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Ultrasound-assisted extraction and characterization of hydrolytic and oxidative enzymes produced by solid state fermentation

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ABSTRACT

Ligninolytic and hydrolytic enzymes were produced with six selected fungi on flax substrate by solid state fermentation (SSF). The extracellular enzyme production of the organisms in two SSF media was evaluated by measuring the soluble protein concentration and the filter paper, endoxylanase, 1,4-β-D-glucosidase, 1,4-β-D-endoglucanase, polygalacturonase, lignin peroxidase, manganese peroxidase and laccase activities of the clear culture solutions produced by conventional extraction from the SSF materials. The SSF material of the best enzyme producer (*Trichoderma virens* TUB F-498) was further investigated to enhance the enzyme recovery by low frequency ultrasound treatment. Performance of both the original and ultrasound macerated crude enzyme mixtures was evaluated in degradation of the colored lignin-containing and waxy materials of raw linen fabric. Results proved that sonication (at 40%, 60% and 80% amplitudes, for 60 min) did not result in reduction in the filter paper, lignin peroxidase and laccase activities of the crude enzyme solution, but has a significant positive effect on the efficiency of enzyme extraction from the SSF material. Depending on the parameters of sonication, the enzyme activities in the extracts obtained can be increased up to 129–413% of the original activities measured in the control extracts recovered by a common magnetic stirrer. Sonication also has an effect on both the enzymatic removal of the lignin-containing color materials and hydrophobic surface layer from the raw linen.

1. Introduction

Solid state fermentation is an attractive alternative method to the widely used submerged fermentation for producing enzymes [1,2]. Since the cultivation conditions significantly influence the growth of culture and the activities of the enzymes, the method is suitable for production of substrate specific enzyme complexes for many industrial applications (e.g. food, feed, pulp and paper, textile and bioethanol industry, etc.). The SSF has several practical and economic advantages especially in those processes, where the crude fermented products may be used directly as enzyme sources [3].

The direct use of the crude fermented products in different technologies such as biopulping and biobleaching of lignocelluloses has been proved to be efficient and cost-effective, since the whole fermented material (residual substrate, fungal biomass and *in situ* enzymes together) from the cultivation is used without a prior downstream processing. Thus, the enzyme complexes are liberated from the SSF material in the bath/system where the target substrate is present, and can act rapidly and efficiently as catalysts [3–5].

However, for most applications, the enzymes adsorbed on the substrate in SSF are usually recovered by extraction. Extraction of enzymes from the crude fermented products is a key-factor in SSF. For achieving highly concentrated enzyme extracts from the SSF substrate, the effect of different solvents, pH, solid to solvent ratio, time and temperature, as well as shaking conditions on the enzyme extraction and concentration was investigated in detail [6–8]. The efficiency of the successive extractions in recovery of hydrolytic and oxidative enzymes was also evaluated [9].

The ultrasound-assisted extraction of bioactive compounds from natural sources has a great potential in the food industry [10,11]. Power ultrasound refers to sound waves with low frequencies (20–100 kHz) and high sound intensities (10–1000 W/cm^2). Its effect can be based on the cavitation phenomenon, which means the formation, growth and collapse of vapor or gas bubbles that occur with ultrasound. In a heterogeneous system, collapse of the

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bubbles near the solid surface results in high velocity micro jets, which accelerate the transport processes and significantly improve the mass transfer. During acoustic cavitation, extremely high local temperatures, high pressure and shearing are created, which can have a remarkable effect on the biomacromolecules and their stability [12–14]. To the best of our knowledge, treatment of solid substrate fermentation materials by ultrasound for improving the efficiency of the extraction of the crude enzymes has not been studied so far. In the field of SSF, only one paper was found on using ultrasound [15]. The authors applied sonication for 30 min in every 12 h in order to stimulate the SSF media, and in the ultrasound assisted fermentation a slightly higher xylanase activity was achieved.

Ligninolytic enzymes can be produced successfully with solid-state fermentation on lignocellulose-containing substrates. Beside ligninolytic enzymes, hydrolytic enzymes such as cellulases and xylanases can also be secreted simultaneously. The main lignin modifying enzymes are oxidoreductases, i.e., two types of peroxidases: lignin peroxidase (LiP) and manganese peroxidase (MnP), and a phenoloxidase: laccase (Lac). Some white-rot fungi may produce all the three enzymes, while others secret only one or two of them [16,17,1].

Ligninolytic enzymes can be applied in different fields for destroying the lignin-holocellulose network in lignocellulose materials, which can be used as feedstock for example in the production of second generation bioethanol or in the pulping process. Biobleaching with ligninolytic enzymes is also based on the degradation of lignin. For biobleaching of linen fabric, which is made from the bast fibers of flax [18], hydrolytic enzymes such as pectinases and xylanases, and oxidative enzymes have proved to be effective in degradation and removal of non-cellulosic substances such as pectin and some hemicelluloses and coloring materials like lignin [19,20].

