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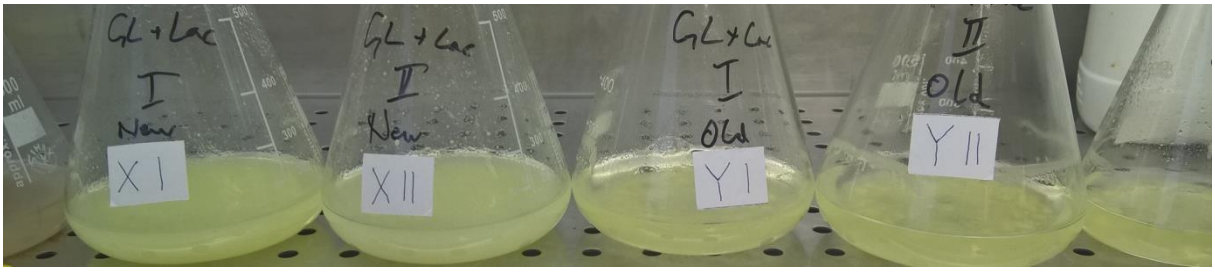

Production and purification of the cellulase enzymatic complex of *Trichoderma reesei Rut-C30*

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Thesis to obtain the Master of Science Degree in

Energy Engineering and Management

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Abstract (EN)

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 *Tricodherma reesei Rut-C30* and the synthesis of its cellulase enzymatic complex will take place in shake flasks in submerged culture conditions. The cellulases produced by this mutant strain are extracellular enzymes and have the advantage of their biosynthesis not being repressed catabolically by high concentrations of glucose in the culture medium. However, the levels of biosynthesis depend on the composition of other nutrients in the culture medium, the presence of inductors, in particular, lactose and their fermentation conditions.

In this phase of the work will seek to optimize the various effects on cell growth and on activation of specific metabolic pathway involved in biosynthesis of the enzymatic complex of cellulase.

The use of raw materials of low economic value, in particular of cellulosic material hydrolysis products and their derivatives as carbon sources and inductors in the culture medium, can be beneficial in cases of "fed-batch" and continuous processes.

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

Resumo (PT)

Com o crescimento das emissões de gases de efeito estufa, como resultado da queima de combustíveis fósseis, um produto de limpeza, é necessário alternativa renovável. Bioetanol, um dos biocombustíveis mais importantes, pode, em parte ou totalmente substituir os combustíveis tradicionais. Ainda assim, a produção de bioetanol compete com a indústria de alimentos, uma vez que se baseia em matéria-prima utilizada para a produção de alimentos. A fim de permitir o uso de materiais de lignocelulose, o processo de hidrólise deve ser melhorada. O objetivo principal deste trabalho consiste na implementação de processos bem sucedidos para produção e purificação do complexo de celulase enzimática de Tricodherma reesei Rut-C30, e seu possível uso no tratamento de subprodutos e materiais de celulose resíduos, em particular resíduos de papel e de cana bagaço.

O estudo da produção do fungo Tricodherma reesei Rut-C30 e a síntese enzimática do seu complexo de celulase será realizado em frascos de agitação em condições de cultura submersa. As celulases produzidas por esta estirpe mutante são enzimas extracelulares e tem a vantagem de a sua biossíntese não ser reprimido catabolically por altas concentrações de glucose no meio de cultura. No entanto, os níveis de biossíntese dependem da composição de outros nutrientes no meio de cultura, na presença de indutores, em particular, a lactose e a sua fermentação condições.

Nesta fase do trabalho irá procurar optimizar os diversos efeitos sobre o crescimento celular e na activação da via metabólica específica envolvida na biossíntese do complexo enzimático de celulase.

A utilização de matérias-primas de baixo valor económico, em particular de produtos de hidrólise de material celulósico e seus derivados como fontes de carbono e indutores no meio de cultura, pode ser benéfico em casos de "fed-batch e contínuos" processos.

A análise do consumo de nutrientes, a produção do fungo, a biossíntese do complexo enzima de celulase e outros dados relevantes da fermentação irá ser investigado a fim de estabelecer uma estratégia para a síntese de proteínas, e a partir de celulase de purificação de complexo enzimático a um nível de fermentação.

Palavras-chave: Celulase; Bioetanol; Trichoderma reesei RUT-C30; Bagaço de cana, cana

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Nomenclature and abbreviations

BGL - β-glucosidase

CBH - cellobiohydrolase

CBM - carbohydrate binding module

CMC - carboxymethylcellulase (endoglucanase)

E.C. number - Enzyme Commission number

EG - endoglucanase

FPU - Filter paper units

HMF - Hydroxymethylfurfural

LA - linear particle accelerator

MSW - Municipal solid waste

NTG - nitrosoguanidine

RCF - Relative centrifugal force

SSF - Solid State Fermentation

SWO - Swollenin

U - Enzymatic units - μmol of glucose produced min^{-1} in standard assay

UV - UV-light

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 rising level of environmental awareness and the negative effects we have on our environment in forced to global community the past decades to cooperate in dealing with these issues. There are several ways to approach this challenging problematic. One major concern stands out when looking into the cause of the growing levels of greenhouse gasses.

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. This supports the idea of a circular economy, where waste streams are returned to be used again.

An obvious and abundant raw material is biomass. Although having many advantages compared to oil - it is present on all populated continents in many shapes and forms, 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.

Here, the concept of biorefineries steps into the spotlight. 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.]

Furthermore, according to data obtained from the U.S. Energy Information Administration [EIA; 2016.], just OECD countries alone consumed about 20 million barrels per day of petroleum and other liquid fuels in 2012., while non-OECD countries are consuming similar quantities, with a predicted growing trend of use.

Biofuels play a key role in hindering this. Improvements in production processes - increasing efficiency, decreasing costs and finding sustainable raw materials for production, all give these alternative fuels a fighting chance in the oil-dominated market.

Bioethanol is one of the most researched and dominant biofuels, with a large presence in the Western world. The importance of this fuel, applying lignocellulosic materials in its production and as a raw material for biorefineries will be discussed.

1.1 Bioethanol - current development and future prospects

In the past century, the oil industry grew rapidly, due to the development of internal-combustion engines and their widespread use in the energy industry. This heavily polluting material, as well as its derivatives, seemed to be an irreplaceable feedstock in the fuel and chemicals industry. This trend can be observed in Figure 1.1.

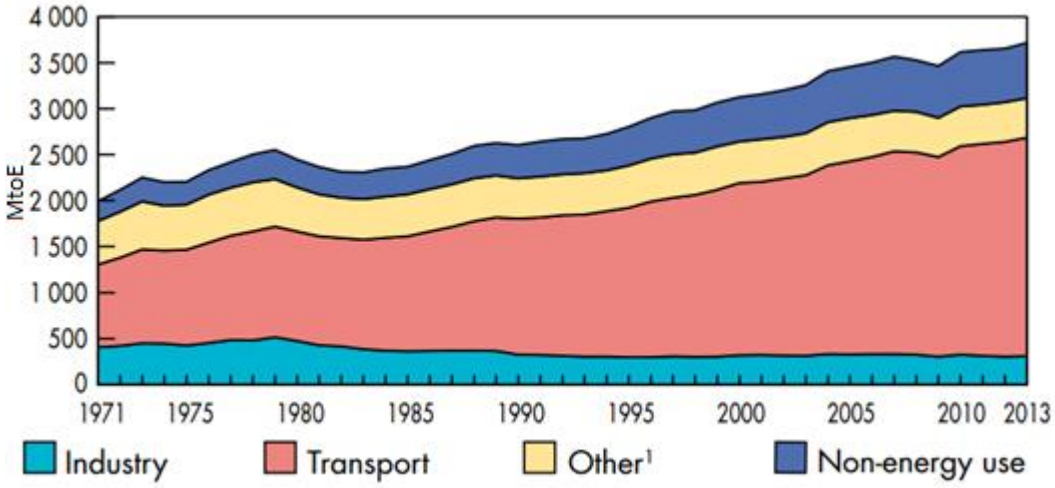

Figure 1.1: Total consumption of oil from 1971 to 2013 by sector (Mtoe) [IEA; 2015.]

It would be unrealistic to expect a rapid switch from these wide-spread energy sources to renewable ones immediately. If this happened, damage could be done to some communities by making energy unavailable and a scarce resource, which is unacceptable. The transition will most-likely take time, since energy is a key element of the world economy that has to adapt to the unavoidable changes in the near future. A more sensible approach would be gradually making the progression, at first partially substituting fossil fuels with cleaner alternatives. This is why the biofuels industry has great potential, in which bioethanol is one of the key elements.[Paris et al.; 2014.]

1.1.1 Bioethanol production from fermentation of biomass-based feedstock

Bioethanol is the main product in the process of anaerobic fermentation. 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.

Although many fungi, bacteria and yeasts are possible working microorganisms in fermentation processes, the yeast *Saccharomyces cerevisia*e is most commonly used for converting glucose into ethanol. It is a well known and thoroughly researched species, in use for millennia and one of the first microorganisms to have its complete genome sequenced.

With the development of genetic engineering processes, new species with superior performance and features. Some of the properties of the working microorganism in this case that could be improved in order to increase the overall success of the process would be increasing resistance to ethanol

concentration, alleviating catabolite repression, as well as developing strains that are able to digest a multitude of carbohydrate groups.

As feedstock for bioethanol production, an array of widely-available feedstock can be used, which is shown in Figure 1.2.

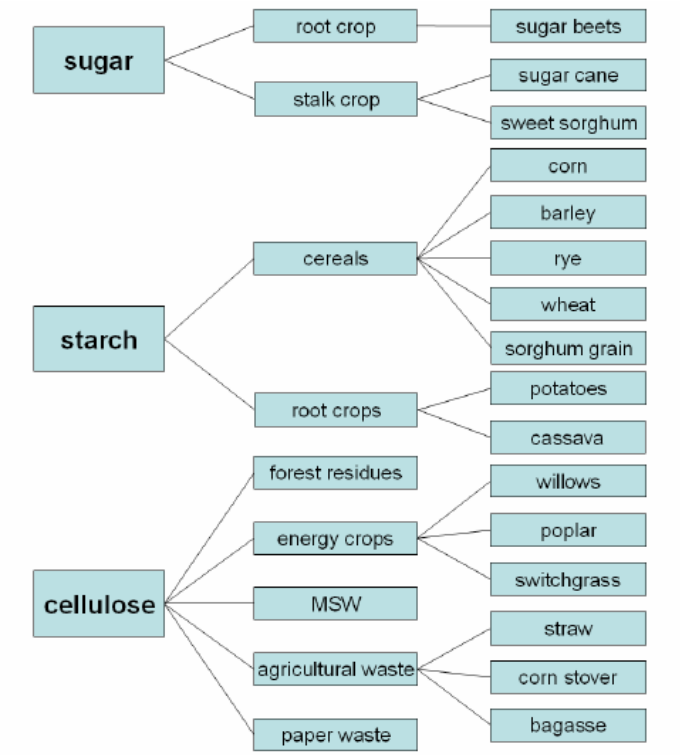

Figure 1.2: Types of feedstock for ethanol production [Rutz and Janssen; 2007.]

While sugar-based materials can be applied directly, starch and cellulose feedstock has to be pretreated and hydrolyzed in order to make it digestible by the microorganisms in the main fermentation. Furthermore, 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 can create undesirable scenarios in which a producer is tempted to sell feedstock to a fuel producer rather than to the food industry, due to a superior economic offer. 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. Adding value to this unavoidable waste gives benefit to the producers, and motivates them to deal with this potentially environmental hazard. 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.

1.1.2 Bioethanol properties and blending

Bioethanol is one of the most promising biofuels due to the favourable properties in utilization.

The comparison of key fuel parameters for bioethanol and petroleum, is shown in Table 1.1.

Table 1.1: Parameters of bioethanol in comparison with petrol [Kemnitz; 2006.]

	Density [kg/l]	Viscosity [mm^2/s]	Flashpoint [°C]	Caloric Value [at 20°C MJ/l]	Caloric value [MJ/l]	Octane number [RON]	Fuel equivalence [l]
Petrol	0.76	0.6	<21	42.7	32.45	92	1
Bioethanol	0.79	1.5	<21	26.8	21.17	>100	0.65

In some cases bioethanol has superior properties, such as higher density and higher octane number. This affects the antiknocking property of the fuels, whether it is used as standalone or as an additive. However, some of the exceptions prevent its wide use in spark-ignition engines. Ethanol has a low vapor pressure, and less evaporative emissions which cause cold start problems in colder climates. Thus, in these cases, ethanol blends with petrol are used. If mixed up to about 40% with gasoline, the two fuels combined have a higher vapor pressure than separately, due to a synergic effect. Furthermore, its energy yield is significantly lower than petrol, with the caloric value of petrol being 32,5 MJ/l, and for ethanol 21.2 MJ/l.

Due to these reasons, as well as in an effort to reduce the overall amount of green-house gas emissions from fossil fuels, bioethanol is blended with gasoline in various ratios. Most common blends are E5, E10, E20, E25, up to E80, E95, and E100, with the number indicating the presence of ethanol in volumetric percentage. Fuel blends with higher ethanol fractions are more common in the Americas (Brazil, USA, both major bioethanol producers). These fuels used for so-called flexible fuel vehicles (FFV) are just entering the European Market, in which diesel motors are dominant, and blends with less ethanol are more common. [Rutz and Janssen; 2007.]

1.2 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.]

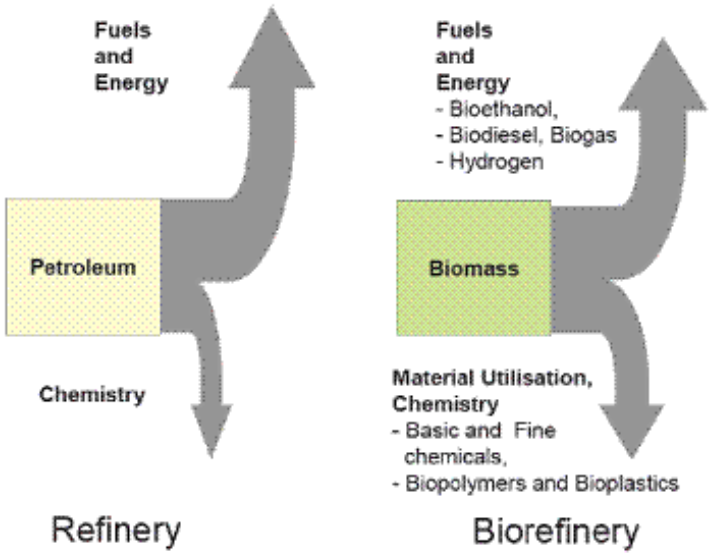

Figure 1.3: Side-by-side comparison of refineries and biorefineries [Dos Santos et.al., 2011.]

