

Research Article

(1→3)- α -D-Glucan from Fruiting Body and Mycelium of *Cerrena unicolor* (Bull.) Murrill: Structural Characterization and Use as a Novel Inducer of Mutanase

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Received 1 December 2016; Accepted 24 January 2017; Published 16 February 2017

Academic Editor: Raffaele Cucciniello

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Water-insoluble, alkali-soluble polysaccharide (marked as ASP) was extracted from the vegetative mycelium and fruiting body of *Cerrena unicolor* strain. Monosaccharide examination of ASP demonstrated that the isolated biopolymer was composed mainly of glucose, xylose, and mannose monomers. The methylation investigation of studied polymers indicated that (1→3)-linked α -D-Glcp is the major chain constituent (92.2% for glucans isolated from fruiting body and 90.1% from mycelium). ¹H NMR, FT-IR, and immunofluorescent labelling determinations confirmed that the polysaccharides isolated from both fruiting body and mycelium of *C. unicolor* are (1→3)- α -D-glucans. The obtained (1→3)- α -D-glucans showed differences in viscosity and similar characteristics in optical rotations. (1→3)- α -D-Glucans extracted from mycelium and fruiting body of *C. unicolor* were also used as potential and specific inducers of mutanase synthesis by *Trichoderma harzianum*. The highest mutanase activity (0.38 U/mL) was obtained after induction of enzyme by (1→3)- α -D-glucan isolated from the mycelium of *C. unicolor*, and this biopolymer has been suggested as a new alternative to streptococcal mutan for the mutanase induction in *T. harzianum*. (1→3)- α -D-Glucan-induced mutanase showed high hydrolysis potential in reaction with dextranase-pretreated mutan, where maximal degree of saccharification and solubilization of this bacterial homoglycan (83.1% and 78.4%, resp.) was reached in 3 h at 45°C.

1. Introduction

Cerrena unicolor is a white rot fungus belonging to the family of Polyporaceae which is able to degrade both polysaccharide and lignin compounds in wood [1]. A crucial role in this process is played by laccases and peroxidases. Fungal laccases are extensively studied for its use in industry, particularly in numerous detoxification processes and waste water regeneration [2]. Potential use in biotechnology of bioactive fractions, laccase, endopolysaccharides, and low molecular weight components with high prooxidant and antibacterial activity, isolated from *C. unicolor*, has also recently published [3].

The main structural components of fungal cell wall are glycoproteins and polysaccharides which determine its rigidity and necessary flexibility during cell growth. The individual elements form the layer structure, wherein the glycoprotein are the outer and polysaccharides (1→3)- β -D-glucans, (1→6)- β -D-glucans, (1→3)- α -D-glucans, and chitin are an interior layer [4]. Glucans are a very diverse group of sugar polymers, whose structure depends on the enzymes responsible for their synthesis. Glucose molecules in polysaccharides chains may be linked to each other by (1→3)- α -, (1→4)- α -, or (1→6)- α - bonds which determines their degree of branching and the spatial distribution in the side chains [5]. These

properties also affect the physical nature of α -D-glucans and their solubility. An important group of α -glucans are (1 \rightarrow 3)- α -D-glucans, which are produced by both the bacterial and fungal cells [6]. (1 \rightarrow 3)- α -D-Glucans that are components of the cell wall fulfill functions as a support cell wall, virulence factor in some pathogens, or reserve material [7, 8]. Some immunomodulating and antitumor activity of (1 \rightarrow 3)- α -D-glucans have been reported [9, 10]. This kind of biopolymers has also been described as a source of effective and specific activators for the synthesis of mutanolytic enzymes [11].

Hydrolytic enzymes group α -(1 \rightarrow 3)-glucanases are capable of acting on (1 \rightarrow 3)- α -D-glucans. Within this group are enzymes which degrade streptococcal mutan, called mutanases [6]. Bacterial mutanases are produced mainly by soil microorganisms (*Streptomyces*, *Bacillus*, *Pseudomonas*, and *Flavobacterium*) whose glucanolytic activities are much lower than those detected in fungal cultures [12]. Currently, the main source of mutanase is filamentous fungi especially of the *Trichoderma* genus. It should be emphasized that the mutanolytic enzymes are important in the fight against tooth decay, because they degrade forming dental plaque sticky, water-insoluble mutans, produced by cariogenic bacteria *Streptococcus mutans* and *S. sobrinus*.

Mutanases are mainly extracellular and inducible enzymes that decompose compounds containing α -(1 \rightarrow 3)-glucosidic linkages. Therefore, the effective biotechnological production of these catalysts can be carried out only in the culture media supplemented in (1 \rightarrow 3)- α -D-glucans as specific stimulants of mutanase synthesis [12]. Until now, the main and most powerful stimulus of mutanase synthesis was streptococcal α -D-glucan known as mutan [6]. Besides the mutan, fungal pseudonigeran, cells of *Schizosaccharomyces pombe*, and alkali-soluble α -D-glucans isolated from *Polyporus tumulosus*, *Phytophthora infestans*, and *Piptoporus betulinus* are also used as mutanase synthesis inducers [13–15]. Additionally, Wiater et al. [11] showed that α -D-glucan isolated from *Laetiporus sulphureus* induced significantly mutanase activity derived from *T. harzianum* cultures.

