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Original article

Cellulolytic activity of biodeteriogenic *Aspergillus* sp. strains

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Abstract

During history, wooden buildings exposed to various agents, either biotic or abiotic, have suffered damage under the influence of living organisms like fungi. The main strategy for degrading wood polymers- cellulose, hemicellulose and lignin is by secreting enzymes that break down the main constituents through lysis.

This study investigates the enzymatic activity of various *Aspergillus* sp. strains identified on the walls of Boz wooden Church in Hunedoara County, Romania, by analysing the influence of different parameters on the production of the cellulolytic enzymes and confirming the presence of a cellulase coding gene fragment. The research primarily examines the total cellulase activity of three *Aspergillus* species—*Aspergillus niger, Aspergillus nidulans* and *Aspergillus sclerotiorum*—isolated from medieval wooden churches in Transylvania, Romania. Variations of several parameters was analysed, such as the incubation time, the influence of incubation temperature on enzymatic digestion, influence of citrate buffer pH, influence of substrate concentration. Additionally, a phylogenetic analysis has been performed based on similarities between the Internal Transcribed Spacer (ITS) regions for the investigated strains.

Keywords

heritage, *Aspergillus*, cellulase, incubation, sequencing.

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Introduction

Fungi, as natural decomposers, produce a wide array of extracellular enzymes that are essential for bioconversion, metabolic processes, and protection against hazardous compounds. Cellulases, enzymes that degrade cellulose, are divided into endoglucanases, exoglucanases, and cellobiohydrolases, which work synergistically to hydrolyse cellulose. Hemicellulose degradation is facilitated by xylanases [1]. Structurally, fungal cellulases share similarities with bacterial cellulases, consisting of a catalytic domain and a carbohydrate-binding module connected by a linker at the N-terminal region [2].

Many species of the *Aspergillus* genus have multiple industrial and medical impact. Being an economically important genus of filamentous fungi, the need to establish a rigorous taxonomy demands significant concern [3]. Therefore, *Aspergillus* probably has the highest number of sequenced genomes. The exploitation of *Aspergillus* enzymes dates to 1894, when Takamine patented a product derived from *A. oryzae*. Since then, a variety of enzymes, including proteases, amylases, lipases, cellulases, glucosamylases, sulfur hydrolases, pectinases, cellobiose, glucose oxidases, catalases, xylanases, proteases, and tannases, have been identified and utilized [4]. In our study, we have investigated the cellulolytic activities of *Aspergillus* strains belonging to three species*: A niger, A nidulans, A. sclerotiorum.*

A. nidulans is a species frequently found in soil, on cereal seeds, oat, wheat maze, rice, with the teleomorph *Emericella nidulans* [5]. It remains one of the most referential models of filamentous fungi in research fields such as hyphal morphogenesis, intracellular transport, development and fungal growth, secondary metabolism, stress response. On the surface, they form radially symmetric superficial colonies, which expand at a constant rate of about 0.5mm/h [6]. It is a versatile fungal resource that has shown the potential to produce different enzymes such as cellulases, β-glucosidase, xylanase, laccase, lipase, protease, β-galactosidase, tannase, keratinase, chitinase and aryl alcohol oxidase [7].

A. niger is a cosmopolitan species found in soil on decomposing debris, roots, fruit, seeds. The mycelium is septate, hyaline, coloured in light yellow. It develops hyphae in the substrate and in the air; conidiophores are not septate and usually grow directly from the substrate, having the hight of 200-400µm and the diameter 7-10µm. The surface of the conidiophores is smooth and at the base they are usually hyaline and change colour in yellow brown towards the apex [5]. It is thermotolerant, both psychrophilic and thermophilic, in a range of 6º - 47 ºC.

First described in 1933, *A. sclerotiorum* belongs to the section *Circumdati* [8]. The metabolites derived were tested and analysed for their structure, bioactivities and biosynthesis, as well as for their technological applications [9]. It has been reported to produce penicillanic acid, xanthomegnin, viomellein and vioxanthin, but also ochratoxins [9,10]. On Czapek Dox agar it has a light-yellow colour with a colony white towards the edges. In 2016, the genome of *A. sclerotiorum* was sequenced as a part of the *Aspergillus* whole-genome sequencing project and the genome assembly size was 37.97 Mbp. The most common fragments sequenced were beta tubulin (BenA), calmodulin, (CaM) and RNA polymerase II second largest subunit RPB II gen [8]. *Aspergillus* comprises 25% of total airborne fungi in the atmosphere, are common soil fungi and decomposing organic matter, releasing a huge number of spores [11]. They are opportunistic pathogens, responsible for aspergillosis in certain conditions.