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

As for chemical methods, a variety of acids, including sulfuric, hydrochloric, hydrofluoric, and nitric acids can be used for hydrolysis of cellulose and hemicellulose. The hydrolysis process is represented by the following simple expressions:

$$\text{cellulose} \rightarrow \text{glucose} \rightarrow \text{HMF} \rightarrow \text{tars}$$

$$\text{xylan} \rightarrow \text{xylose} \rightarrow \text{furfural} \rightarrow \text{tars}$$

If hydrolysis conditions are severe (e.g. high temperatures or acid concentrations) a large fraction of the sugars is degraded to other products, e.g. HMF, furfural, and tars. [Katzen and Schell; 2006.] Recent research found ways of utilizing furfural as a feedstock for production of some useful chemicals. [Huber, 2006.]

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. The presence of lignin, as well as the difference in structure of cellulose and hemicellulose, requires pretreatment operations of separating these components to be made. This increases the overall cost of the process. Waste materials (agricultural, pulp and paper industry, food processing etc.) are a cheap, sustainable resource.

A large variety of important building-block chemicals are produced from lignocellulosic materials, ranging from organic acids (malic, succinic, fumaric), xylitol (which can be hydrated into ethylene glycol and propylene glycol), and many others. 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

In order to utilize biomass as a feedstock for fuel and chemical production, its intricate structure must be broken down to basic building blocks in a clean and efficient way. In order to better understand the weight of this challenge, the structure of cellulose, as well as the interconnections with other important components of biomass, lignin and hemicellulose, will be thoroughly discussed in this chapter.

2.1 Cellulose structure and properties

Cellulose is a virtually inexhaustible polymeric raw material. In nature, cellulose is found in plants, algae and mosses, as the main element of their cell walls. It is also produced by some bacteria and fungi. It is of great importance as an irreplaceable in production of many products, ranging from paper products and textiles, to pharmaceuticals and foods. [Ergun et al., 2016.]

Since cellulose is the most abundant biopolymer on the earth, it is the focus of considerable interest as a renewable energy resource. Cellulosic materials are desirable feedstocks for alternative fuels and energy carriers such as ethanol, biodiesel, or hydrogen since they are renewable and abundant. [Rezaei et al., 2008.]

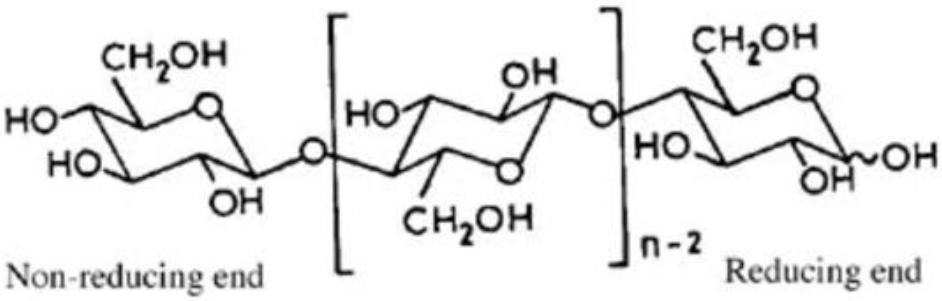

Figure 2.1: Molecular structure of cellulose [http://www.fibersource.com]

Cellulose is a polymer composed of glucose units linked by β-1,4-glycosidic bonds (Figure 2.1.). Hydrolysis of the cellulose chain generates glucose, which is a precursor to valuable chemicals such as biodegradable plastics. Furthermore, glucose derived in this way can be anaerobically fermentated into ethanol. Figure 2.2. shows some of the chemical compounds which can be derived from glucose.

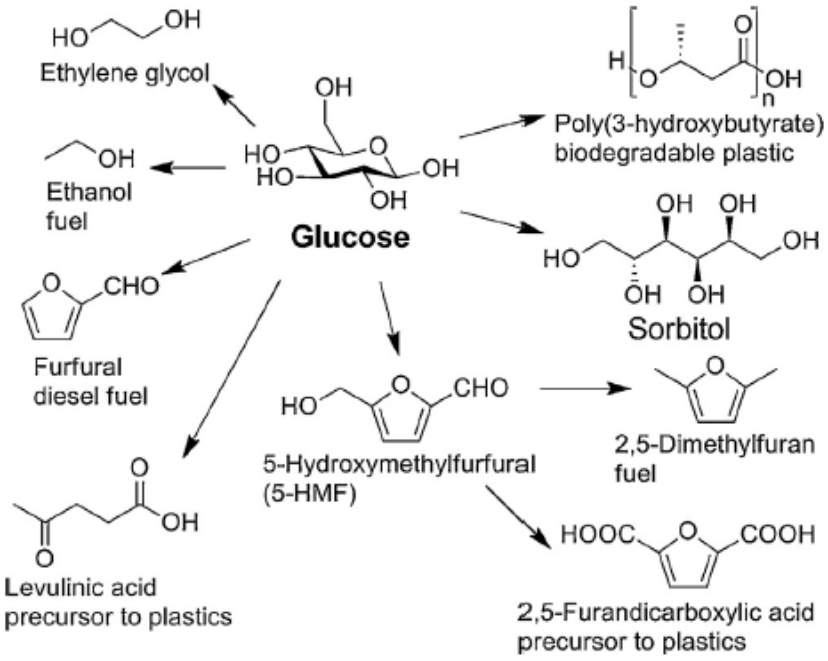

Figure 2.2: Chemicals derived from glucose [Yabushita et al., 2014.]

Most of these compounds are expected to be a base for the bio-based industry. For instance, sorbitol can be converted to polymers and pharmaceuticals, while 5-HMF, gluconic acid, and other derivatives are a base for various products used in the chemical, pharmaceutical and food industry. Some of these are coatings, films, membranes, gums, etc.

All this made cellulose a promising sustainable feedstock for the future. However, before these aspirations become a reality, some key issues regarding the unchaining of the cellulose structure must be overcome.

In a very few cases in nature, cellulose is found in a pure state. Usually, it is a part of a matrix containing hemicellulose and lignin and other cell components. If unaccompanied by these elements, cellulose is prone to degradation. Thus, they play an important role in maintaining the stability of the cell wall. However, although this is a great benefit for the plant, it creates a lot of difficulties for utilization of cellulose, due to relatively poor significance of hemicellulose and lignin, and complicated extraction and purification. Cellulose in a pure form is produced by some bacteria (i.e. *Gluconacetobacter xylinus*), fungi, algae, and invertebrates. [Ergun et al., 2016.]

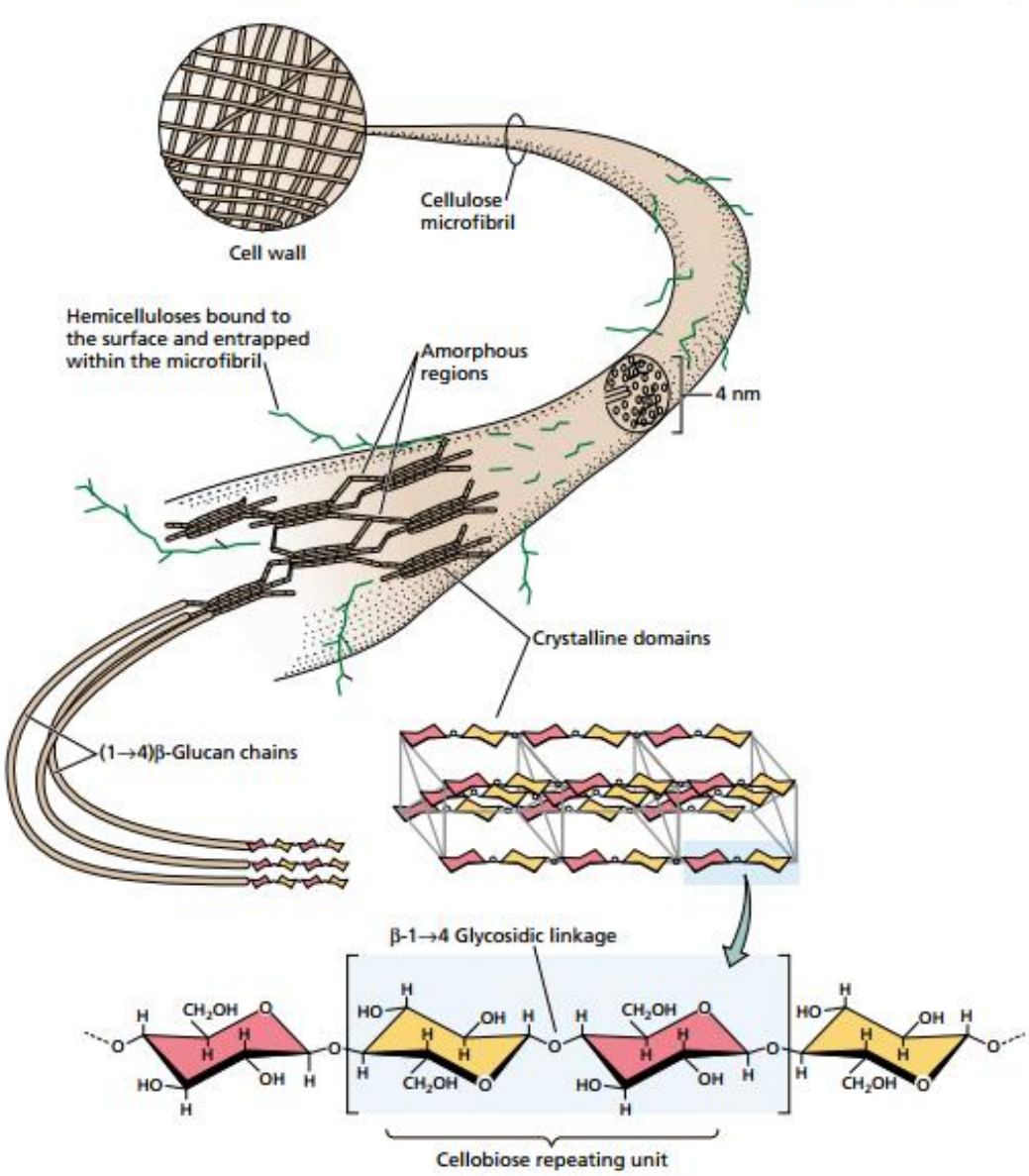

Figure 2.3: Structural model of a cellulose microfibril [Taiz and Zeiger, 1991.]

When building the cell wall, cellulose forms complex microfibril structures, with amorphous and crystalline domains. This structure is presented in Figure 2.3. These different structures are important since they determine the accessibility of the enzymes to the cellulose molecules. In the crystalline domains of the microfibril structure, strong intermolecular hydrogen bonds create a compact cellulose matrix, which prevents cellulase enzymes reacting fully with the polymer, and make the cellulose insoluble in water.

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.] Due to this strong internal hydrogen-bonding, cellulose is highly crystalline in the center of the microfibril, makes molecular modeling difficult. The crystalline superstructure of cellulose is difficult to disrupt even under extreme reaction conditions: cellulose must be heated to 320 °C at a pressure of 25 MPa to remove its cristallinity. [Maurer et al., 2012.]

Although pretreatment is needed to deconstruct the plant cell wall, compounds that are toxic to fermentative microorganisms are normally formed due to the harsh conditions applied. Furfural and furan, which affect cell growth, are formed when applying high temperature and pressure, while formic, acetic, and other weak acids decrease the pH level and affect cell metabolism. Phenolic compounds can be also formed due to lignin breakdown by aggressive chemicals. [Paris et al., 2014.] All of these components pose a threat to the yeast used for fermentation, and their removal (if possible) can be a very expensive inconvenience that can increase the overall cost of the process substantially.

The very promising pathway, on which most of current research is focusing, is enzymatic hydrolysis. A graph showing all the possible routes of cellulose to ethanol conversion are shown in Figure 2.4.

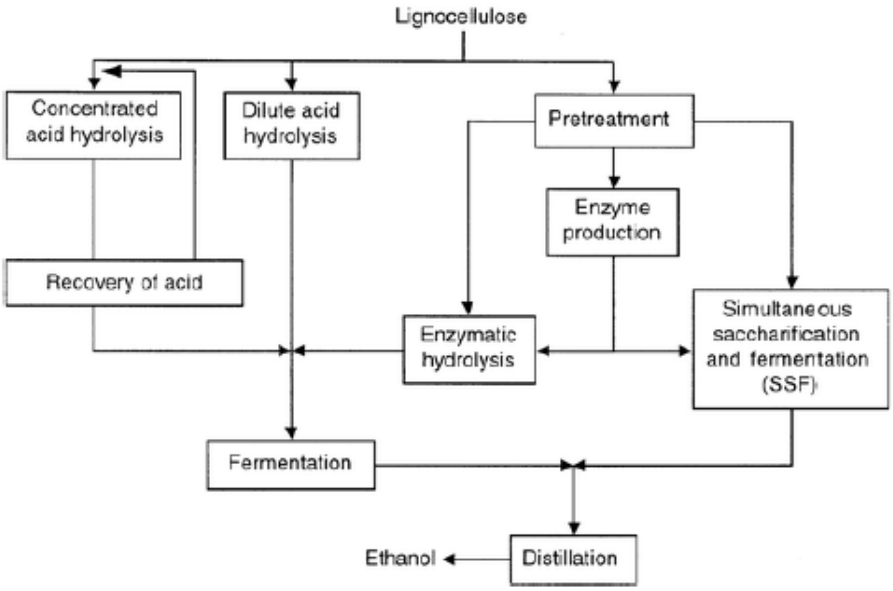

Figure 2.4: Different Cellulosic Ethanol production pathways [Demers et al., 2009.]

2.2 Properties of cellulolytic enzymes - cellulolytic complex performance and production

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.]

A large group of naturally occuring microorganisms, fungi and bacteria, have the ability to degrade lignocellulosic materials to its building components. More than 100 cellulolytic fungi have been reported to date and this number is continually increasing, including (hyphomycetes, ascomycetes, and basidiomycetes), as well as many filamentous bacteria species. [Wyman et al., 2005.] A list of the most significant of these is shown in Table 2.1.

Cellulolytic microorganisms are able to digest a variety of other carbohydrates besides cellulose. Another property of these organisms is the ability of secreting large quantities of extracellular protein into the medium.

Table 2.1: Major microorganisms employed in cellulase production [Singhania, 2009.]

