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DEVELOPMENT OF MICROORGANISM SAMPLING METHODS ON BIO-BASED EARTH PRODUCTS INTENDED FOR HEALTHY, SUSTAINABLE BUILDINGS

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Abstract
The impact of buildings on the environment and on the health of the inhabitants are priority issues nowadays. For many reasons, the demand for building products made of materials such as earth and bio-based materials is increasing. Under certain conditions, mold growth can be observed on the surface of such materials, which raises many questions about their use in buildings. In the framework of the "BIOTERRA" ANR project, the aim of the study was to characterize an earth based material incorporating natural fibers from a biotic point of view. Microorganism sampling methods intended for raw materials and cylindrical specimens were optimized, and the microflora profile of these materials was then obtained. The role of the dry step concerning the presence of molds was also studied. The results showed that raw materials and manufactured specimens contained mainly *Bacillus* sp., *Aspergillus* sp. and *Penicillium* sp. Comparisons among samples taken on the surface or the inside of the dried specimens revealed the survival of some molds despite thermal treatment. Those molds were then able to grow on specimens in high humidity conditions. Sampling methods developed here could also be used to identify the microflora of existing earthen buildings.

Keywords: Bio-based building material, Sick Building Syndrome, mold, sampling methods, earth

1 INTRODUCTION

Recent years have seen renewed interest in ecological houses in industrialized countries, and the impacts of building materials on the health of their inhabitants and on the environment have become priority issues. Some old building materials, such as earth, are being examined from this point of view. Scientific research on earth construction has been expanding significantly for about thirty years. Nevertheless, there are very few publications focusing specifically on unfired earth, although this material is widely used around the world. Nowadays, more than two-thirds of the world's population still live in unfired earth houses [Minke 2000]. This building technique was used in France for centuries, and a large heritage of rammed earth building methods (mud-bricks, cob, etc.) exists in different regions. Earth building has several advantages, such as improving comfort in the house, providing good thermal insulation [Binici et al. 2007] and offering natural regulation of the humidity of indoor air [Pacheco-Torgal and Jalali 2012]. Earth can also be transformed into bio-based materials with the addition of aggregates or fibers of plant matter (straw, flax, hemp, etc.), in order to enhance the thermal insulation and lighten the material. In addition, these materials are low cost and have very low environmental impact.

However, microbial proliferation can sometimes be observed on these materials [Gomes et al. 2012], as in other common building materials. Under certain conditions, such as high and uncontrolled humidity (minimal water activity between 60% and 90%) and a temperature between 10°C and 35°C [Nielsen et al. 2004], molds may grow and form visible mycelia on building walls [Cooley et al. 1998; Lappalainen et al. 2001; Straus et al. 2003; Straus 2011; Andersen et al. 2011]. When molds are visible to the naked eye, development of the mycelium is already very advanced, which can imply health risks. Molds and bacteria may then cause poor indoor air quality, which is one of the most important issues in building. The pollution of indoor air is named Sick Building Syndrome (SBS), and may cause health problems for inhabitants [Stenberg 2011].

The main microorganisms involved in SBS are molds. Fungal development can cause production of allergens, mycotoxins or volatile organic compounds (VOC), and also fungal infections and diseases [Nielsen et al. 1998; Norbäck et al. 2000; Fog Nielsen 2003; Green et al. 2006; Reboux et al. 2010;

Verdier et al. 2014]. Genera involved in health problems are mainly *Aspergillus*, *Cladosporium*, *Penicillium*, *Stachybotrys*, *Ulocladium*, and *Chaetomium* [Andersson et al. 1997; Murtoniemi et al. 2003; Andersen et al. 2011]. Bacterial involvement in these problems is less common or less well known, and there are few studies discussing the problem. The main bacteria identified on wet areas inside buildings are Gram positive bacteria [Rintala et al. 2008], such as *Streptomyces*, and also mycobacteria [Rintala et al. 2002; Rintala et al. 2004; Torvinen et al. 2006]. Adverse effects observed are similar to those of fungi, and include mycobacteria parietal compound in the ambient air, or the production of toxins by *Streptomyces*, which may cause inflammatory reactions [Jussila et al. 2002; Huttunen et al. 2003]. Molds on building materials may be initially present in raw materials, or brought in during the fabrication process or by the outdoor air. Potential origins of microbial contaminations are many and varied, and a large diversity of microorganisms may be encountered.