The main aim of these investigations was isolation, identification, and structural characterization of a new (1 \rightarrow 3)- α -D-glucan derived from laboratory cultured vegetative mycelium of *Cerrena unicolor* and harvested from the natural environment fruiting body of this fungus. Fungal polysaccharides, for example, isolated from laboratory cultivated *C. unicolor* mycelia may express many industrial and biomedical applications [3]. The study also focused on assessing the possibility of use the isolated (1 \rightarrow 3)- α -D-glucan for induction of mutanase synthesis in *T. harzianum* strain. Hydrolytic potential of (1 \rightarrow 3)- α -D-glucan-induced mutanase was evaluated in reaction with a dextranase-pretreated streptococcal mutan.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions. The fruiting body of *Cerrena unicolor* (Bull.) Murrill was collected from deciduous tree *Salix caprea* grown in Drobin, Poland (Table 1). The specimen was identified by molecular biological assay of the internal transcribed region (ITS) of the 5.8S rDNA as described below. Voucher specimen (CU-1A) was

stored in the Department of Industrial Microbiology, Maria Curie-Skłodowska University (Lublin, Poland). *Cerrena unicolor* strain C-139 was obtained from Culture Collection of the Regensburg University (Regensburg, Germany). Examined microorganism was stored on malt agar slants at 4°C. Mycelium of *C. unicolor* was cultured on earlier optimized medium in the growth conditions as described by Rola et al. [16]. *Trichoderma harzianum* strain designated as CCM F-340 (Czech Collection of Microorganisms, Brno, Czech Republic) was used as a culture for mutanase production induced by (1 \rightarrow 3)- α -D-glucan from fruiting body and vegetative mycelium of *C. unicolor*. Stock cultures of *T. harzianum* stored on potato dextrose agar slants at 4°C were used for inoculations. Liquid Mandels medium A (pH 5.3) [17] modified by addition of 0.4% of (1 \rightarrow 3)- α -D-glucan (from fruiting body or mycelium of *C. unicolor*), 0.1% Tween 80, and 0.05% proteose peptone was used for mutanase production. Shaken cultures of *T. harzianum* were performed in 500 mL flasks containing 100 mL of sterile medium. The flasks were seeded with fungal conidia to a final concentration of about 2×10^5 conidia/mL and placed on an orbital rotary shaker (Innova 44, New Brunswick, USA) at 300 rpm and 30°C for 3 days.

2.2. Genomic DNA Isolation and PCR Amplification of the ITS Region. The extraction procedure was performed according to the method described by Borges et al. [18] with our minor modifications. Approximately 20 mg of fruiting body was suspended in lysis buffer (10 mM EDTA, 10 mM β -mercaptoethanol, 4 mM spermidine, 0.1 M NaCl, 0.5% SDS, and 40 mM Tris-HCl, pH 8.0) and incubated at 65°C for 40 min. Prepared in this way fungal fruiting body suspension was extracted with chloroform and phenol, centrifuged for 20 min at 10,000 \times g, and precipitated with ice-cold ethanol. Next, precipitate was washed with 70% ethanol, dried, and redissolved in TE buffer (1 mM Tris-HCl, 0.1 M EDTA, pH 8). Degree of purification and concentration of the DNA sample were estimated using ND1000 spectrophotometer (Thermo Scientific, West Palm Beach, FL, USA). Polymerase chain reaction amplifications (PCR) with primers (ITS 1, ITS 2, ITS 3, and ITS 4) were carried out according to the protocol of White et al. [19]. Reactions were done in a TPersonal thermocycler (Biometra, Germany). Amplified PCR products were quantified by gel electrophoresis (1% agarose gel stained with ethidium bromide), GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific), and purified by microfiltration using Clean-up kit (A&A Biotechnology, Poland). BigDye™ Terminator Cycle Sequencing Kit and ABI PRISM 3730 XL sequencer were used in automatic sequencing (Applied Biosystems, Carlsbad, CA, USA). The GenBank accession number HM357713 was assigned to the nucleotide sequence determined in this study (Table 1).

2.3. Immunofluorescent Labelling of Cell Wall (1 \rightarrow 3)- α -D-Glucan. (1 \rightarrow 3)- α -D-Glucan was localized within *C. unicolor* cell wall using fluorescently labelled antibodies [20]. The samples prepared from fresh mycelia of *C. unicolor* were placed on Lab-Tek II Chamber slides (Nunc, Rochester, USA)

TABLE I: Characteristics of harvested fruiting body of *C. unicolor*.

| GenBank accession number | Geographic origin | Host tree | Fruiting body size | Fruiting body maturity ^a | (1→3)- α -D-Glucan(%) ^b |
|--------------------------|---------------------------|---------------------|--------------------|-------------------------------------|---|
| HM357713 | Drobin (52°44'N, 19°59'E) | <i>Salix caprea</i> | <20 cm | +++ | 46.1 \pm 0.8 |

^aFruiting body maturity: +: immature fruiting body (lack of hymenophore); ++: mature fruiting body with immature spores; +++: mature fruiting body with mature spores. ^bAmount of (1→3)- α -D-glucan in fruiting body dry mass. Results are shown as mean \pm SD of three independent experiments.

