.2 Screening of Hydrolytic Enzyme-Producing Bacteria

Single colonies from fresh LB agar plates were patched onto minimal media (Mm) agar plates (0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.1% KCl, 0.05% yeast extract and 1.5% Bacto agar) [15]. The initial pH of the medium was adjusted to range from pH 6 to 8. For enzymatic activity detection, buffered Mm were supplemented with the following substrates: glyceryl tributyrate 1% (v/v) for esterase activity; olive oil 1% (v/v) containing rhodamine B 0.001% (w/v) for detection of lipolytic activity [16,17]; starch 1% (w/v) for amylase activity [18]; carboxymethylcellulose (CMC) 0.5% (w/v) for CMCase activity; Remazol Brillant Blue-xylan (RBB-xylan) from birchwood 0.5% (w/v) for xylanase activity [15]; apple pectin 1% (w/v) for pectinase activity; polygalcturonic acid 1% (w/v) for polygalacturonase activity; or casein 1% (w/v) for protease activity [19-21]. For the detection of agarase activity, Mm agar plates containing no additional substrate were used.

2.3 Morphological and Physiological Characterization

Colony characteristics (shape, size, pigmentation, etc.), cell morphology (Gram stain) [22], spore formation and motility were determined. Fermentation reactions of pure cultures were performed. For this, phenol red broth base medium (M054) pH 7.4, supplemented with 2% (w/v) of each respective sugar (L-arabinose, D-cellobiose, maltose, D-mannitol, D-xylose, D-sucrose, D-sorbitol, D-glucose, D-galactose, D-fructose) were inoculated with a single colony and incubated overnight at 20℃ or 37℃ depending on their optimal growth temperature. As most end-products from sugar metabolism are organic acids, the resulting decrease in pH turns the phenol red indicator to yellow at acidic pH [23]. In order to detect nitrate reductase enzyme, a denitrification test was carried out. Nitrate broth (0.3% (w/v) beef extract, 0.5% (w/v) peptone, 0.1% (w/v) and 0.1% (w/v) potassium nitrate (KNO_3)) was inoculated with each strain and incubated at 20℃ or 37℃ without agitation to produce anoxic conditions. An inverted Durham fermentation tube was placed in the liquid medium allowing for detection of nitrogen gas resulting from denitrification. Nitrate reduction was monitored after 72 h incubation by addition of Griess reagent (0.8% sulfanilic acid and 0.6% N, N-dimethyl-alpha naphthylamine). Catalase activity was detected following the addition of a few drops of 3% H_2O_2 to the microbial suspension. For oxidase activity, the ability of bacteria to express cytochrome oxidase was determined by using DrySlide oxidase (BD, 231746). The slides, coated with oxidase reagent (N,N,N'N'-tetramethyl-*p*-phenylenediamine dihydrochloride), were rubbed with a bacterial suspension. For oxidase positive bacteria, the slides turn purple within 20 seconds. For the determination of proteolytic activity nutrient gelatin medium was inoculated with fresh bacterial culture and incubated for 2-3 days at the appropriate temperature. Control tubes solidified when placed in ice, whereas those producing proteases do not. The ability of bacteria to utilize sodium citrate as sole carbon source was also tested. Bacteria that utilize citrate convert ammonium phosphate to ammonia (NH_3) and ammonium hydroxide (NH_4OH), both of which tend to alkalinize the agar. The pH change was detected by the change in color due to titration of bromothymol blue.

2.4 PCR Amplification of 16S rRNA Gene and Isolate Identification

The 16S rRNA genes from the fourteen isolates were amplified by PCR using universal primers designed within conserved regions of 16S rRNA gene: 27F (5'-AGAGTTTGATCCTGGCTCAG) and 1522R (5'-AAGGAGGTGATCCAGCCGCA). PCR products were purified using Qiagen minelute PCR purification kit and then quantified by absorption spectrophotometry at 260 nm using Take3 micro-volume plate. Purified PCR products were sequenced with an ABI Prism 3100 automatic sequencer at the Biomolecular analysis platform (Université Laval, QC) using the 27F and 1522R primers. Editing of 16S rRNA gene sequences was performed using Clone Manager professional 7.0 (Sci Ed Centra). Search for sequence similarity was performed using BLAST with the blastn algorithm using the non-redundant nucleotide database GenBank via the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/).