In this work, six selected fungi, that proved to be effective in producing ligninolytic and accompanying hydrolytic enzymes in a previous study [5], were used in SSF on flax fiber as a carbon source for the production of substrate specific enzymes. Subsequently, these enzyme complexes were used in biobleaching of linen. The extracellular enzyme production of the organisms in two SSF media was evaluated by measuring the hydrolytic and oxidative enzyme activities of the clear culture solutions produced by conventional extraction from the SSF materials. For improving the efficiency of the enzyme extraction, low frequency ultrasound at different amplitudes was applied and the recovery of enzymes was characterized by enzyme activities measured in the buffer extracts in the period of 0–60 min. The effect of sonication on the enzyme stability was also evaluated. Finally, the crude enzyme solution and the whole SSF material produced by the best strain were used for removal of the non-cellulosic impurities from raw linen fabric.

2. Materials and methods

2.1. Fungi

The cultures (*Aspergillus giganteus* NRRL 10, *Aspergillus oryzae* NRRL 3485, *Phanerochaete chrysosporium* VKM F-1767, ATCC 24725, NCAIM F-00740 and *Trichoderma virens* TUB F-498) used in this study were purchased from the following microbial culture collections: ATCC (American Type Culture Collection, Manassas, Virginia), NCAIM (National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary), NRRL (Northern Regional Research Center, USDA, Peoria, Illinois). VKM (Russian Culture Collection, Moscow, Russia) and TUB (Technical University of Budapest, Hungary). The strains are stored as freeze-dried cultures. Revitalization from the ampoules was performed on potato-dextrose-agar (PDA) medium in Petri plates supplemented with 100 μg/ml doxycycline hyclate (Sigma–Aldrich, USA) to prevent bacterial contamination during cultivation. After incubation at 30 °C for 4–8 days the properly sporulated cultures were used for inoculation of the sterile media for solid state fermentation.

2.2. Enzyme production in SSF

Two fermentation media were applied in the production of oxidative and hydrolytic enzymes. Both contained 5 g of flax fiber as a carbon source (grown and dew retted in France, and supplied by Hungarolen Kft, Komárom, Hungary). The flax was cut into lengths of 1–2 mm, then wetted with 12 and 15 mL salt solutions (abbreviated by Flax-12 and Flax-15, respectively). The #1 salt solution consisted of (in g/L): NH_4NO_3, 3; $(NH_4)_2HPO_4$, 3; KH_2PO_4, 5; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl 0.5; $CaCO_3$, 0.5. The #2 salt solution contained (in g/L): KH_2PO_4, 1.5; $(NH_4)_2HPO_4$, 2; soybean meal (defatted), 1; corn steep liquor, 50% DM (Sigma–Aldrich, USA), 1; NaCl, 0.5; $CaCO_3$, 1; urea, 0.3; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$, 0.3; and (in mg/L) $FeSO_4 \cdot 7H_2O$, 2.5; $MnSO_4$, 0.8; $CoCl_2 \cdot 6H_2O$, 1; $ZnSO_4 \cdot 7H_2O$, 1.7. The pH of the salt solutions was set to 5.0 by sulfuric acid. The wetted carbon sources with the salt solutions #1 and #2 (abbreviated by Flax-12/1; Flax-12/2; Flax-15/1; Flax-15/2. respectively) were sterilized in 500 mL cotton-plugged Erlenmeyer flasks. They were inoculated with 3 loopful of spores per flask from well sporulating Petri plate cultures. The SSF fermentation was carried out at 30 °C for 4–11 days depending on the growth intensity of the microorganism. All SSF cultures were air-dried at room temperature and stored in sealed plastic bags at 4 °C.

Extraction of the SSF materials was carried out with 0.05 M citrate buffer at pH 5 in the presence of 1 g/L non-ionic surfactant (Tween-80), at 30 °C on a rotary shaker at 220 rpm, for 2 h. The solid-to-liquor ratio was 1:20. After the extraction, the slurry was centrifuged at 8500 rpm for 15 min. The crude and clear enzyme extracts were stored at −20 °C until use.

Surface morphology of the SSF samples was investigated on a JEOL 5500 LV scanning electron microscope in high vacuum mode with a secondary electron detector. The accelerating voltage was 20 kV and the working distance 20–21 mm. The samples were fastened to the copper sample holder by adhesive carbon tape. The surface of the samples was coated to obtain a ca. 5 nm thick Au–Pd metal film.

2.3. Enzyme activity measurements and protein assays

For the production of enzyme of the fungi, eight different enzyme activities were assayed by internationally recognized methods. Filter paper activity (FPA) was determined as described by Ghose [21]. Endoxylanase activity (EXyl) was measured by the colorimetric dinitrosalicylic acid assay applying birch glucuronoxylan (Carl Roth GmbH, Karlsruhe, Germany) as a substrate [22]. The 1,4-β-D-glucosidase activity (βGluc) was determined by using p-nitrophenyl-β-D-glucopyranoside (Sigma–Aldrich. USA) as a substrate [23]. 1,4-β-D-Endoglucanase (EGluc) production was measured by using hydroxyethylcellulose (Fluka) as a substrate [24]. Polygalacturonase (PGal) activity was determined by using polygalacturonic acid (Sigma–Aldrich, USA) as a substrate according to the Minjares-Cassanco method [25]. Laccase (Lac) activity was assayed by using ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonicacid)] (Sigma–Aldrich, USA) as a substrate [26]. Determination of lignin peroxidase (LiP) activity was based on the oxidation of veratryl alcohol to veratraldehyde [27]. The manganese-dependent peroxidase (MnP) activity was measured by the oxidation of phenol red [9].