Production and purification of the cellulase enzymatic complex of *Trichoderma reesei Rut-C30*

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Abstract
With growing emissions of greenhouse gasses as a result of burning fossil fuels, a cleaner, renewable alternative is needed. Bioethanol, one of the most important biofuels, can partially or totally substitute traditional fuels. Still, bioethanol production competes with the food industry since it relies on feedstock used for food production. In order to enable the use of lignocelulose materials, the process of hydrolysis should be improved. The main objective of this work consists of the implementation of successful processes for production and purification of cellulase enzymatic complex of Tricodherma reesei Rut-C30, and its possible use in the treatment of by-products and waste cellulose materials, in particular waste paper and sugarcane bagasse. The study of the production of the fungus will take place in shake flasks in submerged culture conditions. The use of raw materials of low economic value, The analysis of nutrient consumption, production of the fungus, the biosynthesis of enzyme complex of cellulase and other relevant data of the fermentation will be investigated in order to establish a strategy for protein synthesis, and starting enzymatic cellulase complex purification at the fermentation level.
Key words: Cellulase; Bioethanol; Trichoderma reesei RUT-C30; Bagasse, Sugarcane

1. The biorevolution - creating a sustainable future

With growing concern for the severe environmental effects that the utilization of fossil fuels has, particularly greenhouse gas emissions from burning fuels for transportation, developing clean and renewable alternatives became a world-wide priority. However, the substitute to these traditional fuels has to be performance-wise comparable, widely-available and economically sustainable. [Trivedi et al.; 2013.]

A vast majority of chemicals and materials in widespread use, ranging from the textile and automotive industry to the production of pharmaceuticals, fuels and fuel additives, depend on the use of fossil fuels and oil derivatives as their basic feedstock. Modern research aims to deter this harmful practice by providing alternative ways of utilizing nonconventional materials in the production of above-mentioned necessities.

An obvious and abundant raw material is biomass. However, breaking it down into its building blocks in an economically viable and efficient way proved to be a challenge. Another concern is finding new ways of synthesizing traditionally used chemical compounds using this novel feedstock.

Bioethanol production from fermentation of biomass-based feedstock

Bioethanol is one of the most researched and dominant biofuels, with a large presence in the Western world. It is the main product in the process of anaerobic digestion. In this process, microorganisms utilize a carbohydrate-based material in the absence of air, and release significant quantities of ethanol into the medium, which can be separated and purified.

Sugar and starch based materials have a major role in the food-production industry, which is one of the main reasons why lignocellulose-materials got in the spotlight as the feedstock for future bioethanol production.

Growing global population, as well as food scarcity in certain areas of the world makes the use of certain feedstock for energy production unethical, since it competes with food production. This all can be avoided with a widespread use of lignocellulose feedstock, considering that these materials are usually a waste created in agriculture, and are regarded as an inconvenience. The main obstacle of this practice, pretreatment and hydrolysis of the raw material, will be thoroughly discussed further on, as a central theme that this paper aims to resolve.

The Biorefinery concept and the circular economy

The actualization of a circular economy through the use of lignocellulosic wastes as renewable resources can lead to reduce the dependence from fossil-based resources and contribute to a sustainable waste management. The integrated biorefineries, exploiting the overall lignocellulosic waste components to generate fuels, chemicals and energy, are the pillar of the circular economy. [Liguori and Faraco; 2016.]

Producing fuels, chemicals and energy depends on breaking down the raw material using different methods, chemical, physical

and biological. Biological treatment is considered a promising approach for the lignocellulosic waste conversion since it does not require additional energy or chemicals, thus minimizing the negative impact on the environment. This concept works hand in hand with biofuel production, since waste from one can be applied for the other as a raw material.

2. Cellulases - structure and application

Cellulose is a virtually inexhaustible polymeric raw material that is in nature found in plants, algae and mosses, as the main element of their cell walls. Since it is the most abundant biopolymer on the earth, it is the focus of considerable interest as a renewable energy resource. Cellulose is a polymer composed of glucose units linked by β-1,4-glycosidic bonds. Hydrolysis of the cellulose chain generates glucose, which is a precursor to valuable chemicals. Most of them are expected to be a base for the bio-based industry. All this made cellulose a promising sustainable feedstock for the future.

Effective degradation of cellulose is challenging due to the rigidness of its structure, induced by inter- and intra-molecular hydrogen-bonding. [Yabushita et al., 2014.] Proper pretreatment disrupts the intricate biomass structure and enables the hydrolysis process to be done with higher efficiency, or hydrolyses and eliminates hemicellulose and lignin. Pretreatment methods range from simple, mechanical ones, to more complex ones, involving organic solvents and ionic liquids. [Yabushita et al., 2014.] [Badiei et al., 2014.]

