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ISOLATION AND IDENTIFICATION OF FUNGI GROWING ON FIBRE HEMP SHIVE BASED THERMAL INSULATION MATERIALS

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Abstract. Green thermal insulation materials are ecological materials with hemp, linen, jute, wood waste, maize starch, and other types of waste added to polymer-based composites. Such kind of materials are susceptible to the microbial action which can lead to changes of physico-chemical properties of materials, their destruction and also health problems in humans. Here, we isolated and identified several fungal strains that grow on fibre hemp shive based materials. Three isolates were identified as belonging to *Trichoderma* (*Hypocrea*) genus. Our further experiments will be concentrated on the monitoring of growth of newly isolated fungi on and/or inside the materials of interest, isolation and characterisation of the hydrolytic enzymes as well as following the mode of material destruction caused by the fungal growth.

Keywords: hemp fiber shives, thermal insulation materials, biodegradation, fungi, *Trichoderma.*

Intruduction

The construction sector currently uses over 1/3rd of energy available on the market and is largely responsible (ca. 50%) for emissions of CO_2 and other gases into the atmosphere and for generation of waste which poses difficulties in terms of degradation (Medineckienė, Turskis, & Zavadskas, 2010). The production of traditional insulating materials uses large amounts of energy – 7–20 GJ is used in order to produce 1 ton of the mineral wool, resulting in emission of 0.57–0.74 tons of CO_2 to the atmosphere (Ecofys, 2009). The last decade saw rapid development of so called “green construction materials”, the alternative production technologies that contain plant waste from various industries. Production of such materials lowers the emission levels of gases and waste, and also consumption of energy and natural resources thereby significantly decreasing the production costs (Aciu & Colbirzan, 2013). Green materials containing waste are environmental-friendly products as they biodegrade easily compared to the materials produced using the traditional methods, which decreases the costs of their recycling.

Green thermal insulation materials are a type of materials containing hemp, linen, jute, wood waste, maize starch, and other types of waste are added to polymer-based composites (Gonzales–Garcia, Luo, Moreira, Feijoo, & Huppes, 2012; Väisänen, Batello, Lappalainen, & Tomppo, 2018; Balčiūnas, Vėjelis, Lekūnaitė, & Kremensas, 2016). In addition to good insulation and resistance parameters, such materials are also characterised by good acoustic parameters (Kidalova, Stevulova, Terpakova, & Sicakova, 2012). The use of resourses of plant origin allows the production of environmental-friendly materials, but biodestructive factors emerge during exploitation thereof. Due to migration of moisture at the partition walls of the building, the temperature of the dew point and the hygroscopicity of materials of plant origin in the thermal insulation layer of the partition wall, the moisture may accumulate leading to favorable conditions for the growth of microorganisms. Such conditions may result in change of the physical properties of the thermal insulation materials during the exploitation period.

Microorganisms are characterised by a wide range of tolerance to moisture in the environment. Most of them require a substrate moisture level corresponding to water activity of aw = 0.60–0.99 in order to grow actively. The

ability of moulds to germinate and grow was proven under modelled conditions on leather, wood, textile fibres at aw = 0.7–0.8 (Nielsen, K. F., Holm, Uttrup, & Nielsen, P. A., 2004). Xerophilic mould species, e.g. of *Aspergillus* and *Penicillium* genera grow at moisture levels of aw = 0.60, whereas the species prefering high levels of humidity, e.g. *Stachybotrys* grow only at aw = 0.99 (Dedesko & Siegel, 2015). Moulds are divided into three groups based on their need for adequate moisture content in construction materials: primary (optimal aw = 0.8), secondary (aw = 0.8–0.9) and tertiary colonizing species (aw > 0.9) (Grant, Hunter, Flannigan, & Bravery, 1989). It is necessary to maintain an adequate level of moisture on the surface of construction materials only in the initial phase of growth. When moulds form the mycelium, the moisture is retained in its structure and thus prevents further mould growth and metabolic activity.

