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IDENTIFICATION OF ACID- AND THERMOTOLERANT EXTRACELLULAR β-GLUCOSIDASE ACTIVITIES IN *ZYGOMYCETES FUNGI*

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Extracellular β-glucosidase activity of 94 strains, representing 24 species of the genera *Gilbertella, Mucor*, *Rhizomucor*, and *Rhizopus* was evaluated in submerged culture and under solid state fermentation on wheat bran. *Gilbertella persicaria* G1 isolate showed the highest activity (70.9 U ml^{-1}) followed by other *Gilbertella* (58.6–59.0 U ml^{-1}) and *Rhizomucor miehei* isolates (29.2–42.0 U ml^{-1}). Optimum temperature for enzyme production was 25 °C for *Gilbertella* and *Mucor*, and 30 °C for *Rhizomucor* and *Rhizopus* strains. Enzymes of *R. miehei* strains proved to be thermotolerant preserving up to 92.8% residual activity after heating to 75 °C in the presence of cellobiose substrate. Enzymes of *Mucor racemosus* f. *chibinensis, R. miehei* and *Rhizopus microsporus* var. *oligosporus* strains were activated at acidic condition (pH 4). Glucose was a strong inhibitor for each fungal β-glucosidase tested but some of them showed ethanol tolerance up to 20% (v/v). Ethanol also activated the enzyme in these strains suggesting glycosyl transferase activity.

Keywords: Gilbertella – Rhizomucor – Rhizopus – Mucor – β-glucosidase

INTRODUCTION

β-Glucosidases (β-D-glucoside glucohydrolases; EC 3.2.1.21) catalyze the hydrolysis of alkyl and aryl β-glycosides as well as disaccharide glycosides and oligosaccharides of glucose.

The interest in β-glucosidases has been rejuvenated in the last few years because of their great potential in several biotechnological processes from liberating flavours, aromas and isoflavone aglycons to the synthesis of oligosaccharides and alkyl glycosids [3]. Oligosaccharides can be used as therapeutic agents, diagnostic tools, and growth promoting agents for prebiotic bacteria. Alkyl glycosids are non ionic surfactants with high biodegradability and have good antimicrobial properties. Their enzymatic synthesis by the transglycosilation or glycosyltransferase activity of glycoside hydrolases can be performed in one step instead of the several protections – deprotection steps required in chemical synthesis [9, 16].

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Fungal β-glucosidases are generally parts of the cellulose degrading enzyme system, therefore their application for the conversion of cellulose waste material to glucose is an intensively studied area. In winemaking, β-glucosidases play a key role in the enzymatic release of aromatic compounds from glycosidic precursors present in fruit juices, musts and wines [6].

Isoflavone aglycons found in soy-based foods can help preventing several chronic diseases. Production of such foods and beverages from soybeans by fermentation with β-glucosidase-producer strains for the liberation of the aglycons is highly desired [9, 10]. Screening for good β-glucosidase-producer strains focuses on glucose tolerance, acid resistance, high hydrolyzing activity and possible glycosyltransferase and transglycosylase activities. Glucosidases that are acid active and insensitive to glucose are important in the fragrance, aroma, food and beverages industry, in the production of fuel ethanol from cellulosic materials and also in the synthesis of pharmaceuticals [17].

Representatives of the Zygomycetes are widely distributed in soil, in plant debris, on dung and other moist organic matter in contact with soil. Some species cause fungal rots, especially in fresh 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 due to efficient production of extracellular enzymes, mainly proteases and lipases [1, 12, 15].

Several fungal glucosidases have been purified and analyzed [3], but Zygomycetes are poorly characterized from this aspect. Until date, β-glucosidases have been purified only from *Mucor racemosus* [4], *Mucor (Rhizomucor) miehei* [19] and *Rhizopus oryzae* [18]. A linamarase from a *M. circinelloides* strain and its detoxifying activity on plant cyanogenic substrates was also described [13].

In this study, 94 fungal strains belonging in the Zygomycetes were screened for their extracellular β-glucosidase activity to find new producer strains, potentially applicable in further basic studies and biotechnological applications.

MATERIALS AND METHODS

Strains

Isolates of *Gilbertella persicaria* (16), *Rhizomucor miehei* (9), *R. pusillus* (17), *Rhizopus microsporus* var. *oligosporus* (1), *Rh. microsporus* var. *rhizopodiformis* (2), *Rh. oryzae* (13), *Rh. stolonifer* (21) and one isolate of *Mucor circinelloides* f. *jansenii*, *M. circinelloides* f. *lusitanicus*, *M. guillermondii*, *M. indicus*, *M. microsporus*, *M. minutus*, *M. nederlandicus*, *M. psychrophilus*, *M. racemosus* f. *chibinensis*, *M. racemosus* f. *sphaerosporus*, *M. strictus*, *M. amphibiorum*, *M. azygosporus*, *M. fragilis* and *M. zonatus* were screened for extracellular β-glucosidase activity. Fungal isolates involved in the study were deposited in the Szeged Microbiology Collection (SZMC, Szeged, Hungary, http://www.sci.u-szeged.hu/microbiology/).

