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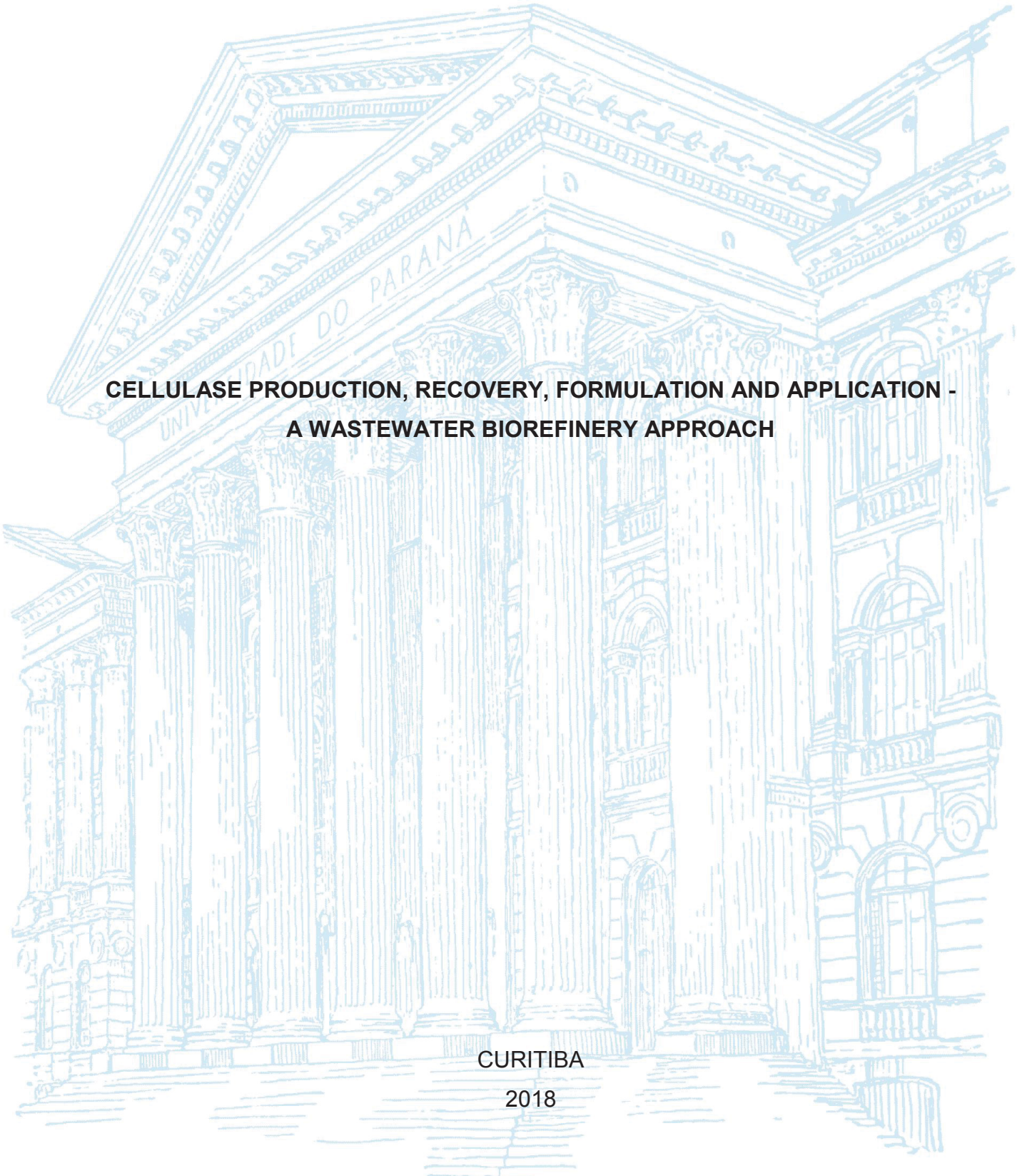

CELLULASE PRODUCTION, RECOVERY, FORMULATION AND APPLICATION - A WASTEWATER BIOREFINERY APPROACH

CURITIBA

2018

NELSON LIBARDI JUNIOR

CELLULASE PRODUCTION, RECOVERY, FORMULATION AND APPLICATION - A WASTEWATER BIOREFINERY APPROACH

Tese apresentada como requisito parcial à obtenção do grau de Doutor em Engenharia, no Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, da Universidade Federal do Paraná.

Orientador: Profª. Drª. Luciana Porto de Souza Vandenberghe
Coorientador: Profº. Drº. Carlos Ricardo Soccol

CURITIBA

2018

FICHA CATALOGRÁFICA ELABORADA PELO SISTEMA DE BIBLIOTECAS/UFPR BIBLIOTECA DE CIÊNCIA E TECNOLOGIA

L694c Libardi Junior, Nelson

Cellulase production, recovery, formulation and application: a wastewater biorefinery approach / Nelson Libardi Junior. – Curitiba, 2018.
185 f. : il. color. ; 30 cm.

Tese - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, 2018.

Orientadora: Luciana Porto de Souza Vandenberghe
Coorientador:Carlos Ricardo Soccol.

1. Celulase. 2. Biorrefinaria. 3. Efluentes sanitários. 4. Biorreator. I. Universidade Federal do Paraná. II. Vandenberghe, Luciana Porto de Souza. III. Soccol, Carlos Ricardo. IV. Título.

CDD: 572

Bibliotecária: Romilda Santos - CRB9/1214

MINISTÉRIO DA EDUCAÇÃO
SETOR TECNOLOGIA
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **NELSON LIBARDI JUNIOR** intitulada: **Cellulase production, recovery, formulation and application - A wastewater biorefinery approach**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 19 de Março de 2018.

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Dedico a Tese aos meus pais,
Sueli Libardi e Nelson Libardi, que sempre
me incentivaram à avançar nos estudos.

AGRADECIMENTOS

Agradeço em especial à Profª. Drª. Luciana Porto de Souza Vandenberghe, pela sua orientação acertiva e pela sua sensibilidade para dar liberdade às minhas escolhas na pesquisa, acreditanto no meu potencial e me apoiando nos momentos difíceis dessa jornada.

Agradeço ao Profº. Dr.º Gustavo Couto pelas suas constribuições e participação como membro externo na banca examinadora de defesa.

Aos professores Drº. Julio Cesar de Carvalho, Drª. Susan Grace Karp e Drª Valcineide Tanobe, pela sua participação e considerações durante a qualificação e defesa desta tese.

Ao Programa de Pós Graduação em Engenharia de Bioprocessos e Biotecnlogia, na pessoa do Profº. Drº Carlos Ricardo Soccol, por liderar a equipe de professores para a construção de um programa de pós graduação de referência, o qual tive a oportunidade de fazer parte.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo financiamento do projeto e da bolsa de pesquisa.

À todos os colegas que cruzaram o meu caminho durante essa jornada.

Somos assim: Sonhamos o voo mas tememos a altura. Para voar é preciso ter coragem para enfrentar o terror do vazio. Porque é só no vazio que o voo acontece. O vazio é o espaço da liberdade, a ausência de certezas. Mas é isso que tememos: o não ter certezas. Por isso trocamos o voo por gaiolas. As gaiolas são o lugar onde as certezas moram.

(DOSTOIÉVSKI, 1879)

RESUMO

A produção de celulase utilizando resíduos como substrato tem sido bem descrita, pois é uma estratégia interessante para reduzir os custos do processo produtivo. Esta pesquisa descreveu pela primeira vez o uso de efluentes sanitários como base para o meio de cultivo para a produção de celulase pela cepa *Trichoderma harzianum*. A lactose e a peptona foram selecionadas como fontes de carbono e nitrogênio. O uso de efluentes sanitários como meio de cultivo proporcionou um aumento na produção de celulases em comparação com o meio sintético. A produção em reatores de coluna de bolha (BCR) e tanque agitado (STR) foi avaliada, sendo que a máxima atividade e produtividade alcançadas foram de 31 U/mL e 645 U/Lh, respectivamente, com o BCR. A demanda química de oxigênio (DQO) e a concentração de nitrogênio foram reduzidas em 98% e 78%, respectivamente, durante o processo de fermentação. A concentração por ultrafiltração levou à recuperação de 73,5% da celulase, usando uma membrana de 30 kDa, resultando em concentração de atividade de 4,23 vezes e fouling de membrana de 19,81%. O processo de concentração por membranas levou à redução da DQO e da concentração de nitrogênio em 81,37% e 52,9%, respectivamente. O pH e a temperatura ótima ficaram entre 4 e 5 e 50 a 70ºC, respectivamente. A constante de Michaelis-Menten (K_m) de endoglucanases e β-glucosidases foram determinados como 63 M e 0.39 mM, respectivamente. A concentração de 10 g/L de glicose inibiu cerca de 45% da atividade da β-glucosidase. A eletroforese em condições desnaturantes (SDS-PAGE) e zimograma revelaram massas moleculares de 48, 43 e 25 kDa. Sorbitol (50% m/v) e ácido benzóico (0,05% m/v) foram adicionados ao extrato líquido e levaram à estabilidade da formulação de celulase em cerca de 100% após 30 dias. O extrato concentrado de enzima foi seco em um spray-dryer com adição de maltodextrina a 20% (m/v) e temperatura de entrada de 120ºC, resultando em maior rendimento em atividade enzimática (40,6%) e estabilidade (85%) após 60 dias. As enzimas formuladas foram aplicadas na hidrólise de cachos vazios de palma (Empty Fruit Bunches - EFB) e resíduo de estação de tratamento de efluentes (ETE). A fração celulósica do resíduo de ETE foi caracterizada (21,3%) e seu potencial para ser recuperado e hidrolisado com uma mistura enzimática avaliada. A aplicação da celulase formulada para a hidrólise dos resíduos EFB e de ETE resultou em rendimentos de glicose de 37,59 $mg/g_{celulose}$ e 21,17 $mg/g_{celulose}$, respectivamente. O resíduo de ETE tem potencial para ser usado como fonte de açúcares fermentescíveis pela hidrólise enzimática, uma vez que grandes quantidades de tais resíduos são produzidas mensalmente na planta de tratamento estudada (4,07 toneladas).

Palavras-chave: Celulase. Biorrefinaria. Efluentes sanitários. Biorreator. Formulação.

ABSTRACT

Cellulase production using residues as substrate has been well described, as it is an interesting strategy of reducing the costs of processes. This research described for the first time the use of sanitary wastewater as a culture base medium for cellulase production by the fungus *Trichoderma harzianum*. Lactose and peptone were selected as carbon and nitrogen sources. The use of domestic wastewater as the culture medium led to an increase of the enzyme activity, compared to synthetic medium. The production in bubble column (BCR) and stirred tank (STR) reactors was tested, where the maximum cellulase activity and productivity of 31 U/mL and 645 U/Lh were achieved, respectively, with BCR. The chemical oxygen demand (COD) and nitrogen concentration were reduced by 98% and 78%, respectively, during the fermentation process. The concentration by ultrafiltration led to cellulase recovery of 73.5% using a 30 kDa membrane, resulting in activity concentration of 4.23-fold with membrane fouling of 19.81%. The membrane process led to the reduction of COD and nitrogen concentration by 81.37% and 52.9%, respectively. The optimum pH and temperature where found to be between 4 and 5 and 50 and 70ºC, respectively. Michaelis-Menten constant (K_m) of endoglucanases and β-glucosidases were determined as 63 M and 0.39 mM, respectively. The concentration of 10 g/L of glucose inhibited around 45% of the β-glucosidases activity. Denaturing gel electrophoresis (SDS-PAGE) and zymogram revealed molecular weights of 48, 43 and 25 kDa. Sorbitol (50% w/v) and benzoic acid (0.05% w/v) were added to the liquid extract and led to cellulase formulation stability at around 100% after 30 days. Concentrated enzyme extract was dried in a spray-dryer with the addition of maltodextrin at 20% (w/v) and inlet temperature of 120ºC, resulting in highest cellulase activity yield (40.6%) and stability (85%) after 60 days. The formulated enzymes were applied in the hydrolysis of palm oil empty fruit bunches (EFB) and wastewater treatment plant residue (WWTP). The cellulosic fraction of the WWTP residue was characterized (21.3%) and its potential to be recovered and hydrolyzed with an in-house enzymatic blend evaluated. The application of the formulated cellulase for the hydrolysis of EFB and WWTP residue resulted in glucose yields of 37.59 $mg/g_{cellulose}$ and 21.17 $mg/g_{cellulose}$, respectively. The WWTP residue has a potential to be used as a source of fermentable sugars by the enzymatic hydrolysis, since huge amounts of such residue is monthly produced in the treatment plant (4.07 tons).

Key-words: Cellulase. Biorefinery. Sanitary wastewater. Bioreactor. Formulation.

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LIST OF ACRONYMS AND ABBREVIATIONS

AC	- Activated Carbon
AMFEP	- Association of Manufacturers and Formulators of Enzyme Products
ANOVA	- Analysis of Variance
ANVISA	- Agência Nacional de Vigilância Sanitária
AXE	- Acetyl Xylan Esterase
BCR	- Bubble Column Reactor
BGL	- Beta-glucosidase
BOD	- Biochemical Oxygen Demand
BSA	- Bovine Serum Albumin
C:N	- Carbon to Nitrogen ratio
CAZy	- Carbohydrate-active enzymes database
CBD	- Cellulose Binding Domain
CBH	- Cellobiohydrolase
CCRD	- Central Composite Rotatable Design
CD	- Catalytic Domain
CIPO	- Canadian Intellectual Property Office
CMC	- Carboxymethylcellulose
CMCase	- Carboxymethylcellulase activity
COD	- Chemical Oxygen Demand
DNS	- Dinitrosalicylic acid
E.C.	- Enzyme Commission
EGL	- Endoglucanase
EPO	- European Patent Office
FP	- Filter Paper
FPase	- Filter paper activity
FPO	- Free Patents Office
GH	- Glucohydrolase
GOD	- Glucose oxydase
HPLC	- High Performance Liquid Chromatography
INPI	- National Institute for Intellectual Property (Brazil)
LPBII	- Laboratório de Processos Biotecnológicos II
LPMO	-Lytic Polysaccharide Monooxygenase

MD - Maltodextrin
MF - Microfiltration
MWCO - Molecular Weight Cut Off
NTK - Total Kjedahl Nitogen
p-NPG - *para*-nitrophenol-β-glucoside
PES - Polyethersulphone
POME - Palm Oil Mill Effluent
RO - Reverse Osmosis
SANEPAR - Sanitation Company of Paraná
SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM - Scanning Electron Microscopy
SmF - Submerged Fermentation
SSF - Simultaneous Saccharification and Fermentation
SSSF - Semi-solid Substrate Fermentation
STR - Stirred Tank Reactor
TGA - Thermal gravimetric analysis
TMP - Transmembrane Pressure
TOC - Total Organic Carbon
TSS - Total Suspended Solids
U_{BGL} - International units for beta-glucosidase
U_{CMC} - International units for carboxymethylcellulase
UF - Ultrafiltation
U_{FP} - International units for filter paper
UNDP - United Nations Development Programme
USPTO - United States Patent Office
vvm - Volume of air per volume of medium per minute
WIPO - World Intellectual Property Organization
WWTP - Wastewater Treatment Plant
XRD - X-ray diffraction

SUMMARY

1. INTRODUCTION

Cellulases are maybe the most reviewed enzymes in the scientific literature, together with the fungus *Trichoderma* due to its ability to produce a complete cellulolytic enzyme complex, grouped in endoglucanases, exoglucanases (cellobiohydrolases) and β-glucosidases acting synergistically in the conversion of cellulosic substrates to glucose.

Developments of the cellulosic biofuel industry boosted the interest on cellulases, reflecting the significance of this enzyme for the industrial sector and for a bio-based economy. The actual worldwide installed cellulosic biofuel production capacity is 480.5 million litres per year of ethanol of which 380.5 million litres per year (or roughly 80%) are produced using *T. reesei* cellulase formulations (BISCHOF et al., 2016).

Cellulase production using alternative substrates has been emerging as an interesting strategy to reduce enzyme production costs, which is still one of the major bottlenecks for application, i.e., second-generation biofuel. Agro-industrial residues, mainly from lignocellulosic nature, such as sorghum bran, corn cob, sugarcane bagasse, wheat straw and elephant grass, have been used for cellulases production.

The use of cellulosic biomass from different sources for the production of biomolecules such as cellulases aiming zero residue generation is the central concept of biorefineries. So, cellulosic biomass and residues from different origins have been also proposed for cellulases production, such as the sludge or the cellulosic matter recovered from wastewater treatment plants (WWTP), which are rich in nutrients, besides containing residual cellulose that would induce the cellulase production. In this way, some researchers proposed the concept of wastewater biorefineries (VERSTER et al., 2014), whereas the WWTP streams and process by-products could be used as water, carbon and nutrient sources to be recovered or directly used for biomolecules' production by fermentation. The wastewater biorefinery concept would promote the development of more sustainable bioprocesses. This strategy could change the status of a simple WWTP process to a high-added value of biomolecules' production. This approach would contribute to the economic viability and sustainability of a production process.

The use of residues for cellulases production brings the challenge of separation of insoluble matters and other residues as well as the concentration of the fermented broth. Membrane technologies have been described as an adequate method for the downstream processing of biological molecules (CHARCOSSET, 2006). Ultrafiltration (UF) processes have also been described as techniques suitable for the purification of wastewater, together with microfiltration (MF), nanofiltration and reverse osmosis. The efficiency of membrane processes could be measured in terms of concentration of cellulases' activity and volume, low fouling effect as well as the reduction of the chemical oxygen demand (COD) and total nitrogen (RYAN et al., 2009). The simultaneous concentration of cellulases and effluent treatment could amplify the interest of using UF processes.

Cellulases' production using residues and the concentration of fermented broth using cheaper technologies are interesting strategies for lowering production costs. However, it is necessary to develop stable cellulase cocktails that could replace the established commercial products in an economical and sustainable way. Most industrial enzymes contain a relatively small fraction of active enzyme (< 10% w/w), and the rest being inactive proteins, stabilizers, preservatives, salts and diluents that allows standardization between production batches. Sometimes it is only the formulation of an enzyme that gives a manufacturer the competitive edge over rival companies (CHAPLIN and BUCKE, 1990).

Agricultural wastes, food processing waste, paper industry waste as well as the municipal solid waste have been reported as cellulosic materials to be enzymatically converted to fermentable sugars and/or biochemicals (LI et al., 2012). The enzymatic hydrolysis of oil palm empty palm fruit bunches (EFB) has been widely proposed as an interesting source of cellulose for producing fermentable sugars (HASSAN et al., 2013). The recovery of cellulosic substances is thought to be a trend of sludge treatment and disposal methods at wastewater treatment plants (HONDA et al., 2000).

The development of a cellulolityc formulation should comprehend the low cost production, the efficiency in separation and concentration and the stability of the liquid and/or dried formulation. However, the sustainability of the process depends on the reduction of pollution potential.

1.1 JUSTIFICATION

The sustainability of industrial processes imposes the development of new alternatives with reduced environmental impact. Cellulase production using residual substrates has been emerging because it is an interesting way to reduce enzyme production costs, which is still one of the major bottlenecks for application, i.e., second-generation biofuel. Wastewaters are an interesting source of nutrients and products to be recovered whereas huge ammounts are daily generated.

The use of domestic wastewater for cellulase production was not yet described in the scientific literature. The authors of this work have already deposited the patent correspondent to this process. The use of membranes with the simultaneous purpose of the cellulases' concentration and the purification of the process efluent could amplify its applicability.

The indenpendency from the international enzymes' suppliers demands studies for the low cost in-house cellulases' formulations, with the development of stable cellulolytic cocktails that could replace the established commercial products in an economical and sustainable way. The opportunity arrives in the development of an efficient cellulases' production process reusing water and nutrients from wastewater with a simultaneous reduction of the pollution potential.

1.2 OBJECTIVES

1.2.1 General objective

This work proposed the simultaneous development of a cellulolitic formulation for application in residues hydrolysis and the reduction of the pollution potential of the process, in a wastewater biorefinery approach.

1.2.2 Specific objectives

a) Evaluation of the use of sanitary wastewater as the basis for the culture medium for cellulase production;
b) Optimization of the culture conditions and selection of carbon and nitrogen sources, as well as inducers for cellulase production in shake-flasks.
c) Biochemical characterization of the produced cellulase;

d) Development of the production process in bioreactor, selecting the best culture conditions and strategies that lead to higher cellulase productivities;
e) Study of the best conditions for the recovery and concentration of the cellulases from the culture broth;
f) Evaluation of the production and recovery steps, in terms of the reduction of the the pollution potential of process effluents;
g) Development of stable dry and liquid cellulase formulations;
h) Application of the cellulolitic formulations for the hydrolysis of residues.

2 REVIEW OF THE PRODUCTION OF CELLULASES FROM *TRICHODERMA*

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ABSTRACT

Cellulases produced by the fungi of the genus *Trichoderma* are one of the most studied enzymes and have a central importance in industrial sector due to its ability to convert cellulosic materials in fermentable sugars for further applications. The massive application of cellulases in different industrial processes is limited by the enzymes' production costs. This fact certainly boosted the research on the use of alternative substrates, such as agro-industrial residues, since they are cheap and environmentally friendly sources of carbon and nutrients. Wastewater has been proposed as an interesting waste stream to be used for the production of biomolecules, through the concept of wastewater biorefinery that was recently proposed by some authors. This review presents some of the most recent and relevant information and advances, since the discovery of cellulases and their impact in the modern society. Some aspects of developed technologies for sustainable processes and exploitation of lignocellulosic biomass as a source of fermentable sugars for biofuels production are discussed. Emphasis will be given for the use of wastewater for added value production of cellulases.

Keywords: cellulase production, wastewater biorefinery, *Trichoderma harzianum*

2.1 INTRODUCTION

Cellulases are maybe the most reviewed enzymes in the scientific literature, together with the fungi of the genus *Trichoderma* due to its ability to produce a complete cellulolytic enzyme complex. Some of the most recent and complete reviews regarding cellulases from *Trichoderma* were published by (SINGHANIA et al., 2010; YAN and WU, 2013; JUTURU and WU, 2014; PAYNE et al., 2015; BISCHOF et al., 2016; GUPTA et al., 2016; DRUZHININA and KUBICEK, 2017; OBENG et al., 2017; SINGHANIA et al., 2017) dealing from their production strategies, substrates, culture conditions and market to the genomic strategies to improve their productivity, application for biomass hydrolysis, fuel production as well as their impact in biorefinery technologies. The relatively recent developments of the cellulosic biofuel industry certainly boosted the interest on cellulases. The actual worldwide installed cellulosic biofuel production capacity is 480.5 million litres per year of ethanol of which 380.5 million litres per year (or roughly 80%) are produced using *T. reesei* cellulase formulations (BISCHOF et al., 2016). According to the authors, more than 100 research articles are published per year related to *Trichoderma* cellulases. All this scientific data reflects the significance of this enzyme for the industrial sector and for a bio-based economy.

Cellulases are cellulolytic enzymes grouped in endoglucanases, exoglucanases (cellobiohydrolases) and β-glucosidases acting synergistically over the conversion of cellulosic substrates to glucose. These complete enzyme complex is mostly produced by the fungi of the *Trichoderma* genus. The cost of this enzyme is still one of the bottlenecks for its application for biomass hydrolysis and bioethanol production. The use of cellulosic residues such as sugarcane bagasse, wheat straw and many others has been an interesting strategy for the low cost production of cellulases, since these enzymes are induced by cellulose. Biomass from different sources is then employed in diverse biomolecules production, aiming for zero residue generation: the central concept of biorefineries. Cellulosic biomass and residues from different origins have been also proposed for cellulases production, such as the sludge from wastewater treatment plants (WWTP), which is rich in nutrients, besides containing residual cellulose that would induce the enzyme production. In this way, some researchers proposed the concept of wastewater biorefineries (VERSTER et al., 2014), whereas the WWTP streams and process by-products could be used as

carbon and nutrient sources to be recovered or directly used for biomolecules' production by fermentation. Raw wastewater and/or the sludge produced from the treatment process are interesting sources of carbon and nutrients, as well as inducers (VERSTRAETE et al., 2009; RUIKEN et al., 2013) for enzymes production by microorganisms from the *Trichoderma* genus (VERMA et al., 2005; DRANI et al., 2011; LIBARDI et al., 2017).

The application of the wastewater biorefineries concept would promote the development of more sustainable bioprocesses. This strategy could change the status of a simple WWTP process to a high-added value of biomolecules production. This approach would contribute to the economic viability and sustainability of a production process.

2.2 MICROBIAL CELLULASE SOURCES

Cellulases are expressed by a wide spectrum of microbes in nature like bacteria, fungi, protozoa and some animal species including termites and crayfish (WATANABE and TOKUDA, 2001; YAN and WU, 2013). Fungi are among the most important and explored cellulase producers. Their natural capacity of colonizing and degrading lignocellulosic materials in terrestrial or aqueous environments contribute as a central player in the carbon biogeochemical cycle. Their deserved industrial interest is due to the high extracellular enzyme secretion in a multi-component and synergistic way (OBENG et al., 2017; SINGHANIA et al., 2017). Examples of aerobic cellulolytic fungi are: *Trichoderma reesei, Penicillium pinophilum, Penicillium funiculosum, Fusarium oxysporum, Aspergillus niger, Sclerotium rolfsii, Humicola sp., Phanerochaete chrysosporium, Talaromyces emersonii, Melanocarpus albomyces,* together with the anaerobic genera *Neocallimastix, Caecomyces,* and *Oprinomyces.* In addition, the thermophilic fungi *Sporotrichum thermophile, Thermoascus aurantiacus, Chaetomium thermophilum, Humicola grisea,* and *Myceliopthora thermophila* were identified as cellulase producers of industrial interest, due to their capacity of producing thermostable cellulases (DOI, 2008).

The anaerobic cellulolytic bacteria are found in the soil, rumens, sewage sludge, decaying plant materials, termite gut, wood-chip piles, compost piles, paper mills and wood processing plants: *Acetivibrio cellulolyticus, Bacteroides cellulosolvens, Butyrivibrio fibrisolvens, Clostridium acetobutylicum, Clostridium*

aldrichii, Clostridium cellobioparum, Clostridium cellulofermentans, Clostridium cellulolyticum, Clostridium cellulovorans, Clostridium herbivorans, Clostridium hungatei, Clostridium josui, Clostridium papyrosolvens, Clostridium thermosuccinogenes, Ruminococcus albus, Ruminococcus flavefaciens, and *Clostridium thermocellum* (MAKI and LEUNG, 2009).

Aerobic cellulolytic bacteria are found in soil, water, plant materials, humus, animal faeces, sugarcane fields and leaf titter. They are: *Bacillus megaterium, Bacillus pumilus, Cellulomonas fimi, Cellulomonas flavigena, Cellulomonas gelida, Cellulomonas iranensis, Cellulomonas persica, Cellulomonas uda, Cellvibrio gilvus, Cellvibrio mixtus, Pseudomonas fluorescens, Streptomyces antibioticus, Streptomyces cellulolyticus, Streptomyces lividans,* and *Streptomyces reticuli* (MAKI and LEUNG, 2009).

Trichoderma is the most explored fungi genus for cellulase production. They are able to excrete a complete cellulolytic complex consisting predominantly of exoglucanases, endoglucanases and lesser amounts of enzymes with other hydrolytic activities. In addition, they have the ability to avoid cellobiose feedback inhibition by the production of low levels of β-glucosidases (PARISUTHAM and KIM, 2014; OBENG et al., 2017). Besides *Trichoderma*, *Aspergillus* genera including species such as *A. niger*, *A. orizae*, *A. unguis*, *A. tubingensis* and *A. fumigatus* are also explored due to their high β-glucosidases expression levels, turning them important for the production of a complete enzyme cocktail for biomass hydrolysis, together with *Trichoderma*.

2.3 *Trichoderma* AND CELLULASE – 71 YEARS OF HISTORY

Around 70 years ago, *Trichoderma reesei* (*Hypocrea jecorina*) was originally isolated during the Word War II at Guadalcanal on the Solomon Island and identified as the cause of massive infection in canvas and other cellulose-containing materials at a US army camp. Samples of the isolated strains were sent to the U.S. Army Quarter Master Research and Development Centre at Natick, Massachusetts. Historically, the wild-type isolate labelled *T. viridae* QM6a, has been used for creating numerous biotechnologically important mutants, particularly those involved in the production of enzymes for lignocellulose hydrolysis, also in industrial scale, and for genome sequencing projects. The *T. viridae* QM6a strain is reported as the strain

from which all the mutants currently used in industry have been derived from (BISCHOF et al., 2016; OBENG et al., 2017). This strain was later named as *T. reesei* in honour of Elwyn Reese who, together with Mary Mandels published their work regarding the production and induction of cellulases by *T. viridae* and the saccharification of biomass to fermentable sugars (MANDELS and REESE, 1957). This strain was later improved by radiation mutagenesis originating the modern industrial strains. During the 1970's efforts by mutagenesis programs conducted by the Rutgers University were performed for the increase of the cellulase productivity by 20-fold (MANDELS et al., 1979) originating the mutant RUT-C30, which is available to the public domain (PETERSON, 2012). From the 1990's many publications used the specie name *Hypocrea jecorina* instead of *T. reesei* since Kuhls et al. (1996) discovered evidences that *H. jecorina* is the sexual form of *T. reesei*. In 2008 *T. reesei* had its genome sequenced by Martinez et al., (2008), opening the way for the large-scale application of genome studies.

The cellulase production by the *Trichoderma* strains promoted a great impact in the cellulosic biofuel production. The worldwide installed cellulosic biofuel production capacity is 480.5 million of litres per year of ethanol, from which 380.5 million litres per year (roughly 80%) are produced using *T. reesei* enzyme formulations such as Accellerase and Cellic CTec (BISCHOF et al., 2016). Around 11% of all technical enzyme formulations that are registered by the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) are produced using *T. reesei*. *T. reesei* still maintains its importance in research, exemplified by more than 100 published research articles dealing with the fungus or its enzymes each year (BISCHOF et al., 2016).

2.4 GENERAL ASPECTS

Cellulases are multienzyme complexes capable of hydrolysing the β-1,4-glucosidic linkages of cellulose, producing as primary products glucose, cellobiose and cello-oligosaccharides. The enzymatic complex acts synergistically with the action of the endo-glucanases (EGL), exo-glucanases, also named as cellobiohydrolases (CBH) and β-glucosidades (BGL). EGL produce nicks in the cellulose polymer exposing reducing and non-reducing ends. CBH acts upon these reducing and non-reducing ends to liberate cello-oligosaccharides and cellobiose

units. BGL cleaves cellobiose to liberate glucose, thereby, completing the hydrolysis (SINGHANIA et al., 2010, 2017).

2.5 CELLULASE CLASSIFICATION

According to the Carbohydrate-Active enZymes Database (CAZy), EGL are found in the glycoside hydrolase (GH) families 5-8, 12, 16, 44, 45, 48, 51, 64, 71, 74, 81, 87, 124 and 128, while CBH's are found in GH families 5-7 and 48. The BGL are found in GH families 1, 3, 4, 17, 30 and 116. According to the Enzyme Commission, the cellulases are classified as endoglucanases (E.C. 3.2.1.4), reducing exoglucanases (E.C. 3.2.1.176), non-reducing exoglucanases (E.C. 3.2.1.91) and β-glucosidades (E.C. 3.2.1.21) (JUTURU and WU, 2014). Some authors (SINGHANIA et al., 2017) even describe a different classification, the BGL from CBH and EGL, since their substrate (cellobiose) is different from the cellulose polymer. In fact BGL is a part of the cellulase cocktail for a complete degradation of cellulose to glucose, but their mechanism of action is different.

2.6 CELLULASES' APPLICATIONS

Cellulases have a wide spectrum of applications ranging from detergent and laundry to animal feed processing and food and brew production, textile processing and paper and pulp manufacture. In the detergent industry cellulase-containing enzyme cocktails are added to the detergent formulations, especially for those applied in clothes washing. In animal feeds cellulases are used for the production of animal and poultry feeds, partly hydrolysing the cellulose and hemicellulose contents, increasing its digestibility. In textile industry cellulases are used in the processing and finishing of the cellulose based textiles, removing hairiness of the cellulose threads, development of smooth and glossy appearance and imparting colour brightness. In food process industries, cellulases are applied in combination with other enzymes such as xylanases and pectinases, for the extraction of juices, in the reduction of viscosity, improvement of stability and aromatic properties as well as for the reduction of the membrane fowling during juice filtration. In addition, cellulases have been also applied for the removal of bacterial biofilms from surfaces and pipes from industries and water distribution systems (JUTURU and CHUAN, 2014).