2.5 Phylogenetic Analysis of 16S rRNA Gene Sequences

For construction of a phylogenetic tree, additional 16S rRNA gene sequences were retrieved from GenBank database. The 16S rRNA gene of *Geoarcheota* sp. Str. WT 2007-339-2 (accession number: KT453558) was used as outgroup. Multiple sequence alignments were performed with CLUSTAL-W and phylogenetic analysis was inferred with MEGA 6 program [24] downloaded from the NCBI database [25]. The evolutionary history was inferred using the Neighbor-Joining method [24]. The evolutionary distances were computed using the Maximum Composite Likelihood method [26] and are expressed as the number of base substitutions per site. The stability of relationships was assessed by a bootstrap analysis based on 1000 resamplings of the neighbour-joining dataset [27].

2.6 GenBank Deposition

The 16S rRNA sequences data have been deposited electronically and registered in GenBank under the following accession numbers: MMB1 (KR493003), MMB2 (KR493004), MMB3 (KR493005), MMB4 (KR493006), MMB5 (KR493007), MMB6 (KR493008), MMB7 (KR493009), MMB8 (KR493010), MMB9 (KR493011), MMB10 (KR493012), MMB11 (KR493013), MMB12 (KR493014), MMB13 (KR493015) and MMB14 (KR493016).

3. RESULTS AND DISCUSSION

3.1 Identification of the Strains Based on 16S rRNA Gene Sequencing

The universal 27F and 1522R primers were used to amplify 16S rRNA genes from 14 different isolates. The 16S rRNA sequences were aligned and compared with other 16S rRNA genes in GenBank by using BLAST. Phylogenetic relationships to previously identified species are shown in Fig. 1. The isolates clustered into three phyla (*Actinobacteria*, *Firmicutes* and *Bacteroidetes*) and represented 4 different genera: *Microbacterium*, *Chryseobacterium*, *Bacillus* and *Lysinibacillus*. The percentage of identity to the nearest relative identified by a BLAST analysis and the DSM "Type strain" are also provided when known (Table 1). Note that *Bacillus* sp. JU2 (closest relative to MMB8 and 9) was first reported by Kumar and Pratibha in 2010 [28]. It was found in drinking water in India, but similar strains (99% identity in 16S rRNA gene sequence) were also found in water samples from China and Germany [29,30]. The bootstrap value for the *Bacillus* JU2 clade (which includes MMB8 and MMB9) is rather low (0.61) and its separation from *Lysinibacillus fusiformis* on the basis of 16S rRNA gene sequences is far from certain. In fact, BLAST analysis of *Bacillus* JU2 (or related *Bacillus* JUN-3) supports an assignment to *Lysinibacillus*. Phenotypic characteristics (shown below) also provide additional evidence to substantiate the placement of MMB8 and MMB9 into the genus *Lysinibacillus.*

3.2 Morphological Characterization

Isolates were characterized on the basis of colony colour, colony morphology (shape, margin, elevation and texture), cell morphology (Gram staining), and ability to grow at various temperatures (Table 2). Thirteen out of 14 isolates belong to genera which were Gram-positive. The 14 isolates were purified, streaked on LB agar plate and photographed after growth (Fig. 2).

The isolate MMB1 is related to *Microbacterium oxydans* DSM-20578 (a reclassification of *Brevibacteriurn oxydans* [31]) which is a bacterium registered with the Centers for Disease Control. This Gram-positive bacterium produces a greenish pigment. The strain identified here was found to be photo-chromogenic, i.e. its colour appeared when grown in the presence of light (Fig. 2) as reported by Trutko et al. [32]. This isolate grew from 4 to 30℃ on plates. It was nonmotile, a feature observed for a strain of *M. binotii* but not for *M. oxydans* as reported by Huang et al. [33].

The isolates related to *Bacillus thuringiensis* MMB2, *Bacillus* sp MMB3 *and Bacillus cereus* MMB4 were similar for most phenotypic properties, being the only three strains which could grow under anaerobic or aerobic conditions. They had a moderate growth rate at 37°C. Only one Gram-negative strain was found (MMB5), and it belonged to the genera *Chryseobacterium* which includes risk level 2 strains. This species formerly known as *Flavobacterium* (family of *Cytophagaceae*) is non motile, oxidase positive, non-fermentative, and Gram negative, as reported earlier by Fraser and Jorgensen [34]. As shown in Fig. 2, MMB5 colonies appeared orange in color.