The use of *Aspergillus* in biotechnology begun approx. one century ago when James Currie, a food chemist discovered that *A. niger* was able to produce citric acid, a food and beverage additive. *A niger* produces several enzymes used in food and feed production, such as glucoamylases, proteases and phytases [12], α-amylase, amyl glucosidase, glucose-oxidase, glucose dehydrogenase, β-glucosidase, β-glucanase, β-galactosidase, lipase, pectinase, metalloproteinase and glycerol oxidase [13]. In green chemistry, filamentous fungi are regarded as robust microorganisms for the biotransformation of natural products, but nonetheless, the screening of microorganisms is crucial for finding the most effective strains [14]. Enzymes have a long history in human civilisation, being mentioned in Odyssey and Iliad (cheese making enzymes). In antiquity, they were used in different recipes, being of plant or animal provenience, or resulted from the activity of microorganisms. In the context when biomasses are major sources of renewable energy, that provide for biofuel and bioethanol production, microorganisms such as bacteria, fungi, yeast can be used as biological sources of extracellular enzymes that can be used in industrial processes to fragment carbon compounds in solid state fermentation (SSF) [15].

In order to overproduce microbial enzymes needed in industries, there is a need to optimise the fermentation. Nitrogen sources such as ammonium nitrate, potassium nitrate, sodium nitrate ammonium acetate, ammonium sulphate are documented to increase bacterial enzymes production [16].

Materials and methods

Sample collection and identification

Fungal biodeteriorating strains were collected during autumn 2018 from the following four wooden church

monuments in Hunedoara county, Romania: Church of the Assumption of Mother Mary, Lunca Motilor, 17th century, Pious Parascheva, Tarnavita, 17th century, St. George wooden church from Boz, 17th century, Church of the Assumption of Mother Mary, Alun, 17th century. Samples were collected from the interior walls using sterile cotton swabs and the content was immediately discharged into a PDB (Potato Dextrose Broth) sterile tube. The surfaces analysed were chosen from the interior spaces of the monuments, covering a wide variety of textures, such as: wood painting, construction wood, wooden objects used for religious service. The broth content was inoculated onto Potato Dextrose Agar plates using serial dilutions from up to 10^{-4} with distilled water and several passages for isolation were performed. The isolates were analysed using optical microscopy and by observing the colony features. Species were identified using MALDI-ToF system.

Three *Aspergillus* strains were subjected to comparison in terms of cellulolytic enzyme production. Mandel Reese basal medium (g/l) KH_2PO_4 2, $(NH_4)_2SO_4$ 1.4, $MgSO_4x7H_2O$ 0.3, $CaCl_2$ 0.3, yeast extract 0.4, $FeSO_4x7H_2O$ 0.005, $MnSO_4$ 0.0016, $ZnCl_2$ 0.0017, $CoCl_2$ 0.002, $CuSO_4x5H_2O$ 0.001. supplemented with 5% carboxymethyl cellulose (CMC) was inoculated with a loop of fungal culture and incubated for growth 7 days at room temperature. Growth was stopped and enzymes were collected by centrifugation at 10000 rpm and filtration through 2 nm nanopore filter. The resulted filtrate was subjected to 3-5- dinitrosalicylic acid test to assess enzyme concentration of the filtrate and to optimise the production parameters. Variation of several parameters such as incubation time, incubation temperature, influence of citrate buffer pH, substrate concentration and filtrate concentration impacted the enzymatic activity. Variation of incubation time was analysed by assessing the release of reducing sugars at several times: 0 min, 30 min, 60 min, 90 min, 120 min, 150 min, 180 min. The substrate concentration has been varied between 0-1 mg/ml CMC. Incubation temperature has been variated at different values: 20, 30, 40, 50, 60, 70°C. The citrate acid buffer's pH was variated at different values. 3-5-DNS test was performed on each sample for assessment of free sugars released from enzymatic treatment.