and fixed with a 3% (v/v) formaldehyde solution in distilled water at 65°C for 30 min. The next, fixed fungal cells were washed three times in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.4) before infiltration by 1% (v/v) Tween 20 in PBS buffer (PBS-T). The presence of the (1→3)- α -D-glucan was detected by use of 150 μ L of solution of mouse IgM MOPC-104E (0.1 mg/mL in PBS buffer) (Sigma, St Louis, MO, USA) as the primary antibody and in the same amount of Alexa Fluor 488 goat anti-mouse IgM (μ -chain specific) (Sigma, St Louis, MO, USA) as the secondary antibody. The samples were incubated as follows: primary antibodies overnight at 4°C in a wet chamber and secondary antibodies by 2 h in dark at 37°C. (1→3)- α -D-Glucan was observed on fluorescence microscope (Olympus BX 51, Germany), at an emission wavelength of 525/550 nm and excitation wavelength of 470/500 nm.

2.4. Isolation of Alkali-Soluble Polysaccharides (ASPs). Vegetative mycelium and fresh fruiting body and of *C. unicolor* were lyophilized and milled, and the dried materials were used for the isolation of the alkali-soluble polysaccharides according to a method described by Wiater et al. [11]. Both the dried materials of mycelium (100 g) and fruiting body (100 g) were milling and treating the resulting powders with water at 121°C for 1 h ($\times 3$). Wall materials were removed by centrifugation (10,000 rpm for 30 min) and freeze-dried. To isolate the alkali-soluble fraction, freeze-dried materials were suspended in 1 M NaOH under constant stirring. After an overnight incubation at room temperature, the supernatants were neutralized with 1 M HCl. The insoluble fractions were collected by centrifugation, washed with water ($\times 3$), and lyophilized to give the white powders [ASPs, purified (1→3)- α -D-glucan preparations].

2.5. Carbohydrate Analysis. For sugar analysis, the polysaccharides were hydrolyzed with 2 M TFA (100°C, 4 h). For absolute configuration of monosaccharides, analysis of acetylated *R*-(-)-2-butylglycosides was used [21]. The studied sugars were modified into alditol acetates form [22]. Glucans were methylated by the method of Hakomori [23] and purified on a Sep-Pak C₁₈ cartridge according to the method of York et al. [24]. The resulting material was hydrolyzed in 2 M TFA (100°C, 4 h) and reduced with NaBD₄. The partially methylated alditols were transformed to acetate derivatives. Prepared alditol acetates and partially methylated alditol acetates were analyzed using GC-MS analyses on a Hewlett-Packard gas chromatograph (model HP5890A, Germany) equipped with a mass selective detector (MSD model HP5971). Separations were accomplished in a capillary

column (HP-5MS, 30 m \times 0.25 mm) with helium as the carrier gas. The temperature program was 150°C (5 initial min) and then raised to 310°C at a ramp rate of 3°C/min and final time 20 min. ¹H NMR spectra of the polysaccharide dissolved in 1 M NaOD in D₂O were recorded with a Bruker Avance (300 MHz) spectrometer. The ¹H chemical shift was obtained using acetone ($\delta_{\text{H}} - 2.225$ ppm) as the internal standard. FT-IR spectroscopy was recorded with a Perkin Elmer FT-IR spectrophotometer (Model 1725X, Wellesley, MA, USA) in the wavelength range between 400 and 4000 cm⁻¹. A specimen was prepared by the KBr-disk method. Specific rotation [α]_D²⁵ (*c* 1 M sodium hydroxide) was determined at 589 nm using a Perkin Elmer Automatic Polarimeter model 341 LC (Wellesley, MA, USA). The viscosity of polysaccharides (*c* 1 M sodium hydroxide) was measured with a Brookfield viscometer model DV 3 (Stoughton, MA, USA) at 20°C.

2.6. Mutanase Assay. Mutanase activity was estimated using the method described by Wiater et al. [11]. The reaction mixture contained 0.5 mL of 0.2% (w/v) dextranase-pretreated mutan (DTM) in 0.2 M sodium acetate buffer (pH 5.5) and 0.5 mL of the suitably diluted enzyme solution. The samples were incubated for 1 hour at 45°C, and next the reducing sugars released were quantified by the Somogyi-Nelson method [25, 26]. Appropriate substrate and enzyme blanks were included to correct any free reducing group not emanating from DTM. One unit of mutanase activity (U) was calculated as the amount of enzyme hydrolyzing dextranase-pretreated mutan (DTM) to yield reducing sugars equivalent to 1 μ mol of glucose/min. Mutanase activity was expressed as units per mL of culture (U/mL).

2.7. Preparation of Dextranase-Pretreated Mutan (DTM). For the determination of the mutanase activity, dextranase-pretreated mutan (DTM) substrate (50 U of dextranase/mg of native mutan was incubated at pH 6.0, 37°C, 3 \times 24 h) was prepared. Native mutan was synthesized according to the procedure described by Wiater et al. [12]. Dextranase of *Penicillium* sp. (enzyme activity of 12.9 U/mg preparation, Sigma-Aldrich (St. Louis, MO., USA)) was used in described method. As demonstrated using ¹H NMR method the linkage structure of the native and the dextranase-pretreated mutan have mixed-linkage (1→3)- α - and (1→6)- α -biopolymers but a greater proportion of (1→3)- α - than (1→6)- α - linkages was observed.

