

Isolation of amylolytic, xylanolytic, and cellulolytic microorganisms extracted from the gut of the termite *Reticulitermes santonensis* by means of a micro-aerobic atmosphere

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Abstract The aim of this work was to isolate enzyme-producing microorganisms from the tract of the termite *Reticulitermes santonensis*. The microorganisms were extracted from the guts and anaerobic (CO₂ or CO₂/H₂) and micro-aerobic atmospheres were used to stimulate growth. Three different strategies were tried out. First, the sample was spread on Petri dishes containing solid media with carboxymethylcellulose, microcrystalline cellulose or cellobiose. This

technique allowed us to isolate two bacteria: *Streptomyces* sp. strain ABGxAviA1 and *Pseudomonas* sp. strain ABGxCeA. The second strategy consisted in inoculating a specific liquid medium containing carboxymethylcellulose, microcrystalline cellulose, or cellobiose. The samples were then spread on Petri dishes with the same specific medium containing carboxymethylcellulose, microcrystalline cellulose, or cellobiose. This led to the isolation of the mold *Aspergillus* sp. strain ABGxAviA2. Finally, the third strategy consisted in heating

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the first culture and spreading samples on agar plates containing rich medium. This led to the isolation of the bacterium *Bacillus subtilis* strain ABGx. All those steps were achieved in controlled atmospheres. The four enzyme-producing strains which were isolated were obtained by using a micro-aerobic atmosphere. Later, enzymatic assays were performed on the four strains. *Streptomyces* sp. strain ABGxAviA1 was found to produce only amylase, while *Pseudomonas* sp. strain ABGxCeIIA was found to produce β -glucosidase as well. *Aspergillus* sp. strain ABGxAviA2 showed β -glucosidase, amylase, cellulase, and xylanase activities. Finally, *B. subtilis* strain ABGx produced xylanase and amylase.

Keywords Termite · *Bacillus* · *Pseudomonas* · *Streptomyces* · *Aspergillus* · Atmosphere

Introduction

Termites are able to break the lignocellulosic complex. Lignocellulose is composed of lignin, cellulose, and hemicelluloses. Cellulose is a polymer of glucose units bound together by hemicelluloses. These macromolecules are more complex and made of various monomers: D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose, and D-glucuronic acid. Hemicelluloses are linked to lignin by ester or ether bonds. Lignin is a complex macromolecule resulting from the polymerization of *p*-coumaryl, coniferyl, and sinapyl alcohols (Breznak and Brune 1994).

The termite *Reticulitermes santonensis*, a wood-eating termite, contains a highly diversified microflora composed of bacteria, mycetes, and protists. These microorganisms act as a microbial consortium which is able to break the lignocellulose components. In Yang et al. (2005) published an excellent review about the strains living in the gut of *R. santonensis*. The termite gut is divided into three parts: foregut, midgut, and hindgut. That study concluded that there are differences in microbial communities in accordance with the location considered as well as the distance from the gut wall. The major phyla of prokaryotes which were identified were *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, and *Proteobacteria*. Tartar et al. (2009) identified the fungi genera *Aspergillus*, *Neurospora*, *Verticillium*, *Trichoderma*, and *Thermomyces*.

The conditions relating to pH and O₂ in the gut of *R. flavipes*, which can be considered as *R. santonensis* (Austin et al. 2005), were studied previously (Brune et al. 1995). The pH values in the gut vary from 6 to 7. However, the repartition of oxygen is more complex. It can penetrate the peripheral hindgut up to 50–200 μ m. The central zone of the hindgut was found to be completely anoxic because of the oxygen consumption by the hindgut microflora (Brüne and Stingl 2006).

This study relates to the isolation of enzyme-producing strains isolated from the gut of the termite *R. santonensis*.

Specific atmospheres were used to simulate micro-aerobic and anaerobic environments in order to copy the conditions which can be found inside the termite gut. The most interesting microorganisms able to synthesize enzymes were identified on the basis of 16S and 28S rDNA sequences.

Materials and methods

Termites

Reticulitermes santonensis Feytaud (Rhinotermitidae) was harvested on the Island of Oleron (France). The termites were kept in darkness at a temperature of 27 °C with a relative humidity of 70 %. The termites were first fed on pinewood which was gradually replaced by poplar wood.

