IDENTIFICATION OF UNKNOWN FILAMENTOUS FUNGI FROM WILLOW WOOD AND SORGHUM CHIPS

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Abstract

Molecular biological methods are generally applied in the identification processes of microorganisms. We aimed to isolate numerous cellulolytic filamentous fungi strains from willow wood and sorghum chips, and attempted to identify them with polymerase chain reaction (PCR).

Modified Czapek-Dox medium was used with the addition of microcrystalline cellulose and carboxymethyl cellulose (CMC) as a source of carbon, in order to isolate cellulolytic filamentous fungi strains. Through sequence-based identification, representatives of the genera *Trichoderma, Aspergillus* and *Fusarium* were identified.

Introduction

Filamentous fungi create a diverse and often well-described group in the kingdom Fungi, although a high number of unidentified species exists. The classical, morphology-based identification of filamentous fungi often proves to be inadequate due to the morphological similarities among the different species. Therefore, molecular biological methods are usually required along with macro- and micromorphological studies in order to identify these diverse microorganisms [1].

Filamentous fungi have been known as proficient colonizers of lignocellulosic plant tissues and have been recognized as producers of exceptionally rich and diverse hydrolytic enzymes [2], [3]. This characteristic makes them valuable candidates for the biological pre-treatment of substrates before anaerobic digestion (AD). AD is a four-stage process including hydrolysis, acidogenesis, acetogenesis and methanogenesis. In this process, a wide range of organic wastes can be degraded, and a carbon-neutral, renewable energy carrier, biogas is produced [4]. Pre-treatment before AD can enhance not only the biogas yields during AD but the amount of degraded organic materials as well. Physical and chemical pre-treatments can be applied, biological pre-treatments are the most environmentally friendly methods.

Experimental

Willow wood and sorghum chips colonized by filamentous fungi were selected randomly.

Microcrystalline cellulose and carboxymethyl cellulose (Merck, Darmstadt, Germany) were applied respectively as a source of carbon in modified Czapek-Dox medium during isolation (https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium130.pdf), in order to isolate cellulolytic filamentous fungi. The medium contained 100 µg/ml of antibiotics (ampicillin and streptomycin, respectively) to prevent bacterial growth.

Stereomicroscopic images were taken with a Nikon SMZ745T stereo microscope (Fig. 1-4.). After several re-plating on the cellulose-rich media, pure isolates were obtained. Pure cultures

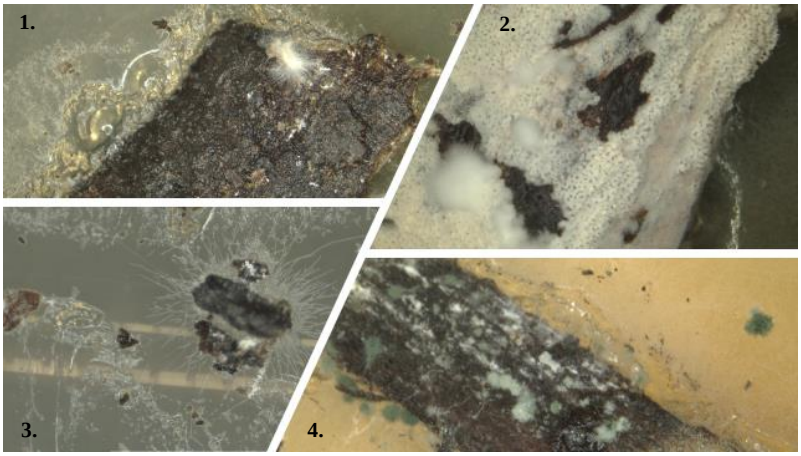

Figure 1-4.

were maintained on Potato Dextrose Agar (Sigma-Aldrich, St. Louis, USA). Plates were incubated at 25°C during both isolation and maintenance.

Micromorphology of the pure isolates was examined using Lactophenol Cotton Blue stain, and images were taken with an Olympus BX53 microscope.

The identification process included various molecular biological methods such as DNA isolation, polymerase chain reaction (PCR), agarose gel electrophoresis, DNA purification, and DNA sequencing.

