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Isolation of cellulolytic fungi from waste paper gradual recycling materials

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Recycling plants convert waste paper into pulp and subsequently into paper (cartoon), mainly tissue paper products. Materials taken from the gradual recycling process were analysed as a source of screening of high cellulase producers. The plate-clearing assay method and enzyme assay methods recommended by International Union of Pure and Applied Chemistry (IUPAC) were used in the investigation. The number of fungal isolates (cfu · g^{-1} or ml^{-1} of substrate), the percentage of cellulolytic strains in the total number of isolated fungal strains and the activities of two enzymes – filter paper cellulase (FPCase) and carboxymethyl cellulase (CMCase), were detected and analysed. Quantitative cellulase activity measurements using phosphoric acid swollen-cellulose and CMC as a carbon source, selected 26 species of the filamentous fungi as potential cellulase producers. Twelve fungal strains (from the 139 isolated) were chosen for the CMCase and FPase activity assay. Three strains of the fungi investigated – *Aspergillus niger* DPK-cl-12, *Gliomastix murorum* var. *murorum* (= *Acremonium murorum*) DPK-cl-44 and *Stachybotrys chartarum* DPK-cl-111 – were selected as potentially able to secrete fight exocellular cellulases (overall FPClase and endoglucanase – CMCase). Cellulase enzymatic activity of these fungal strains determined under study conditions were higher than those of the fungal strains of the genus *Trichoderma*, known as high cellulase producing fungi.

Key words: cellulolytic fungi, isolation, selection, endoglucanase, waste-paper, recycling

INTRODUCTION

Cellulolytic enzymes play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi, bacteria, actinomycetes and protozoa. In industry, these enzymes have found novel applications in the production of fermentable sugars and ethanol (Olson, Hahn-Hagerdahl, 1996; Levy et al., 2002; Van Wyk, Mohulatsi, 2003), organic acids (Luo et al., 1997), detergents and other chemicals (Oksanen et al., 1998). They have been used in the pulp and paper industry, e. g., in deinking of fiber surfaces and in improving pulp drainage (Oksaren et al., 2000; Suurnäkki et al., 2004), in the textile industry (Cavaco-Paulo, Gübitz, 2003; Nierstrasz, Warmoeskerken, 2003; Miettinen-Oinonen et al., 2002, 2004), animal feed (Ishikuro, 2000), and even in the food industry (Penttilä et al., 2004; Urlaub, 2002), for the processing of paper and cellophane, as well as for biotransformation of waste cellulose to fermentable sugars (Van Wyk, Mohulatsi, 2003). As lytic enzymes, they are of prime importance is the protoplast production (Davis, 1985; Mandels, 1974; Bhat, 2000).

The demand for more thermostable, highly active and specific cellulases is on the increase. Fungi are well-known agents of decomposition of organic matter in general and cellulose substrates in particular (Lynd et al., 2002). Fungal cellulases are inducible enzymes that are usually excreted into the environment (Bhat, Bhat, 1997) and depend on cellulose type (amorphous or crystalline) acting on the organism (Ortega et al., 2001). The role of the fungi *Acremonium* spp., *Chaetomium* spp., *Trichoderma reesei, Trichoderma viride, Penicillium pinophilum, Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*), *Fusarium solani, Talaromyces emersonii, Trichoderma koningii, Fusarium oxysporium, Aspergillus niger* and *Rhizopus oryzae* in the cellulose degradation process in various environments has been well documented (Kuzmanova et al., 1991; Teerei, Koivala, 1995; Bhat, Bhat, 1997; Schülein, 1997; Murashima et al., 2002; Mach, Zeilinger, 2003).

This study concentrates mainly on fungi isolated from waste paper raw material and substrates collected at the different stages of waste paper recycling to cardboard, on fungal ability to utilize cellulose as a carbon source and on the screening of hyper-producers of cellulase.

MATERIALS AND METHODS

Samples of the different substrates – raw materials (waste paper), waste paper after primary re-sort, waste paper after deep resort (paper pulp), pulp after clarification; primary effluent mud, waste water from biological treatment reservoir; waste water from the sedimentation reservoir after six months of sedimentation and waste water after five years of exposure in the sedimentation reservoir – were collected in 2006 at the "Klaipėdos kartonas" cardboard factory located in the Lithuanian seaport. In

laboratory, 50 g of each substrate (some of which needed to be milled) was added to the conical flask containing 450 ml of NaCl solution (0.75%, w/v) and mixed for 10 min on a rotary shaker (120 rpm). Samples of sludge and waste water were added as 50 ml to 450 ml of NaCl solution. The supernatants were separated by centrifugation, and ten-fold dilutions up to 10^5 colony forming units (CFU) ml^{-1} were prepared. Two dilutions (10^3 and 10^4 CFU ml^{-1}) were used for the analysis of total complexes of fungi present in the samples, and 10^5 (CFU ml^{-1}) dilutions were used for isolation of cellulolytic fungi.

Plate screening

A medium containing Mandels' mineral salts solution (Mandels et al., 1974) with addition of 17.5 g · l^{-1} agar and 5.0 g · l^{-1} phosphoric acid-swollen cellulose and 0.5% L-sorbose as a colony restrictor and inducer of cellulose production (Wang et al., 1995) was used for the isolation of cellulolytic fungi. The cellulose substrate used in the clearing test was prepared according to the procedure as recommended by Stewart et al. (1982). The plates were seeded with 100 µl of stock suspension (10 plates for each sample) and incubated at 25 °C for 4–7 days, followed by 24 h at 50 °C, after which clear zones could be observed only around colonies of the active fungal strains. Cellulose activities of the more active fungal isolates were determined using a carboxymethyl cellulase activity assay (CMCase). Basal medium containing (g · l^{-1}): CMC – 10, $NaNO_3$ – 6.5, K_2HPO_4 – 6.5, yeast extract – 0.3, KCl – 6.5, $MgSO_4 \cdot 7H_2O$ – 3.0, glucose – 0.65 and agar – 17.5, was used for plate screening. Agar blocks (5 mm in diameter) from one-week-old colonies grown on PDA plates were cut and inoculated in the centre of the plates. The plates were incubated at 25 °C for three to five days, followed by 18 h at 50 °C in the dark. Cellulolytic strains were selected on the basis of the diameter of the hydrolysis zone surrounding the colonies. For observations, plates were stained with 1% Congo red dye (15 min), followed by destaining with 1 M NaCl solution for 20 min. Cellulose activity on carboxymethyl cellulase (CMC, sodium salt medium viscosity, SIGMA) agar was recorded as the Index of Relative Enzyme Activity (I_{CMC}) was recorded as clear zone ratios = clear zone diameter / colony diameter (Teather, Wood, 1982; Bradner et al., 1999).