Kingdom	Genus	Species
Fungi	*Aspergilus*	*A. niger*
		A. nidulans
		A oryzae
		A. aculeatus
	Fusarium	*F. solani*
		F. fusosporioum
	Humicola	*H. insolens*
		H. griesa
	Melanocarpus	*M. albomyces*
	Neorospora	*N. crassa*
	Phanerochaete	*P. chrysosporioum*
	Penicillium	*P. brasilianum*
		P. occitanis
		P. decumbans
		P. purpurogenum
		P. janthinellum
	Talaromyces	*T. emersonii*
	Trichoderma	***T. reesei***
		T. harzianum
		T. longibrachiatum
Bacteria	*Acidothermus*	*A. cellulolyticus*
	Bacillus	*B. subtilis*
	Clostridium	*C. thermocellum*
		C. acitobutylicum
		C. cellulovorans
	Pseudomonas	*P. cellulose*
	Rhodothermus	*R. marinus*
	Cellulomonas	*C. fumi*
		C. uda
Actinomycetes	*Streptomyces*	*C. drodzowiczii*
		S. lividans
	Thermomonospora	*T fusica*
		T curvata

However, cellulase activity *in vivo* is not mediated by a single enzyme. Rather, it is a complex of several different enzymes which act in synergy. The cellulase complex forms a unique structure which has been called a cellulosome and appears to be ubiquitous among cellulolytic microorganisms [Lamed et al., 1987].

The cellulosome includes:

- endoglucanase which hydrolyzes β-1,4 linkages between adjacent glucose molecules within the amorphous regions of the cellulose polymer thereby breaking the chain in the middle. A clear indicator of their activity is a decrease in viscosity; (E.C.3.2.1.4)
- exoglucanase which degrades the nicked cellulose chains from their non-reducing ends producing glucose, cellobiose and/or cellotriose. These liberate D-glucose from 1,4-β-D-glucans and hydrolyze D-cellobiose slowly; (E.C.3.2.1.91)
- cellobiohydrolase, another type of exoglucanase which removes larger polysaccharides from the non-reducing end of the cellulose molecule; EC 3.2.1.91
- β-glucosidase, or cellobiase, which converts cellobiose to glucose. [Coughlan, 1985]. (E.C.3.2.1.21) [Polaina, MacCabe; 2006.]

Enzymatic hydrolysis of natural lignocellulosic materials is a very slow process because cellulose hydrolysis is hindered by structural parameters of the substrate, such as lignin and hemicellulose content, surface area, and cellulose crystallinity. [Mansfield et al., 1999]

Since hemicellulose can have a significant presence in the material, a "cocktail" of both cellulolytic and hemicellulolytic enzymes should be applied. Degradation of hemicellulose along with cellulose is essential for an efficient hydrolysis of lignocellulosic biomass. In fact, co-hydrolysis of xylan can improve cellulose accessibility to the cellulases as a result of xylan solubilization. [Paris et al., 2014.]

The complex mechanism of the hydrolysis of a typical hemicellulose structure - arabinoxylan, is shown in Figure 2.5.

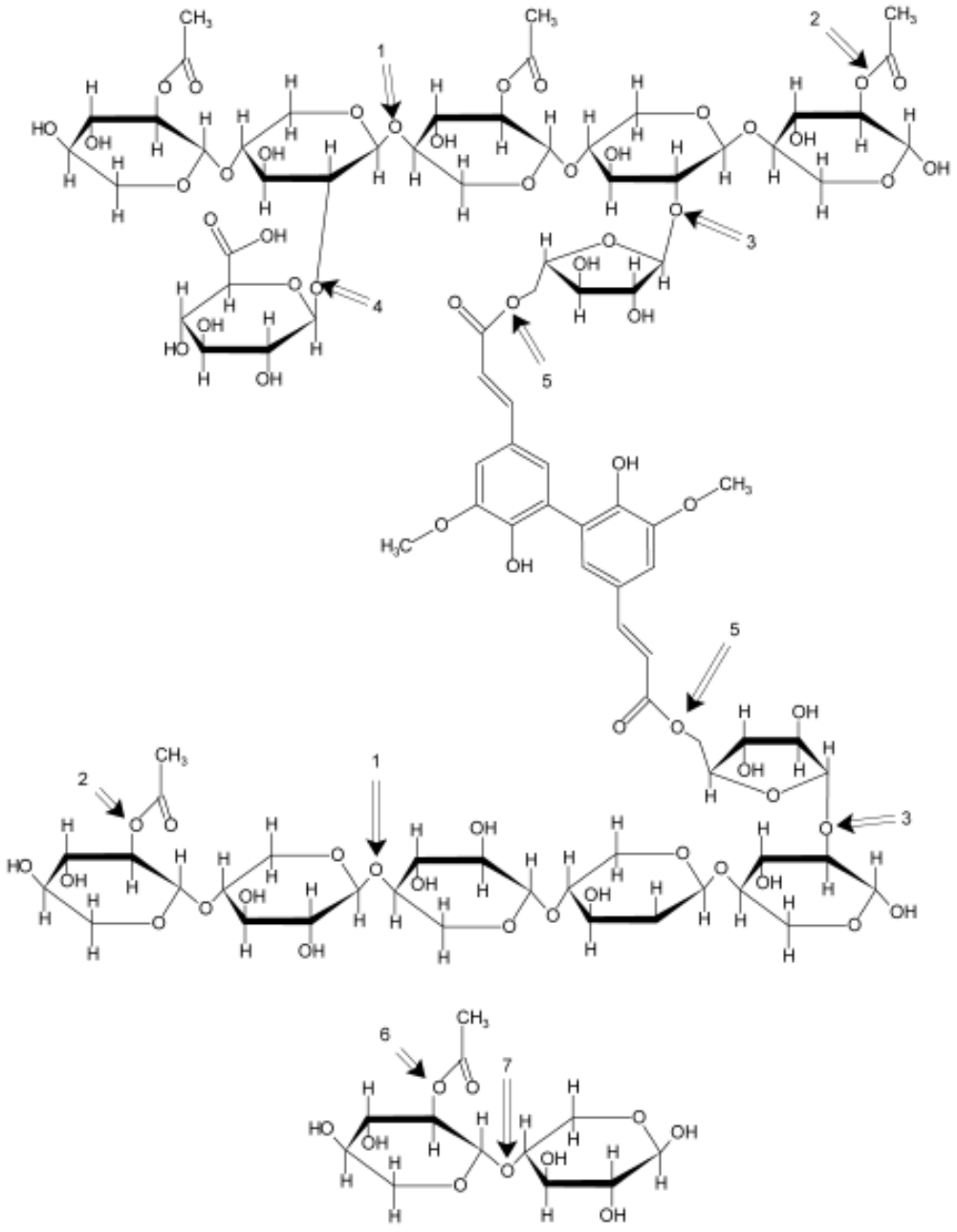

Figure 2.5: Enzyme activities in depolymerization of arabinoxylan. The structure is a generalized diagram of arabinoglucuronoxylan. Enzyme activities are (1) endoxylanase, (2) acetyl xylan esterase, (3) a-L-arabinofuranosidase, (4) a-D-glucuronidase, (5) ferulic acid esterase, (6) acetyl esterase, and (7) h-xylosidase. [Wyman et al., 2005.]

The main obstacle with enzymatic hydrolysis lies in the enzyme itself. Commercial enzymes are widely available, but have a considerably high price. Also, since their selectivity is quite low, in the prolonged time that is needed for full sacharification of the cellulose, formation of inhibitors can occur. In order to overcome these issues, cheaper production of more efficient enzymes must be developed.

A majority of reports on cellulase production utilizes submerged fermentation technology. This enables better monitoring of the process, easier handling and simplifies downstream treatment, as opposed to

the naturally occurring process that resembles solid state fermentation. [Sukumaran et al., 2005] However, solid state fermentation is gaining interest for processes that rely on simultaneous cellulose saccharification and alcoholic fermentation.

Many fungi can grow in submerged culture in different forms ranging from dispersed filaments to pellets, depending on culture conditions and the strain of organism used. Pellets are spherical or ellipsoidal masses of hyphae with variable internal structure, ranging from loosely packed hyphae, forming ‘‘fluffy’’ pellets, to tightly packed, compact, dense pellets [Papagianni, 2004]. The impact of pellet formation on cellulase yield is discussed briefly in Chapter 2.2.1.

Various factors determine the yield of cellulase from the fermentation. Certain enzyme production inducers are known, although the exact mechanisms of their influence are not fully understood. These are carbon sources that are more challenging to metabolize than glucose (usually cellulose derivatives, such as cellobiose and sophorose), since easily utilizable sugars repress production.. Cellulosic biomass, like cereal husk or straw, can be used as a cellulase inducer. The most commonly used one in commercial enzyme production is lactose. It is believed that the intracellular galactose-1-phosphate levels might control the enzyme secretion. [Sukumaran et al., 2005]

Complete utilization of lignocellulosic material is limited due to lack of microorganisms capable of fermenting a variety of sugars obtained by hydrolysis of lignocellulosic materials. *S. cerevisiae*, for instance, is widely used as fermentative organisms, efficiently ferments hexoses, but is not naturally able to ferment pentoses such as xylose and arabinose. [Paris et al; 2014]

2.2.1 Fermentation operational conditions and fermentation types

Temperatures between 45°C and 50°C are characteristic of enzymatic hydrolysis while the optimal performance temperature for fermentation using S. cerevisiae is around 30°C. SSF is normally carried out at 38°C being hydrolysis the rate-limiting process [15]. To enhance SSF performance, commercially available cocktail enzymes, capable of hydrolyzing the substrate at lower temperature, or thermotolerant microorganism, such as K. marxianus, should be considered. [Paris et al; 2014]

Proper agitation is necessary for proper growth of the working microorganism. Agitation enables access of fresh substrate to the microorganism, and decreases the concentration of metabolic products in the immediate environment around it. Propeller stirrers are most commonly used, but with moderation, in order to prevent damage to the filamentous morphology of the microorganism. Usually, aeration and stirrers combined provide sufficient agitation in the medium.

For some microorganisms, poor agitation creates the possibility of formation of pellets, which decrease the overall efficiency of enzyme production. Sections inside the pellet have poorer growth conditions, lower availability of nutrients and oxygen levels, which drops the yield of extracellular enzymes. However, with excessive agitation, poor extracellular enzyme yield is also observed, probably due to shear stress on the microorganism cells [Lejune and Baron, 1995.] [Domingues, 2001.]

As mentioned before, there is a possibility of performing saccharification and fermentation separately or simultaneously. Generally, hydrolysis is carried out in a separate vessel, since the cellulolytic enzymes and yeasts used in the fermentation demand different operational conditions. However, if these conditions are similar, the two processes could be carried out simultaneously. The main advantage of this process is that the sugars generated by hydrolyzing cellulose can be directly metabolized in anaerobic digestion to produce ethanol. This prevents inhibition by product of the cellulase enzymes, since glucose concentration remains low due to the yeast activity. [Paris et al; 2014]

The alternative, separating these two phases, although more technologically demanding and costly, has certain benefits. That way, both the hydrolysis and fermentation process can be conducted with optimal operational conditions, without compromise. Usually the cellulose enzymes are separated and transferred to another environment, where biomass hydrolysis takes place.

Adding the lignocellulosic material directly to the fermentation broth where cellulase enzymes are generated is possible. Immediately after enzyme production, the saccharification process starts. The product is a broth containing sugars and nutrients remaining in the medium after the production the cellulolytic complex, that were not utilized by the microorganism, as well as the sugars originating from the hydrolyzed biomass. This broth can be sterilized and inoculated with the yeast. This way, waste generation from the process is minimized.

The relationship between fungal growth conditions and cellulase production has been discussed. From theoretical assumptions it has been suggested that to be commercially successful it is necessary to produce 1100 filter paper units L^{-1} h^{-1}. This amount of enzyme requires a 70 g L^{-1} h^{-1} growth rate of the culture. Under most favorable conditions fungal cultures produce 35 g L^{-1} h^{-1} of cell mass. This gap could be closed if the chosen microorganism would show any one or both of the following properties: (i) a high-enhanced capacity for cellulase production; (ii) the ability to produce enzymes with a high specific activity. These desirable properties may be achieved by either new strain selection and/or strain improvement. [Gosh and Gosh, 1992]

2.2.2 Cellulase recovery and purification

After the production of the cellulolytic enzymatic complex, it has to be separated and purified in order to obtain a stable product. The separation process in this case involves removal of cellulolytic enzymes from the medium components, polysaccharides, small molecules and proteins other that cellulolytic ones.

The purification process involves four main stages:

- Removal of insoluble components (solids),
- Recuperation of the protein and purification in order to increase their concentration

- Separation of different fractions of the extracellular enzymes (usually chromatographical methods)
- Preparation of the pure enzyme, in a final form that is ready for commercial use [Domingues, 2001.]

Separation and purification processes vary greatly when it comes to performance, price, and applicable scale. As defined by Stanbury et al. [Stanbury et. al, 1984.], there are several criteria when it comes to determining the optimal recovery process:

1. Location of the product: intra- or extracellular
2. Concentration of the product in the fermentation broth
3. Physical and chemical properties of the product
4. Intended use of the product
5. Minimal acceptable purity level
6. Magnitude of bio-hazardous elements in the broth
7. Impurities in the fermentor broth
8. Product's marketable price

The same source mentions that price of product recovery can range from 15 to 70% of total costs. Although the source is not very recent, not much progress on costliness of these processes has been made since. It is also stated that protein purification processes correspond belong to the group of the more expensive ones. This makes sense when taking into consideration the points made in the list above.

In the case of cellulase purification, there are just a few mitigating factors that reduce the overall process cost. These are the extracellular location of the enzymes (cell disruption is not necessary, even undesirable), as well as the low demand for purity, since the enzymes are not used in the food of pharmaceutical industry. However, thermal and mechanical sensitivity of the enzymes, low concentration in the broth, as well as the low marketable price, limit the number of possibly applicable separation techniques, and are the reason for the low cost effectiveness of the process. This is an issue that has to be solved, and is one of the major problems hampering efficiency of cellulase production.

Two possible approaches on overcoming costliness issues could be made. Firstly, the problematic properties themselves could be moderated, for instance production microorganisms with the ability to secrete higher concentrations of enzymes could be developed. Alternatively, processes that are currently used in these cases could be made more efficient and cheaper, influencing in that way the overall cost-effectiveness of the purification process.

In table 2.2., some most commonly used enzyme separation and purification techniques are listed, along with the exact property of the enzyme that is the basis on which the separation is made.

Table 2.2: Most commonly used purification techniques and enzyme property that is basis for separation [Domingues, 2001.]

