Efficient Cellulases Production by *Trichoderma atroviride* G79/11 in Submerged Culture Based on Soy Flour-Cellulose-Lactose

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The microbiological hydrolysis of lignocellulose waste materials, using enzymatic biopreparations and its anaerobic processing, is a promising strategy for the efficient use of renewable energy sources. This article presents the optimization of microbiological media to improve cellulase production by *Trichoderma atroviride* G79/11 for further application as a cellulolytic biopreparation. The characterization of *T. atroviride* phenotypic microarrays was performed using the Biolog® PM plates approach. The optimization investigations were aimed at increasing the efficiency of the cellulolytic enzyme production involved in the solid-state fermentation type medium. This medium was based on dried sugar beet and wheat bran, as well as two liquid media based on mineral and soy flour, cellulose, and lactose (MSCL). The basic components of the MSCL were optimized. The study involved optimization of the content of the carbon and nitrogen source and the detergent additive. In order to increase the cellulase production, proper *T. atroviride* G79/11 culture conditions were established.

Keywords: Cellulolytic activity; Enzyme preparation; Trichoderma atroviride

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INTRODUCTION

The biological aspects of cellulosic biomass processing are a significant research area. This field of study includes matters regarding cellulolytic enzymes and the microorganisms that produce these biomolecules. The problems that need attention in the area of cellulosic biomass decomposition are not limited to cellulase production, but focus on understanding the physiology of cellulolytic microorganisms. The high efficiency of formulations in waste-origin lignocellulose decomposition is determined by several factors. These factors can be divided into those associated with substrate biodegradation or those related to hydrolytic activity (Alvira *et al.* 2010). To improve the efficiency of the biodegradation process, it is necessary to combine scientific achievements and technological approaches. Certain aspects that should be considered comprehensively to ensure reduction of the enzyme production cost and simultaneous maintenance of high enzymatic activity include: 1) development of technologies for preconditioning cellulosic materials to facilitate their microbial decomposition; 2) development of efficient production of cellulolytic enzymes; 3) optimization of the hydrolytic pretreatment of biomass; with the use of cellulolytic preparations in order to obtain the highest possible yield of hydrolysis; 4) development of genetically modified producers of enzymes; 5) protein engineering aimed at improving the catalytic specificity or stability of the enzyme protein; and 6) optimization of culture conditions and new substrates (Alvira *et al.* 2010).

Many technologies of cellulolytic enzyme production are based on genetic mutants, but the costs of commercial applications of the enzymes derived from cultures of modified

microorganisms remains high and unprofitable (Sukumaran *et al.* 2005). Therefore, the biotechnological production of cellulolytic enzymes based primarily on the empirical optimization of the process variables (culture conditions and the composition of the production medium) is still relevant and necessary. Most reports on cellulases describe the production of enzymatic proteins in liquid media, and *Trichoderma reesei* is the most widely studied microorganism in this field (Sukumaran *et al.* 2005).

An alternative strategy is the production of cellulases using isolates from a particular type of waste. A literature review shows that little work has been accomplished in this area. In the few published studies (Tengerdy and Szakacs 2003; Jourdier *et al*. 2013), filamentous fungi were used to obtain hydrolytic degradation of lignocellulose biomass. The strains were isolated from materials subjected to bioconversion. Nevertheless, the medium used for dry fermentation (solid state fermentation; SSF) was based on waste that may be beneficial on an industrial scale (Jourdier *et al.* 2013). Another strategy is to gain effective cellulolytic enzymes originating from mixed cultures of microorganisms. Diversification of enzyme producers, *e.g*., phylogenetically different microorganisms, can lead to more effective degradation of the lignocellulose substrate. This is probably due to the diverse kinetic properties of the enzymes that they produce (Hansen *et al.* 2015). Some microorganisms secrete unique enzymes, which may compete or supplement the effect of a commercial enzyme preparation. This special ability results in a faster and more complete hydrolysis of biomass and uses a lower amount of enzymes (Juturu and Wu 2014). An efficient enzyme system, which breaks down cellulose fibrils, requires the presence of hydrolases such as endo- and exoglycanases and *β*-glucosidases. The cellulolytic enzymes acting on native cellulose first attack the amorphous parts of the cellulose fibers and break them up into smaller crystalline fragments. In the next folding step, the crystalline fibrils are fragmented into smaller pieces (Cheng *et al.* 2012). Microorganisms can produce various types of cellulases, each with a different mode of catalytic attack and substrate specificity. In addition, cellulose-producing microorganisms use different strategies for cellulose degradation (Kuhad *et al.* 2016). However, non-specific cellulolytic activity (FPU) is a good measure of the total activity of cellulases. The simple FPU method for colorimetric determination is widely accepted (McIntosh and Vancov 2010; Song *et al.* 2014; Matsakas and Christakopoulos 2015).

The first steps in searching for efficient enzyme producers is the optimization of the reaction conditions, *i.e*., temperature, pH, oxygenation, and the selection of a suitable type and concentration of the substrate. These factors, alone or in combination, affect cellulase biosynthesis. Since the assumption of the biopreparation is to constitute variable enzymes, it is required for microbiological media to include enzymes production inducers such as cellulose, lactose, and soy flour to induce cellulases, lactases, and proteases, respectively. Given its low cost and availability, lactose is a suitable carbon source; however, soluble carbon sources can inhibit the synthesis of cellulases (Mathew *et al.* 2008). The second critical stage of development during biopreparations is the optimization of the media for the production of cellulolytic enzymes.

Certain substances in the culture medium may act as potent inducers or inhibitors of enzyme production, affecting enzyme activation or weakening the expected effects. However, the traditional methods for determining the potential activators or substances that inhibit the growth and development of microorganisms are time consuming and expensive. An excellent alternative in this regard is a phenotypic microarray (Phenotype Microarrays, Biolog® PM). This method simplifies the quantitative analysis of a number of phenotypic traits of microorganisms by combining it into a single test. This analysis encompasses a set of characteristics related to the catabolic properties and chemical sensitivity of microorganisms and plays a key role in optimizing the culture conditions for microbial sporulation or production of secondary metabolites (Nowak *et al.* 2012; Kuan *et al.* 2015).

The selection of relevant culture conditions and substrate compositions can significantly shorten the time needed to achieve the maximum cellulolytic activity. The cost of production for cellulolytic enzymes is highly important. Production costs include the price of the media components and all its expenditures to ensure the necessary conditions for culturing microorganisms. The efficiency of enzyme biopreparations may vary depending on the type of biomass subjected to hydrolysis. Therefore, optimum conditions for cellulolytic activity of obtained enzymes is also important to be evaluated. The goal of this study was to optimize the microbiological media to improve the production of *Trichoderma atroviride* G79/11 cellulases for further application as a cellulolytic biopreparation (Oszust *et al.* 2017). Moreover, the phenotypic microarray characterization of *T. atroviride* was performed using the Biolog® PM plates approach.

EXPERIMENTAL

Materials

Trichoderma atroviride G79/11

The G79/11 strain was selected from dairy sewage sludge (dairy wastewater treatment plant in Krasnystaw, Poland) and identified as *Trichoderma atroviride*. The D2 LSU genomic region was analyzed by comparative sequencing with universal primers. MicroSEQ® ID software was used to assess the raw sequence files and to perform sequence matching to the MicroSEQ® ID-validated reference database (Frąc *et al.* 2014).

The nucleotide sequence of segment D2, located within the large ribosomal subunit (LSU), was deposited in the GenBank database under the accession number KT333455. The G79/11 strain was deposited in the International Culture Collection of Microorganisms of the Industrial Biotechnology Agricultural and Food Institute in Warsaw, Poland, under the number KKP 2056p. An isolate with high hydrolytic potential was selected in the Laboratory of Molecular and Environmental Microbiology, Institute of Agrophysics, Polish Academy of Sciences in Lublin.

Methods

Phenotypic microarray of T. atroviride G79/11

Phenotype microarrays for filamentous fungi (PM Biolog®, Hayward, CA, USA) were used to characterize the *T. atroviride* G79/11 strain. To indicate which carbon sources were metabolized by the G79/11 strain, PM1-PM2 plates were used. The following plates were used to assess its nutritional requirements: phosphorus and sulfur, PM4; supplements, PM5; and nitrogen, PM3 and PM6-PM8. The chemical sensitivity of the G79/11 strain was determined with a phenotypic microarray panel from PM21 to PM25. The arrangement of the individual compounds on the PM plates are described at www.biolog.com. The G79/11 strain was cultured for 14 days at 27 °C in soy flour, cellulose, and lactose (MSCL) medium before the Biolog® PM procedure for filamentous fungi was employed. A homogenous suspension of spores in inoculation fluid (IF-FF, Biolog®) was prepared by obtaining 62% of transmittance (*T*) measured with a turbidimeter (Biolog®). The inoculum was processed using an Ultra Turax IKA® homogenizer for 30 s and then prepared for each PM by adding appropriate supplements, according to the manufacturer's instructions. The plates were inoculated by introducing 100 mm^3 of the various suspensions into each well of the individual PM plates. The plates were incubated for 360 h at 27 °C in the OmniLog® system, which was automatically read every 15 min. The kinetic curves for the G79/11 growth were associated with the use of the compounds placed on the PM plates. The sensitivity of the strain to the chemicals used in the PM panel was presented in units of OmniLog (Biolog®).