The ANR collaborative project “BIOTERRA” aims to identify, characterize and provide solutions to microbial growth on earthen bio-based products (bricks and coatings) used in the construction and renovation of healthy, sustainable buildings. This project will also aim to develop and validate innovative methodologies for the identification of microbial strains and the study of their growth on building products. With this in mind, a preliminary study was carried out in order to characterize an earth based material incorporating natural fibers from a biotic point of view. Microorganism sampling methods intended for raw materials and building products were set up and optimized. The presence of microorganisms was also observed during some important steps of the fabrication process. Finally, microbial isolates were characterized, and a first microbial profile of these materials was obtained.

2 MATERIAL AND METHODS

2.1 Material

Quarry Fines from Washing Aggregate Sludge (FWAS) were used for this investigation. These fines have a high proportion of limestone (around 60%) and only around 20% of clay. Before being used, they were stored in plastic bags at room temperature. Barley straw was also tested in the earth matrix. The length was between 10 and 30 mm. Straw was also stored in plastic bags at room temperature.

2.2 Manufacturing

Two different mixtures were prepared for the various tests: (i) specimens made of FWAS only, marked FWAS-S0% and (ii) specimens made of FWAS containing 3% of straw (weight content) marked FWAS-S3%. The water content of the mixtures, determined by the Proctor test, was around 14% for FWAS-S0% and 19% for FWAS-S3%. To manufacture the specimens, earth and straw fractions were poured into a blender and mixed by hand. Then, water was added and the materials were mixed mechanically in the blender until a homogeneous mix was obtained. The raw materials were mixed the day before pouring.

Cylindrical specimens 5 cm in diameter and 5 cm high (Φ5H5) (Fig. 1) intended for biotic tests were manufactured by doubled static compression at the Proctor density.

The specimens were first dried at 40°C for 24 hours, then the temperature was increased by 0.1°C/min up to 100°C and stayed at 100°C until the weight became constant (weight variation less than 0.1%). The specimens were then transferred from the manufacturing room to the microbiological laboratory under sterile conditions, and stored at room temperature.

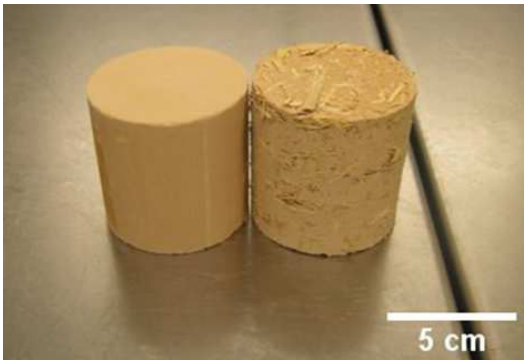

Fig. 1: Cylindrical specimens FWAS-S0% and FWAS-S3%.

2.3 Microbial sampling and characterization methods

In order to sample the microorganisms contained in raw materials, the materials were suspended in aqueous sterile saline solutions and the influence of some key parameters (shaking time, addition of detergent, etc.) was evaluated. Sampling was more difficult on manufactured specimens because microorganisms were included in the matrix. A specific method, using adhesive sterile tape, had to be set up and optimized. All microbial assays were conducted under controlled conditions. Each assay was performed in triplicate in 2 independent tests.