While cellulases and hemicellulases are widely used in several industries, the

major future application of these biocatalysts is expected to be the conversion of plant biomass into sugars for further fermentation to ethanol and other energy carriers as well as to useful platform chemicals (VIIKARI et al., 2012). The increased demand of the production of biofuels and chemicals from renewable resources pushes the technological development of processes for the depolymerisation of plant biomass. The enzymatic conversion of biomass to fermentable sugars is environmentally friendly and is totally integrated to the concept of biorefinery. The manufactured bioethanol must maintain a competitive price versus the conventional fuels, and the current dosage of enzymes account for a significant part of the ethanol manufacturing costs (VIIKARI et al., 2012). To ensure low-cost, reliable and flexible delivery, the enzyme production facility would preferably be situated on-site or near-site. It has been suggested the elimination of cells' separation, concentration and formulation costs and the use of simultaneous hydrolysis/fermentation processes for lowering costs.

The conversion of lignocellulosic materials to fermentable sugars requires three sequential steps comprising size reduction, pre-treatment and enzymatic hydrolysis. Sequentially the released sugars could be fermented and converted to ethanol. Modifications of the traditional simultaneous saccharification and fermentation process (SSF) including the liquefaction as well as the consolidation of the enzyme production and hydrolysis are presented in Figure 1.

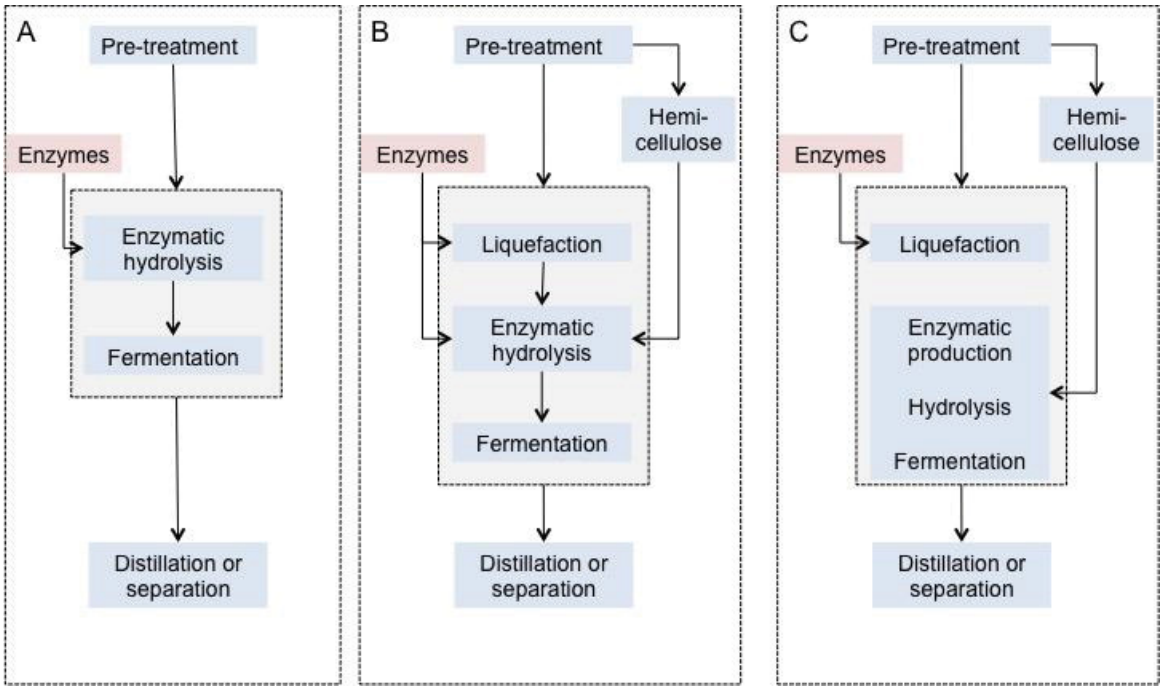

Figure 2.1 – Lignocellulose to ethanol process concepts. a) Traditional SSF; b) SSF with pre-liquefaction; c) consolidated process with pre-liquefaction. Adapted from Viikari et al., (2012).

The mechanism for cellulose hydrolysis consists of the synergistic action of EGL, CBH and BGL. The enzymatic depolymerisation performed by EGL and CBH is the rate-limiting step of cellulose hydrolysis process. The secondary step occurs in the liquid phase involving the conversion of cellobiose to glucose, which is performed by the BGL. The synergy means that the measured activities exceed the added activities of the individual enzymes. The optimum ratios of cellulases for cellulose hydrolysis based on protein load are: CBHI 50-60%; CBHII 10-30%, EGL 10-30%, Xylanases 5-10% and BGL less than 1% (VIIKARI et al., 2012). The addition of non-cellulolytic accessory enzymes is also reported to increase the biomass hydrolysis, i.e. endoxylanases, ferulic acid esterase and acetyl xylan esterase (AXE) (VIIKARI et al., 2012). The supplementation of cellulases with biological or non-biological additives for biomass hydrolysis improvement has been proposed. Supplementary addition with hemicellulases, laccases as well as lytic polysaccharide mono-oxygenases (LPMO's) has been reported, together with the addition of non-hydrolytic accessory proteins named expansins and swollenins. Chemical additives such as EDTA, $CaCl_2$, $MgCl_2$, Tween and Triton X-100 are added for their action as metal cofactors or activators, yielding significant effects on enzymatic activities by assisting

in the biochemical reactions (OBENG et al., 2017).

Besides being important for cellulase cocktails for biomass hydrolysis to obtain fermentable sugars for ethanol production, BGL are applied in a range of biotechnological processes. The applications of BGL are reported based on their hydrolysis activity and the transglycosylation activity (SINGHANIA et al., 2017). The enhancement of the digestibility of cellulose-based feed for single-stomach animals like chickens and pigs, the enzymatic release of aromatic compounds in winemaking and release of oil content in tea beverages (SUB et al., 2010), the reduction of bitterness of citrus juices by the hydrolysis of naringenin to prunin (ROITNER and SCHALKHAMMER, 1982), the hydrolysis of isoflavones from soy-based foods to aglycone, due to their phytoestrogenic activity for reduction of menopausal symptoms (HU et al., 2009), the detoxification of cassava by its treatment with BGL (PETRUCCIOLI et al., 1996), the extraction of phenolic compounds from cranberry and pineapple pomace (VATTEM, 2003; CORREIA et al., 2004). The transglycosylation activity of BGL is used for the production of synthetic oligosaccharides and alkylglycosides. Oligosaccharides can be used as therapeutic agents, diagnostic tools and grow-promoting agents. Alkylglycosides are non-ionic surfactants with potential application in pharmaceutical, chemical, cosmetic, food and detergent industries (BANKOVA et al., 2006).

2.7 THE DIVERSITY OF CELLULASE ENZYMES

The same microorganism is able to secrete more than one type of cellulase. Fungi, such as *T. reesei* and *A. niger,* can secrete 12 and 8 hemicellulases, respectively, in high concentrations (YAN and WU, 2013). *T. reesei* produces two cellobiohydrolases (CBHI and CBHII), two endoglucanases (EGI and EGII) as well as seven types of β-glucosidases (BGLI to BGLVII). The CBHI and CBHII together with EGI and EGII contribute to 90% of the secreted enzymes, in a ratio of 60:20:10:10, while all the seven EGs constitute up to 1% of the secreted protein (SINGHANIA, et al., 2017). According to Garvey et al. (2013), EG contribute up to 80% and CBH up to 15% of the total excreted proteins. *T. reesei* possesses low levels of intracellular BGL to avoid effects of cellobiose feedback inhibition during cellulose hydrolysis (PARISUTHAM and KIM, 2014). This low BGL expression in *T. reesei* has been described as a bottleneck, since it has an important function for the synergistic

cellulose hydrolysis. Other fungi species produce different cellulase compositions. *A. niger,* for example*,* is a strong BGL producer comparing to *T. reesei*. *Humicola insolens* cellulase system lacks a cellulose-binding domain and *Humicola grisea* produces a thermostable cellulase. A very complete review of the fungal cellulases diversity and structures is presented by Payne et al. (2015).

A variety of aerobic fungi species including *Phanerochaete* and *Penicillium*, as well as the aerobic bacteria *Thermobifida* are known to have a non-complex cellulase system and are the most used for industrial applications. Contrarily, bacterial strains like *Clostridium cellulovorans* and *Clostridium thermocellum* produce cellulolytic enzymes in a multienzyme complex named cellulosome, which is associated with the degrading cell wall. The cellulosome allows the microorganism to adhere on the substrate for an efficient competition for cellulose hydrolysis and uptake of the soluble oligosaccharides (JUTURU and WU, 2014; SINGHANIA, et al., 2017).

The two-domain structures of most of the microbial cellulases are characterized by a catalytic domain (CD) containing the catalytic site and the cellulose-binding domain (CBD) that facilitates the binding of the enzyme to the cellulosic substrate. CDB anchors to the cellulose substrate allowing the CD to perform the catalytic function (BAYER et al., 1998; JUTURU and CHUAN, 2014).

2.8 CELLULASE SECRETORY PATHWAY AND INDUCTION

The secretion of proteins only accounts for a fraction of microbial activities. The secretion of proteins in microorganisms requires a series of operations between the subcellular locations, from the protein synthesis to their transport to the extracellular matrix. Cellulases are located in vesicles that are derived from the endoplasmic reticulum, which has ribosomes and is attached to the outside surface of the membrane. For example, BGL are grouped according to their location as intracellular, cell wall-associated and extracellular (YAN and WU, 2013).

While the bacteria such as *Clostridium themocellum* excrete cellulases constitutively, fungal cellulases are produced in the presence of cellulose as substrate. So, the fungal cellulases are inducible enzymes. However cellulose itself is not able to trigger the induction because of its insolubility. It is too large to be transported into the cells and, an inducer able to pass through the cell wall is necessary to activate the induction for the enzyme expression. To overcome all this

biochemical processes, previously to the cellulase induction, there is an effort to screen cellooligosaccharides and their derivatives that could be used as inducers, such as cellobiose, sophorose, lactose, xylobiose, gentiobiose, laminaribiose, naribiose, thiocellobiose, thiogentiobiose. Lactose has become a very common cellulase inducer for industrial application for economic reasons (YAN and WU, 2013; SINGHANIA et al., 2017). Basal levels of constitutive cellulases are bound in the surface of the conidia of *T. reesei*, mainly CBHII, degrading cellulose into soluble cellooligosaccharides, allowing them to enter the cell and be converted into an inducer that triggers the cellulase induction (SUTO and TOMITA, 2001; SINGHANIA et al., 2017). A schematic model of the cellulase induction mechanism proposed by SUTO and TOMITA (2001) is presented in the Figure 2.

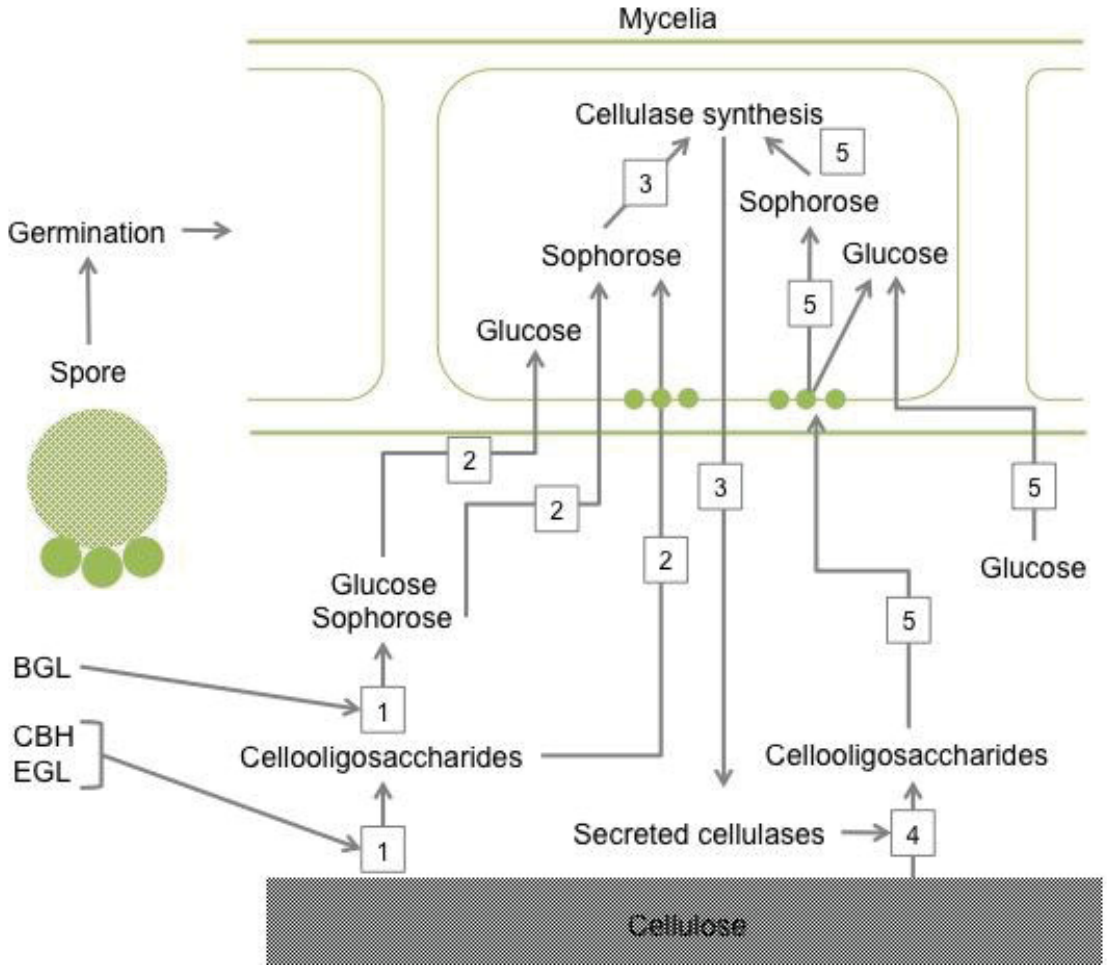

Figure 2.2 – Schematic model of cellulase induction in *T. reesei*. The numbers inside squares are related to each cellulase excretion step. Adapted from Suto and Tomita (2001).

The following steps explain the cellulase secretion mechanism, according to Suto and Tomite (2001). *Step 1*: Conidia spores come into contact with cellulose, cellobiohydrolase on the spore surface, degrading the cellulose to cellooligosaccharides, which are then hydrolysed to glucose and transglucosylated to form sophorose by a constitutive BGL. *Step 2*: The conidia spores germinate and the glucose, as a carbon source, and sophorose, as an inducer, enter the cell. *Step 3*: Sophorose induces the production of cellulase that is secreted to outside of the cell. *Step 4*: The secreted cellulase degrades cellulose, causing the increase of cellooligosaccharides and glucose concentration. *Step 5*: Glucose is assimilated; cellooligosaccharides are hydrolysed to glucose and transglucosylated to form sophorose by a constitutive BGL. *Step 6*: Return to step 3 and the cycle continue until the cellulose is exhausted form de substrate.

Cellulase production is induced by cellulose or other substrates such as cellulose derivatives and, on the other side, is susceptible to repression by a readily metabolizable carbon source such as glucose, glycerol or fructose (MORIKAWA et al., 1995). For this reason glucose derepressed mutant strains such as *T. reesei* Rut-C30 were developed to allow the production of increasing cellulase titters using easy biodegradable substrates like glucose. The catabolic repression is observed when an easily assimilable carbon source, such as glucose is present in the substrate. In *T. reesei* the cellulase genes *cbh1*, *cbh2*, *egl1*, *egl2* and *egl5* are repressed by glucose at the transcriptional level (SUTO and TOMITA, 2001). According to Suto and Tomita (2001), several positive (XYR1, ACE2 and HAP2/3/5) and negative (ACE1 and CRE1) regulators are involved in the regulation of cellulase gene expression. These regulators are zinc finger proteins, binding to cellulase gene promoters to regulate cellulase expression. ACE1 knockout strains showed increased expression of cellulases and hemicellulases, speculating that the *ace1* protein act as a repressor for cellulase and hemicellulose gene expression. The *ace2* knock-out strains reduced the transcriptional levels of cellulase mRNAs by 30% and cellulase activity by 70%. Deletion of *xyr1* gene eliminates cellulase induction both on cellulose and sophorose (SUTO and TOMITA, 2001). Lactose is used as substrate because it relieves the fungus from catabolic repression, being slowly hydrolysed by the fungus (MORIKAWA et al., 1995).

2.9 CELLULASE PRODUCT INHIBITION

One of the most problematic aspects concerning the application of cellulases for biomass hydrolysis is the product inhibition. This phenomenon is observed for EGL and CBH, which is caused by cellobiose retarding the overall conversion of cellulose to glucose. EGL and CBH suffer a non-competitive inhibition by cellobiose and this behaviour deserves attention since the majority of the commercial cellulase cocktails for biomass hydrolysis are mainly constituted by CBH. The presence of the BGL in the enzymatic cocktail is the most commonly used strategy to prevent the CBH and EGL inhibition. In fact the presence of BGL has a double benefit, it cleaves cellobiose to glucose, getting rid of the inhibitor and transforming it into the desired biomolecule for further application. Strategies have been developed to overcome the product inhibition in industrial processes by removing the product through membrane filtration, with the retention and recycling of enzymes and the permeation of the product, as well as the conversion of cellobiose via cellobiose dehydrogenase (CDH). Other inhibitory effect related to cellulases could be caused by lignin, ethanol, glucose, lactose, various ions, buthanol, acetone, mannose, galactose, xylose, xylans, xylooligomers and xylo/gluco-oligosaccharides (PAYNE et al., 2015).

However, the most cited undesirable effects of the enzymatic biomass hydrolysis processes is the BGL feedback inhibition caused by the produced glucose from the cleavage of cellobiose. The presence of glucose inhibits the BGL blocking the active site or the leaving of the hydrolysed substrate. Besides the membrane filtration, the use of engineered strains is also a strategy for preventing the feedback inhibition effect. The gene *cre1* is responsible for the catabolic repression regulation and the inhibitory effect that glucose has on cellulase production. The deletion of this gene in the mutant strain RUT C-30 gives to them the ability to grow on glucose for cellulase production, in contrast to the parental strain QM6a (PAYNE et al., 2015; SINGHANIA et al., 2017).

2.10 CELLULASE KINETICS

Modelling enzymatic hydrolysis kinetics is necessary to better characterize and understand the interactions between cellulases and cellulose, to integrate

present and future enzyme improvements, and to design optimized process reactors and equipment.

Kinetics and classification of models for cellulases involves more steps than classical enzyme kinetics. These major steps include (1) the binding of cellulases to the substrate via the cellulose binfing domain (CBD), (2) the recognition of the bond susceptible to hydrolysis by the catalytic domain (CD) (non-reducing end of the oligomer chain, in the case of CBH/BGL and internal glycosidic bond in the amorphous region, in the case of EGL), (3) the formation of enzyme–substrate complex to initiate hydrolysis of cellulose, (4) the hydrolysis of the β-glycosidic bond and simultaneous forward movement of the enzyme along the cellulose chain, (5) the release of cellulase from the substrate or repetition of step 4 or steps 2 and 3, in the case if only the catalytic domain detaches from chain and, finally, the (6) hydrolysis of cellobiose to glucose by BGL. Product inhibition and changes in the substrate properties during the course of hydrolysis should be taken into account (JUTURU and CHUAN, 2014). Based on the behaviour of the cellulose degradation mechanisms, Bansal et al., (2009) presented 4 kinetic models proposed in the literature: (1) empirical models, (2) Michaelis–Menten based models, (3) models for adsorption and (4) models developed for soluble substrates. Empirical models (1) have been generally used to correlate hydrolysis with either the structural properties of the substrate or with time. These models help in understanding the interactions between the substrate properties such as crystallinity, lignin content, or acetyl content and in estimating initial rate reactions important for resuspension experiments and Lineweaver–Burk plots used in the Michaelis–Menten models. The Michaelis–Menten based models (2) consider the mass action laws that are applied for homogenous reaction conditions and, hence, cannot be directly applied to the heterogeneous reaction conditions of enzymatic hydrolysis of insoluble cellulosic substrates. Cellulose hydrolysis is a heterogeneous reaction occurring on the substrate surface. For heterogeneous reaction systems, classical chemical kinetics assumption of uniformly mixed systems does not hold. The conversion of cellobiose to glucose by BGL, however, can be modelled by Michaelis-Menten kinetics since it is a homogeneous reaction. The models of adsorption (3) consider that the incorporation of adsorbed cellulase is performed by two ways, the Langmuir adsorption isotherm or the kinetics equations. Finally, the models of soluble cello-oligosaccharides (4) explain their effects on enzyme activity over soluble cello-

oligosaccharides. However, models developed for soluble substrates cannot be extrapolated for insoluble substrates. This is due to heterogeneous action of cellulases on insoluble cellulose.

Busto and Ortega (1996) tested the kinetic parameters of *T. reesei* cellulases using carboxymethylcellulose as substrate in concentrations ranging from 0.25 to 2%. The authors obtained the Michalis-Menten constant (K_m) and the maximum velocity os substrate conversion (V_{max}) of 1.32% and 405.5 µmol of glucose/mLh^{-1}, respectively, by linear regression using a Lineweaver-Burk inversion. Kinetic parameters are greatly influenced by the kind of substrate, temperature, microbial source and presence of inhibitors. The kinetic parameters of commercial BGL's with different substrates such as Novozymes S188 BGL (*A. niger*) with pNPG as substrate, at 50ºC, demonstrated a K_m value of 0.46 mM (HIMMEL et al., 1993). *T. reesei* cellulases from a culture broth presented a K_m of 0.182 mM in the same conditions whereas using the natural substrate cellobiose presented a K_m of 1.25 mM (CHIRICO, 1987).

2.11 CELLULASE ACTIVITY

The universal cellulase assay proposed by Mandels and Andreotti, (1976) and "officialised" by Ghose, (1987) helped to reduce the well-known problems associated to the different methods of cellulase quantification. Different methods give different results for cellulase quantification, making the comparison questionable. The different existing cellulase assays and the proposition of adaptations were reviewed (XIAO and STORMS, 2004; EVELEIGH et al., 2009; DASHTBAN et al., 2010; CAMASSOLA and DILLON, 2012).

The most popular and used assay to measure the total cellulase activity is the Filter Paper Assay (FPA). This is the "standard" method proposed by Mandels and Andreotti (1976) and Ghose (1987). This assay measures the reducing sugars produced from the synergistic action of EGL, CBH and BGL in a standard cellulosic substrate, a 1 x 6-cm strip of Whatman filter paper nº 1. The International Unit of the filter paper activity is the FPU or U_{FP} (Filter Paper Unit) whereas the assay is named FPase. This unit is defined as the micromole of reducing sugars liberated per minute. Xiao and Storms (2004) proposed the micro-plate based assay to measure the cellulase activity, which was modified by Camassola and Dillon (CAMASSOLA and

DILLON, 2012). This method is performed in 96-well microplates instead of test tubes, allowing for more samples, using less reagents and reducing the wastes, with similar results to that obtained by the standard method.

The released reducing sugars are measured by the dinitrosalicylic acid (DNS) method (MILLER, 1959). The original method takes into account that 0.5 mL of diluted enzymes releases about 2.0 mg of glucose equivalents in 60 minutes. The addition of DNS reagent stops the enzymatic reaction and, then samples are incubated for 5 minutes at 100ºC, and the absorbance is read at 540 nm. Other colorimetric method that is used is the Somogy-Nelson, which employs alkaline cooper as an inorganic oxidant. Besides the colorimetric methods, reducing sugar could also been assayed with the use of high-performance liquid chromatography (HPLC) as well as glucose peroxidase reagent (GOD) (DASHTBAN et al., 2010).

The EGL activity is measured using soluble cellulose substrate with high degree of polymerization such as carboxymethylcellulose (CMC). Mandels and Andreotti (1976) proposed this method in which the enzymatic assay is named CMCase (U_{CMC}), in which enzyme samples are incubated for 30 minutes under 50ºC with CMC 2% (GHOSE, 1987).

CBH (exoglucanases) activity determination uses as substrate the microcrystalline cellulose, named Avicel. Avicel has a low degree of polymerization and it is relatively inaccessible to EGL. Other substrates, such as paranithophenyl-*ρ*-D-cellobiose, methylumbelliferone-beta-cellobiose and bacterial microcrystalline cellulose, can also be used for CBH activity determination (DASHTBAN et al., 2010).

BGL's activity determination (U_{BGL}) is mainly performed by the use of *ρ*-nitrophenol-β-glucoside (*ρ*NPG). The enzyme activity is stopped after 15 min incubation by the addition of sodium carbonate and the absorbance is read at 405 nm. One unit of BGL is defined as the amount of enzyme that liberates 1 µmol of *ρ*-nitrophenol per minute (SINGHANIA et al., 2017). Other substrates such as cellobiose can also be used whereas the liberated glucose can be evaluated by the glucose oxidase (GOD) method. Cellulase activity determination assays are presented in Figure 3.

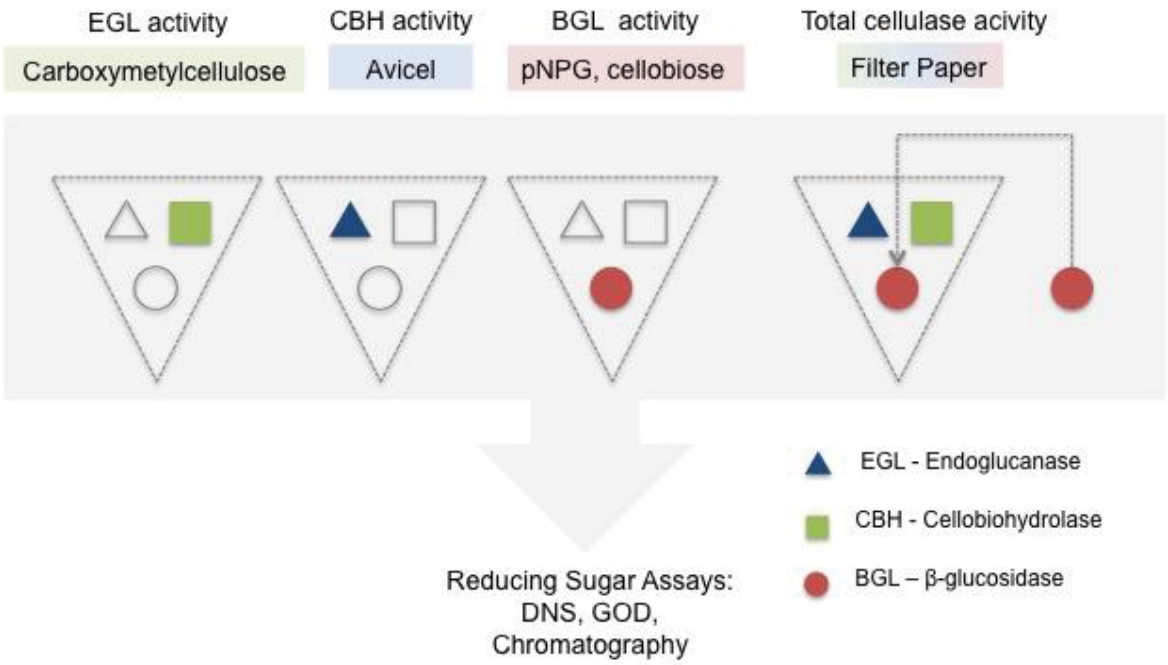

Figure 2.3 - Scheme of the cellulase activity assays.

Novel approaches for the measurement of cellulase activity have been developed as the quartz crystal microbalance, the fluorescent microfibrils from bacterial cellulose and the miniaturization and automation of the assays processes (DASHTBAN et al., 2010).

2.12 CELLULASE MARKET

According to Singhania et al. (2017), cellulases are the third largest industrial enzyme and its demand increased since 1995 in several industrial applications like detergent, textile, animal feed, food, paper and biofuel industry. The return of the commercial interest in the second-generation ethanol also increased the importance of this enzyme in the global market.

The world company leaders involved in cellulase production are the Danish Novozymes and the North American Genecor, recently taken over by DuPont. The massive investments in research of these companies are bringing down the cost of cellulases. DuPont replaced the Accelerase 1500 (produced with a genetically modified *T. reesei* strain) and Accelerase XY (xylanase cocktail to be used in combination Accelerase 1500) by Accelerase TRIO, the first enzyme cocktail for biomass hydrolysis. Novozymes commercializes a wide range of cellulases for different applications such as Cellusoft AP and Cellusoft CR for textile industrial

processes, Carezyme and Celluclean for laundry detergents and Denimax for surface modification of denim. The company developed the Celluclast for biomass hydrolysis, a cocktail of EGL and BGL, to be used in combination with the BGL Novozyme 188L for the complete hydrolysis of cellulose to glucose. Later the company also presented a complete enzyme cocktail family named Cellic Ctec and Cellic HTec. Other important players in the cellulase market are the Japanese Amano Enzyme, Inc., and the Indian AdvancedEnzymes. A list of commercial cellulases is presented in the Table 2.1.

Table 2.1 – Commercial cellulases in the market and their general characteristics. Adapted from Singhania et al (SINGHANIA et al., 2010; 2017).

Commercial product	Supplier	Country	Enzyme activity (U_{FP}/mL)	Microbial source	Use pH	Use Temperature (ºC)	Form available
Celluclast	Novozymes	Denmark	56	*T. longibrachiatum/A. niger*	5.0	50	Liquid
Novozymes 188	Novozymes	Denmark	-	*A. niger*	5.0	50	Liquid
Cellic CTec2	Novozymes	Denmark	-	*Not informed*	-	50	Liquid
Cellic CTec3	Novozymes	Denmark	-	*Not informed*	-	50	Liquid
Cellulase 2000L	Rhodia-Danisco	France	10	*T. longibrachiatum/ T. reesei*	5.0	50	Liquid
Rohament CL	Rohm-AB	Finland	50	*T. longibrachiatum/ T. reesei*	5.0	50	Liquid
Viscostar 150 L	Dyadic	USA	33	*T. longibrachiatum/ T. reesei*	5.0	50	Liquid
Multifect CL	Genecor	USA	64	*T. reesei*	5.0	50	Liquid
Bio-feed Beta L	Novozymes	Denmark	<5	*T. longibrachiatum/ T. reesei*	5.0	50	Liquid
Energex L	Novozymes	Denmark	<5	*T. longibrachiatum/ T. reesei*	4.5	50	Liquid
Ultraflo L	Novozymes	Denmark	<5	*T. longibrachiatum/ T. reesei*	5.0	50	Liquid
Viscozyme L	Novozymes	Denmark	<5	*T. longibrachiatum/ T. reesei*	5.0	50	Liquid
Cellulyve	50L Lyven	France	24	*T. longibrachiatum/ T. reesei*	5.0	50	Liquid
GC 440	Genecor-Danisco	USA	<5	*T. longibrachiatum/ T. reesei*	5.0	50	Liquid
GC 880	Genecor	USA	<5	*T. longibrachiatum/ T. reesei*	5.0	50	Liquid
Spezyme CP	Genecor	USA	49	*T. longibrachiatum/ T. reesei*	4.0	50	Liquid
GC 220	Genecor	USA	116	*T. longibrachiatum/ T. reesei*	5.0	50	Liquid
Accelerase 1500	Genecor	USA	-	*T. reesei*	4.0-5.0	50-65	Liquid
Cellulase AP30K	Amano Enzyme	Japan	0,17	*A. niger*	4.5	60	Powder
Cellulase TRL	Solvay Enzymes	India	95	*T. longibrachiatum/ T. reesei*	4.5	50	Powder
Econase CE	Alko-EDC	USA	40	*T. longibrachiatum/ T. reesei*	5.0	50	Liquid
Cellulase TAP106	Amano Enzyme	Japan	0.42	*T. viriddae*	5.0	50	Powder
Biocellulase TRI	Quest Intl.	USA	68	*T. longibrachiatum/ T. reesei*	4.5	50	Liquid
Biocellulase A	Quest Intl.	USA	0,29	*A. niger*	5.0	55	Powder
Ultra-low microbial	Iogen	USA	88	*T. longibrachiatum/ T. reesei*	-	-	Liquid
Accelerase TRIO	DuPont-Genecor	USA	-	*T. reesei*	-	50	Liquid

2.13 STRAIN IMPROVEMENT

The three main methods described for strains' improvement for increased cellulase production are the mutagenesis and selection, the genome shuffling and the gene cloning. The mutagenesis and selection, also named as classical strain improvement is performed by a variety of chemical or physical mutagens. The genome shuffling is related to the protoplast fusion. The recombinant DNA technology involves the cloning of the cellulase encoding genes and the expression in industrial strains. Many fungal cellulases have been cloned and expressed for the production of high titres of cellulase (SINGHANIA et al., 2017). Cre1 sites in the *T. reesei* genome and other fungal strains have been silenced through several strategies such as RNA interference resulting in increased cellulase activity. Zhang et al., 2010) have developed a *T. reesei* strain by overexpression of BGL under the control of the cbh1 promoter. The resultant recombinants produce high levels of BGL and filter paper activity. Druzhinina and Kubicek (2017) published a very complete review regarding the genetic engineering of the *T. reesei* cellulases.

