JOURNAL OF ENGINEERING annals OF FACULTY OF ENGINEERING HUNEDOARA

PRODUCTION OF CELLULOLYTIC ENZYMES ON AGRICULTURAL WASTE BY DIFFERENT ZYGOMYCETES

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Abstract:
Filamentous fungi are good producers of different extracellular enzymes. Due to this feature, some of them assumed to play an important role in the decomposition of plant and other organic materials. Several members of the class Zygomycetes are involved in different biotechnological applications, in consequence of their efficient extracellular enzyme production. Because of the increasing interest in the microbial biodegradation, isolates of *Mucor corticolus* (syn.: *M. circinelloides* f. *corticolus*) and *Gilbertella persicaria* were screened for their secreted cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase) and beta-glucosidase activities. These enzymes are key-players in the microbiological degradation of cellulose biomass. The aim of the present study was to evaluate the production of these cellulolytic enzymes on a cheap, exploitable agricultural waste. To investigate the production of cellobiohydrolase and beta-glucosidase, solid-state fermentations were performed on chopped corn-stalks and corn leaves as carbon source. Cultures incubated at 25°C for 12 days were repeatedly sampled to monitor changes in the enzyme activities. Results show that isolates of both species showed intensive growth on these substrates, and high activities of the investigated enzymes were observed during the fermentation period. The potential application of these fungi for biodegradation and enzyme production is discussed.

Keywords:
Gilbertella, *Mucor corticolus*, solid-state fermentation (SSF), cellobiohydrolase, beta-glucosidase

1. INTRODUCTION

Corn is a major crop in the eastern European countries and therefore large amount of corn stalk arise as agricultural waste. Bioconversion may convert corn stalk to ethanol, which is a clean burning fuel and chemical feedstock. Utilization of this resource requires hydrolyzes of cellulose to fermentable reducing sugars in the first step. Cellulases, responsible for the hydrolysis of cellulose, are complex mixture of enzyme proteins with different specificities to hydrolyze the β-1,4-glycosidic linkages bonds. Three major enzyme activity classes are found in the cellulose enzyme complex [5]: endoglucanases (EC 3.2.1.4), cellobiohydrolases (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91) and beta-glucosidases (β-D-glucoside glucohydrolase; EC 3.2.1.21). Cellobiohydrolase is the major component of the fungal cellulase systems accounting for 40–70% of the total cellulase proteins [4]. Cellobiohydrolases remove monomers and dimers from the end of the glucan chain. Beta-glucosidase hydrolyzes glucose dimers and in some cases cellulose oligosaccharides to glucose [2].

Zygomycetes fungi are widely distributed in soil and plant debris, on dung and other moist organic matter in contact with soil. Some species cause fungal rots, especially in fruits and vegetables, while others are important as spoilage microorganisms of certain foods. Several members of this fungal group are well known from biotechnological applications in consequence of its effective extracellular enzyme production [13-16]., e.g. mainly proteases and lipases [12, 1]. Solid-state fermentation (SSF) is a process carried out in the absence or

near absence of any fluid in the space between particles [9]. In comparison with other processes used for enzyme production, SSF has the advantage that it allows the usage of solid agricultural and agro-industrial residues as a substrate for microbial growth [10, 11]. Such residues have yielded good results in the production of cellulases and xylanases [3, 8]. Currently, the rapidly evolving biotechnological applications require the isolation and characterization of new cellulose-degrading microorganisms. The aim of the present study was to investigate the production of cellulolytic enzymes by *Gilbertella persicaria* and *Mucor corticolus* on corn stalks and corn leaves as sole carbon source.

2. THE STUDY

Strains and culture conditions. In this study, *Gilbertella persicaria* (G1) and *Mucor corticolus* (M21; syn.: *M. circinelloides* f. *corticolus*) strains were used. Isolates were maintained on malt extract agar slants (0.5% malt extract, 0.5% yeast extract, 0.5% glucose, 1% KH_2PO_4, 1.5% agar) at 4 °C. For the solid-state fermentation, Cultivation of fungi was performed in 250 ml Erlenmeyer flasks; the culture medium contained 5 grams of chopped corn stalks and corn leaves moistened with 5 ml distilled water. Autoclaved media were inoculated with 10^6 spores and incubated at 25 °C for 12 days.