PCR and electrophoresis

DNA extraction was performed using Zymo Research extraction kit. Extracted DNA was subjected to PCR amplification of the ITS region, using the following reaction mix: for 25 µl of mix, there were used 12.5µl DreamTaq Green DNA Polymerase (1min/1kb), 1µl of each primer, 8.5µl nuclease free water (Table 1). ITS is a non-coding region of the nuclear ribosomal DNA located between the small and the large subunit ribosomal RNA genes, which is especially useful for elucidating relationships among closely related species. The average length of ITS region is 550 bp in the fungal kingdom but varies markedly among lineages. Between the forward and reverse primers, there are to be amplified 18S SSU, ITS1, 5.8S, ITS2 and 28S LSU, as part of the fungal ribosomal RNA structure. Because of a higher enzymatic activity, strains of *A. niger* were selected to confirm the presence of a cellobiohydrolase gene fragment.

After amplification, amplicons were subjected to electrophoresis in agarose gel 1% TAE, for 25 min at 100V and gels were examined in UV light.

Results

Results from analysing the enzymatic behaviour in terms of variation of incubation time, incubation temperature, pH and substrate concentration are presented in Figures 1-4.

Incubation time: During a timespan of 120 min, all three samples showed a peak in the digestion of cellulose and releasing of reducing sugars, followed by a downfall because of the reducing of the substrate. A lag period is achieved between time 0 and time 40 min for all three samples. A maximum value is achieved for *A. niger* filtrate after

Table 1. PCR parameters.

Initial denaturation	95º C	120 sec	Primers	Expected Fragment length
Denaturation	95º C	30 sec		
Annealing	57ºC for ITS	30 sec	Forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3 Reverse ITS4 (5'- TCCTCCGCTTATTGATATGC-3')	630bp.
	51ºC for partial cds	30 sec	Cellulase forward (AGCAGCTAATTTTTCTATCTTCA) Cellulase reverse (CGGTGAGAGTGTGACATCGT)	258 bp
Extension	72ºC	20 sec for cellulase cds 45 sec for ITS		
Final extension	72ºC	8 min		

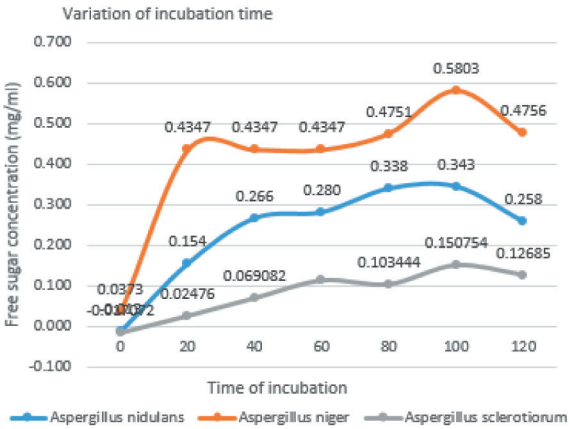

Fig. 1. Variation of enzymatic activity depending on incubation time.

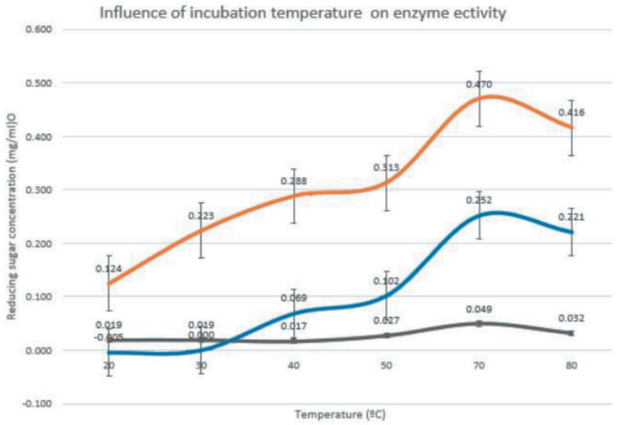

Fig. 2. Variation of enzymatic activity depending on incubation temperature.

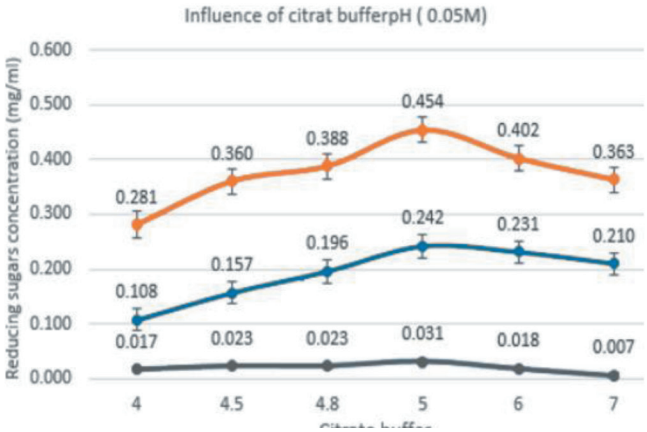

Fig. 3 Variation of enzymatic activity depending on pH.