Process	Property
Gel filtration chromatography	Size and shape
Ultrafiltration	Size
Ion exchange chromatography	Electric charge
Isoelectric focusing	Isoelectric point/charge
Precipitation with ammonium sulphate	Solubility/Hydrophobicity
Hydrophobic Interaction	Hydrophobicity
Reversed-phase chromatography	Hydrophobicity
Phase separation	Solubility
Partition Chromatography	Solubility
Affinity Chromatography	Specific interactions

3. *Trichoderma reesei* - history of use and strain improvement

The filamentous fungus *Trichoderma reesei* has a long history of use for production of hydrolytic enzymes. It was isolated during the Second World War from the Solomon Islands, at the US Army QuarterMaster Research and Development Center at Natick, Massachusetts, after the military personnel noticed the fungus was degrading cotton garments and canvases. Originally named "strain QM6a" and thought to be a *T. viride* species, later was given a new species name after the Natick researcher Elwyn T. Reese. [Mandels and Reese, 1957.] [Simmons, 1977.]

After the potential of such a microorganism was realised, 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.] All modern commercially used *T. reesei* strains descend from the original QM6a strain.

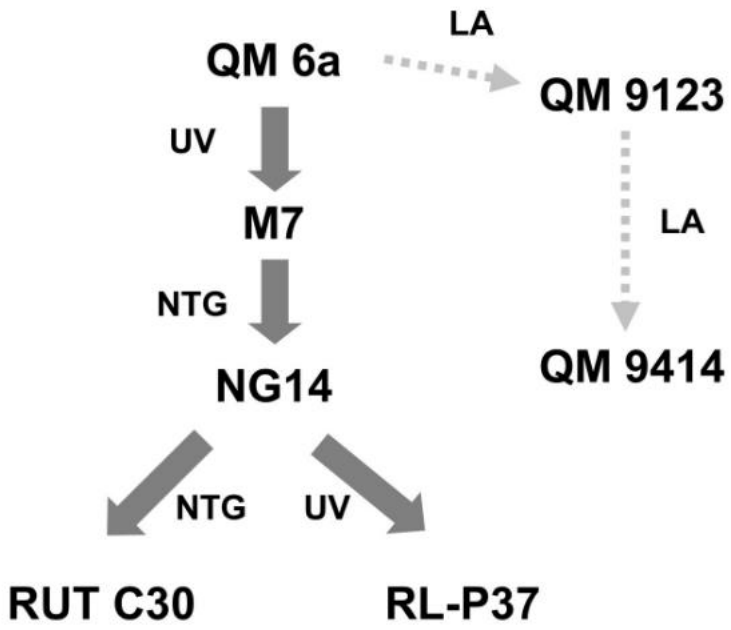

Figure 3.1: Lineage of the QM6a strain. [Seidl et al., 2008.]

Throughout its lineage, as new mutated strains were derived from the original one, improvements were made in the ability to secrete extracellular protein and improve enzymatic activity. Some of these properties for various important mutants are presented in Table 3.1.

Table 3.1: The extracellular protein and enzyme activities of wild-type *T. reesei* QM6a and mutants QM9414, MCG77, NG-14 and RUT-C30, following growth on 6% (w/v) roll-milled cotton in a 10 l fermenter for 14 days [Ryu & Mandels, 1980]

Strain	Soluble protein (mg/ml)	FPU (U/ml)	Productivity (FPU/lh)	CMC (U/ml)	βGl (U/ml)
QM6a	7	5	15	88	0.3
QM9414	14	10	30	109	0.6
MCG77	16	11	33	104	0.9
NG-14	21	15	45	133	0.6
RUT-C30	19	14	42	150	0.3

Recent investigations discovered that the *T. reesei* is a asexual anamorph of the sexually propagating fungus *Hypocrea jecorina*. Although by the "holomorph concept", the name of the sexual for should be used for the species, due to its historical significance, the *Trichoderma reesei* is used as a species name, making an exception to the conventional practise. [Seidl et al., 2009] [Seiboth et al, 2011.]

T. reesei is widely used in the textile, food and feed industry, since it was given the GRAS status (Generally Recognized As Safe) by the U.S. Food and Drug Administration. It serves as a model organism for the group of cellulolytic microorganisms, due to its long history of use and extensively researched genome sequence.

3.1. *T. reesei* cellulolytic system

A variety of cellulolytic enzymes is needed to degrade cellulose to its building blocks. The *T. reesei* cellulosome is composed out of the several typical enzyme groups, which can be derived into four enzymatic groups, explained in more detail in Chapter 2.2.

Figure 3.2 is a graphical representation of the synergistic performance of different groups on breaking down the cellulose chain. Besides the three main groups, other important components are presented. For instance, some of the endoglucanases and cellobiohydrolase are connected to carbohydrate binding modules, as they form a bipartite structure connected via a flexible linker region. Also, non-enzymatic proteins such as swollenin (SWO) can be seen disrupting the crystalline structure, exposing the individual polymer chains to the enzymes and improving the overall efficiency of the hydrolysis process. [Seiboth et al., 2011.]

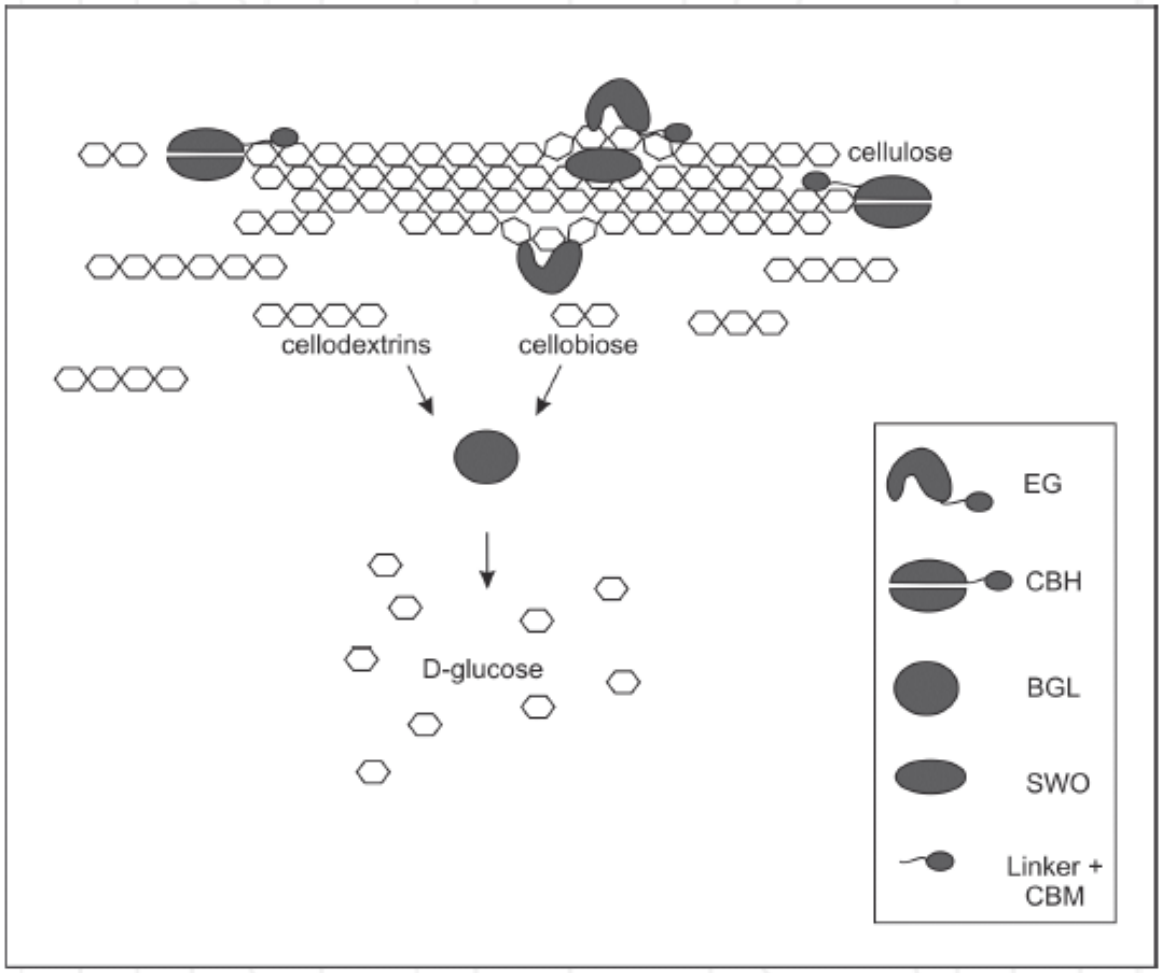

Figure 3.2: Degradation of cellulose by cellulases and non-enzymatic proteins of *T. reesei* [Seiboth et al., 2011.]

3.2 *T. reesei* RUT-C30

At Rutgers University, New Jersey, a separate line of high-producing mutants was generated. Among these strains, RUT-C30 has become the most frequently used cellulase hyperproducer in the public domain. [Seiboth et al, 2011.]

This strain was obtained through a three-step procedure. First, mutagenesis by UV light and screening for catabolite derepression led to the isolation of strain M7. Further mutagenesis by N-nitroguanidine led to the isolation of NG14, a partially derepressed strain that produced approximately twice the extracellular protein, five times the filter paper activity, twice the β-glucosidase activity and twice the endoglucanase activity of QM9414. Following another round of UV mutagenesis and screening for high cellulase activity and catabolite derepression by resistance to the antimetabolite 2-deoxyglucose (2DG), RUT-C30 was isolated. [Peterson and Nevalainen, 2012.]

RUT-C30 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} ,15–20 times higher than that of QM6a when grown in shake flasks.

The strain possesses certain physiological differences compared to QM6a. These exquisite ultrastructural properties are connected to its superior potential of enzyme production. Microscopic analysis exposed a six- to sevenfold higher endoplasmic reticulum (ER) content in RUT-C30 during secretion than in QM6a. Phospholipid content of the mycelium and the concentration of the nucleoside diphosphatase, which is a ER marker enzyme, is double that of the QM6a. Mycelial protein content was about five times higher, and β-glucosidase and endoglucanase activity was three and thirty times higher, respectively. The RUT-C30 strain lacked the typical Golgi apparatus, and instead contained saccules associated with the ER. It was undetermined if these saccules were Golgi bodies with an unusual morphology, or the strain exhibited a Golgi-independant secretory pathway. [Ghosh et al., 1984.] [Glenn et al., 1985.] [Ghosh et al., 1982.]

Another, more recent study, by Peterson and Nevalainen [Peterson and Nevalainen, 2012.] showed that this strain lacks annulate lamellae that are abundant in the wild-type QM6a, and possess excessively transverse parallel cisternae and a small amount of punctate-like bodies, all indicating cellular stress. As for the strain phenotype, the conidia of RUT-C30 are of a lighter green colour and the bottom view of colonies growing on an agar plate lack the yellow color (pigment) typical for the wild-type.

Finally, 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.

Since the strain's genetics are thoroughly researched, homologous recombination could be a major approach for producing knockout strains in *T. reesei*. Recent advances in method development have resulted in high-throughput generation of knockout/gene replacement strains by more efficient strategies, increasing the efficiency of homologous recombination and allowing sequential gene deletions. While these strategies have so far been applied to the members of the *T. reesei* family tree, they could similarly be applied to the RUT-C30 strain. Sexual crossing can also be used as a fast and efficient method of eliminating detrimental mutations by complementation with wildtype fungi and combining favourable features expressed by different successful mutant strains. [Peterson, Nevalainen; 2012.]

4. Materials and Methods

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.

4.1 Cellulase complex production

4.1.1 Strain

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.

Fungal spores preserved in a cryogenic vial, kept at −80 °C in 20% (v/v) glycerol, were used to inoculate potato dextrose/agar (Difco) slants, which were incubated at 28 °C. Five days after inoculation, the spores were harvested and washed with sterilized water and the spore concentration was determined in a Neubauer counting chamber after appropriate dilution [Domingues et al., 2000].

4.1.2 Culture media

Cultivations are carried out several different cultivation media, with slight modifications and variations. The basis for the three main kinds of liquid media used are a modification of the Trichoderma minimal medium (TMM) [Penttilä et al., 1987], the Mandels solution [Mandels and Reese, 1957], traditionally used for solid state production, and the third being the one showing pulpy growth in literature [Domingues et al., 2000.] This media contains a similar content of salts to the TMM, while additionally containing peptone, similarly to the Mandels solution. However, it is supplemented with yeast extract as one of its components, and thus, just for clarification purposes, this medium will be regarded as the "supplemented" medium. The composition of all media bases, without the carbon sources, is given in Table 4.1.

Table 4.1: Content of the modified Trichoderma Minimal medium, Mandels solution and the "Supplemented" medium used in the experiments

Component in g/l	Trichoderma minimal	Mandels solution	"Supplemented"
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

A list of all the chemicals used in preparation of the different media is presented in Table 4.2. Details about the manufacturer and purity level are also laid out.

All chemicals used for experimets were of >99% purity. The peptone used is Himedia Bacteriological Peptone. The yeast extract used for the media is Difco Ultrafiltered Yeast Extract.

As for the carbon sources, glucose was mainly used, while lactose, cellobiose and wheat bran were added in smaller quantities as enzymatic complex production inducer.

4.1.2.1 Preculture media

Preculture media contained media bases from Table 4.1, and 30 g/l of glucose as the sole source of carbon.

4.1.2.2 Main fermentation

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).

4.1.2.3 Variations

In an effort to determine optimal fermentation conditions, experiments with variations of the media were made. In contrast to the solely synthetic substrate that is the Trichoderma Minimal Medium, semi-synthetic mediums, Mandels and Supplemented medium, were used, containing peptone and

yeast extract. Nevertheless, an effort was made to maintain the needed amount of nutrients for the growth and enzyme production by the microorganism, as well as maintain the concentration of iron in the media at adequate levels, since it proved to be a key element influencing mycelial structure and pellet growth [Domingues et al., 2000.]

4.1.3. Sterilization of materials and equipment

All materials and equipment used for the fermentations (components of the media along with Erlenmeyer flasks, measuring cylinders, pipette tips, etc.) were sterilized in an autoclave prior to use, at a temperature of 121°C, at 104 kPa pressure for 20 min. The medium bases from Table 4.1 were sterilized separately from carbon sources, in order to prevent creation of products of the Mailard reaction, which lowers the content of N and C sources available to microorganism, as well as possibly affect its metabolism [Kim and Lee, 2003]. This phenomenon is well known, at it became common practise to separate these components separately. After attempting to sterilize the mixed components, a dark yellow coloration appeared in the flasks. Figure II.ii from Appendix II to this thesis shows a similar occurrence that was observed after a shake flask with the fungal biomass and medium was sterilized.