Sampling and quantification on raw materials

Several techniques were used on the raw materials in order to optimize microorganism sampling methods. Each material (FWAS: 1g; straw: 0.25g) was mixed with 10 mL of sterile Phosphate Buffer Saline (PBS) at room temperature. Sterile detergent (Tween80) was added to make the sampling of conidia easier. Different final detergent concentrations (1%, 5% and 10%) were tested. Suspensions were shaken at 300 rpm for 10 minutes. Shaking time was also extended to 30 minutes with a final detergent concentration of 5%. Three masses of FWAS in different volumes of buffer (1g/10mL, 5g/20mL and 25g/100mL) were also tested with 30 minutes’ shaking time. After homogenization by vortex, a range of dilutions of suspension were prepared in sterile distilled water. The suspensions and dilutions were deposited on various nutrient media, which were incubated at different temperatures: Tryptone Soy Agar (TSA) medium was incubated for 2 days at 32.5°C to enumerate aerobic and aero-anaerobic bacteria; Potato Dextrose Agar (PDA) with 0.05 mg/mL of chloramphenicol (Cm) was incubated for 5 days at 22°C to enumerate fungi. After incubation, the colonies formed were counted (CFU: Colony Forming units). Another method to sample microorganisms on

straw was tested, using a Smasher™ blender (AES Laboratories). Straw (2.5 g) was placed in a sterile bag with a membrane inside to separate solid particles from liquid after blending. Then, 100 mL of PBS with detergent was added at room temperature. Two final concentrations of detergent (1% and 5%) were tested. The bag contents were blended for 2 minutes. Blending time was extended to 5 minutes with a 5% final concentration of detergent. A range of dilutions was used and CFU were enumerated as described above.

Evaluation of release of microorganisms from adhesive dressing

An adhesive dressing was artificially contaminated in order to estimate the release of microorganisms from it. Adhesive sterile dressings (Hydrofilm®) were cut into pieces (about 4 cm x 4 cm). One milliliter of a suspension of *A. brasiliensis* (*niger*) (ATCC 16404 / CBS 733.88) spores (10^7 CFU/mL) was deposited on each piece of adhesive dressing. Then the pieces were put under a laminar flow in a Biosafety Cabinet (BSC) until the water had completely evaporated. Adhesive dressings were put into a tube and 10 mL of PBS with a 5% final concentration of detergent was added. They were agitated by a vortex for 5 or 10 minutes. The suspension and a range of dilutions were deposited on nutrient medium (PDA with 0.05 mg/mL of Cm) and were incubated at 22°C for 5 days. After incubation, the colonies formed were counted.

Sampling and quantification on manufactured specimens

Sampling with pieces of adhesive dressing was carried out on Φ5H5 FWAS-S0% specimens and FWAS-S3% specimens at 2 different times of the manufacturing process: before the drying stage, directly after the compression step, and after the drying stage, when specimens were taken out the thermal chambers. Adhesive dressings were pressed on to manufactured specimens for 5 minutes. Then, the same protocol as described in section 2.3.2 (with 5 minutes' agitation and without the evaporation step) was used to put the sampled microorganisms in suspension. The range of dilutions and the enumeration were as described in section 2.3.1.

Two dried cylindrical specimens of each composition were broken horizontally using a 100 kN capacity hydraulic press. Samples were taken with pieces of adhesive dressing on the surface and inside the broken specimens. Dressings were deposited on PDA-Cm medium and incubated for 7 days at 22°C to observe fungi.

Simulation of mold growth on manufactured specimens

Under controlled conditions, a sterile clay brick was placed in a sterile bowl. A piece of sponge covered the clay brick and three Φ5H5 dried specimens of the same composition were placed on the sponge. The bowl was filled with water to the brick height, which enabled humidification of the specimens by capillarity without dissolving them (Fig. 2). Then, the bowl was incubated under controlled conditions at room temperature for two months. One bowl was prepared for each of the two different compositions. Specimens were observed during the incubation time.

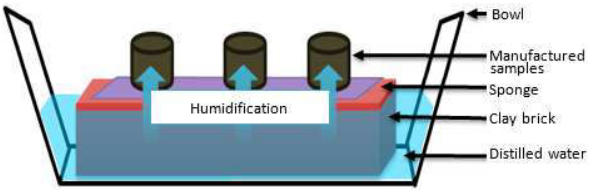

Fig. 2: Schematic diagram of the humidification system.