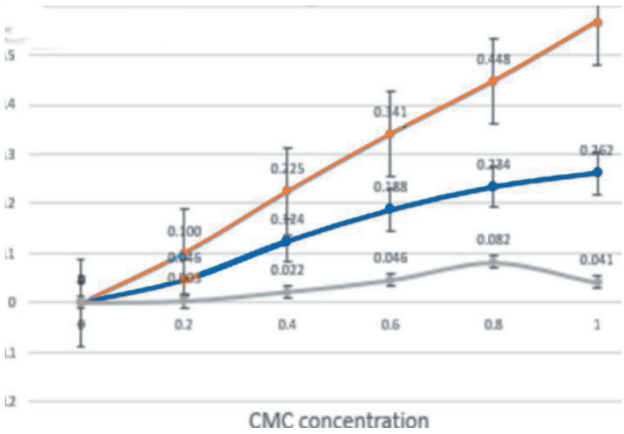

Fig. 4. Variation of enzymatic activity depending on substrate concentration.

100 min of incubation, and the lowest value after 120 min for *A. sclerotiorum* filtrate (Fig. 1).

Incubation temperature: The growth in concentration of the CMC substrate is directly correlated with the amount of reducing sugars resulted. A maximum degradation of the CMC is obtained at 60ºC, followed by a rapid decrease, probably due to protein denaturation. The highest degradation rate is achieved for *A. niger*, and the lowest growth rate is achieved for *A. sclerotiorum* (Fig. 2).

Citrate buffer pH variation: the pH was varied between 4 and 7. The highest enzymatic digestion was recorded for *A. niger* at pH 5 and the lowest for *A. sclerotiorum* at pH 7. An increase in digestion activity is recorded between pH 4 and 5 and a decrease between pH values 5 and 7 (fig. 3).

Substrate concentration was adjusted from 0 to 1 mg/ml, and it was concluded that there is a liniar corespondence between substrate concentration and release of free sugars, without a relevant tendency for substrate inhibition (Fig. 4).

Results from electrophoresis migration of the PCR amplicons of ITS region and cellulase gene are presented in Fig. 5. Sequencing results for ITS amplicons and the resulted phylogenetic tree is presented in Fig. 6.

Discussions

A. niger is particularly noted for its extensive enzyme production, which is crucial for the degradation of plant polysaccharides like cellulose, xyloglucan, galactomannan, and pectin. These enzymes facilitate the conversion of complex carbon sources into simpler molecules that the fungi can absorb [17]. *A. niger* has also been documented for its ability to degrade agricultural residues through SSF and microbial transformation of biological material, including substrates such as bagasse, sawdust, corn cobs, wheat bran, and sugar beet pulp [15]. Various carbon sources, including flakes of different origins like flour, soybean, agro-industrial wastes, starch, glucose, or maltose, can serve as substrates for producing enzymes like CMCases, avicellulases, lipases, β-xylosidases, and β-glucosidases. This microorganism is a prolific producer of a diverse range of extracellular proteins, including α-amylase, oxidase, catalase, dehydrogenase, hydrolase, cellulase, and pectinase [18]. *A. niger* is widely used in biotechnology for various processes, such as the production of antibiotics, enzymes, food products, industrial acids, and alcohol [19]. Amylase production has been observed on

Fig. 5. Left- electrophoresis result for ITS amplicon, for the 4 tested strains. PCR results for cellulase gene, revealing a length between200 and 300bp. 68 *_Aspergillus nidulans*, 10*_Aspergillus niger*,183*_ Aspergillus niger* (Tarnavita), D 11-Densus-*Aspergillus niger.*