2.14 GENERAL ASPECTS OF CELLULASES PRODUCTION

The enzyme production costs is still one of the most important bottlenecks for its application in a larger scale, i.e., for biomass hydrolysis in biorefineries. A diversity of methods are applied to improve the enzyme production, taking hand of better bioprocess strategies, the use of cheaper raw materials or even residues as substrates, strain engineering and improvement, etc.

The majority of the commercially available hydrolases are produced by submerged fermentation (SmF), overexpressing selected genes in either native or heterologous microbial hosts. However, there are still many research publications testing and comparing SmF with solid state-fermentation (SSF) processes, as an attempt for enzymatic productivity improvement, reducing its costs and improving the viability for commercial application.

The production of cellulases is traditionally carried out in SmF processes, mainly using the fungi of the genus *Trichoderma* and *Aspergillus*. The production of cellulase in SmF permits a better handling and monitoring of the process when compared to SSF and this aspect is important since their expression is highly

influenced by many parameters including the nature of cellulosic substrate, the temperature and pH of the medium, the nutrient availability, the presence of inducers and others (SINGHANIA et al., 2017).

The majority of cellulase production processes is conducted under the batch mode. A general behaviour of fungal cellulase production in a typical batch strategy of production comprises a lag phase after inoculation with a pH rise, particularly if peptone is present. The consumption of carbohydrate substrate leads to pH decrease untills about 3.0 and then cellulase is excreted to the medium. The enzyme production ceases with the carbon source consumption and the pH rises again (MANDELS and WEBER, 1969; BAILEY and TÄHTIHARJU, 2003).

The necessity of the control of the repression caused by the accumulation of reducing sugars in the culture medium pushes the use of strategies such as the use of lactose as carbon source, coupled with fed-batch operational mode which results in very high productive processes (WARZYWODA and LARBRE, 1992). The fed-batch also allows the step-feed of glucose for biomass growth followed by lactose-feed for cellulase production. A patented strategy proposes the feeding of 15 g/L of glucose followed by 250 g/L of lactose, achieving a final cellulase concentration of 18.5 U_{FP}/mL (COHEN, 2012). Continuous processes are described as problematic for fungal fermentation since it is difficult to maintain the physiological state of the microorganism at its optimum for enzyme production, in a steady-state behaviour, with a fixed dilution rate. The fungal metabolism could change between productive and non-productive cell-growth or unwanted sporulation could occur, also affecting the enzyme productivity. The balance between undesirable physiological states and the maximum enzyme productivity on the basis of a fixed, time-dependent feeding rate over a long period of time is rather sensitive (BAILEY and TÄHTIHARJU, 2003). Control parameter strategies have been employed to overcome the fluctuations of microbial metabolism based on the dynamic of the pH of the culture medium, since it is an indicator of the stage of the growth and cellulase production. Bailey and Tähtiharju, (2003) proposed an automatic addition of new medium to the reactor based on the pH rise after the carbon source starvation, during the production of cellulase using *T. reesei* on a lactose medium in continuous cultivation.

2.15 CELLULASE RECOVERY AND FORMULATION

Membrane technologies are adequate for the processing of biological molecules because they operate at low temperatures and pressures and involve no phase changes or chemical additives, thereby minimizing the denaturation, deactivation and degradation of the products. A common application of ultrafiltration (UF) in downstream processing is for product concentration (CHARCOSSET, 2006). The use of UF for cellulase concentration was described using membranes with a molecular weight cut-off (MWCO) varying from 5 to 100 kDa (QI et al., 2012) for retaining cellulases, whose molecular weight ranges from 60 to 90 kDa (MORES et al., 2001). The UF allows for the concentration of cellulase between 50 to 75 FPU/mL (ESTERBAUER et al., 1991). Before the UF step, microfiltration (MF) can be used for the clarification of the culture broth and to retain suspended solids > 0.2 μm (MORES et al., 2001). Rodrigues et al. (2014) reported the recovery of 38% of a commercial cellulase from hydrolysed wheat straw. Qi et al. (2012) reached a protein recovery of 89.4%, 73.9% and 79.7% using MWCO of 5, 10 and 30 kDa. Although the use of UF has been described as an interesting way to ensure cellulase concentration, there are some operational problems related to the fouling and concentration polarization, which lead to the reduction of the permeate flux and possibly damage of the membrane. These processing problems may increase the cleaning costs, lowering the competitiveness of this process (SHI et al., 2014).

Formulation is an art and often the precise details of the methods used to stabilise enzyme preparations are kept secret or revealed to customers only under the cover of a confidentiality agreement. Sometimes it is only the formulation of an enzyme that gives a manufacturer the competitive edge over rival companies. It should be remembered that most industrial enzymes contain relatively little active enzyme, the rest being due to inactive protein, stabilisers, preservatives, salts and the diluent which allows standardisation between production batches of different specific activities (CHAPLIN and BUCKE, 1990). The formulations should enhance the enzyme stability against the physical (thermal and chemical) denaturation, catalytic-site deactivation and proteolysis. The use of sugars is common as a water-exclusion agent. Besides the enzymatic stability, the formulation should guarantee the preservation against microbial contamination, avoidance of physical precipitation

or haze formation, minimization of sensitising dusts or aerosol and optimize esthetical criteria such as color and odour (AEHLE, 2004).

Liquid cellulase formulations are obtained by the concentration of the culture broth by ultrafiltration in most of the cases and the addition of adjuvants and stabilizers. According to the Table 2.1, most of the commercial cellulases are delivered in liquid formulations containing additives in order to maintain the proteins stability and prevent contaminations. Preservatives like biocidal agents such as benzoic acid are products added to the enzyme formulations to maintain their stability. Most of the safety rules regarding the formulation of enzymes are related to the food and health industry and not to technical enzymes such as cellulases (AMFEP). The available information about enzymatic formulations is very scarce due to its inherent industrial interest.

A solid cellulase formulation can be obtained by lyophilisation or by spray drying the already concentrated broth. Besides the use of additives such as sugars, polyalcohols or sodium chloride to stabilize the enzyme, drying processes require the use of encapsulating agents such as maltodextrin, microcrystalline cellulose, starch, gelatin, as well as antiumectants as colloidal silicon dioxide (FERREIRA et al., 2016). In spray-drying processes the temperature of the injected air must be below 150ºC, to avoid the inactivation of some cellulases (ESTERBAUER et al., 1991). The use of the spray drying process for the drying of enzymes is still underexplored in the academic literature, since much of its development occurs in the industrial scope.

Blending of cellulolytic enzyme preparations from different fungal sources has been also proposed, in order to prepare a complete enzymatic complex formulated according to its application. For the hydrolysis of cellulose a blend of CBH, BGL and EGL is prepared according to desirable proportions of the enzymes (ADSUL et al., 2014).

2.16 CULTURE MEDIUM FOR CELLULASE PRODUCTION

When developing an industrial bioprocess and designing a fermentation process it is of central importance the definition of the medium composition that can significantly affect product concentration, yield and volumetric productivity. The mostly used medium for the production of cellulases by *T. reesei* is the basal medium proposed by Mandels and Weber (1969), with or without modifications. Other culture

medium compositions were proposed aiming the increase of biomass growth and cellulase production in industrial scales with lower costs. Besides the use of macronutrients and trace elements, the carbon and nitrogen source are the major concern associated to the production of cellulases. It is possible to describe the cellulose and lactose as the most used and effective carbon sources for the production of fungal cellulases from *Trichoderma* strains. These carbon sources prevents the glucose-repression effect presented by other simpler sugars like glucose or fructose and are known as natural cellulase inducers. The presence of simple sugars in the culture medium lead to a fast biomass growth, but represses cellulase expression. The use of lactose or cellulose increases the lag phase, but induces the cellulase production (AHAMED and VERMETTE, 2009). Together with the use of lactose, commercial cellulosic materials such as Solka-Floc and Avicel were used since the 1980 decade for cellulase production from *Trichoderma* and extensively reviewed by Esterbauer et al. (1991). The authors presented the use of lactose as inducer, ranging from 2 to 10%, combined or not with cellulosic materials, achieving enzyme activities up to 30 U_{FP}/mL.

The great majority of the hydrolases published data are related to bench-scale production processes. Only few reports deal with the results and problems related to cellulase production in a pilot plant, or scale-up process parameters. Pourquie et al. (1988) performed cellulase production in a 3000-L fermenter achieving the productivity of 243 U_{FP}/Lh. Esterbauer et al. (1991) already reported cellulase production in 3000 L bioreactor reaching cellulase activities of 18 U_{FP}/mL, using a *T. reesei* mutant. Miettinen-Oinonen et al. (2004), working with a 700-L fermenter, achieved 1,160 nkat/mL of cellulase after 72 hours. Unfortunately, many authors do not inform the obtained protein concentrations, what would certainly allow the calculation of enzyme concentrations in g/L basis. High-level cellulases production greatly depends on inducers and costly carbon sources such as pure cellulose, lactose or sophorose, which make enzymes' final costs too expensive for using in a cellulosic ethanol process.

Lignocellulosic materials can be used as carbon source and/or inducers for enzyme production (ELLILÄ et al., 2017). The use of agro-industrial residues is an interesting strategy for costs reduction, which is mainly associated with the culture medium formulation. In the last decades there has been an increasing trend towards efficient utilization of agro-industrial residues in fermentative bioprocesses. Cellulosic

residues serving as carbon sources in the majority of the commercial cellulase fermentations are cellulosic biomass including straw, spent hulls of cereals and pulses, rice or wheat bran, bagasse, paper industry waste, and various other lignocellulosic residues that induce cellulase production. In the same way, cheese whey has been used as the most economical source of lactose replacing the use of the pure lactose (DAHOD et al., 2010; SINGHANIA et al., 2017).

The majority of the cellulosic agro-industrial residues are in the solid form, what suggests the development of SSF processes for enzyme production. However there are increasing examples in the literature of the use of such residues in suspension in SmF processes that is also called semi-solid state fermentation (SSSF) processes. In addition, the natural fungal ability to adhere on the solid particles facilitates the use of SSSF with the advantage of releasing the extracellular enzymes in the liquid phase.

Various residues used for cellulases production in SmF and the wide range of enzymatic activities and productivities achieved are exemplified in Table 2.2.

Table 2.2 *Trichoderma* cellulases' production in SmF using agro industrial residues.

Strain	Residue	Process conditions	Enzyme activity (U/mL)	Productivity ($U/L \cdot h^{-1}$)	Reference
T. reesei	Soda pulp from Japanese cedar	28 ºC, 300L	13.6 U_{FP} 11.4 U_{BGL}	94.4 79.16	(SHIBUYA et al., 2015)
T. reesei	Corn cob residue 4%	30 ºC, 30m^3 reactor	5.25 U_{FP}	54.68	(LIMING and XUELIANG, 2004)
T. reesei (RUT- C30)	Destarched wheat bran 2%	30 ºC, 120 rpm	0.12 U_{CMC} 0.01 U_{FP} 0.054 U_{BGL}	1.0 0.08 0.45	(PRÉVOT et al., 2013)
T. reesei (RUT-C30	Sugar beet pulp 5%	28 ºC, 350 rpm	0.6 U_{CMC}	5.68	(LISBETH et al, 2003)
Trichoderma harzianum	Partially delignified cellulignin 0.75%	30 ºC, 200 rpm	0.09 U_{FP} 0.55 U_{CMC} 0.7 U_{BGL}	0.93 5.72 7.29	(CASTRO et al., 2010)
Trichoderma harzianum	Steam exploded Aspen 0.75%	28 ºC	2.3 U_{FP}	15.97	(SADDLER et al., 1985)
Trichoderma reesei	Wheat bran 2%	25.5 ºC	13.7 U_{FP}	91.33	(WARZYWODA et al., 1983)
Trichoderma reesei	Sugarcane bagasse 1% + Wheat bran 1%	28 ºC, 90 rpm	0.31 U_{FP}	0.58	(LAN et al., 2013)
Trichoderma viridae	Sugarcane bagasse 1%	30 ºC, 180 rpm	0.88 U_{FP} 33.8 U_{CMC} 0.33 U_{BGL}	3.66	(ADSUL et al., 2004)

Besides the use of agro-industrial residues, an emerging approach for biomolecules production is the use of wastewater treatment facilities by-products, such as sludge resulting from waste treatment processes. This kind of residues are gaining attention due to their high composition in carbon, nitrogen, phosphorous and other compounds that could be used for bioprocesses such as enzymes production that will further be discussed.

Sanitary wastewater sludge (0.75%) supplemented with wheat flour (2%) was reported for cellulases production in 500 mL Erlenmeyer flasks at 32.5 ºC, 175 rpm, pH 5.0, and 2% (v/v) inoculum, reaching 10.2 U_{FP}/mL after 3 days of fermentation,

corresponding to the productivity of 141 U_{FP}/Lh (ALAM et al., 2008). Alkaline pre-treated sanitary sludge was reported as a culture medium for the production of cellulases at the concentration of 30 g/L of suspended solids. The bioprocess was carried out in a 15L reactor for 96 hours, using the strain *T. viridae*, reaching maximum cellulase activity (150 U_{FP}/mL) after 36 hours of processing (VERMA et al., 2007). Effluent sludge (0.5%) supplemented with microcrystalline cellulose (Avicel - 2% (w/v)) and empty palm bunches (0.2%) were used for cellulases production in Erlenmeyer flasks containing 50 mL of culture medium, at 30 ºC and 150 rpm, for 120 hours, using *Trichoderma* Rut-C 30 strain, reaching the enzymatic activity of 0.384 U_{FP}/mL, leading to a productivity of 3.2 U_{FP}/Lh (DRANI et al., 2011).

A patent search (Table 3) revealed the numbers of deposits with the use of by-products from wastewater treatment plants (WWTP) for cellulase production.

Table 2.3 - Results from national (Brazil) and international patents databases registers related to the use of WWTP by-products for cellulase production. Data collection in 07.2017.

Key-word	Data base					
Cellulase production with	WIPO	INPI	EPO	USPTO	CIPO	FPO
Domestic wastewater	0	0	0	52	0	94
Wastewater	14	0	16	243	0	409
Domestic sludge	0	0	1	67	0	117
Sludge	0	0	12	414	0	607
Sewage	8	0	11	281	0	391

WIPO – World Intellectual Property Organization
INPI – National Institute for Intellectual Property (Brazil)
EPO – European Patent Office
USPTO – United States Patent Office
CIPO – Canadian Intellectual Property Office
FPO – Free Patents Office

The patent US 0178144A1 (ALAM et al., 2012) from Malaysian authors reports the use of sanitary effluent sludge as the basis for culture medium. Sludge was obtained after the sedimentation process in an effluent treatment plant. Authors reported the use of a culture medium composed of cellulose (1.5%), peptone (0.5%), polysorbate 80 (0.2%), sucrose (1%), potassium phosphate dihydrate (25%) and

magnesium sulfate heptahydrate (0.03%). The bioprocess was carried out in a 30-L bioreactor, mechanically agitated at 100 rpm, and air flow at 0.5 to 1 volume of air per volume of medium per minute (vvm). The maximum cellulase production reached 21 U_{FP}/mL, with pH values ranging from 3 to 3.5, in 169 hours, leading to maximum productivity of 125 U_{FP}/Lh.

Most of the results presented in Table 3 report the use of sanitary effluent sludge. No record of either patent or scientific publication was found reporting the use of raw sanitary effluent as the basis for the culture medium for the production of cellulases. The first description was published (LIBARDI et al., 2017) reporting the use of sanitary wastewater as culture medium component for cellulases production using the strain *Trichoderma harzianum* TRIC03. The authors performed the process in a bubble column reactor achieving the productivity of 10.2 U_{FP}/Lh for FPase and 64.6 U_{CMC}/Lh for CMCase.

2.17 CONCEPT OF WASTEWATER BIOREFINERY

The use of agro-waste materials as substrate for valuable biomolecules production is a strategy that has been already used for some decades in the bioprocess industry, aiming to reduce the production costs and to develop sustainable processes. Recently, this research topic gained more attention with the development of the bio-based processes. Significant efforts are being devoted to the development of bio-based sustainable processes to decrease the use of non-renewable fossil resources that are depleting very quickly. The sustainable feedstock supply is one of the key issues for the evolution towards the bio-based economy. The bio-based economy can be defined as the sector that employs waste biomass derived from crop residues of food and feed production, forestry residues, fermentation process wastes, food/beverage process wastes, marine crops and processing wastes, municipal solid and liquid wastes manure and animal products, for the production of process and products. Since it is characterized by the higher efficiency in terms of energy and materials together with the reduction of environmental unfriendly wastes production, the bio-based processes are clearly advantageous (KAUR et al., 2014). The biorefinery concept is totally integrated to the development of a bio-based economy. According to the International Energy Agency (INTERNATIONAL ENERGY AGENCY, 2008), the biorefinery is the sustainable

processing of biomass into a spectrum of marketable products and energy. Cherubini (2010) defines biorefinery as a facility (or network of facilities) that integrates biomass conversion processes and equipment to produce transportation biofuels, power and chemicals from biomass. Being an integral upstream, midstream and downstream processing of biomass into a range of products, the biorefineries are characterized by the use of mechanical pre-treatments (extraction, fractionation, separation), thermochemical, chemical, enzymatic and microbial (aerobic and anaerobic fermentation) conversions. In addition, several economic and environmental issues are related to biorefineries such as the global warming, energy conservation, security of supply and agricultural policies (JONG and JUNGMEIER, 2015). These aspects are on the focus due to the continuous and fast depletion of the conventional energy and material resources and the awareness and concern regarding the environmental effects of their utilization (KAUR et al., 2014).

The Wastewater Biorefinery is the application of the Biorefinery concept to the Wastewater Treatment Plants (WWTP). Wastewater biorefineries are based on aspects of the environmental engineering and bioprocess engineering fields, integrating the improvement of the natural environment and the development of processes for the manufacture of bioproducts (VERSTER et al., 2014). The focus is the integration of a wastewater treatment process to the production of value-added products, considering the wastewater as raw material, the WWTP by-products as end products to be recovered and the WWTP bioreactors as a biotechnological industry. Some of the key characteristics of wastewater biorefineries are: the use of the wastewater as a substrate for the microbial community and its complexity compared to a defined culture medium; the use of a non-sterile microbial community instead of pure cultures; the diluted nature of the nutrients in the wastewater; the non-sterile condition of the bioreactor; the necessity of biomass retention to compensate the dilution of the nutrients (VERSTER et al., 2014).

Different unit processes are divided in preliminary, primary, secondary and even tertiary treatments that compose most of the WWTP's. The streams with material inputs and outputs are useful sources of raw material for an intended bioprocess, or a valuable material to be recovered. Figure 2.4 is an example of the possible nutrients and by-products that are recovered from each stream and the possibilities for their use.

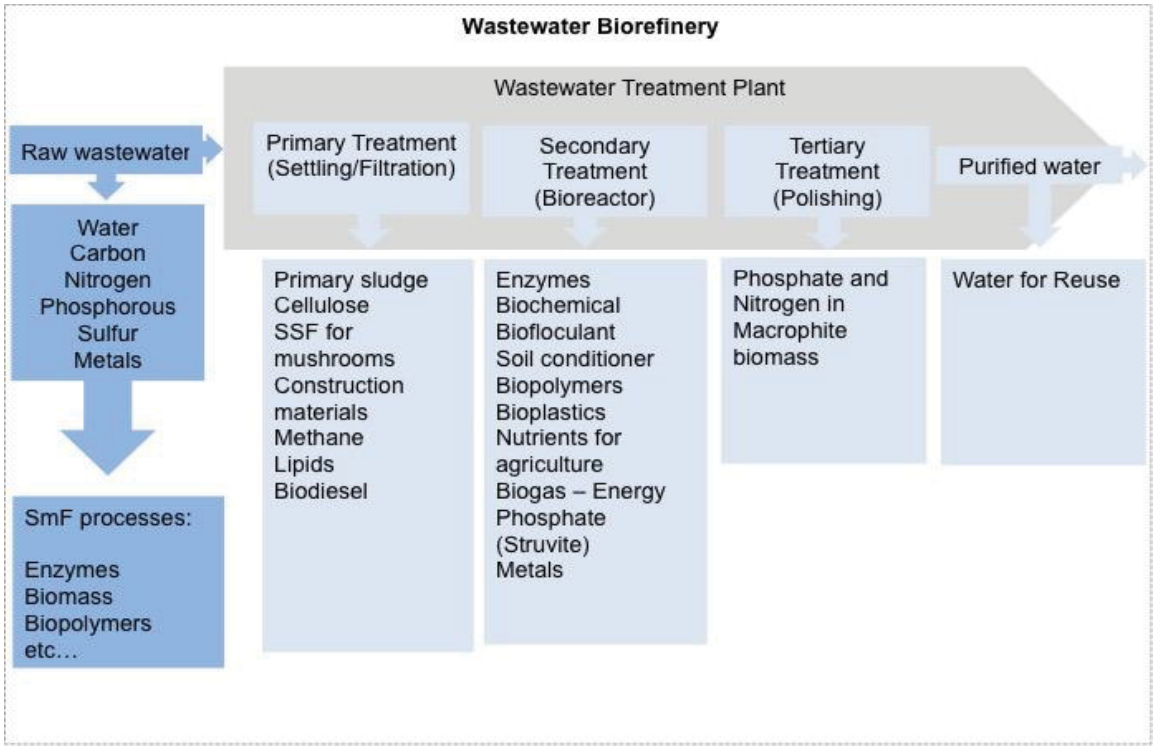

Figure 2.4 - A proposal for a wastewater biorefinery. Adapted from Verster et al. (2014).

The raw wastewater entering the WWTP is a nutrient rich stream that could be used to feed an industrial bioreactor for the production of biomolecules such as organic acids, enzymes, biomass and others. The other treatment steps are also source of nutrients and other resources as it can be observed in Figure 2.4. The primary settling tank is a source of biosolids with high carbon content, useful for composting or anaerobic digestion for biogas and energy production as well as for solid-state fermentation (SSF), as potential process for the use of these biosolids for the production of value-added mushrooms. From WWTP's biological conversions, it is possible to recover different products such as enzymes, biochemicals, bioflocculants, soil conditioners, biomaterials, bioplastics and others. The tertiary treatment process is a source of other materials and, finally, the treated water can be reused for other processes with low water quality standards.

In a general industrial bioprocess, pure strains are inoculated in a bioreactor filled with concentrated culture medium at sterile conditions, where the substrate is converted by microorganisms into biomass and/or bioproducts. On the other hand, in an environmental WWTP bioprocess, a non-sterile mixed culture is fed to a sanitary wastewater medium, which contains the nutrients for microbial growth under diluted

conditions. The diluted nature of the wastewater is an important difference when considering it as the substrate for a bioprocess. For instance, some authors disagree that the Monod equation that is developed to describe the bacterial growth kinetics could be used to describe the kinetics of a wastewater treatment. This is due to substrate concentration, which is very diluted and a mixture of microorganisms of natural occurrence are developed in the reactor (METCALF AND EDDY, 2003).

2.18 WASTEWATER FOR CULTURE MEDIUM

The potential of the use of domestic wastewater as a source of water and nutrients for industrial processes is enormous. According to Verstraete et al. (2009), (2009), from 1 m^3 of sanitary wastewater it is possible to recover 0.05 kg of nitrogen, 0.14 m^3 of methane, 0.1 kg of organic fertilizer and 0.01 kg of phosphorous. The Athlone Municipal Treatment Plant, one of the 23 unities of the City of Cape Town – South Africa, generates around 70 tons of carbon, 6 tons of nitrogen, and 1 ton of phosphorous per day, treating 120,000 m^3 of effluent per day (VERSTER et al., 2014). The treatment capacity of a WWTP is directly related to the contributing population. According to Henze and Comeau (2008), the worldwide definition of effluent contribution per person per day is 0.2m^3, which is also defined as person equivalent. In addition, the person load is defined in terms of biochemical oxygen demand (BOD), total suspended solids, total nitrogen and total phosphorous, and varies from country to country. The BOD load in Brazil ranges from 20 to 25 kg per person per year, in India is 10–15 kg, in United States is 30–35 kg and in Germany is 20–25 kg. According to the Brazilian Water and Wastewater Services National Diagnosis (BRASIL, 2018), 4.06 billions of m^3 of sanitary wastewater are generated annually in the country, considering that 80% of the produced water is transformed in wastewater. From this amount, only 59.7% is collected, and only 44.9% is treated. The environmental and social impact of such a scenario has induced local and global initiatives. In 2015, the United Nations Organization (UNDP, 2017) proposed its Sustainable Development Goals, with 17 goals to be achieved by 2030, one of which is the sustainable management of sanitation, including the development of technologies of wastewater treatment and reuse.

One of the fundamental aspects to evaluate the viability of using domestic wastewater in a bioprocess is the comparison between the culture medium of a

specific bioprocess and the composition of the wastewater itself. This first evaluation probably reveals the necessity of supplementation of wastewater to achieve the minimum requirements for an intended bioprocess.

The order of magnitude between a typical culture medium compared to a domestic wastewater concentration was reported by Verster et al. (2014). According to the authors, a culture medium for poly-glutamic acid production is around 100 times concentrated in comparison to the sanitary wastewater in terms of carbohydrate concentrations. A typical composition of raw municipal wastewater is presented in Table 2.4.

Table 2.4 – Composition of raw domestic wastewater.

Nutrients	Concentration (g/L)
Chemical oxygen demand	0.750
Biochemical oxygen demand	0.350
Total Nitrogen	0.060
Ammonia-Nitrogen	0.045
Total Phosphorous	0.015
Total suspended solids	0.400
Volatile suspended solids	0.320
Metals	**Concentration (mg/L)**
Aluminum	0.600
Cadmium	0.002
Copper	0.070
Lead	0.060
Zinc	0.200

Adapted from Henze and Comeau (2008).

The biological oxygen demand (BOD) as well as the chemical oxygen demand (COD) refers to the organic matter that is present in wastewater. Bailey and Tähtiharju (2003) proposed the use of 40 g/L of lactose for the cellulases' production from *Trichoderma* strain. The use of carbohydrate content, which is around 50 times higher than the carbohydrate content in raw domestic wastewater, suggests the necessity of other carbon sources supplementation to provide enough energy for enzyme production. Henze and Comeau (2008) described the typical COD/Total nitrogen ratio of sanitary wastewater that is between 8 to 12. Even with diluted carbon and nitrogen concentrations in the wastewater, this ratio is in accordance with

the elementary composition for microbial biomass in terms of dry cell weight (DAHOD et al., 2010).

Wastewater sludge was already proposed as a component for culture medium, since it is an interesting source of nutrients. According to Verma et al. (2005), it is very rich in nutrients and metals (g/kg of sludge): 404 total carbon, 53 total nitrogen, 1.21 total phosphorous, 1.7 Al, 15.5 Ca, 0.004 Cd, 0.35 Cu, 0.58 Zn, 9.239 Fe, 2.41 K and 3.23 Na. From these data, sludge's C:N ratio is around 8. Alam et al. (2008) also described the composition of domestic wastewater sludge: 32% of carbon, 3.8% of nitrogen, 1.6% of phosphorous, 0.05% of magnesium, 0.15% of potassium and trace elements. Liming and Xueliang (2004) tested the C:N ratios of 6, 7, 8 and 9 using corn cob residue (40 g/L) as carbon source with other compounds according to the medium proposed by Mandels et al. (1981), obtaining the best results in terms of enzymatic activity (5.25 U_{FP}/mL) with a C:N ratio of 8.

2.19 COUPLING RECOVERY AND FILTRATION

Membrane technologies have been described as a suitable technique for wastewater purification, including microfiltration, nanofiltration and reverse osmosis. These technologies are gaining more popularity due to the guarantee of achieving a specific water quality and the recent lower costs of the membranes (RYAN et al., 2009; VERSTRAETE et al., 2009). The use of membrane-based technologies for wastewater nutrient recovery besides the water purification has been reviewed (XIE et al., 2016) and proposed as a promising technology for the development of wastewater biorefineries, taking advantage of product productivity maximization in combination with the reduction of effluent concentration (KLEEREBEZEM and LOOSDRECHT, 2007). Membrane filtration is a technology suitable for separation of suspended, colloidal and soluble impurities from water. Despite the evolving performance of membranes, limited publications report their use in primary treatment in order to tackle the environmental footprint issue of wastewater treatment (VERSTRAETE et al., 2009). Membrane technologies are also reported as one of the most interesting technologies for the downstream processing of biological molecules. The concomitant use of membranes for wastewater purification and the concentration of high added value bioproducts demonstrate the feasibility of the integration of technologies, contributing for the development of wastewater biorefineries.

3 DOMESTIC WASTEWATER AS SUBSTRATE FOR CELLULASE PRODUCTION BY *Trichoderma* sp.

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This chapter was published in the journal Process Biochemistry (2017).

Doi: 10.1016/j.procbio.2017.03.006

(Attachment A)

ABSTRACT

Cellulase production using residues as substrate has been well described, as it is an interesting method of reducing the costs of processes, one of the main bottlenecks for the production of enzymes. This research describes for the first time the use of raw domestic wastewater, which is largely and continuously generated, as a culture base medium for cellulase production. The strain *Trichoderma harzianum* TRIC03 was selected according to the highest activity produced for FPase (5.4 U_{FP}/mL) and CMCase (8.2 U_{CMC}/mL). Peptone was selected as a nitrogen source and microcrystalline cellulose as the inducer for cellulase production, resulting in FPase activities of 5.6 and 5.0 U_{FP}/mL and CMCase activities of 12.0 and 14.4 U_{CMC}/mL. The use of domestic wastewater as the culture medium led to an increase of 1.41 and 1.14 fold of FPase and CMCase production, respectively, compared to the water-based medium. Production was also carried out in a bubble column reactor in which the maximum productivities achieved 10.2 U_{FP}/Lh and 64.6 U_{CMC}/Lh. The presented results demonstrate the feasibility of the use of domestic wastewater for cellulases production, thereby contributing to the development of a sustainable process for reusing wastewater with a significant reduction in environmental impact.

Keywords: cellulase production, domestic wastewater, *Trichoderma harzianum*

3.1 INTRODUCTION

Cellulases production using alternative substrates has been well studied, as it is an interesting way to reduce enzyme production costs, which is still one of the major bottlenecks for its application, i.e., second-generation biofuel. The composition of the culture medium significantly affects product concentration, yield, and volumetric productivities, which is of central importance for economically viable production. The use of agro-industrial residues is an interesting strategy for the reduction of costs associated with the culture medium formulation of cellulase production. There has been an increasing trend toward efficient utilization of agro-industrial residues in cellulase production by means of submerged and solid-state fermentation, such as wheat straw (ROMERO et al., 1999), sugarcane bagasse (ADSUL et al., 2004; LAN et al., 2013), palm oil extraction residues (ALAM et al., 2009), corn cob (LIMING and XUELIANG, 2004), sorghum bran (BAILEY and POUTANEN, 1989), paper pulp (BELGHITH et al., 2001), and elephant grass (VISSER et al., 2013).

In addition to the importance of productivity, the sustainability of the cellulase production process or any bioprocess should also be taken into account. Besides the use of agro industrial residues, another approach is the use of effluents in such processes. Wastewaters are an interesting source of nutrients and products to be recovered such as carbon, nitrogen, phosphorous, organic fertilizers, methane and others. Its composition can vary according to the place, the period of the year, and the pluviometric index. In addition, cellulose, which is the main cellulase inducer, is also present in the raw sanitary wastewater (HENZE and COMEAU, 2008). The presence of these macronutrients greatly contributes to the costs associated with the wastewater treatment plants (WWTP). The nutrient loads in municipal wastewaters are diluted but still add up to significant daily loads because of the massive volumes generated in urban populations (VERSTER et al., 2014). Verstraete et al. (2009) presents that from 1 m^3 of sanitary wastewater it is possible to recover 0.05 kg of Nitrogen, 0.14 m^3 of Methane, 0.1 kg of organic fertilizer and 0.01 kg of phosphorous. According to Verster et al., (2014) the Athlone Municipal Treatment Plant, one of the 23 unities of the City of Cape Town, generates an estimated 70 tons of carbon, 6 tons of nitrogen, and 1 ton of phosphorous per day, and treats 120,000 m^3 of effluent per day. The Atuba WWTP, a water-treatment unity of Curitiba, Brazil, also treats

around 120,000 m^3 of effluent per day. The treatment capacity of a WWTP is directly related to the contributing population. According to Henze and Comeau, (2008), the worldwide definition of effluent contribution per person per day is 0.2 m^3, which is also defined as person equivalent. In addition, the person load is defined in terms of biochemical oxygen demand (BOD), total suspended solids, total nitrogen and total phosphorous, and varies from country to country. The BOD load in Brazil is 20-25 kg per person per year, India 10-15 kg, United States 30-35 kg and Germany 20-25 kg.