Characterization of microorganisms

Some aspects (color, size, relief) of the colonies and mycelia were first observed on the isolation medium. Bacterial isolates were Gram stained and mold isolates were stained with cotton blue. Then, the aspect of cells and hyphae was observed by optical microscopy (X400 to X1000) so that bacilli/cocci Gram+/Gram- could be distinguished for the bacteria, and molds could be identified at the genus level.

Statistic tools

Averages and standard deviations were calculated for each condition. A Student test was performed to compare means. The tests were carried out on R software. A p-value below the threshold for statistical significance (0.05) is shown by an asterisk above the means concerned in figure 3 to 6.

3 RESULTS

Preliminary assays showed that the microflora of the raw materials was mainly composed of bacterial spores and fungal conidia. Therefore only these two types of microorganisms were considered in the following assays.

3.1 Sampling on raw materials

Various parameters were assayed in order to enhance microorganism sampling on FWAS (Fig. 3) or straw (Fig. 4). For FWAS, an increase of the shaking time (Fig. 3.A) did not improve the recovery of bacterial and fungal spores. FWAS was easily suspended in the buffer by a simple vortex and FWAS particles in suspension could be directly deposited on a medium or diluted. Similarly, the detergent concentration (Fig. 3.B) did not have any effect on the recovery of bacterial spores. However, the use of detergent at 5% final concentration doubled the recovery of fungal conidia. Finally, an increase in the FWAS / volume of buffer had no effect on the recovery of the bacterial and fungal spores (data not shown). Therefore, the conditions chosen were a shaking time of 30 minutes, a final detergent concentration of 5% and a ratio of mass/buffer volume of 1g/10mL.

Regarding the straw, a 30-minute shaking time (Fig. 4.A) significantly improved (by about 1 log10) the recovery of both bacterial spores and mold conidia. Straw was rougher than FWAS and microorganisms could be blocked on it, so were not easily suspended. As observed previously, the use of detergent (Figure 4.B) had no significant effect on the sampling of bacterial spores. However, in the case of molds, an addition of detergent significantly enhanced the recovery of conidia by about 1 log10, even with a final concentration of detergent of 1%. The use of a surfactant enabled better suspension of conidia, thanks to lipophilic interaction with conidia membrane and hydrophilic interaction with PBS [Barnes and Gentle 2011].

Figure 4.C presents the results of the sampling when a Smasher™ was used. The detergent still did not have any significant effect on the recovery of bacterial spores but it increased the recovery of fungal conidia as observed with the previous method. In addition, a 5-minute blending time (instead of 2 minutes) did not have any effect on the recovery of microorganisms. Compared to the shaking method, the blending method enhanced the recovery of bacterial spores by a factor of 4 but no difference occurred for fungal samples. The use of the Smasher™ for straw was therefore advantageous in comparison to a shaking step, with a better recovery of bacterial spores and a shorter processing time.

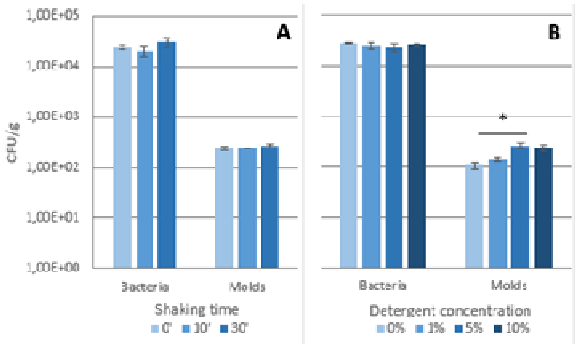

Fig. 3: Colony-forming units (average ± standard deviation; 2 independent assays in triplicate) sampled per gram of FWAS according to shaking time (A) or detergent concentration (B).