2.8. Protein Estimation. The protein content was measured at 595 nm by the method of Bradford [27], using bovine serum albumin as a reaction standard in the range of the concentrations from 40 μ g/mL to 400 μ g/mL.

2.9. Reducing Sugars Estimation. The reducing sugars concentration was determined by the Somogyi-Nelson colorimetric method [25, 26]. The test solution (0.5 mL) was mixed with 0.5 mL alkaline copper reagent and then boiled in water bath for 20 min. After cooling, with 0.5 mL of chromogenic reagent (arsenomolybdate) and 1.5 mL of distilled water, the absorbance was measured at a wavelength of 520 nm. Concentration of reducing sugars was calculated based on the calibration curve with glucose as a standard. The amount of reducing sugars was expressed in $\mu\text{g/mL}$.

2.10. Induction of *T. Harzianum* Mutanase by (1 \rightarrow 3)- α -D-Glucan. The synthesis of *T. harzianum* mutanase was induced by (1 \rightarrow 3)- α -D-glucans isolated from the mycelium and fruiting body of *C. unicolor* strains. Flask submerged cultures of the fungus *T. harzianum* were carried out by 3 days on Mandels medium, pH 5.3, containing (g/L) KH_2PO_4 2; $(\text{NH}_4)\text{SO}_4$ 1.4; urea 0.3; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 0.3; CaCl_2 0.3; Bacto-peptone 0.5; Tween 1 mL/L; and microelements solution 1 mL/L with tested (1 \rightarrow 3)- α -D-glucan (in an amount of 0.4 g in 100 mL of medium) as a mutanase inducer. After cultivation, the mutanase activity, protein concentration, and pH value were estimated.

2.11. Hydrolysis Experiments. Hydrolysis was performed in plugged Eppendorf tubes using 0.05% NaN_3 as a preservative. The reaction mixtures contained 1 mg of dextranase-pretreated mutan (DTM) and *T. harzianum* mutanase induced by (1 \rightarrow 3)- α -D-glucan isolated from mycelium of *C. unicolor* (1 U/mL) in 1 mL of 0.2 M sodium acetate buffer (pH 5.5). The samples were incubated for 24 h at 45°C and agitated at 300 rpm. Next, mutan hydrolyzates were withdrawn at various intervals of up to 24 h and heated at about 100°C for 5 min to stop the reaction. Total reducing sugars were analyzed by the Somogyi-Nelson method [25, 26]. The calculation included enzyme and substrate blanks. The percentage of mutanolysis was calculated by the following formula: saccharification (%) = [reducing sugars formed (mg) \times 0.9/mutan (mg)] \times 100. Additionally, the turbidimetric analysis (at 560 nm) the residual insoluble glucan was determined. The degree of mutan solubilization was calculated and expressed as a percentage.

2.12. Statistical Analysis. Data were analyzed using one-way ANOVA followed by a post hoc Tukey test. All the results are expressed as mean \pm SD from three experiments ($n = 3$). Values of $P \leq 0.05$ were only reported as statistically significant.

3. Results and Discussion

3.1. Morphological and Genetic Characteristics of *C. unicolor* Strains. The strain of *Cerrena* whose fruiting body was collected from *Salix caprea* was identified at the species level by analysis of their ITS region. One product (length 648 bp) was obtained from PCR with ITS1-ITS4 primers and subsequently by direct sequencing. The complete sequence of this product indicated 100% identity to the *Cerrena unicolor*

ITS sequences. The obtained sequence was deposited in GenBank under HM357713 accession number (Table 1). For comparative testing of the mycelium, *C. unicolor* 139 (ITS sequence deposited in GenBank under accession number DQ056858) obtained from Culture Collection of the Regensburg University (Regensburg, Germany) was used [28]. Based on the available genome sequence of *C. unicolor* future analysis of genome sequences and their expression is much easier. This also applies to the enzymes, for example, laccase *C. unicolor* of biotechnological interest and bioactive fraction having antioxidant, antibacterial, antitumor, or immunostimulatory activity [3].