According to the Brazilian Water and Wastewater Services National Diagnosis of 2014 (BRASIL, 2018), 4.06 billions of m^3 of sanitary wastewater is generated annually in the country, considering that 80% of the produced water is transformed in wastewater. From this amount, only 59.7% is collected, and only 44.9% is treated. The environmental and social impact of such a scenario has induced local and global initiatives. In 2015, the United Nations Organization proposed its Sustainable Development Goals, with 17 goals to be achieved by 2030, one of which is the sustainable management of sanitation, including the development of technologies of wastewater treatment and reuse (UNDP, 2017).

The use of domestic wastewater for cellulase production was not yet described in the scientific literature. Soccol et al. (2014), the authors of this work, have already deposited the correspondent patent. The use of sanitary wastewater sludge in combination with other compounds has already been described (VERMA et al., 2005, 2007; ALAM et al., 2008; DRANI et al., 2011). The production of cellulase from domestic wastewater could lead not only to the reuse of its nutrients but also to the reuse of water itself, as it is an important and scarce resource.

The aim of this study was to produce cellulase by means of submerged fermentation with the *Trichoderma* sp. strain, using domestic wastewater as the culture medium. The aim is to reduce production costs and create an environmentally friendly process.

3.2 MATERIALS AND METHODS

3.2.1 Microorganisms

Eleven filamentous fungi strains from the culture collection of the Bioprocess Engineering and Biotechnology Department of UFPR and State University of Feira de

Santana were screened for the cellulase production, *Colletotrichum aeschynomenes* (TRIC02), *Trichoderma harzianum* (TRIC03, TRIC05 and TRIC06), *Colletotrichum gloeosporioides* (TRIC04), and *Trichoderma* sp. (TRIC193 and TRIC221).

Microorganisms were grown on potato dextrose agar (PDA) tubes for 7 days at 30 ºC. Slants were maintained at 4 ºC for up to three months or until their use for inoculum preparation.

3.2.2 Inoculum preparation

Spores were harvested from agar tubes using an extraction solution composed of distilled water and Tween® 80 (0.1%). An aliquot of 1 mL of the spore suspension was inoculated in 500-mL Erlenmeyer flasks with 100 g of rice previously cooked for 2 minutes. Erlenmeyer flasks were incubated for 7 days at 30 ºC. An inoculum suspension of approximately 1 x 10^7 spores/mL was obtained.

3.2.3 Wastewater sampling and characterization

The raw domestic wastewater used for the culture medium composition was collected at the Atuba Wastewater Treatment Plant from the Sanitation Company of Paraná (SANEPAR), located in Curitiba, Brazil. For comparison, the sanitary wastewater was replaced by distilled water, for the composition of a water-based medium. Samples were collected at the parshall flume in different periods. Samples collected on 12.11.15, 03.12.15, and 07.12.15 were characterized by ion chromatography using the equipment Metrohm Compact IC, model 761,817, equipped with a cation column Metrosep C3 (0.9 mL/min; 3.5mM HNO3; 40 ºC) and an anion column Metrosep A supp 5 (0.7 mL/min; 1 mmol/L sodium bicarbonate and 3.2 mmol/L sodium carbonate; room temperature). Other parameters were provided by SANEPAR and were analyzed according to standard methods (APHA, 1989), (Table 3.1).

The cellulose content in wastewater was estimated by means of the enzymatic degradation of the solid fraction separated from wastewater. A total of 100 L of raw domestic wastewater, collected from the Parshall flume, was sieved with a fine-mesh sieve (20 mm). The solid material was dried, and the wet weight was measured. Enzymatic hydrolysis was performed in 250-mL Erlenmeyer flasks using the enzyme Celluclast® (Novozymes) at 2% (v/v) in contact with the solid material at a

concentration of 7.5% (m/v) in citrate buffer solution at pH 4.8 under 50ºC and 120 rpm of agitation over 48 h.

3.2.4 Screening of cellulase producers

The selection of the cellulolytic strain was carried out using a culture medium composed of the following (g/L): peptone-5.0, microcrystalline cellulose (Avicel®)-5.0, KH_2PO_4-2.5, $MgSO_4.7H_2O$-0.5, and raw domestic wastewater, with a final pH of 6.0 (ALAM et al., 2012). The cellulase production was conducted in 250-mL Erlenmeyer flasks incubated in a shaker with 120 rpm, at 28ºC, for 7 days. Assays were carried out in duplicate.

Table 3.1 - Physical-chemical composition of different raw domestic wastewater.

Parameter	SANEPAR*, Curitiba-Brazil	VESTER et al. (2014)	HENZE AND COMEAU (2008)	RUIKEN et al. (2013)
pH	7.2	7.25		
[1]COD (mg/L)	331.6	880	750	
[2]BOD (mg/L)	184.4		350	
[3]TSS (mg/L)	126.1		400	
[4]SS (ml/L)	2.2	13		
Alkalinity (mg/L)	175.4	275		
Sodium (mg/L)	40.04			
Magnesium (mg/L)	14.8			
Ammonium (mg/L)	25.68			
Potassium (mg/L)	13.86			
Calcium (mg/L)	45.28			
Fluorine (mg/L)	0.254			
Chlorine (mg/L)	16.86	211		
Bromine (mg/L)	2.412			
Phosphate (mg/L)	3.704			
Sulphate (mg/L)	17.77			
Cellulose (%)	21.3			79

*Culture medium for cellulase production
[1]Chemical Oxygen Demand
[2]Biochemical Oxygen Demand

[3]Total Suspended Solids
[4]Sedimentable Solids

Table 3.2 - Estimated physical-chemical composition of carbon sources added to domestic wastewater for cellulase production.

%	Sucrose	Sugarcane molasses	Soybean bran	Corn bran
Protein			48.8	5.5
Reducing sugars		11		
Sucrose	99.0	34		
Carbohydrates (starch)			5.5	12.5
Fiber			74.0-82.0	50.0-90.0

Sources: SINDHU et al. (2016); THAKUR AND HORNBURGH (2007); OLIVEIRA JUNIOR et al. (2014).

3.2.5 Influence of wastewater's concentration on cellulase production

The influence of wastewater's concentration on fungi metabolism and, consequently, on enzyme synthesis was carried out using different dilutions of 2, 4, 8, 20, and 50 fold with deionized water supplemented with (g/L) peptone-5.0, microcrystalline cellulose (Avicel®)-5.0, KH_2PO_4-2.5, and $MgSO_4.7H_2O$-0.5. A positive control was performed with domestic wastewater at its original concentration (undiluted), and the negative control was composed of deionized water. Assays were carried out in duplicate in 250-mL Erlenmeyer flasks incubated in a shaker with 120 rpm, at 28ºC, for 7 days.

3.2.6 Influence of carbon and nitrogen sources and inducers on cellulase production

The culture medium composition definition started with one-factor-at-a-time experiments for the choice of the best carbon source, nitrogen source, and cellulase inducer. The influence of supplementary carbon sources, sugarcane molasses, soybean bran, corn bran and sucrose (5 g/L), was tested. These supplementary agro

industrial carbon sources were added to the culture medium without any previous pretreatment. All of them were sterilized together with the culture medium. Sugarcane molasses was provided by Usina de Santa Teresinha, Maringá-PR, Brazil, corn and soybean bran were obtained from the local market. The estimated composition of each is presented in Table 3.2. Peptone, yeast extract, urea, and ammonium sulphate were tested as the nitrogen sources (5 g/L). Microcrystalline cellulose (Avicel), carboximethylcellulose (CMC), sugarcane bagasse, palm-Empty Fruit Bunches (EFB), cheese whey, and palm oil mill effluent (POME) were tested as cellulase inducers (5 g/L and 30 g/L). These agro-industrial inducers were added to the culture medium without any previous pretreatment. All of them were sterilized together with the culture medium. They were provided by industries of the region as follows: sugarcane bagasse (Usina de Santa Teresinha, Maringá-PR, Brazil), EFBs and POME (Biopalma, Belém-PA,Brazil) and cheese whey (Cooperativa Agroindustrial Witmarsum, Palmeira-PR, Brazil). Cellulase production was carried out in 150-mL Erlenmeyer incubated in a shaker, in duplicate, at 28ºC with 120 rpm for 7 days. Assays were conducted in duplicate.

3.2.7 Optimization of medium composition for cellulase production

A full factorial central composite rotatable design (CCRD) with 3 factors, 4 central points, and 6 axial points, for a total of 18 runs, was applied to test the effect of KH_2PO_4, $MgSO_4.7H_2O$, and microcrystalline cellulose on cellulase production (Table 3.3). These factors were previously selected as significant in a Plackett-Burman test (data not shown). Supplementary carbon sources did not present a positive effect on the process, which is why they were not included in further studies. The selected nitrogen source concentration, peptone, was fixed at 5 g/L. The software Statistica 7.0 (Statsoft, USA) was employed to generate the experimental matrix for use in the statistical analyses.

3.2.8 Cellulases production in bubble column bioreactor

Cellulases production was conducted in a 5-L bubble column reactor (BCR), with a 120 mm diameter and a 500 mm height, with a working volume of 3 L (Figure 3.1). Medium composition was defined previously. The bioreactor was maintained at 28ºC using a water bath, with an aeration rate of 0.3 volumes of air per volume of

medium per minute (vvm) (1 L of filtered air per minute) over 160 h. Samples of 15 mL were withdrawn every 24 hours and were centrifuged at 3000 rpm (2146 *g*) for 10 minutes. The supernatant was used for cellulases activity determination.

3.2.9 Cellulase activity determination

Total cellulase activity was measured according to the Filter Paper Assay (FPase) standard method described by Mandels and Reese (1957) and Ghose (1987), adapted for deep-well microplates according to Camassola and Dillon (2012). Filter paper stripes (0.6 x 1.0 cm) were employed as the substrate and 50 µL of samples were appropriately diluted in sodium citrate buffer (100 µL) (50 mM; pH4.8) and were incubated for 60 min at 50ºC. For the measurement of endoglucanases, a carboximethylcellulase assay (CMCase) was used, according to Ghose (1987), also adapted for deep-well microplates. Aliquots of 20 µL of carboxymethylcellulose 2% (CMCase) were used as substrate and 20 µL of the samples were appropriately diluted in the same buffer and incubated for 30 min at 50 ºC. After enzymatic reaction, the reducing sugars were measured by means of the dinitrosalicylic acid (DNS) method (MILLER, 1959). One unit of cellulase activity was defined as the amount of enzyme producing 1 µmol of reducing sugars per minute.

According to Dashtban et al. (2010), the FPase method was used to measure the total cellulases activity, and the CMCase method was used to determine the endoglucanases activity. All analytical determinations of CMCase and FPase activities were performed in triplicate.

Table 3.3 - Central Composite Rotational Design (CCRD) experimental design with 3 factors plus 4 center points: Influence of medium composition on FPase activity.

Run	KH_2PO_4 (g/L)	$MgSO_4.7H_2O$ (g/L)	Avicel® (g/L)	FPase (U/mL)
1	2.5(-1)	0.5(-1)	5.0(-1)	7.83 ± 1.46
2	2.5(-1)	0.5(-1)	13(+1)	4.37 ± 0.3
3	2.5(-1)	0.9(+1)	5.0(-1)	8.36 ± 0.08
4	2.5(-1)	0.9(+1)	13(+1)	4.60 ± 0.99
5	6.5(+1)	0.5(-1)	5.0(-1)	6.10 ± 0.57
6	6.5(+1)	0.5(-1)	13(+1)	2.67 ± 0.27
7	6.5(+1)	0.9(+1)	5.0(-1)	6.47 ± 0.55
8	6.5(+1)	0.9(+1)	13(+1)	3.43 ± 0.81
9	1.13(-1.68)	0.7(0)	9.0(0)	6.21 ± 0.83
10	7.86(+1.68)	0.7(0)	9.0(0)	4.23 ± 0.76
11	4.5(0)	0.36(-1.68)	9.0(0)	5.03 ± 0.53
12	4.5(0)	1.03(+1.68)	9.0(0)	5.59 ± 1.03
13	4.5(0)	0.7(0)	2.27(-1.68)	-----
14	4.5(0)	0.7(0)	15.7(+1.68)	2.64 ± 0.2
15(C)	4.5(0)	0.7(0)	9.0(0)	5.58 ± 0.15
16(C)	4.5(0)	0.7(0)	9.0(0)	4.71 ± 0.78
17(C)	4.5(0)	0.7(0)	9.0(0)	4.19 ± 1.00
18(C)	4.5(0)	0.7(0)	9.0(0)	4.87 ± 0.41

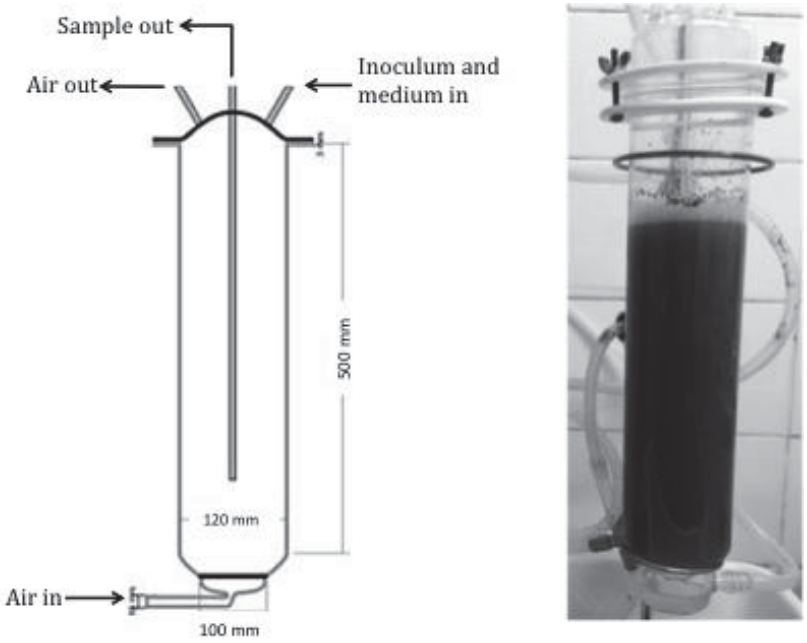

Figure 3.1 - Bubble column reactor for cellulase production: scheme and image of the reactor during fermentation process.

3.3 RESULTS AND DISCUSSION

3.3.1 Domestic wastewater characterization

The Sanitation Company of Paraná – SANEPAR provided the characterization data for the raw sanitary effluent, including the following parameters: pH, chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS), suspended solids (SS), and alkalinity. These data are related to the average values analyzed from monthly sampling, which occurred from January to May 2015, and are presented in Table 3.1. The other parameters are related to the average values from three samples quantified by the authors.

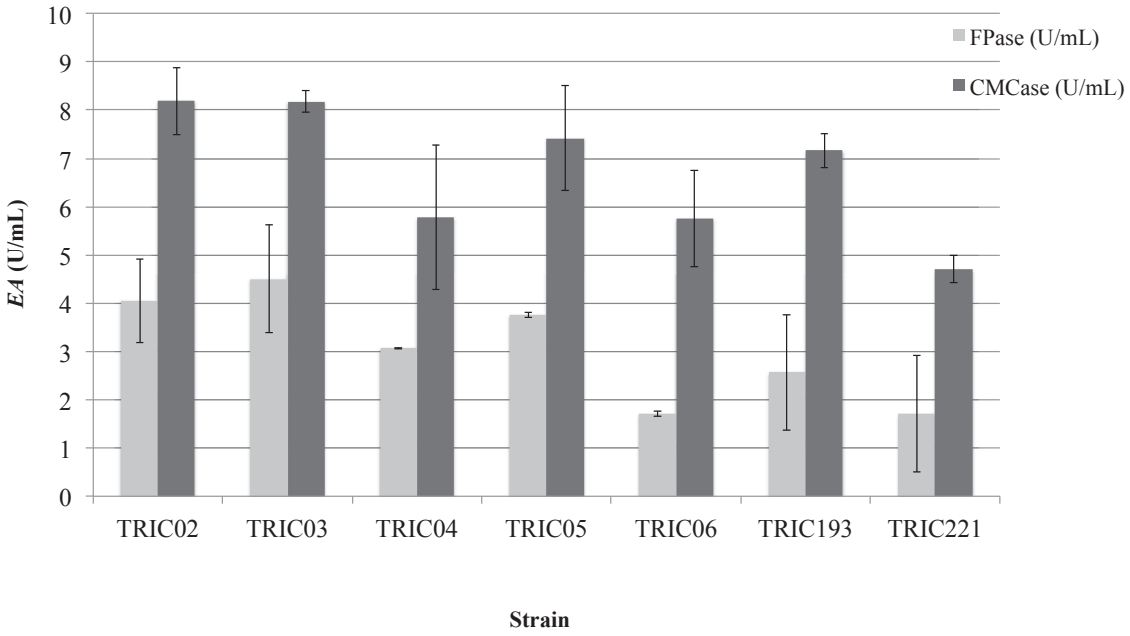

Figure 3.2 - Cellulase activities (*EA* - FPase and CMCase) produced by the tested strains using domestic sanitary wastewater.

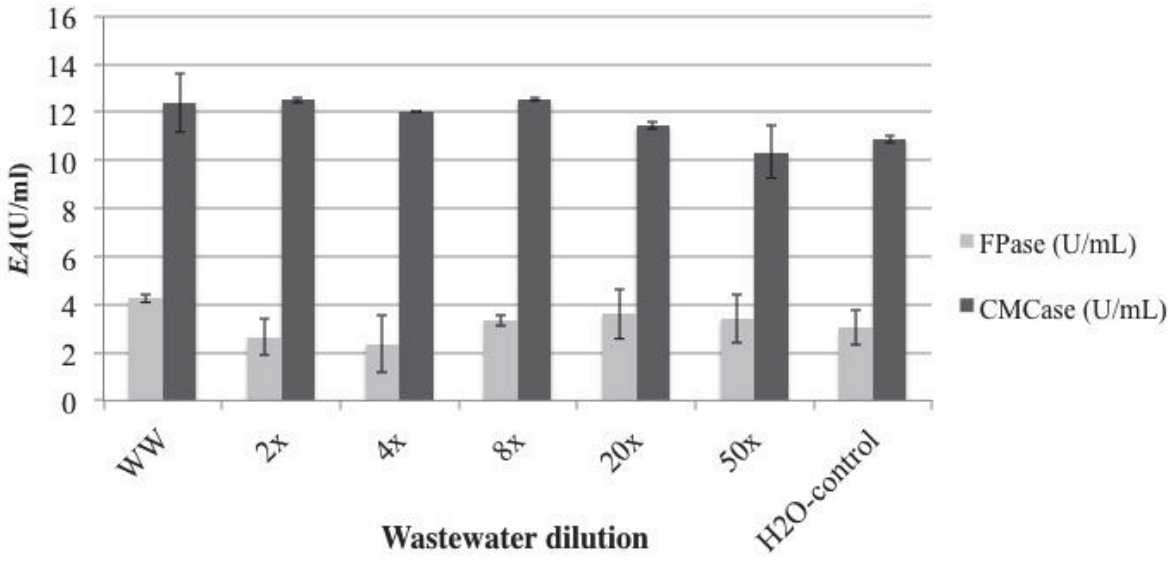

Figure 3.3 - Influence of different concentrations of domestic sanitary wastewater on cellulase production by *Trichoderma harzianum* TRIC03, in terms of FPase and CMCase. WW – undiluted domestic wastewater (positive control); H_2O – water-based medium (negative control).

The levels of the COD, BOD, and TSS of the evaluated domestic wastewater are lower than the levels for typical domestic wastewater (750, 350, and 400 mg/L, respectively) reported by Henze and Comeau, (2008) and the Athlone WWTP reported by Verster et al. (2014). This may relate to the dilution of wastewater, which is caused by the infiltration of rainwater into the municipal collection system.

The enzymatic hydrolysis of the solids collected from the wastewater revealed that 21.3% of the total mass was cellulose-like material after the weight-loss involved in its degradation. This fraction corresponds to 4.17 mg/L of cellulose-like material in the wastewater, suggesting its possible influence as an inducer in cellulase production when used as a culture medium formulation. Based on these results and considering the Atuba WWTP flow (1700L/s), 18.37 tons of cellulose-like material is estimated to be present per month in the raw effluent, revealing the potential of this material to be reused for biotechnological processes. A complete study should be conducted in order to obtain more reliable data on the concentration of cellulose in WWTPs. According to Ruiken et al. (2013), 79% of the total mass collected from sieved raw wastewater (0.35 mesh) could be considered cellulose after thermographic analysis of samples from the Blaricum WWTP (Netherlands).

The composition of wastewater is greatly influenced by the wastewater collection system. Thus, the analytical data suggests that the use of sanitary wastewater for the composition of the culture medium for bioprocesses should be carefully evaluated. The use of any residue and subproduct for enzyme production requires its characterization and the evaluation of the effect of its composition fluctuation on enzyme production and microbial metabolism.

3.3.2 Screening of cellulase producers

The selection of the fungal strains was mainly based on the enzyme activity obtained after the 6 days of fermentation. According to Figure 3.2, the strain *Trichoderma harzianum* TRIC03 showed the highest FPase (4.5 U_{FP}/mL) and CMCase (8.2 U_{CMC}/mL) activities and a good capacity for biomass aggregation in pellets with an approximated diameter of 0.25 cm (visual observation). Although the strain *Trichoderma harzianum* TRIC03D produced comparable FPase activities (3.9 U_{FP}/mL) and higher CMCase activities (8.9 U_{CMC}/mL), a very low *pellet* formation was

observed. In this case there was no correlation between *pellet* formation and enzyme production. The formation of *pellets* is a matter of interest with regard to the biomass separation from the supernatant. Also, according to Ahamed and Vermette, (2009), the fungus morphology affects the rheology of the fermentation and the production of enzymes. These authors described that the physiology and morphology of filamentous microorganisms in submerged cultures are dependent on the concentration of inoculum. High spores concentration inoculum leads to filamentous mycelium whereas low concentrations of spores produce pellets. In this work a high concentrated spore solution was used as inoculum and, for almost all tested strains, pellets were formed. This result suggests that other factors are affecting the morphology, as the agitation, temperature and culture medium. The strain *Trichoderma harzianum* TRIC03 was chosen for further experimentation. Another interesting candidate for further studies was the strain *Trichoderma harzianum* TRIC03, which achieved activities of 3.7 U_{FP}/mL and 7.4 U_{CMC}/mL, with satisfactory homogeneous pellet formation (visual observation).

3.3.3 Influence of the domestic wastewater concentration on cellulase production

According to the results presented in Figure 3.3, the concentration of domestic wastewater has no apparent influence on cellulase synthesis by *Trichoderma harzianum* TRIC03. Cellulase activity reached 4.2 U_{FP}/mL and 12.4 U_{CMC}/mL when using undiluted wastewater as the culture medium preparation. Using deionized water (control) as the solvent for the culture medium the cellulase activities were 3.0 U_{FP}/mL and 10.9 U_{CMC}/mL. These results show that sterilized domestic wastewater could be used as the basic solvent for cellulase production medium without creating any interferences, contamination, or inhibition in the tested concentrations. In addition, slightly higher cellulase activities were obtained using wastewater as the medium solvent compared to deionized water for the production medium. This fact is probably due to the presence of micronutrients in the domestic wastewater and to the increase in C, N, and P content, which is very interesting for microorganism's growth and, though, the production of different biomolecules.

Trichoderma harzianum TRIC03 showed its great capability of using municipal wastewater sludge as substrate and source of nutrients. In fact, the genus *Trichoderma* was already described by Drani et al. (2011), who used it in submerged

fermentation, as well as described by Verma et al. (2005). It is possible to affirm that these microorganisms can degrade the cellulosic part of municipal solid residues, aiming to improve a possible composting process after a previous separation of the inorganic part. Although, it would be necessary to understand how these microorganisms act in a non-sterile environment. It is also interesting to have in mind that some strains also produce other enzymes that could be explored in combination with cellulases aiming their application in different industrial processes.

3.3.4 Influence of supplementary carbon sources on cellulase activities

According to Figure 3.4, sucrose was the most influencing carbon source (8.0 ± 0.3 U_{FP}/mL; 11.6 ± 0.5 U_{CMC}/mL). Nevertheless, the control experiment, which did not have any supplementary carbon source, also resulted in high cellulases' activity (7.8 ± 1.0 U_{FP}/mL; 11.7 ± 0.7 U_{CMC}/mL), which was similar to that found when sucrose was added. Sugarcane molasses, corn and soybean bran did not present a positive influence, leading to lower cellulases' activities. With the aim of reducing the costs of the culture medium, the presented result is very positive. Also, the absence of an easily degradable substrate, such as sucrose, is interesting due to the reduction of possible contamination.

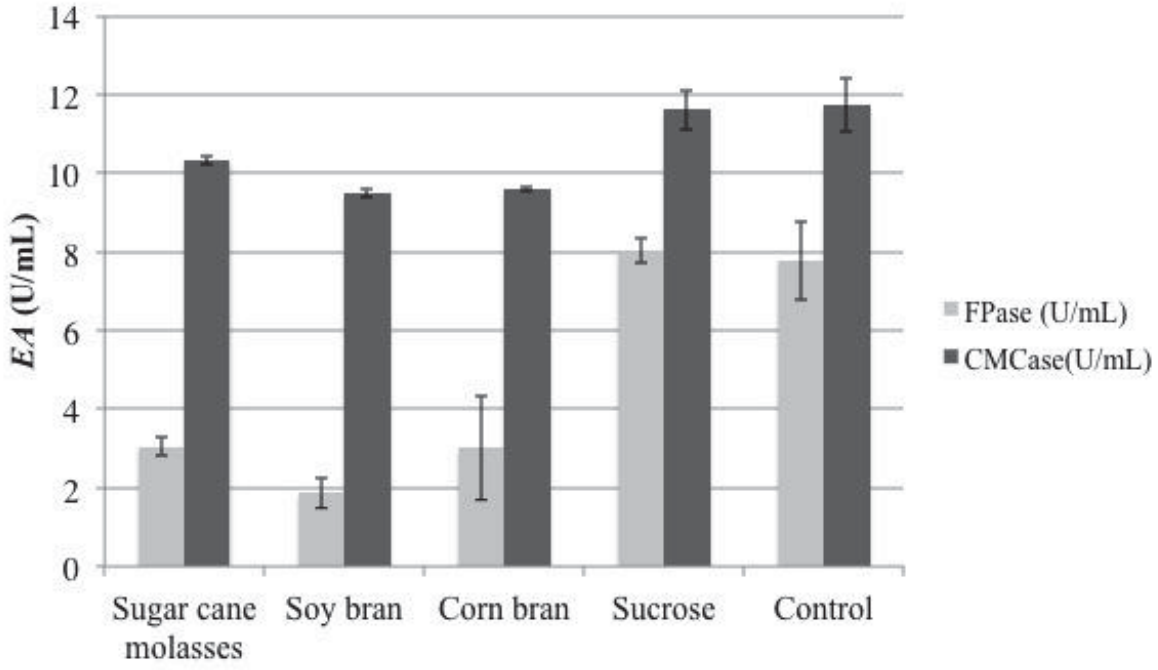

Figure 3.4 - Influence of different carbon sources added to domestic sanitary wastewater as base medium for cellulase production by *Trichoderma harzianum* TRIC03.

3.3.5 Influence of supplementary nitrogen sources on cellulase production

Peptone provided the highest cellulase activities (5.6 ± 0.4 U_{FP}/mL; 12.0 ± 0.3 U_{CMC}/mL), which were approximately 4.8 times superior to FPase and 1.9 times superior to CMCase activities obtained with ammonium sulfate, which were the second highest activities (1.1 ± 0.02 U_{FP}/mL; 6.4 ± 0.4 U_{CMC}/mL), as it can be seen in Figure 3.5. The use of urea was not positive in terms of cellulase activity (0.3 ± 0.007 U_{FP}/mL; 2.2 ± 0.07 U_{CMC}/mL), even when compared to the control experiment (0.3 ± 0.06 U_{FP}/mL; 2.9 ± 0.56 U_{CMC}/mL), which did not have an additional nitrogen source. So, peptone was chosen as the nitrogen source for further studies. Other nitrogen sources will also be tested in further assays, as peptone is a high cost component.

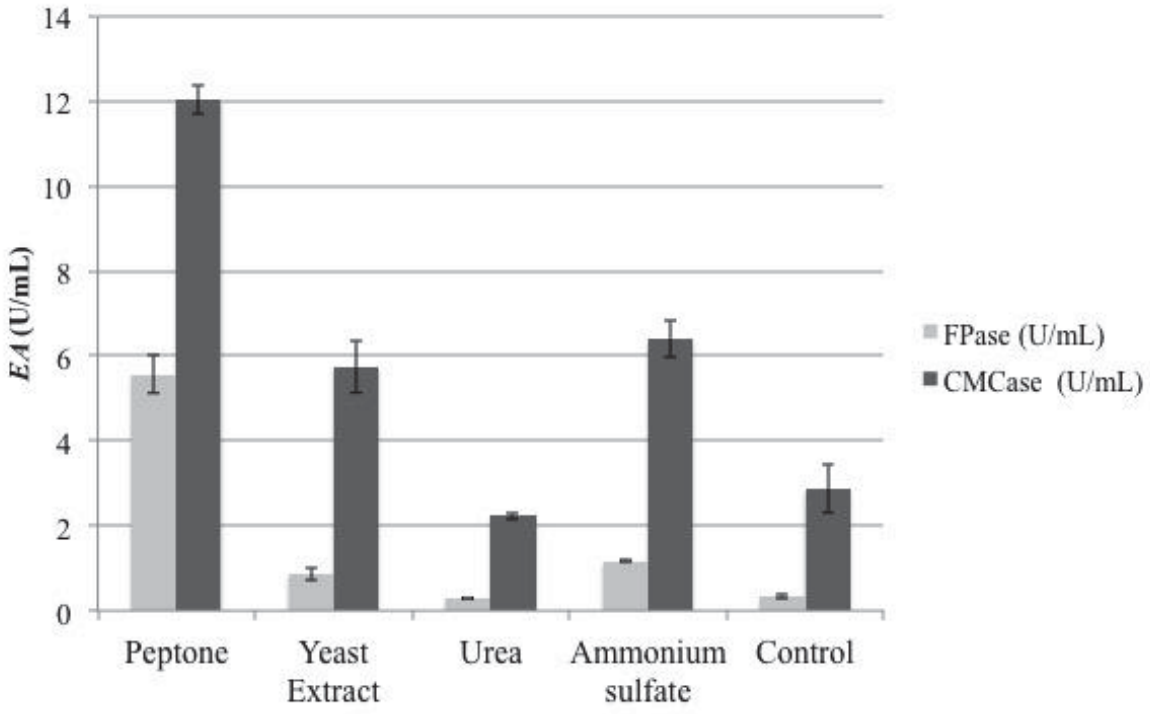

Figure 3.5 - Influence of nitrogen sources added to domestic sanitary wastewater as base medium on cellulase production by *Trichoderma harzianum* TRIC03.

3.3.6 Influence of different inducers on cellulase production

As presented in Figure 3.6, microcrystalline cellulose (Avicel®) was the best inducer in terms of promoting the highest enzyme activities (5.0 ± 0.6 U_{FP}/mL; 14.4 ± 0.4 U_{CMC}/mL). Liming and Xueliang (2004) obtained similar results (5.25 U_{FP}/mL) using sanitary wastewater sludge (0.5%) supplemented with microcrystalline cellulose (24.6 g/L) and corn cob (40 g/L), which were added to the medium proposed by Mandels and Reese (1957), composed of 11 compounds. In this work, only the compounds KH_2PO_4, MgSO4, peptone, and microcrystalline cellulose were added to the sanitary wastewater (instead of purified water).

