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Cellulase production and morphology of *Trichoderma reesei* in different experimental conditions

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SUMMARY. Cellulases production involving alternative substrates has been intensely researched, because it offers perspectives for lowering the costs for enzyme production, costs which are a major obstacle for the development of this field. The concentration, turnout, effectiveness of the enzyme production depends on the content of culture medium. In order to find a way to increase cellulase production, two culture media with different cellulosic substrates in submerged culture experiments were tested. We used a promising off-corn growth media that was efficient and the cellulases output due to substrate action was calculated. The reduction of the sugars released by the enzyme was noticed. On off-corn medium the measurements reached 0.188 mg of released glucose/mL/min/50ºC, while on PSM medium the strains reached the release of only 0.118 mg glucose/mL/min/50ºC. The abundance of the fungi and the pellet morphology were microscopically compared by optic and electronic means. Mycelia with hyphae and spores were also visible in these circumstances, suggesting that when the environment of the mycelium alters, part of the mycelium autolyses and spores are released to propagate. The present study also included the use of newly synthesized cellulases in order to obtain a plant protoplast culture. This study proves that cellulolytic enzymes with further application in laboratory can be provided by less expensive techniques.

Keywords: cellulase activity, off-corn culture media, *Trichoderma reesei*

Introduction

Trichoderma reesei represents an efficient producer of extracellular proteins, therefore it is widely employed in cellulase production. Cellulases play a central role in the biological conversion of lignocellulosic materials to fermentable sugars. Three types of cellulases are known to be produced by *T. reesei* and to interact releasing glucose,

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namely: cellobiohydrolases or exo-β-glucanase (EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and β-glucosidases EC 3.2.1.21) (Ma *et al*., 2013; Gupta *et al*., 2016). *T. reesei* also provides xylanase for the digestion of hemicellulase, resulting xylose. The genome of *T. reesei* comprises at least 200 genes involved in encoding glycoside hydrolases (Häkkinen *et al*., 2012), enzymes which free fermentable sugars from lignocellulose. Accessary proteins have a support role, getting involved alongside cellulases, to assist degradation of lignocellulosic biomass, and are also subjected to increasing interest (Gao *et al*., 2011; Zhang *et al*., 2018). These components act synergistically in the conversion of cellulose to glucose (Muthuvelayudham and Viruthagiri, 2006).

Cellulose is the most abundant component of the plant cell wall. If its structure is broken down a wide variety of sugars would become available for other organisms or for life/carbon cycles. Fungi of the genus *Trichoderma* have become very popular due to their capacity to produce a wide range of cellulolytic enzymes (cellobiohydrolases, endo-β-1,4-glucanases and β-glucosidases) (Zhang *et al*., 2012; Somerville *et al*., 2004; Caroll and Somerville, 2009).

Trichoderma reesei has become the most important source of cellulolytic enzymes (Schuster and Schmoll, 2010) and subsequently (has become) a key point in producing second generation biofuels (Sukumaran *et al*., 2005; Bharathiraja *et al*., 2017; Wang *et al.,* 2014). Fuel crisis around the world will be a distant issue to deal with, if an alternative to fossil combustibles is going to be found. New strains of *Trichoderma reesei* have already an improved ability to increase the cellulose production more than 15–20 folds, compared to the wild type strain (Kubicek, 2013; Xia *et al.,* 2018). Even though many authors pursue the goal of producing industrial cellulases (Ahamed and Vermette, 2008; Jourdier *et al*., 2012), the goal of our work was to use a *Trichoderma reesei* strain to perform further laboratory scale experiments in plant/bacterial biotechnology area. Another study highlighted that not only the carbon source is affecting the co-regulation of biomass-degrading enzymes but also the structural characteristics of the substrate (Foreman *et al*., 2003; Peciulyte *et al*., 2014; Zhang *et al*., 2017).

We started our work in order to extend the knowledge on *T. reesei* liquid culture and reduce laboratory costs when cellulases are needed. Two culture media with different cellulosic substrates as carbon and energy source in submerged culture experiments were tested in order to find a way to increase celullase production. We found an off-corn growth media that was efficient, and we calculated the yield of cellulases production. Microscopic observations by optic and electronic means were carried out and a comparison between the fungi frequency and pellet morphology was made. An attempt to use the newly synthesized cellulases to obtain a plant protoplast culture is a part of our work. This study shows that cellulolytic enzymes with further application in laboratory can be obtain with low cost expenses.