DNA isolation

Pure filamentous fungi strains were inoculated into Potato Dextrose Broth (Sigma-Aldrich, St. Louis, USA), respectively. After 5 days of incubation at 25°C, DNA samples were prepared with the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, CA, USA).

Polymerase Chain Reaction

Isolated DNA samples were amplified in polymerase chain reactions (PCR). Three different sets of primers were applied in various reactions. In the table below, the circumstances of each PCR step and the primers are described (Table 1.).

Name of the primer	**CMD5/6** [5]	**RPB2** [6]	**FU2/3** [7]
Forward primer sequence	5' - CCGAGTACAAGGAGGCCTTC - 3'	5'- GAYGAYCGKGAYCAYTTCGG-3'	5' - GGTCGCCGTAAGAGGGGTTGG - 3'
Reverse primer sequence	5' - CCGATAGAGGTCATAACGTGG - 3'	5'- CCCATRGCYTGYTTRCCCAT-3'	5' - CGAGCCCGGTACCATGGACG - 3'
1. First denaturation	95 °C - 5 min	95 °C - 3 min	94 °C - 3 min 30s
2. Denaturation	95 °C - 30 s	95 °C - 30 s	94 °C - 1 min
3. Annealing	56 °C - 30 s	56 °C - 30 s	60 °C - 30 s
4. Elongation	72 °C - 30 s	72 °C - 1 min 20 s	72 °C - 2 min
5. Final extension	72 °C - 2 min	72 °C - 3 min	72 °C - 5 min
Number of cycles (step 2-4)	35x	35x	35x

Table 1.

Agarose Gel Electrophoresis

After PCR, the amplicons were separated by their sizes using agarose gel electrophoresis. In this experiment TAE (Tris-Acetate-EDTA) buffer and 1% agarose gels were used, and the agarose gel electrophoresis lasted 35 min at 90V.

DNA extraction

Appropriately sized DNA fragments were cut from the agarose gel and extraction of the amplicons was carried out with the GeneJet Gel Extraction Kit (ThermoFisher Scientific, Waltham, MA, USA), following the manufacturer's protocol.

DNA sequencing

Samples were sequenced at the Molecular Genomics Centre of the Biological Research Centre (Szeged) on a 3500 Series Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

The DNA sequences were filtered and analysed using BLAST against the NCBI database.

Results and discussion

After the series of experiments, six strains of filamentous fungi were identified, belonging to the genera *Trichoderma, Aspergillus* and *Fusarium.*

Two strains proved to be *Trichoderma harzianum*. Identification was based on the segment of the calmodulin gene, which was amplified using the primers CMD5/6. The isolates showed 100.00% and 95.81% sequence similarity to deposited sequences, respectively, and were studied micromorphologically as described above (Fig. 5.).

Figure 5.

Aspergillus clavatus from the genus *Aspergillus* has also been identified. The *A. clavatus* isolate has shown 98.77% sequence similarity to deposited sequences, based on the amplicons of the second largest subunit of the nuclear DNA-dependent RNA polymerase II (with RPB2 primers). Macromorphological characteristics of *A. clavatus* support the result of molecular biological methods (Fig. 6.).

From the environmental samples, *Fusarium solani* and other representatives of genus *Fusarium*

Figure 6.

have been identified with the application of FU2/3 primers, which primers amplify a partial β-tubulin gene fragment. These fungi are known as pathogenic strains, therefore the samples were eliminated from further experiments.

Conclusions

Filamentous fungi have a key role in the deconstruction of the lignocellulosic biomass of various origins due to their remarkable enzyme synthesizing ability. The bioavailability of resistant plant cell wall biopolymers can be considerably improved by fungal pre-treatment, which makes the identification of cellulolytic filamentous fungi crucial for future applications. The identified fungal strains in the present study, belonging in the genera *Trichoderma* and *Aspergillus*, can serve as relevant candidates in enzyme activity assays, and in future experiments including fungal pre-treatment with the aim of enhancing biogas production.

Acknowledgements
This work was supported by the Hungarian National Research, Development and Innovation Fund (grant number NKFI-PD 128345), 2020-112-PIACI-KFI-2020-00117, and the domestic grant GINOP-2.2.1-15-2017-00081.
We owe special thanks to Dr. László Galgóczi and Dr. Sándor Kocsubé.

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