Identification of fungal isolates

Fungi were isolated as mono-cultures on three media: malt extract agar (MEA, Liofilchem S.r.l., Italy), potato dextrose agar (PDA, Liofilchem S.r.l., Italy) and Czapek's agar (CA, Liofilchem S.r.l., Italy). Fungi were identified to the species according to the current universal keys of identification (Raper, Fennell, 1977; Domsch et al., 1980; Samson et al., 2000; Pitt, 1979; Carmichael et al., 1980; Kiffer, Morelet, 2000).

Production of extracellular enzymes in shake flasks

Isolates for the production of extracellular enzymes were chosen according to the plate-clearing assay. The production of extracellular enzymes was induced in submerged fungal cultures. Agar blocks from one-week-old colonies of fungi grown at 25 °C on PDA plates were inoculated in conical flasks (250 ml) containing 100 ml of the medium used to determine cellulose digestion by specific fungi. The medium consisted of 1.1 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g NH_4NO_3 per liter and 5 g · l^{-1} of the appropriate carbon source. Cultures were incubated in an orbital shaker incubator for 14 days at 25 °C. After 14 days of cultivation, culture aliquots were centrifuged at 5000 × g to remove solids. The supernatants were assayed for their enzymatic activity.

Carbon sources

For the production of endo-1,4-β-glucanases, liquid cultures were carried out using 5 g · l^{-1} carboxymethyl cellulose (sodium salt medium, low viscosity, SIGMA) 5 g · l^{-1} cellulose powder (ball-milled filter paper) as a sole carbon source and inducer of cellulases. Either 2% CMC (β-1,4-endoglucanase activity assay) or Whatman N° 1 filter paper strip – 1.0 × 6.0 (= 50 mg) (filter paper activity as overall cellulolytic activity assay) was used in reaction mixtures for enzyme assay.

Enzyme assay

Total cellulase activity was determined by measuring the amount of reducing sugar formed from filter paper. Endoglucanase (β-1,4-endoglucanase – EC3.2.1.4) activity was assayed by measuring the amount of reducing sugar from carboxymethyl cellulose (CM-cellulose). Enzymes' activity was assayed according to the methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) Commission on biotechnology (Ghose, 1987). Endoglucanase (CMCase) activity was determined by incubating 0.5 ml of the supernatant with 0.5 ml of 2% carboxymethyl cellulose (low viscosity CMC, SIGMA) in 0.05 M sodium citrate buffer (pH 4.8) at 50 °C for 30 min. Filter paper degrading activity (FPCase) was determined by incubating 1.0 ml of the supernatant with 1.0 ml 0.05 M of the sodium citrate buffer (pH 4.8) containing Whatman N° 1 filter paper strip – 1.0 × 6.0 (= 50 mg). After incubation for 24 h at 50 °C, the reaction was terminated by adding 3 ml of 3,5-dinitrosalicylic acid (DNS) reagent to 1 ml of the reaction mixture. In these tests, reducing sugars were estimated colorimetrically with 3,5-dinitrosalicylic acid after Miller (1959) and Onsori et al. (2005), using glucose as standards. The enzymatic activity of total (FP-cellulase) and endoglucanase (CM-cellulase) was defined in the International Units (IU). One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol reducing sugars (measured as glucose) per ml per min.

Statistical analysis

Statistical data were presented using Microsoft Excel (Čekanavičius, Murauskas, 2000). The concentration of fungal isolates was determined as a mean of 5–8 independent tests and expressed as colony-forming units in 1 g of dry weight (d.w.) or 1 ml of the substrate. Significance differences among the replicates were presented as Dunkan's multiple range tests in the form of probability (p) values.

RESULTS AND DISCUSSION

In most investigations, members of the fungal genus *Trichoderma* Pers. have been extensively studied due to their ability to secrete cellulose-degrading enzymes. Most of the works have been carried out on *T. aureoviride* Rifai, *T. viride* Pers., *T. reesei* E. G. Simmons, *T. harzianum* Rifai strains and their mutants evaluating their ability to produce extracellular cellulolytic enzymes (endoglucanases, exo-

glucanases and cellobiase) which act synergistically in the conversion of cellulose to glucose. The cellulases secreted by *Trichoderma* have received widespread industrial interest leading to commercial applications (Olson, Hahn-Hagerdahl, 1997; Oksanan et al., 2000; Mach, Zeilinger, 2003; Cavaco-Paulo, Gübitz, 2003, Nierstrasz, Warmoeskerken, 2003; Van Wyk, Mohulatsi, 2003; Penttila et al., 2004). However, despite the efforts of many laboratories, no commercially efficient enzyme complex has been produced. In addition, the *Trichoderma* cellulase system is deficient in cellobiase, causing the accumulation of disaccharide cellobiose which produces repression and end product inhibition of the enzymes (Gruno et al., 2004). The demand for more thermostable, highly active and specific cellulases is on the increase, therefore, cellulase systems of the other fungi have been also investigated (Updegraff, 2004; Hanif et al., 2004; Kamal, Mathur, 2005; Gopinath et al., 2005; Ikram-ul-Haq, Khan, 2006). The studies were concentrated mainly on soil fungi (Lynd et al., 2002). Most fungi can be adapted to anthropogenic substrates such as natural or waste cellulose or to substrates containing high amounts of cellulose. Waste paper recycling factories convert waste paper into pulp and subsequently into paper (or cartoon), mainly tissue paper products. Almost any household paper can be recycled, including used newspapers, cardboard, packaging, stationery, direct mail, magazines, catalogues, greeting cards and wrapping paper. All raw materials are contaminated by microorganisms (bacteria, fungi and actinomycetes) whose viability and activity depend on the conditions to which substrates are exposed. During our investigation, microbiological analysis of the samples collected at the cardboard factory from the gradual wastepaper recycling process showed different levels of contamination by fungi and the percentage of fungal isolates able to grow in the medium with the sole carbon source – cellulose. The number of viable fungi isolated on the Mandels' salt medium with glucose (control) varied from 2 to 84.7 thousands CFU · g^{-1} d.w. or ml^{-1} sample. When Mandels' salt medium with the sole carbon source, cellulose, was used, only the fungi than can utilize cellulose were isolated (Fig. 1, A). The numbers of the fungi growing on the CMC and phosphoric acid-swollen cellulose media varied from 0.34 to 14.7 thousands CFU · g^{-1} d.w. or ml^{-1} and from 0.18 to 13.7 thousands CFU · g^{-1} d.w. or ml^{-1}, respectively,