Materials and methods

1. The microorganism. The microorganism used in this study was *Trichoderma reesei*, which was maintained on potato dextrose agar culture media (M129 culture media) (Göbel *et al.,* 2004) and grown and recultivated by culture plate method on PSM culture medium with agar (Mandel's agar media) (Mandels, 1975). This fungus was incubated at 30ºC, for 5–7 days, and after that the colonies with evidence of cellulose digestion were selected to be further assayed for cellulose activity.

2. Decomposition of cellulose (filter paper) by Trichoderma reesei. To emphasize cellulose decomposition, *Trichoderma reesei* inoculum is cultivated on a specific medium and incubated at 28ºC for 4 weeks. The characteristics of growth, the color of the paper and the part of the strip indicating disintegration are periodically observed. At the end, the number of test tubes, for each dilution, in which growth took place is determined, and the probable number of cellulosolytic microorganisms is calculated. The degree of paper deterioration is observed, and the phase of paper deterioration is assessed, compared to control (Carpa *et al*., 2014).

3. Cellulase activity at Trichoderma reesei in submerged system. For the submerged system the experiment consisted in two sets. In one set, the mineral culture media (PSM) contained mineral salt solution and the study was carried out according to Mendel's method (Mandels and Reese, 1957; Mandels, 1975). The second set was done on off-corn culture media. The citrate buffer 0,05M, pH=4 was used to dissolve mineral PSM medium and the off-corn medium.

In 250 mL Erlenmeyer flasks, 100 mL of sterilized culture medium were inoculated with 5 mL of fungal suspension in triplicates. The flasks were incubated in orbital shaker incubator (150 rpm) at 30 ºC, for 96 hours. At every 12 hours, the fermented broth was centrifuged at 3,000 rpm for 15 minutes and the supernatant (crude enzyme) was used for further analysis.

The comprehensive activity of cellulase was determined using the filter paper assay by the method recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Li *et al*., 2013). The overall cellulolytic activity was determined by 3,5-dinitrosalicylic acid (DNS), Miller's method (1959). The absorbance was measured at 600 nm using UV/VIS Jasco spectrophotometer. Sugar content was measured using a calibration curve of glucose. Enzymatic activity of cellulase complex was expressed in International Units/mL defined as the amount of enzyme which releases one micro mole of reducing sugar expressed as glucose per minute at 50 °C (Shafique *et al.,* 2009).

4. Microscopic investigations. By optic microscopic investigation of *Trichoderma reesei* species, using simple staining the shape and morphology of *T. reesei* were easy observed. A smear colored with 1% phenicated methylene blue is made and it is examined with immersion lens (100×) (Carpa *et al.,* 2014).

Scanning electron microscopy (SEM) analysis was performed using a Hitachi SU8230 High Resolution Scanning electron Microscope equipped with a cold field

emission gun. For morphological analysis the samples were deposited on aluminum stubs and sputter-coated coated with 10 nm gold on a Q150T ES Quorum. EDX System (X-Max N80TLE Silicon Drift Detector (SDD) from Oxford Instruments.

5. Digestion of plant material. *Arabidopsis thaliana* seedlings were used as plant material to test the enzymes produced by *T. reesei*. The 4 weeks plant material of *Arabidopsis thaliana* was obtained from seeds sterilized and cultured in a growth chamber (temperature of 21 °C and photoperiod of 16 h light and 8 h darkness) on MS1/2 medium (Murashige and Skoog, 1962).

Results and discussion

1. Macroscopic and microscopic investigations. The cellulase synthesizing strain *Trichoderma reesei* was cultured on M129 and PSM solid culture media. The inoculation technique used was to punch the medium in the center. The Petri dishes were incubated 5–7 days at temperature of 30°C. It was noticed that on M129 medium the growth of the hyphae was much stronger compared to the ones on PSM medium (Fig. 1A, B).