Selection of cellulase production media

Trichoderma atroviride G79/11 was cultivated in shaken cultures (productive media) in two types of liquid media, *i.e.*, soy flour-cellulose-lactose (MSCL) and mineral (MA), and in solid fermentation medium based on wheat bran and beet pulp (OPWB). The composition of MSCL, MA, and OPWB is presented in Table 1. For the liquid media, the culture was performed for 5 days to 10 days in 250-mL Erlenmeyer flasks (27 °C, 100 rpm). The cellulolytic activity in media filtrates (MSCL and MA) or extract (OPWB) was determined during cultivation at 24 h intervals at 37 °C and 50 °C in pH 4.5 and 7.0 to evaluate optimal conditions for cellulolytic activity of enzymes obtained during cultivation in productive media. The MSCL and MA filtrates were obtained after separation of the mycelium biomass and insoluble components of the medium by filtering through sterile gauze and centrifugation (4000 rpm, 20 min). The extraction of the cellulases from OPWB was carried out as follows. First, 250 mL of sterile demineralized water was added to each flask with culture. The contents of the flask were ground and shaken for 2 h. Once extracted, the contents of the flask were filtered through sterile gauze and centrifuged (4000 rpm, 20 min) to obtain a clear supernatant.

Cellulolytic activity

Cellulolytic activity was determined based on non-specific cellulolytic activity (saccharifying cellulase) (filter paper unit; FPU) (Mullings 1985; King *et al*. 2007). The absorbance of the colored reaction products were read with a spectrophotometer microplate reader (INFINITE M200PRO, TECAN, Zürich, Switzerland), with a wavelength of λ = 540 nm against the blank. The amount of enzyme, which during 1 min at 50 °C/37 °C liberates 1 µM of glucose, was assumed as a FPU activity unit (Weldesemayat 2011). The analysis was performed in triplicate for each of the biological duplicates. Hence, the optimization of the composition of the medium and culture conditions for *T. atroviride* strain G79/11 was carried out in six replicates (n = 6). The results are expressed as a percentage of the average non-specific cellulolytic activity. The % activity was calculated relative to the highest value non-specific activity for each optimization experiment. The statistical analysis of the results was performed using *Statistica* 10.0 (StatSoft, Inc., Tulsa, OK, USA) based on ANOVA with a significance level of α = 0.05. The Tukey test (p < 0.05) was used to determine significant differences between the mean values of the studied parameters.

Optimization of soy flour-cellulose-lactose medium (MSCL) and culture conditions of T. atroviride G79/11 for enhancement of cellulolytic activity

The optimization of culture conditions, including the composition of media for *T. atroviride* G79/11, was carried out to receive a sufficient production of cellulases in aerobic conditions. The production took place in conical flasks with a capacity of 50 mL and contained 12 mL of medium. The flasks were shaken at 27 °C on a reciprocating shaker at a speed of 150 rpm. The starting point of the culture medium was sterilized soy flour-cellulose-lactose (MSCL) with a pH 4.5, and inoculum having 1×10^9 ($\pm 0.2 \times 10^9$) spores of a 17-day culture on the MSCL medium supplemented with agar (35 g dm^{-3}). The initial composition of MSCL is given in Table 1. The analyses were performed in a biological duplicate with 8 steps. Each step of optimization was based on the optimized former variant in which the highest cellulolytic activity was observed.

Because the sources of carbon and nitrogen (cellulose, lactose, soy flour, and ammonium sulfate (VI)) are the critical components for the production of cellulases, the optimization stages consequently included the selection of appropriate content in the medium.

Table 1. Cellulolytic Media

Solid Medium Based On Wheat Bran And Beet Pulp (OPWB)		Mineral Medium Mandels And Andreotti With Modifications (MA)		Soy Flour-Cellulose-Lactose Medium (MSCL)	
Beet pulp	13.8 g dm^{-3}	0.1 M citrate buffer pH 5.6	480 cm^3 dm^{-3}	Lactose	17.5 g dm^{-3}
Wheat bran	6.9 g dm^{-3}	$(NH_4)_2SO_4$	2.8 g dm^{-3}	Microcrystalline cellulose	11.8 g dm^{-3}
Microcrystalline cellulose	15 g dm^{-3}	KH_2PO_4	4.0 g dm^{-3}	Soy flour	17.5 g dm^{-3}
1% $(NH_4)_2HPO_4$	30 cm^3 dm^{-3}	$MgSO_4$	0.6 g dm^{-3}	KH_2PO_4	6.3 g dm^{-3}
Microelements solution	20 cm^3 dm^{-3}	$CaCl_2 \cdot 2H_2O$	100 g dm^{-3}	$(NH4)_2SO_4$	9.92 g dm^{-3}
		Peptone	2.0 g dm^{-3}	$CaCl_2 \cdot 2H_2O$	0.82 g dm^{-3}
		Tween 80	0.5 g dm^{-3}	$MgSO_4 \cdot 7H_2O$	0.82 g dm^{-3}
		Microcrystalline cellulose	10 g dm^{-3}	Tween 80	0.15%
		Microelements solution	20 cm^3 dm^{-3}	Antifoam B	5 cm^3 dm^{-3}
				Microelements solution	20 cm^3 dm^{-3}
Microelements	(mg dm^{-3})	Microelements	(mg dm^{-3})	Microelements	(mg dm^{-3})
$FeSO_4 \cdot 7H_2O$	513	$FeSO_4 \cdot 7H_2O$	250	$FeSO_4 \cdot 7H_2O$	513
$MnSO_4 \cdot H_2O$	166	$MnSO_4 \cdot H_2O$	80	$MnSO_4 \cdot H_2O$	166
$ZnSO_4 \cdot 7H_2O$	8.5	$ZnSO_4 \cdot 7H_2O$	70	$ZnSO_4 \cdot 7H_2O$	8.5
$CoCl_2 \cdot 6H_2O$	204			$CoCl_2 \cdot 6H_2O$	204

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The components of the medium were optimized in pairs by analyzing cellulose and lactose content in the first step, and soy flour and ammonium sulfate (VI) ($(NH_4)_2SO_4$) in the second step. The optimization of the synthesis of cellulases by *T. atroviride* G79/11 was carried out with 1, 5, 10, 15, and 20 g dm^{-3} concentrations of cellulose and 1, 5, 10, 15, and 20 g dm^{-3} concentrations of lactose at the same time. After selecting the potent concentrations of cellulose and lactose, the content of soy flour and ammonium sulfate, at a concentration range of 5, 10, 15, 20, and 25 g dm^{-3} and 0.5, 2.5, 5, 7.5, and 10 g dm^{-3}, respectively, was optimized. The next step of optimization involved the selection of a suitable source of nitrogen. The study included three nitrogen sources: sodium nitrate (V) ($NaNO_3$), ammonium nitrate (V) (NH_4NO_3), and ammonium sulfate (VI). Next, an analysis of the effect of an addition of Tween 80 to the substrate to increase the activity of the cellulases was carried out. For this step, the detergent concentrations used were 0%, 0.025%, 0.5%, 0.1%, 0.15%, 0.2%, 0.5%, and 1%. The fifth step in optimization of the substrate induction consisted of an assessment of the effect of pH at the values of 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5. The pH was determined using a 1 M hydrochloric acid (HCl) solution and 10% ammonia water (NH_4OH). The sixth step was employed to determine the effect of temperature at the values of 22 ºC, 27 ºC, and 32 ºC on the cellulolytic activity. The seventh step of the optimization procedure included an evaluation of the effect of inoculum radiation with white light. The inoculum was illuminated from the eighth day of culture with fluorescent lamps (OSRAM 30W/77 FLUORA) used as a source of artificial white light. The control mycelium for the inoculum was grown in the dark. In the last, eighth step, the amount of the inoculum used was optimized. The study involved five variants: 8, 10, 12, 14, and 16 x 10^9 spores. Determination of the density of the inoculum was performed in a Thom's chamber. The spores were collected from a 17-day culture on a MSC agar medium induced by white light. The activity of cellulases was measured at 24 h intervals for the 18 days of culture.

MSCL filtrates were obtained after separation of the biomass mycelium and insoluble components of the medium by filtering through sterile gauze and centrifugation (4000 rpm, 20 min) in order to prepare a clear solution for the assay.