Thus, the media bases mentioned above (components without C sources) were prepared in a 200% concentration, while solutions of glucose and lactose were prepared as 20% and 7.5% solutions, respectively. After sterilization, the separate components of the media were mixed and diluted with sterilized water to the adequate concentration required for (pre)fermentation, before being finally inoculated. Separated components of the media were stored in a cold room, 4°C prior to use.

4.1.4 Production in shake flasks

A 250 ml Erlenmeyer flask containing 50 ml of medium was inoculated with 50 µl of a spore suspension of 10^7 spores ml^{-1}, made using a Bürker green counting chamber, which served as a pre-culture [Domingues et al., 2001]. The pre-culture was incubated for 48 h at 34°C on an orbital shaker at 200 rev/min^{-1}.

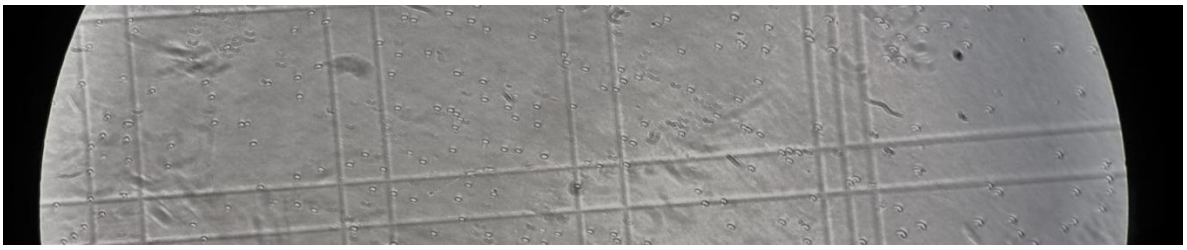

Figure 4.2: Microscope view of the spores during the counting process (magnification 100x) - spores visible

The flasks used for cellulase production, containing the production media, were inoculated with a 10% inoculum from the pre-culture. These flasks were incubated at 28° for 5 days, on an orbital shaker at 150 rev/min^{-1} [Kadam and Kreutzer; 1995.]

A scheme of the procedure is given in Figure 4.3.

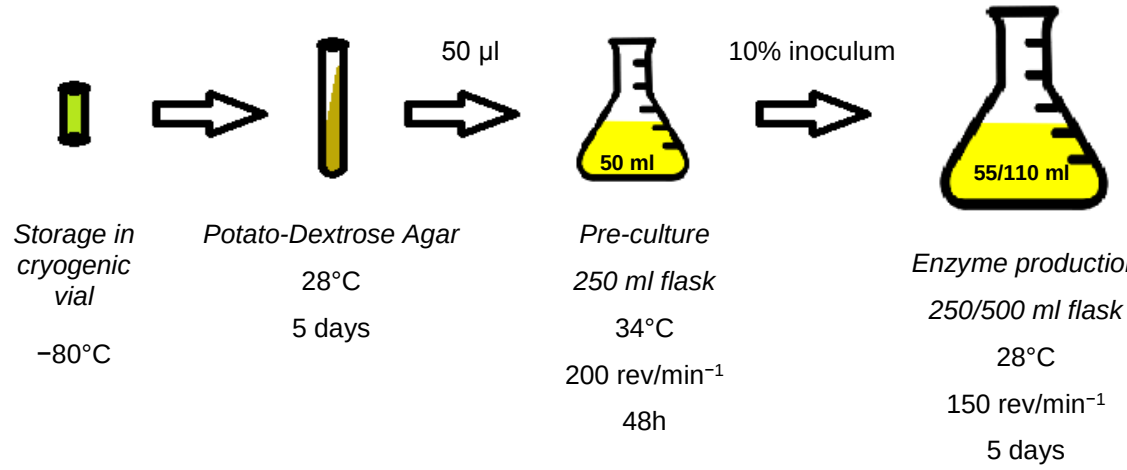

Figure 4.3: Overall scheme of fermentation procedure

Sampling of was done in a laminar flow chamber, sterilized prior to use for 15 minutes using a UV lamp. This is illustrated in Figure 4.4.

Figure 4.4: Flasks with fermentation media (during sampling procedure) - note the difference in homogeneity of the flasks on the left (Supplemented media) and flasks on the right (TMM). Homogeneity and sedimentation occurred due to difference in size of pellets.

4.1.4 Solid-state production

The *Trichoderma reesei* was grown on wheat bran in 100 ml Erlenmeyer flasks. 3 grams of wheat bran was covered with 5 ml of Mandels solution, which was separately sterilized. This step was used to induce the enzyme production of the fungus after an extended period in cryogenic conditions.

The growth of the fungus on the solid medium is illustrated in Figure 4.5.

Figure 4.5: Solid-state production on wheat bran (dark green spore coloration visible)

4.1.5 Commercial cellulase

The commercial cellulase used for some tests was cellulase from *Trichoderma longibrachiatum* - Sigma C9748. The enzyme activity has originally 1 FPU/mg of powder.

4.2 Hydrolysis of lignocelulosic materials

In order to test the performance of the produced cellulolytic enzymatic complex, several experiments with conventional feedstocks for bioethanol production were made. The enzymatic kinetics of the reaction between the cellulolytic complex and several raw materials were examined. These materials, as well their moisture content are presented in Table 4.2.

Table 4.2: Raw materials used for hydrolysis testing, and dry matter content

Material	Dry matter
Untreated sugarcane bagasse	99%
Sugarcane bagasse,	99%
Sugarcane shell, pretreated	99%
Sugarcane marrow,	99%
Paper pulp	44.20
Pine wood, untreated	90.41
Cow dung	92.73

The sugarcane baggase samples (untreated, pretreated, marrow and shell) were acquired from Univeristy of Sao Paolo, Brazil. The pine wood, cow dung and paper pulp samples were obtained from the Department of Chemical engineering of IST, University of Lisbon.

Dry matter conversion rate was calculated from difference in the weight of the dry sample and after hydrolysis. After 72h of hydrolysis, the liquid was centrifuged at 18500 RCF (12000 r/min) for 8 minutes at 4 °C. The supernatant was removed, and the solids were filtered using a vacuum pump with Whatman No. 1 filter paper. The paper with the sample was dried at 60 °C for 24h, and the weight was measured.

4.2.1 Pretreatment of sugarcane bagasse

The biomass used in the experiments (bagasse from sugarcane) was obtaned from the Lacanga located in Lacanga, São Paulo, Brazil. It was obtained in early 2014, and originated from the 2013/2014 harvest. The pretreatment of bagasse was carried out in collaboration with the physical organic chemistry group coordinated by Prof. Dr. Antonio da Silva Curvelo Aprígio (IQSC-USP).

The sugarcane residue was collected directly from the alcohol production line. In order to remove any remaining sugars in it, water extraction was done with the Soxhlet solvent, refluxed for about 8 cycles. The residue was placed to dry in a convection oven at a temperature of about 40 °C, for 2 days, until it contained a relative humidity percentage below 10% m/m and was subsequently stored at room temperature. The moisture content was measured using a "Topray" thermobalance produced by "BEL Engineering".

The different parts of the sugarcane, the bark and the marrow, were separated using a stationary drill. These fractions were treated identically as the bagasse in the lignification process.

4.2.1.2 Delignification method

For delignification, a reactor with a 304-stainless steel screw cap was used, of total volume 195 cm^3 (5.0 x 4.0 x 15.5 cm). 10 g of dried material (bagasse, marrow and skin) was dissolved with 16% Na_2O solution (w/w) and 0.15% anthraquinone solution (w/w) with solid-liquid ratio 1:10 . The method was customized for laboratory conditions by the staff of University of Sao Paolo, Brazil.

The heating was done using a heat bath containing glycerol as the heating fluid, with digital temperature control, while the reactors was immersed in the bath only when the fluid has reached the desired temperature (160 °C). Reactions were terminated after 100 min, after which the reactors were immediately placed in a water bath with ice. After cooling the pulp liquors were separated by filtration at atmospheric pressure.

The pulp was subjected to a refining procedure using a shredder for about 5 min. In this procedure, the cellulose fibers were separated from each other with a water solution. Then the pulp was filtered under vacuum and washed with distilled water until obtaining a neutral pH in the filtrate. Subsequently, the slurries were dried at room temperature and, after weighing and determining the moisture, passed through the mill "Solab" SL31, and stored at room temperature.

4.2.2 Determination of unhydrolysed material fraction

In order to determine the hydrolysis rate of the lignocellulosic materials, the residual content of dry, solid biomass was determined. This, alongside results showing reducing sugar concentration increase, is an indicator of the successfulness of the hydrolysis process.

After 72h of the reaction, the content of the flasks was filtered through Whatmans no. 1 filter paper, using a Büchner flask. Vacuum in the flask was created using a laboratory vacuum pump. This equipment is presented in Figure 4.6.

Figure 4.6: Equipment used for separating the residual solid matter after 72h of hydrolysis: Büchner flask connected to vacuum pump

After vacuum filtration, the sample cake on the (previously dried and measured) filter paper was dried in a Petri dish for 48h at 65 °C. After drying, the filter paper was weighed on an analytical scale and the percentage of residual solid mass was determined.

4.3 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.

Despite a few alternatives were possible, these methods were used due to their precision and reproducibility of results, as well as the possibility of processing small samples. This was imperative in case of production in shake flasks, since substantially decreasing the total volume by taking many large samples disrupts the process parameters.

4.3.1 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 9600 RCF (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.

For determining the total dry biomass content of a flask for any day of the fermentation, the whole content of the Erlenmeyer flash was transferred into 50ml Falcon tubes, centrifuged for 8 minutes at 9600 RCF (12000 r/min) (as described in Chapter 4.4.1.) and dried in a Petri dish (that was previously dried and weighed). After drying, the pellet was weighed and the content of dry biomass per ml of medium was calculated. This method proved to give more replicable results with lower variation, but required a separate flask to be preordained for this method for each day of fermentation.

4.3.2 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.] All Measurements are done in triplicate.

The exact procedure is as follows:

- 100 µl of sample is transferred to wells in 1 ml well plate. Sample is diluted, if needed, so concentration of reducing sugars falls into the method range of 0.5 - 5 g/l;
- 100 µl of DNS reagent is added to the well;
- Well plate is heated for 5 minutes in 100 °C water bath; (Coloration of the samples is presented in Figure 4.7.
- 500 µl of Milli-Q water is added to each well;
- 200 µl of sample from each well is transferred to a transparent well plate, used for measuring;
- The well plate is transferred to a microplate spectrophotometer, where absorbance is recorded for each well.

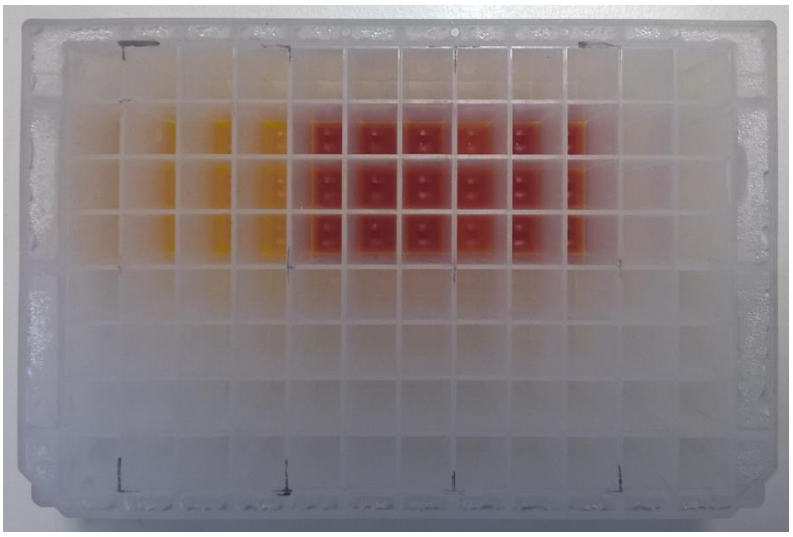

Figure 4.7: Well plate with DNS-treated samples

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).

4.3.3 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, identical to the one described in chapter 4.2.2. As a blank, the concentration of fermentable sugars in the sample was used.

The general equation for calculating the Filter Paper Unit is showed in Figure 4.8.

$$FPU = \left(Concentration\ of\ sugar\ released\ \left[\frac{mg}{ml}\right]\right) * \frac{(total\ volume\ of\ assay\ [ml])}{Glu.M\left[\frac{mg}{\mu mol}\right] * sample\ vol.[ml] * duration\ [min]}$$

Figure 4.8: General equation for calculating enzymatic activity in mmol ml^{-1} min^{-1}

In this part of the experimental work, there were several issues and inconsistencies encountered. These are elaborated in Chapter 5.1.5, as well as in Appendix I.

4.3.4 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. Also called Smith's

assay, it is a modification of the Lowry protein assay. It is a spectrophotometric method in which assays change colour from light green to purple, based on the concentration of protein in the sample. A calibration curve was formed, and used for calculation of protein. All samples were tested in triplicates.

4.4 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.

4.4.1 Centrifugation

Samples of the whole content of the Erlenmeyer flask was transferred into 50ml Falcon tubes, centrifuged for 8 minutes at 18500 RCF (12000 r/min) and the firm pellet was separated from the supernatant.

4.4.2 Precipitation with Ammonium-sulphate

For obtaining the extract of the enzymatic complex, ammonium-sulphate was added in small increments until reaching saturation. 50 ml of supernatant of the fermentation was used (obtained using the process from Chapter 4.4.1.).

4.4.3 Ultrafiltration

The equipment used for ultrafiltration was a stirred concentrator produced by Diaflo, with the capacity of 50 ml. The membrane used in this experiment was a Millipore polysulphonic membrane with pore diameter equivalent to 5000 Da. Pressure of 3 atm was used, and the equipment was kept at room temperature.

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. To be specific, the original idea was to test the cellulase production performance of the fungus in shake flasks and proceed to production in the stirred fermenter. Then, a set of experiments on separating and purifying the cellulase complex was to be made. 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. Also, problems with low reproducibility of results in some analytical techniques (determination of enzymatic activity, determination of dry biomass content) demanded additional time to be spent on determining the reasons for these difficulties, as well as overcoming them.

5.1 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.

In order to stimulate the enzymatic complex production, the spore solution from the cryotubes was grown on solid medium (as described in Chapter 4.1.4.). This was done in order tovercome poor enzymatic activity that was noticed in the initial laboratory work stages. After this, spores obtained from the solid medium were propagated on slants in test tubes, and used for inoculating all further fermentations.