The problem of mould attacks on construction materials is widely described in the literature; it is estimated that atmospheric air and deposits of dust are the main sources of moulds (Korpi, Pasanen, Pasanen, & Kalliokoski, 1997). Incorrectly stored construction materials without protection against weather factors may be attacked by moulds easily. It was shown that moulds may use dust deposited in higher humidity as food for their growth (Korpi et al., 1997). Under such conditions, moulds are able to metabolize actively, producing numerous volatile compounds and mycotoxins (Pasanen, A., Kujanpää, Pasanen, P., Kalliokoski, & Blomquist, 1997; Gutarowska & Czyżowska, 2009.) Water adsorption and deposition of dust and microorganisms cause changes of material surface parameters, followed by successive growth of subsequent microbial populations. This results in the development of microorganisms and biodeterioration of the material.

It has been determined that synthesis of specific extracellular enzymes and organic acids by moulds is stimulated by the composition of the construction material. Moulds change the composition of the enzymes in construction environment, making it possible for them to use the available substrates (Beech, Otlewska, Skora, Gutarowska, & Gaylarde, 2016). In such environment, their metabolism is directed towards formation of acids, such as oxalic, citric, gluconic and succinic (Gutarowska & Czyżowska, 2009). Due to the metabolic activity of moulds, the materials change their colour, and also loose the resistance, brittleness and other original properties. Moulds growing on construction materials not only destroy the construction materials, they also create health hazards. The predominant diseases include mycoses, allergies, mycotoxicoses, SBS (sick building syndrome), lung hemosiderosis ((Marinicioni & Altamirano-Medin, 2017). Growth of moulds on surfaces of synthetic polymers is possible at high moisture content in the material and with available carbon source. Synthetic materials used as thermal insulating materials (polyurethane foam, styrofoam, mineral wool) are also characterised (in addition to low water sorption) by expanded specific surface area, cellular structure (styrofoam) or fibrous structure (mineral wool), very coarse surface, potentially supporting mould growth. Possible growth of species such as *Paeciliomyces variotii*, *Trichoderma harzianum*, *Penicillium sp.* on polyurethane foams has already been described in the literature (Webb et al., 2000). However, the isolation and characterisation of the microorganisms (moulds) that grow on the green thermal insulation materials has not been previously performed. In this work we used such materials overgrown with microorganisms in order to isolate pure cultures.

Materials and Methods

Green thermal insulation materials

The following materials were used in order to isolate fungi that grow on the materials of interest: fibre hemp shives (hereafter – FHS); FHS with the acrylate resin binder Acrodur 9501; FHS bonded with naturally present lignin; FHS with the corn starch binder.

Isolation of fungal strains

To isolate fungal strains, FHS panels (2.5×2.5×1 cm) were spread with 5 ml of sterile water and left at room temperature (22 °C) for one week. The moulds from these panels were inoculated by microbiological loops on the Petri dishes with solid PDA media (Visagie et al., 2014) and grown at 28 °C for several days.

The GTM panels of interest (20 g) were placed in 50 mL of sterile water for 2 hours with shaking at 120 rpm. Outwashes were centrifuged at 12000 g for 20 min, and precipitates were stored at +4 °C until subsequent analysis.

DNA extraction, amplification and identification

Total DNA from pure cultures was isolated using a Genomic DNA purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) in accordance with the manufacturer's instructions. The quality of extracted DNA was