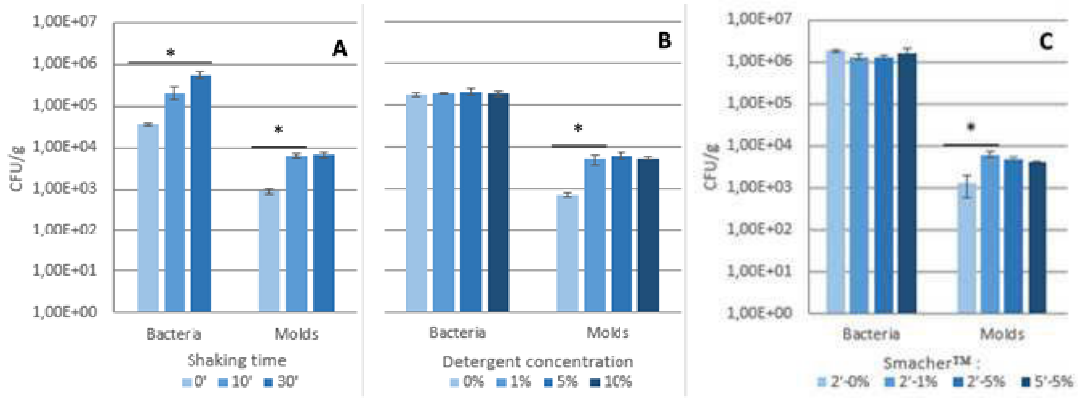

Fig. 4: Colony-forming units (average ± standard deviation; 2 independent assays in triplicate) sampled per gram of straw according to shaking time (A), detergent concentration (B) and Smasher™ blending (C).

3.2 Evaluation of the release of microorganisms from the adhesive dressing

To determine the influence of the vortexing time on the release of microorganisms from the adhesive dressing, fixed conidia of A. brasiliensis were vortexed for 5 or 10 min. The results are presented in Figure 5. When only homogenization by vortex was used, as few as 3.65×10^5 conidia were recovered even though 8.7×10^6 conidia were deposited. With a longer vortexing time (5 or 10 minutes), recovery of conidia increased significantly by around 1 log10, and 2.8×10^6 conidia were recovered. No significant difference was observed between 5 or 10 minutes of vortexing, so a 5 minute vortexing time seemed enough to release fixed conidia, although almost 1 log10 conidia were not recovered.

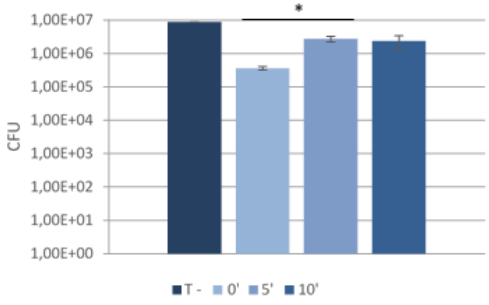

Fig. 5: Colony-forming units (average ± standard deviation; 2 independent assays in triplicate) of A. brazilensis *recovered according to vortexing time.*

3.3 Sampling on manufactured specimens

Figure 6 presents CFU enumerated after sampling on cylindrical FWAS-S0% or FWAS-S3% specimen surfaces before or after the drying stage. Undried FWAS-S3% specimens contained 1 log10 more bacteria and fungi than undried FWAS-S0% specimens. Although FWAS-S3% specimens contained only 3% of straw, the addition of plant fibers led to detection of a significant quantity of microorganisms on the surface of the manufactured specimens. Molds could not be detected with FWAS-S0% specimens. The initial concentration of fungi in raw materials (2×10^2 CFU/mg) was too low to be determined with this sampling method. Dried specimens contained 3 log10 less bacteria than undried specimens. Same difference of counted bacteria between FWAS-S0% and FWAS-S3% specimens was observed after the drying stage. No enumeration was presented for fungi on dried specimens because too few isolates were obtained.

In order to confirm the absence of molds in dried manufactured specimens, sampling was carried out on the surface and inside the cylindrical specimens (Fig. 7). Very few molds were isolated by surface sampling: less than one per dressing, and only on FWAS-S3% specimens. But in samples taken inside the Φ5H5 specimens, various molds were observed. These results suggested that some microorganisms survived the drying step, especially at the heart of the manufactured specimens.