Isolation of microorganisms

Some termites were washed in ethanol to prevent surface contaminations, then in water and held with two dissection forceps, as described before (Tarayre et al. 2013). Intestines were extracted under sterile conditions and put into an Eppendorf tube containing 200 μ l of YPD medium (10 g/l peptone, 5 g/l yeast extract, and 10 g/l glucose). The sample was kept at 40 °C for 24 h. The intestines were then sterilely crushed and the volume was adjusted to 1.5 ml with sterile YPD medium. The volume was divided into three parts each of which was put into a tube containing 5 ml of medium. The first tube was saturated with an atmosphere composed of 16 % of CO₂ and 84 % of air to simulate a micro-aerobic atmosphere (atmosphere A). The second atmosphere consisted of 100 % of CO₂ (atmosphere B) and the third atmosphere containing 85 % of H₂/15 % CO₂ (atmosphere C) were used to create anaerobic conditions. The tubes were put on an agitator at 30 °C for 6 days. The first group of microorganisms was isolated by spreading 100 μ l of various dilutions of the contents of each tube on agar plates containing modified GBG medium (Lynd and Grethlein 1987). Different substrates were added as carbon sources to this medium: carboxymethyl-cellulose 2.5 g/l (CMC), microcrystalline cellulose 2.5 g/l (MC), or cellobiose 2.5 g/l (CELL). The agar plates were kept in darkness at 30 °C and the microorganisms were grown in the atmospheres A, B or C for 3 days before isolation. The second group of microorganisms was obtained by pouring the contents of the tubes into flasks containing liquid GBG medium with the three carbon sources described above. The aim of this step was to enrich the microbial population able to hydrolyze cellulose or cellobiose. After 14 days, 100 μ l of different dilutions of each liquid medium were spread on agar plates containing

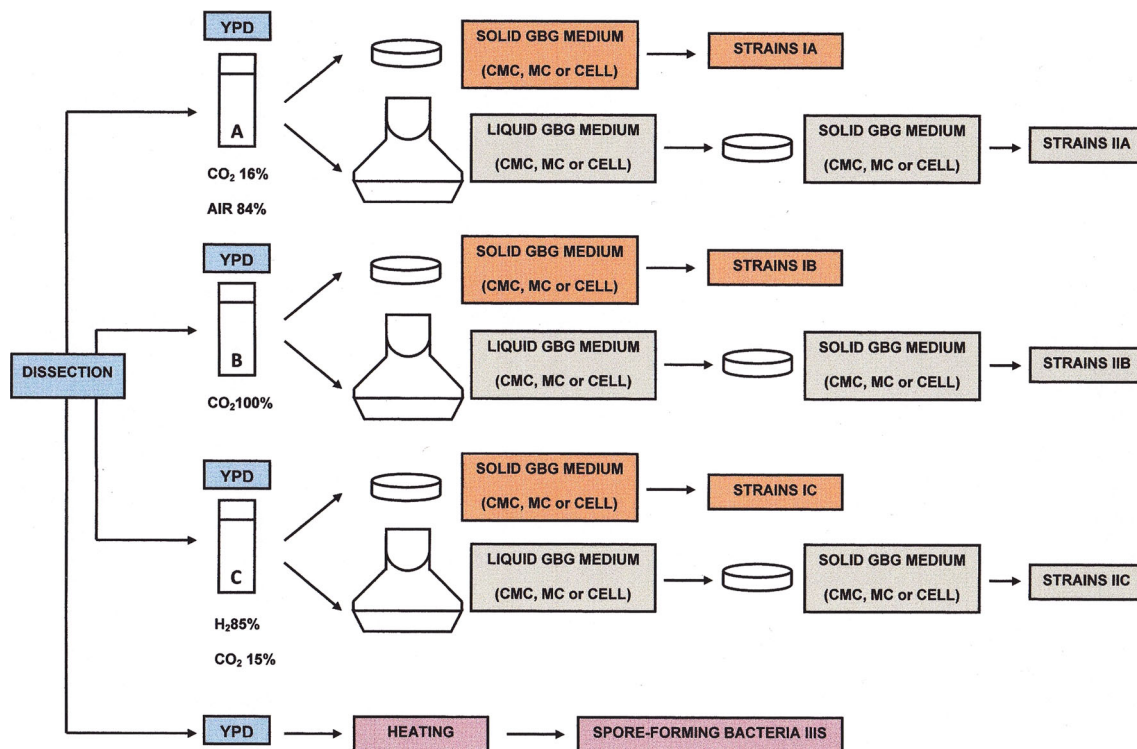


Fig. 1 Strategies of isolation of enzyme-producing microorganisms from the gut of *R. santonensis*. The isolation of microorganisms was performed in different atmospheres and in solid or liquid media added