3.2. Isolation and Structural Analysis of Alkali-Soluble Polysaccharides. Analysis of composition of *C. unicolor* fruiting body showed the presence of water (50%), water-insoluble (1 \rightarrow 3)- α -D-glucan (23.1%), and the remaining part composed of other structural ingredients of cell wall. The ASPs were obtained from the lyophilized fruiting body and mycelium of *C. unicolor* by alkaline solution extractions with the yield of 46.1% from fruiting body and 9.5% from mycelium. As it was described earlier, the α -glucans content in the fungal cell walls varies depending on the species [4]. Fruiting bodies of *Laetiporus sulphureus* are the richest source of (1 \rightarrow 3)- α -D-glucan (56.3%) [11]. In the case of *Aspergillus niger* mycelium, this water-insoluble biopolymer constituted only 9% of its composition [29] whereas it was absent in the *Saccharomyces cerevisiae* and *Candida albicans* cell walls [4]. For the detection of (1 \rightarrow 3)- α -D-glucan in the *C. unicolor* mycelium cell walls, the immunofluorescent labelling with specific antibodies was done. This method was previously used by Fujikawa et al. [20] to visualize the location of (1 \rightarrow 3)- α -D-glucans in the phytopathogenic fungus *Magnaporthe grisea* where these polysaccharides including chitosan are the main component of cell integument. The presence of (1 \rightarrow 3)- α -D-glucan in the cell wall of *C. unicolor* was highlighted by use of the fluorescent microscope (Figure 1). In the present report, a detailed structural analysis of (1 \rightarrow 3)- α -D-glucans was conducted using ^1H NMR and FT-IR spectra technique. ^1H NMR analysis of ASPs isolated from fruiting body and mycelium of *C. unicolor* showed the presence of glucose molecules linked by (1 \rightarrow 3)- α -glucosidic bonds (Figure 2). It may be clearly observed by the existence of singlets at 5.6408 and 5.6283. Both of these values corresponded to the region of 5.200 to 5.650 ppm which characterize glucose α -anomers. Similar results were obtained earlier in the study of glucans isolated from *L. sulphureus* where 5.33 ppm resonance signal was observed [11]. Structural analysis of ASPs by FT-IR spectra was made in the wavelength range of 4000 to 500 cm^{-1} . As shown in Figure 3, FT-IR spectrum of ASPs displays absorption bands at 3336.01 cm^{-1} and 3313.04 cm^{-1} (for glucans isolated from fruiting body and mycelium, resp.) which indicates the presence of -OH group, characteristic of polysaccharides chains [30]. The band between 1000 and 1100 cm^{-1} (i.e., 1012.32 cm^{-1} and 1009.26 cm^{-1}) indicated the presence of O-substituted glucose residues and β -linkages in the glucosidic chain [31]. Additionally, characteristic α -linked glycosyl residues at 847.72 cm^{-1} and 822.00 cm^{-1} were

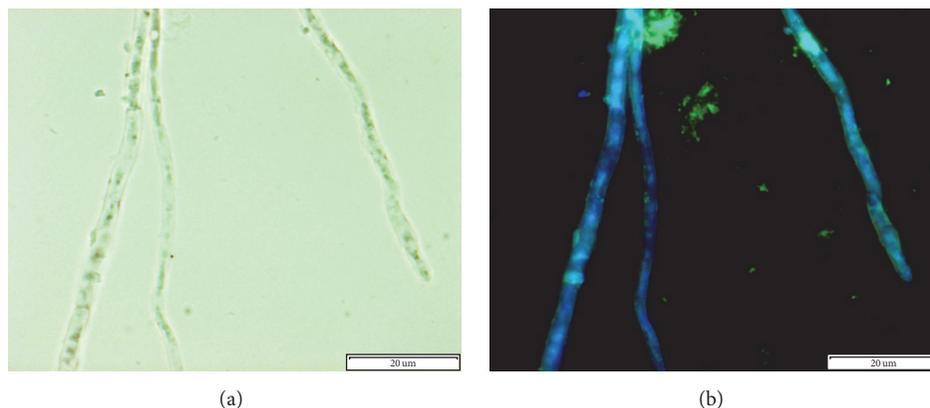


FIGURE 1: Detection of (1→3)- α -D-glucan in the hyphae of *C. unicolor* strain C-139 by means of fluorophore-labelled antibodies (Clone IgM MOPC-104E). (a) Filaments in the light microscopy, (b) fluorescent image of the same filaments. Twenty samples were observed and typical images are presented. Scale bar = 20 μ m.

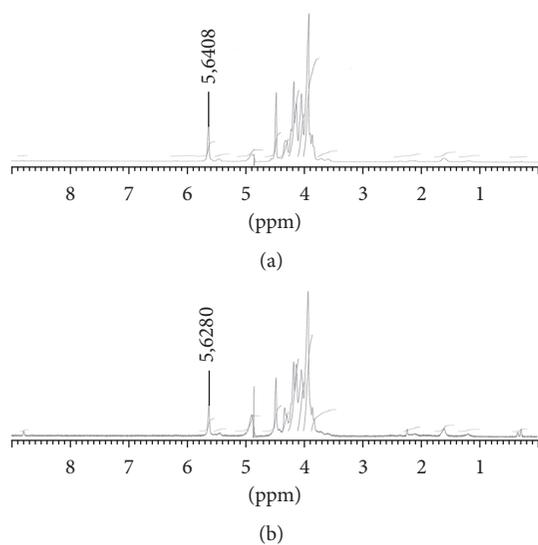


FIGURE 2: ^1H NMR spectra of alkali-soluble water-insoluble polysaccharides (ASPs) obtained from fruiting body (a) and vegetative mycelium (b) of *C. unicolor*.