Cellulolytic enzymes are the third most important industrial enzyme due to its versatile applications in various industries such as paper and pulp, textile and detergent industry. The resurgence in utilization of biomass for bio-ethanol and other value added organic compounds production has attracted major attention of researchers globally towards cellulases. [Singhania, 2009.]

Cellulase activity is mediated by a complex of several different enzymes which act in synergy. The cellulase complex (cellulosome) includes endoglucanase, exoglucanase, cellobiohydrolase, and β-glucosidase, or cellobiase.

Commercial enzymes are widely available, but have a considerably high price. What causes this is the cost of protein purification processes, which can make up to 70% of total cost.

3. *Trichoderma reesei* RUT-C30-cellulolytic system

The filamentous fungus *Trichoderma reesei* has a long history of use for production of hydrolytic enzymes. An effort was made through strain development programs using random mutagenesis of the wild species aiming at developing strains with superior cellulolytic enzyme production capabilities. [Eveleigh, 1982.]

Among these strains, RUT-C30 has become the most frequently used cellulase hyperproducer in the public domain. [Seiboth et al, 2011.] It has the ability to secrete a large amount of extracellular protein. Literature sources [Eveleigh, 1982] [Bisaria and Ghose, 1981.] reported that the strain produced 20 mg of extracellular protein ml^{-1} and displayed cellulase activity of 15 filter paper units ml^{-1}.

What makes this strain a promising enzyme producer is, as stated above, is hyperproduction of cellulase with resistance to catabolite repression. This trait enables submerse production, which greatly reduces downstream processing costs and simplifies these processes.

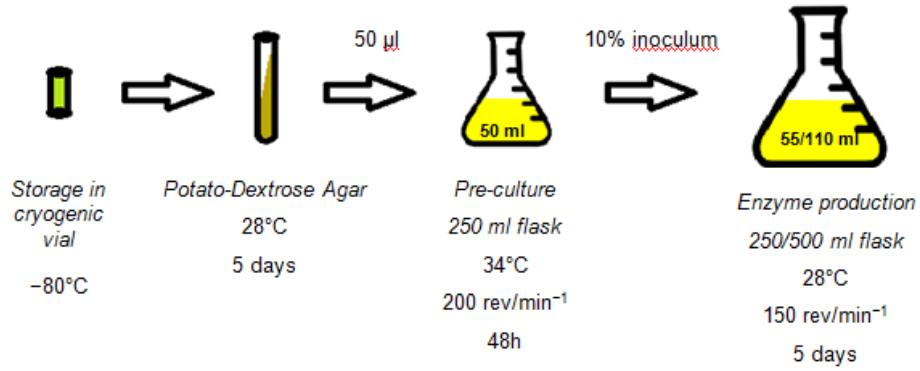

Figure 2: Overall scheme of fermentation procedure

4. Materials and Methodology

Methods and materials applied in the experiments connected to this thesis were selected based on the availability of the equipment and working conditions in the laboratories where the experimental work took place. These are The Institute for Bioengineering and Biosciences (IBB) at Instituto Superior Técnico (IST), and University of São Paulo, where bagasse pretreatment was done.

Cellulase complex production

The strain used in all the experiments is *Trichoderma reesei Rut C-30* from the microbial strain catalogue of the Institute for Bioengineering and Biosciences (IBB) at Instituto Superior Técnico (IST), Universidade de Lisboa (UL). The strain was originally a gift from Dr M. Pentillä (VTT, Finland). The properties of this mutant strain are discussed in more detail in chapter 3.1 of this paper.

Cultivations are carried out several different cultivation media, with slight modifications and variations. The composition of all media bases, without the carbon sources, are given in Table 4.1.

Preculture media contained media bases from Table 4.1, and 30 g/l of glucose as the sole source of carbon. The composition of production medium for cellulases was the same as that of the corresponding pre-culture medium, except that it was supplemented with specific enzymatic complex production inducers (15 g/l of lactose, 20g/l of cellobiose or 10g/l of wheat bran).

A scheme of the fermentation procedure is given in Figure 2.

Table 4.1: Content of the media used in the experiments

Component in g/l	TMM	Mandels solution	"Supp." medium
KH_2PO_4	15	2	15
$(NH_4)_2SO_4$	5	1.4	5
$MgSO_4*7H_2O$	1.23	0.3	1.23
$CaCl_2*2H_2O$	0.8	0.4	0.8
$FeSO_4*7H_2O$	0.0035	0.0035	0.0035
$MnSO_4*H_2O$	0.0015	0.0015	0.0015
$Zn(O_2CCH_3)_2*H_2O$	0.0015	0.0015	0.0015
$CoCl_2*6H2O$	0.003	0.003	0.003
Urea	/	0.3	/
Peptone	/	0.75	0.75
Yeast extract	/	/	0.3

In an effort to determine optimal fermentation conditions, experiments with variations of the media and operational conditions were made.