with carboxymethylcellulose (CMC), microcrystalline cellulose (MC), or cellobiose (CELL). Spore-forming bacteria were isolated after heating the sample

solid GBG medium with the three carbon sources (same concentrations). The agar plates were kept in the same conditions as before, prior to isolating the second group of strains. The third group of microorganisms was obtained by heating the microbial suspension stemming from the crushed intestines (10 min, 80 °C). Then, 100 µl of culture at different dilution rates were spread on agar plates containing rich solid medium (glucose 20 g/l, yeast extract 10 g/l, casein peptone 10 g/l, agar 17 g/l). This method led to the isolation of spore-developing bacteria. The whole method is illustrated in Fig. 1.

Investigation of enzymatic activities

The following activities were observed in each strain: endo-1,4-β-D-xylanase, endo-1,4-β-D-glucanase, β-glucosidase, and α-amylase. The medium used for β-glucosidase investigation consisted of casein peptone (8 g/l), esculin sesquihydrate (1 g/l), ammonium ferric citrate (1 g/l) and agar (17 g/l), pH 7.4. The basic medium used to detect all the other activities was derived from Kasana et al. (2008) and composed of NaNO₃ (2 g/l), MgSO₄·2H₂O (0.5 g/l), KCl (0.5 g/l), casein peptone (0.2 g/l), agar (17 g/l). Trace elements were added to the medium (2.86 mg/l H₃BO₃, 1.81 mg/l MnCl₂·4H₂O, 0.222 mg/l ZnSO₄·7H₂O, 0.39 mg/l

1 NaMoO₄·2H₂O, 0.079 mg/l CuSO₄·5H₂O, and 0.0494 mg/l Co(NO₃)₂·6H₂O). The following components were used as carbon sources: 0.5 g/l AZCL-xylan medium (endo-1,4-β-D-xylanase activity), 0.5 g/l AZCL-HE-cellulose medium (endo-1,4-β-D-glucanase), 0.5 g/l AZCL-amylose medium (α-amylase activity). AZCL media were supplied by Megazyme (Megazyme International Ireland, Bray Business Park, Bray, Co. Wicklow, Ireland). All the strains were first cultivated in rich liquid media. Bacteria were grown in the following medium: glucose 20 g/l, yeast extract 10 g/l, casein peptone 10 g/l. Molds were cultivated in potato dextrose broth (24 g/l) containing chloramphenicol (0.5 g/l). After 24 h of cultivation, 80 µl of each culture were spot plated on the different media (three replicates) under the atmospheres chosen in accordance with the conditions of isolation. The plates were incubated for 3 days at 30 °C.

Identification of enzyme-producing microorganisms

DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega Benelux BV Branch Office, Schipholweg 1, 2316 XB LEIDEN, The Netherlands). The primers were provided by Eurogentec (Eurogentec S.A., Belgium). They were the universal primers 357F and 1100R, 8F, and 1492R (Lane 1991; Longnecker and

Reysenbach 2001), and LR0R and LR6 (Vilgalys and Hester 1990). The primers 357F/1100R and 8F/1492R were used to amplify 16S rDNA of bacteria, while the primers LR0R/LR6 were used to amplify 28S rDNA of mycetes. The program used for the amplification of 16S rDNA (primers 357F/1100R) and 18S rDNA (primers LR0R/LR6) consisted of 5 min of denaturation (95 °C), 25 cycles of amplification composed of 30 s at 95 °C, 30 s at 54 °C, 2 min at 72 °C, and a final extension of 10 min at 72 °C. The program used for 16S rDNA (primers 8F/1492R) amplification included 10 min of denaturation (95 °C), 30 cycles of amplification composed of 1 min at 95 °C, 1 min at 54 °C, 2 min at 72 °C, and a final extension of 10 min at 72 °C. PCR products were used for the sequencing reaction. Sequencing was achieved by Progenus[®] (Rue des Praules 2, 5030 Sauvenière, Belgium) with a Genetic Analyzer 3130 designed by Applied Biosystems[®]. The sequences were aligned with the Vector NTI[®] program and the homologous sequences present in the GenBank database were identified using the NCBI (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>) BlastN program (Altschul et al. 1990).