determined using 1% agarose gelectrophoresis. In order to identify the isolated fungi, internal transcribed spacers (ITS) of rDNA were amplified using the fungi specific primers ITS1 forward (TCCGTAGGTGAACCTGCGG) and ITS4 reverse (TCCTCCGCTTATTGATATGC) (Visagie et al., 2014). The PCR was performed in a total reaction of 50 µL, consisting of the kit DreamTaq green PCR Master Mix (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) and 1 µL of DNA template (5 ng), according to the manufacturer's instructions. PCR amplification was carried out by Bio-rad thermocycler under the following conditions: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min 30 s and 72 °C for 2 min. The final extension was carried out at 72 °C for 10 min. The PCR products were purified using a GeneJet PCR purification kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) and then transformed to pJet 1.2 plasmid whith the kit CloneJET PCR Cloning Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) according to the manufacturer's instructions. Then plasmid digested with XbaI and BglII enzymes and 1% agarose gelectrophoresis was run. The ITS inserts were sequenced using pJet1.2 forward and/or reverse primers at BaseClear (Leiden, Netherlands). The obtained sequences were compared with those found at the National Center for Biotechnology Information (NCBI) usig the BLAST sequence analysis tool (Altschul et al., 1997).

Results and discussion

In this work we attempted to isolate and identify the fungal species capable of growth on green thermal isolation materials. As the starting material, fibre hemp shives samples with or without binding material, and with visible microbial activity, were used (Figure 1).

Using the PDA growth medium, several morphologically different strains were isolated (Figure 2). The isolates were stored at –80 °C with 15% glycerol. In order to establish the identity of isolated fungi, we have isolated genomic DNA from each strain, performed PCR (Figure 3), and cloned the obtained DNA fragments into pJet 1.2 plasmid. The restriction analysis using XbaI and BglII restriction enzymes confirmed the presence of the desired insert (Figure 4). Subsequently, the ITS of rDNA regions 1 and 2 as well as 5.8S rDNA were sequenced, and the results obtained were compared with known ITS sequences in NCBI GenBank. Our results revealed the 99% identity with *Hypocrea* genus of fungi, represented in Figure 2 A and Figure 2 C. In addition, the comparison of

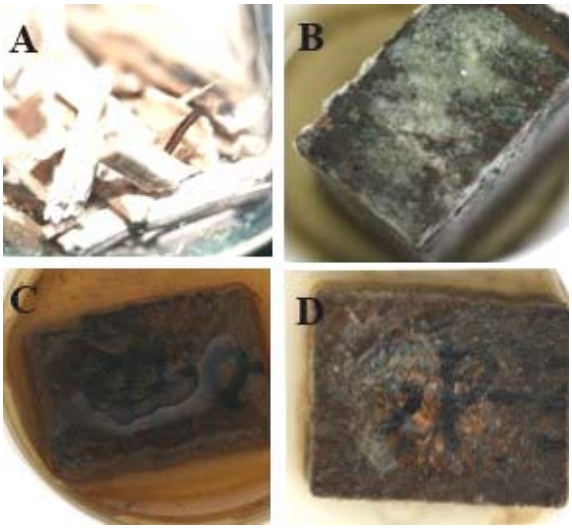

Figure 1. Thermal isolation materials used for the isolation of fungi. a) FHS; b) FHS with the acrylic resin binder resins; c) FHS are bonded with naturally contained lignin; d) FHS SP–3-10 with the corn starch binder

ITS sequences revealed 99% identity with *Trichoderma* genus of the fungi represented in Figure 2 B, as well as 84% identity with the *Mucor* genus of the fungi represented in Figure 2 D.

Teleomorphs (the sexual reproductive stage) of *Trichoderma* are considered as species of the ascomycete genus *Hypocrea*. Recent changes in fungi nomenclature proposed to keep the name *Trichoderma* for both species (Rossman, Seifert, & Samuels, 2013). This genus is well known fungi growing on the rotten wood. *Trichoderma fungi* produce several types of hydrolytic enzymes, cellulases, xylanases, and β-glucosidases (Horta et al., 2018), contributing to the decay process. Our further studies will be focused on the isolation and characterization of such enzymes isolated from the above described cultures of fungi. In addition, the growth of fungi will be monitored at different temperatures and relative humidity conditions using the climatic camera. The growth of fungi on the surface and/or inside the thermal insulation materials will be monitored using the scanning electron microscopy (SEM). It is important to understand the action of fungi towards the thermal insulation materials in order to protect them from biodeterioration during their use for the insulation of buildings as well as to biodegrade them after use. A recent remarkable discovery in this field is the identification of bacterium *Ideonella sakaiensis*, capable of degradation of polyethylene terephthalate (PET) (Yoshida et al., 2016). We hope that our research will also contribute both to creation of new materials for the construction industry and their environmentally friendly biodegradation after the expiration of shelf-life.

