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UTILIZATION OF OILSEED RESIDUES AND OAT BRAN AS SUBSTRATES FOR β-GLUCOSIDASE PRODUCTION BY ZYGOMYCETOUS FUNGI

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ABSTRACT

Utilization of agro- and food-industrial residues has special importance today. One possible way of the applications is the production of industrially interesting microbial hydrolases, for which, solid-state fermentation is a low-cost and environmental friendly biotechnological technique. Since most filamentous fungi are able to biodegrade the cellulosic content of these natural materials, high-yield production of extracellular β-glucosidases can be obtained during the fermentation. In this study, zygomycetous fungal strains representing *Rhizomucor miehei*, *Rhizopus stolonifer*, *Mucor corticolus*, *Mortierella echinosphaera* and *Umbelopsis autotrophica* were grown in solid cultures containing various oilseed residues and oat bran as carbon sources to assess the production of their β-glucosidases. Among the oilseed residues, pumpkin seed proved to be the best inductor for β-glucosidase activity. Enzyme production of *R. miehei* and *U. autotrophica* was further enhanced when oat bran was used as support. Effect of minerals salts on the enzyme yield was also assayed, in which β-glucosidase production of the investigated strains was generally stimulated after moisturizing the substrates by mineral salt solution.

Keywords: plant residues, filamentous fungi, solid-state fermentation, extracellular β-glucosidase

INTRODUCTION

Agricultural and food industrial processes produce large amount of by-products and waste materials which can be utilized for various microbiological-biotechnological applications. Besides the bioethanol preparation (SARKAR ET AL., 2012), high-yield production of microbial enzymes is also useful direction to exploit these substrates efficiently. Since filamentous fungi can produce a variety of hydrolases (e.g. cellulases, lipases, proteases), fermentation using these microorganisms is a well applicable method for decomposing the plant-derived materials. As part of the cellulase enzyme system, β-glucosidases (EC 3.2.1.21) play crucial role in the enzymatic hydrolysis of cellulosic content (GAO ET AL., 2013; SINGHANIA, 2009). These enzymes catalyze the hydrolysis of cellodextrines as well as the endo- and exoglucanase inhibitor cellobiose to glucose (LYND ET AL., 2002). The hydrolyzing activity of β-glucosidases can be utilized in various applications, such as enzymatic degradation of olive wastewater, or liberation of aroma and antioxidative phenolic compounds from plant-derived products (KHOUFI ET AL., 2011; ACOSTA-ESTRADA ET AL., 2014). Under certain conditions, β-glucosidases are able to transfer glycosyl groups to saccharides and alcohols resulting in the formation of pharmaceutically important oligosaccharides, alkyl-glycosides, and different glycoconjugates (SMAALI ET AL, 2007).

Zygomycetes fungi are a remarkable group of filamentous fungi. Many species, especially those belonging to the order Mucorales, have successfully been used for production of cellulase enzymes (FERREIRA ET AL., 2013). However, compared to other filamentous

fungal group, we know little about how these enzymes are induced and formed in solid conditions using plant-derived residues. In our previous work, β-glucosidase activity of several zygomycetes grown in liquid and solid media was measured and some strains showed intensive extracellular enzyme activity on wheat bran (TAKÓ ET AL., 2010). This study presents the β-glucosidase production of zygomycetes strains using oilseed residues such as hempen-, line-, poppy- and pumpkin-seed and oat bran in solid-state fermentation (SSF). Five strains of the genera *Rhizomucor*, *Rhizopus*, *Mucor*, *Umbelopsis* and *Mortierella* were tested and the effect of mineral salts and olive oil on the product yield was also assessed. To our best knowledge, *Umbelopsis* and *Mortierella* isolates have never been investigated in this regard.

MATERIAL AND METHOD

Fungal strains and media for fermentation

Rhizomucor miehei (SZMC 11005; SZMC - Szeged Microbiological Collection, Szeged, Hungary), *Rhizopus stolonifer* (SZMC 13609), *Mucor corticolus* (SZMC 12031), *Mortierella echinosphaera* (SZMC 11251) and *Umbelopsis autotrophica* (SZMC 11276) strains were used for SSF. The hempen-, line-, poppy- and pumpkin seed residue substrates were remained after extraction of the plant-seed oils (Solio Ltd.). Crude fiber content of them was determined by the producer. The oat bran was purchased in a local market (Natura Ltd.).

Culture conditions for β-glucosidase production

To investigate the production of β-glucosidases on the plant residues and under different conditions, five grams of substrate were taken in 100-ml Erlenmeyer flasks and moistened with 5 ml distilled water (medium 1) or 9.5 ml mineral salt medium (medium 2; % in w/w substrate in distilled water: 0.75% $(NH_4)_2SO_4$, 0.34% NH_2CONH_2, 1.8% NaH_2PO_4, 0.3% KH_2PO_4, 0.045% $MgSO_4$ x $7H_2O$, 0.0375% $CaCl_2$). The culturing media were autoclaved, and the flasks were inoculated with a spore suspension containing 10^6 sporangiospores/ml. Cultures were grown for 7 days at desired temperatures depending on the culturing requirements of the tested strain (20 °C: *U. autotrophica*, 25 °C: *M. corticolus*, *Rh. stolonifer*, *M. echinosphaera*, 37 °C: *R. miehei*). All fermentation tests were carried out in three independent experiments.