obtained for ASPs isolated from mycelium of *C. unicolor*. In the case of biopolymer derived from fruiting body of *C. unicolor* we also showed, similarly to Kozarski et al. [32], two peaks at 845.64 cm^{-1} and 821.52 cm^{-1} , which indicate that the isolated material contained (1→3)- α -D-glucan. In the study of FT-IR spectra of glucans isolated from cell walls of *Piptoporus betulinus*, *Aspergillus nidulans*, and *L. sulphureus* the similar profile of the spectrum was observed [11, 33]. Monosaccharide analysis of ASPs isolated from fruiting body and mycelium of *C. unicolor* demonstrated that the basic monomer building these polysaccharides was glucose (93.5%, fruiting body, and 95.3%, mycelium) (Table 2). In addition, a small percentage of other monosaccharides, xylose (2.3 and 3.6%) and mannose (2.4 and 2.9%), were found. There was

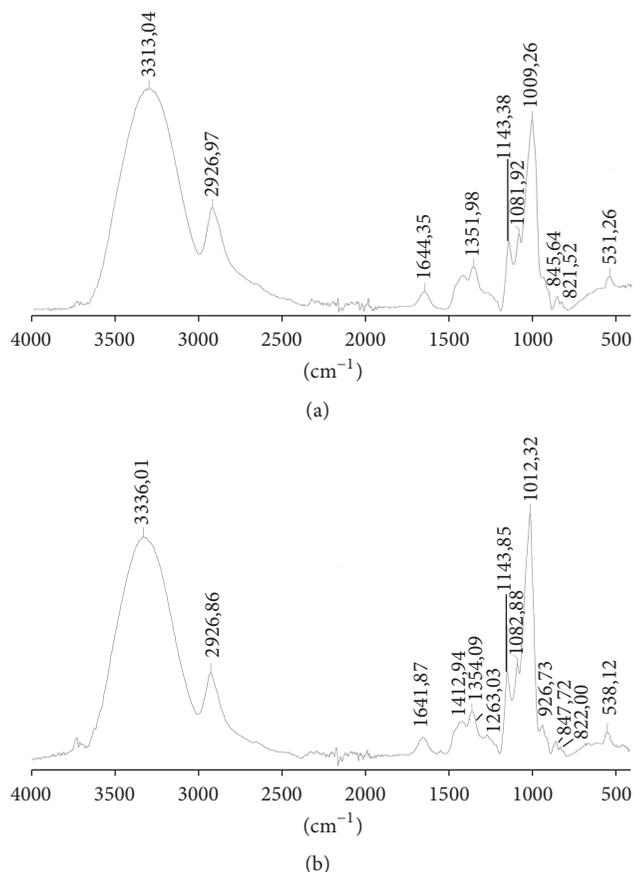


FIGURE 3: FT-IR spectra of alkali-soluble water-insoluble polysaccharides (ASPs) obtained from fruiting body (a) and vegetative mycelium (b) of *C. unicolor*.

no significant differences in the percentage of the various monosaccharides isolated from the *C. unicolor* mycelium and fruiting body. A similar composition (with glucose, xylose, and mannose) of α -glucans isolated from the cell wall of

TABLE 2: Monosaccharide analysis (Glc: glucose; Xyl: xylose; Man: mannose) of ASP (alkali-soluble) preparations extracted from fruiting body and vegetative mycelium of *C. unicolor*.

| Origin | Monosaccharide amounts (%) | | |
|---------------|----------------------------|-----------|-----------|
| | Glc | Xyl | Man |
| Fruiting body | 95.3 ± 1.0 | 2.3 ± 0.2 | 2.4 ± 0.1 |
| Mycelium | 93.5 ± 1.1 | 3.6 ± 0.3 | 2.9 ± 0.1 |

L. sulphureus was reported by Grün [4]. Methylation analysis of glucans extracted from fruiting body and vegetative mycelium of *C. unicolor* showed that (1→3)-linked α -D-Glcp is the major chain constituent (92.2% in fruiting body and 90.1% in mycelium) while (1→4)-linked α -D-Glcp (4.4% and 7.4%, resp.) is the minor one (Table 3). The obtained results proved that larger percentage of (1→4)-linked α -D-Glcp occurs in the glucan extracted from the mycelium which makes it even more branched. Furthermore, the structure of studied glucans contained one type of doubled substituted glucose residues (1→; →3, 4)- α -D-Glcp. The studied (1→3)- α -D-glucans preparations showed differences in viscosity and similar characteristics of their optical rotations (Table 4). The specific optical rotations of the α -glucans are in the range of +200° to +206°. The values of viscosity fluctuated between 2.12 and 7.55 mPa·s. Different viscosity of the tested α -glucans may indicate differences in their molecular mass. Based on the analysis of all the above data it can be concluded that the water-insoluble, alkali-soluble polysaccharides isolated both from fruiting body and mycelium of *C. unicolor* contained predominantly (1→3)- α -D-glucan.