Submerged culture

Of each isolate, 5×10^5 spores were inoculated in 15 ml liquid minimal medium (0.15% $(NH_4)_2SO_4$, 0.15% Na-L-glutaminate, 0.05% yeast nitrogen base), supplemented with 1% cellobiose (Sigma) for β-glucosidase activity induction. Cultivation was performed under continuous shaking (200 rpm) for six days at 25 or 37 °C depending on the culturing requirements of the tested strains. For sample preparation, every day, 500 µl samples were collected and filtered to remove insoluble particles. The filtrates were centrifuged at 13,000 rpm for 30 min and the supernatant was used for enzyme activity measurements (diluted 1 : 10 with distilled water if necessary).

Solid-state fermentation

Five grams of wheat bran and 5 ml distilled water were mixed in a 100 ml Erlenmeyer flask, autoclaved, inoculated with 10^6 spores, and incubated at different temperatures (e.g. 25, 30, 40 and 50 °C) for six days. For sample preparation, every day, an Erlenmeyer flask was taken and extracted with 30 ml distilled water at 4 °C for 3 h. After filtration of the extract, 1 ml filtrate was centrifuged at 13,000 rpm for 10 min. The supernatant was diluted to 1 : 1000 with distilled water and analyzed for total protein concentration and β-glucosidase activity.

Glucosidase activity measurement

β-glucosidase activity was determined by *p*-nitrophenyl-β-D-glucopyranoside (PNPG, Sigma). Twenty µl of 7 mM PNPG was added to 180 µl diluted extract, and incubated for 30 min at 50 °C. The reaction was stopped by 50 µl of 0.1 M sodium carbonate, and the *p*-nitrophenol release was measured at 405 nm. One enzymatic unit was defined as the amount of enzyme that releases 1 µmol of *p*-nitrophenol in 1 minute under the assay conditions. Enzyme activities were measured in 96-well microtiter plates using an ASYS Jupiter HD (ASYS Hitech) microplate reader.

Protein assay

Total protein content in the correspondingly diluted extracts was determined by means of a Qubit™ Fluorometer (Invitrogen) according to the manufacturer's recommendation. The determination was done in triplicate on each sample and the mean was reported.

Table 1
Fungal strains showing significant β-glucosidase activities in submerged culture and after solid-state fermentation on wheat bran

Isolate	Code[a]	Original code[b]	Volumetric activity		Yield		
			U ml^{-1}		U g^{-1} cellulosic substrate		U g^{-1} wheat bran
			Submerged culture	Solid state fermentation	Submerged culture	Solid state fermentation	Solid state fermentation
G. persicaria	G1	ATCC 201107	2.54	70.9	254	3864	425.4
G. persicaria	G2	ATCC 201108	2.74	59	274	3215	354
G. persicaria	G14	SZMC 11098	3.16	58.6	316	3193.7	351.6
M. amphibiorum	M81	CBS 763.74	1.04	1	104	54.5	6
M. azygosporus	M82	CBS 292.63	0.77	1.6	77	87.2	9.6
M. fragilis	M13	CBS 236.35	0.85	41.6	85	2267.2	249.6
M. guillermondii	M83	CBS 174.27	1.78	1	178	54.5	6
M. racemosus f. *chibinensis*	M84	CBS 636.67	0.24	6.2	24	337.9	37.6
R. miehei	C15	SZMC 11029	1.25	29.7	125	1618.7	178.6
R. miehei	R8	NRRL 5282	3.70	38.3	370	2087.4	229.8
R. miehei	R11	NRRL 5901	0.46	29.2	46	1591.4	175.2
R. miehei	R17	ETH M4918	2.15	42	215	2289	252
Rh. microsporus var. *oligosporus*	Rh31	NRRL 2710	0.85	18.4	85	1002.8	110.4
Rh. oryzae	Rh28	NRRL 2908	28.15	17.8	2815	970.1	106.8
Rh. stolonifer	Rh40	CBS 403.51	9.92	15.1	992	823	90.6