All materials and equipment used for the fermentations were sterilized in an autoclave prior to use, at a temperature of 121°C, at 100 kPa pressure for 20 min.

In order to test the performance of the produced cellulolytic enzymatic complex, several experiments with conventional feedstocks, sugarcane baggase and others, for bioethanol production were made.

Analytical techniques

Analytical procedures used for the experiments were in accordance with commonly used methods from literature, suitable for the Institute for Bioengineering and Biosciences laboratory conditions.

Determination of dry biomass content
Content of dry biomass was determined by centrifuging a 1 ml sample of the fermentation medium in an 1.5 ml Eppendorf tube, for 8 minutes at 12000 r/min, separating the supernatant and washing the pellet with 1 ml isotonic saline water 3 times, then drying it at 60 °C for 24 hours. The weight of the pellet was used to calculate the content of dry biomass per ml of medium.

Determination of fermentable sugars concentration
The concentration of the fermentable sugars present in the media was determined using the 3,5 dinitrosalicylic acid reagent (DNS) method, used for quantitative analysis of reducing sugars. [Miller, 1959.] The results were presented as glucose equivalents. Because of this, results showing concentration of disaccharides in the media (primarily lactose and cellobiose) were lower than their real values (by about 80% for lactose and 60% for cellobiose).

Determination of enzymatic activity
A modification of the Filter Paper assay for Cellulase by Montenecourt [Montenecourt et al., 1978] was used for determining the activity of the cellulase complex. This activity was determined by the release of reducing sugars produced in 60 min from a mixture of 0.2 ml of an appropriately diluted enzyme solution, 0.8 ml of 0.1 M acetate buffer, pH 4.8 and 20 mg Whatman No. 1 disks filter paper, incubated at 50 °C. One international filter paper unit (FPU) was defined as the amount of enzyme that releases 1 μmol glucose min^{-1}. The concentration of the reducing sugars in the assay after cellulase activity was determined using the DNS method. The general equation for calculating the Filter Paper Unit is showed in Figure 3.

$$FPU = \left(Crs\left[\frac{mg}{ml}\right]\right) * \frac{(Vat[ml])}{Gl.M\left[\frac{mg}{mmol}\right] * Vs.[ml] * t[min]}$$

Figure 3: General equation for calculating enzymatic activity in mmol ml^{-1} min^{-1} (Crs - concentration of released sugars; Vat - total volume of assay; Gl.M. - molecular weight of glucose; Vs - sample volume; t - duration of assay)

Determination of extracellular protein concentration
For determination of enzyme concentration (which are a significant part of the whole extracellularly secreted protein concentration), BCA (bicinchoninic acid assay) method was used. It is a spectrophotometric method in which assays change colour from light green to purple, based on the concentration of protein in the sample. All samples were tested in triplicates.

Enzyme separation and purification techniques

After fermentation, enzyme separation and purification techniques aim to separate enzymes from other components of the media, as well as the fungus itself, all the while preserving their activity. Due to available equipment and other circumstances, three basic techniques were used: centrifugation, precipitation with Ammonium-sulphate and ultrafiltration.

5. Results and Discussion

The group of experiments that was originally planned to be part of this work was different, and the work plan had to be adapted due to some unexpected issues. However, in the first stages of work a very low enzymatic activity was observed, which is attributed to loss of activity of the fungus during extensive time in cryotube storage.

Production in shake flasks

Production of the cellulolytic enzyme complex was performed in order to examine production in a batch cultivation, as well as optimizing the production process. Initially, an attempt was made to replicate the procedure and results by F.C. Domingues [Domingues et al., 2001.]. However, results for enzymatic activity were considerably lower compared to those stated in literature (roughly 10 times lower, [Domingues et al., 2001.]).
In the next set of experiments, shake flask production was made using 500 ml Erlenmeyer flasks in order to compare the performace of the three fermentation media: Trichoderma Minimal Medium, Mandels solution and the Supplemented Medium.

Table 2: Results for fermentation in 500 ml flasks

Media	TMM	Mandels solution	Supp. medium
Ferm.sugars (final c) [g/l]	27.7	26.19	25.56
Extracellular protein (final c) [g/l]	0.28	0.38	0.41
FPA (final c) [FPU/ml]	0.10	0.115	0.136

It can be concluded that Supplemented medium created better growth conditions for the fungus, which resulted in a greater reduction of the initial concentration of sugar in the medium, more extracellular proteins produced, as well as higher enzymatic activity.