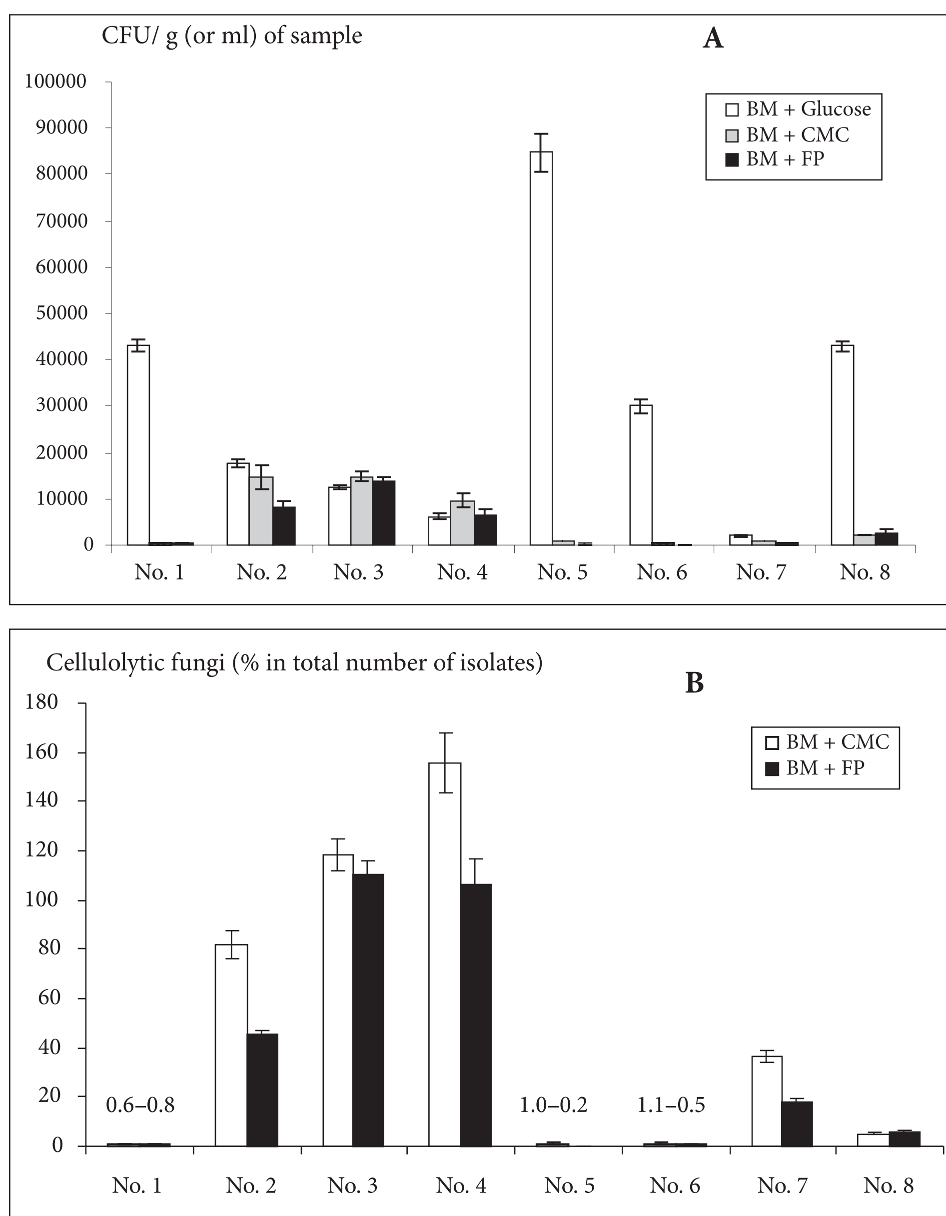

Fig. 1. The number of viable fungi (*A*, as CFU) isolated on Mandels' salt medium (basal medium, BM) with glucose, basal medium with carboxymethyl cellulose (BM + CMC) or basal medium with phosphoric acid-swollen filter paper cellulose (BM + FP) and the percentage of cellulolytic fungi (*B*). Substrates investigated: raw materials (waste paper) (No. 1), waste paper after primary re-sort (No. 2), waste paper after deep re-sort (paper pulp) (No. 3), pulp after clarification (No. 4), primary effluent mud (No. 5), waste water from the biological treatment reservoir (No. 6), waste water from the sedimentation reservoir after 6 months (No. 7) and waste water from the sedimentation reservoir after 5 years (No. 8). Each bar represents the mean of five–eight replicates

and differed significantly ($p < 0.02$) from their numbers in the control medium.

Significant differences ($p < 0.01$) were observed in the abundance of fungi in samples of raw materials, primary effluent mud, waste water from the biological treatment reservoir, waste water after five years of exposure to the environment and other materials. However, despite the high contamination of these samples by fungi, very low numbers of fungi were able to grow on the cellulose media (Fig. 1, A). The percentage of cellulolytic fungi in the total number of the isolated strains under investigation conditions was very low and made only 0.6–5.67% (Fig. 1, B).

The highest numbers of cellulolytic fungal strains were isolated from waste paper after primary re-sort, waste paper after deep re-sort and primary effluent mud (8 to 14.6 thousand CFU, 13.7 to 14.7 thousand CFU and 6.8 to 9.6 thousand CFU, respectively) (Fig. 1, A). Upon revealing the percentage of cellulolytic fungi (Fig. 1, B), it became clear that the highest percentage of the waste paper after primary re-sort, waste paper after deep re-sort and primary effluent mud were more valuables sources of cellulolytic fungi. The percentage of cellulolytic isolates was significantly higher ($p < 0.01$) in these than in the other substrates. The high concentrations (CFU $\cdot$ g^{-1} d.w. or ml^{-1} sample) of fungi determined in raw materials (waste paper) and waste water after exposure to the environment may be due to the total contamination by fungi. As demonstrated by this experiment, the number of cellulolytic fungi determined by the plate screening method is low because of the inaccuracy of the method of waste paper treatment and the abundance of incidental contaminants and their antagonism in the media used in the investigation.