Activities presented were measured at the 6th day of fermentation except for *M. azygosporus* (M82) and *M. fragilis* (M13) measured at the 4th and the second day, respectively. In the liquid cultures, fungi were grown at their optimum temperatures that was 25 °C for all *Gilbertella* and *Mucor* isolates, *Rh. oryzae* Rh 28 and *Rh. stolonifer* Rh40 and 37 °C for all *Rhizomucor* isolates and *Rh. microsporus* var. *oligosporus* Rh31. In case of the solid-state fermentations, the highest activities are shown irrespectively from the culturing temperature. [a]A code which is used throughout this paper for clarity. [b]Abbreviations: ATCC, American Type Culture Collection, CBS, Centraalbureau voor Schimmelcultures, ETH, Swiss Federal Institute of Technology Culture Collection, NRRL, Agricultural Research Service Culture Collection; SZMC, Szeged Microbial Collection.

Effect of glucose and ethanol on the extracellular β*-glucosidase activity*

The standard reaction mixture contained 0.1 ml of 7 mM PNPG, 0.8 ml sodium acetate buffer (pH 5), 0.1 ml crude enzyme extract, and different concentrations of the appropriate inhibitor. The measurement was done as described above.

Effect of acidic pH and heat treatment on enzyme activity

To identify the β-glucosidases active under acidic pH, residual activity in 100 mM acetate buffer at pH 4 was determined as percentage of the activity measured at pH 6. Heat treatment was performed heating the crude enzyme extract in the absence or presence of cellobiose (5 mM) substrate to 75 °C for 5 min. After cooling the solution to room temperature, enzyme activities were determined as described above.

RESULTS

Screening of Zygomycetes for β*-glucosidase activity*

Among the 94 isolates tested, 15 strains showed significant extracellular β-glucosidase activity in submerged culture and/or in solid-state fermentation (Table 1). Isolates showing less than 1 U ml^{-1} volumetric activity in solid state culture were excluded from further investigations. Enzyme activities in solid-state fermentation were higher than in liquid culture, except for *M. amphibiorum* (M81)*, M. guillermondii* (M83) and *Rh. oryzae* (Rh28).

In case of 10 isolates, effect of cultivation temperature on enzyme production was also tested (Fig. 1). Isolates of *R. miehei* were thermotolerant; while they showed scant activity growing on wheat bran at 20 °C; only they were able to produce the enzyme in considerable amount at 50 °C.

Effect of temperature on enzyme activity

Heating the crude extracts to 75 °C for 5 min resulted in activity loss up to 100% in case of the *Mucor* and *Gilbertella* strains (Table 2). The highest residual enzyme activity was observed in the extract of the *R. miehei* R8 strain (29.1%). It is worth to mention that enzymes of the *R. miehei* strains R8 and R17 proved to be thermostable at 60 °C preserving 100% of their activity after the heat treatment (data not shown).

Since the substrate may protect an enzyme from heat inactivation by retaining the correct protein conformation, heat treatments were performed again in the presence of 5 mM cellobiose (Table 2). In this system, the enzyme extracts of *R. miehei* strains proved to be thermotolerant preserving up to 92.8% residual activity.