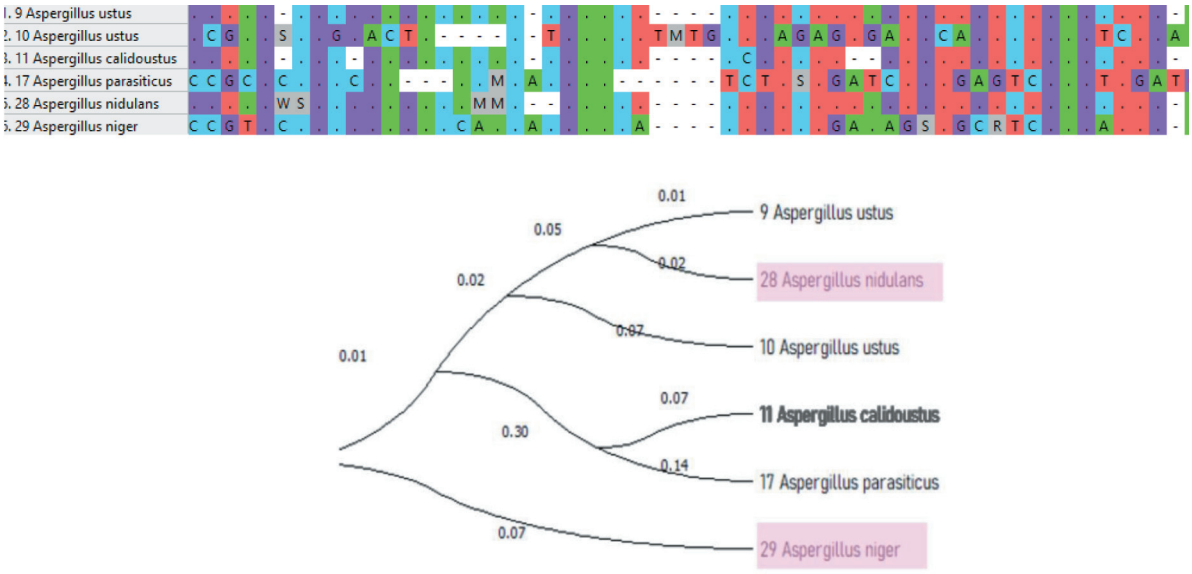

Fig. 6. Alignment of ITS sequences in MEGA 11 and generated phylogenetic three.

rye flakes and is particularly abundant in rye medium with glucose [20]. Asparaginase, an enzyme derived from a genetically modified nonpathogenic strain of *A. niger*, catalyses the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia. This enzyme is utilized to reduce free L-asparagine levels, a major precursor in the formation of the food contaminant acrylamide [15]. Enzymes also facilitate the reutilization of straw as a biomass resource [21]. The pH variation of enzymatic incubation shows an increase in digestion activity from pH 1 to 6, followed by a decline, with peak cellulase activity observed at 30°C. The highest cellulase activity was recorded on white sawdust, followed by rice bran, white bran, and sorghum [1]. Solid-state fermentation and biofilm fermentation, both dependent on surface adhesion, are governed by a concept termed surface adhesion fermentation, proposed in 2003. The adhesion process induces a specific cascade of gene expression based on substrate specificity [22].

Aspergillus also secretes proteases, phospholipases, and esterases, which can be considered virulence factors in tissue infections. These enzymes can be studied through induction in specific media and identified using qRT-PCR techniques[23].

Conclusions

The enzymatic analysis of the *A. niger, A. nidulans* and *A. sclerotiorum* filtrates revealed that all filtrates exhibited peak cellulolytic activity after 100 minutes of digestion at a temperature of approximately 65°C, in a buffer with a pH of 5, and with a CMC concentration of 1 mg/ml. Among the filtrates, *A. niger* demonstrated the highest activity in terms of releasing reducing sugars.

Fungal enzymes have a wide range of applications, including lignocellulose degradation, saccharification, biobleaching, biopolishing, protein stain removal (proteases), dehairing, organic pollutant remediation, juice clarifica-

tion (amylase), fiber splitting, and stain removal. Additionally, fungal enzymes contribute to the production of single-cell proteins, antifungal biocontrol, antifungal treatments, cancer treatment, and the formation of sophorose through transglucosylation by glucosidase [23].

To meet the growing global demand for secondary metabolites and industrial enzymes, synthetic biology has enabled the development of cell factories as a sustainable and efficient solution for green production [24]. A thorough understanding of the fungal strains used is crucial for this approach. Investigating the parameters that influence fungal enzyme activity is a promising strategy for advancing the production of renewable energy from reconverted cellulose debris and expanding the market for industrial enzymes [25].

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Conflicts of interest

The authors declare no conflict of interest.

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