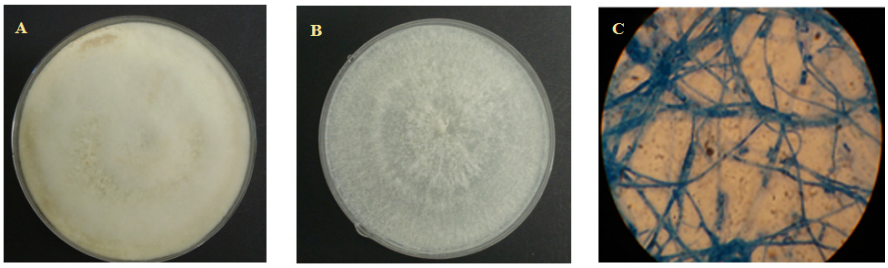

Figure 1. *Trichoderma reesei* cultured on M129 culture media (A) and PSM culture media (B). Dispersed mycelial morphology generated by growing *Trichoderma reesei* in off-corn culture media. The images were captured by optical microscope (C).

From the cultures grown on solid medium microscopic preparations colored with phenicated methylene blue were obtained. By optical immersion microscopy blue colored hyphae specific to *Trichoderma reesei* were highlighted (Fig. 1C). *Trichoderma reesei* is used at a large scale to produce biomass degrading enzymes and is also used in research (Martinez *et al*., 2008; Peterson and Nevalainen, 2012).

The best developed cultures were further used to assess the cellulase activity.

2. Cellulose decomposition (filter paper) by Trichoderma reesei. In order to emphasize the capability of *Trichoderma reesei* to decompose cellulose three experimental versions were tested (with 0.5 mL, with 1.0 mL and with 1.5 mL inoculum). These were observed for 4 weeks. Afterwards the test tubes in which the deterioration of Watmann paper occurred were counted, within each dilution. The most probable number of cellulosolytic microorganisms was next calculated

(Carpa *et al*., 2014). The medium number of cellulosolytic microorganisms was 0.17 x 10^3/mL inoculum and 26 x 10^3/mL inoculum. It was noticed that, at all the experimental versions relative to the control, the strain of *Trichoderma reesei* developed mycelium which extended on the surface of the paper strip. It was also noticed that, the volume of the inoculum influenced its development (Fig. 2A).

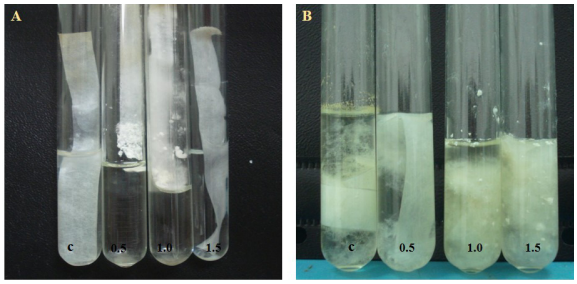

Figure 2. Tubes with filter paper and *Trichoderma reesei* inoculum after 4 weeks of incubation (A), and after shaking (B).

In order to assess the degree of paper deterioration one positive test tube was taken from each dilution and was strongly shake for 1 minute. The same action was repeated in a sample with no inoculum and then, by comparison with the control, the degree of paper deterioration was assessed (Carpa *et al*., 2014). After agitation, in Fig. 2B is visible that at the control the paper remained intact (built up in a ring form in the first test tube). At the experimental alternative with 0.5 mL inoculum, almost 50% of the paper was degraded, while at the versions with 1 mL and 1.5 mL inoculum the paper was 100% deteriorated.

3. Cellulase activity at Trichoderma reesei in a submerged system. For assessing the cellulase activity at *Trichoderma reesei* two types of nutritive medium were used: PSM medium (mineral Mendel), marked with A, and medium with off-corn, marked with B. The cellulase activity was assessed in experimental triplicates. In each Erlenmeyer flask 5 mL of *Trichoderma reesei* suspension were inoculated. Then all the experimental alternatives were incubated for 84 hours in an incubator with shaking of 150 rpm at 30ºC (Fig. 3).

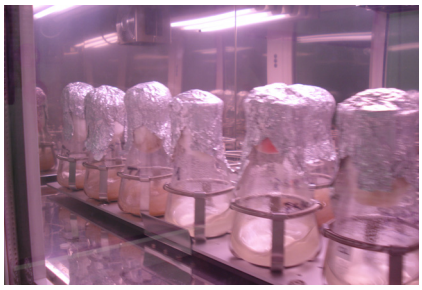

Figure 3. Samples cultivated in the shaking incubator.