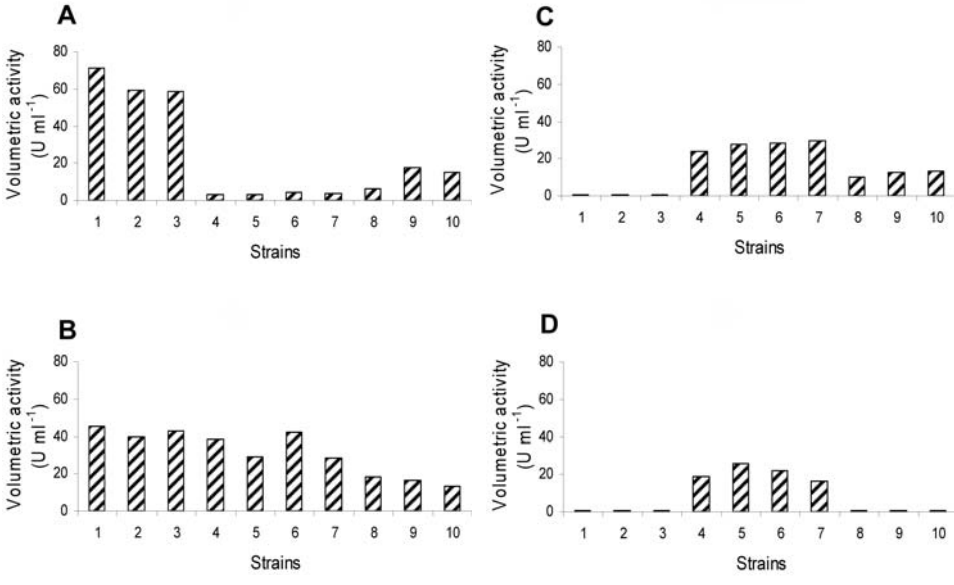

Fig. 1. Comparison of the highest extracellular β-glucosidase production of 10 zygomycetes in solid-state fermentation on wheat bran at different temperatures (A: 25 °C; B: 30 °C; C: 40 °C; D: 50 °C). 1 – *G. persicaria* G1; 2 – *G. persicaria* G2; 3 – *G. persicaria* G14; 4 – *R. miehei* R8; 5 – *R. miehei* R11; 6 – *R. miehei* R17; 7 – *R. miehei* C15; 8 – *Rh. microsporus* Rh31; 9 – *Rh. oryzae* Rh28; 10 – *Rh. stolonifer* Rh40

Effect of glucose and ethanol on enzyme activity

Glucose substantially inhibited the enzyme activity of the *Gilbertella, Rhizopus* and *Rhizomucor* strains (Table 2). Some *Mucor* isolates produced enzymes that proved to be more tolerant to glucose preserving up to 30.7% residual activity. Addition of ethanol to 15% (v/v) increased the enzyme activity in cases of all *R. miehei* strains suggesting glycosyltransferase activity of the enzyme. Effect of ethanol on the enzyme activities in various concentrations can be seen in Fig. 2. Enzymes of *M. fragilis* (M13), some *Rhizopus,* and all *R. miehei* strains were activated by ethanol.

DISCUSSION

In this study, several strains belonging to the genera *Gilbertella*, *Mucor*, *Rhizomucor*, and *Rhizopus* have been examined to identify good β-glucosidase producers.

Solid-state fermentation generally resulted in significantly higher enzyme activities than the liquid cultures. Growth in solid-state culture is closer to natural conditions and enzyme secretion resembles enzyme production under natural conditions.

Table 2
Effect of glucose, acidic pH and heat treatment on the enzyme activity of the isolates. Residual activity is given as percentage of the untreated control activity (100%)

Isolate	Glucose[a]	pH 4[b]	75 °C[c]	75 °C[d]
G. persicaria (G1)	8	43.3	2.9	2.2
G. persicaria (G2)	7.5	44.1	0.5	7.5
G. persicaria (G14)	8.3	44.2	1.0	0.0
M. amphibiorum (M81)	25	79.4	5.7	12.8
M. azygosporus (M82)	22	62.4	6.4	0.0
M. fragilis (M13)	30.2	98.4	2.1	0.7
M. guillermondii (M83)	30.8	27.0	7.4	13.3
M. racemosus f. *chibinensis* (M84)	15.7	119.6	6.8	0.36
R. miehei (C15)	13	89.6	10.7	92.8
R. miehei (R8)	13.8	79.7	29.1	60.0
R. miehei (R11)	12.8	105.6	7.5	82.8
R. miehei (R17)	13.9	113.9	4.4	79.8
Rh. microsporus v. *oligosporus* (Rh31)	14.7	104.2	8.0	26.1
Rh. oryzae (Rh28)	13.5	88.5	10.5	11.1
Rh. stolonifer (Rh40)	6.2	45.1	12.6	8.1

[a]Glucose concentration: 10 mg ml^{-1}. [b]Residual activity is given as percentage of the activity at pH 6. [c]Crude enzyme extract was heated for 5 min. [d]5 mM cellobiose was added to the crude extract and heated together for 5 min.