3.3. Induction of *T. harzianum* Mutanase by (1→3)- α -D-Glucan. Mutanase is an inducible enzyme decomposing polymers containing in their structure (1→3)- α -glucoside bond. Therefore intensified production of this catalyst takes place only in a medium containing a specific inducer such as (1→3)- α -D-glucan [10]. In order to choose the best inducer for the mutanase synthesis by *T. harzianum* strain, shaken fungal cultures have been grown on the Mandels medium (pH 5.3) for 3 days. In the present work (1→3)- α -D-glucan from the mycelium and fruiting body of *C. unicolor* were used as a potential inducers of mutanase. The results obtained showed that the highest activity of mutanase by *T. harzianum* was detected in the cultures supplemented with (1→3)- α -D-glucan from the mycelium of *C. unicolor* (Table 5). The addition of this polysaccharide to the medium gave the maximal enzyme yield of 0.38 U/mL. Wiater et al. [11] obtained higher mutanase activity (0.82 U/mL) after induction of its synthesis by (1→3)- α -D-glucans obtained from the harvested fruiting bodies of *L. sulphureus*. On the other hand, the result achieved by us is slightly better than that (0.33 U/mL) given by Wiater et al. [34], who used a bacterial mutan to induce mutanase in *T. harzianum* F-470 strain. In turn, there were significantly lower levels of enzyme production by *T. harzianum* OMZ 779 (0.16 U/mL) shown by Guggenheim and Haller [35] in shaken flask cultures with 1% mutan. In the case of bacterial mutanases, Meyer and Phaff [14] reached

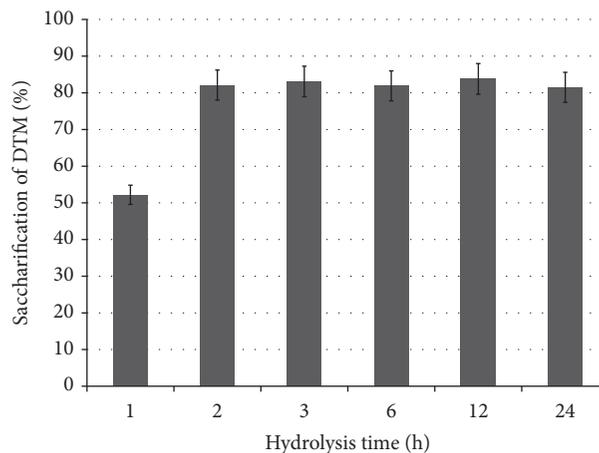


FIGURE 4: The kinetics of saccharification of dextranase-pretreated mutan (DTM) using *T. harzianum* mutanase induced by (1→3)- α -D-glucan isolated from mycelium of *C. unicolor*.

the maximal mutanase activity (0.31 U/mL) in cultures of *Bacillus circulans* WL-12 supplemented with whole cells of *Schizosaccharomyces pombe* or purified (1→3)- α -D-glucan from *A. niger*.

After using for the induction of mutanase the pure (1→3)- α -D-glucan isolated from the fruiting body of *C. unicolor*, a high enzyme productivity (0.26 U/mL) was also noted. Sanz et al. [36] showed intensified production of mutanase by *T. asperellum* after induction of this culture by α -D-glucans isolated from *Botrytis cinerea* mycelium and Ait-Lahsen et al. [37] used as a mutanase activator the mycelium from *A. niger*. In sum, the present study reveals that the (1→3)- α -D-glucan preparations, isolated from mycelium and fruiting body of *C. unicolor*, effectively induced mutanase in *T. harzianum* and fully substituted streptococcal mutan. In contrast to mutan, these new and powerful stimulants for inducible mutanase synthesis are inexpensive, easily available, and safe for humans. Therefore, they will be very useful to facilitate the mutanase production on a larger scale and at relatively low costs acceptable for oral applications. From the biotechnological point of view the use of *C. unicolor* mycelium as a source of (1→3)- α -D-glucan seems to be more efficient and promising for economic reasons. Additionally, the production of mutanase would become independent of the time for picking up fruiting bodies of this fungus from the environment.

3.4. Hydrolysis Experiments. Enzymatic hydrolysis of dextranase-pretreated streptococcal mutan (DTM) was carried out with the use of partially purified mutanase induced by (1→3)- α -D-glucan isolated from mycelium of *C. unicolor*. Experimental results were obtained by monitoring enzymatic saccharification of DTM and its solubilization (Figures 4 and 5). In both cases, hydrolysis reaction rates increased linearly in the initial phase of the process and later became almost constant. The maximum degree of mutan saccharification

TABLE 3: Summary of methylation analysis of glucans extracted from fruiting body and vegetative mycelium of *C. unicolor*.

| Origin | Methylated sugar | Linkage type | Molar amounts (%) |
|---------------|---|-----------------------------|-------------------|
| Fruiting body | 2,3,4,6- <i>O</i> -Me ₄ -D-glucose | α -D-Glcp-(1→ | 0.2 |
| | 2,4,6- <i>O</i> -Me ₃ -D-glucose | →3)- α -D-Glcp-(1→ | 92.2 |
| | 2,3,6- <i>O</i> -Me ₃ -D-glucose | →4)- α -D-Glcp-(1→ | 4.4 |
| | 2,6- <i>O</i> -Me ₂ -D-glucose | →3,4)- α -D-Glcp-(1→ | 3.2 |
| Mycelium | 2,4,6- <i>O</i> -Me ₃ -D-glucose | →3)- α -D-Glcp-(1→ | 90.1 |
| | 2,3,6- <i>O</i> -Me ₃ -D-glucose | →4)- α -D-Glcp-(1→ | 7.4 |
| | 2,6- <i>O</i> -Me ₂ -D-glucose | →3,4)- α -D-Glcp-(1→ | 2.5 |

The glucan preparation was methylated before being hydrolysed, reduced, and acetylated. Therefore, the methylated sugars mentioned refer to permethylated ailditol acetates identified by GC-MS (e.g., 2,3,4,6-*O*-Me₄-D-glucose refers to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol).