At intervals of 12 hours 1 mL of the medium (supernatant) which was centrifuged at 3000 rpm for 15 min is taken for enzyme analysis. After centrifugation the supernatant and the carboxymethylcellulose solution are put into Eppendorf tubes (v/v) and incubated to 50°C for 15 min. The content of the tubes is moved into glass test tubes. 3 mL of 3,5-Dinitrosalicylic acid (DNS) are added on theme and the test tubes were placed into a beaker of hot water. Boiling for 5 min follows. After boiling the content of the test tubes must be yellow (control) and yellow-browny (samples) (Fig. 4). After cooling the content of the test tubes is subjected to spectrophotometer measurements at 640 nm.

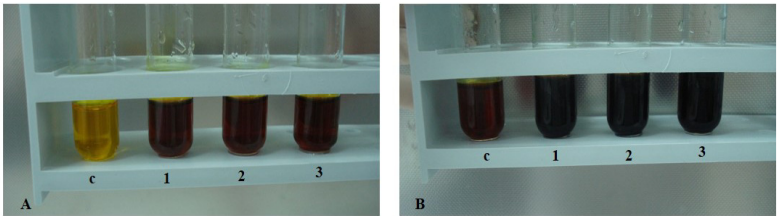

Figure 4. Content of test tubes at PSM medium (A) and off-corn medium (B); c=control, 1, 2, 3=samples.

It was noticed that, both on PSM medium (A) and off-corn medium (B), initially a decrease of optic density to – 0.0092 for PSM medium and – 0.0455 for the alternative on off-corn (B) occurred. Subsequent, on both culture media growth of the species of interest can be observed, noticing that on off-corn medium (B) the growth values are double comparing to the ones on PSM (A) (Fig. 5).

Likewise, there's a difference between the two media at the decrease of the optical density, which was stronger at the culture on off-corn medium (B) after about 60 hours. Regarding the culture on PSM medium (A), the decrease occurred only after 72 hours have passed and was much slighter.

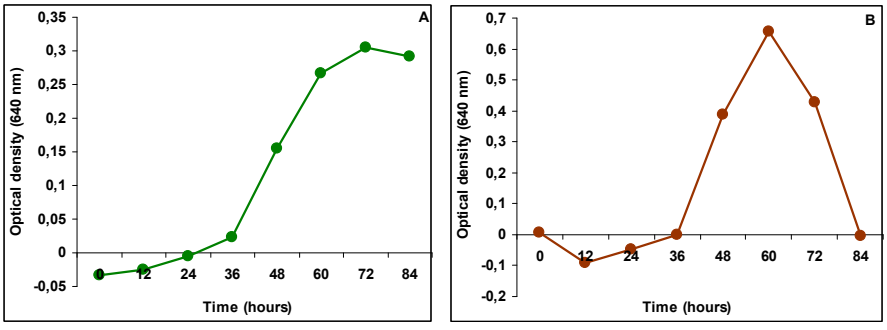

Figure 5. Decreasing sugars at *Trichoderma reesei* in PSM medium (A) and in off-corn medium (B).

The comprehensive activity of cellulase was determined using the filter paper assay by the method recommended by the IUPAC. Following the assessing of cellulase activity it was found that at each experimental alternative, due to the activity of the substrate, the reduction of the sugars released by the enzyme occurred. At the strain grown on off-corn medium the value obtained was 0.188 mg released glucose/ mL/min/50ºC, while at the strain grown on PSM medium the value was 0.118 mg released glucose/mL/min/50 ºC (Fig. 6).