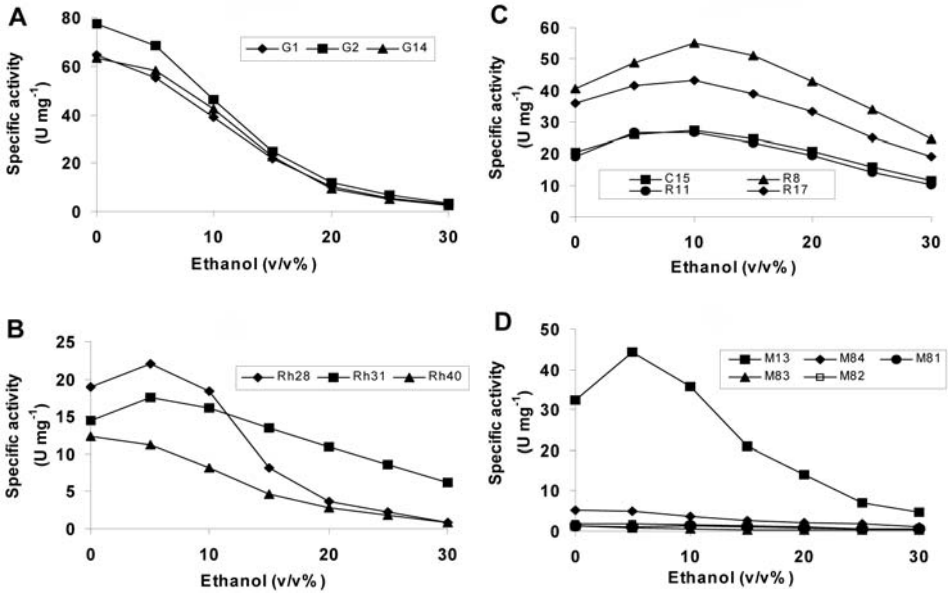

Fig. 2. Specific activities of β-glucosidase enzymes of *Gilbertella* (A), *Rhizopus* (B), *Rhizomucor* (C), and *Mucor* (D) strains after addition of ethanol in various concentrations

Under solid-state fermentation hydrolytic enzymes can be released from the catabolic repression and this may lead to high level expression of these enzymes [9]. In the case of *Aspergillus oryzae*, it is revealed that, in submerged culture, β-glucosidase is trapped in the cell wall, while it is secreted to the medium under solid-state fermentation and the glucosidase release is controlled by transcriptional regulation [11].

In a recent study, cellobiase (β-glucosidase) production of *A. niger* NIAB 280 in solid-state fermentation was investigated using different substrates, such as cellobiose, corncobs, α-cellulose and wheat bran [14]. In this system, wheat bran resulted in the highest enzyme production (35 U ml^{-1} at the fourth day), giving a product yield of 462.0 U g^{-1} substrate. It is worth to mention, that in our tests, *G. persicaria* isolates showed also high activities on the same substrate reaching similar product yields up to 425.4 U g^{-1} wheat bran (Table 1). Among the tested Zygomycetes, *Rh. oryzae* isolate showed significantly higher β-glucosidase activity in submerged culture than in solid-state fermentation. The measured 28.15 U ml^{-1} enzyme activity can be considered as significant; for comparison, enzyme activity of a *Trichoderma viride* strain was found to be 14 U ml^{-1} in submerged culture [7]. Calculating production yields on the basis of cellobiose content of liquid medium or on the cellulose content of wheat bran (11 w/w%) [2, 8], more than tenfold increase in production yield can be observed by *G. persicaria, R. miehei, M. fragilis* and *Rh. microsporus* var. *oligosporus* using wheat bran for enzyme production (Table 1).

Enzyme extracts of *G. persicaria* isolates had high volumetric activity under solid-state fermentation but showed medium acid tolerance and no glucose- and thermotolerance. *Mucor* species were characterized with low enzyme activities under the tested conditions, except *M. racemosus,* and *M. fragilis*. Glucose at 10 mg ml^{-1} concentration was less inhibitory to the enzymes of *M. fragilis* and *M. guillermondii* than to those of the other investigated strains. Glucosidases of the *Rhizopus* and *Rhizomucor* isolates showed excellent acid tolerance. Isolates of *R. miehei* were able to produce β-glucosidase even at 50 °C; moreover, in the presence of a protective substrate, these enzymes proved to be thermotolerant. The *R. miehei* (described as *M. miehei*) strain isolated from manure showed the highest activity on wheat bran also at 50 °C [19]. The same temperature was the optimum of the β-glucosidase purified from *Rh. oryzae* MIBA348 testing on CMC [18], while a linamarase from *M. circinelloides* had a temperature optimum of 40 °C [13]. All β-glucosidase producing zygomycetes known from the literature have a pH optimum of around 5 or higher with a relatively broad pH stability suggesting, that like some of our isolates, they can be used under acidic conditions in various biotechnological processes [4, 13, 18, 19]. Alcohol and acid tolerance is an important feature of enzymes used for aroma liberation in wine making.

R. miehei enzymes could be activated by ethanol suggesting that they have glucosyltransferase activity. Alcohols also activated the β-glucosidase of *Fusarium oxysporum* and catalyzed the synthesis of methyl-, ethyl-, propilglucosides [5]. We suppose that ethanol was an acceptor for glucose in these strains, decreasing the amount and hereby the inhibitory effect of free glucose.

Until now, Zygomycetes were poorly characterized from the aspect of β-glucosidase production. Our results suggest that the β-glucosidases of Zygomycetes, especially from *R. miehei,* could be potentially applicable in biotechnological processes. Further investigations will be taken for detailed characterization of the enzymes of these strains.

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