The strains related to *Lysinibacillus* identified as MMB6 and MMB7 were very similar for most morphological traits reported here. They were Gram positive spore formers in agreement with a previous report, but differed from the strains isolated by Wang et al. [35] which were dark yellow. *Bacillus* JU2 strains MMB 8 and 9 were very similar to *Lysinibacillus* strains described above. At variance with *Lysinibacillus* strains MMB6 and 7, they did not sporulate, a trait which is common to most *Bacillus* strains found in this environmental sample.

Finally, the *Bacillus subtilis* group (MMB 10 to 14) was rather homogenous, except for MMB12 which had different colony characteristics. The colony appearance of MMB12 differed from the other strains from this group as shown in Fig. 2.

3.3 Hydrolytic Activities

Apart from catalase, agarase and esterase, no secreted enzymatic activities were detected for MMB1 (Table 3). Because of the absence of CMCase and gelatinase activities, this strain appears to differ from a strain affiliated to *M. oxydans* observed in the gut of root-feeding larvae of *Holotrichia parallela*, a beetle found in China [33]. No lipase activity was detected in this strain, at variance with an earlier analysis of *M. oxydans* by Hantsis-Zacharov [36]. However, one has to keep in mind that such discrepancies may be attributed to differences in the exact substrate molecule and detection method used for enzymatic activity detection (here the enzymes have to be secreted for detection to occur).

B. thuringiensis MMB2 strain displayed a larger set of enzymatic activities than *M. oxydans*, including CMCase, lipase, protease and gelatinase as shown in Table 3. Das et al. [37] reported a *B. thuringiensis* strain with lipase, amylase and protease activities. We also found amylase and protease activity, but no lipase or xylanase activity. Examination of genome sequence information (NCBI) predicts the presence of xylanase deacetylase activity in MMB2, but no xylanase *per se*.

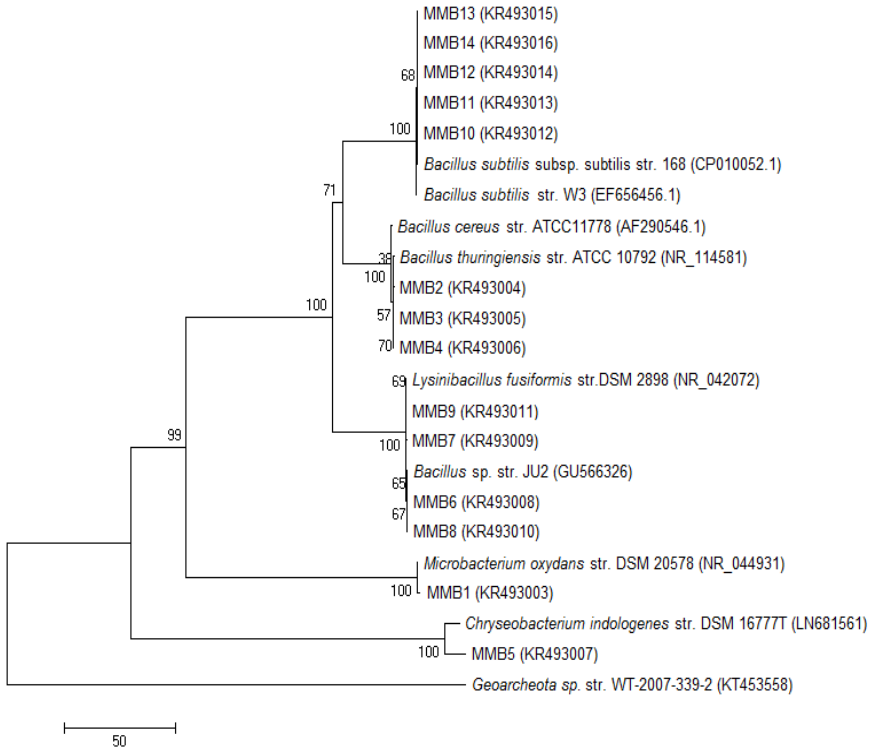

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences

The tree was generated from the alignment of 16S rRNA gene sequences and shows the evolutionary relationships between the isolated strains and previously characterized species. Type strains are indicated by a T. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of nucleotide base differences. The analysis included 23 nucleotide sequences and was performed with MEGA 6

Fig. 2. Photos of bacterial strains grown on LB agar plate showing the colony morphology