RESULTS AND DISCUSSION

Trichoderma atroviride G79/11 Phenotypic Characterization

Trichoderma atroviride G79/11 was characterized by a panel of phenotypic microarray plates (PM). As shown in Fig. 1, the G79/11 strain decomposed the following carbon sources located on plates PM1 and PM2: *α*-D-lactose, L-arabinose, D-trehalose, D-mannose, L-fucose, D-xylose, D-mannitol, D-ribose *α*-D-glucose, uridine, D-cellobiose, *p*-hydroxyphenylacetic acid, n-hydroxyphenylacetic acid, L-lyxose acid, D-galacturonic, dextrin, gelatin, laminarin, pectin, D-arabinose, D-arabitol, arbutin, *i*-erythritol, gentiobiose, palatinose, D-raffinose, 2-hydroxybenzoic acid, quinic acid, sorbic acid, and dihydroxyacetone. The capability of the G79/11 strain to metabolize of disaccharides such as cellobiose, lactose, or dextrin observed in the PM analysis indirectly indicates that the isolate exhibits *β*-glucosidase, lactase, and amylase activity, respectively. The ability to utilize gelatin and pectin suggests that the test strain has the capability to produce proteolytic and pectinolytic enzymes as well. The PM approach revealed that the *T. atroviride* G79/11 strain degraded cellulose and starch. However, the PM method was more sensitive and demonstrated a wide range of catabolic abilities. Due to the complex nature of their mixture and a wide range of organic compounds, organic wastes undergoing anaerobic digestion can be a source of potential bioproducts and biocomponents used in biotechnology.

The PM studies showed that G79/11 mainly utilized 2',3'-phosphate, monocyclic

adenosine disposed on plate PM4, for which the level of catabolism was 12.7% higher than in the control, as a phosphorus source (Fig. 1). However, the presence of nitrate (V), urea, and D-glucosamine resulted in the complete inhibition of G79/11 growth. Other phosphorus compounds showed no effect on its growth. No source of sulfur (PM4) (Fig. 1) inhibited the activity of G79/11. Only thiosulphate, tetrathionate, phosphorothioate, and taurine and its derivatives, as well as D, L-lipoamide, and sulfone derivatives, inhibited the activity of the G79/11 strain, but only up to 50%. The presence of such substrates as derivatives of methionine, glutathione, and lanthionine resulted in a 35% growth of the analyzed strain. The strain was further found to have the ability to grow in L-glutamine acid, D, and L-α-hydroxybutyric acid as well as D, L-carnitine supplements (PM5) (Fig. 1). The strain exhibited a growth inhibition in the presence of 9% and 10% sodium chloride (NaCl), 6% NaCl with the addition of L-carnitine, 6% NaCl, trigonelline, and 4%, 5%, and 6% sodium formate. Lactose is considered a good inducer of the production of cellulolytic enzymes (Aro *et al.* 2005). On the other hand, the phenotypic study revealed a high level of utilization of this substrate by *T. atroviride* G79/11. Therefore, the aim of the investigation was to optimize the medium targeted for G79/11.

The utilization of nitrogen sources by the strain is shown in Fig. 2. G79/11 showed the ability to grow in the presence of nitrate(III), nitrate(V), L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-glutamic acid, L-glutamine, glycine, L-leucine, L-phenylalanine, L-pyroglutamic acid, L-proline, L-serine, L-valine, D-valine, L-homoserine, L-ornithine, N-amylamine, ethanolamine, agmatine, acetamide, formamide, mannosamine, N-acetyl-D-glucosamine, adenosine, guanine, guanosine, inosine, xanthine, allantoin acid, and γ-amino-n-butyric acid. A literature review shows that urea and ammonium sulfate are most commonly used as a nitrogen source for cellulase production (Wesołowska-Trojanowska and Targoński 2014). The study results indicate that urea has an inhibitory effect on the growth of G79; however, the strain exhibited an efficient growth rate in the presence of nitrates and other nitrogen sources during the optimization of the MSCL medium, with the exception of ammonium sulfate. Ammonium nitrate and sodium nitrate salts were also tested. Furthermore, G79/11 grew relatively well on a number of amino acids. This clearly demonstrates the need for organic nitrogen sources in the analyzed test strain. Soy flour is an excellent source of organic nitrogen in the form of amino acids (methionine, proline, serine, valine); additionally, it is relatively inexpensive and rich in vitamins and trace elements (Shin *et al.* 2013, 2014). The PM results, including a set of characteristics related to the catabolic properties and chemical sensitivity of microorganisms, play a key role in optimizing microbial culture conditions, spawning, and the production of secondary metabolites (Panek *et al.* 2016). Thus, in further stages of the research, the induction medium for the *T. atroviride* G79/11 strain was optimized in terms of this ingredient.

The PM21-PM25 phenotype microarray assay (Fig. 3) showed that the *T. atroviride* G79/11 strain was inherently sensitive to the following compounds: promethazine hydrochloride, dodecyltrimethylammonium bromide, sodium dichromate, copper sulfate (II), trifluoperazine, diamide, thiourea, chloride zinc hydroxamate, L-glutamic acid, caffeine, hydroxamate, L-arginine, glycine hydroxamate, potassium iodide, 3-amino-1,2,3 triazole, lithium chloride, boric acid, benzamidine, acetate, thallium (I), paromomycin, benzethonium chloride, chlorpromazine, dequalinium chloride, glycine hydrochloride, hydroxylamine chloride, chromium (III) hexahydrate, cuprous chloride dihydrate, sodium metaborate dihydrate, sodium periodate, sodium azide, sodium selenite, thioridazine hydrochloride, isethionate, pentamidine, hydrated methyl chloride, aluminum sulfate, hydroxyurea, tetrazolium violet, amitriptyline hydrochloride, mechlorethamine hydrochloride, 5-fluoro-2'-deoxyuridine, clomiphene citrate, fluorouracil, and ibuprofen. The chemical sensitivity analysis indicated appropriate trace elements to be added to the media. Some of these features are typical of the species *Trichoderma atroviride*.

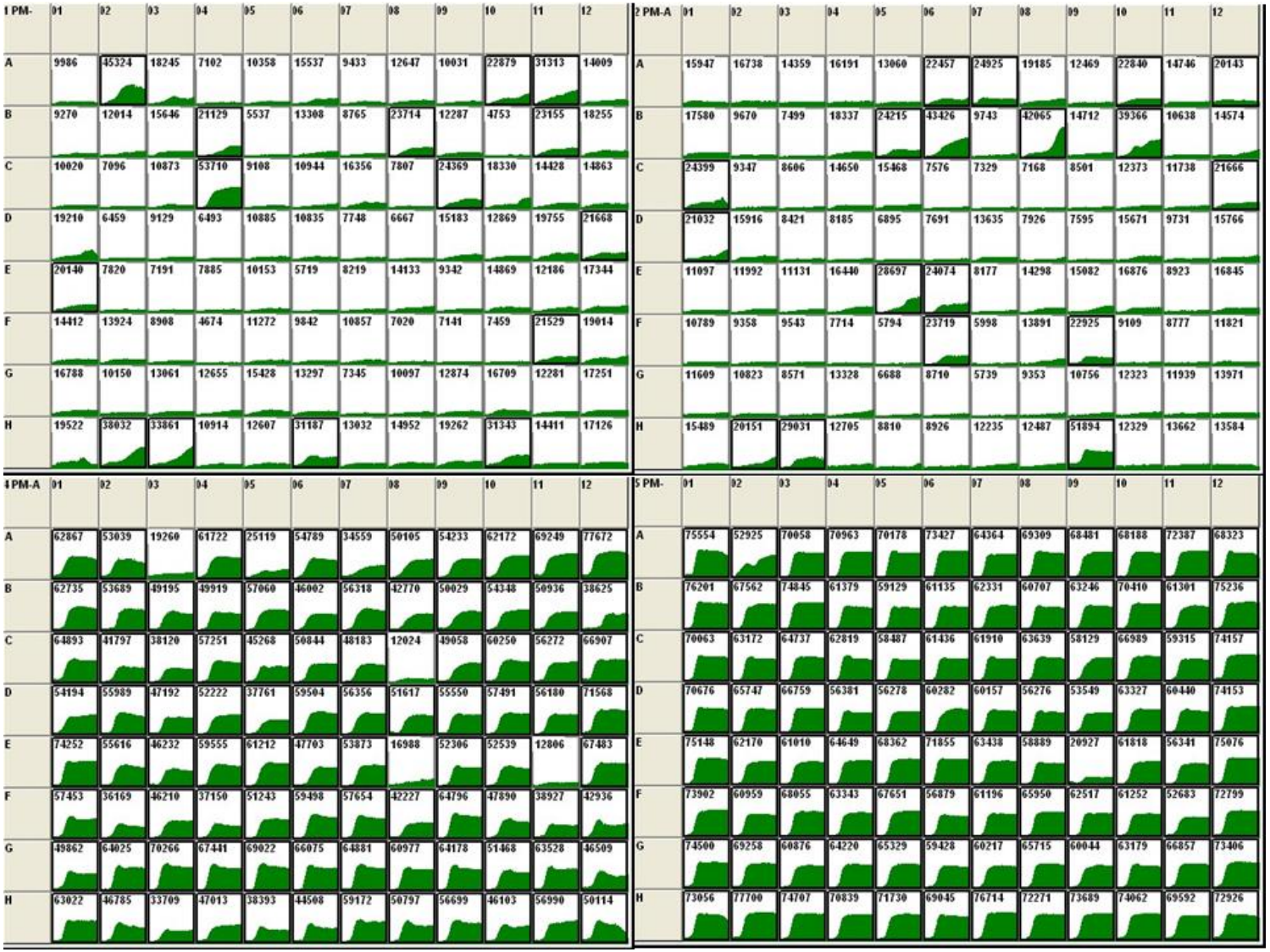


Fig. 1. Carbon, phosphorus, and sulfur supplements utilization by *T. atroviride* G79/11 following Biolog® Phenotype Microarray