TABLE 4: The data of viscosity and optical rotation analysis of (1→3)- α -D-glucans extracted from fruiting body and vegetative mycelium of *C. unicolor*.

| Strain | Viscosity (mPa·s) | Optical rotation [α] _D ²⁵ (°) |
|---------------|-------------------|--|
| Fruiting body | 2.12 ± 0.1 | 206 ± 5 |
| Mycelium | 7.55 ± 0.3 | 200 ± 3 |

Results are shown as mean ± SD of three independent experiments.

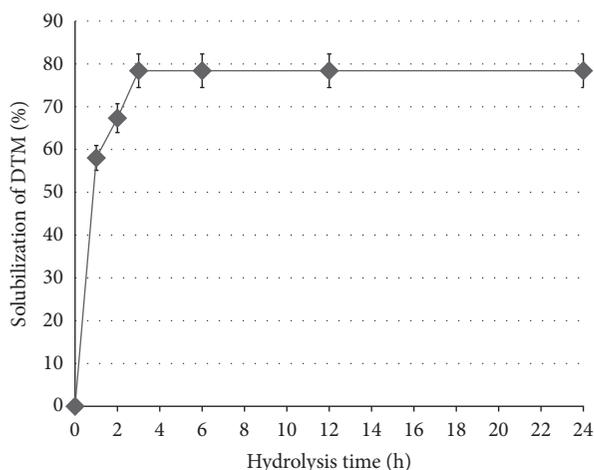


FIGURE 5: The kinetics of solubilization of dextranase-pretreated mutan (DTM) using *T. harzianum* mutanase induced by (1→3)- α -D-glucan isolated from mycelium of *C. unicolor*.

(83.1%) and its solubilization (78.4%) was reached after 3 h of hydrolysis. Having considered that dextranase-pretreated mutan, prepared in our laboratory, was a mixed-linkage glucan with 79.8 mol% of (1→3)- α -glucosidic linkages, it is possible to conclude that almost all of these glucosidic bonds were degraded during specific action of mutanase on this biopolymer. Wiater et al. [11] obtained similar results for *T. harzianum* mutanase induced by fruiting body of *L. sulphureus* where hydrolysis of substrate at a level of 80% was achieved after 3 h. In turn, Kopec and Vacca-Smith [38], during hydrolysis of insoluble mutan, obtained only

15.3% saccharification with mutan-induced mutanase of *T. harzianum* after 4 h of incubation at 30°C. Similar results (hydrolysis yield of up to 20% after 48–64 h at 37°C) were obtained for Dextranase 501 (a commercial preparation containing several hydrolytic activities) and glucanohydrolase with dextranolytic and amylolytic activity from *Lipomyces starkeyi* [39, 40]. Thus, efficient solubilization of mutan in a short period of time gives a chance to use the *T. harzianum* mutanase induced by (1→3)- α -D-glucan of *C. unicolor* in preventive dentistry as a preparation in the fight against dental caries. High efficiency of mutan solubilization (83% after 6 hours) was also received by Pleszczyńska et al. [41] after using mutanase from *Paenibacillus* sp. MP-1-induced by *L. sulphureus* mycelium.

4. Conclusions

In this study, the new (1→3)- α -D-glucan preparations isolated from mycelium and fruiting body of *Cerrena unicolor* were exactly identified and characterized. Both tested glucans proved to be effective and easily accessible inducers of mutanase synthesis in *T. harzianum*. The simple culturing of *C. unicolor* mycelium and intensification the scale of its production are very interesting from technological point of view. In fact, obtaining large amounts of (1→3)- α -D-glucans from this source could significantly accelerate the production of mutanase which would increase the chance of getting the product that could be used in the prevention of dental caries. Mutanase could be used as an active additive to preparations intended for oral hygiene, such as mouthwashes, toothpastes, and dental gels, and also for washing and storage of prosthesis and prosthetic devices for removal of denture plaque located on their acrylic surfaces. As active ingredient, mutanase could become useful supplement to mechanical cleaning of teeth and dentures with a toothbrush, dental sticks, and dental floss.

Competing Interests

The authors declare that there is no conflict of interests.

TABLE 5: Effect of (1→3)- α -D-glucan inducers on mutanase production by *T. harzianum* strain cultivated in shaken flask cultures.

| Source of (1→3)- α -D-glucan | Activity of mutanase [U/mL] | Protein [μ g/mL] | pH medium |
|-------------------------------------|-----------------------------|-----------------------|-----------|
| Fruiting body | 0.26 \pm 0.004 | 103.2 \pm 1.6 | 5.23 |
| Mycelium | 0.38 \pm 0.007 | 120.6 \pm 1.3 | 6.23 |

Results are shown as mean \pm SD of three independent experiments.

Acknowledgments

This work was supported by the National Science Centre (Poland) based on the Decision no. DEC-2013/09/B/NZ9/01829 and the Research Program BS/UMCS.

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