Table 1. Assignment of isolates found in decaying wood

Isolate code	Phylum/class	Closest relative from BLAST analysis	Closest relative acession number	% Sequence identity
MMB1	Actinobacteria	*Microbacterium oxydans*	KF870422	99
MMB2	Firmicutes	*Bacillus thuringiensis*	KJ49638	99
MMB3	Firmicutes	*Bacillus thuringiensis*	KJ49638	99
MMB4	Firmicutes	*Bacillus cereus*	KM391419	99
MMB5	Bacteroidetes	*Chryseobacterium indologenes*	KJ806354	99
MMB6	Firmicutes	*Lysinibacillus fusiformis*	KP192021	99
MMB7	Firmicutes	*Lysinibacillus* sp.	AB84789	99
MMB8 and MMB9	Firmicutes	*Bacillus* sp. *JU2*	GU566326	99
MMB10 - MMB12	Firmicutes	*Bacillus subtilis*	CP007173	99
MMB13	Firmicutes	*Bacillus subtilis*	JN366756	99
MMB14	Firmicutes	*Bacillus subtilis*	KJ210578	99

MMB3 displayed 9 enzymatic activities out of the 14 assessed here, including CMCase, agarase, protease, amylase and gelatinase activities (Table 3). We found no xylanase activity, despite the availability of published xylanase structures for several *Bacillus* sp. strains. Esterase or lipase activities were not detected for MMB3, which is different from a related thermotolerant strain [38]. The enzyme profiles of MMB2, MMB3 described above, and that of MMB4 related to *Bacillus cereus*, were very similar. The strain *B. cereus* and *B. thuringiensis* are closely related and in the opinion of many researchers they should be considered as a single species based on genetic and genomic features [39-41]. The complete analysis presented here (phylogeny, morphology, enzymatic activities and sugar metabolism as detailed in Fig. 2 and Tables 2, 3 and 4, supports the previously reported similarities among these species.

MMB5 strain showed catalase, oxidase, agarase, protease, amylase and gelatinase activities. No CMCase activity was detected making these different from other previously characterized *Chryseobacterium* strains. However, in the presence of agarase activity, one can mistakenly assign CMCase or cellulase activity to a microbe when Gram's iodine is used for the agar plate analysis [15]. We did not detect any xylanase activity, despite the fact that a putative protein predicted by genomic analysis would share 60% identity to an active xylanase in *Chryseobacterium* [42].

Bacterial isolates MMB6 and MMB7 related to *Lysinibacillus* DSM-2898 were positive for catalase, oxidase and gelatinase activities as tested here (Table 3). These observations are similar to those reported recently by Ahmad 2014 [43], except that these authors did not detect gelatinase activity. Pandey et al. [44] detected cellulase activity, while Alves-Prado et al. [45] detected xylanase activity in *Lysinibacterium* strains, but we did not for MMB6 and MMB7. Once again, this may depend on the exact methods used for enzyme screening.

As mentioned above, strains MMB8 and MMB9 are of special interest because they have not been characterized despite their first identification in 2010 [29,30]. Their enzymatic profile is very similar to the *Lysinibacillus* profile but with the exception that MMB8 and 9 exhibited activity on tributyrin substrate and weak lipase activity on olive oil agar, while they showed no gelatinase activity.

Although MMB8 and MMB9 are identified as *Bacillus* sp. according to the phylogenetic analysis, they are devoid of cellulase and agarase activities, both activities that were found in MMB2, MMB3 and MMB4. They are completely different from the *Bacillus subtilis* block (MMB10 to MMB14) as well, having no protease, amylase or pectinase activities. The analysis of enzymatic activity for MMB8 and 9 leads to a similar observation as mentioned above (morphological analysis of Table 2) these strains are more similar to *Lysinibacillus*. The transfer of several strains previously assigned to *Bacillus* to *Lysinibacillus* has been suggested previously [46,47] and we concur that MMB8 and MMB9, while previously identified as *Bacillus* sp., should be assigned to *Lysinibacillus*.