The number of fungi isolated from paper pulp and from paper pulp after clarification in the medium with both celluloses (CMC and phosphoric acid-swollen cellulose) were higher than of fungi isolated in control medium with glucose (Fig. 1, A). The number of fungi isolated from paper pulp was 14.7 and 13.7 thousands of CFU $\cdot$ g^{-1} on the medium with CMC and phosphoric acid-swollen cellulose, respectively, and the cellulolytic fungi comprised 118.07% and 110.44% of the total number of isolates in the control medium. The number of fungi isolated from paper pulp after clarification was 9.6 and 6.6 thousands of CFU $\cdot$ g^{-1} in the medium with CMC and phosphoric acid-swollen cellulose, respectively, and cellulolytic fungi comprised 155.64% and 106.45% of the total number of isolates in the control medium. The higher numbers of colony-forming units on cellulose media than in the control medium can be explained by the fact that most fungal propagules were lost during waste paper gradual recycling, and real cellulolytic fungi which are resistant to the various factors were left in the complexes of the recovered fungi. A negligible increase in fungal concentration and their recovery in the cellulose medium were observed in wastewater after six months of sedimentation. Large numbers of fungi were isolated from primary effluent mud (up to 84.75 thousands CFU $\cdot$ ml^{-1} sample), however, the number of cellulolytic fungi was very low (1.0–0.2%) (Fig. 1, A and B).

Carboxymethyl cellulose was a more favourable carbon source for screening the cellulolytic fungi. Significantly higher numbers of cellulolytic fungi ($p < 0.05$) were isolated from samples of waste paper after primary re-sort and after deep re-sort when a medium with CMC was used, as compared with the medium containing phosphoric acid-swollen cellulose.

A total of 412 isolates were obtained from the analyses of the samples of eight substrates collected in the waste paper recycling factory. The identification of these isolates resulted in 37 species and varieties plus 11 sterile fungi. Among the identified species, six belonged to *Aspergillus* P. Micheli ex Link, nine to *Penicillium* Link, three to *Mortierella* Coem., two to each genus of *Gliocladium* Corda, *Trichoderma* Pers. and *Mucor* Fresen., and one to each genus of *Acremonium* Link, *Arthrobotrys* Corda, *Botryotrichum* Sacc. & Marchal, *Cladosporium* Link, *Chaetomium* Kunze, *Epicoccum* Link, *Geomyces* Traaen, *Fusarium* Link, *Gliomastix* Guég., *Lecanicillium* W. Gams & Zare, *Paecilomyces* Bainier, *Stachybotrys* Corda, and *Sporotrichum* Link. A total of 269 isolates were able to grow in the medium with cellulose as a sole carbon source. They belonged to 26 species (Table). The species with the greatest number of cellulolytic strains were *Penicillium funiculosum* (the ratio to the total number of cellulolytic fungi = 21.1%), *P. simplicissimum* (15.7%), *P. brevicompactum* (16.2%), *Stachybotrys chartarum* (7.5%) and *Aspergillus repens* (4.7%). The indices of relative enzyme activity (I_{CMC}) determined on CMC medium (Table) presented preliminary endoglucanase activity characteristics of the fungal isolates belonging to different species. The index equal to 1 or > 1 was determined for fungi of 15 species (57.69% of all cellulolytic species detected). Fungal strains belonging to two species –*Aspergillus niger* var. *nige* and *Gliomastix murorum* var. *murorum* – showed the highest CM-cellulase activity ($I_{CMC} = 2.21 \pm 0.97$ and 2.07 ± 0.85, respectively). The lowest CM-cellulase cellulase activity was shown by fungal strains belonging to *Mucor racemosus* and *Penicillium fellutanum* ($I_{CMC} = 0.28 \pm 0.02$ and 0.54 ± 0.22, respectively). Isolates of the fungi capable of decomposing cellulose vary physiologically even within the given species. Relative enzymatic activity of *Aspergillus niger*, *Epicoccum nigrum*, *Gliomastix murorum* var. *murorum*, *Paecilomyces lilacinus* and *Penicillium fellutanum* strains was highly different (standard deviation calculated from the data of the strains made 43.89%, 50.56, 41.06, 46.29 and 40.74%, respectively). Some strains of *Aspergillus niger* and *Gliomastix murorum* var. *murorum* were characterized by a very high relative enzymatic activity index (I_{CMC} – 2.5 to 3.5) and were chosen for the further investigation. Fast-growing fungi could not been detected as hypercellulose-producing fungi following the analysis of the relative activity index. The amount of the enzyme depends on the biomass, therefore, when the fungus colony consists of a sparse clearing of the cellulose medium, it is detectable only in a close contact or in an area close to the mycelium.

Twelve strains of the fungi screened as potential cellulase producers were investigated in the shake flask culture using CMC or cellulose powder as a carbon source in the culture medium. After 14 days of cultivation, enzymatic activity was measured by the production of sugar reducing end group, which is taken to be an indication of cleavage of cellulose molecules. Two standard substrates were used for the determination of cellulase activity in terms of overall (FPCase) and endoglucanase (CMCase) contents (Ghose, 1987). Filter paper was used as a standard substrate to measure the total cellulose activity (Wu et al., 2006). The filter paper activity, termed as FPase, expressed

Table. **Cellulolytic fungi detected by the plate screening method and the Index of Relative Enzyme Activity (I_{CMC}) determined on CMC medium**