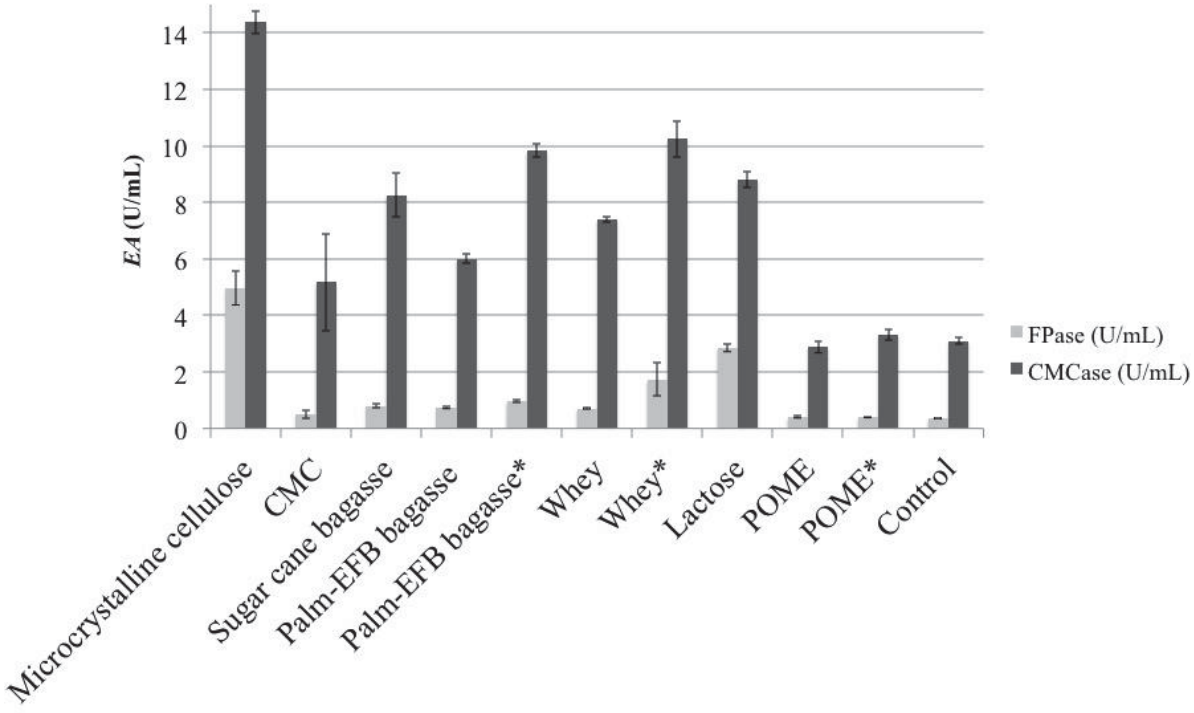

Figure 3.6 - Influence of inducers (5 g/L and *30 g/L) on cellulase production by *Trichoderma harzianum* TRIC03 in combination with domestic sanitary wastewater).

Between the other tested inducers, those of cellulosic composition, such as sugarcane bagasse and Palm Empty Fruit Bunches-EFB, promoted enzyme activities comparable to those that were achieved with the use of lactose, a well-known cellulase inducer (ADSUL et al., 2004). Dairy whey also seems to be an interesting candidate for cellulase induction as it is a by-product of the dairy industry. The

increase of dairy whey concentration in the culture medium from 5 g/L to 30 g/L led to an increase of 147% in FPase and 39% in CMCase activities. The same behavior was observed in the case of the Palm-EFB bagasse as cellulase inducer, with an increase of 30% in FPase and 63% in CMCase activities.

The presence of cellulose in the culture medium is recognized by constitutive conidial cellulases, which release small amounts of cellooligosaccharides from the cellulose. These are then assimilated by the fungus and act as inducers for further synthesis of large amounts of cellulases (AHAMED and VERMETTE, 2009). Although the use of cellulosic substrates, such as sugarcane bagasse and Palm-EFB, resulted in lower cellulase activities compared to the use of microcrystalline cellulose, the high costs related to this inducer should be taken into account. The re-use of wastes or subproducts as substrate also contributes for a reduction in the environmental impact of their disposal.

3.3.7 Optimization of medium composition for cellulase production - Central Composite Rotatable Design (CCRD)

According to the Pareto chart (data not shown), Avicel® was the most significant factor on cellulase production. Higher cellulase (FPase) activities were obtained in runs 1 and 3 (7.83 and 8.36 U_{FP}/mL, respectively) using lower concentrations of KH_2PO_4 (2.5 g/L) and Avicel® (5.0 g/L), as it can be seen in the Table 3.3. However, lower values of cellulase (FPase) activities were achieved in runs 6 and 14 (2.6 and 2.6 U_{FP}/mL, respectively), with higher concentrations of KH_2PO_4 (4.5 and 6.5 g/L) and Avicel® (13.0 and 15.7 g/L). The influence of $MgSO_4$ concentration was not significant on cellulase activity.

The reason why higher cellulase activities were obtained using lower Avicel® and KH_2PO_4 concentrations in the culture medium, was probably due to the presence of these components in the sanitary wastewater. After optimization, the final culture medium formulation was the following (g/L): peptone-5.0, Avicel-5.0, KH_2PO_4-2.5, and $MgSO_4.7H_2O$-0.5. The effects of two independent variables (Avicel® and $MgSO_4$) on cellulase production were predicted by the following polynomial regression equation:

$$Z \text{ (cellulase activity, U/mL)} = 12.48 - 0.61Y + 0.003Y^2 - 0.66X + 0.009X^2 + 0.153*0.7Y + 0.01YX + 0.01*7X - 0.429$$

where cellulase activity (U/mL) is a function of KH_2PO_4 (Y) and Avicel® (X). The coefficient of determination (R^2) was 0.961, which ensures satisfactory data and indicates that approximately 96.1% of the variability in the dependent variable, cellulase activity, could be explained by the model. Alam et al. (2008) incremented the enzymatic activity in 48% after the statistical optimization of the culture conditions, achieving 10.2 U_{FP}/mL (0.14 U/mLh) using sanitary wastewater sludge (0.75% w/w) supplemented with wheat flour (2% w/w) with the fungus *Trichoderma harzianum* in Erlenmeyer flasks.

3.3.8 Kinetics of cellulase production in bubble column bioreactor

Cellulase production was performed in a bubble column reactor (BCR) using the conditions determined before. The maximum cellulase activity was achieved after 144 hours for CMCase (8.3 U_{CMC}/mL) and for FPase (1.46 U_{FP}/mL), as shown in Figure 3.7. Maximum productivities were observed after 120 hours, resulting in 64.6 U_{CMC}/Lh and 10.2 U_{FP}/Lh for CMCase and FPase, respectively. In this work, the results were 218.7% higher in terms of productivity and 284.2% in terms of enzymatic activity compared to the results obtained by Drani et al. (2011). These authors achieved 0.38 U_{FP}/mL of cellulase activity and a productivity of 3.2 U_{FP}/Lh using sanitary wastewater sludge (0.5%) supplemented with microcrystalline cellulose (2%) and Palm-EFB (0.2%) in experiments performed in Erlenmeyer flasks with 50 mL of fermentation medium over 120 h at 30 ºC and 150 rpm, using the fungus *Trichoderma* RUT-C 30. Verma et al. (2007) also used the sludge of the sanitary wastewater treatment process (0.03% w/v) after alkaline hydrolysis as a component of the culture medium, reaching 160 U_{FP}/mL (4.44 U_{CMC}/mLh) for CMCase. In this case, the hydrolysis was probably responsible for the higher results on cellulase activity and productivity, since it promotes hemicellulose degradation, with the release of pentoses, and the susceptibility of cellulose of the sludge that was certainly efficiently used by the employed strain for cellulase production. The sanitary wastewater, presented in this work, needs additional sources of peptone, cellulose, phosphate and magnesium. Even though, it is still a very important issue. The

mixture of the sanitary wastewater with sludge, in an appropriate proportion, could also be an interesting approach for cellulase production. Liming and Xueliang, (2004) produced cellulases with *Trichoderma reesei* ZU-02 in 500-mL Erlenmeyer flasks and in a 30 m^3 stirred tank bioreactor. These authors achieved 31.3 U_{FP}/Lh of maximum productivity in Erlenmeyer flasks and 57 U_{FP}/L.h in a stirred tank bioreactor over 96 hours. These authors worked with corn cob residue, as part of the substrate, but used it together with the medium proposed by Mandels et al. (1981), which has compounds that strongly influence cellulase production. Saini et al. (2015) achieved the maximum cellulase activity of 1.29 U_{FP}/mL after 120 hours, resulting in a maximum productivity of 10.7 U_{FP}/Lh using a 7-L stirred tank bioreactor maintained at 28ºC, pH 5.0, and 1.5 vvm, with the fungus *Penicillium oxalicum* using a synthetic culture medium.

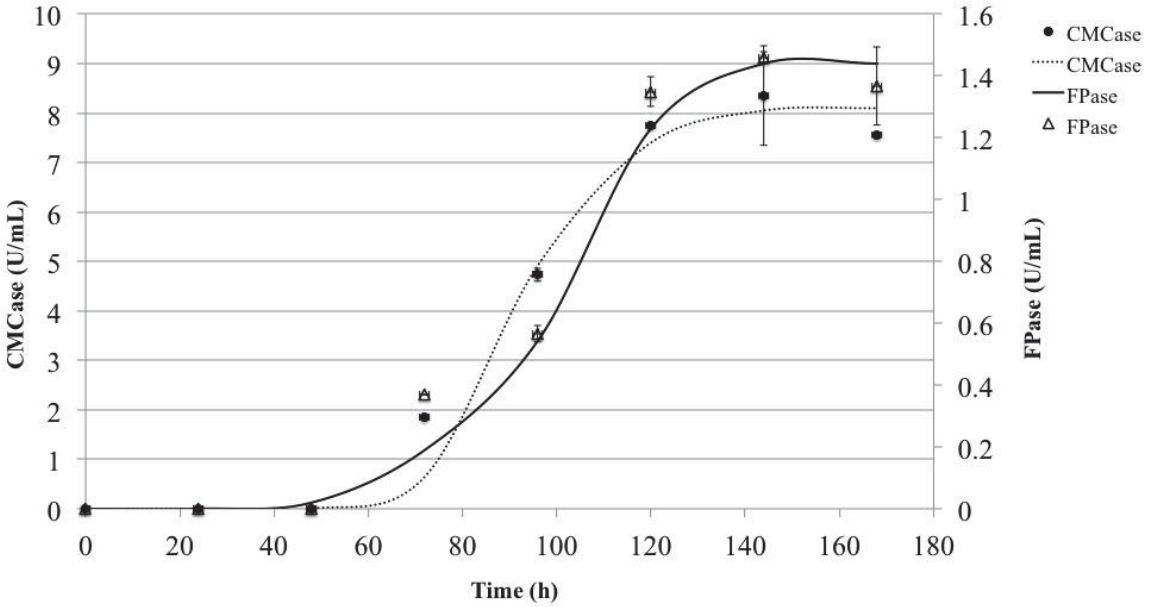

Figure 3.7 - Kinetics of cellulases production in BCR by *Trichoderma harzianum* TRIC03 using domestic sanitary wastewater: FPase and CMCase evolution.

The results obtained in this work are not comparable to some commercial cellulases available in the market, since they are concentrated and formulated (Table 3.4). The cellulases's crude extract, with CMCase and FPase activities, was not concentrated by ultrafiltration or formulated. These procedures are normally performed for commercial enzymes, which lead to very high activities and stabilities.

The sequence of experiments that were carried out in this work is presented in Table 3.5, which involved: Strain screening, study of the influence of carbon and

nitrogen sources addition, optimization of medium composition and, finally, the kinetics of cellulases production in a BCR. It is possible to conclude that there was an increase of enzyme activity with the sequence of experiments with very good productivities when these results are compared with literature. The final culture medium composition, after optimization, is similar to the starting culture medium defined by Alam et al. (2012) for cellulase production, except the use of domestic wastewater instead of the sludge that was proposed by the authors.

Table 3.4 - Reported cellulases activities and productivities obtained with different microorganisms and medium composition conditions.

Microorganism	Substrate	Bioprocess condition	Enzyme activity	Productivity	Reference
Trichoderma harzianum	raw sanitary wastewater	30 ºC; 1 [1]vvm; 144 h	1.4 FPU/mL	64.0 U/L.h (CMCase) 10.1 U/L.h (FPase)	THIS WORK
Trichoderma rut-c 30	Sanitary wastewater sludge + microcrystalline cellulose + palm-efb	30 ºC;150 [2]rpm; 120 h	0.38 FPU/mL	3.2 U/L.h	RANI et al., (2011)
Trichoderma viride	Sanitary wastewater sludge	200 [2]rpm; [3]d.o 30%		4444 U/L.h	VERMA et al., (2007)
Trichoderma reesei zu-02	corn cob residue	96 h	5.5 FPU/mL	57 U/L.h	LIMING AND XUELIANG, (2004)
Penicillium oxalicum	synthetic medium	28 ºC;120 h; 1.5 [1]vvm	1.3 FPU/mL	10.7 U/L.h	SAINI et al., (2015)
Trichoderma viride	sugar cane bagasse	30 ºC; 180 [2]rpm	1.0 FPU/mL	3.66 U/L.h	ADSUL et al., (2004)
Trichoderma harzianum	delignified cellulose	30 ºC; 200 [2]rpm		0.93 U/L.h	DE CASTRO et al., (2016)
Trichoderma viride	sugar cane bagasse + wheat bran	28 ºC; 90 [2]rpm		0.58 U/L.h	LAN et al., (2013)
Celluclast 1.5 l (novozymes)			56 FPU/mL		SINGHANIA et al., (2010)
GC880 (genecor)			<5FPU/ mL		SINGHANIA et al., (2010)
Cellulase 2000l (rhodia-danisco)			10 FPU/mL		SINGHANIA et al., (2010)

[1]volume of air per volume of medium per minute
[2]revolutions per minute
[3]dissolved oxygen

Table 3.5 - Steps of cellulases production: From strain screening to the kinetics in bubble column reactor.

Experiment	Medium composition	Bioprocess condition	Tested condition	FPase	CMCase	Result
1. Strain screening	peptone-5.0; Avicel®-5.0; KH_2PO_4-2.5; $MgSO_4$-0.5; raw domestic wastewater	28 ºC; 120 [5]rpm; 168 h	11 strains	4.5 U/mL	8.2 U/mL	Selection of *Trichoderma harzianum* TRIC03 strain
2. Influence of domestic wastewater concentration	peptone-5.0; Avicel®-5.0; KH_2PO_4-2.5; $MgSO_4$-0.5;	28 ºC; 120 [5]rpm; 168 h	Diluted raw domestic wastewater 0, 2, 4, 8, 20, 50 fold	4.3 U/mL	12.4 U/mL	Undiluted wastewater
3. Influence of carbon source	peptone-5.0; Avicel®-5.0; KH_2PO_4-2.5; $MgSO_4$-0.5; raw domestic wastewater	28 ºC; 120 [5]rpm; 168 h	Absence of carbon source, sugarcane molasses, soybean bran, corn bran and sucrose (5 g/L)	7.8 U/mL	11.7 U/mL	Absence of supplementary carbon source
4. Influence of nitrogen source	Avicel®-5.0; KH_2PO_4-2.5; $MgSO_4$-0.5; raw domestic wastewater	28 ºC; 120 [5]rpm; 168 h	Absence of nitrogen source, peptone, yeast extract, urea, and ammonium sulphate	5.6 U/mL	12.0 U/mL	Peptone (5g/L)
5. Influence of cellulase inducers	peptone-5.0; KH_2PO_4-2.5; $MgSO_4$-0.5; raw domestic wastewater	28 ºC; 120 [5]rpm; 168 h	Avicel®, CMC[1], sugar-cane bagasse, palm-EFB[2], whey, and POME[3] (5 and 30 g/l)	5.0 U/mL	14.4 U/mL	Microcrystalline cellulose (5g/L)
6. Bubble column reactor production	peptone-5.0; Avicel®-5.0; KH_2PO_4-2.5; $MgSO_4$-0.5; raw domestic wastewater	28 ºC; 0.3 [4]vvm; 144 h		1.4 U/mL; 10.2 U/L.h	8.3 U/mL; 64.6 U/L.h	----

[1]CMC: carboximethylcellulose
[2]Palm-EFB: Palm-Empty Fruit Bunches
[3]POME: palm oil mill effluent
[4]volume of air per volume of medium per minute
[5]revolutions per minute

This is the first publication that has specifically reported the use of raw sanitary wastewater as culture medium for cellulase production. It is also important to point out the great advantages presented by this process, which employs a simple and cheap culture medium, and the possibility of installing *in situ* small-to-medium capacity cellulase production unities linked to urban wastewater treatment plants. Although many research studies have focused on the use of alternative substrates, such as wastewater sludge or agro industrial residues as a cheap source of nutrients, the reuse of domestic wastewater itself as the medium solvent is extremely important, as water has become a scarce and expensive resource, mainly for the industrial sector.

3.3.9 Future perspectives for a cellulolytic complex

The presented work focused in the evaluation of the viability of cellulases production (endo and exo-glucanases) using domestic wastewater as substrate. However, the application of the produced enzymes dictates the technological route for its production as well as the requirements for its application. In a wide range of applications of cellulases, the hydrolysis of lignocellulosic biomass for biofuel production has been the focus of many researchers and companies around the world, looking for the development of sustainable processes.

An efficient cellulolytic system for lignocellulosic biomass degradation requires, basically, the presence of three classes of enzymes acting synergistically: cellobiohydrolases (or exo-1,4-beta-glucanases) (EC 3.2.1.91), endo-1,4-beta-glucanase (EC 3.2.1.4) and β-glucosidades (EC 3.2.1.21). The endo-glucanases hydrolyzes the amorphous regions of cellulose, decreasing its degree of polymerization. Cellobiohydrolases hydrolyse the cellulose polymer from the ends, releasing cellobiose. Finally, the β-glucosidades hydrolyse the cellobiose to glucose (SØRENSEN et al., 2013). The conversion of cellobiose to glucose performed by β-glucosidases is related as the limiting step in the production of biofuels from lignocellulosic materials and it is one of the bottlenecks for a viable biomass based biofuel production (TIWARI et al., 2013).

Trichoderma species are well known as efficient cellulose degraders and have been widely studied. *T. reesei* is related as a cellulase producer, where

cellobiohydrolases account for 60 - 85% of the total secreted proteins, followed by endoglucanases accounting for 10 -15% of the total secreted proteins (MA et al., 2011). Although *Trichoderma* is a well-established cellulase producer strain, β-glucosidases are excreted in very low concentrations. *Trichoderma* do not produce sufficient β-glucosidase concentrations for a complete and efficient industrial cellulose hydrolysis (SØRENSEN et al., 2013). In addition, β-glucosidases have an anti-inhibitor effect in the hydrolysis process through the inhibition of endo and exo-glucanases activity. Strategies to overcome this limitation have been emerging, as the addition of an external source of β-glucosidades, produced by another microorganism (ABDELLA et al., 2016) as well as the co-cultivation of two fungal strains producing endo, exo-glucanases and β-glucosidase together (WEN et al., 2005) and the heterologous expression of β-glucosidase gene in a host microorganism (MA et al., 2011).

Commercial enzymes for biomass hydrolysis were initially prepared as separate fungal fermentation products that needed to be combined for efficient hydrolysis. The combination of the commercial products Celluclast (a *T. reesei* cellobiohydrolase and endo-glucanase) and Novozymes 188 (an *A. niger* β-glucosidase) by Novozymes is an example of enzyme cocktail for cellulosic biomass hydrolysis. Recently, enzyme companies as Novozymes and Genecor are replacing this combination of two products for a single product containing the full array of enzymes for cellulosic biomass hydrolysis (SØRENSEN et al., 2013). In addition the commercial cellulolytic cocktails for biomass hydrolysis are enzyme preparations from genetically modified *Trichoderma reesei* (TIWARI et al., 2013).

Abdella et al. (2016) achieved the highest productivity of β-glucosidase (1.78 U_{BGL}/mLday) and enzyme activity (9.6 U_{BGL}/mL) using a rotating fibrous bed bioreactor operated at repeated fed batch mode using the fungus *Aspergillus niger*. Lan et al. (2013) also performed experiments in a rotating fibrous bed bioreactor although testing the fungus *Trichoderma viride*. By this way it was possible to achieve 0.22 U_{BGL}/mL of β-glucosidase and a productivity of 0.01 U_{BGL}/mLd, lower values than that obtained by Abdella et al. (2016).

Wen et al. (2005) performed the simultaneous production of cellulase and β–glucosidase co-cultivating the fungi *Trichoderma reesei* and *Aspergillus phoencis* with dairy manure. Higher β-glucosidase activities were achieved with the mixed

culture (0.64 U_{BGL}/mL) in comparison to the production with *T. reesei* alone (0.1 U_{BGL}/mL).

The heterologous expression of the β-glucosidase gene from *Penicillium decumbers* in a *Trichoderma reesei* strain improved its activity in 8-fold, from 4.37 U_{BGL}/mL (*T. reesei* parental strain) to 34.31 U_{BGL}/mL (MA et al., 2011). Wang and Xia, (2011) demonstrated that the β-glucosidase gene from *A. niger* was cloned and expressed in *T. reesei*, resulting in an enzyme activity of 5.3 U/mL, showing an increase of 106-fold in β-glucosidase activity compared to the host strain (0.05 U_{BGL}/mL).

The production of an efficient enzyme cocktail for lignocellulosic biomass hydrolysis surely passes through the evaluation of other strategies for the production of higher β-glucosidases activities. According to scientific data, the use of other microorganisms than the *Trichoderma* strain employed in this work could be tested. In this way, a future perspective would be the evaluation of β-glucosidases production using domestic wastewater as substrate, by an individual *Aspergillus* strain culture with further enzymatic preparation with endo and exo-cellulases from *Trichoderma*, or a co-cultivation of *Aspergillus* strains with the already tested *Trichoderma* strain.

3.4 CONCLUSIONS

Domestic sanitary wastewater was employed as an alternative fermentation medium for cellulase production, a medium-value bioproduct, in submerged fermentation with high activities (8.3 U_{CMC}/mL; 1.46 U_{FP}/mL) and productivities (64.6 U_{CMC}/Lh; 10.2 U_{FP}/Lh). It was possible to define the most suitable sources of carbon (none), nitrogen (peptone), and inducer (microcrystalline cellulose) for this process. The process was developed in BCR-type bioreactors, showing the possibility of easy scaling-up and operation. It is possible to conclude the viability of the use of sanitary wastewater as an alternative substrate for cellulase production, thereby contributing to the development of sustainable processes for enzyme production.

4 SIMULTANEOUS CELLULASE PRODUCTION, RECOVERY AND EFFLUENT TREATMENT - A WASTEWATER BIOREFINERY APPROACH

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ABSTRACT

The production of important biomolecules using effluents from wastewater treatment plants is emerging as an alternative for their concomitantly re-use and treatment. Raw domestic wastewater was used as a culture medium for the cellulases production in bubble column (BCR) and stirred tank (STR) reactors using the strain *Trichoderma harzianum* TRIC03-LPBII. Maximum cellulase activity and productivity of 31 U/mL and 645 U/Lh were achieved, respectively, with BCR. The chemical oxygen demand (COD) and nitrogen concentration were reduced by 98% and 78%, respectively, during the fermentation process. The fermented broth was subjected to microfiltration (0.45 μm) and ultrafiltration procedures with membranes of different cut-offs (5, 10 and 30 kDa). In addition, a pretreatment with activated charcoal was also tested. The separation process led to a cellulase recovery of 73.5% using a 30 kDa membrane, resulting in activity concentration of 4.23-fold with membrane fouling of 19.81%. The membrane process led to the reduction of COD and nitrogen concentration by 81.37% and 52.9%, respectively. The results showed the great advantage of producing cellulases using domestic wastewater with a significant reduction of its environmental impact. These results contribute to the development of sustainable bioprocesses approaching the biorefinery concept.

Keywords: Cellulase production, Domestic wastewater*, Trichoderma harzianum*, Ultrafiltration, Bioreactor

4.1 INTRODUCTION

Increasing attention paid to the development of sustainable industrial processes imposes the development of new processes with reduced environmental impact. Organic residues have been used for the production of biomolecules such as enzymes in the biotechnology industry. Some residues are carbon, nitrogen and other nutrients sources, and their use contributes for the reduction of the production costs and the sustainability of the process. Cellulase production using alternative substrates has been emerging because it is an interesting way to reduce enzyme production costs, which is still one of the major bottlenecks for application, i.e., second-generation biofuel. The composition of the culture medium significantly affects product concentration, yield and volumetric productivities, which are of central importance for economically viable production. A wide range of cellulase production reports is available in the literature with the use of agro-industrial residues, mainly from lignocellulosic nature, such as sorghum bran, corn cob, sugarcane bagasse, wheat straw and elephant grass, resulting in cellulase activities of 0.1 - 23.0 filter paper units per millilitre (U_{FP}/mL) (ESTERBAUER et al., 1991). Other cellulosic and non-cellulosic carbon sources or cellulase inducers have been also described such as solka floc, microcrystalline cellulose, lactose, sucrose and sophorose (MORIKAWA et al., 1995). Lactose is reported as one of the most effective carbon sources, in combination or not with cellulosic residues, for fungal cellulases production.

The recovery of nutrients from wastewater treatment plants (WWTP) has been emerging as a strategy for the production of biomolecules, since there are huge amounts of carbon, nitrogen and phosphorous in its composition. The use of WWTP residues such as sludge for cellulase production has been described by some authors (ALAM et al., 2008). Libardi et al. (2017) reported the feasibility of using sanitary wastewater for cellulase production by the fungus *Trichoderma harzianum*. The use of wastewater as part of the culture medium for cellulases production allows the simultaneous production of an added-value product and the reduction of the pollution charge of the effluent through its consumption as carbon and nutrient sources for microbial growth and energy production. Other authors (ALAM et al., 2003) evaluated the reduction of the COD as an indicator of the organic matter

reduction during fermentation processes using wastes like cheese whey, starch processing wastes, apple distillery waste and olive mill wastewaters.

However, the use of such residues brings a new challenge: the culture medium presents many insoluble matters, unwanted metals and other components. So, the use of wastewater adds further substances to the complex fermented broth, which already contains proteins, biomass and other biomolecules. In this way, an efficient separation and purification process must be developed for enzyme recovery.

Membrane technologies are adequate for the downstream processing of biological molecules because they operate at low temperatures and pressures and involve no phase changes or chemical additives, thereby minimizing the denaturation, deactivation and degradation of the products. A common application of ultrafiltration (UF) in downstream processing is for product concomitant separation and concentration (CHARCOSSET, 2006). The use of UF for cellulase separation and concentration was described using membranes with a molecular weight cutoff (MWCO) varying from 5 to 100 kDa (QI et al., 2012) for cellulases with molecular weight ranging from 60 to 90 kDa (MORES et al., 2001). The UF allows for the concentration of cellulase between 50 and 75 U_{FP}/mL (RODRIGUES et al., 2014). Before the UF step, microfiltration (MF) can be used for the clarification of the culture broth and to retain suspended solids > 0.2 μm (MORES et al., 2001). Rodrigues et al., (2014) reported the activity recovery of 38% of a commercial cellulase from wheat straw hydrolyzate. Qi et al. (2012) reached a protein recovery of 89.4%, 73.9% and 79.7% using MWCO of 5, 10 and 30 kDa. Although the use of UF has been described as an interesting way to ensure cellulase separation and concentration, there are some operational problems related to the fouling and concentration polarization, which lead to the reduction of the permeate flux and possibly damage of the membrane. These processing problems may increase the cleaning costs, lowering the competitiveness of this process (SHI et al., 2014).

UF processes have also been described as techniques suitable for the purification of wastewater, together with MF, nanofiltration and reverse osmosis (RO) (RYAN et al., 2009). The efficiency of these processes could be measured in terms of the reduction of the COD, as well as total nitrogen. The COD has been employed to determine the total amount of organic matter in domestic wastewater (WAN et al., 2016). Authors have reported some restrictions of using UF regarding the fouling effects of the filtration of secondary effluent of domestic WWTPs, which implies an

increase of associated process costs that may restrict its application (VERSTRAETE et al., 2009). However, the use of separation and concentration of high added value products, such as cellulases, could amplify the interest of using UF processes. This approach is in accordance with wastewater biorefinery concepts, taking advantage of product productivity maximization in combination with the reduction of effluent concentration (KLEEREBEZEM and LOOSDRECHT, 2007). In a UF process, where the product of interest is in the retentate fraction, the permeate fraction could be seen as the effluent of the separation process, requiring adequate treatment and destination.

This work aims to demonstrate the production of cellulases using domestic wastewater as substrate in Bubble Column (BCR) and Sirred Tank Reactor (STR) followed by its recovery and concentration from the culture broth using MF and UF. Simultaneously, the reduction of the pollution charge of the effluent was evaluated by monitoring the COD and nitrogen as environmental parameters.

4.2 MATERIAL AND METHODS

4.2.1 Production of cellulases using domestic wastewater

Cellulase production was performed using *Trichoderma harzianum* (TRIC03) from the Federal University of Paraná Culture Collection, Bioprocess Engineering and Biotechnology Department (LPBII-UFPR). The culture medium was composed of lactose (11.9 g/L), peptone (5.0 g/L), KH_2PO_4 (2.5 g/L), and $MgSO_4.7H_2O$ (0.3 g/L), which were added to the raw domestic wastewater. Libardi et al. (2017) proposed the use of domestic wastewater as a culture medium base for cellulase production. The raw domestic wastewater was collected at the Atuba Municipal Wastewater Treatment Plant from the Sanitation Company of Paraná (SANEPAR), located in Curitiba, Brazil. A fungal spore suspension of approximately 10^7 spores/mL was used as inoculum.

Cellulases production was tested in 4 bioreactors systems: (R1) 1-L bubble column reactor (BCR); (R2) 1-L immobilized biomass fixed bed BCR; (R3) 4-L stirred tank reactor (STR) and (R4) 3-L BCR. The R2 system had 80% of its volume filled with K1 Kaldness support (Veolia Water Technology, France), which is generally used for wastewater treatment purposes promoting biological biofilm formation over

its surface, with a surface area of 500 m^2/m^3. The STR had a total volume of 5 L, equipped with flat blade impellers and temperature control, with agitation rate of 120 rpm.

All the bioreactors were maintained at 28ºC with an aeration rate of 0.3 vvm (1L of filtered air per minute) during 120 h. Samples of 15 mL were withdrawn every 24 h and were centrifuged at 3000 rpm (2146 *g*) for 10 min. The supernatant was used for cellulases activity determination, protein concentration, reducing sugars, COD, nitrogen and elementary analysis. The separated solids fraction was employed for biomass determination by dry weight.

4.2.2 Cellulase separation and purification procedures

Cellulase separation and purification by MF and UF were performed using a VivaFlow 200 (Sartorius Stedim, Germany) crossflow filtration system. The system was equipped with a peristaltic pump connected to a flow restrictor and the pressure indicator to polyethersulfone (PES) membrane cassettes and reservoirs. The membranes have a surface area of 200 cm^2. The MF membrane has a pore size of 0.2 µm, and the UF membranes' Molecular Weight Cut-Off (MWCO) are 5 kDa, 10 kDa and 30 kDa.

Before experiments, membranes were rinsed by pumping approximately 400 mL of ultrapure water, since they are stored with a solution of 10% ethanol, according to the manufacturer. After use, membranes were cleaned by recirculating 250 mL of 0.5 mM NaOCl: 0.5 M NaOH for 30-40 minutes, according to the manufacturer's instructions. After fermentation, the cellulase-containing crude broth produced in the BCR was subjected to a vacuum filtration using a Whatman no. 1 filter paper. The filtrate then passed through MF and UF. The experiments were performed at room temperature, and the samples in the feed tank, retentate and permeate reservoirs were maintained and cooled (in an ice bath). A sample volume of 250 mL was used for each experiment. All experiments were carried out with a flow rate of 220 mL/min and a transmembrane pressure (TMP) of 1 bar. Graduated reservoirs were used, in which it was possible to monitor the volumes of permeate and retentate during the process.

As an alternative process, the cellulase crude extract was treated with powdered activated charcoal (AC) (Carbomafra, Brazil), with surface area between

500 to 1200 m^2/g, at a concentration of 20 g/L (POLETTO et al., 2015), aiming to evaluate its influence on the MF and UF processes. The pretreatment was performed in 500-mL Erlenmeyer flasks with 250 mL of crude extract and 5 g of activated charcoal. Flasks were incubated in a rotatory shaker at 120 rpm for 30 min at 25 ºC. Samples were centrifuged at 3034*g* (6000 rpm) for 10 minutes, and the collected supernatant was filtered through Whatman paper no 1. Samples were then microfiltrated (0.2 µm) and ultrafiltrated (30 kDa membrane).