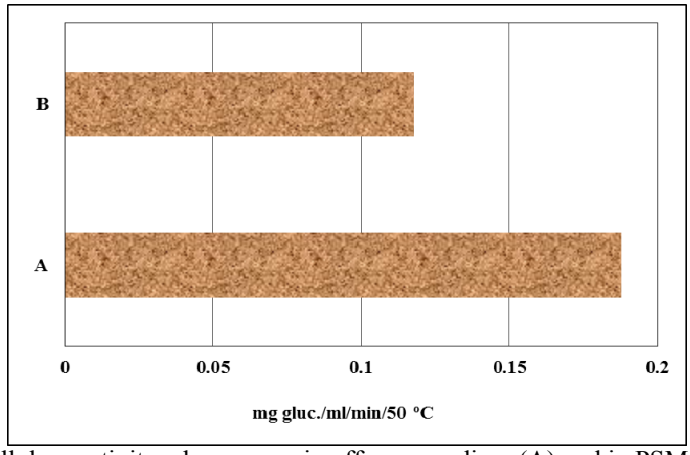

Figure 6. Cellulase activity when grown in off-corn medium (A) and in PSM medium (B).

Also, in this case the stimulating action of off-corn in the culture medium can be seen. Thus, following the experiments it can be concluded that the off-corn medium represents a culture medium richer than the PSM medium and is also preferred by *T. reesei.*

After 84 incubation hours at each experimental version the presence of numerous granules was noticed in the culture medium. At controls these granules were missing. In the samples containing PSM medium, the granules were smaller and fewer (Fig. 7). *T. reesei* granules grown on PSM medium generated difficulties at the supernatant extraction after centrifugation.

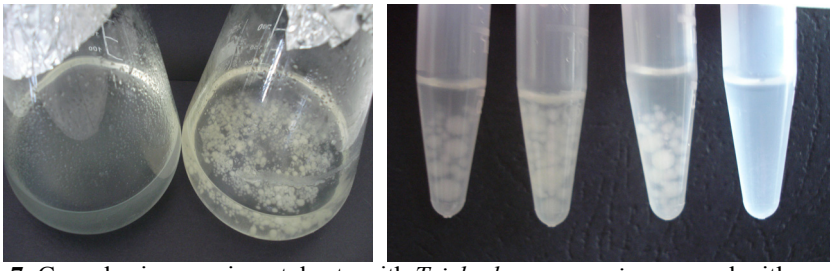

Figure 7. Granules in experimental sets with *Trichoderma reesei* compared with control on PSM medium, after 84 hours incubation.

At the versions on off-corn medium these granules were larger and much numerous than at the versions incubated on PSM medium (Fig. 8). This is correlated with the spectrophotometric values obtained at the kinetics of cellulase production at *Trichoderma reesei.* The control does not contain granules but presents a small quantity of off-corn extracted while pipetting. The presence of numerous granules on off-corn medium would be explained by the fact that this substrate is much better for cellulase production than PSM medium.

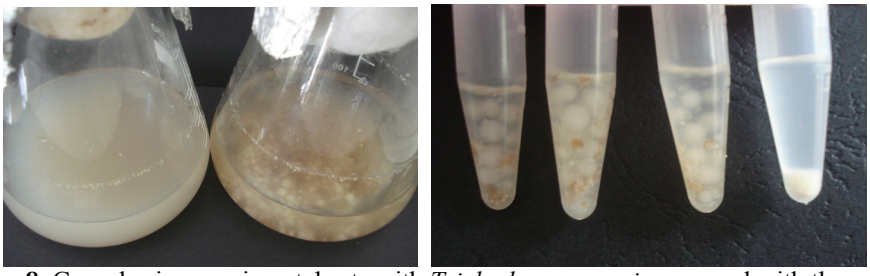

Figure 8. Granules in experimental sets with *Trichoderma reesei* compared with the control left picture, left Erlenmeyer flask; right picture, 1st Eppendorf in the right side) on culture medium with off-corn, at 84 incubation hours.

At the end of the cellulase assessments, microscopic preparations were made out of each experimental version. Thus, granules were taken from each type of tested medium and were pressed on microscopic slides. They were colored with methylene blue. Hyphae specific for *Trichoderma reesei* were observed in all versions investigated with the immersion objective lens (Fig. 9).