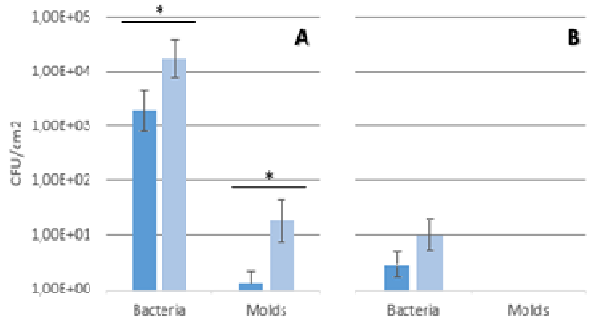

Fig. 6: Colony-forming units (average ± standard deviation; 2 independent assays in triplicate) of microorganisms recovered by adhesive dressing sampling on undried (A) and dried (B) specimens.

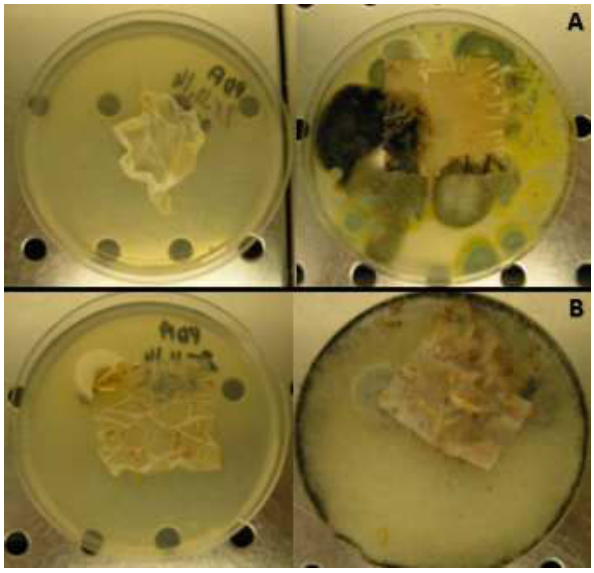

Fig. 7: Samples obtained with adhesive dressing on the surface and inside FWAS-S0% (A) and FWAS-S3% (B) specimens, after 7 days of incubation at 22°C.

3.4 Simulation of mold growth on manufactured specimens

Cylindrical dried specimens were observed for two months in (i) humid conditions and (ii) dry conditions. No mold was observed on specimens in the dry condition. At one month with high humidity, a few white molds appeared for both compositions and grew slowly. At 6 weeks, a white mold was more developed on one FWAS-S0% specimen (Fig. 8.A). For FWAS-S3% specimens (Fig. 8.B), one specimen presented a black mold spread on its surface. The growth of molds on dried specimens confirmed the observations of section 3.3, i.e. the presence of molds in the heart of dried specimens. However, no other noteworthy growth was observed on the other specimens of the triplicate. This suggested that, even if a few molds survived the thermal treatment in some specimens, this resistance to heat was random, and the majority of specimens did not contain enough fungi to form appreciable mycelia on the surface under assay conditions.

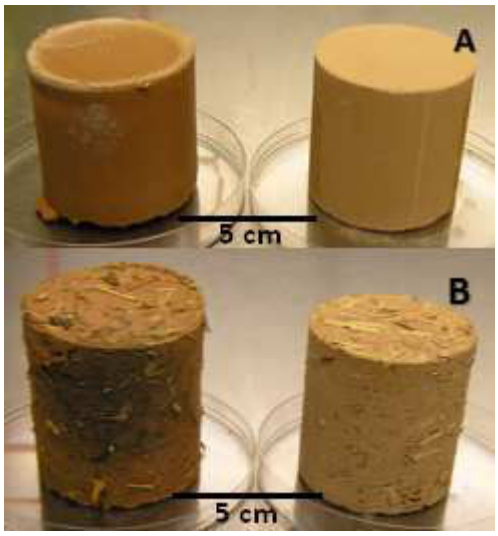

Fig. 8: Observations of FWAS-S0% specimens (A) or FWAS-S3% specimens (B) after incubation at room temperature for two months in humid conditions and dry conditions.