The permeate flux J (L/m^2h) was determined and is defined as the flow of a volume of sample V (L) through the membrane surface area A (m^2) during a time interval t (h), according to equation 1 (RASHID et al., 2013).

$$J = \frac{1}{A}.\frac{dV}{dt} \quad (1)$$

The hydraulic permeability was determined with the pumping of pure water through the system before and after the samples' processing. The TMP and the volumes of the reservoirs were monitored, and the permeate flux was calculated. The water permeability of the membrane Lp (L/m^2hbar) was expressed as the permeate flux of deionized water (L/m^2h) at a specific TMP (bar), according to equation 2 (QI et al., 2012).

$$Lp = \frac{J}{TMP} \quad (2)$$

Irreversible fouling (IF), which is defined as the decrease of the membrane pure water permeability after filtration (Lpa) divided by the pure water permeability before filtration (Lpb), was determined according to equation 3 (QI et al., 2012).

$$IF\ (\%) = \frac{Lpb - Lpa}{Lpb} \times 100 \quad (3)$$

The volume concentration ratio (VCR) is the ratio between the feed volume Vf (L) and the concentrate volume Vc (L) (QI et al., 2012) and was expressed by equation 4.

$$VCR = \frac{Vf}{Vc} \quad (4)$$

The recovery yield (%) represents the performance of the UF process, relating the enzyme activity of the feed (Af) and the concentrate (Ac), both in U/mL, and the volumes of feed (Vf) and concentrate (Vc) streams, which was determined by equation 5 (POLETTO et al., 2015).

$$Recovery\ yield\ (\%) = \frac{Ac \times Vc}{Af \times Vf} \quad (5)$$

The fold purification (equation 6) is another way to represent the efficiency of the UF process. This is the ratio between the concentrate specific activity and the crude broth (initial feed) specific activity (POLETTO et al., 2015).

$$Fold\ purification = \frac{specific\ activity\ in\ the\ concentrate}{specific\ acitivy\ in\ the\ crude\ broth} \quad (6)$$

The fold activity concentration is simply the relation between Ac and Af, according to equation 7 (POLETTO et al., 2015).

$$Fold\ activity\ concentration = \frac{Ac}{Af} \quad (7)$$

4.2.3 Analytical methods

The crude and fermented broth as well as the retentate and permeate of each step of MF and UF were evaluated for cellulase activity, protein concentration, reducing sugars concentration, biomass, COD and nitrogen concentration. Total cellulase activity was measured according to the filter paper assay (FPase) standard method described by Mandels and Reese, (1957) and Ghose, (1987), adapted for deep-well microplates according to Camassola and Dillon, (2012). Filter paper stripes (0.6 x 1.0 cm) were employed as the substrate, and 50 µL of samples were appropriately diluted in sodium citrate buffer (50 mM; pH 4.8) and incubated for 60 min at 50 ºC. After the enzymatic reaction, the reducing sugars were measured by the dinitrosalicylic acid (DNS) method (MILLER, 1959). One unit of cellulase activity was defined as the amount of enzyme producing 1 µmol

of reducing sugars per minute. Protein concentration was determined by the Bradford method, using bovine serum albumin as a standard (BRADFORD, 1976) and the reagents of the QuickStart Bradford kit (Biorad – USA). COD was analysed through spectrophotometry, and the total organic nitrogen was evaluated using the Kjeldahl method according to the standard method of the American Public Health Association (APHA, 1989). The biomass was gravimetrically determined. The total organic carbon (TOC) and Total Nitrogen were evaluated by the elemental analysis performed in a CHNS Vario Macro analyser (Elementar, Germany)

The general scheme of experimental steps including cellulase production and recovery is presented in Figure 4.1.

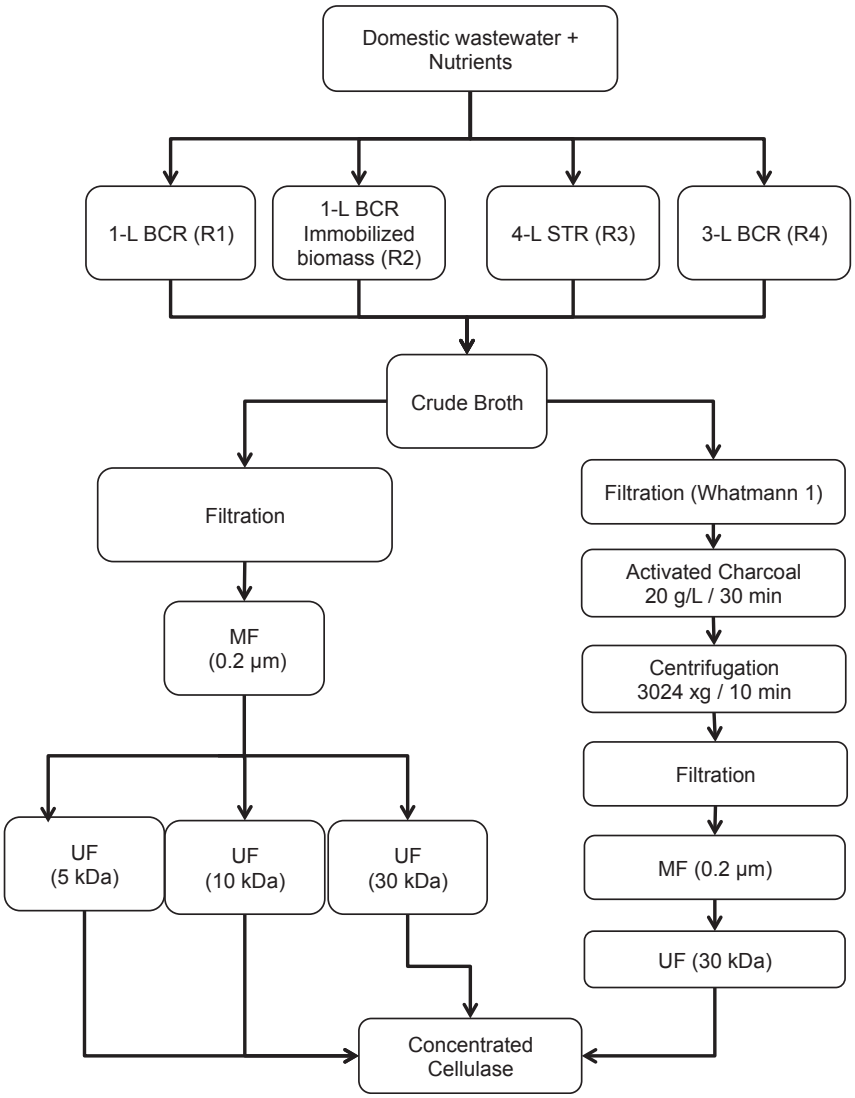

Figure 4.1 - Steps of cellulase production, separation and purification.

4.3 RESULTS AND DISCUSSION

4.3.1 Cellulase production strategies using domestic wastewater

The maximum cellulase activity was achieved in the 3L-BCR (R4), with 31.0 U_{FP}/mL, after 48 h as shown in Figure 4.2. The results in terms of yields and productivities (Table 4.1) were also higher for this reactor. The immobilization of the biomass in the R2 reactor halved the enzyme activity (5.79 U_{FP}/mL) when compared to the reactor R1 (10.27 U_{FP}/mL), and reduced all the measured kinetic parameters (Table 4.1), whereas the biomass concentration in both reactor were similar (10.2 g/L and 12.5 g/L, respectively). Even if the immobilization has stimulated biomass growth, probably due to the reduction of shear stress caused by the aeration (Figure 4.6), it had a negative impact on cellulase production. On the other hand, the R3 (STR) reactor highly favoured cellulase activity (17.0 U_{FP}/mL), with a yield of conversion of substrate in biomas ($Y_{EA/S}$) of 897.6 U_{FP}/g and maximum productivity ($Q_{pmáx}$) of 236.5 U_{FP}/Lh, but with lower values in terms of biomass concentration (5.53 g/L), probably associated to the brakeage of the fungal hyphae by the impellers.

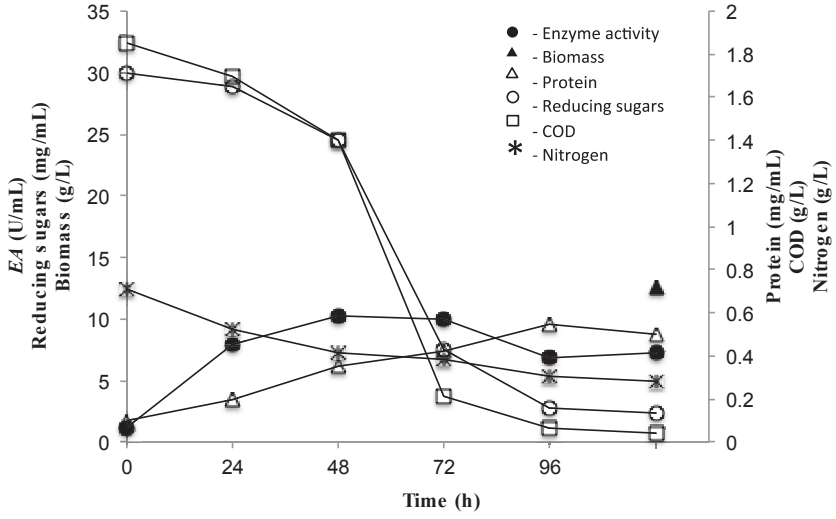

Figure 4.2 – Kinetics of cellulase, biomass and protein production and consumption of reducing sugars, COD and nitrogen for the 1L-BCR. Enzyme activity (●) reducing sugars (○); biomass (▲); protein (△); COD (□); Nitrogen (∗).

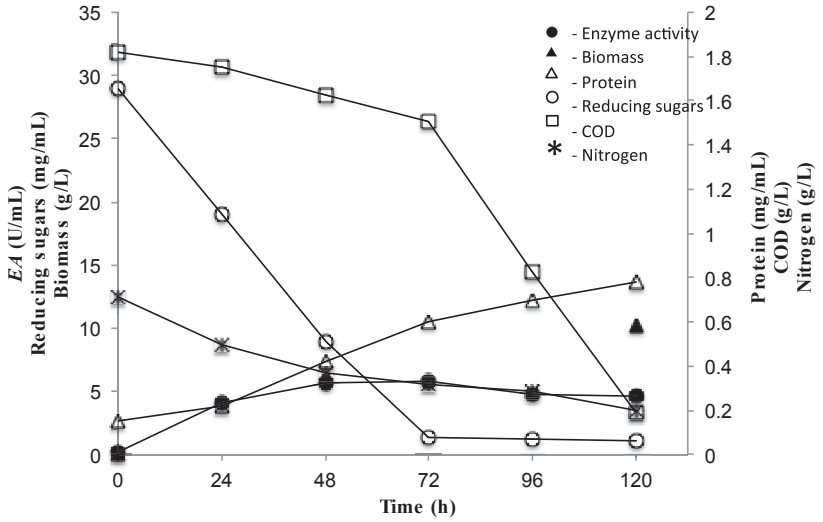

Figure 4.3 – Kinetics of cellulase, biomass and protein production and consumption of reducing sugars, COD and nitrogen for the immobilized biomass fixed bed 1L-BCR. Enzyme activity (●) reducing sugars (○); biomass (▲); protein (△); COD (□); Nitrogen (∗).

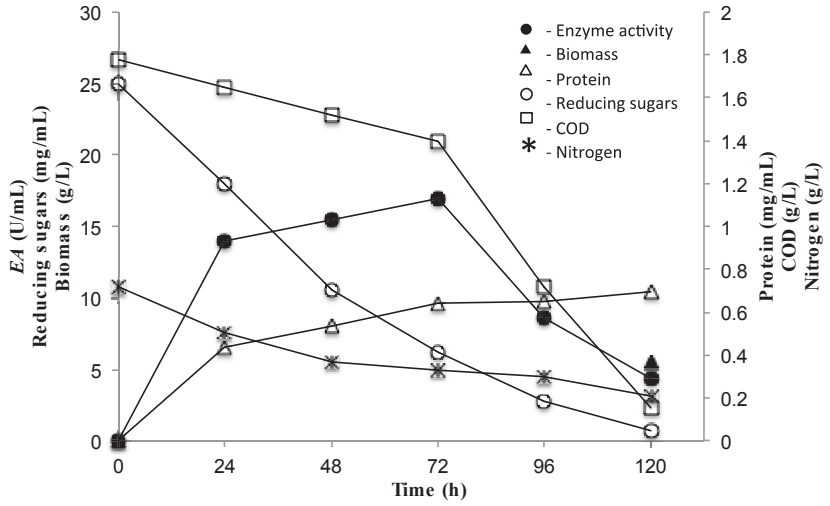

Figure 4.4 – Kinetics of cellulase, biomass and protein production and consumption of reducing sugars, COD and nitrogen for the 4L-STR. Enzyme activity (●); reducing sugars (○); biomass (▲); protein (△); COD (□); Nitrogen (∗).

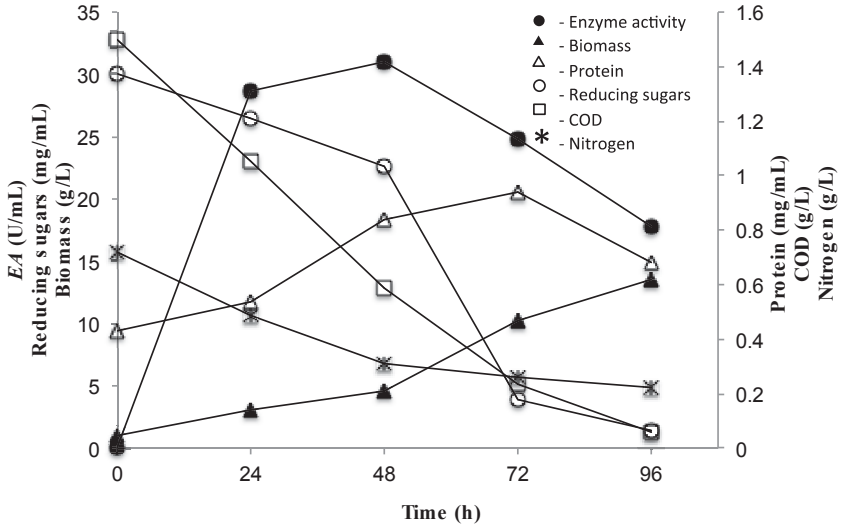

Figure 4.5 – Kinetics of cellulase, biomass and protein production and consumption of reducing sugars, COD and nitrogen for the 3L-BCR. *EA* (●); reducing sugars (○); biomass (▲); protein (△); COD (□); Nitrogen (∗).

In all the tested reactors systems, the cellulase activity achieved a maximum value followed by a decrease, probably associated to proteases attack or denaturation processes. Alam et al. (2008) also verified that the maximum activity (10.2 U_{FP}/mL) was achieved after 72 h followed by a reduction to 2.6 U_{FP}/mL at 168 h, in the fermentation process using wastewater sludge as substrate for *Trichoderma harzianum* fermentation. The authors achieved a $Y_{EA/COD}$ of 21.42 U_{FP}/g_{COD}.

Table 4.1 – Kinetic parameters of cellulase production in different bioreactor systems

Parameters	R1 1-L BCR	R2 Imm. BCR	R3 4-L STR	R4 3-L BCR
EA_{max} (U_{FP}/mL)	10.27	5.79	17.02	31.0
Biomass (g/L)	12.5	10.2	5.53	13.52
$Qp_{máx}$ (U_{FP}/Lh)	214.0	80.4	236.5	645.4
Qp (U_{FP}/Lh)	60.3	38.3	36.9	186.0
$Y_{EA/S}$ (U_{FP}/g)	342.3	196.1	897.0	4206.2
$Y_{EA/COD}$ (U_{FP}/g_{COD})	20.2	17.9	44.3	34.0
$COD_{removal}$ (%)	97.7	89.6	85.7	98.1
$Nitrogen_{removal}$ (%)	60.3	71.9	70.6	77.7

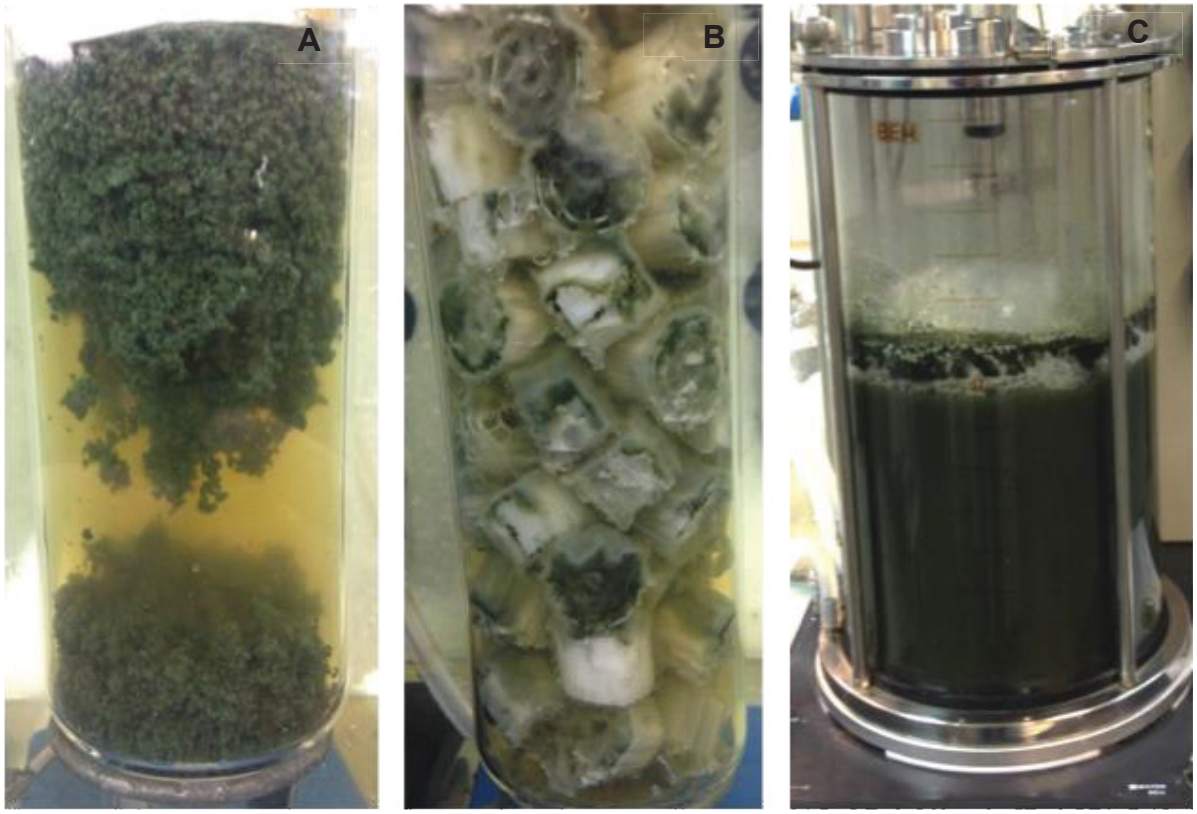

Figure 4.6 – Morphological differences of the fungal biomass during cellulase production using domestic wastewater in: 1L-BCR (A), fixed bed 1L-BCR (B) and 4L-STR (C).

The maximum enzyme productivity achieved in the 3-L BCR (R4) (645.4 U/Lh) was about 63 times superior compared to that obtained by Libardi et al. (2017) (10.2 U/Lh). This would be probably related to the differences between the reactors regarding the length-to-diameter ratio and superficial gas velocities. While the R4 has a length-to-diameter ratio of 4.16, the R1 present a 3.75. The superficial gas velocity of the R4 and R1 were 0.44 cm/s and 0.99 cm/s. The R4 reactor presented a homogeneous (bubbly flow) regime, with uniform small size bubbles and rise velocities, resulting in a gentle mixing over the entire cross-sectional area of the column. The R1 presented a more heterogeneous regime (churn-turbulent), with a turbulent motion of the larger bubbles and liquid circulation. According to Kantarci et al. (2005), the gas-liquid mass transfer coefficient is lower at heterogeneous regime, compared to homogeneous flow.

Besides the reactor dimensions and characteristics, the addition of lactose in the culture medium certainly boosted the enzyme production since it is a carbon source and cellulase inducer, replacing microcrystalline cellulose that was previously proposed (LIBARDI et al., 2017). Lactose has been described as an interesting cellulase inducer since it prevents catabolic repression that may be provoked by easily metabolized carbon sources such as glucose, glycerol or fructose (MORIKAWA et al., 1995). High cellulases activities have been already described with the use of lactose as carbon source, ranging from 2 to 10 %, combined or not with cellulosic materials, achieving cellulase activities up to 30 U_{FP}/mL (ESTERBAUER et al., 1991). Pourquie et al., (1988) reached the productivity of 243 U_{FP}/Lh, a similar value found in this work with the R1 system (214 U_{FP}/Lh) and 2.6 times lower than R4 (645.4 U_{FP}/Lh), with a 3000-L fermenter using a *Trichoderma* strain. These results show the great potential of scaling-up this process for even higher productivities. The higher published results have been related to the use of synthetic carbon source such as lactose, with or without the combination with cellulosic residues. The use of lignocellulosic residues for the production of *Trichoderma* cellulases resulted in enzyme activities and productivities of 5.25 U_{FP}/mL and 54.68 U_{FP}/Lh using corn cob residue at 4% (LIMING and XUELIANG, 2004); 13.7 U_{FP}.mL and 91.33 U_{FP}/Lh using wheat bran at 2% (WARZYWODA et at., 1983). Ivanova et al (2013) discovered that lactose permeases (transporter) are present in *Trichoderma* strains and are regulated by the presence of lactose in the culture medium, what answer why lactose is the only carbon source that induces the production of cellulases on an industrial level without inhibition.

Other WWTP residues than the raw wastewater have been used for cellulases' production such as sludge. Normally a pre-treatment of the sludge is necessary to hydrolyze the recalcitrant compounds and turn them more accessible to the microorganisms. Drani et al. (2011) tested the WWTP sludge for cellulase production achieving maximum productivities of 3.2 U/Lh with *Trichoderma* strains in submerged fermentation.

4.3.2 COD and N removal during the fermentation processes

The maximum reduction of 98% for COD content and 78% for nitrogen, which was achieved in the 3-L BCR (R4), shows that the lactose and peptone were

efficiently used for the production of fungal biomass and metabolites. According to Figure 4.7, the reduction of COD concentration is accompanied by low reducing sugars concentrations, indicating that reducing sugars content is related to COD.

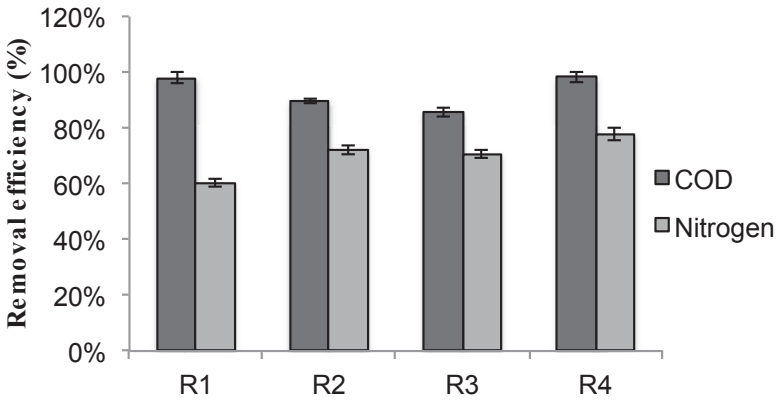

Figure 4.7 – Efficiency of COD and Nitrogen removal for the 1-L BCR (R1), 1-L immobilized biomass fixed bed BCR (R2), 5-L STR (R3) and the 3-L BCR (R4).

Alam et al. (2008) evaluated COD consumption during *Trichoderma harzianum* fermentation with sanitary wastewater sludge for cellulases production . The COD concentration was reduced in 96% after 7 days of fermentation, achieving a cellulase activity of 10.2 U_{FP}/mL. Alam et al. (2003) obtained 98% of COD removal for domestic wastewater sludge fermented with a mixed culture of *Aspergillus niger* and *Penicillium corylophilum*.

The evaluation of COD reduction content has been evaluated in fermentation processes using various wastes such as cheese whey, starch processing wastes, apple distillery waste and olive mill wastewaters (ALAM et al., 2003). Ghaly and Kamal (2004) achieved 90.6% of COD content reduction for protein production using cheese whey as medium for *Kluyveromyces fragilis* fermentation. Bo et al., (1999) achieved a 95% of COD reduction during the production of fungal biomass protein using starch processing wastewater as culture medium for *Aspergillus* and *Rhizopus* strains.

The Table 4.2 presents the analysis of the total organic carbon (TOC) and nitrogen fractions during the fermentation process for the 1L-BCR (R1).

Table 4.2 - Analysis of carbon and nitrogen fractions during cellulase production in 1L-BCR (R1).

Process time (h)	N%	C%	C/N
0	0.04	0.32	8
24	0.08	0.16	2
72	0.07	0.10	1.42

The TOC suffered a reduction of 68.7% after 72 hours of fermentation (Table 4.2). This reduction was also observed for COD and reducing sugars concentration that can be seen in Figure 4.2. This fact demonstrates that the COD can be used as an indirect measurement of the soluble organic matter in the culture medium and its reduction is regarded to the consumption of the organic matter from wastewater, but mostly from lactose, by the fungi.

In Table 4.2 it is also observed that the total nitrogen increases from 0.04 to 0.08% during the first 24 h of fermentation process and this value is maintained until 72 h. The increase and/or the maintenance of the total nitrogen would be related to the release of proteins that are produced by the fungal strain in the fermented broth, including the enzymes, which are directly associated to the release of organic nitrogen. The same phenomena was observed by Ghaly and Kamal (2004), in cheese whey fermentation for single-cell protein production using the yeast *Kluyveromyces fragilis*.

The C:N ratio of 8 is in accordance with the elementary composition for fungi biomass in terms of dry cell weight, (BALTZ, et al., 2010). Xia and Shen (2004) tested the C:N ratios of 6, 7, 8 and 9 using corn cob residue (40 g/L) as carbon source with other compounds according to the medium proposed by Mandels et al. (1981), obtaining the best results in terms of enzymatic activity (5.25 U_{FP}/mL) using the ratio of 8.

4.3.3 Cellulase recovery and concentration

Membrane systems are certainly an excellent strategy for separating and purifying enzymes and proteins. They can also concentrate enzyme activity to interesting levels for further applications. The cellulase crude extract was

microfiltrated to remove particles and cell debris. As presented in Table 4.3, the cellulase activity was reduced to 3.71 U/mL, resulting in a recovery of 58%, presenting a permeate flux of 172 L/m^{2}h, with an efficient clarification of the enzymatic extract. Table 4.3 summarizes the main results of each step of the studied MF and UF. The best results in terms of efficiency were clearly achieved when using the 30 kDa membrane without activated charcoal as a pretreatment.

Table 4.3 – Separation, purification and concentration of cellulases by MF and UF using different membranes.

Step	Volume (mL)	Enzyme Activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Total activity (U_{FP})	Total Protein (mg)	Cellulase Recovery (%)	Protein Recovery (%)	Fold activity conc.	Fold Purification	Flux (L/m^2h)	Time (min)
Crude Broth	250	5.92	1.41	4.2	1480	352.5	100%	100%	1.00	1.00	-	-
[1]MF – 0.2 µm	230	3.71	1.52	2.4	853.3	349.6	58%	99%	0.63	0.58	172	4
[2]UF - 5 kDa	40	0.32	1.79	5.8	412.8	71.6	48.4%[a]	23%	2.78	1.37	14.48	9
UF - 10 kDa	40	3.57	1.83	7.4	542.8	73.2	63.6%[a]	20.1%	3.66	1.77	94	5.33
UF - 30 kDa	40	5.68	1.89	8.3	627.2	75.6	73.5%[a]	14.8%	4.23	1.98	109	5
UF - 30 kDa - [3]AC	30	4.51	1.24	3.6	135.3	37.2	39%[a]	11%	1.69	0.48	94.5	3

[a]The presented recovery values are related to the comparison with the MF.
[1]Microfiltration
[2]Ultrafiltration
[3]Activated carbon

In Figure 4.8 it is possible to observe the enzymatic activity and protein concentration obtained after each step of UF. The enzymatic activity in the retentate fraction increases as the membrane MWCO increases. The permeate fraction was not affected in the same way, meaning that the losses are higher with smaller membrane pore sizes. However, the proteins in the permeate fraction increase with higher-MWCO membranes, indicating that there are proteins smaller than the membrane's pores. Figure 4.8 indicates that most of the proteins have a molecular weight higher than 5 kDa.

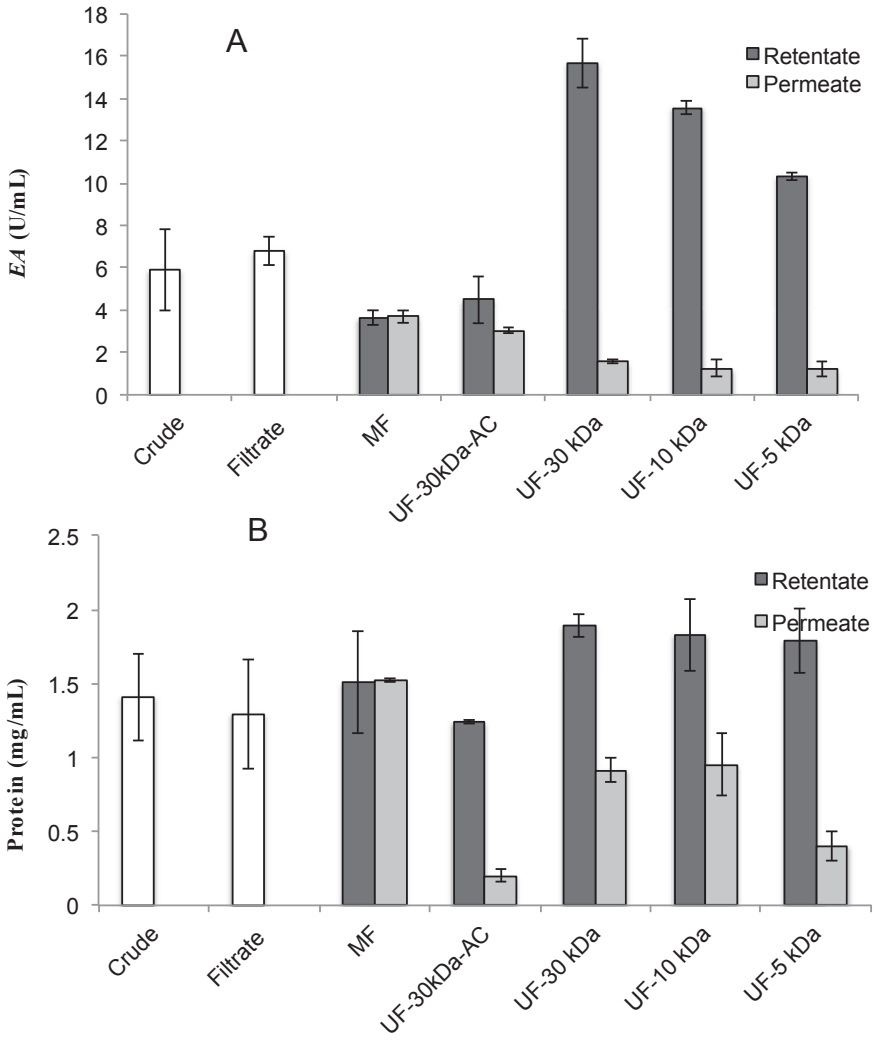

Figure 4.8 - (A) Enzymatic activity and (B) protein concentrations of the crude, filtered, microfiltered broth and of the retentate (dark grey bars) and permeate (light grey bars) samples obtained after using UF membranes with different MWCO values (with and without AC pretreatment)

According to Figure 4.9-a, higher-MWCO membranes lead to higher cellulase-recovery percentages. The highest recovery percentage (73.5%) was obtained with the 30 kDa MCWO membrane. This means that 26% of the cellulase was detected in the permeate fraction and 0.5% of losses were verified, probably due to other causes such as fouling or enzyme denaturation. The 10 kDa membrane promoted cellulase recovery of 63.6%. However, the loss of cellulase activity was higher than that of the 30 kDa membrane (11.8%), and the cellulase activity in the permeate fraction was 24.6%. The UF with a 5 kDa membrane presented the highest loss of cellulase activity (31.6%), with lower values in the retentate (48.8%) and permeate (20%) fractions.

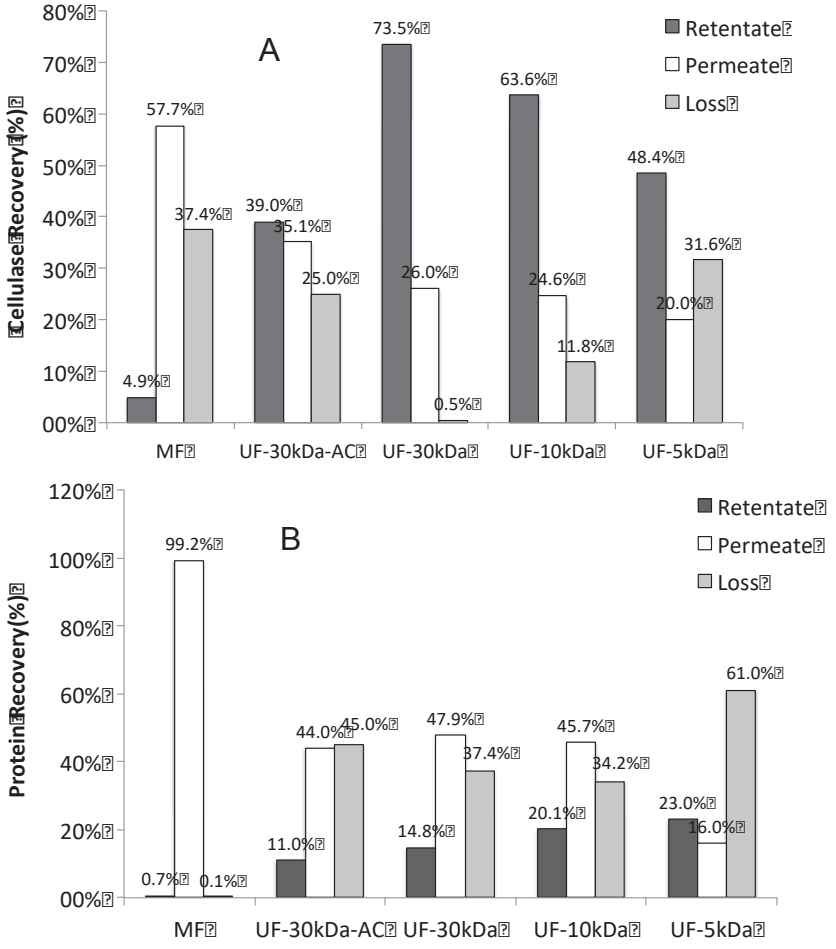

Figure 4.9 - (A) Cellulase recovery and (B) protein recovery percentages in the UF permeate (white bars) and retentate (dark grey bars) fractions and the losses (light grey bars) during the processes.