Fungal species	Ratio to total number of cellulolytic* (%)	Number of strains tested on CMC medium	(I_{CMC})
Acremonium strictum W. Gams	0.8	6	1,32 ± 0.08
Arthrobotrys oligospora Fresen.	0.2	2	0,42 ± 0,03
Aspergillus fumigatus Fresen	0.6	4	0.73 ± 0,04
Aspergillus niger var. *niger* Tiegh.	2.1	8	2.21 ± 0,97
Aspergillus repens (Corda) Sacc.	4.7	9	1,54 ± 0,54
Botyrotrichum piluliferum Sacc. & Marchal	0.9	2	0,97 ± 0,11
Cladosporium cladosporioides (Fresen.) G.A. de Vries	1.5	5	0,97 ± 0,07
Chaetomium globosum Kunze	0.5	2	0.88 ± 0.04
Geomyces pannorum (Link) Singler & J.W. Carmich. (= *Chrysosporium pannorum* (Link) S. Hughes)	1.3	6	1.29 ± 0.31
Epicoccum nigrum Link (= *E. purpurescens* Ehrenb.)	0.2	3	0.89 ± 0.45
Fusarium oxysporum Schltdt.	2.1	2	1.31 ± 0.27
Geotrichum candidum Link	0.5	1	0.83 ± 0.14
Gliocladium roseum Bainier (= *Clonostachys rosea* f. *rosea* (Link) Schroers, Samuels, Seifert & W. Gams	1.4	3	1.37 ± 0.25
Gliomastix murorum var. *murorum* (Corda) S. Hughes) (= *Acremonium murorum* (Corda) W. Gams)	3.6	8	2.07 ± 0.85
Lecanicillium lecanii (Zimm.) Zare & W. Gams (= *V. lecanii* (Zimm.) Viégas)	0.7	4	0.66 ± 0.03
Mucor racemosus Fresen.	0.1	1	0.28 ± 0.02
Myrothecium verrucaria (Alb. & Schwen.) Ditmar	1.1	2	1.04 ± 0.26
Paecilomyces lilacinus (Thom) Samson	1.9	3	1.62 ± 0.75
Penicillium brevicompactum Dierckx	16.2	11	1.31 ± 0.14
Penicillium fellutanum Biourge	3.5	4	0.54 ± 0.22
Penicillium funiculosum Thom	21.1	12	1.87 ± 0.13
Penicillium purpurogenum Stoll	5.9	5	1.45 ± 0.21
Penicillium simplicissimum (Oudem.) Thom	15.7	13	1.11 ± 0.04
Trichoderma harzianum Rifai	4.3	7	1.00 ± 0.12
Trichoderma longibrachiatum Rifai	1.6	4	0.98 ± 0.04
Stachybotrys chartarum (Ehrenb.) S. Hughes	7.5	6	1.85 ± 0.23

* Cellulolytic on the medium with the phosphoric acid-swollen cellulose.

summations of a simultaneaus synergistic action of endoglucanases, cellobiohydrolases and β-glucosidase in a cellulase preparation. Soluble cellulose (carboxymethyl cellulose, CMC) was used as a substrate for determining endoglucanase activity. Significant differences in activity ($p < 0.01$) were detected for two enzymes – CMCase and FPase (Fig. 2). The overall cellulase activity levels were lower than the CMCase activity level for all fungi (except *Stachybotrys chartarum* strain DPK-cl-111). The determination of cellulase activity is a complicated process, because the hydrolysis of insoluble cellulose (filer paper in our case) may not be linear with enzyme amount and/or reaction time. Very large differences of those two enzymes were observed for *Stachybotrys chartarum* DPK-cl-100, *Penicillium funiculosum* DPK-cl-19 and *Gliocladium roseum* DPK-cl-102. Perhaps, like in the fungi from the genus *Trichoderma* (as reviewed by Gruno et al., 2004), the cellulase system could be deficient in cellobiase, causing the accumulation of disaccharide cellobiose which produces repression and end product inhibition of the enzymes. CMCase activity of the fungus *A. niger* DPK-cl-12 was statistically significantly ($p < 0.01$) highest among all the twelve fungi investigated. Comparative CMCase under investigation showed fungi *S. chartarum* DPK-cl-100, *P. funiculosum* DPK-cl-19, *M. verucaria* DPK-cl-06, *G. murorum* var. *murorum* DPK-cl-03 and *G. roseum* DPK-cl-102 whose activity varied from 1.5 to 2.5 $U \cdot mL^{-1}$ (Fig. 2). Endoglucanase activity of those fungi was significantly higher than that of T*richoderma* strains (DPK-cl-36 and DPK-cl-51). Trichoderma is known as a very good producer of cellulases, perhaps due to the different adaptability of fungi to the anthropogenic substrates and different resistance to the factors affecting fungal populations during the recycling procedures.

Although 12 isolates showed a good filter paper distintegration and CMC utilization within 14 days, not all of them can be recommended as potential hypercellulase producers. We were interested in the isolates that showed a high FPCase activity. Not all results are presented in this work, but some fungal species attracted our attention to their cellulase systems and deserve further investigation. Among them are *Aspergillus niger*, *Gliomastix murorum, Myrothecium verrucaria*, *Paecilomyces* spp., *Penicillium funiculosum* and *Stachybotrys chartarum.* These our findings are comparable with some results obtainen by other investigators (Updegraff, 2004; Kluczek, Turpeinen et al., 2006).

Four fungi – *Aspergillus nige*r DPK-cl-12, *Gliomastix murorum* var. *murorum* (= *Acremonium murorum*) DPK-cl-44, *Penicillium funiculosum* DPK-cl-19 and *Stachybotrys chartarum* DPK-cl-111, grown for 14 days in the medium with CMC, were