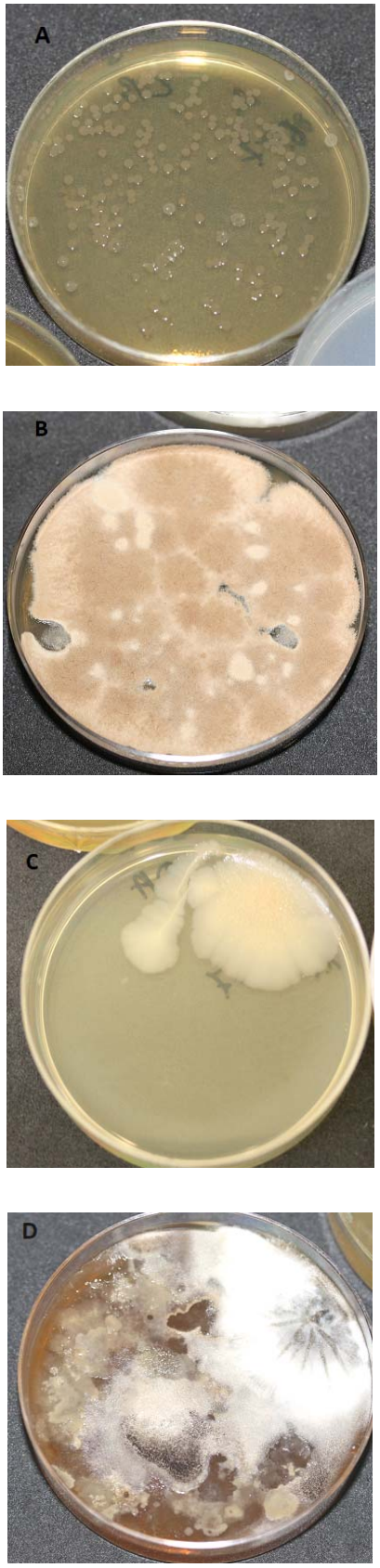

Figure 2. Morphology of some fungi isolated using green thermal insulation materials. A to D corresponds to the strains isolated from FHS with different binding materials

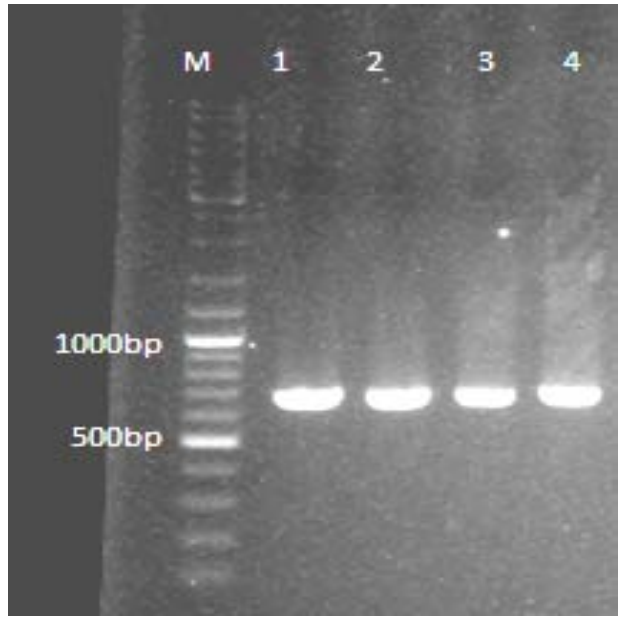

Figure 3. PCR products of approximately 550 to 600 bp of ITS region. M- MW marker; 1 to 4 – PCR species corresponding to the fungi shown in Figure 2