Impact of fermentable sugar concentration on determining enzymatic activity

In order to examine the reason to poor enzyme activity of the fungus, compared to values in literature, a test was made to insure that the enzymatic activity measuring procedure itself isn't problematic. In literature, there is a lot of ambiguity on the exact procedures used in the Filter paper assay method. What is observed from these results is an almost logarithmic dependency of dilution of the sample on activity - lower enzyme concentration resulted in higher apparent enzymatic activity.

The peak activity for the Supplemented mediumis on day 3. After that, the specific activity drops and remains in a constand steady drop. Compared to results obtained in literature [Domingues et al., 2001.], activity in this case is significantly lower - 10 to 15 times worse. This spoilage is more prominantly visible here than when comparing values of results for enzymatic activity and extracellular protein concentration.

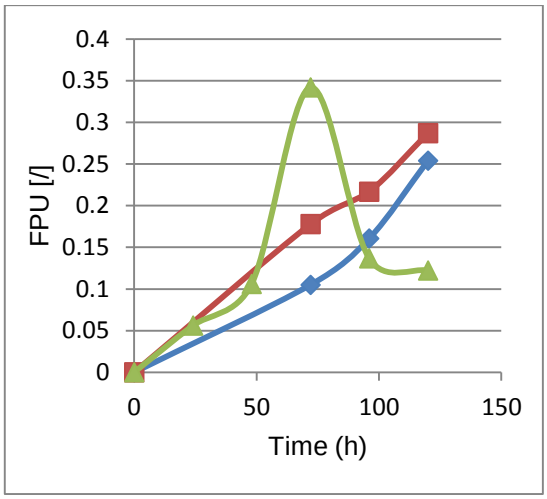

Figure 6: Enzymatic activity of T reesei grown the three media (-♦- TMM , -■- Mandels solution, -▲- "Supplemented" medium)

Sugarcane baggase hydrolysis kinetics

Testing of the *Trichoderma* enzymatic complex on untreated, pretreated sugarcane baggase, its parts and other waste materials was made. Figure 7 shows these results.

A solution of the supernatant in a 0.05M citrate buffer (pH 4.75) was made. The sample was diluted to obtain 1 FPU/mg of dry material. Samples were centrifuged at 12000 r/min for 5 minutes to separate the remaining solids and the supernatant was used to determine the concentration of sugar using the DNS method. All samples were made in duplicate, and a blind was made with omitting baggase. A separate flask was used for determining the dry mass after 72h.

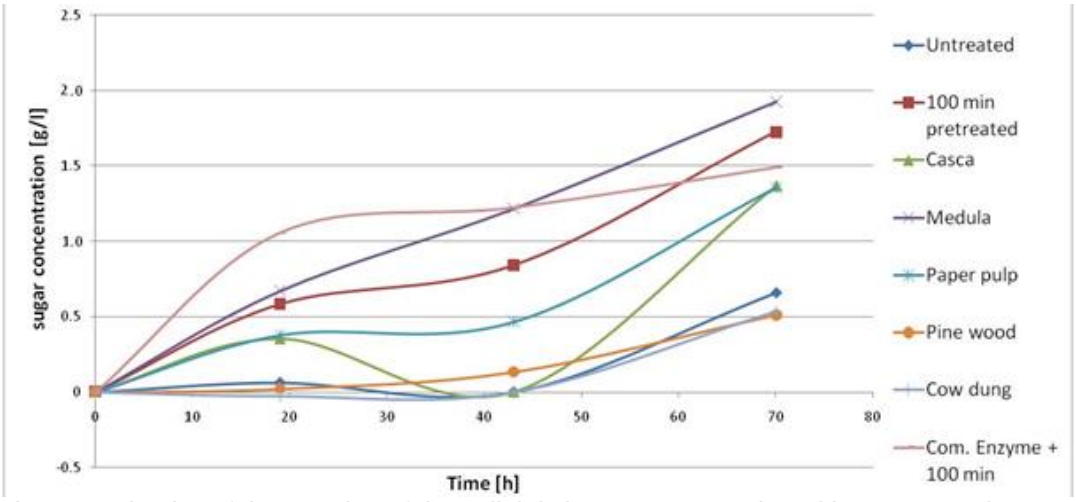

Figure 7: Kinetics of the reaction of the cellulolytic enzyme complex with sugarcane bagase

Separation and purification of the enzymatic complex

In order to prove the presence of cellulolytic enzymes, gel electrophoresis was used on samples of supernatant collected from fermentation. These results indicate that most of the extracellular enzymes produced fall into the category of cellulases, due to the similarities between the samples obtained in the laboratory and the commercial enzyme. In Figure 9, results of this experiment are shown.