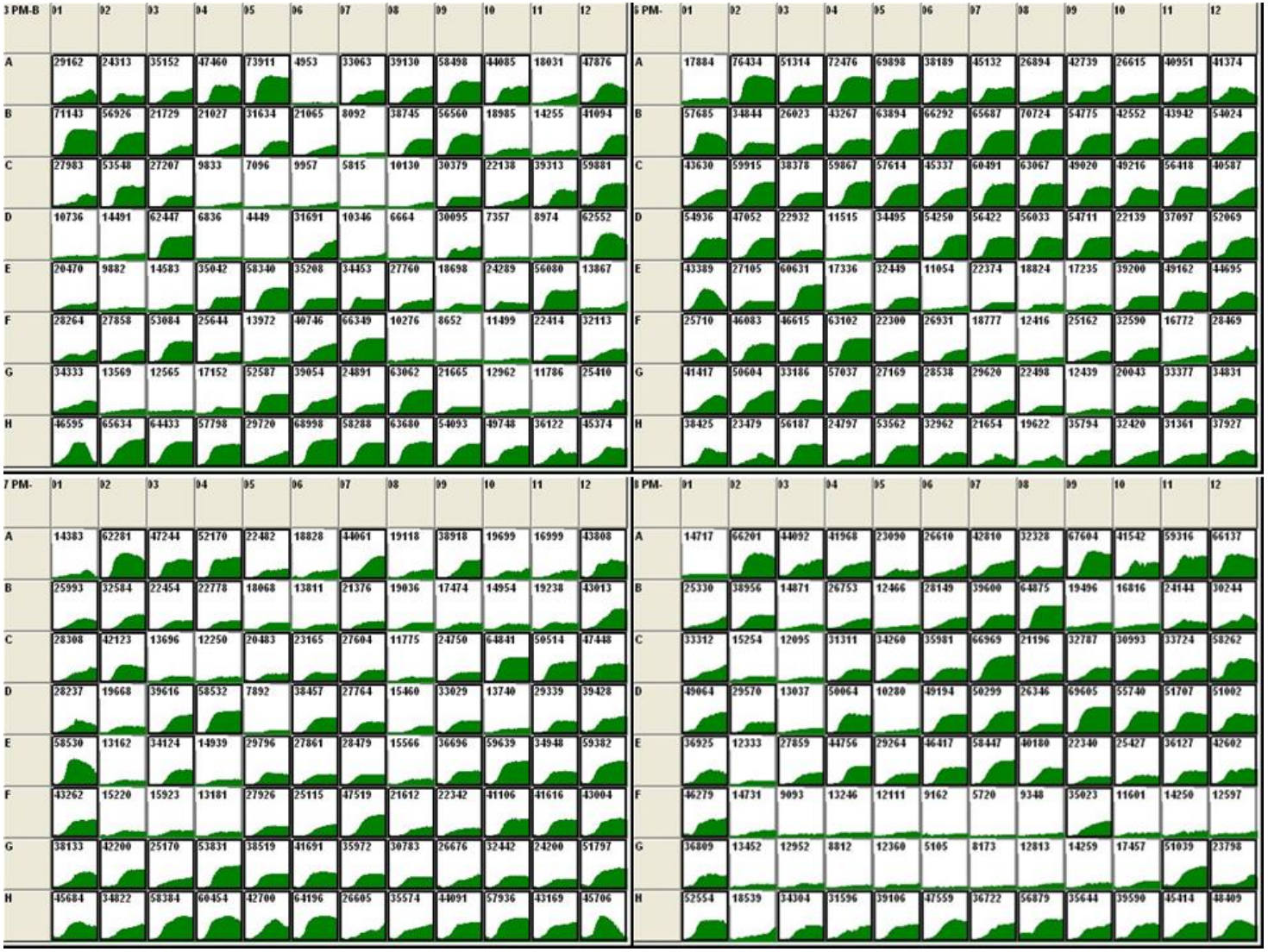


Fig. 2. Nitrogen compounds utilization by *T. atroviride* G79/11 following Biolog® Phenotype Microarray

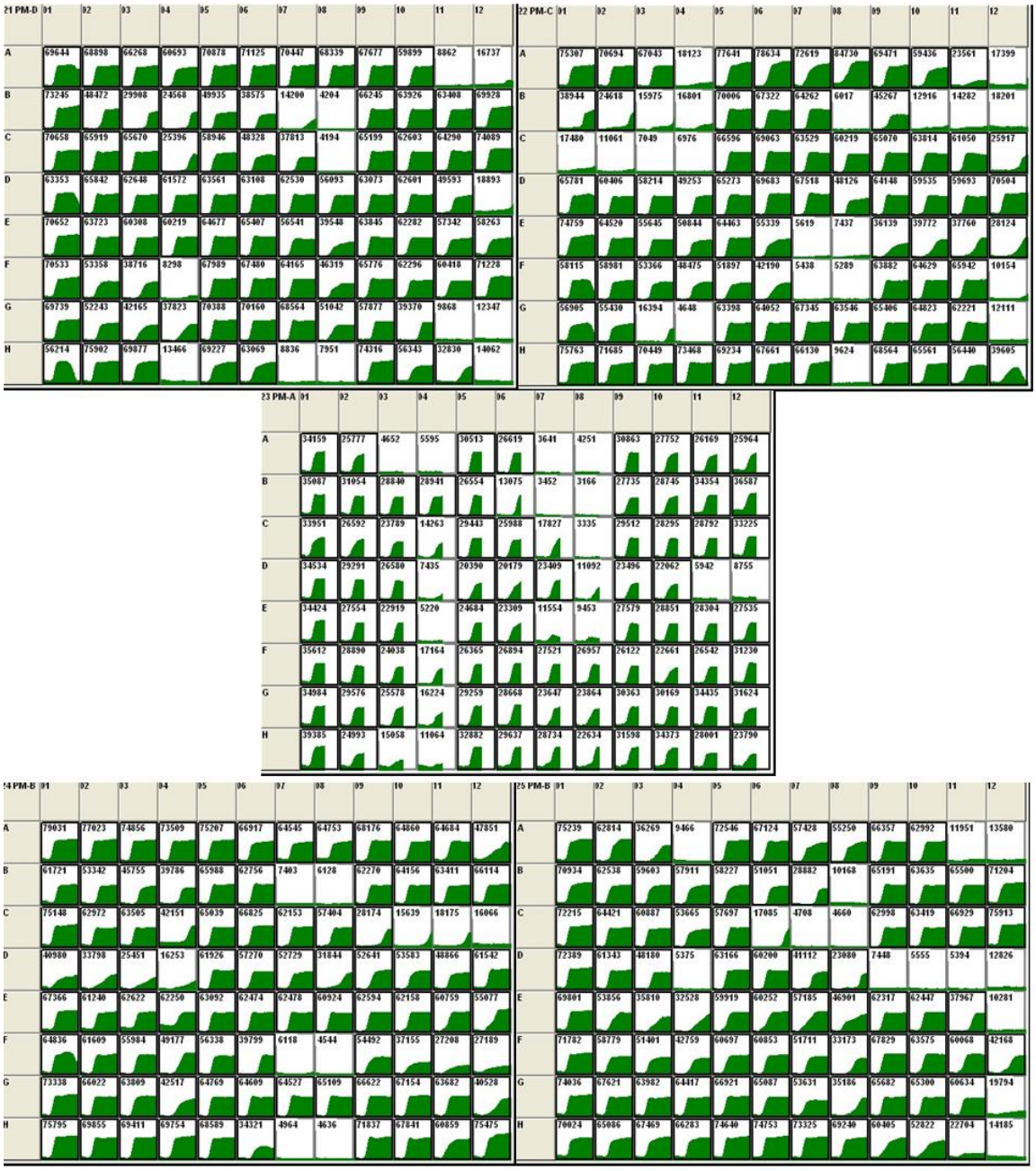


Fig. 3. The sensitivity of *T. atroviride* G79/11 to the chemicals following Biolog® Phenotype Microarray

The results from the phenotypic characterization of the *T. atroviride* G79/11 strain, including the ability to utilize carbon and nitrogen sources, growth on a variety of supplements, and chemical sensitivity, provided the base information needed for further research on the production of cellulases.