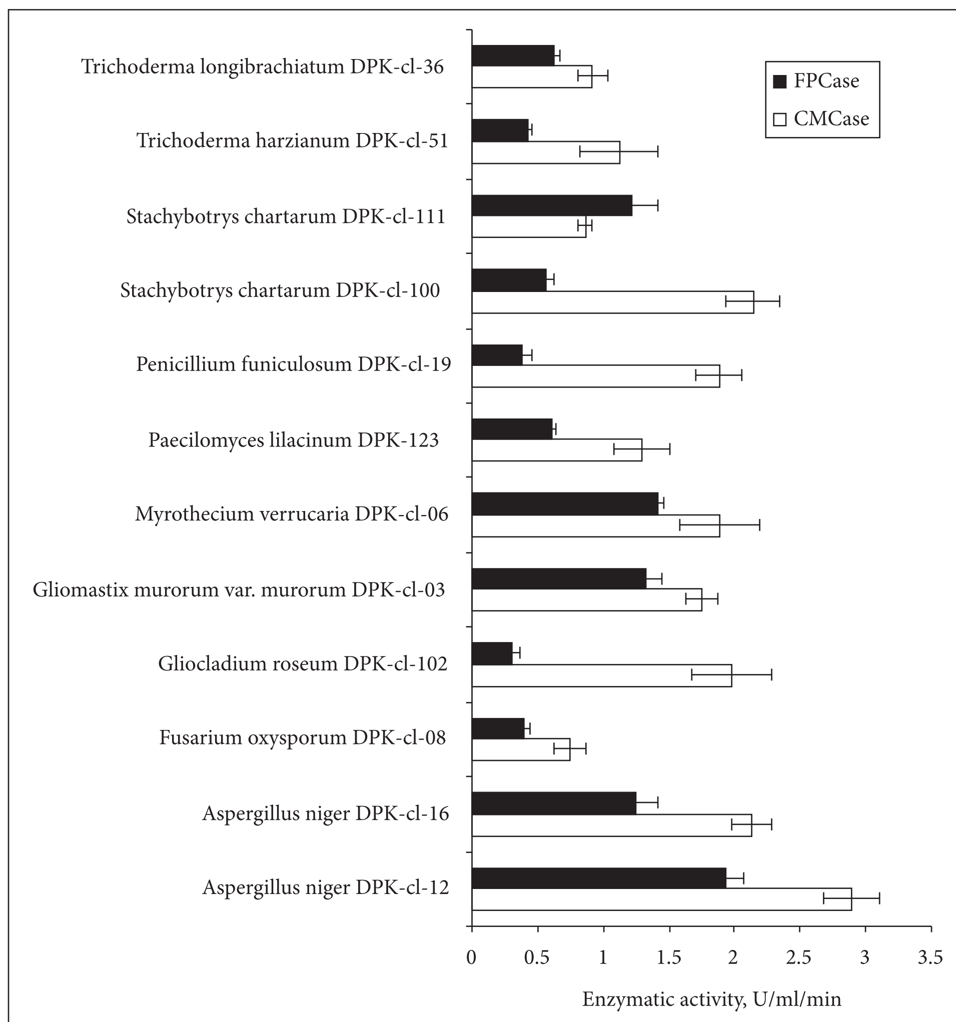

Fig. 2. Exocellular endoglucanase (carboxymethyl-cellulase, CMCase) and total filter paper cellulase (FPCase) activity of fungi after 14 days of cultivation in the medium with carboxymethyl cellulose or filter paper powder cellulase as a sole carbon source. Each bar represents the mean of three replicates of enzymatic activity (U · ml^{-1} · min^{-1})

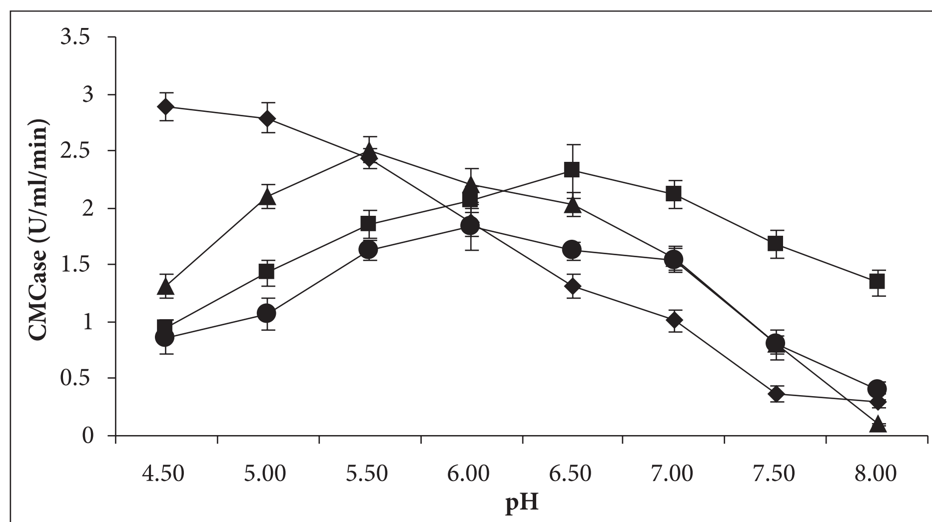

Fig. 3. Effect of pH on CMCase (endo-β-1,4-glucanase) activity of three fungal strains: *Aspergillus niger* DPK-cl-12 (◆), *Gliomastix murorum* var. *murorum* (= *Acremonium murorum*) DPK-cl-44 (▲), *Penicillium funiculosum* DPK-cl-19 (●) and *Stachybotrys chartarum* DPK-cl-111 (■) at 30 °C

used to determine the pH effect on endoglucanase activity. *Aspergillus niger* DPK-cl-12 shows the highest CM-cellulase activity (2.89 ± 0.12 U · ml^{-1} · min^{-1}) which has an optimum pH 4.5 when assayed at 30 °C (Fig. 3).

The CMCase of *G. murorum* var. *murorum* DPK-cl-44 showed its optimum pH to be 5.5, and its activity fell to 50% (from 2.4 to 1.2 U/ml) when the pH ranged from 5.5 to 4.5 and 7.0. The CMCase activity of *A. niger* DPK-cl-12 and *S. chartarum* DPK-cl-111 showed their optimum pH to be 4.5 and 6.5, respectively. Despite the CMCase activity of *P. funiculosum* DPK-cl-19 was lower than that of other three fungi, its CMCase activity showed its stability at pH from 5.5 to 7.0. However, at pH 8.0 the activity

of fungi *A. niger*, *G. murorum* var. *murorum* and *P. funiculosum* was almost los; the activity of *S. chartarum* at that pH remained high enough and showed its alkali stability. Endoglucanase activity of *G. murorum* var. *murorum* DPK-cl-44 and *S. chartarum* DPK-cl-111 at the optimal pH was 2.51 ± 0.12 U · ml^{-1} · min^{-1} and 2.32 ± 0.24 U · ml^{-1} · min^{-1}, respectively. Figure 2 presents CMCase activities measured at pH 4.8 (as recommended by the method (Ghose, 1987). As one can see from Fig. 2, CMCase activity of the fungi *Gliomastix murorum* var. *murorum* DPK-cl-44, *Penicillium funiculosum* DPK-cl-19 and *Stachybotrys chartarum* DPK-cl-111 were three times lower than that of fungus *A. niger* DPK-cl-12. Analysis of data presented in Fig. 3 showed that when the pH was changed, CMCase activity raised to the level close to the CMCase activity of the fungus *A. niger* DPK-cl-12.

CONCLUSIONS

The research covered some distribution and biochemical characteristics of the fungi adapted to the anthropogenic substrates, such as cellulose containing materials.

• A total of 412 isolates were obtained from the analyses of samples of eight substrates collected in the waste paper recycling factory. The identification of these isolates resulted in 37 species and varieties plus 11 sterile fungi.

• A total of 269 isolates were able to grow in the medium with cellulose as a sole carbon source. Twenty-six species of fungi were described as potential cellulolytic species.