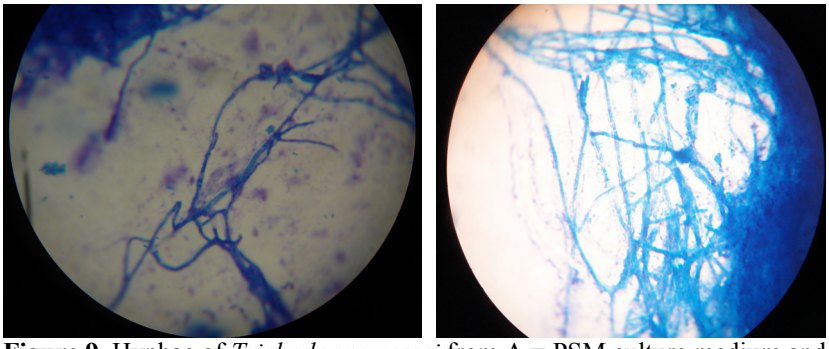

Figure 9. Hyphae of *Trichoderma reesei* from A = PSM culture medium and B = off-corn medium, at 100x optical microscope.

These granules are *T. reesei* fungal hyphae conglomerations that built-up as "bundles", while shaking. These granules were also investigated by Scanning electron microscopy (SEM), whereby both off-cornched mycelia and hyphae, off-cornched conidiophores with sporangia heads, were distinguished (Fig. 10).

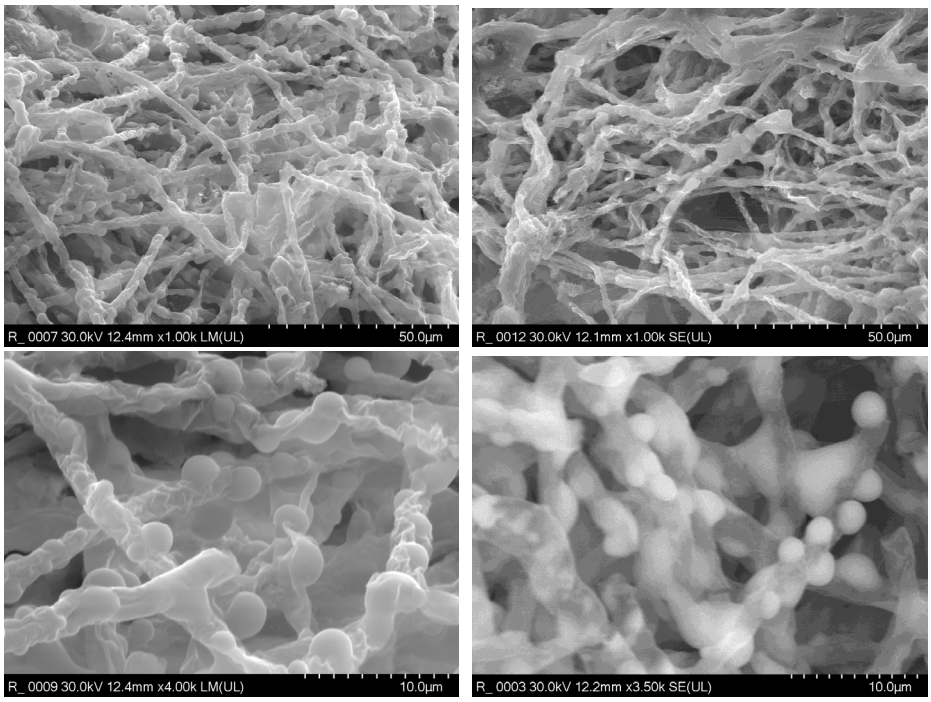

Figure 10. Scanning electron microscopy (SEM) pictures revealing morphological characteristics of *Trichoderma reesei* in off-corn culture medium under submerged fermentation conditions. (A) Highly off-cornched mycelia. (B) Hyphae, off-cornched conidiophores with sporangia heads.

Long and ramified hyphae would increase the surface area of the fungus, possibly enhancing the interaction with the substrate, and thus improving the enzyme productivity. The presence of spores could be also observed under this condition (Fig. 10), which might suggest that the environment of mycelium growth deteriorate, part of mycelium autolyzed and spores were released in order to adapt to the environment.

4. Digestion of the plant material. Cellulose, the major polymer in plant cell walls, is a very stable compound and it is known to be a recalcitrant molecule (Peciulyte *et al*., 2014). The digestion of the plant material occurred after 2.5 h, and this was noticed by macro- and microscopic observations. The main purpose of this experiment was to obtain viable plant protoplasts which could be used in future plant physiology or genetics experiments. The surface of the leaf lamina used as plant material (*Arabidopsis thaliana*) in the experiment was about 1.5 cm (Fig. 11A). The enzyme solution used was extracted by centrifugation out of *T. reesei* culture.