Extraction and determination of β-glucosidase activity

Preparation of crude samples was carried out by an extraction with 30 ml of 0.1 M sodium acetate buffer (pH 6.0) at 4 °C for 24 h. The extracts were filtrated and then centrifuged at 16.200*g* for 15 min. The resulted clear supernatants were designated as crude extracts and used for enzyme activity assay. The β-glucosidase activity was determined by using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG; Sigma) as substrate in a reaction mixture containing 180 µl diluted enzyme solution and 20 µl of 7 mM *p*NPG. Reaction mixtures were incubated at 50 °C for 30 min and the reaction was stopped by adding 50 µl of 10% (w/v%) Na_2CO_3. *Para*-nitrophenol release was monitored at 405 nm in 96-well microtiter plates using an ASYS Jupiter HD microplate reader (ASYS Hitech). One unit of β-glucosidase activity (U) was defined as the amount of enzyme that released 1 µmol of *p*-nitrophenol per min under assay conditions. Enzyme activity values were expressed in U/g dry weight substrate (U/gds).

Statistical analysis

Standard deviations of means were performed with Microsoft Excel software from the Microsoft Office package. Enzyme activity values are averages counted from three independent measures.

RESULTS

Solid-state fermentation is a process when growth/cultivation of microorganisms is performed on water insoluble or sparingly water-soluble materials. Since solid medium could simulate the natural habitat of filamentous fungi, this fermentation technique is frequently used to produce inducible enzymes (MITCHELL and BEROVIČ, 2006). In this study, β-glucosidase production of five filamentous fungal strains was investigated in solid cultures using various oilseed residues and oat bran. The *R. miehei* and *M. corticolus* strains were selected on the basis of our previous screening researches (TAKÓ, 2011). The *Rh. stolonifer*, *M. echinosphaera* and *U. autotrophica* isolates showed intensive growth on these substrates in a recent assay (KOTOGÁN ET AL., 2013).

Detection of β-glucosidase activity on oilseed residues

Hempen-, line-, poppy- and pumpkin seed residues were used as substrates and moistened with distilled water (medium 1) or mineral salt solution (medium 2). The results in *Table 1* provide a summary of the β-glucosidase production obtained for each substrate and isolate. The tested isolates exhibited various enzyme yields during the fermentation tests; however, results showed that these plant residues are potential substrates for β-glucosidase production by the investigated fungi. The *R. miehei* and *M. corticolus* isolates proved to be the best producers on these substrates presenting from 1.32 to 8.16 and 0.99 to 4.75 U/gds of β-glucosidase, respectively. In general, enzyme yields were positively influenced by the addition of mineral salt solution as support; especially, in case of the *M. corticolus* isolate, at which the product yield has been enhanced for each substrate. At least 1.15 fold increases in the β-glucosidase activity could be detected by this strain.

Table 1. β-Glucosidase yield of the tested zygomycetes fungi on oilseed residues moisturized with distilled water (medium 1) or mineral salt solution (medium 2)

Substrate	β-Glucosidase activity (U/g dry substrate) [a]				
	Rhizomucor miehei	*Rhizopus stolonifer*	*Mucor corticolus*	*Umbelopsis autotrophica*	*Mortierella echinosphaera*
Hempen seed					
medium 1	4.19 ± 0.15 [b]	0.038 ± 0.003	1.29 ± 0.08	0.56 ± 0.01	0.013 ± 0.001
medium 2	4.58 ± 0.32	0.050 ± 0.003	4.75 ± 0.16	0.95 ± 0.03	0.017 ± 0.001
Poppy seed					
medium 1	6.90 ± 0.29	0.009 ± 0.001	0.99 ± 0.08	0.26 ± 0.01	0.005 ± 0.001
medium 2	1.32 ± 0.11	0.021 ± 0.001	1.15 ± 0.02	0.57 ± 0.03	0.004 ± 0.001
Line seed					
medium 1	2.50 ± 0.21	0.056 ± 0.007	2.11 ± 0.11	1.16 ± 0.06	0.034 ± 0.003
medium 2	4.69 ± 0.27	0.047 ± 0.006	2.69 ± 0.15	0.88 ± 0.06	0.033 ± 0.001
Pumpkin seed					
medium 1	3.72 ± 0.23	0.036 ± 0.002	1.10 ± 0.04	0.87 ± 0.09	0.041 ± 0.002
medium 2	8.16 ± 0.79	0.069 ± 0.001	1.51 ± 0.08	1.18 ± 0.03	0.032 ± 0.003

[a] Activities presented were measured at the 7th day of the fermentation.
[b] Values are averages calculated from the data of three independent measurements ± standard deviation.