The length of the prefermentation (48h) proved to be optimal, since it proved to be sufficient for formation of short (2-5 mm) filamentous strands of mycelium to form, enabling equal amounts of fungus to be transferred to fermentation flasks during inoculation. If the prefermentation time was prolonged for another 24 hours, the fungus would form large pellets, making the inoculum less homogeneous.

Initially, an attempt was made to replicate the procedure and results by F.C. Domingues [Domingues et al., 2001.]. Results of this set of experiments are presented in Figure 5.1.

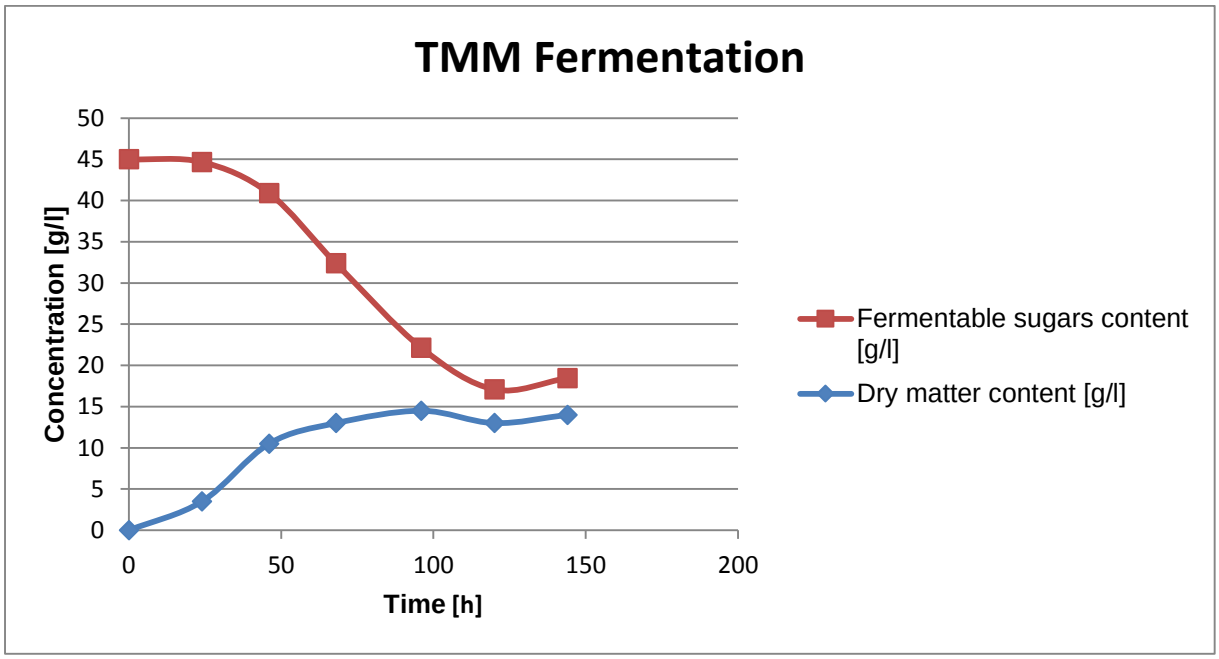

Figure 5.1: Results for dry mass and fermentable sugar content for Trichoderma Minimal Medium, shake flask production according to procedure by Domingues et al., 2001.

These values and trends seem reasonable, compared to the values obtained in literature. Although residual fermentable sugar concentrations are quite high to those in large bioreactors, this is understandable due to inferior production conditions in 250 ml shake flasks.

At this stage, no valid results were obtained for protein concentration and enzyme activity, and thus these results are omitted from the figure above. Certain discrepancies were observed in the DNS techniques used for determining sugar concentration after enzymatic activity. These are explained in Chapter 5.1.5.

5.1.1. Effect of fermentation volume on Filter paper activity

In order to enable taking a higher number of larger samples, an analysis was made to test the effect of increasing the total volume of the fermenattion liquid. Shake flasks of 250 and 500 ml with 55 and 110 ml of fermentation liquid, respectively, were used. Results of this experment are presented in Table 5.1. Except the shake flask size, all other parameters are identical to the first set of exeriments.

Table 5.1: Results for fermentation in 250 and 500 ml shake flasks

Flask size	500 ml (110 ml of liquid)	250 ml (55 ml of liquid)
Fermentable sugars (final concentration) [g/l]	21.72	27.70
Extracellular protein (final concentration) [g/l]	0.28	0.21
Filter paper activity(final concentration) [FPU/ml]	0.101	0.051

After this conclusion, 500 ml flasks were used for the main fermentation stage. Also, first results for enzymatic activity were obtained, since alterations were made to the technique in order to increase the quality of results (more information in Appendix 1).

However, results for enzymatic activity were considerably lower compared to those stated in literature (roughly 10 times lower, [Domingues et al., 2001.]). As there were a few possibilities for the reason of this occurance, which will be discussed later, further testing has been made.

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. The Mandels solutioncontains urea and peptone, and is not purely synthetic like TMM (detailed content of both media is presented in Table 4.1.). As source of carbon, 30 g/l of glucose was used, as well as 15 g/l of lactose as cellulase production inducer. Results for this set of experiments are presented in Table 5.2.

Originally, only the TMM and Mandels solution were considered for testing. Mandels solution is usually used with solid-state production, where a complex source of carbon is used. In this case, only pure sugars (lactose and glucose) are added. This hinders the presence of additional ammounts of phosphorus and nitrogen originating from the feedstock. In order to eliminate any suspicions that optimal amounts of phosphorus and nitrogen are added, another set of experiments was made.

In search for optimal growth conditions and medium composition, a study of the influence of culture conditions on mycelial structure and cellulase production [Domingues et al., 2000.] showed that a "Supplemented" media, that contains amounts of inorganic salts like in TMM, while still containing peptone and urea like Mandels solution, showed best enzymatic activity

5.1.2 Effect of different media on activity

Table 5.2: Results for fermentation in 500 ml flasks using TMM, Mandels solution and the Supplemented medium.

Media	TMM	Mandels solution	Supplemented medium
Fermentable sugars (final concentration) [g/l]	27.72	26.19	25.56
Extracellular protein (final concentration) [g/l]	0.28	0.38	0.41
Filter paper activity(final concentration) [FPU/ml]	0.101	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, while specific enzymatic activity did not vary as much.

This phenomenon might me due to formation of pellets in the case of TMM, while in Supplemented medium the fungus grew more loosely. Mandels solution also prevented pellet formation, while poorer enzymatic performance might be atributed to lower concentration of phosphorus and sulphate. In Figure 5.2, differences between the two shake flasks, TMM and the Mandels solution, can be observed.

Pellet formation is an important characteristic which greatly affects enzymatic activity. Due to lack of oxigen and poor material transfer inside the pellets, growth conditions aren't optimal and fungus activiy is lower. Factors that determine the appearance of pellets in the growth of *Trichoderma reesei* are inoculum size, and concentration of iron, among others. [Domingues et al., 2000.] The decicive factor in this case seems to be iron, which might be present in a higher concentration in the Mandels solution due to the addition of peptone.

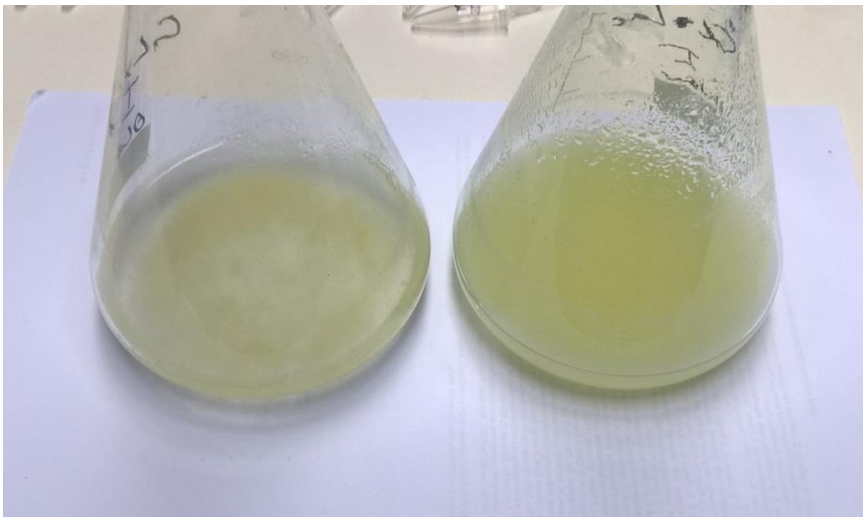

Figure 5.2: Pelletisation in shake flask production with TMM (left) and Mandels solution (right) - note precipitiated flocules of mycelium in the flask with TMM, while growth is more pulpy and hevenly distributed in the other flask

5.1.3. Cellobiose and wheat bran as cellulase production inducers

Before continuing with further research, a test was made of two alternative inducers: cellobiose and wheat bran. However, in both cases results were relatively poor. Figure 5.3. shows the change of fermentable sugar concentration with time for both cases.

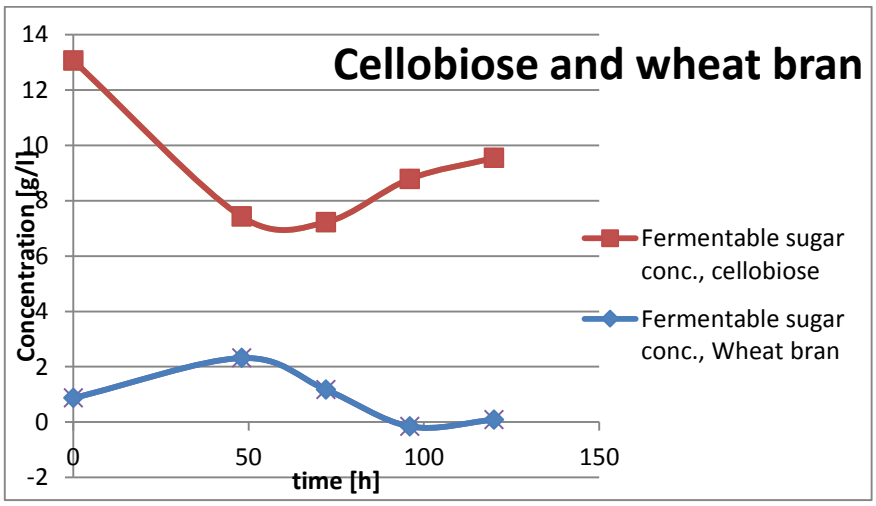

Figure 5.3: Fermentable sugars conc. for cellobiose and wheat bran

Fermentation conditions were identical to the previous set of experiments, except glucose and lactose were substituted with 20 g/l of cellobiose and 1 g/l of wheat bran. Results of concentration of cellobiose in the graph are lower than real values, as is explained in Chapter 4.3.2.

In the case of production with cellobiose, initial concentration of fermentable sugars shows about 13 g/l of glucose equivalent, despite 20 g/l of cellobiose added. This shows that 1 gram of this

disaccharide is detected by the DNS method as roughly 0.65 grams of glucose equivalent. However, after the third day of fermentation, there is an increase in sugar concentration, due to hydrolysis of cellobiose.

As for wheat bran, a small initial concentration of sugar originates from the prefermentation where glucose is present, since 10% of inoculum was used. In the following days, an increase of sugar concentration occurs due to some cellulolytic activity, but after 4 days the sugars released are metabolysed. As stated above, activity was poor in both cases compared to results obtained in previous experments. Results are presented in Figure 5.4.

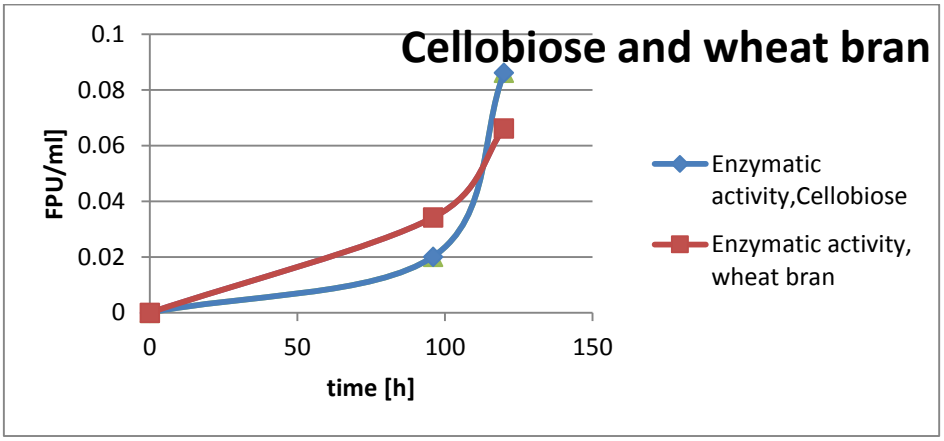

Figure 5.4: Enzymatic activity in FPU for cellobiose and wheat bran

5.1.4. Effect of the addition of Tween 80 on enzymatic activity

In literature, the comercial surfactant Tween 80 is commonly added to the producion media in order to prevent pellet growth and enable higher enymatic activity. However, our results haven't showed any noticable impact of the addition of 0.5 g/l of Tween 80 on enzymatic activity. Also, there were issues with sterilisation of Tween solutions, and they were prone to contamination. In order not to overextend the span of experimental work, this phenomenon was not studied further.

5.1.5 Impact of fermentable sugar concentration on determining enzymatic activity in Filter paper assay

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. The exact method for calculating the enzymatic activity is also rather vaguely explained in literaure. What is generally mentioned is that the samples should release of 2 mg reducing sugar/h in the assay conditions. [Montenecourt and Eveleigh, 1978.]

The sample of the supernatant from the fermentation was diluted and activity was measured. Diluted solutions contained 1:1, 1:3 and 1:7 of sample to water ratio, respectively. Results of this experiment are present in Figure 5.5.