Table 2. Morphological and physiological characteristics of bacterial strain

Isolate code	**MMB1**	**MMB2**	**MMB3**	**MMB4**	**MMB5**	**MMB6**	**MMB7**	**MMB8**	**MMB9**	**MMB10**	**MMB11**	**MMB12**	**MMB13**	**MMB14**
Strain assignment	***Microbacterium oxydans***	***Bacillus thuringiensis***	***Bacillus* sp**	***Bacillus cereus***	***Chryseobacterium indologenes***	***Lysinibacillus fusiformis***	***Lysinibacillus* sp.**	***Bacillus* sp. *JU2***	***Bacillus* sp. *JU2***	***Bacillus subtilis***	***Bacillus subtilis***	***Bacillus subtilis***	***Bacillus subtilis***	***Bacillus subtilis***
Gram staining	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Colony Texture	smooth cream	smooth cream	smooth cream	smooth cream	smooth cream	smooth cream	smooth cream	smooth cream	smooth cream	smooth mucoid	smooth mucoid	rough dry	smooth mucoid	smooth mucoid
Pigmentation of colonies	green	colorless	colorless	colorless	orange	colorless	colorless	colorless	colorless	colorless	colorless	whitish*	colorless	colorless
Cell shape	circular	circular	circular	circular	circular	circular	circular	circular	circular	irregular	irregular	rhizoid	irregular	irregular
Optical properties	glistening	opaque	opaque	dull	dull	glistening	glistening	glistening	glistening	glistening	glistening	dull	glistening	glistening
Margin	entire	entire	entire	undulate	entire	entire	entire	entire	entire	undulate	undulate	curled	undulate	undulate
Elevation	raised	flat	flat	flat	convex	flat	flat	raised	raised	convex**	convex**	flat	convex**	convex**
Motility	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Sporulation	-	-	-	+	-	+	+	-	-	-	-	-	-	-
Aerobic	obligate	facultative	facultative	facultative	obligate	obligate	obligate	obligate	obligate	obligate	obligate	obligate	obligate	obligate
Growth Temp (℃)														
4	$+^{s}$	-	-	-	$+^{s}$	$+^{s}$	$+^{s}$	-	-	-	-	-	-	-
22	$+^{7-10}$	$+^{6-10}$	$+^{6-10}$	$+^{6-10}$	$+^{6-8}$	$+^{6-10}$	$+^{6-10}$	$+^{6-8}$	$+^{6-8}$	$+^{6-8}$	$+^{6-8}$	$+^{6-8}$	$+^{6-8}$	$+^{6-8}$
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37	-	$+^{6-8}$	$+^{6-8}$	$+^{6-10}$	$+^{6-8}$	$+^{6-10}$	$+^{6-10}$	$+^{6-10}$	$+^{6-10}$	$+^{6-10}$	$+^{6-10}$	$+^{6-10}$	$+^{6-10}$	$+^{6-10}$
50	-	-	-	-	-	-	-	-	-	$+^{6-8}$	$+^{6-8}$	$+^{6-8}$	$+^{6-8}$	$+^{6-8}$

*s: slow; superscript numbers show growth pH range; +: positive reaction; -: no reaction; *: colony devoid of color at center; ** colony convex with white grooves*

Table 3. Enzymatic activities of bacterial strains. Hydrolytic activities were detected after growth on minimal media agar plates containing relevant substrates

Isolate code	**MMB1**	**MMB2**	**MMB3**	**MMB4**	**MMB5**	**MMB6**	**MMB7**	**MMB8**	**MMB9**	**MMB10**	**MMB11**	**MMB12**	**MMB13**	**MMB14**
Enzyme activity	***Microbacterium oxydans***	***Bacillus thuringiensis***	***Bacillus* sp**	***Bacillus cereus***	***Chryseobacterium indologenes***	***Lysinibacillus fusiformis***	***Lysinibacillus* sp.**	***Bacillus* sp. *JU2***	***Bacillus* sp. *JU2***	***Bacillus subtilis***	***Bacillus subtilis***	***Bacillus subtilis***	***Bacillus subtilis***	***Bacillus subtilis***
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxydase	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	-	-	+	-	-	-	-	-	-	+	+	+	+	+
Nitrate	-	+	+	+	-	-	-	-	-	+	+	+	+	+
CMCase	-	+	+	+	-	-	-	-	-	+	+	+	+	+
Agarase	+	+	+	+	+	-	-	-	-	+	+	+	+	+
Xylanase	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Esterase	w	w	-	-	-	w	w	+	+	+	+	+	+	+
Lipase	-	-	-	-	-	-	-	w	w	+	+	+	+	+
Protease	-	+	+	+	+	-	-	-	-	+	+	+	+	+
Amylase	-	+	+	+	+	-	-	-	-	+	+	+	+	+
Endopolygalacturonase	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Pectinase	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Gelatinase	-	+	+	+	+	+	+	-	-	+	+	+	+	+