Sample preparation: Fungal cultures were extracted with 50 ml distilled water at 4 °C for 3 h. After filtration, extracts were centrifuged (10.000 x g, 20 min, 4 °C) and the supernatant was assayed for enzymatic activities.

Enzyme assay: Beta-glucosidase and cellobiohydrolase activities were measured using the appropriate *p*-nitrophenyl-β-D-glucopyranoside (pNPG, Sigma) and *p*-nitrophenyl-β-D-cellobioside (pNPC, Sigma) as substrates. Reaction mixture consisted of 0.1 ml of 7 mM substrate, 0.8 ml of sodium acetate buffer (pH 5.0) and 0.1 ml of crude extract. After incubation at 50 °C for 30 min, the reaction was stopped with 2 ml of 0.1 M sodium carbonate. The released *p*-nitrophenol was measured spectrophotometrically (DU®-65, BECKMAN) at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of *p*-nitrophenol per min under the described assay conditions.

3. ANALYSIS AND DISCUSSION

The fungal isolates used in this study were selected in previous experiments in which wheat bran was used as substrate. The present assay used corn stalks, corn leaves as carbon source, and strains were grown for 12 days at 25 °C: enzyme activities were determined from the crude water extracts obtained every second day. Both isolates showed intensive growth on these substrates, but they revealed high differences in the production of the cellulolytic enzymes. Extracellular beta-glucosidase activities of these fungi were found to be higher than their cellobiohydrolase activities; similar observation was recorded for mixed cultures of *Aspergillus ellipticus* and *A. fumigatus* in solid-state fermentation [6].

Cellulolytic enzyme production of *M. corticolus* are shown in Fig. 1. The highest beta-glucosidase and cellobiohydrolase activities were reached on the twelfth day after the inoculation (10.4 U/ml and 2.5 U/ml, respectively). Amounts of both enzymes were permanently increased during the fermentation period, and remarkable rises in the activities were detected at the tenth and twelfth culturing day. It is worth to mention that beta-glucosidase activity of *M. corticolus* used in the present analysis is comparable to those of a *Trichoderma viride* wild type and a mutant strain reported by a recent study [7]. These isolates produced the enzyme within a range of 4-15 U/ml on wheat bran.

In contrast to *M. corticolus*, *G. persicaria* had the maximum yield of both enzymes on the eighth day of the cultivation (Fig. 2). In this fermentation, the highest value for beta-glucosidase and cellobiohydrolase activity was 3.1 U/ml and 1 U/ml, respectively. Further increase of the incubation period resulted in decreased enzyme production. It is supposed that the lower extracellular enzyme production led to the reduction in the enzyme activities at *G. persicaria*, and longer fermentation period is required to produce higher amounts of cellulolytic enzymes in case of *M. corticolus* on corn-stalks as substrate.