Selection of Cellulase-Production Medium for the *T. atroviride* G79/11 Strain

The results shown in Fig. 4. revealed that the MSCL medium was the most conducive to the production of cellulolytic enzymes by the *T. atroviride* G79/11 strain. Therefore, MSCL was selected for the next stages of the research. The OPWB medium

was not conducive to the production of cellulolytic enzymes by the G79/11 strain. Only cellulases derived from the culture of the *T. atroviride* G79/11 fungus from MA on the fifth and seventh days of culture showed activity (80% and 70% of the maximum activity obtained for cellulases from the MSCL culture medium), and that too only at a pH 4.5 and a temperature of 37 °C. This indicates a limited range of operating conditions that can be used for obtaining enzymes, in comparison to the *T. atroviride* G79/11 culture on a MSCL medium. On the fourth day of culture, effective cellulase production was recorded at a temperature of 37 °C. However, on the second and third day, G79/11 showed the highest cellulase production at 50 °C. In terms of the possibility of the use of cellulases for biomass hydrolysis, this is a real advantage, and a step contributing to the final improvement of the efficiency of the methane fermentation process. Thus, hydrolysis based on enzymes derived from the fungal G79/11 strain culture can be carried out in both mesophilic and thermophilic conditions under a wide range of pH values. These results primarily indicate the cost-effective use of *T. atroviride* G79/11 enzymes in biomass conversion or degradation.

The variability effect from the cellulolytic activity, of the G79/11 strain cultured on various media (OPWB, MA, MSCL) depending on pH and temperature conditions, may be associated with modifications of cellulase proteins by photolytic enzymes. Proteases can influence cellulolytic enzymes in three ways: they control the secretion of extracellular enzymes, affect their activity and stability, and participate in production thereof by modifying cellulase-derived inactive forms. Proteases can function as post-translational modulators that convert inactive zymogens into fully active enzymes (Janas *et al.* 2004).

The cellulase system of various strains of cellulose-degrading fungi has been extensively studied in recent years (Bischof *et al.* 2016). Cellulose deconstruction is a complex biological process and is achieved through the sequential order of multiple carbohydrate-active enzymes *e.g.*: endoglucanases, exoglucanases, β-glucosidases, cellobiohydrolases, which may also occur as isoenzymes (Witte *et al.* 1990). The concept of enzymatic saccharification of cellulose by a synergistic combination of different cellulase activities was proposed. Therefore, the complete cellulose saccharification occurs due to the secreted cellulase mix of *Trichoderma* G79/11. Moreover, cellulase synthesis changes during fungus cultivation and FPU method is not highly specific (it detects activities of all cellulose degrading enzymes). Therefore, the changes in the total cellulolytic activity may be the result of changes in activity of particular enzymes and their isoforms during the time. In addition, crude enzyme preparation was analyzed, therefore enzymes activity may be selectively inhibited or increased.

Optimization of the MSCL Medium Composition

Cellulolytic enzyme activity in fungi largely depends on the composition of the medium ingredients. Productive media must contain all the nutrients necessary to meet the requirements for energy and carbon that are essential for cell function, as well as trace elements and vitamins.

The basic criterion for a medium is to contain sources of carbon and nitrogen. Other cardinal factors determining the production of cellulolytic enzymes include the pH of the culture medium, temperature, presence of light, addition of a detergent, and amount of inoculum introduced into the medium (Aehle 2007).

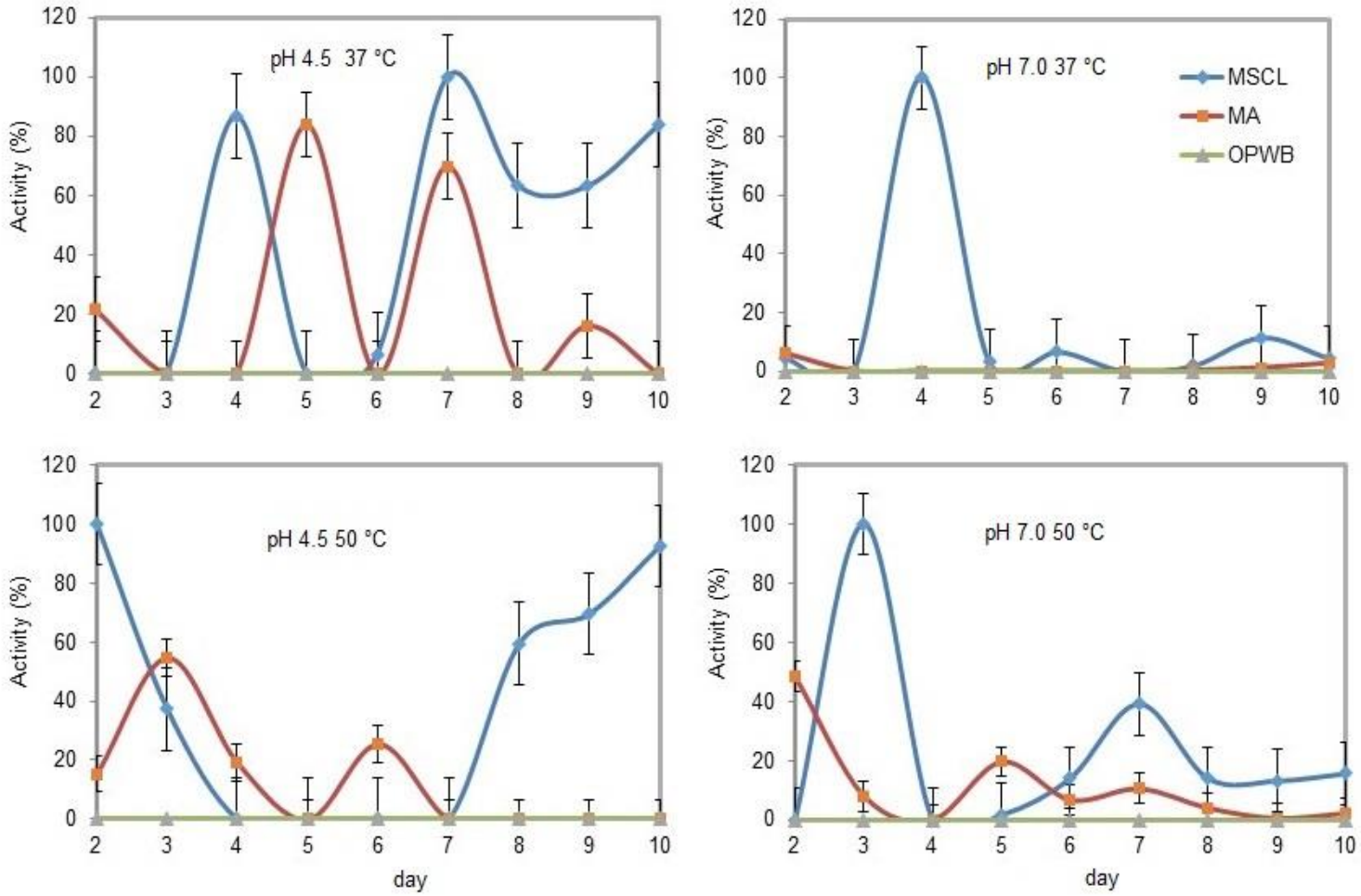


Fig. 4. The non-specific cellulolytic activity of *T. atroviride* G79/11 on the three productive media depending on the pH and temperature. Explanation: MSCL is the soy flour-cellulose-lactose medium, MA is the mineral medium, and OPWB is the medium based on sugar beet and wheat bran. The error bars represent the standard error.

Nutrients are usually added to the medium in an amount of 5 g dm^{-3} to 25 g dm^{-3} and are provided as a yeast extract. Ammonium sulfate or urea is commonly used as a nitrogen source (Wesołowska-Trojanowska and Targoński 2014). Additionally, ingredients added to the culturing media are often enzyme production inducers. While developing media for cellulolytic activity, it is important to evaluate the ability of the microorganism to absorb their ingredients.