(+: positive reaction; -: no reaction, w: weak)

Table 4. Carbohydrate fermentation profiles of bacterial strains

Isolate code	MMB1	MMB2	MMB3	MMB4	MMB5	MMB6	MMB7	MMB8	MMB9	MMB10	MMB11	MMB12	MMB13	MMB14
Strain Assignment	***Microbacterium oxydans***	***Bacillus thuringiensis***	***Bacillus* sp**	***Bacillus cereus***	***Chryseobacterium indologenes***	***Lysinibacillus fusiformis***	***Lysinibacillus* sp.**	***Bacillus* sp. *JU2***	***Bacillus* sp. *JU2***	***Bacillus subtilis***	***Bacillus subtilis***	***Bacillus subtilis***	***Bacillus subtilis***	***Bacillus subtilis***
L-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	+	-
D-Cellobiose	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Maltose	+	+	+	+	-	-	-	-	-	+	+	+	+	+
D-Mannitol	+	-	-	-	-	-	-	-	-	+	+	+	+	+
D-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-D-Sucrose	-	-	-	-	-	-	-	-	-	+	+	+	+	+
D-Sorbitol	-	-	-	-	-	-	-	-	-	+	+	+	+	+
D-Glucose	-	+	+	+	-	-	-	-	-	+	+	+	+	+
D-Galactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Fructose	-	+	+	+	-	-	-	-	-	+	+	+	+	+

+: positive reaction; -: no reaction

Our results suggest that strains related to *Lysinibacillus* (MMB6 to MMB9) have a very limited set of secreted enzymes, and their growth may be dependent on the earlier colonisers of wood including bacteria or fungi. In contrast, the strains associated with *Bacillus subtilis* (MMB10 to 14) were positive for all enzymatic activities tested here. This is in agreement with the abundant literature describing this species.

3.4 Sugar Metabolism

The ability to break down wood polymers is often associated with the ability to metabolize the resulting smaller saccharides. The results shown in Table 4 confirm this. Apart from L-arabinose, D-xylose and D-galactose, all sugars analysed were metabolized by the *Bacillus subtilis* group. This is in line with the several hydrolytic enzymes detected for strains MMB10 to MMB14. At the other end of the spectrum, strains MMB5 to MMB9 were unable to metabolize any of the sugars tested.

This is also compatible with the observation that they did not secrete most hydrolytic enzymes tested. The *Bacillus* related strains MMB2, 3 and 4 did metabolize glucose, which is compatible with their CMCase activity. D-xylose was not used by any of the strains studied here. This supports the absence of secreted xylanase activity for strains MMB1 to 9, but is difficult to reconcile with the enzymatic activity shown by the *Bacillus subtilis* group.

3.5 Applicability of Strain

Our applied research program is aimed at identifying new strains (i.e. having measurable differences from their closest relatives) that produce extracellular enzymes with potential application for the modification of wood fibre polymers. In this regard, the strains found in the decaying wood stump can be divided into three groups depending on their potential source of industrial enzymes. The *B. subtilis* group (MMB10 to MMB14) produced many enzymes of relevance for wood-derived materials modification or hydrolysis. They are also better adapted to higher temperatures (50ºC) a feature compatible with several pulp and paper processes. The *Bacillus* group of MMB2 to MMB5 lacked some enzymatic activities (especially xylanase and pectinase) but still showed CMCase activity, useful for wood polymer treatment. Other enzymes such as lipases and esterases have applications in mechanical pulp production and in paper recycling. Apart from the *Bacillus subtilis* group, it appears that strains MMB8 and MMB9 also produce lipolytic enzymes and deserve to be further investigated. Regarding applications in other sectors, several strains (the *Bacillus subtilis* group and MMB2 to MMB5) expressed amylase and as such deserve to be further considered for their possible application in biofuel production.

4. CONCLUSION

In this study we isolated and characterized a community of fourteen bacterial strains from wood decay. Isolated bacterial strains were identified on the basis of 16S rRNA gene sequences and were related to *Microbacterium*, *Chryseobacterium*, *Lysinibacillus,* and *Bacillus* gene. *Microbacterium* strain was found to be photochromogenic bacteria. Only *Bacillus subtilis* strains produced many enzymes of relevance for wood-derived polymers modification. This group deserves to be further considered for their possible application in biofuel production and pulp and papers industry.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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