Protein recovery presented an inverse profile (Figure 4.9-b). Smaller-MWCO membranes led to higher protein-recovery percentages in the retentate fraction.

Protein concentration decreased from the 5 kDa membrane to the 30 kDa membrane, whereas the concentration of proteins in the permeate increased. In concentration experiments with cellulases, Rashid et al. (2013) achieved a total protein percentage of 34.67% in the retentate fraction using a 10 kDa membrane, with 43.9% of the total protein in permeate and 21.44% in losses. According to Figure 4.9, the treatment with activated charcoal negatively affected the enzyme concentration. The enzyme activity in the retentate was reduced by 71.23% after using activated charcoal as a pretreatment for the samples, compared to the sample subjected to the 30 kDa membrane without previous pretreatment. The total enzyme activity of the crude broth was 1350 U, which was reduced to 347.1 U after the pretreatment with activated charcoal. This means a reduction in total cellulase activity by 3.8 times without a significant reduction of volume (150 to 130 mL). This result indicates the possibility of cellulase being adsorbed by the activated charcoal. In addition, the MF membrane retained fragments of activated charcoal. A similar behaviour was observed regarding the protein concentration, with a reduction from 1.89 to 1.24 mg/mL in the retentate with versus without the AC pretreatment for the 30 kDa MCWO membrane, respectively, indicating that the total protein content was also influenced by the use of activated charcoal. AC pretreatment negatively affected cellulase and protein recoveries by UF. It is possible to infer from Figure 4.9 that losses during the process caused the low recovery rates. The loss of 0.5% observed with the 30 kDa MCWO membrane increased to 25% when the AC pretreatment was applied, meaning that cellulases were possibly adsorbed into the AC.

It is clear that the 30 kDa membrane promoted the highest increase in activity concentration (4.23-fold), compared to the 10 kDa and 5 kDa membranes, which resulted in 3.66- and 2.78-fold increases in activity concentrations, respectively (Table 4.3). Rashid et al. (2013) presented values of cellulase activity in terms of fold concentration, achieving 9.09-fold increase using a 10 kDa MWCO membrane.

The use of higher-MWCO membranes promoted higher cellulase activity recovery in the permeate and lower losses. Rodrigues et al. (2014) recovered a commercial cellulase (Celluclast 1.5 FG L – Novozymes) from a wheat straw hydrolyzate using a 10 kDa plate membrane in a cross-flow UF system. The authors optimized the process conditions and achieved values of cellulase recovery from 32% to 67%. However, the comparison should take into account that commercial cellulases are formulated to maintain their activity, which may prevent denaturation

during UF. Poletto et al. (2015) concentrated pectinases using a cross-flow (hollow fibre) UF system and achieved protein recovery of 80.4%, which Rodríguez-Fernández et al. (2013) also observed, with a pectinase recovery rate of 86.14%. According to Powell and Timperman (2005), the primary cause of protein loss through the membrane is the pore size distribution. Shear forces also contribute by producing smaller fragments. Some proteins are close to the MCWO and could pass through the membrane and into the filtrate. Figure 4.9 shows that considerable fractions of proteins are present in the permeate fraction, especially in the 10 and 30 kDa membranes, indicating the low molecular weight character of some proteins. Therefore, the low protein recovery values should be related to the fraction of proteins with lower molecular weight than the membrane MWCO that pass through it and are detected in the permeate. As can be observed in Figure 4.9, the higher protein-loss percentages occurred with the 5 kDa membrane and the lower protein content was obtained in the permeate fraction, indicating that most of the proteins are retained in the membranes with a MWCO between 5 and 10 kDa.

Rodrigues et al. (2014) observed losses of around 20% in UF of commercial cellulase using a 10 kDa MWCO membrane. The authors observed the same losses when small volumes of samples were processed at lab scale, which influences the recovery percentages. This fact would not be as impactful at the industrial scale, where the volumes are higher and small losses do not have the same proportion of impact. According to Table 4.3, it is possible to observe an increase of the fold purification values as the membrane MWCO increased. This is in accordance with what was previously discussed and the observation that proteins are retained in the 5 kDa membrane. When the 30 kDa membrane is used, more proteins pass through it to go to the permeate fraction, increasing the purification degree of the retentate fraction (1.98-fold). This characteristic is especially important when the target product is in the retentate fraction, as is the case for the cellulases presented in this work. The use of MF followed by the 30 kDa UF resulted in a global recovery of 42.3%, when compared with the crude broth from the fermentation process. The results should be compared to those of other authors with caution, since there is a diversity of UF systems such as hollow fibre, flat-sheet cassettes, spiral-wound cartridges, tubular modules and other enhanced mass transfer devices such as charged membranes and electric or ultrasonic fields (SAXENA et al., 2009). In addition, very few publications specifically describe the UF of non-commercial cellulases, as

described in this work. The commercial cellulases used by some authors in MF and UF processes have been previously formulated to ensure their stability during storage. According to Esterbauer et al. (1991), sugars such as glucose and lactose as well as glycerol or polyalcohol could be added to prevent the loss of enzyme activity. The interference of these compounds in the culture broth would contribute to the differences in cellulase recovery percentages when comparing commercial and non-commercial cellulases. UF processes have become more attractive because they offer the advantages of lower costs and ease of scale-up for commercial production, when compared to chromatographic methods, e.g., size-exclusion chromatography for protein concentration (SAXENA et al., 2009). Since the aim of this work was to produce cellulases with a low to medium degree of purification, the proposed process could certainly be adopted. Additionally, the cellulases were produced with domestic wastewater as a substrate to reduce costs for the process steps from enzyme production to separation and recovery.

4.3.4 Analysis of the permeate flux and membrane fouling

After the MF step, the enzymatic extract (permeate) was processed through UF. The behaviour of the permeate flux as a function of time for the three tested membranes (5, 10 and 30 kDa) was evaluated, and the results are presented in Figure 4.10. For all tested membranes, two different stages of the permeate flux were observed: first, an expressive drop of permeation flux (around 24 L/m^{2}h for the 5 and 10 kDa membrane and 18 L/m^{2}h for the 30 kDa membrane) within the first minute of the process, and afterwards, a reduced variation. Figure 4.10 indicates that the 30 kDa membrane showed the highest permeate flux, followed by the 10 kDa and 5 kDa membranes, respectively. The permeate flux behaviour of the 5 kDa membrane (14.5 L/m^{2}h) is 86.7% lower than the permeate flux of the 30 kDa membrane (109 L/m^{2}h) and 84.5% lower than that of the 10 kDa membrane (94 L/m^{2}h).

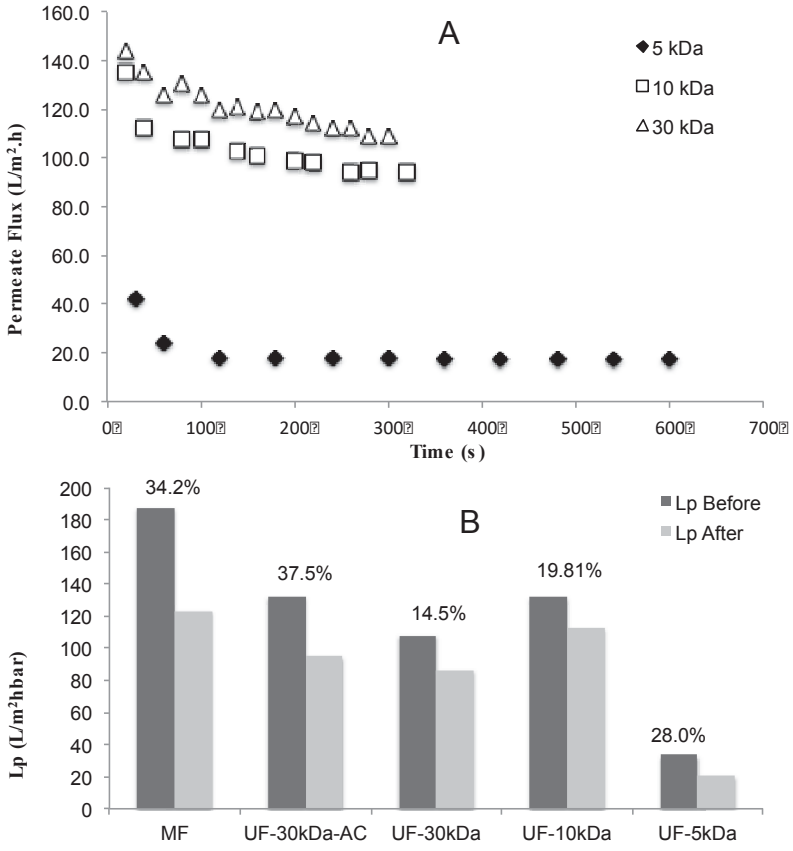

Figure 4.10 - (A) Permeate flux in 5 (closed diamond), 10 (open square) and 30 (open triangle) kDa ultrafiltration membranes of crude cellulase extract pretreated by microfiltration and (B) permeability of pure water before (dark grey bars) and after (light grey bars) the ultrafiltration, and the fouling (%) of the membranes of different MWCO.

The best anti-fouling performance between the UF membranes was observed in the 10 kDa membrane (14.5%), followed by the 30 kDa membrane (19.81%). Contrarily, the highest fouling effects were observed in the 5 kDa and MF membranes. This effect could be explained by the MF membrane retaining the larger particles of the crude broth, i.e., the fungal spores that made the membrane green. On the other hand, the pores of the 5 kDa membrane were possibly blocked by the smaller particles of the crude broth that passed through the MF membrane. It is also important to point out that the use of activated charcoal promoted increased fouling. There was a 28.0% reduction in Lp with the AC pretreatment. The use of activated charcoal was expected to reduce the fouling effect, since the suspended particles in

the crude broth would be adsorbed. However, its use also released some particles in the culture broth that were retained by the membrane resulting in the dark appearance of the membrane after the UF process.

MF has been described as an important step to increase the efficiency and permeate flux of the subsequent UF. Poletto et al. (2015) incremented the pectinase recovery after UF from 59% to 81.4% by adding a prior MF step in a hollow-fibre filtration system. According to the authors, the MF step halved the fouling in the UF step, reducing the permeate flux from 22 to 11 L/m^{2}h. Rashid et al. (2013) tested MF membrane pore sizes of 0.2 and 0.45 μm in a hollow-fibre system for the filtration of cellulases. The authors concluded that, although the 0.2 μm membrane suffered a higher fouling (19.37%), compared to the 0.45 μm membrane (3.8%), the cellulase activity in the permeate was 16% higher, indicating that the fouling could also be favourable for the process because it rejects suspended matter and high molecules that could interfere in the target product.

Two different stages of the permeate flux were observed during the UF process. The same behaviour was observed by Rodríguez-Fernández et al. (2013) when performing the UF process of phytases. The authors reported that this could be due to the fouling effect as well as to the increment in viscosity of the feed with its consequent concentration. In fact, the flux decline during the whole process for both membranes (MF and UF) is a typical behaviour of the membrane separation process, which promotes a reduction in the filtration driving force. This fact is due to the presence of different compounds of different sizes in the permeate.

Samples that passed through the 10 kDa and 30 kDa membranes were concentrated from 250 to 40 mL in only 5 minutes, while with the 5 kDa membrane, 29 minutes were necessary to achieve the same volume of 40 mL. The operational costs of the UF process are inversely proportional to the permeate flux. Results from several authors are presented in Table 4.4.

Table 4.4 – Examples of enzymes recovery and concentration by MF and UF processes parameters.

General information	MWCO	MP (bar)	Flux (L/m^2h)	Fouling (%)	Recovery [a,b] (%)	Fold activity conc.	Ref.
Cross-flow-Plate membrane; Cellulase; Domestic wastewater	0.2 µm 5 kDa 10 kDa 30 kDa		172 14.5 94 109	31.2 37.5 14.5 19.81	58[a] 48.4[a] 63.6[a] 73.5[a]	0.63 2.78 3.66 4.23	This work
Cross-flow-hollow fiber; Cellulase; Wastewater sludge	0.2 µm 0.4 µm 5kDa 10kDa 30 kDa	1.025 1.025	8.12 - - - -	21.44 - - - -	- - - - -	9 - - - -	Rashid et al. (2013)
Dead-end-stirred cell Commercial cellulase; Lignocellulosicb hydrolyzate	5 kDa 10 kDa 30 kDa	3.5	26.5 26.5 26.5	50.8 43.7 66.6	89.4[b] 73.9[b] 79.7[b]	- - -	Qi et al. (2012)
Cross-flow-plate membrane Commercial cellulase; Wheat straw hydrolyzate	10 kDa		-	-	38 [a]	-	Rodrigues et al. (2014)
Cross-flow-hollow-fiber Pectinase Wheat bran + nutrients	0,4 µm 10 kDa	2.5	18 24	57 -	95.2 [a] 80.4 [a]	0.95 19.8	Poletto et al. (2015)

[a] Enzyme recovery ; [b] Protein recovery

It is not simple to make a direct comparison, as can be seen in Table 4.4, since the permeate flux behaviour is dependent on the membrane surface area and porosity, as well as the time of the process. Poletto et al. (2015) obtained a permeate flux of 24 L/m^2h using a 10 kDa membrane in a hollow-fibre system. A very similar value was obtained by Qi et al. (2012) using the 10 kDa membrane (26.5 L/m^2h) in a

dead-end filtration system. It should be noted that the use of the 5 kDa membrane in this work resulted in a low value (14.5 L/m^{2}h). In addition, the increase of the MWCO from 5 to 10 kDa resulted in a strong difference of permeate flux, rising from 14.5 to 94 L/m^{2}h. This same increase is not observed when comparing the 10 and 30 kDa membrane permeate fluxes, from 94 to 109 L/m^{2}h. This behaviour could be explained by the fact that most of the proteins and other residual matter have a molecular weight lower than 10 kDa but higher than 5 kDa. This phenomenon was also observed in the evaluation of the membrane fouling, whereas the 5 kDa membrane resulted in higher values of this parameter.

Anti-fouling performance is one of the most important factors to consider when choosing a suitable membrane for the process. The fouling effect can be classified as reversible or irreversible. Reversible fouling is related to the effects of concentration polarization and gel-layer formation and can be removed by a simple backwashing. However, irreversible fouling is caused by particles being deposited into the membrane's pores and is removed by chemical cleaning (QI et al., 2012). The Lp values of the 10 and 30 kDa membranes obtained in this work were 3 and 2.4 times higher than that obtained by Qi et al. (2012) using 10 kDa (43.7%) and 30 kDa (48.7%) Polyethersulfone (PES) membranes, respectively, for cellulase concentration from an enzymatic hydrolyzate of steam-exploded wheat straw, using a hollow-fibre filtration system. A preliminary MF step certainly contributes to lowering the fouling effect in UF membranes. Poletto et al. (2015) observed this effect when testing the UF process (10 kDa MWCO) with and without a previous MF of the crude broth. While the previous use of a MF membrane decreased Lp by 50.6%, the use of the crude broth directly on the UF membrane resulted in a 72.5% decrease in Lp. The higher fouling effect presented by these authors could be related to the residual solid substrate particles suspended in the medium, even after centrifugation before the UF process.

Poletto et al. (2015) did not observe a strong reduction in fouling when using activated charcoal as a pretreatment for recovering pectinases from crude broth. The authors achieved a 42.5% reduction in the Lp using an activated charcoal pretreatment (5 g/L) and a 50.6% reduction without the use of activated charcoal. As discussed before, this result is related to the solid substrate fermentation used by these authors, which could have released more particles into the culture broth than

the submerged fermentation presented in this work did, which efficiently removed the particles through the activated charcoal treatment.

The cellulase transmission (defined as the enzyme concentration in the permeate divided by the enzyme concentration in the feed) increased as the MWCO increased, as expected. Mores et al. (2001) also reported the presence of cellulase in permeate fractions of 2% and 6% using PES membranes with 30 and 50 kDa MWCO, respectively. Some hypotheses for this phenomenon are that some cellulase isoforms may be smaller than 30 kDa and/or the membrane cut off may not be so selective.

4.3.5 Analysis of the COD and N concentration of the membrane fluxes

Evaluating the effluents generated during the UF is a matter of central importance when developing a sustainable biotechnological process. Since the retentate fraction contains the product of interest (the concentrated cellulase), the permeate is the effluent of this process and should therefore be evaluated for correct management and disposal. Although the objective of this UF process from an “industrial biotechnology” point of view is to maximize cellulase recovery, the central task from an environmental perspective is to minimize the effluent concentration. By this reasoning, the COD of the retentate and the permeate fractions of the MF and UF were evaluated, as shown in Figure 4.11. Cassini et al. (2010) recovered the proteins present in the wastewater of an isolated production process for soy protein. The use of UF with 5 kDa membranes retained 52% of the proteins and reduced the COD concentration of the final effluent by 30%, showing the viability of coupling a resource-recovery process to a wastewater treatment technology. Alonso et al. (2001) achieved COD reduction of 47% with a 50 kDa UF spiral membrane and a 46% reduction using a 0.2 μm MF membrane, respectively, when filtering sanitary wastewater. The higher efficiencies obtained in this work compared to those of other authors indicate that the majority of the macromolecules contained in the fermented broth are larger than 30 kDa and that the tested membranes are efficient for retaining the COD.

The MF process led to a reduction in COD concentration from 36 to 32 mg/L (11.36%). So, MF alone is not efficient to remove the chemically oxidable matter in the fermented broth. According to Figure 4.11, a completely different behaviour was

observed when UF membranes were used, with a very good separation and concentration of the chemically oxidazable matter (COD) that were mainly detected in the retentate fractions. Low concentrations of COD were present in the permeate fractions. The efficiencies of COD removal by each UF membrane were very similar. A removal of COD from 68 to 9 mg/L (86%) was observed when using the 5 kDa membrane, from 64 to 8 mg/L of COD (86%) with the 10 kDa membrane and from 72 to 13 mg/L of COD (81%) with the 30 kDa membrane. The efficiency of COD removal obtained using the 10 kDa membrane (86%) was higher than the highest efficiency obtained by Rashid et al. (2013) when using a 10 kDa membrane (30.78%) for cellulase concentration from a culture broth containing palm oil mill effluent as part of the culture medium.

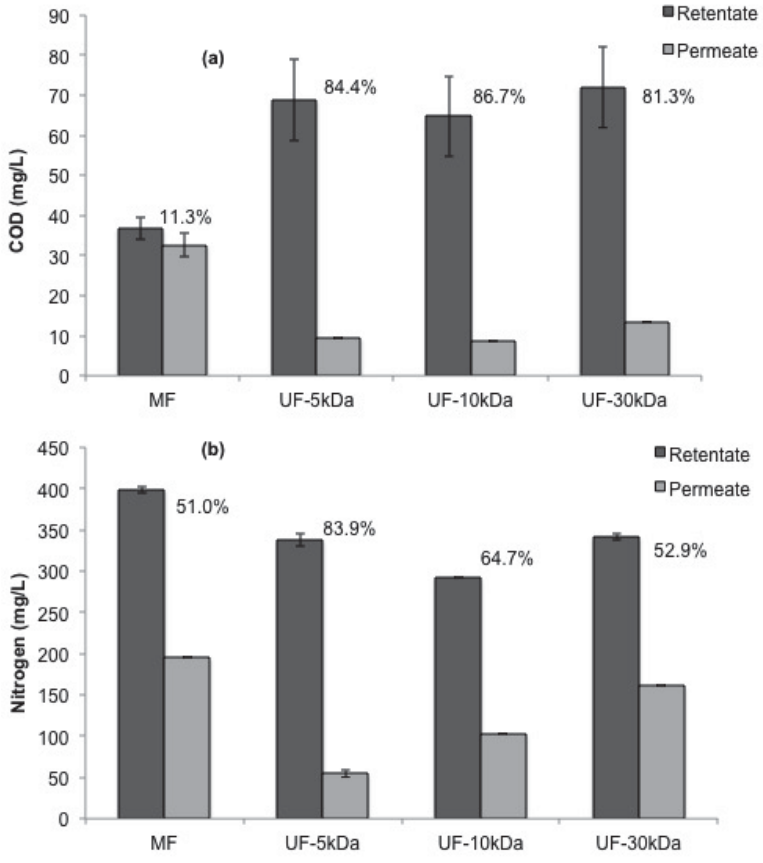

Figure 4.11 - (a) COD and (b) nitrogen concentration and percentage of reduction of the retentate (dark grey bars) and permeate (light grey bars) fractions for different MF and UF membranes (5, 10 and 30 kDa).

Another important process parameter was the total organic nitrogen concentration of the permeate and retentate fractions that were also tested, as

shown in Figure 4.11. The 5 kDa membrane led to the highest efficiency of nitrogen removal (83.9%) from the permeate compared to the retentate fraction, followed by the 10 kDa (64.7%) and 30 kDa (52.9%) membranes. The efficiency of nitrogen removal by the MF membrane (51%) was similar to that presented by the 30 kDa membrane. This same behaviour was observed in the protein recovery concentration presented in Figure 4.9. Alonso et al. (2001) achieved nitrogen removal of 12% using a 50 kDa membrane in a spiral membrane system to filter pre-filtered domestic wastewater.

The similarity in nitrogen removal efficiency when comparing the UF (30 kDa) to the MF membranes was not observed for COD removal. According to Alonso et al., (2001), this is related to the N-association with organic macromolecular compounds, which are found in colloidal suspension and are retained by the UF and MF membranes. This is not observed for COD, which is probably associated with dissolved matter. The COD concentrations in the permeate fractions of all of the tested UF membranes were very low. Considering that the product of interest is in the retentate fraction, the permeate was the effluent of this concentration process. In this way, this UF process resulted in an effluent with COD concentrations below the acceptable limit of 125 mg/L and total nitrogen removal efficiencies close to 70-80% for sanitary wastewater disposal, according to European Union legislation on wastewater treatment for sanitary wastewater disposal. The reduction of COD and nitrogen concentration achieved in this work suggests that the resulting effluent from this production process just needs simpler treatment steps to be disposed of in nature. Other parameters should be evaluated for this assumption; however, the low COD concentration and high nitrogen removal already mean reduced costs for treating the resulting wastewater from this enzyme production process. A fact that increases the environmental importance of evaluating the COD content of the permeate fraction from this UF process is that it corresponds to a larger volume for the process and this effluent must be correctly disposed. The permeate fraction corresponds to 84% of the volume of fermented broth passed through the MF and UF membranes.

4.4 CONCLUSION

The use of lactose as carbon source for cellulase production combined with sanitary wastewater in a 3-L BC reactor increased by 10 times the enzyme productivity (645.4 U_{FP}/Lh) compared to previous works. In addition, the maximum COD and nitrogen removal efficiencies of 98 and 78% were achieved during the fermentation process, respectively. Besides cellulosic wastes have been related as interesting carbon sources for cellulase production, the higher productivities achieved by the use of lactose are still incomparable. The combination of cellulosic residues with lactose or lactose-containing residues like cheese whey may be interesting for lowering the costs and maintain high productivities.

The cellulase productivity indicates that the use of BC reactors is adequate for this process, whereas the biomass pellets where maintained and easily separated from the fermented broth only by a simple sedimentation. The stirred reactor presented higher productivities, but lower biomass production, probably due to breakage by the impellers.

The behaviour of MF and UF processes for recovering and concentrating cellulase were evaluated. The use of MF followed by the 30 kDa UF membranes resulted in recovery percentages above 70%, which are in accordance to some of the best results found in the literature. In addition, COD and nitrogen removal efficiencies of 81 and 52.9 % were achieved. Since the aim of this work was to produce cellulases with a low to medium degree of purification, the UF could certainly be adopted as they present lower costs and ease of scale-up for commercial production. Also, the reduction of the pollution charge achieved demonstrated double benefit of using UF as a suitable technique.

This work proposed the insertion of a traditional biotechnology process, comprised of bioreactor production and membrane separation, in the broader spectrum of the wastewater biorefinery. This strategy seems to be interesting for the coupling of the wastewater treatment/reuse and biomolecules production. This work also proposes the reduction of the production costs by the use of sanitary wastewater as a source of nutrients and water. The final effluent presented organic matter concentration in accordance to the legislation, reducing the costs associated to wastewater treatment processes.

5 CHARACTERIZATION AND FORMULATION OF CELLULASES' EXTRACT - DEVELOPMENT OF LIQUID AND POWDER FORMULATIONS

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ABSTRACT

Raw domestic wastewater was used as a culture medium for cellulases' production in a bubble column reactor using the strain *Trichoderma harzianum* TRIC03-LPBII. Cellulase's crude extract was separated and concentrated by micro and ultrafiltration membranes. The concentrated cellulases presented optimum pH and temperature between 4 and 5 and 50 and 70ºC, respectively. The Michaelis-Menten constant (K_m) of endoglucanases and β-glucosidases (BGL) were determined as 63 and 0.39 mM, respectively. The concentration of 10 g/L of glucose inhibited around 45% of the BGL activity. Denaturing gel electrophoresis (SDS-PAGE) and zymogram revealed molecular weights of 48, 43 and 25 kDa and the identification of each protein band is being processed. Sorbitol (50% w/v) and benzoic acid (0.05% w/v) were added to the liquid extract and led to cellulase formulation stability at around 100% after 30 days, at 4ºC. Enzyme concentrated extract was dried in a spray-dryer with the addition of maltodextrin at 20% (w/v) and inlet temperature of 120ºC, resulting in a highest yield (40.6%) and stability (85%) after 60 days. The produced enzyme powder presented the form of irregular spheres, which is typical for the use of maltodextrin as encapsulation agent.

Key Words: Enzyme Formulation; Spray-dryer; Cellulase Stability.

5.1 INTRODUCTION

Cellulases consist of enzymatic complexes that act synergistically with the action of endo-glucanases (EGL), cellobiohydrolases (CBH) and β-glucosidades (BGL). *Trichoderma* strains may produce two cellobiohydrolases (CBHI and CBHII), two endoglucanases (EGI and EGII) as well as seven types of β-glucosidases (BGLI to BGLVII) (SINGHANIA et al., 2017). Cellulases from different sources have distinct profiles as they exhibit specific optimum pH and temperature, thermal stability, affinity by certain substrates as well as inhibitory effects, influencing their kinetic parameters. BGL feedback inhibition caused by glucose production from the hydrolysis of cellobiose is a limiting factor for biomass hydrolysis, one of the most interesting cellulase applications.

Cellulases' production using agro-industrial residues and other wastes as substrates is emerging as an interesting alternative for lowering its production costs. The on-site small-scale cellulase production is an interesting strategy for consumers' independency from international suppliers, what brings a new challenge for efficient development of stable cellulase cocktails that could replace the established commercial products in an economical and sustainable way.

In the early 1990's, the first standard cellulase preparations were developed through concentration of microbial fermented broth and addition of stabilizers for storage. Nowadays, these formulations consist of a mixture of several enzymes and unknown compounds. Formulations of commercial cellulases are not totally available and their production is often discontinued in favour of new and improved variations (HIMMEL et al., 2017). Commercial cellulases are highly concentrated with further formulation with specific additives for protein stability during storage. They can be found as stabilized liquid concentrates or as particulate solids. However, the majority of the commercial cellulases are prepared as liquid formulations (SINGHANIA et al., 2017). Most industrial enzymes contain a relatively small fraction of active enzyme (< 10% w/w), and the rest being due to inactive proteins, stabilizers, preservatives, salts and diluents that allows standardization between production batches. Sometimes it is only the formulation of an enzyme that gives a manufacturer the competitive edge over rival companies. Enzyme formulations are kept secret or revealed to customers only under the cover of a confidentiality agreement (CHAPLIN and BUCKE, 1990).

The primary task of formulation is to minimize losses in enzymatic activity

during transport, storage, and use. Formulations enhance stability by counteracting the primary forces of deactivation: denaturation, catalytic site deactivation, and proteolysis (BECKER et al., 1997). Denaturation occurs by physical unfolding of an enzyme's tertiary protein structure under thermal or chemical stress. Once an enzyme begins to unfold it becomes dramatically more vulnerable to deactivation and proteolysis. To minimize unfolding, the formulator can alter the protein's environment so as to induce a compact protein structure; this is done most effectively by a preferential exclusion of water from the protein surface by adding water-associating compounds such as sugars, polyhydric alcohols, and lyotropic salts (PAZ et al., 2000).

Other compounds, which are added to enzymes before commercialization, may consist of substrates, such as thiols, to create a reducing environment, or antibiotics, benzoic acid and esters that are used as contamination preservatives for liquid enzyme preparations, inhibitors of enzyme activity and chelating agents. These type of additives must, certainly, be compatible with the final application of the enzymatic product. To prevent microbial contamination, biocides can be effective, but the group of acceptable chemicals, which could be employed for controlling or killing microbes is increasingly limited by health and safety regulations. According to the Association of Manufacturers and Formulators of Enzyme Products (AMFEP), enzymatic products may contain preservatives, which may be regulated by the Biocidal Product Regulation. In 2015, the AMFEP got the authorization for using sodium benzoate as a biocidal product that is classified as product-type number 6, which includes preservatives that must be employed for products during storage according to the EU regulation nº 528/2012. This regulation concerns their availability on the market and the use of biocidal products. According to this regulation, biocidal products are referred as products that are used for preservation of manufactured products, other than foodstuffs, feeding stuffs, cosmetics or medicinal products or medical devices by the control of microbial deterioration to ensure their shelf life.

Drying is one interesting way to produce more stable and storable enzymes. The removal of water reduces the freedom of movement of protein molecules inhibiting conformational changes that lead to the loss of activity. Spray-drying has been used as a cost effective technique for the solid cellulase powder production in comparison to the freeze-drying technique (BELGHITH et al., 2001; SELIVANOV, 2005). The spray drying process normally requires the use of an adjuvant for the

microencapsulation during the dry process, such as maltodextrin, arabic gum, starch, microcrystalline cellulose, lactose and others. In addition, technological adjuvants such as colloidal silica could be used as a desiccant and as a non-stick agent for hygroscopic powders, as well as anti-caking and anti-humectant (FERREIRA et al., 2016). The drying process is influenced by many operational factors including the type of nozzle atomizer, the inlet and outlet temperature, feeding flow, pressure, and physical properties of the material to be atomized (SAMANTHA et al., 2015).

This work aimed to perform the partial characterization of cellulases produced using domestic wastewater as culture medium, followed by the evaluation of the stability and characteristics of liquid and powdered formulations.

5.2 MATERIAL AND METHODS

5.2.1 Cellulase production and concentration

Cellulase production was performed using the *Trichoderma harzianum* (TRIC03) strain from the Federal University of Paraná Culture Collection, Bioprocess Engineering and Biotechnology Department. The culture medium was composed of lactose (11.9 g/L), peptone (5.0 g/L), KH_2PO_4 (2.5 g/L), and $MgSO_4.7H_2O$ (0.3 g/L), prepared with raw domestic wastewater collected from the Atuba Wastewater Treatment Plant from the Sanitation Company of Paraná (SANEPAR, Brazil). A fungal spore suspension of approximately 10^7 spores/mL was used as inoculum. Cellulase production was conducted by submerged fermentation in a 1.5-L BCR with a working volume of 1 L, at 28 ºC with an aeration rate of 0.3 vvm (1 L of filtered air per minute) during 122 h. The fermented broth was filtered in a Whatman paper nº 1, followed by microfiltration and ultrafiltration using 0.2 µm and 30 kDa poliethersulphone (PES) membrane cassettes, respectively, with a surface area of 200 cm^2. Micro and ultrafiltration were performed in a VivaFlow 200 (Sartorius Stedim, Germany) crossflow filtration system.

5.2.2 Enzyme characterization

The concentrated enzymatic broth was used for the characterization experiments. The pH-enzyme activity curves were determined using the enzyme substrates, which were dissolved in proper ratio in different buffer solutions (citrate

buffer with pH ranging between 3.0 and 6.0; phosphate buffer with pH ranging from 7.0 to 8.0) for enzyme assays. The enzymes' optimum temperature was determined by the incubation of the enzyme-substrate preparation in temperatures ranging from 30 ºC to 70 ºC, with the use of controlled water baths.