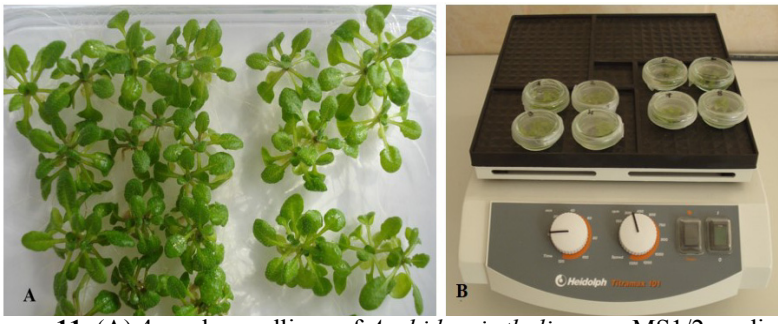

Figure 11. (A) 4 weeks seedlings of *Arabidopsis thaliana* on MS1/2 medium; (B) Leaf fragments in enzyme solution, on shaker.

The plant material was weighed and incubated with the enzyme in Petri dishes of 35 mm. The ration between plant mass and the enzyme solution was about 50 mg at 1 mL solution, with two exceptions (Table 1). The leaf explants were sectioned before being submerged in the enzyme solution and were shaken at 300 rpm, at room temperature, for 2.5 h, using the device Titramax 101 (Fig. 11B).

Table 1.

The enzyme solution types used for the digestion of the plant material

Samples No.	Sample types	Description	Observation
1	Negative control	Distillated water 2 mL	
2	Positive control	Cellulase Onozuka R-10 1% + Macerozyme 0.5% + 0.4 M sucrose	
3	EP + Macerozyme	Enzymatic solution EP 2 mL + Macerozyme 0.5%	
4	ET + Macerozyme	Enzymatic solution ET 2 mL + Macerozyme 0.5%	
5	EP + Macerozyme	Enzymatic solution EP 4 mL + Macerozyme 0.25%	Double EP enzyme quantity compared to sample 3.
6	ET + Macerozyme	Enzymatic solution ET 4 mL + Macerozyme 0.25%	Double EP enzyme quantity compared to sample 4.
7	EP + Cellulase Onozuka R-10 + Macerozyme	Enzymatic solution EP 2 mL + Cellulase Onozuka R-10 0.5% + Macerozyme 0.5%	
8	EP + Cellulase Onozuka R-10 + Macerozyme	Enzymatic solution ET 2 mL + Cellulase Onozuka R-10 0.5% + Macerozyme 0.5%	

EP – Enzyme isolated from *T. reesei* grown on PSM medium; ET – Enzyme isolated from *T. reesei* grown on off-corn medium.

The experimental versions are described in Table 1. At the described samples, Macerozyme R-10 was used as a source of pectinase and Cellulase Onozuka R-10 was used as a supplementary source of cellulase. The enzyme solutions which were separated by centrifugation at the end of the experiment on the growth of *T. reesei* culture in a submerged system were marked with EP (enzyme isolated from *T. reesei* grown on PSM medium) and ET (enzyme isolated from *T. reesei* grown on off-corn medium). The centrifugation was done at 4000 rpm, for 30 min., at 4 °C.

The leaflets were submerged in the enzyme solutions. After 2.5 hours of shaking at room temperature, the digestion of plant material and its dispersion in the enzyme solution could be observed (Fig. 12).

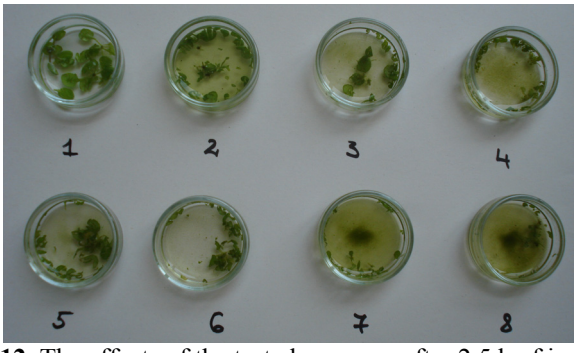

Figure 12. The effects of the tested enzymes after 2.5 h of incubation with shaking, at room temperature.