• Active cellulase producers were selected among isolates of fungi belonging to the genera *Aspergillus*, *Gliomastix*, *Gliocladium*, *Myrothecium Paecilomyces*, *Penicillium*, *Tricho-derma* and *Stachybotrys*.

• Three strains – *Aspergillus nige*r DPK-cl-12, *Gliomastix murorum* var. *murorum* (= *Acremonium murorum*) DPK-cl-44, *Penicillium funiculosum* DPK-cl-19 and *Stachybotrys chartarum* DPK-cl-111 – were screened as more active in cellulose hydrolysis and as the potentially high extracellular cellulose producers.

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References

1. Bhat M. K. Cellulases and related enzymes in biotechnology. *Biotechnology Advances*. 2000. Vol. 18. P. 355–383.
2. Bhat M., Bhat S. 1997. Cellulose degrading enzymes and their potential industrial applications. *Biotechnol. Adv*. Vol. 15. P. 583, 620.
3. Bradner J. R., Gillings M., Nevalainen K. M. H. 1999. Qualitative assessment of hydrolytic activities in Antarctic microfungi grown at different temperatures on solid media. *World J. Microbiol. Biotechnol.* Vol. 15. P. 131–132.
4. Carmichael J. W., Kendrick W. B., Conners I. L., Sigler L. 1980. *Genera of Hyphomycetes*. Edmonton, Alberta, Canada: The University of Alberta Press. 386 p.
5. Cavaco-Paulo A., Gübitz G. 2003. Catalysis and processing. In: Cavaco-Paulo A., Gübitz G. (ed.). *Textile Processing with Enzymes*. England, Woodhead Publishing Ltd. P. 86, 119.
6. Čekanavičius V., Murauskas G. 2000. *Statistika ir jos taikymai*. I. Vilnius: TEV, 239 p.
7. Davis B. 1985. Factors influencing protoplast isolation. In: Peberdy J. F., Ferenizy L. (eds.). *Fungal Protoplasts: Applications in Biochemistry and Genetics.* Marcel Dekker, New York. 356 p.
8. Domsch K. H., Gans W., Anderson T.-H. 1980. *Compendium of Soil Fungi*. London, New York, Torroute, Sydney, San Francisco: Academic Press. 869 p.
9. Ghose T. K. 1987. Measurement of cellulose activities. *Pure Appl. Chem*. Vol. 59. P. 257–268.
10. Gopinath S. C., Anbu P., Hilda A. 2005. Exracellular enzymatic activity profiles in fungi isolated from oil-rich environments. *Mycoscience.* Vol. 46. N 2. P. 119–126.
11. Gruno M., Väljamäe P., Pettersson G., Johansson G. 2004. Inhibition of the Trichoderma reesei cellulase by cellobiose is strogly dependent on the nature of the substrate. *Biotechnol. Bioeng*. Vol. 86. P. 503–511.
12. Hanif A., Yasmeen A., Rajoka M. I. 2004. Induction, production, repression, and de-repression of exoglucanase synthesis in *Aspergillus niger*. *Bioresource Technology*. Vol. 94. N 3. P. 311–319.
13. Ikram-ul-Haq M. M. J., Khan T. S. 2006. An innovative approach for hyper production of cellulolytic and hemicellulolytic enzymes by consortium of *Aspergillus niger* MSK-7 and *Trichoderma viride* MSK-10. *African Journal of Biotechnology*. Vol. 5. N 8. P. 609–614.
14. Ishikuro E. 1993. Feed additives. *Modern Media*. Vol. 46. P. 289–296.
15. Kamal L., Mathur S. N. 2005. Cellulolytic activities of *Chaetomium globosum* on different cellulosic substratas. *World Journal of Microbiology and Biotechnology*. Vol. 5. N 1. P. 23–26.
16. Kiffer E., Morelet M. 2000. *The Deuteromycetes. Mitosporic Fungi. Classification and Generic Keys*. U.S.A.: Science Publishers Inc. 273 p.
17. Kluczek-Turpeinen B., Maijala P., Tuomela M., Hofrichter M., Hatakka A. 2005. Endoclucanase activity of compost-dweling fungus Paecilomyces inflatus is stimulated by humic acids and other low molecular mass aromatics. *World Journal of Microbiology and Biotechnology*. Vol. 21. N 8–9. P. 1603–1609.
18. Kuzmanova S., Vandeska E., Dimitrovski A. 1991. Production of mycelial protein and cellulolytic enzymes from food waste. *Journal of Industrial Microbiology and Biotechnology*. Vol. 7. N 4. P. 257–261.
19. Levy I., Shani Z., Shoseyov O. 2002. Modification of polysaccharides and plant cell wall by endo-1,4-b-glucanase and cellulose-binding domains. *Biomolecular Engineering*. Vol. 19. P. 17–30.
20. Luo J., Xia L. M., Lin J. P., Cen P. L. 1997. Kinetics of simultaneous saccharification and lactic acid fermentation processes. *Biotechnol. Progr*. Vol. 1. P. 762–767.
21. Lynd L. R., Weimer P. J., van Zyl W. H., Pretorius I. S. 2002. Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiology and Molecular Biology Reviews*. Vol. 66. N 3. P. 506–577.
22. Mach R., Zeilinger S. 2003. Regulation of gene expression in industrial fungi: *Trichoderma. Appl. Microbiol. Biotechnol*. Vol. 60. P. 515–522.