Results and discussion

Isolation and identification of microorganisms

This strategy enabled us to isolate 12 strains. However, the identification was only performed with the strains displaying enzymatic activities. All the assays were performed in culture media with low concentrations of nitrogen to reconstitute the conditions found in the termite gut. No strain was isolated in anaerobic atmospheres (CO₂ 100 % and H₂ 85 %/CO₂ 15 %). This observation may be due to acidification caused by CO₂. Another gas could have been used to check that, such as N₂. On the other hand, it was established that oxygen penetrates the outer part of the hindgut lumen by 50–200 μm. Consequently, anoxic conditions are limited to the dilated compartments of the central hindgut portion, which is mainly colonized by anaerobic protists in lower termites (Brüne and Stingl 2006). Another point is that it is particularly difficult to prevent oxygen transfers during the crushing step of the intestines. Therefore, it is not surprising to isolate only aerobic strains from the termite gut.

Three strains were isolated in micro-aerobic atmospheres and one spore-forming bacterium was found after heating the sample. It is important to note that no anaerobic strain was isolated. All the strains were identified on the basis of rDNA sequences. The first strain belonged to the group of *Strains IACELL* (isolated in micro-aerobic atmosphere, on solid GBG medium containing microcrystalline cellulose, see Fig. 1). It was identified as *Streptomyces* sp., and was named

Streptomyces sp. strain ABGxAviA1 (97 % identity with *S. sampsoni*, *S. somaliensis*, *S. exfoliatus*, *S. albus*, *S. coelicolor*, *S. lividens*, *S. griseus*, *S. viridochromogenes*; GenBank ID: KF297884). The second strain was isolated in the group of *Strains IACELL* (isolated in micro-aerobic atmosphere, on solid GBG medium containing cellobiose, see Fig. 1) and was identified as *Pseudomonas* sp., and further named *Pseudomonas* sp. strain ABGxCella (99 % identity with *P. citronellolis*, *P. aeruginosa*, *P. delhiensis*, *P. knackmussii*, *P. denitrificans*, *P. nitroreducens*, *P. azelaica*, *P. multiresivorans*; GenBank ID: KF297883). The third strain was a mold isolated in the group of *Strains IIAMC* (isolated in microaerobic atmosphere, on liquid GBG medium containing microcrystalline cellulose, see Fig. 1) and was found to be an *Aspergillus* sp. The strain was given the name *Aspergillus* sp. strain ABGxAviA2 (95 % identity with *A. fumigatus*; GenBank ID: KF297885). The identity of the strain was confirmed by the mycothèque of Louvain-la-Neuve (Croix du sud 2, bte L7.05.06, 1348 Louvain-la-Neuve, Belgium). The spore-developing bacterium (*Group III*, see Fig. 1) was identified as a *Bacillus* sp. (99 % identity with *B. subtilis*, *B. methylotrophicus*, *B. tequilensis*, *B. licheniformis*). Further DNA analysis was described in another article (Tarayre et al. 2013). Gyrase (GenBank ID: JX545344) and xylanase (GenBank ID: JX545345) sequences confirmed that the strain was a *Bacillus subtilis*. The strain was named *B. subtilis* strain ABGx (16S GenBank ID: KF297882).

Enzymatic activities

All the strains which were isolated were aerobic. Consequently, the enzymatic assays were performed in aerobic conditions. The following enzymatic activities were tested on the isolated strains: endo-1,4-β-D-xylanase, endo-1,4-β-D-glucanase, β-glucosidase, and α-amylase. The results are presented in Table 1. Many examples of α-amylase- and β-glucosidase-producing strains of *Pseudomonas* can be found in literature. For instance, Kimura et al. (1988) studied the maltotetraose-producing strain of *Pseudomonas stutzeri*. Rickard et al. (1989) studied the production of a cloned β-glucosidase from *Pseudomonas* sp. strain PS2-2. *Pseudomonas* species have already been found in the termite gut, such as *P. aeruginosa* and *P. cepacia* (König et al. 2006). Pourramazan et al. (2012) isolated an endo-1,4-β-D-glucanase-producing strain of *Pseudomonas* sp. from the xylophagous termite *Microcerotermes diversus*. *Pseudomonas* sp. strain ABGxCella was isolated on a medium containing cellobiose. Although it was logical to detect a β-glucosidase activity hydrolyzing esculin, an amylase activity was also observed.