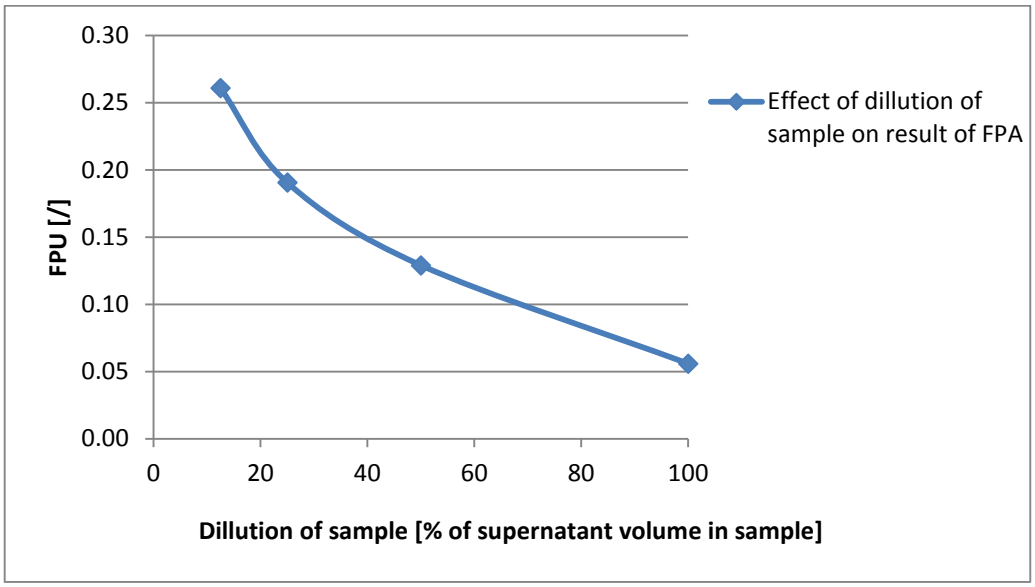

Figure 5.5: Effect of dilution of sample on result of Filter paper assay (percentage representing the content of supernatant in diluted sample)

What is observed from these results is an almost logarithmic dependency of dilution of the sample on activity. There are three possible reasons for this phenomenon, and most probably all three contribute in its occurence more or less.

First, there is a high concentration of sugar is present in the sample (30 g/l of glucose and 15 g/l of lactose are used in the main fermentation), even after 5 days of growth, due to poor growth conditions in shake flasks. The DNS method, used for determining the concentration of sugar in the samples ranges from 0.5 to 5 g/l of glucose equivalent. Due to this reason, samples of the supernatant are diluted prior to measurement 5 to 10 times. The units of enzymatic activity are calculated based on the difference of sugar concentration in the assay where the cellulase complex hydrolised filter paper and the blank, which contains only the supernatant with the enzymes. Diluting these assays in order to make them adequate for the DNS method also "dilutes" the concentration of sugars resulting from enzymatic activity, which can cause higher standard deviation and low precision in measurement.

Secondly, a high concentration of glucose in the sample can cause inhibition of enzymatic activity by product. Since a significant amount of the product of the desired reaction is already present in the solution, the kinetics of the hydrolysis reaction can be affected.

Finally, another reason for higher activity in diluted samples is due to a larger contact surface being available per enzymatic unit. This enables higher activity, since a smaller fraction of the enzymes remaines inactive due to lack of binding locations on the paper surface.

After these conclusions, in further experiments samples of the supernatant were diluted 4 times prior to application in the enzymatic assay.

Another group of flask fermentations was made, this time applying the above mentioned correction to the analytical method. This time, the "Supplemented" media showed higher activity than previously. These results are presented in Figure 5.6.

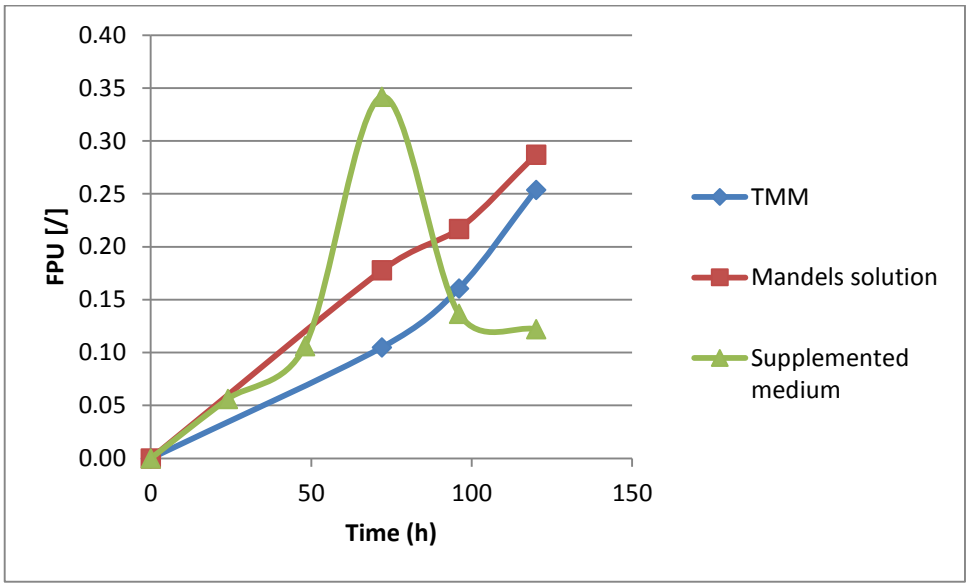

Figure 5.6: Enzymatic activity of *T. reesei* grown the three media

It can be observed from the graph in Figure 5.6 that although, the activity of the TMM and Mandels solution continued to grow up to 120 hours, the maximum activity for the Supplemented medium proved to be just after three days of fermentation, and at that point higher that the maximum activity for the other two media. Lower fermentation time is preferable, and in this case the Supplemented medium proved supperior. This also coresponds to literature data [Domingues, 2001.]

5.1.6 Specific activity of the enzymatic complex

Although concentration of extracellular protein and filter paper activity are good indicators, specific activity of the enzymatic complex gives us a better understanding of the enzymes ability of hydrolyzing cellulose. This value represents the activity of an enzyme per milligram of total protein (expressed in μmol mg^{-1}). Specific activity gives a measurement of enzyme purity in the mixture.

For this work, samples were taken every 24h, and enzymatic activity and protein content were measure. These results are presented in Figure 5.7.

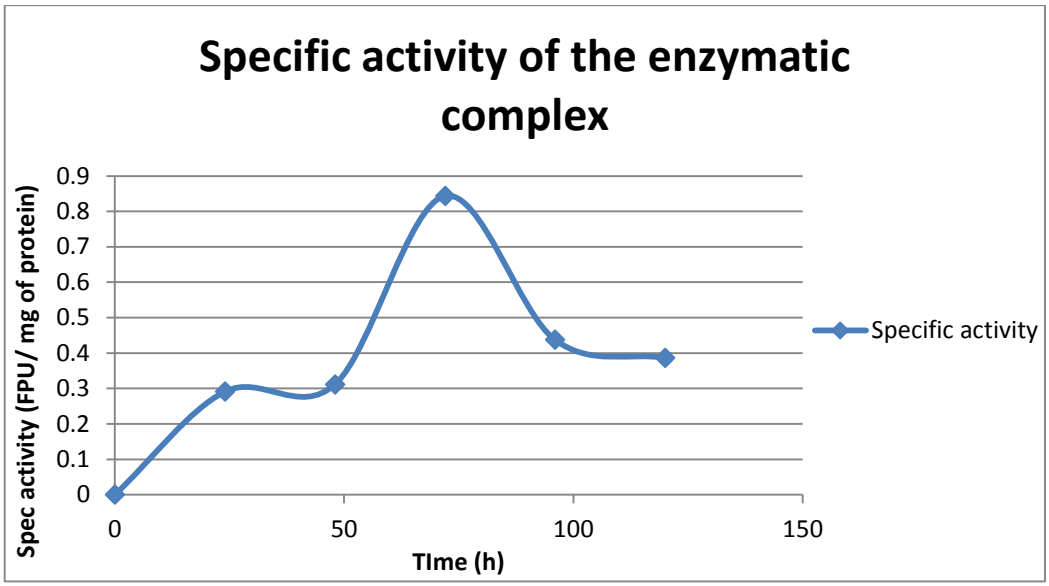

Figure 5.7: Specific activity of cellulase enzymatic complex (Supplemented medium)

Similarly to what is seen is figure 5.6, the peak activity is 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.], specific activity in this case is significantly lower - 10 to15 times worse. This is another indicator that the fungus's ability to secrete working cellulolytic enzyme was compromised. This spoilage is more prominantly visible here than when comparing values of results for enzymatic activity and extracellular protein concentration. The possible reasons for these unwanted effects will be discussed in the conclusions chapter of this paper.

5.2 Sugarcane baggase hydrolysis kinetics

Testing of the *Trichoderma* enzymatic complex on untreated, pretreated sugarcane baggase, as well as different parts of the sugarcase were made. Several other waste materials were used, pine wood, paper pulp and cow dung. 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. Thus, 0.1g of dry material was suspended in a total volume of 50 ml.

For this experiment, the Supplemented medium was used, since it proved to provide the highest enzymatic activity. The fermentation was stopped after after 3 days, when the activity is highest, by centrifuging a sample of the medium. This supernatant was used for preparing solutions.

Samples were taken periodically, centrifuged at 9600 RCF (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.

The materials were compared to a comercial cellulolytic enzymatic complex dissolved in the buffer to adequate concentration and bagasse pretreated for 100 min.

The kinetics of the reaction of the *Trichoderma* cellulasic enzymatic complex with bagase for the duration of 72h is presented in Figure 5.8.

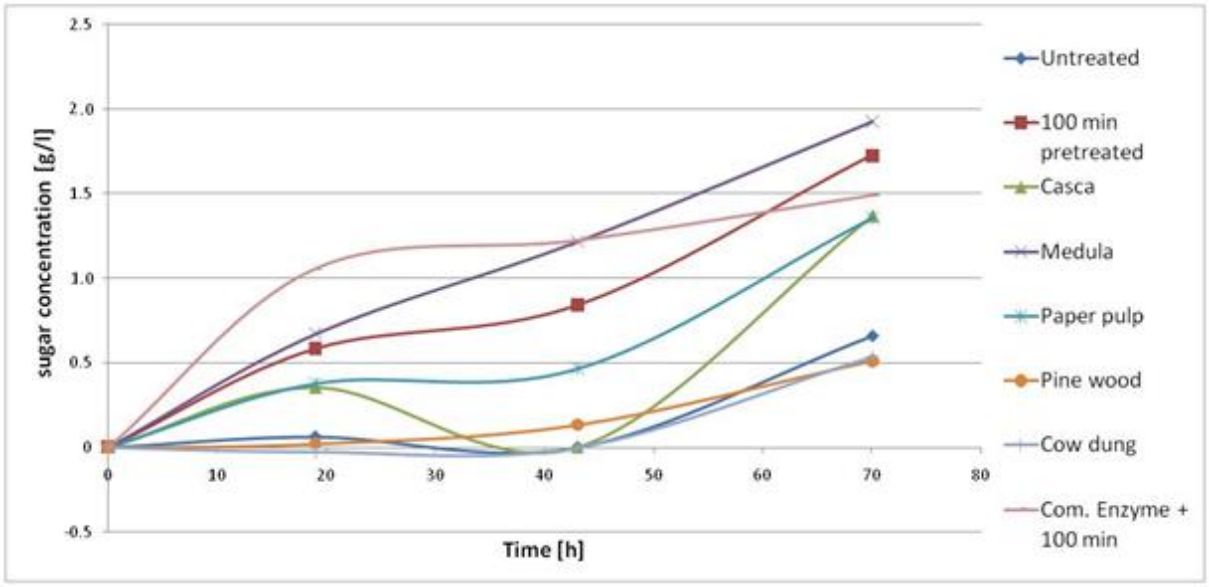

Figure 5.8: Kinetics of the reaction of the *Trichoderma* cellulolytic enzyme complex with sugarcane bagase

The chemical structure of some of the materials in Figure 5.8. is presented in Table 5.3. As it can be noticed, untreated bagasse had a significantly higher level of Lignin, which was greatly reduced in the process of pretreatment. This exposed the cellulose molecules to the enzymes and resulted in a higher glucose yield in pretreated samples.

Table 5.3: Structure of sugarcane bagasse, untreated, pretreated, marrow and stalk.

Samples	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ashes (%)	Total (%)
Untreated bagasse	41,73±2,52	26,01±0,92	26,54±1,83	0,26	94,5±1,6
Treated bagasse (100 min)	63,77±3,16	26,24±0,23	9,99±7,16	2,96	102,9±10,1
Medula (100 min)	64,67±5,27	26,40±1,42	9,38±4,98	0,42	100,8±11,7
Casca (100 min)	69,97±2,39	25,67±0,44	9,05±5,49	0,22	104,9±7,4

Another important parameter is the reduction of the amount of dry matter as a result of hydrolysis. The weight reduction was measured as a comparisson of dry matter weight prior to hydrolysis and after hydrolysis and separation of liquid by filtration. Data for eatch material is presented in Figure 5.9.

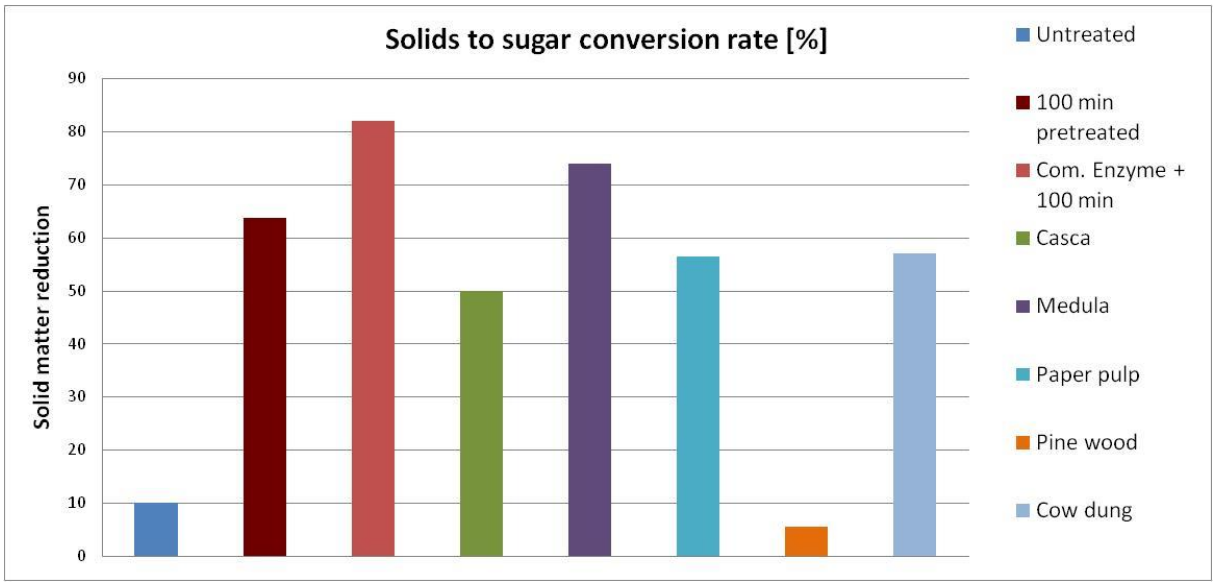

Figure 5.9: Reduction of solid matter content [%] after 72h of hydrolysis

As for sugar concentration increase, the pretreated sugarcane marrow had highest overall yield. This is understandable, since the structure of this part of the sugarcane stalk had porous structure with a higher cellulose to hemicellulose ratio. Second best are the flasks with 100 min pretreated bagasse. One with the commercial enzyme had higher activity after 24h, while for the Trichoderma-produced enzymatic complex yield gradually increased over the period of 72 hours. Poorest yield was with untreated bagasse and pine wood.