The production of cellulases is dependent upon the presence of culture medium ingredients and is repressed by the presence of a readily assailable carbon source, *e.g*. glucose (Aro *et al.* 2005; Wang *et al.* 2014). In turn, cellulose with a suitable crystal structure is a good inducer of cellulolytic enzyme production. In many of the culturing media for cellulase production that is proposed in literature, cellulase production inducers include carboxymethylcellulose (CMC), filter paper, α-cellulose, and Avicel cellulose, as well as lignocellulose waste biomass, *e.g*., sugar cane, wood chips, straw, rape and wheat, and lactose from whey. The materials are relatively cheap, readily available, and contain the nitrogen and mineral salts or vitamins necessary for the growth and functioning of microorganisms (Kuhad *et al.* 2016). Pure cellulose can be replaced with lignocellulose biomass in solid-state fermentation media, which is beneficial from an environmental and economic point of view. This is one way for the management of organic waste, but can also lead to reduction of culturing costs related to the high cost of cellulose. In this case, the wheat bran and sugar beet pulp waste products, *i.e*., the main components of the OPWB medium, were analyzed in this study. However, due to poor growth of the mycelium on the OPWB, and consequently the poor production of cellulases even during the 10 days of culture, the OPWB was abandoned in further studies and MSCL was optimized. The synthesis of cellulases by *T. atroviride* G79/11 was tested for different concentrations of cellulose (1 to 20 g dm^{-3}) and lactose (1 to 20 g dm^{-3}) in the MSCL medium. Fig. 5a shows the non-specific cellulolytic activity of the tested strain on the fifth day of culturing. The results indicate that the highest efficiency of cellulase production was obtained for a MSCL containing 5 g dm^{-3} of cellulose and 10 g dm^{-3} of lactose. This step yielded an approximately 2-fold increase in the production of cellulases by the G79/11 strain (6.4 cm^3 U), in comparison to the initial MSCL composition.

Supplementation of the nutrient composition in the MSCL medium with soy flour was also proposed. This component is readily available and relatively cheap, compared with yeast extract. A literature review shows that such an ingredient has been used in the biotechnological production of cellulases for a strain of *Trichoderma longibrachiatum* (Xie *et al.* 2015) and *Aspergillus fumigatus* (Kahil and Hassan 2015), but there are few studies where its quantity is subjected to optimization in order to increase the production of cellulases. Therefore, this step involved optimization of cellulase production using soy flour in the range of 5 to 25 g dm^{-3} and ammonium sulfate ($(NH_4)_2SO_4$) (0.5 to 10 g dm^{-3}) as a nitrogen source (Fig. 5b). The maximum cellulolytic activity was detected when the content of ammonium sulfate reached 5 g dm^{-3} and soy flour 20 g dm^{-3}. However, in all the analyzed concentrations of ammonium sulfate and soy flour at 15 to 20 g dm^{-3}, the strain also showed relatively high cellulolytic activity (5.8-6.4 U cm^{-3}). Based on these results and taking into account the economic aspects of production for cellulolytic biopreparations, the costs of the development of a relatively productive medium in successive stages of optimization, with 5 g dm^{-3} of ammonium sulfate and 20 g dm^{-3} of soy flour, should be decreasing. At the optimized content in the medium, already 1 g dm^{-3} of ammonium sulfate and 15 g dm^{-3} of soy flour had contributed to a relatively high enzyme activity.

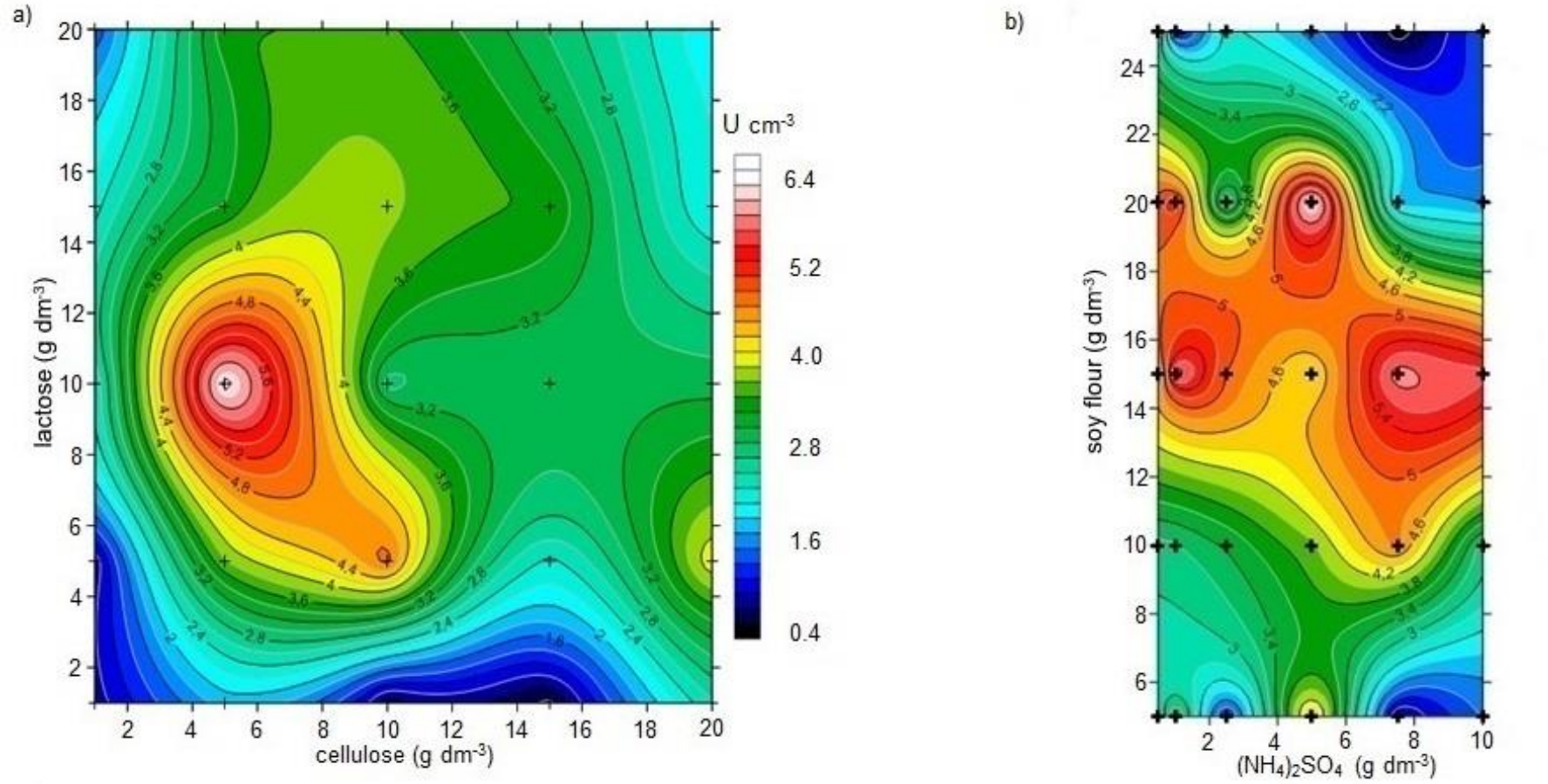
The effects of the type of nitrogen source (sodium nitrate, ammonium nitrate, and

ammonium sulfate) on the cellulolytic activity of G79/11 are shown in Fig. 5c. The nitrogen source was added at the level of 5 g dm^{-3}. Depending on the day of culture, different effects were observed with the different types of nitrogen sources. The highest activity was recorded on the fifth day of the culture for all the nitrogen compounds tested. By that time, the cellulolytic activity had increased by 10% for ammonium nitrate in comparison to the ammonium sulfate control.

The addition of surfactants such as Tween 80 into the media has a greater impact on efficient production of cellulases (Deswal *et al.* 2011). Therefore, the effects of Tween 80 content in the MSCL medium on the efficient production of cellulolytic enzymes was checked in this study during the five days of culturing (Fig. 5d). The highest values of cellulolytic activity for the tested Tween 80 variants were noted on the fifth day. The addition of this ingredient in an amount of 0.025%, 0.05%, 0.5%, and 1% caused a 10% decrease in the cellulolytic activity of the G79/11 strain in relation to the variant without the addition of the detergent. In contrast, addition of 0.1% to 0.2% of Tween 80 to the media caused an increase in the production of cellulose on the fifth day of the culture by approx. 10% compared to the variant without the added detergent. The highest cellulase activity was achieved after an application of 0.15% Tween 80. Thus, its amount was maintained at this level. However, the 0.2% Tween 80 additive shifted the maximum secretion of cellulases on the fourth day of the culture and provide 96% cellulolytic activity, which made a positive contribution in terms of development of a cellulolytic biopreparation of *T. atroviride* G79/11.