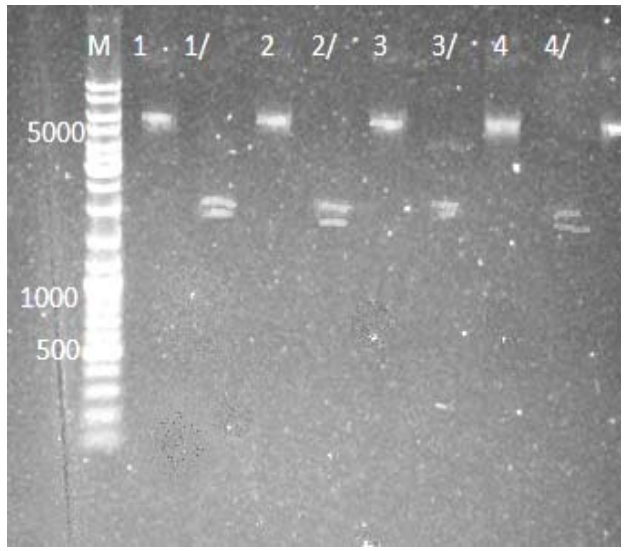

Figure 4. The restriction analysis of pJet 1.2 plasmids with the cloned respective PCR products, corresponding to the fungi shown in Figure 2. M- MW marker, 1 to 4 – undigested plasmids; 1/ to 4/ – plasmids digested with both XbaI and BglII restriction enzymes. The restriction profile corresponds to the expected one with the presence of the insert

Conclusions

1. Several fungal strains that are capable of growth on green thermal insulation materials made of fibrous hemp shives were isolated.
2. The identity of fungi was determined by the PCR and sequencing of internal transcribed spacers (ITS).
3. Three of the isolates belong to well known wood destroying *Trichoderma* (*Hypocrea*) genus.

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MIKROORGANIZMŲ, AUGANČIŲ ANT TERMOIZOLIACINIŲ MEDŽIAGŲ, KURIŲ PAGRINDAS YRA PLUOŠTINIŲ KANAPIŲ SPALIAI, PAIEŠKA

D. Vasiliauskienė, G. Balčiūnas, J. Urbonavičius

Santrauka

Gamtinės kilmės termoizoliacinės medžiagos yra nekenksmingos aplinkai, todėl jas siekiama kuo plačiau naudoti. Iš tokių medžiagų, kaip pluoštinės kanapės, linai, džiutas, medienos atliekos, kukurūzų krakmolas, kartu su polimerais formuojami kompozitai. Tokias medžiagas veikia aplinkos mikroorganizmai, o tai gali lemti šių medžiagų struktūros pokyčius, jų degradaciją bei gali turėti įtakos žmonių sveikatai. Mes išskyrėme ir identifikavome keletą grybelių kamienų, kurie auga ant medžiagų, kurių pagrindas yra pluoštinių kanapių spaliai. Buvo nustatyta, kad trys iš išskirtųjų kamienų priklauso *Trichoderma (Hypocrea*) genčiai. Mūsų vėlesniais tyrimais bus siekiama toliau tirti išskirtųjų grybelių augimą ant termoizoliacinių medžiagų paviršių bei jų viduje, apibūdinti galimus hidrolitinius fermentus, dalyvaujančius šiuose destrukciniuose procesuose, taip pat tirti medžiagų irimo dėl grybelių augimo procesus.

Reikšminiai žodžiai: kanapių pluoštai, šiluminės izoliacijos medžiagos, biologinis skaidymas, grybai, trichoderma.