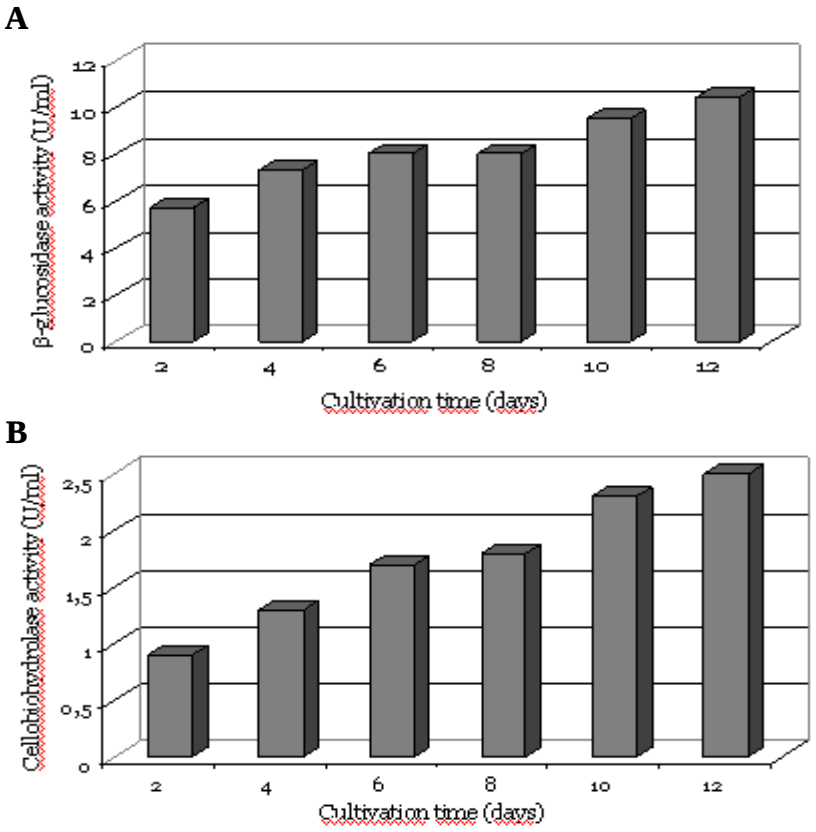

Figure 1. Time course profiles of beta-glucosidase (A) and cellobiohydrolase (B) production by *M. corticolus* by using corn-stalks and corn leaves alone as substrate.

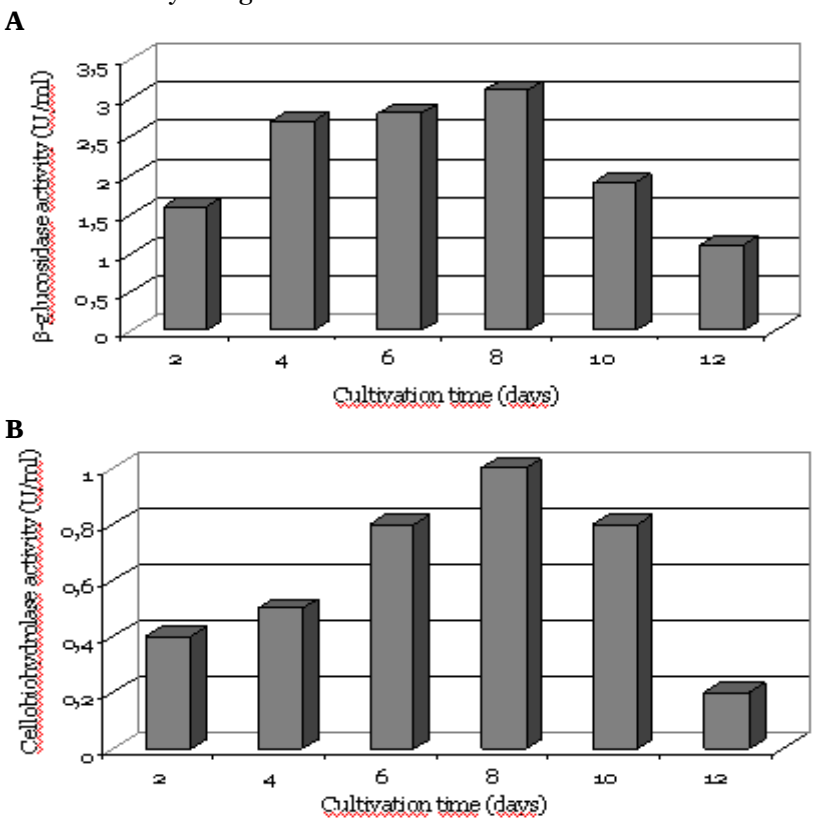

Figure 2. Time course profiles of beta-glucosidase (A) and cellobiohydrolase (B) production by *G. persicaria* by using corn-stalks and corn leaves alone as substrate.

ACKNOWLEDGEMENTS

This research was supported by ETT grants (214/2006; 261/2006) and the J. Bolyai Research Scholarship of the Hungarian Academy of Sciences.

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