The effect of pH on the production of cellulolytic enzymes by the G79/11 strain was studied. Figure 6a shows the relationship between the cellulolytic activities of the strain and the pH of the MSCL medium. The highest activity was observed when the strain was cultured in an acidic medium (pH in the range of 3.5 to 5.5). Substantially higher activity values were observed when the medium had a pH value of 4.5. In turn, 50% and 90% inhibition of cellulase activity were observed at pH 6.5 and 7.5, respectively. At pH 8.5, there was no production of cellulolytic enzymes. The optimum pH for the production of fungal cellulases is in the range of 4.5 to 5.5, which was also confirmed in investigations of cellulases from *Aspergillus phoenicus* (Kim *et al.* 2009). The authors showed the maximum adsorption of cellulases for the pH range of 4.8 to 5.5, which also corresponded to the highest enzyme activity. Changing the pH values alters the properties of the substrate by modifying the content of ionic components in the medium. For these reasons, the pH profile may be different in the case of cellulosic co-substrates of various origin or composition, which is important at the stage of enzymatic hydrolysis of the composed biomass.

The relationship between the non-specific cellulolytic activity of G79/11 and temperature conditions over the five days of culture in the MSCL medium is shown in Fig. 6b. In each day, there were differences in the activity for the 22 °C, 27°C, and 32 °C temperature variants. The maximum activity was recorded at the temperature of 22 °C, while the lowest value was detected at 32 °C, which may be related to the amount of enzymes produced or the involvement of different forms of active and inactive enzymatic proteins. The greatest variation between the studied temperature variants was observed on the fourth day. In comparison to 22 °C, a 42% reduction of cellulase production was found at 27 °C. In turn, at a temperature of 32 °C, only 10% of the production was found on the fourth day of culturing relative to the value obtained at a temperature of 22 °C.


a)
lactose (g dm-3)
cellulose (g dm-3)
U cm-3
6.4
5.2
4.0
2.8
1.6
0.4
b)
soy flour (g dm-3)
(NH4)2SO4 (g dm-3)

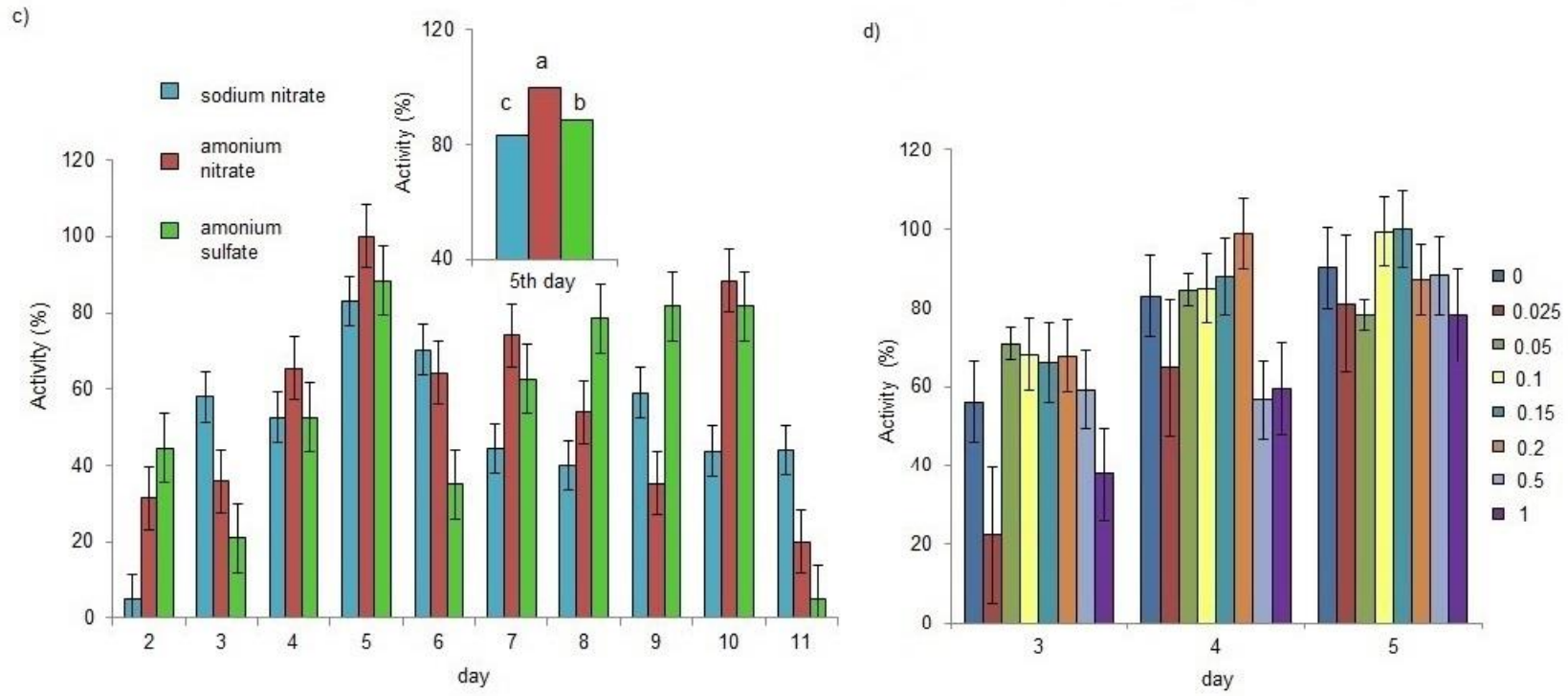


Fig. 5. The non-specific cellulolytic activity of *T. atroviride* G79/11 depending on (a) the lactose and cellulose content, (b) the soy flour and ammonium sulfate content, (c) nitrogen source, and (d) Tween 80 content in the soy flour-cellulose-lactose medium (MSCL). Different letters above the bars indicate errors on the significance of differences (α = 0.05) between the mean values of the studied parameters (Tukey's test ($p < 0.05$))

The optimum temperature for cellulolytic enzyme biosynthesis by fungi of the *Trichoderma* genus, in culture directly on the lignocellulose, was determined as 30 °C (Hawrot-Paw and Izwikow 2016). Other researchers reported the maximum production of cellulolytic enzymes at 45 °C (Gautam *et al.* 2010). The ability of the *T. atroviride* G79/11 strain to secrete enzymes at lower temperatures is an advantage. Therefore, industrial extraction of enzymes from G79/11 cultures will not require high expenditures.

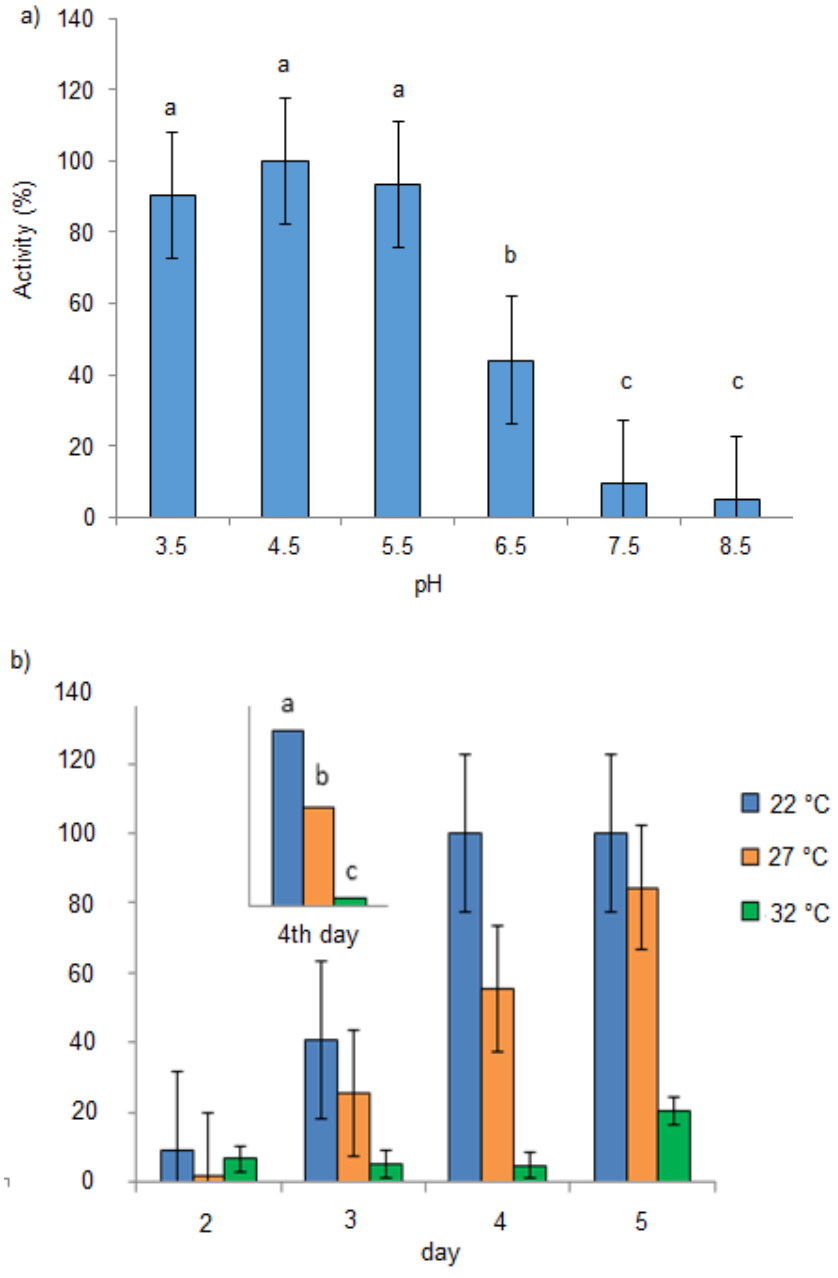


Fig. 6. The non-specific cellulolytic activity of *T. atroviride* G79/11 depending on (a) the pH of soy flour-cellulose-lactose medium (MSCL) and (b) temperature of the culture. Different letters above the bars indicate errors on the significance of differences (α = 0.05) between the mean values of the studied parameters (Tukey's test ($p < 0.05$))