Streptomyces species producing xylanase were also found in *R. santonensis* (Mattéotti et al. 2012), and α-amylase-producing strains were studied previously

Table 1 Enzymatic activities detected in the four enzyme-producing strains

Strain	Endo-1, 4-β-D- xylanase	Endo-1, 4-β-D- glucanase	β- Glucosidase	α- Amylase
<i>Streptomyces</i> sp. strain ABGxAviA1	–	–	–	Yes
<i>Pseudomonas</i> sp. strain ABGxCe1A	–	–	Yes	Yes
<i>Aspergillus</i> <i>fumigatus</i> strain ABGxAviA2	Yes	Yes	Yes	Yes
<i>Bacillus subtilis</i> strain ABGx	Yes	–	–	Yes

(Vukelić et al. 1992). Watanabe et al. (2003) isolated actinomycetes from the termites *Coptotermes formosanus*, *Reticulitermes speratus*, *Neotermes koshunensis*, *Odontotermes formosanus* and *Hodotermopsis japonica*. The strains which were isolated were closely related to *Streptomyces* and able to degrade lignin and carboxymethylcellulose. Pasti and Belli (1985) extracted actinomycetes belonging to the genera *Streptomyces* and *Micromonospora* from the hindgut of the termites *Macrotermes*, *Armitermes*, *Odontotermes* and *Microcerotermes* spp. In the present case, *Streptomyces* sp. strain ABGxAviA1 was only able to produce amylase, although it had been isolated on a medium containing microcrystalline cellulose.

Another strain of *B. subtilis* was extracted from *R. santonensis* (König et al. 2006). Bashir et al. (2013) also isolated cellulolytic strains of *B. subtilis*, *B. cereus*, *B. pumilus* and *B. licheniformis* showing endo-1,4-β-D-glucanase activity from termite guts. Dheeran et al. (2012) isolated a xylanolytic strain of *Paenibacillus macerans* from wood-eating higher termites. In another study, it was possible to identify by DGGE some strains of *Bacillus* able to degrade xylan and carboxymethylcellulose from the gut of the termite *Odontotermes formosanus* (Mathew et al. 2012). It was also possible to identify a symbiosis between *Bacillus* and *Clostridium*. Cellulolytic *Bacilli* were found to create the anaerobic environment necessary for the growth of *Clostridium* (Mathew et al. 2013). However, this is the first time that a strain of *B. subtilis* has been extracted from the gut of a termite and showed xylanase and amylase activities.

Other strains of *Aspergillus fumigatus* were found to produce amylase, cellulase, and xylanase (Wase et al. 1985; Planchot and Colonna 1995). As far as we know, this is however the first time that this strain has been isolated from the gut of this termite. Other filamentous fungi

belonging to the *Aspergillus* genus, such as *A. niger* and *A. oryzae*, have been found to be excellent enzyme producers (Barbesgaard et al. 1992; Schuster et al. 2002). Moreover, Tartar et al. (2009) also identified a xylanase-producing strain of *A. nidulans* in the gut of *R. flavipes* by transcriptomics. This is nevertheless the first time that a strain close to *A. fumigatus* (95 % of identity) has been found inside termite guts. Several species of *Aspergillus* cause the disease of aspergillosis. *A. fumigatus* is considered as the most common etiological agent in that disease (Barbesgaard et al. 1992).

This article provides a new method of isolation of microorganisms focusing on a specific group of strains living in the termite gut. Although it was used for termites, such a method can apply to other sources. The main problem encountered was the difficulty in simulating the original conditions of the termite gut. This is particularly complex because it is a culture medium the composition and the microflora of which depend on location. It can be considered as a natural continuous-flow fermentor divided into organized sections. Therefore, termites offer huge prospects as regards the isolation of enzyme-producing microorganisms.

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