The following versions can be recorded as being efficient for the digestion of the plant material: 7, 8 and 4. At version 1 the digestion did not occur, because this was the negative control.

Differences were recorded between the enzymes extracted from PSM medium and those on off-corn medium, the last ones being more efficient. The versions 4 and 8 are the ones containing enzymes extracted from the off-corn medium. When the ratio between the enzyme solution and the plant material used was modified, no improvement of the digestion efficiency was noticed, but on the contrary, a decrease of it. This case can be observed in Fig. 12, at versions 5 and 6.

Out of the microscopic observations it was concluded that the regulation of osmolality is an essential factor for obtaining viable protoplasts, but these do not obviously influence the digestion of the plant material (Fig. 13). Similar to the version 3, depicted in the image, protoplasts were also obtained in the other versions but the viability was low.

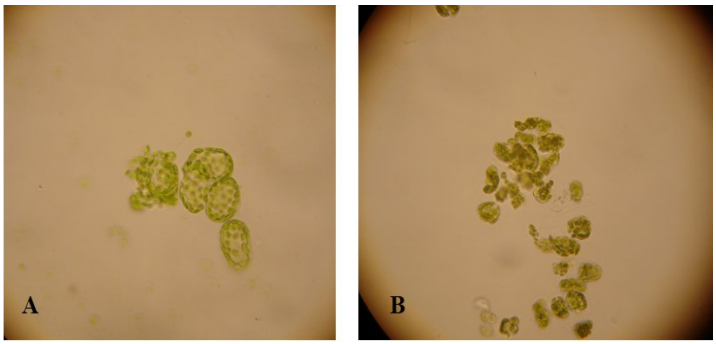

Figure 13. Microscopic images of the protoplasts, version 2 = positive control (A) and version 3 = cellulase obtained from culture grown on PSM medium with 0.5% Macerozyme R-10 (B) (40x).

Thus, obtaining cellulase implies also other determinant factors as the concentration of the enzyme in use, the type of enzyme, pH (Li *et al*., 2013) but also osmolality, whose regulation is very important.

It is essential to enhance the activity of enzymes from *Trichoderma reesei* in the economical production of biofuels from plant materials as a new cost-effective approach. Carbon source plays a vital role in enzyme production because carbohydrates and their derivatives have the ability to induce most of cellulolytic enzymes (Zheng *et al*., 2017).

Conclusions

Initially, *Trichoderma reesei* was recultivated on culture medium with potatoes (M129) plates, and then was transferred on PSM medium with agar, using the culture plate method. These samples grown on plates were further used to obtain the *T. reesei* inoculum for submerged cultures in PSM and off-corn media. Using the microscopic techniques, the morphology of *T. reesei* could be observed, by microscopic preparations with simple phenicated methylene 1% stain.

The composition of the culture medium strongly influenced the development of the studied species, most preferred culture medium for *T. reesei* proving to be the submerged medium based on off-corn.

The action of the cellulosic enzymes on cellulosic materials was observed by testing the enzymes extracted from the submerged *T. reesei* cultures on stripes of filter paper. In time (after 4 weeks), the digestion of cellulose was observed by comparing the samples containing the enzyme with the control, the difference signaling that *T. reesei* produced enough cellulase to cause the degradation of cellulose.

The digestion of the plant material by the enzyme extracted from *T. reesei* cultures was also observed, by obtaining *Arabidopsis thaliana* viable protoplasts.

This method could be improved and further used in plant genetics, plant cytology, physiology and other fields. The cellulase activity was also assessed and a decrease of the sugars freed by the enzyme was noticed.

Trichoderma reesei proved to be a good producer of cellulase, and thus its degradation action is seen as a very valuable property, in the context of its capacity to hydrolyze biomass rich in cellulose.

Trichoderma reesei represents a very important species of fungi due to the bioethanol production, in the context of the environmental problems protruding at the beginning of 21th century, regarding the greenhouse gases emissions. Thus, attention should be focused on developing technologies based on biological techniques.

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