The results for both sets of experiments show that values for pretreated bagasse is the mean of values for the shell and marrow, since the bagasse sample is composed out of a mix of these two sugarcane parts.

5.3 Separation and purification of the enzymatic complex

5.3.1 Results of gel electrophoresis of extracellular proteins obtained during fermentation

In order to prove the presence of cellulolytic enzymes, gel electrophoresis was used on samples of supernatant collected from fermentation. In Figure 5.10, results of this experiment are shown. 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. Samples in 3 and 4 are duplicates.

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. The upper band in lanes 3 and 4 does not co-align to any otherband present in the commercial enzyme. However, it can represent any other group of extracellular protein secreted by the fungus, since cellulases are not solely present in the media.

Individual fractions separated by the gel electrophoresis could be isolated using column chromatography and tested separately. This was not done due to limited time available.

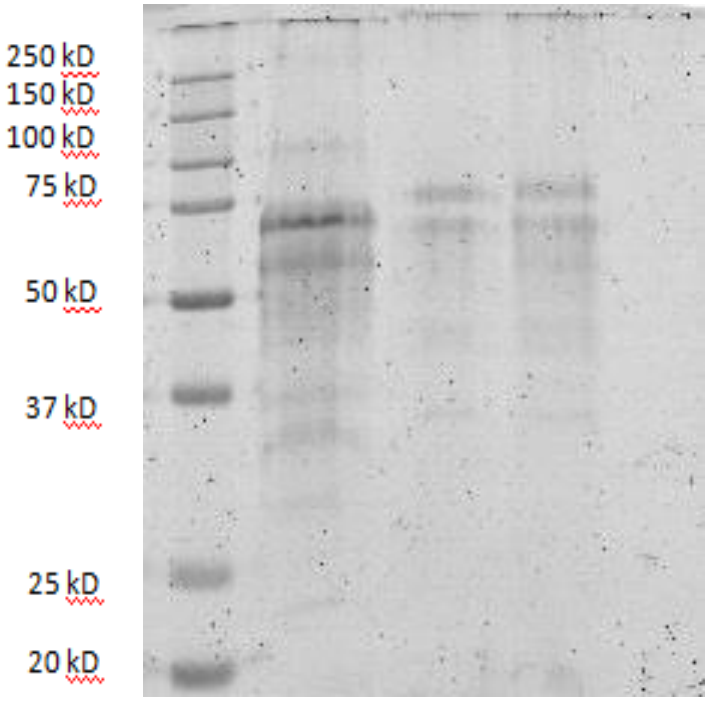

Figure 5.10: Results of gel electrophoresis of extracellular proteins obtained during fermentation. The samples according to lanes are, from left to right: Lane 1 - markers, Lane 2 - comercial cellulase enzyme, Lane 3 and 4 - *Trichoderma reesei* fermentation supernatant samples.

5.3.1 Precipitation with Ammonium-sulphate - 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 no successful increase of enzyme concentration was achieved. The precipitate was disolved with an adequate amount of 0.1M acetate buffer, pH 4.8. Results of the experiment are show in Table 5.4.

Table 5.4: Comparison of activity of supernatant acquired collected from fermentation (Supplemented medium, 4th day) and solution of precipitate generated with the addition of ammonium sulphate.

	Enz. activity [FPU/ml]
Supernatant from fermentation	0.28
Precipitate	0.09

5.3.2. Ultrafiltration results

Ultrafiltration proved to be the most effective out of the techniques applied in this work which aim to concentrate the secreted enzymes. The procedure applied is described in detail in chapter 4.4.3. Figure 5.11. represents the equipment used for this experiment.

Figure 5.11: Equipment used for concentrating the supernatant obtained from fermentation

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 5.5.

Table 5.5: 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. As for the protein in the permeate, 83.3% of the protein that passed through the membrane lost their activity.

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. This was noticed in the first stages of research, when experimental conditions from literature [Domingues et al., 2001.] were replicated using the same strain. Preliminary results in Filter Paper activity, extracellular protein concentration and consumption of nutrients in media indicated that the activity of the fungus was compromised.

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. Although this improved the results greatly compared to the values obtained initially, still they were 2-10 times lower than what was expected based on information from literature.

Reasons for this are hard to determine with limited time and equipment availability. Since the spores of the *T. reesei* fungus were stored in a cryogenic vial, in a deeply frozen state, for 15 years, this might cause the poor performance of the fungus. Although results for extracellular protein concentration are slightly lower than literature results, cellulolytic activity of the enzymes is disproportionally lower. This indicates that either the enzymes are secreted, but are inactive for some reason, or they are inactivated due to the action of proteases.

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.

7. Scope for future research

Since there is many reasons to believe that biomass is a promising feedstock for production of biofuels and chemicals, certain improvements in the technology of cellulase production have to be made. Research should be focused on key issues that affect the cost-effectiveness of the process. Overcoming them would enable this technology to be competitive to conventional methods of (bio)fuel and chemical production.

Firstly, the expenses on the growth media could be lowered by researching how new substrates would affect cellulase production. Waste streams from certain industries could be used as a basis for the fermentation media, not only as a source of carbon, but of other macro- and micronutrients as well. Also, although applying lower nutrient concentration decreases enzyme production, it results in lower residual concentrations of medium components after fermentation. This increases efficiency of production (nutrients used / nutrients spent) and simplifies downstream processing.

Submerged production is beneficial compared to solid-state, and improvements should be made in this process. Pellet formation affects enzyme production efficiency greatly, and more data about this poorly understood phenomenon should be collected.

Integrating cellulase hydrolysis with fermentation is promising, since it is logistically simpler and time-effective. The transition between these two very different steps in the process should be managed by compromising operational parameters without affecting overall performance.

What greatly affected the structure of this thesis significantly is the obvious loss of activity of the working microorganism during storage. This indicates that not only stability of mutated strains should be improved, but an effort should be made to discover new wild species with better properties and performance. Then, with improving genetic modification technologies, these favourable traits could be further improved and refined.

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. For same samples a range of enzymatic activity values was obtained dependant on the dilution of the samples. 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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APPENDIX I

Issues with determining cellulolytic activity

Measuring cellulolytic activity is a paramount method for this work, since it is used for obtaining key results necessary for fulfilling predetermined objectives. Sadly, it is in these method where problems were encountered, which greatly redefined the course of research.

At the very beginning of the experimental work, first results obtained from what was a seemingly simple, straightforward method indicated significantly lower enzymatic activity than what was expected from results in similar conditions obtained in literature sources, with a standard deviation at times reaching 100%. Before taking into consideration the less probable option that the cellulase production ability of the fungus was compromised, an assumption made that there was an error in performing the assay.

An effort was made to study more deeply this method and correct the flaw. Acording to some literature sources [Montenecourt et al., 1978.] [Domingues, 2001.] in the applied method, 0.5 ml of appropriately diluted sample was added to 2 ml of 0.1M acetate buffer pH 4.8 and 50 mg of Note No.1 Whatman filter paper discs and incubated in a thermostated bath at 50, with a shaking frequency 75U / min for 60 minutes. The first mentioned source is the one referred to in a lot of modern scientific articles dealing with cellulose activity. The activity was calculatied using the formula in Figure 4.1. The problematic line the abovementioned source is "appropriately diluted", and the overall vague explanation of the methodology.

As already discussed in Chapter 5.1.5. of this thesis, the DNS method is used for determining reducing sugar concentration. As part of the method, there is six instances of using pipettes to transfer various volumes (adding the sample, the buffer to dilute it, the DNS reagent, transferring from test tube to microplate). A lot of manipulation increases the total standard deviation.

For determining reducing sugar using the DNS method, a concentration of 0.5 - 5 g/l is needed. Since in the samples of the fermentation broth contained 20-40 g/l, they need to be diluted before measurement.

One possibility was to dilute the samples after hydrolysis of filter paper and then multiply the difference in sugar concentration in the assay and the blank. This is problematic, since the assay is made with a sample that has a significant concentration of sugar that can suppress the reaction. Also, by diluting the blank and sample with hydrolysed filter paper after incubation, we "dilute" the difference that is the basis of determining enzymatic activity, decreasing preciseness and inducing a large standard deviation.

The other possibility is to dilute the sample of the fermentation broth prior to performing the Filter Paper Assay, which has the benefit of removing the possibility of anabolic repression. Also, a lower

concentration of enzyme could have higher productivity by having an increased ratio of contact surface area available per enzyme.

Nevertheless, there is space for development: of a more precise, fast method of determining sugar concentration, as well as of a replicable cellulase assay with low standard deviation that can work with smaller samples.

Other possibilities for error, although unlikely, were tested. The effect of the presence of the paper in the sample on sugar concentration was tested. Sugar concentration in samples was measured when briefly inserting paper, and removing it, without any positive results. Also, solutions of sugar in the buffer used for the Filter Paper Assay were made and incubated with and without paper, without any effect on sugar levels. Different tubes (glass test tubes, plastic Eppendorf tubes), as well as different heaters (incubators, heated baths) were tested, in an attempt to determine if the conditions in the assay were different from what was intended, without any indication that these factors affected the ability to determine the enzymatic activity of the enzymatic complex.

APPENDIX II

Issues with contamination

Contamination in biotechnological practice is always a looming threat. Generally, good laboratory practice and care in preparation and conduction of the experiment can prevent any unwanted microorganisms from jeopardizing the growth of the working organism.

However, resilience of a microorganism in conditions where a contamination is present is a preferred trait. In case of the used *Trichoderma reesei*, the strain proved to be quite sensitive and unable to compete with bacterial contaminations. During the experimental work connected to this thesis, there were several instances when certain flasks showed alterations in appearance that indicated that a contamination occurred.

In indicator that a contamination was present was discoloration (absence of bright yellow pigmentation characteristic for the fungus at hand), lack of growth or formation of large pellets, 1-5 cm in diameter.

In one instance, pH value of a group of flasks with unreasonably large pellets was measured after sterilization (Figure II.i). Normally, the pH value of the media was 4.6-4.8, and was not controlled during fermentation in shake flasks, and would normally decrease to 4.2-4.4 after 120h. This was due to acidic products of metabolism of the fungus. However, the flask, which would later be affirmed to be contaminated, had a significantly lower pH value of 3.85.

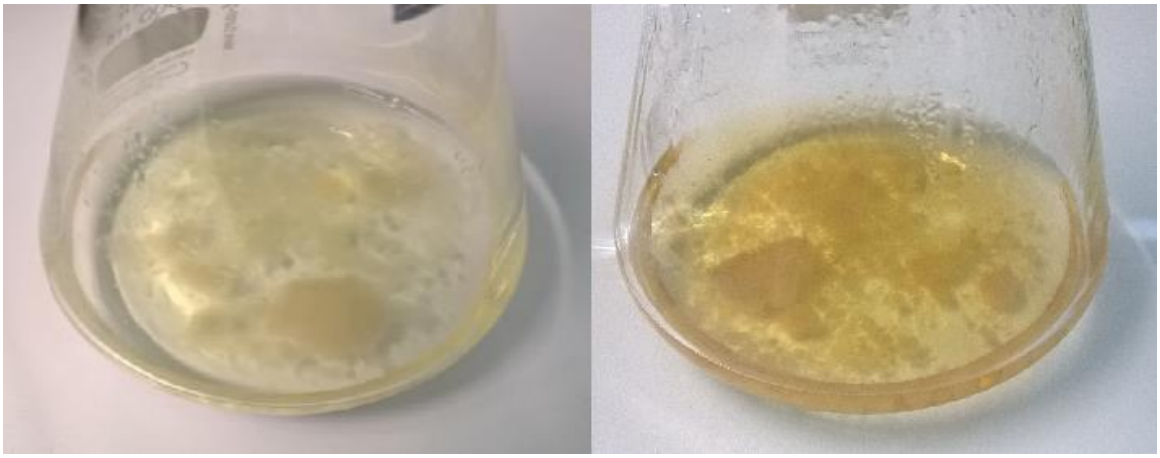

Figure II.i: Contaminated medium, before (left) and after (right) sterilization. Note the darker yellow colour in the flask after sterilization due to what is most probably the creation of Maillard reaction products

A sample of the fermentation medium was examined under a microscope, and significant presence of bacterial cells was observed (Figure II.ii). Most probably, the presence of the bacteria lowered the pH value more intensely than what is normal for *T. reesei*. The fungus formed pellets as a reaction to the stressful environment created by the contamination.

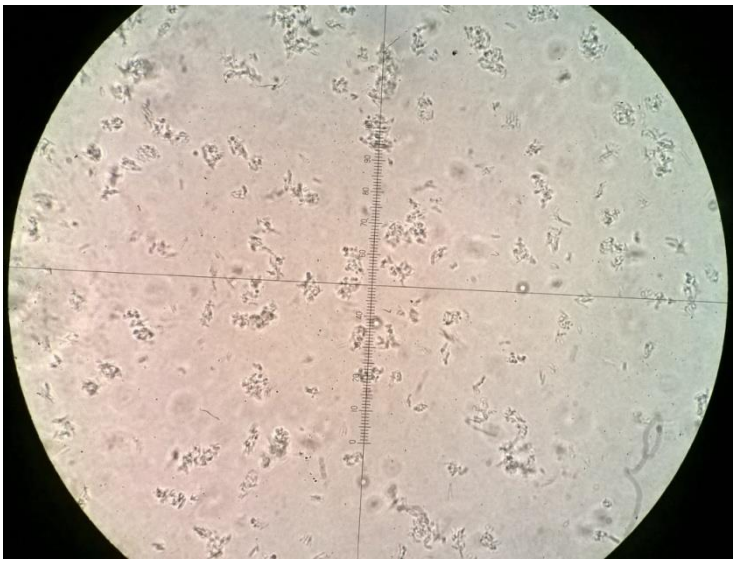

Figure II.ii: Sample of fermentation medium with bacteriological contamination (200x magnification)

This corresponds to observations from literature [Ferreira et al., 2009], that low ph values (around 4) of the media triggered formation of fluffy or compact pellets.