Interestingly, there was no general correlation between the β-glucosidase activities developed on each substrate and the crude fibre content of the seeds (*Table 2*). Except in the case of *M. corticolus*, maximum β-glucosidase production could be recorded on pumpkin seed residue, which contains the lowest amount of crude fibre (3-3.5%) among the oilseed materials. One possible explanation for this phenomenon may be that the crude fibre content of this substrate is more accessible for the exoglucanases and endoglucanases produced by the fungi. To avoid the end-product inhibition effect of cellobiose (MURPHY ET AL, 2013), higher yield of β-glucosidase is required. Additionally, since certain carbohydrates (e.g. glucose, cellobiose) have strong inhibitory effect on β-glucosidases (EYZAGUIRRE ET AL., 2005), enzyme activity may also be influenced by the sugar content of the plant-seed substrates. The producer indicated up to 5 and 5.5% sugar and starch content in case of the hempen seed and poppy seed residues, respectively. The slight lower enzyme activity by *Rh. stolonifer*, *U. autotrophica* and *M. echinosphaera* on these substrates may be due to the inhibitory effect of the carbohydrates presented in the crude extract. Because of moderate tolerance to glucose has been reported for *R. miehei* and *M. corticolus* β-glucosidases (KRISCH ET AL., 2010), these enzymes could exhibit considerable activity at higher sugar concentrations. Another explanation for the variable yields would be the different extractive (e.g. flavonoids, tannins, terpenes, etc.) content of the substrates which may cause, for example, microbial growth inhibition and fibre hygroscopicity reduction (ANG ET AL., 2013); however, chemical composition analysis of the oilseed residues has not been performed.

Table 2. Crude fibre content of the oilseed residues (as given by the producer)

Oilseed residue	Crude fibre content (w/w %)
Hempen seed	13.2
Poppy seed	10
Line seed	9-10
Pumpkin seed	3-3.5

Detection of β-glucosidase activity on oat bran

While the enzyme activity of the other strains obtained on oat bran was comparable to that presented on oilseed residues, *R. miehei* was outstanding in its β-glucosidase activity using this substrate (*Table 3*). The maximum 30.39 U/gds β-glucosidase activity recorded in this research was 2.5 and 6.7 fold higher compared to the enzymes produced by *Aspergillus fumigatus* on wheat straw (SHERIEF ET AL., 2010) and *A. fumigatus* SK1 on oil palm trunk (ANG ET AL., 2013), respectively. However, the presented enzyme activity is less significant as compared with our previous study in which 229.8 U/gds product yield could be reached for the same isolate using wheat bran based SSF (TAKÓ ET AL., 2010).

Table 3. β-Glucosidase yield of the tested zygomycetes fungi on oat bran moisturized with distilled water (medium 1) or mineral salt solution (medium 2)

	β-Glucosidase activity (U/g dry substrate) [a]				
	Rhizomucor miehei	*Rhizopus stolonifer*	*Mucor corticolus*	*Umbelopsis autotrophica*	*Mortierella echinosphaera*
medium 1	26.04 ± 0.52 [b]	0.048 ± 0.002	1.19 ± 0.05	1.85 ± 0.03	0.038 ± 0.001
medium 2	30.39 ± 0.55	0.068 ± 0.001	1.69 ± 0.04	3.35 ± 0.11	0.027 ± 0.001

[a] Activities presented were measured at the 7th day of the fermentation.
[b] Values are averages calculated from the data of three independent measurements ± standard deviation.

Using oat bran as substrate, *U. autotrophica* was able to produce β-glucosidase with higher yield compared to other research on oilseed residues (see *Table 1* and *Table 3*). The exhibited 3.35 U/gds enzyme activity is significant since it was 3 fold higher than to that presented by *M. corticolus* which had been identified as strong β-glucosidase producer in our previous examinations on wheat bran (TAKÓ, 2011). It is worth to mention that there have been no previous studies on *Umbelopsis* species for their β-glucosidase activity. Activity data presented here suggest that it would be advantageous to screen these fungi for β-glucosidase production in future researches.

CONCLUSIONS

Successful production of industrially important microbial enzymes on agro- and food-industrial residues is a promising and eco-friendly approach to utilize these materials. Here, we studied the feasibility of some oilseed residues and oat bran to produce high level β-glucosidases through solid-state fermentation using zygomycetes. The investigated five fungal strains grow well during the cultivation, and they can be used for efficient utilization of these plant residues for production of extracellular β-glucosidases. In case of most fungi, pumpkin seed proved to be the best substrate for enzyme production; maximum enzyme activity was achieved by *R. miehei*. It can be concluded that the β-glucosidase yield generally increased by moisturizing of the substrate with mineral salt solution. This study also highlighted the potential of *U. autotrophica* for β-glucosidase production.

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