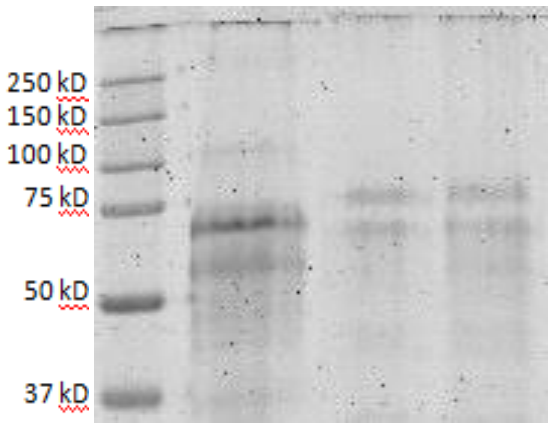

Figure 9: Results of gel electrophoresis of extracellular proteins obtained during fermentation. The samples according to lanes are, from left to right: Lane 1 - markers, Lane 2 - comercial cellulase enzyme, Lane 3 and 4 - *Trichoderma reesei* fermentation supernatant samples.

Increasing enzyme concentration - results

Due to the low concentration of extracellular protein produced by the fungus, such a small amount of precipitate was obtained after ammonium sulphate addition and centrifugation, so no successful increase of enzyme concentration was achieved.
Ultrafiltration proved to be the most effective out of the techniques applied in this work which aim to concentrate the secreted enzymes.
The supernatant was obtained from a shake flask fermentation using the "Supplemented" medium, after 4 days of incubation. After ultrafiltration, the permeate and the retentate fractions were used to determine protein concentration and enzymatic activity. These results are presented in Table 3.

Table 3: Results of analysis of retentate and permeate samples obtained from ultrafiltration.

Fraction	Volume [ml]	Enz. activity [U/ml]	Protein content [g/l]
Supernatant before ultrafiltration	48	0.147	0.38
Retentate	12	0.135	0.48
Permeate	36	0.02	0.31

From the results from the table above, we can conclude that although a large amount of protein passed through the membrane (61.1%), an increase in protein concentration was achieved. However, since 27,3 % of the enzymes lost their activity, the number of FPU/ml in the retentate solution is lower, despite the larger concentration of protein. In conclusion, although this method was the only one in which protein concentrating was achieved, enzymatic activity of the concentrated solution was lower.

6. Conclusions

What greatly determined the course of the experimental work related to this thesis was the obvious reduction of activity of the fungus that was used for production of the cellulase enzymatic complex. In order to determine the reason for these issues, many variations to the original setup were tested. Size of fermentation flasks, different media, sample volume, various enzyme production inducers and media additives were tested and selected for further experiments based on their performance. Reasons for this are hard to determine with limited time and equipment availability.
Nonetheless, based on the setup and experimental conditions that showed best performance in enzymatic activity, samples of the enzymatic complex were obtained and tested. Some hydrolytic reactions of various samples containing cellulose were observed, but these were hindered due to low enzyme concentration. What greatly reduced the ability to properly determine the activity of these enzymes were high values of residual sugar in the media. This caused the slight variations in glucose concentration increase (due to hydrolysis of cellulose) to be measured with less accuracy.
In order to overcome these problems, tests were made on applying various methods of enzyme purification. These attempts proved to be unsuccessful, due to the same general

issues with the fermentation: low protein content and high residual sugar concentration.
Finally, although the initial expectations for this thesis were mostly unachievable, attempts were made to work with existing conditions in ways that would maximize results. Although these findings might not be of scientific importance for improvement of cellulase production efficiency, it might have a didactic value to it. It could show to a younger researcher which paths to take when faced with unexpected results, and to stay encouraged, since in science all knowledge gained is precious.
As scope for future research, research on lowering the expenses of proudction by researching how new substrates would affect cellulase production. Waste streams from certain industries could be used as a basis for the fermentation media. Also, integrating cellulase hydrolysis with fermentation is promising, since it is logistically simpler and time-effective. With improving genetic modification technologies, favourable traits could be further improved and refined on selected strains.
Finally, a more well-defined and universally applicable method for measuring enzymatic activity should be developed. In the work that is on display above, enzyme concentration, as well as the amount of residual sugars in the sample after fermentation, greatly influence the apparent filter paper assay results. This can create confusion among researchers when trying to replicate results. Clarity and brevity is of paramount importance in scientific research.

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