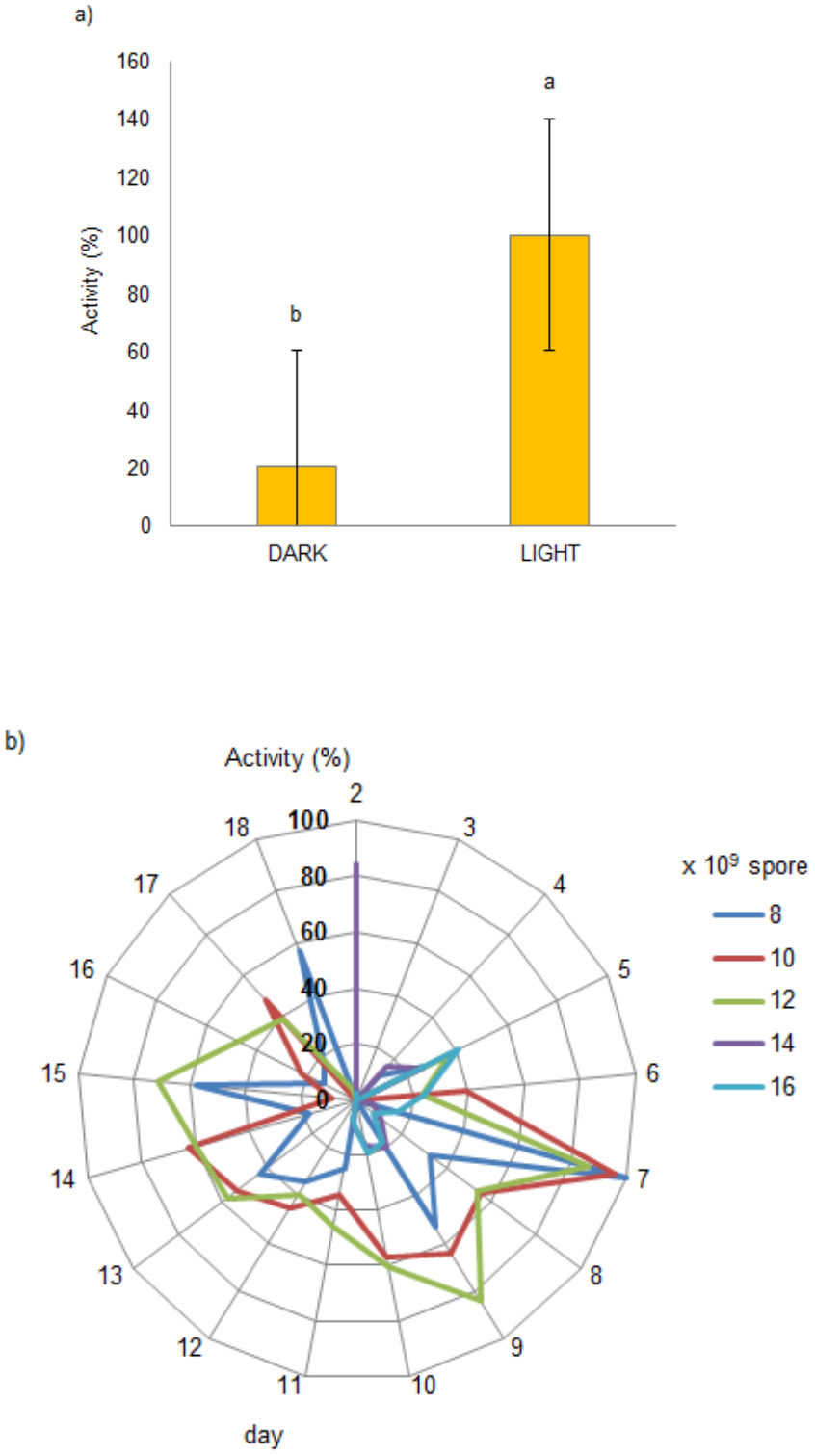


Fig. 7. The non-specific cellulolytic activity of *T. atroviride* G79/11 depending on inoculum radiation with white light (a) inoculum amount, and (b) the culture time. Different letters above the bars indicate errors on the significance of differences (α = 0.05) between the mean values of the studied parameters (Tukey's test (p <0.05))

In this study, cellulase production by the G79/11 strain was improved when the inoculum was radiated (Fig. 7a). According to Steyaert *et al.* (2010), light prompts sporulation in *Trichoderma* fungi. Cellulase activity was approximately 75% higher when the inoculum was treated with white light starting from the eighth day of culture, compared to the variant without exposure to light (dark). The high cellulolytic activity was probably related to the amount of enzyme activated for the germination of conidial spores. Germinated spores taken from inoculum adapted in the proper production media and fulfilled the expected function. However, spores (inoculum) radiated with linearly polarized white light result in increased specific activity and hydrolysis of microcrystalline cellulose in comparison to the native enzyme (Nowak *et al.* 2012). Therefore, some activated enzymes may have been transferred with the inoculum to the MSCL medium, thereby resulting in an increased cellulolytic activity.

An interesting question is how long the enzymes remain active and what factors suppress them. Some studies (Aro *et al.* 2005; Hsieh *et al.* 2014) indicate that accumulation of a cellulose hydrolysis product results in the inhibition of cellulolytic enzymes. Zhao *et al.* (2016) demonstrated the tolerance of cellulolytic microbial strains to high contents of glucose in the medium and synergistic action of cellulase mixtures derived from phylogenetically distant strains. After adsorption of cellulases to a substrate, the enzymatic reaction starts. The pH value, temperature, and inoculum quality and quantity are key determinants of enzymatic activity. In this study on the optimization of the amount of inoculum, daily fluctuations in the cellulolytic activity for the inoculum variants of 8, 10, and 12×10^9 spores (Fig. 7b) were observed. The study showed that the lowest amount of the inoculum caused greater shifts in the production of cellulases. From an econometric point of view, the lowest amount of the inoculum that yields the highest production of cellulases can bring tangible benefits. However, it can also cause problems with capturing the moment with the highest enzyme secretion. The variant with the lowest number of spores (8×10^9) was selected for further analysis.

CONCLUSIONS

1. The MSCL medium was optimized for increased cellulase production of *T. atroviride* G79/11. The culture filtrate was used to obtain the metaferm biopreparation in liquid and lyophilizate forms. The phenotype microarray, based on Biolog® PM plates, was a useful tool for preselecting ingredients to compose the proper medium.
2. Cellulase production increased at the composition of 10 g dm^{-3} lactose, 5 g dm^{-3} microcrystalline cellulose, 20 g dm^{-3} soy flour, 6.3 g dm^{-3} KH_2PO_4, 9.92 g dm^{-3} $(NH_4)_2NO_3$, 0.82 g dm^{-3} $CaCl_2 \times 2H_2O$, 0.82 g dm^{-3} $MgSO_4 \times 7H_2O$, 0.15% Tween 80, 5 cm^3 dm^{-3} Antifoam B, 513 mg dm^{-3} $FeSO_4 \times 7H_2O$, 166 mg dm^{-3} $MnSO_4 \times H_2O$, 8.5 mg dm^{-3} $ZnSO_4 \times 7H_2O$, and 5.4 mg dm^{-3} $CoCl_2 \times 6H_2O$ and in the following culture conditions: media with a pH of 4.5, temperature of 22 ºC, and *Trichoderma atroviride* G79/11 inoculum (8×10^9 spores) radiated with white light.

ACKNOWLEDGEMENTS

This work was funded from the budget for science by The National Centre for Research and Development in Poland (LIDER 048/L-2/10 Programme 2011-2014).

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Article submitted: July 10, 2017; Peer review completed: September 17, 2017; Revised version received: September 20, 2017; Accepted: September 21, 2017; Published: September 26, 2017.
DOI: 10.15376/biores.12.4.8468-8489