3.5 Characterization of isolates

The isolates obtained during assays were characterized by macroscopic and microscopic phenotypes. On FWAS, most of the bacterial isolates (more than 85%) were Gram+ sporulated bacilli able to grow in aerobic conditions and so considered as *Bacillus* sp. Fungal genera observed were mainly *Penicillium* (around 40%), *Aspergillus* (35%), *Cladosporium* (10%) and more rarely, *Rhizopus* (2%) and *Ulocladium* (2%) (Fig. 9). As for FWAS, bacterial isolates from straw were mainly Gram+ sporulated bacilli (more than 85%), and mold isolates mainly belonged to *Aspergillus* (around 95%) genera, with some *Penicillium* (2%) and *Rhizopus* (2%) isolated. The bacterial and fungal phenotypes observed on straw showed only half the diversity of those obtained on FWAS. Before packaging in plastic bags, FWAS specimens were stored outdoors, which implied potential contamination by the ambient air or by rain. Moreover, FWAS could offer a more varied medium for the growth of microorganisms.

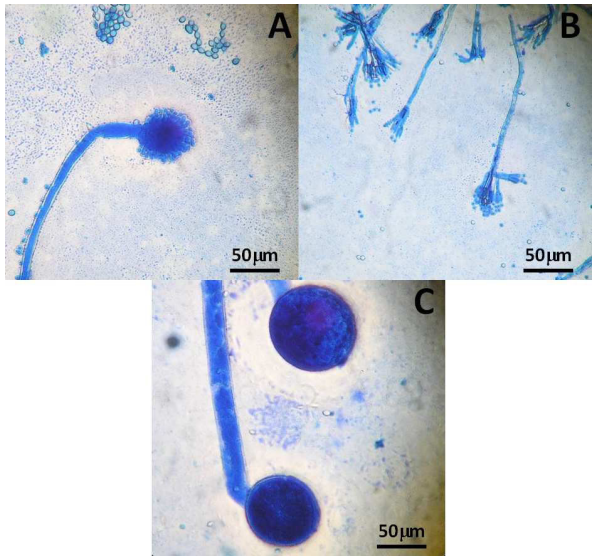

Fig. 9: Observations by optical microscopy of Aspergillus *sp. (A),* Pencillium *sp. (B) and* Rhizopus *sp. (C) isolates after staining with cotton blue.*

Finally, isolates obtained on cylindrical specimens were compared with those obtained on raw materials. Although few mycelia were sampled, they were the same as those observed on raw materials.

4 CONCLUSION

The optimization of techniques to sample microorganisms showed that a longer shaking or blending time increased the recovery of microorganisms on straw, and the use of a blender improved the sampling. An addition of detergent also appeared to be very important to the recovery of fungal conidia. As expected, most fungal isolates on raw materials belonged to the *Aspergillus* and *Pencillium* genera, molds which are common in the environment. Most bacterial isolates were *Bacillus* sp, but isolation of anaerobic bacteria will be carried out in further work to extend the types of microorganism explored. First sampling using adhesive dressing did not recover enough conidia to ensure the characterization of the microflora of manufactured specimens. Despite this limit of quantification, this technique was a non-destructive sampling method, which would enable direct sampling in houses or constructions. The purpose of this method was to detect microorganisms at a contamination level with visible molds on the material. The thermal treatment used to manufacture specimens did not remove all the microorganisms in the heart of the specimen, especially molds. Even though few specimens were concerned, the remaining molds could grow in conditions of high humidity. Raw materials contained initial harmful microorganisms, which were not totally inactivated during the manufacturing process. A higher drying temperature could be tested in order to “sterilize” materials efficiently.

On the basis of these sampling methods, the next steps of this work will be the identification and characterization of microbial diversity and proliferation on earth constructions and bio-based earthen products. Specimens will be collected *in situ*, on existing earth buildings. Then, microflora profiles will be obtained by using microbial isolations and genomic approaches (high-throughput DNA sequencing). The proliferation and adhesion of mycelia and biofilms on bio-based manufactured materials will also be studied to determine the environmental conditions favorable to their growth (temperature, relative humidity).

5 ACKNOWLEDGMENT

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