23. Mandels M. L., Hontz L., Nistrom J. 1974. Enzymatic hydrolysis of waste cellulose. *Biotechnology and Bioengineering*. Vol. 16. P. 471–493.
24. Miettinen-Oinonen A., Londesborough J., Joutsjoki V., Lantto R., Vehmaanperä J. 2004. Three cellulases from *Melanocarpus albomyces* with applications in the textile industry. *Enzyme and Microbial Technology.* Vol. 34. P. 332–341.
25. Miller G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Annl. Chem*. Vol. 31. P. 426–428.
26. Murashima K., Nishimura T., Nakamura Y., Koga J., Moriya T., Sumida N., Yaguchi T., Kono T. 2002. Purification and characterization of new endo-1,4-β-D-glucanases from *Rhizopus oryzae. Enzyme Microb. Technol*. Vol. 30. P. 319–326.
27. Nierstrasz V., Warmoeskerken M. 2003. Process engineering and industrial enzyme applications. In: Cavaco-Paulo A., Gübitz G. (eds.). *Textile Processing with Enzymes.* England, Woodhead Publishing Ltd. P. 120–157.
28. Oksanan T., Peeabilaniana J. 1998. Alkaline detergent enzymes from alkaliphilic enzymatic properties, genetics and structures. *Extremophiles*. Vol. 2. N 3. P. 185–190.
29. Oksanan T., Pere J., Paavilainen I., Büchert J., Viikari L. 2000. Treatment of recycled craft pulps with *Trichoderma reesei* hemicellulases and cellulases. *J. Biotechnol.* Vol. 78. P. 39–48.
30. Olsson L., Hahn-Hagerdahl B. 1997. Fermentation of lingocellulose hydrolisates for ethanol production. *Enzyme Microb. Technol*. Vol. 18. P. 312–331.
31. Onsori H., Zamani M. R., Matallebi M., Zarghami N. 2005. Identification of over producer strain of endo-β-1,4-glucanase in *Aspergillus* Species: Characterization of crude carboxymethyl cellulose. *African Journal of Biotechnology*. Vol. 4. N 1. P. 26–30.
32. Ortega N., Busto M. D., Perez-Mateos M. 2001. Kinetics of cellulose saccharification by Trichoderma reesei cellulases. *International Biodeterioration and Biodegradation.* Vol. 47. P. 7–14.
33. Penttilä M., Limon C., Nevalainen H. 2004. Molecular biology of *Trichoderma* and biotechnological applications. In: Arora D. (ed.). *Handbook of fungal biotechnology*. Marcel Dekker, Inc. P. 413–427.
34. Pitt J. I. 1979. *The Genus Penicillium.* London, New York, Toronto Sydney, San Fracisco: Academic Press. 635 p.
35. Raper K. B., Fennell D. I. 1977. *The Genus Aspergillus*. New York: Robert Erieger Publishing Company Huntimgton. 685 p.
36. Samson R. A., Hoekstra E. S., Ftrisvald O. 2000. *Introduction to Food-and Airborne Fungi.* Ultrecht: Centraalbureau Vooor Schimmelcultures. 383 p.
27. Schülein M. 1997. Enzymatic properties of cellulases from *Humicola insolens. J. Biotechnol*. Vol. 57. P. 71–81.
38. Stewart J. C., Stewart C. S., Heptinstall J. 1982. The use of tritiated cellulose in screening for cellulolytic microorganisms. *Biotechnology Letters*. Vol. 4. N 7. P. 459–464.
39. Suurnäkki A., Niku-Paavola M-L., Buchert J., Viikari L. 2004. Enzymes in pulp and paper processing. In: Aehle W. (ed.). *Enzymes in Industry.* Weinheim, Wiley-VCH. P. 232–244, 437–439.
40. Teather R. M., Wood P. J. 1982. Use of congo red-polysacharide interactions in enumeration and characterization of cellulolytic bacteria in the bovine rumen. *Appl. Environm. Microbiol*. Vol. 43. N 4. P. 777–780.
41. Teeri T., Koivula A. 1995. Cellulose degradation by native and engineered fundal cellulases. *Carbohydr. Eur.* Vol. 12. P. 28–33.
42. Updegraff D. M. 2004. Utilization of cellulose from waste paper by *Myrothecium verrucaria*. *Biotechnology and Bioengineering*. Vol. 13. N 1. P. 77–97.
43. Urlaub R. 2002. Enzymes in fruit and vegetable juice extraction. In: Whitehurst R., Law B. (eds.) *Enzymes in food technology*. Sheffield, Academic Press, CRC Press. P. 145–183.
44. Van Wyk J. P. H., Mohulatsi M. 2003. Biodegradation of waste-paper by cellulose from *Trichoderma viride*. *Bioresource Technology*. Vol. 86. P. 21–23.
45. Wang D., Qu Y. B., Gao P. J. 1995. The mechanism of increasing cellulase biosynthesis rate in *Trichoderma* by L-sorbose. *Acta Mycol. Sinica*. Vol. 14. P. 143–147.
46. Wu B., Zhao Y., Gao P. J. 2006. A new approach of measurement of saccharification capacities of crude cellulase. *BioResources*. Vol. 1. N 2. P. 189–200.

Dalė Pečiulytė

CELIULIAZINIŲ MIKROMICETŲ IŠSKYRIMAS IŠ POPIERIAUS ATLIEKŲ PALAIPSNINIO PERDIRBIMO SUBSTRATŲ

Santrauka

Antrinio žaliavų perdirbimo gamykloje surinktos popieriaus atliekos perdirbamos į kartoną arba popieriaus audinius. Tyrėme palaipsnio popieriaus atliekų perdirbimo medžiagas, aiškindamiesi galimybę šiuose substratuose surasti perteklinės celiuliazės producentus. Tyrimui taikėme celiuliozės terpių skaidrinimo metodą ir Tarptautinio taikomosios chemijos komiteto (IUPAC) rekomenduojamus celiuliazių nustatymo metodus. Ištyrėme mikromicetų koncentraciją (kolonijas sudarančių vienetų skaičių (KSV) 1 grame arba 1 ml tiriamo pavyzdžio) palaipsnio perdirbimo medžiagose, celiuliaziniu aktyvumu pasižyminčių kamienų procentą bendrame išskirtų kamienų skaičiuje ir dviejų fermentų (filtrinio popieriaus celiuliazė – FP-celiuliazė, carboksimetilceliuliazė – CM-celiuliazė) aktyvumą skystose auginimo terpėse. Palyginamųjų tyrimo rezultatų pagrindu išaiškinome dvidešimt šešias celiuliaziniu aktyvumu pasižyminčių mikromicetų rūšis. Atrinkę dvylika iš 139, aktyvesnių hidrolizuojant celiuliozę, mikromicetų kamienų nustatėme jų CM-celiuliazinį ir FP-celiuliazinį aktyvumus. Išaiškinome tris mikromicetų kamienus – *Aspergillus niger* DPK-cl-12, *Gliomastix murorum* var. *murorum* (= *Acremonium murorum*) DPK-cl-44 ir *Stachybotrys chartarum* DPK-cl-111, intensyviau sekretuojančius egzoląstelinę bendrąją (FP-celiuliazę) ir CM-celiuliazę. Trijų kamienų celiuliazinis aktyvumas, įvertintas šio tyrimo sąlygomis, yra didesnis nei *Trichoderma* genties grybų, žinomų kaip perteklinių celiuliazių producentų.

Raktažodžiai: celiuliozę skaidantys mikromicetai, išskyrimas, atranka, endogliukonatas, popieriaus atliekos, perdirbimas