The synthetic substrate *ρ*-NPG (*para*-nitrophenol-β-glucoside) was used in concentrations raging from 0.008 to 2 mM for kinetic parameters determination of BGL. Carboxymethylcellulose (CMC) concentrations ranging from 0.5 to 3.5 % (w/v) were used for EGL evaluation. The glucose inhibition effect over BGL was evaluated using concentrations of 0, 2.7, 5.5, 16 and 55 mM.

Kinetic parameters were evaluated according to the Michaelis-Menten model. Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) were determined from the double reciprocal Lineweaver-Burk linearization method (Equation 1) using the software Origin 7.0 (OriginLab, USA).

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

The turnover number or molecular activity K_{cat} (s^{-1}) was determined according to equation 2, where [E] refers to the total enzyme concentration.

$$K_{cat} = \frac{Vmax}{[E]} \quad (2)$$

The enzyme-catalyzed reaction activation energy E_a (kJ/mol) was obtained from the equation 3, where A is a pre-exponential factor, R is the universal gas constant (8.314 J/mol/K) and T is the temperature (ºK).

$$K_{cat} = Ae^{-Ea/RT} \quad (3)$$

The inhibition constant K_i (M) was determined from equation 4, where [I] refers to the inhibitor concentration (M), and α is the value obtained from the division of K_m constant with inhibition by K_m constant without inhibition.

$$K_i = \frac{[I]}{\alpha - 1} \quad (4)$$

The enzyme half-life = $t_{1/2}$ (min) was calculated by the determination of thermal-decay constants at 50ºC, where K_D is the denaturation constant.

$$t_{1/2} = \frac{\ln 2}{K_D} \quad (5)$$

The catalytic efficiency was calculated by the division of K_{cat} and K_m.

5.2.3 SDS-PAGE and Zymogram

Previously filtered enzymatic extract was microfiltered (0.22 μm) and concentrated (30 kDa) using a Vivaflow® 200 system (Sartorius, Germany). The concentrated samples were mixed with loading buffer (SCHÄGGER, 2006) and subjected to electrophoresis (LAEMMLI, 1970) at 30 V for approximately 1 h. Then, 10% (w/v) SDS-PAGE gels were submitted to 80 mA for approximately 2 h using a running buffer containing glycine 192 mM, 0.1% (w/v) SDS and Trizma base 25 mM. Samples with denaturant buffer were boiled for 3 min prior to electrophoresis. After SDS-PAGE gel was fixed, stained and washed (SCHÄGGER, 2006). For the zymogram analysis, two sets of samples were prepared with 10% (w/v) SDS-PAGE gel containing 1% (w/v) of carboximetylcellulose, which was washed thrice with distilled water and incubated at 50ºC with citrate buffer pH 4.8 (0.05M) for 1h. After it was stained in 1% (w/v) Congo red for 1.5 h and destained with 1 M NaCl (BÉGUIN, 1983). The molecular weights of the identified bands of the gel were calculated with the software Gelanalyzer 2010.

5.2.4 Liquid formulation and stability

The pre-selection of the additives and their tested concentrations for the cellulase formulation were based on previous works (ANVISA, 1988; GOELZER, 2015; MONTIBELLER, 2015). The tested additives are grouped according to Table 5.3.

The cellulase formulations were added to amber glass flasks maintaining at least 1/3 of headspace. The stability experiments were conducted in triplicate, with the incubation of the flasks at 50ºC during 14 days. Cellulase (U_{FP}) activity was

determined at the initial and final incubation times, or until the decrease of more than 50% of cellulase activity.

In the second step of the experimental procedure, 3 additives were selected for repetition of the stability test against a control sample without additives, in the same described conditions.

In the third step, three selected additives (sorbitol, benzoic acid and sodium chloride) were combined in a full factorial experimental design 2^3 + 3 central points for a total of 11 runs, (Table 5.4) for the evaluation of their effect on cellulase formulation stability. The software Statistica 7.0 (Statsoft, USA) was employed to generate the experimental matrix and statistical analyses.

Finally, the selected additives (sorbitol and benzoic acid) were employed in the long-term stability tests performed during 60 days at 4ºC.

5.2.5 Spray-Drying assays

The spray-drying procedure was performed in a bench-scale spray dryer Lab Plant SD-05 (Labplant™, England), with outlet temperature maintained at 70 ºC ± 10 ºC, a nozzle atomization system with 0.5mm diameter and compressor air pressure of 0.06MPa. The concentrated enzyme broth was formulated with maltodextrin (Corn Products, USA) as encapsulation agent. Different concentrations of maltodextrin (5%, 12.5% and 20% (w/v)) were tested in combination with inlet temperatures of 120 ºC, 150 ºC and 180 ºC in a full factorial experimental design with 2^2 + 2 central points (Table 5.1). The dried powders were collected and kept in glass bottles until further analysis.

Table 5.1 – Coded and un-coded parameters of full factorial experimental design 2^2 + 2 central points.

Experiment code						
Maltodextrin (%)	5	20	5	20	12.5	12.5
Inlet temperature (ºC)	120	120	180	180	150	150

The mass recovery and cellulase activity yield were determined according to Belghith et al. (2001). The total solids (TS) content (g) and the powder yield (%) was calculated from Eq. (6) and (7), respectively:

$$Ts\ (g) = ((ES \times vol) + (MD \times vol)) \quad (6)$$

$$Mass\ Recovery\ (\%) = \frac{Wt}{Ts} \times 100 \quad (7)$$

Where: ES represents the concentrated cellulase broth dry matter (0.45%), MD the percentage of Maltodextrin used (%, m/v), vol volume of fermented extract (400 mL) and Wt powder mass (g) of the samples obtained after drying.

The cellulase activity yield, also described as encapsulation efficiency was obtained based on the total enzyme activity before and after spray drying process. The total activity of the concentrated cellulase broth was 7200 U_{FP} (18.0 U_{FP}/mL in 400 mL) and 9100 U_{FP} (18.0 U_{FP} /mL in 500 mL) for the Aerosil® tests. Aerosil® is a commercial colloidal silica tested as an adjuvant to increasing desirable properties for powder formulations. The final cellulase activity was measured diluting 0.2 g of cellulase powder in 2 mL of deionized water. The cellulase activity yield and the total mass activity were calculated according to the Eq. (8) and (9).

$$Cellulase\ activity\ yield(\%) = \frac{(Total\ mass\ final\ activity(U) \times 100)}{Total\ mass\ initial\ activity(U)} \quad (8)$$

$$Total\ mass\ activity(U) = \frac{Mass\ activity\ (U.g)}{Powder\ mass\ (g)} \quad (9)$$

5.2.6 Cellulase powder characterization

The morphological properties of the spray-dried powders were observed in a Scanning Electronic Microscope (SEM) (JEOL, JSM-6360LV).

The powder samples were fixed to the SEM stubs of 12 mm diameter and then subjected to metallization with a thin layer of gold. Samples were observed with magnifications of 300 x, 750 x and 1500 x. An acceleration potential of 10 kV and backscattering electrons mode was used during micrograph.

The average size of the dried samples in aqueous medium was used to determine the hydrodynamic diameter by DLS (Brookhaven, NanoDLS). A small sample was suspended in purified water using magnetic agitation, and the particle size distribution was monitored during each measurement until successive readings became constant.

The diffractogram of the samples were examined using a X-ray diffractometer XRD-7000 (Shimadzu). Operational conditions of 40 kV of potency and 20 mA were used. Scanning conditions were 2 theta, scan range between 5 – 50 and 2.0 degree.min^{-1}scan speed.

Powders' moisture content was measured gravimetrically by infrared moisture balance Top-Ray (Bel Engineering®, Italy). Samples of approximately 3 ± 0.5 g, were placed evenly on the balance pan. The temperature was adjusted to 105°C and the results were recordered as moisture percentage.

5.2.7 Cellulase activity and sugars determination

Total cellulase activity was measured using a filter paper assay (FPase, stated as U_{FP}) standard method described by Mandels and Reese, (1957) and Ghose, (1987), adapted for deep-well microplates according to Camassola and Dillon, (2012). Filter paper stripes (0.6 x 1.0 cm) were employed as the substrate, and 50 µL of samples were appropriately diluted in sodium citrate buffer (50 mM; pH 4.8) and incubated for 60 min at 50 ºC. For the evaluation of endoglucanases (EGL, stated as U_{CMC}), the carboximetylcellulase assay (CMCase) was used (GHOSE, 1987), also adapted for deep-well microplates. Aliquots of 20 µL of carboxymetylcellulose 2% were used as substrate and 20 µL of the samples were appropriately diluted in the same buffer and incubated for 30 min at 50ºC. The reducing sugars were measured by the dinitrosalicylic acid (DNS) method (MILLER, 1959). One unit of cellulase activity was defined as the amount of enzyme producing 1 µmol of reducing sugars per minute.

Beta-glucosidase (BGL, stated as U_{BGL}) activity determination was performed by the use of 10 mM *ρ*-nitrophenol-β-glucoside (*ρ*NPG). After 15 minutes of incubation at 40 ºC, the enzyme activity is stopped with sodium carbonate and the absorbance read at 405 nm. One unit of BGL is defined as the amount of enzyme that liberates 1 µmol of *ρ*-nitrohpenol per minute (SINGHANIA et al., 2017).

5.3 RESULTS AND DISCUSSION

5.3.1 Enzyme characterization

Optimum pH and temperature of different cellulases from the enzyme extract are presented in Figure 5.1.

A narrow optimum pH profile was observed for FPase and BGL, between 4 and 5. However the same pattern was not observed for the EGL, which is determined as CMCase activity, where maximum relative cellulase activity occurs with acid pH conditions and decreases with the increase of pH. Other authors (BUSTO and ORTEGA, 1996) reported a very narrow optimum pH for CMCase activity that reaches its maximum at pH 5.0. BGL has higher activities at pH 4 – 5 according to Figure 5.1, which is reported by other authors (CHEN et al., 2011; TIWARI et al., 2013).

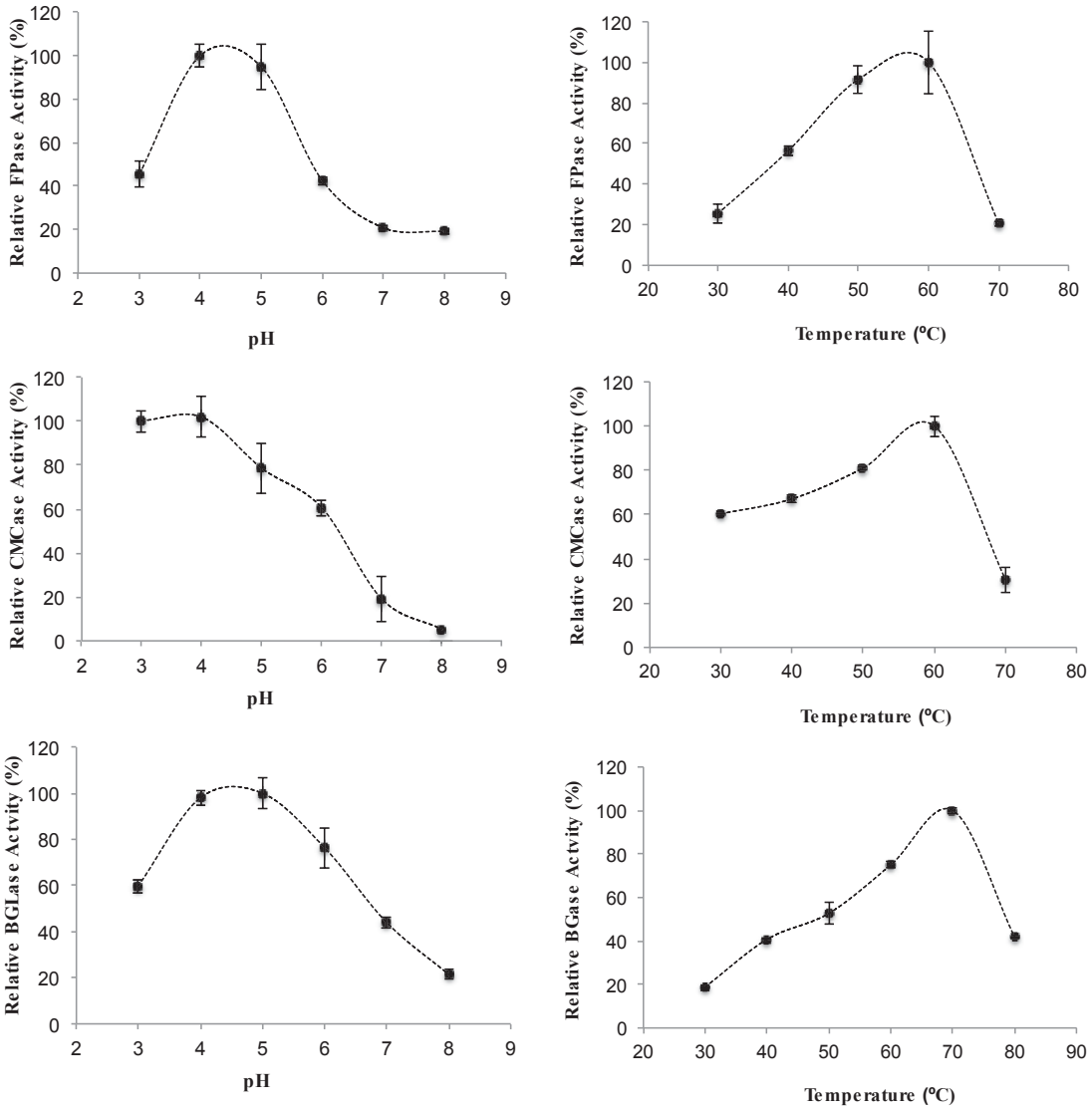

Figure 5.1 - Optimum pH (left column) and temperature (right column of FPase, CMCase and BGLase.

All the information about pH profiles are especially important for cellulases' application in pre-treated lignocellulosic biomass, since alkali and acid pre-treatment are the most used methods.

According to the Figure 5.1, right column, the optimum temperature between 50 and 60ºC was observed for FPase and CMCase, respectively, whereas temperatures between 60 and 70 ºC were observed for BGL, with a sharp enzyme activity decrease with temperatures above 70 ºC. According to Sørensen et al. (2013), mesophilic fungi produce BGL with optimum temperatures around 65-70ºC. Yun et al. (2001) performed the biochemical characterization of BGL from *T. harzianum* determining the optimum activity at pH 5.0 and 45 ºC. Chandra et al. (2013), found the optimum values as pH 5.5 and 55 ºC for a BGL from *T. citrinoviride*.

The temperature and pH behavior presented in the Figure 5.1 indicate that they may be applied for hydrolysis, since most of the commercial cellulosic enzymes are applied in hydrolysis processes at 50 ºC and pH 5.0. Residual acid or alkali from the pre-treatments of cellulosic residues may interfere in the enzymatic saccharification leading to the necessity of a previous pH adjustment. Regarding the temperature, the increase of 10 ºC double the enzymatic reaction rate until the denaturation and irreversible inactivation occurs (SØRENSEN et al., 2013).

5.3.2 Kinetic parameters

Michaelis-Menten plots of the cellulases (EGL and BGL) using CMC and *p*-NPG as substrate were evaluated and the results are presented in Figure 2.

CMCase (EGL) presented a K_m of 1.29 % (m/v) (63 M) and V_{max} of 1.206 µmol/mLmin. Busto and Ortega (1996) found a K_m of 1.32% and V_{max} of 6.75 µmol/mLmin, for EGL produced from a *T. reesei* culture (Table 5.2).

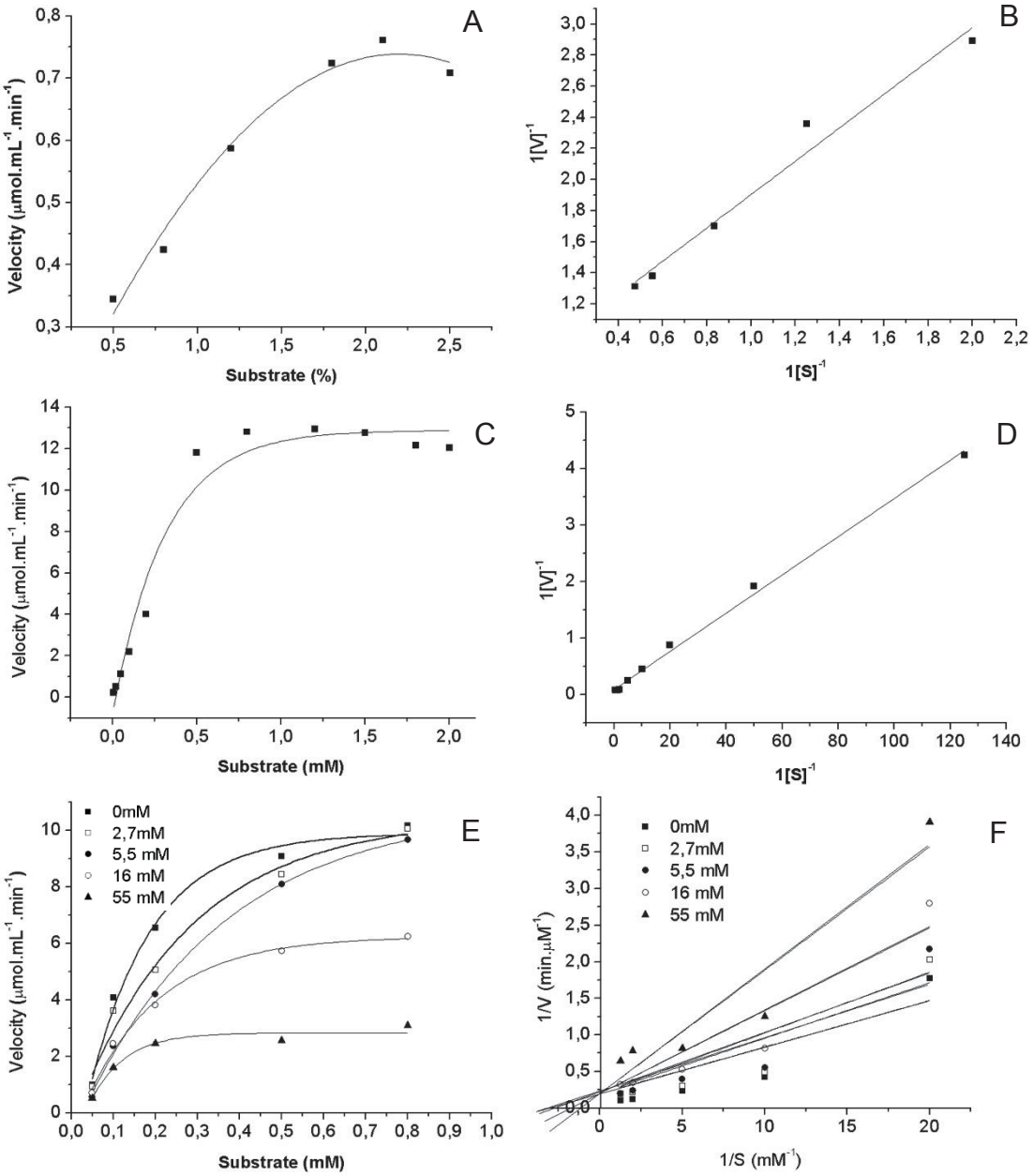

Figure 5.2 – Michaelis-Menten and Lineweaver-Burk plots for EGL using carboximethylcelulose as substrate (A, B), BGL using *p*-NPG as substrate (C, D), and the inhibition effect of glucose on BGL (E, F), respectively.

Michaelis constant (K_m) value of 0.39 mM and V_{max} of 12.04 µmol/mL.min were determined for BGL. A similar value (0.38 mM) was obtained by Chauve et al., (2010) using the same substrate for *T. reesei* BGL. The authors compared the kinetic parameters of BGL from *A. niger* and *T. reesei* using a purified broth by fast protein liquid chromatography. *A. niger* BGL presented a K_m of 0.57 mM. For cellobiose as substrate the authors found higher K_m values, indicating the higher affinity by the

synthetic substrate pNPG. Other authors found the K_m values of 0.182 mM (CHEN et al., 1992) and 0.102 mM (CHIRICO, 1987) of BGL's from *T. reesei* strains using pNPG as substrate. Decker et al. (2000) presented the K_m value of 0.64 mM for BGL from *A. niger*.

Table 5.2 – Kinetic properties of the cellulases over different substrates.

		Substrate	
Property	Filter Paper	CMC	*p*-NPG
K_m (mM)		63	0.39
V_{max} (µmol/mL.min)		1.206	12.04
E_a (kJ/mol)	39.0	14.2	37.2
K_{cat} (s^{-1})	20	100	100
K_{cat}/K_m ($s^{-1}.M^{-1}$)		1.58	2.56
$t_{1/2}$ (min)	123	141	19.8
K_i $(M)_{gluc.\ 55mM}$			0.0330
K_i $(M)_{gluc.\ 16mM}$			0.0192
K_i $(M)_{gluc.\ 5.5mM}$			0.0165
K_i $(M)_{gluc.\ 2.7mM}$			0.0162

Fungal BGL are classified, according to their affinities towards their substrates cellobiose and pNPG, into three groups: BGLI (aryl β-glucosidases) with lower K_m for pNPG; BGLII (true cellobiases) with lower K_m for cellobiose and BGLIII (broad substrate specificity) similar affinities for both substrates, in which most of the BGL belongs. K_m values could range from 0.031 to 340 mM for cellobiose and from 0.055 to 34 mM for pNPG, for a variety of fungal species (TIWARI et al., 2013).

Commercial BGL's Novozymes S188 (*A. niger*) and Sigma (*A. niger*) have K_m values of 0.46 mM (HIMMEL et al., 2017) and 1.03 mM (DEKKER, 1986), respectively.

In Figure 5.2-F, according to Chauve et al. (2010), it is identified the classical common interception on *y*-axis with different glucose concentrations, confirming the competitive inhibition behavior. The competitive inhibition involves the formation of an inactive enzyme-product complex and this seems to be independent of the substrate nature, since the authors tested both natural (cellobiose) and synthetic (pNPG) substrates. Authors found a more sensitive behavior of BGL for glucose inhibition in

the case of the *A. niger* BGL, presenting an inhibition constant of 2.7 mM in comparison to a BGL from a *T. reesei* with 3.25 mM. The presence of 55 mM (10 g/L) of glucose inhibited BGL's activity by 45%. Chauve et al. (2010) found that the presence of 30 g/L of glucose inhibited BGL's activity by 85%.

The equations from the Arrhenius plot (Figure 5.3) were used for the calculation of the enzyme energy of activation presented in the Table 5.2.

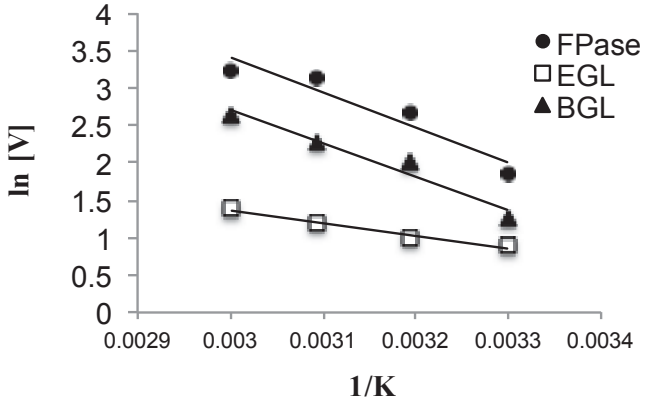

Figure 5.3 – Arrhenius plots for the cellulase reaction with the substrates Filter Paper (Total cellulase), CMC (EGL) and *ρ*-NPG (BGL).

The values of energy of activation of the 39.0, 14.2 and 37.2 kJ/mol were determined for FPase, CMCase and BGLase, respectively, which are in accordance to classic enzymatic reactions (CHAUVE et al., 2010). According to Whitaker (1994) the energy of activation for the enzymatic transformation of reagents to products is comprehended between 25 and 62 kJ/mol. Chauve et al. (2010) determined an energy of activation of 53.2 kJ/mol for an *A. niger* BGL for temperatures between 30 and 60ºC.

The $t_{1/2}$ values indicated that the BGL lost the activity earlier (19.8 min) than total cellulase (123 min) and EGL (141 min). Around 70% of the BGL activity was lost in the first 10 minutes of incubation (Figure 5.4), whereas the total cellulase activity and EGL activity presented losses of 14 and 9 %, respectively. The sharp enzyme activity decrease probably reduced the $t_{1/2}$ value for BGL. The $t_{1/2}$ value of 69 min was determined for an *A. niger* mannanase identified by Montibeller (2015) and 58.7 min for an *A. niger* xylanase, at the same tested temperature (GOELZER, 2015).

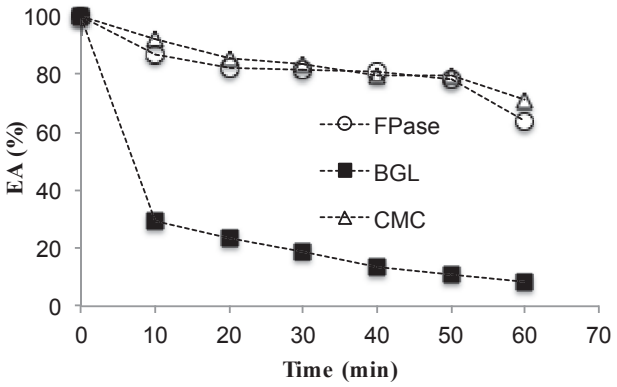

Figure 5.4 – Loss of enzyme activity (*EA*) during 60 minutes of incubation at 50ºC for FPase, BGL and EGL.

5.3.3 SDS-PAGE and Zymogram

The SDS-PAGE presented two bands with 48 and 43 kDa and another with 25 kDa. Other less expressive bands with 71 kDa, 37 kDa, 30 kDa, 27 kDa and 26 kDa were also observed. The Zymogram revealed a clear band with approximately 27 kDa and other two bands around 47 kDa (Figure 5.5).

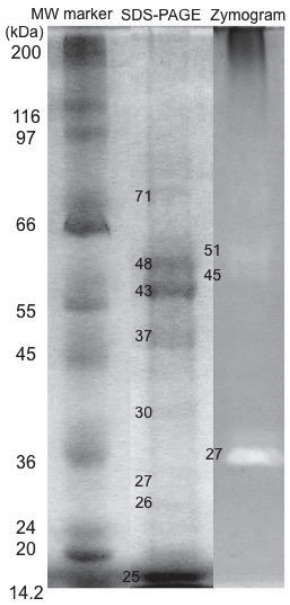

Figure 5.5 – SDS-PAGE and Zymogram of the culture concentrates.

The *Trichoderma* genus is able to secrete five different EGL's (MIETTINEN-OINONEN, 2004), the Cel7B (EGL-I) with 50-55 kDa (PENTTILÄ et al., 1986), representing 5-10% of the secreted protein; Cel5A (EGL-II) with 48 kDa (SALOHEIMO et al., 1988); Cel12A (EGL-III) with 25 kDa (WARD et al., 1993; OKADA et al., 1998); Cel61A (EGL-IV) with 34 kDa (SALOHEIMO et al., 1997); Cel45A (EGL-V) with 23 kDa (SALOHEIMO et al., 1994). Marco et al. (2007) identified two EGL's with 29 and 36 kDa from *Trichoderma harzianum* in cultures containing chitin as the sole carbon source.

In the same way, the *Trichoderma* genus is able to secrete two EGL's and two BGL's, named Cel7A (CBH-I) with 59-68 kDa (SHOEMAKER et al., 1983; TEERI et al., 1983); Cel6A (CBH-II) with 50-58 kDa (TEERI et al., 1987) and BGL-I with 75 kDa and BGL-II with 52 kDa (FOREMAN et al., 2003).

Th 27 kDa band identifyied in the zymogram is in accordance to EGL IV and V, according to Saloheimo et al. (1997). Other authors (ROCHA et al., 2013) found the zymogram for CMCase activity around 60 kDa, which may be more related to the most common EGL (Cel7A) for the *Trichoderma* genus.

The secretome of *Trichoderma harzianum* is very complex and composed mainly (51.36%) by glycoside hydrolase family, including chitinases, endo-N-acetylglucosaminidases, hexoaminidases, galactosidases, xylanases, exo-1,3-glucanases, endoglucanases, xylosidases and others. Proteases contribute with 8.9% of the secreted proteins, 17.6% are intracellular proteins, and 22.14% are unknown proteins (VALE et al., 2012). Vale et al. (2012) evaluated the secretome of *T. harzianum* grown on cellulose as the sole carbon source and chitinases were shown to be the majority of the secreted hydrolases, instead of cellulases that were expected. Gómez-Mendoza et al. (2014) found that the use of sugar-cane bagasse as carbon source induced the highest cellulolytic and xylanolytic activities in *T. harzianum* cultures. According to SDS-PAGE analysis, the molecular mass of BGL from *T. harzianum* is 75 kDa (YUN et al., 2001); A 90-kDa BGL was identified by MALDI-TOF analysis from an enzymatic extract from *T. citrinoviride*, consisting of a single polypeptide chain (CHANDRA et al., 2013).

5.3.4 Study of the stability of cellulases' liquid formulation

The first step of the study of enzymatic stability extract included the selection of the best compounds to be added in cellulases' liquid formulation under accelerated conditions is presented in Table 5.3. The tested additives and theirs concentrations were selected based on their function and maximum limits for application, according to ANVISA (1988).

Table 5.3 – Coded and un-coded additives for the cellulases' formulation and their residual cellulase activity at accelerated stability conditions.

Group	Code	Function	Additive	Concentration (w/v) %	Residual enzyme activity (%)
Group 1		Salts	Sodium chloride	0.5	31.6
			Sodium citrate	1.0	27.7
			Potassium sorbate	0.15	17.7
			Sodium benzoate	0.25	21.9
Group 2		Polyols	Glycerol	27.6	56.5
			Sorbitol	54.6	82.4
			Xylitol	45.6	77.8
			Mannitol	5.0	34.9
Group 3		Poliethyleneglycol	PEG400	2.5	21.0
			PEG800	2.5	18.9
Group 4		Acids	Benzoic acid	0.05	56.0
			Citric acid	1.0	32.4
			Ascorbic acid	0.05	45.3
Group 5		Proteins	BSA	2.5	34.8
		Aminoacids	Methionine	1.0	32.7
		Synthetic aminoacids	EDTA	0.05	35.3

Sorbitol and benzoic acid, a polyol and an acid, maintained the highest relative cellulase activities of 82.4% and 55.9%, respectively, after 216 hours of incubation under 50 ºC. Xylitol also provided good results (77.8%). However, it is an expensive compound for industrial applications. Among the group of salts, considered as

stabilizers and preservatives, sodium chloride and sodium citrate maintained around 30% of the initial cellulase activity. Sorbitol, benzoic acid and sodium chloride were chosen to be tested in a further experiment, with a control, consisting of a non-formulated concentrated enzyme extract (Figure 5.6).

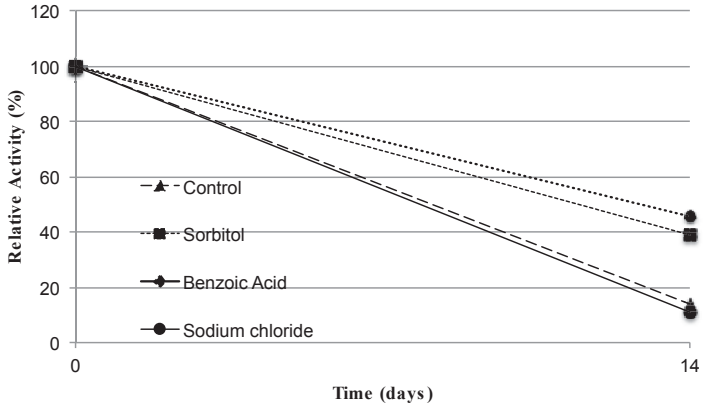

Figure 5.6 – Effect of the compound in the cellulase stability incubated during 14 days at 50 ºC.

Sorbitol and sodium chloride maintained the highest relative activities of the enzymatic extract (38.77 and 45.66%, respectively) after 14 days under 50 ºC. Sodium chloride presented no effect on relative cellulases activity when compared to the non-formulated enzymatic extract. Several papers claim to have discovered thermostable BGL's, however, activities are only reported at its optimal temperature for a short duration of time. For biomass hydrolysis processes that are typically carried out for several hours or even days, it is very important the stability of the enzyme at specified temperatures (SØRENSEN et al., 2013).

The third step of enzymatic formulation was the study of the interaction of three independent variables in different combinations (Sorbitol + Benzoic acid and Sorbitol + Sodium chloride) on cellulase production is presented in Table 5.4.

The coefficient of determination (R^2) was 0.74, which indicates that approximately 74% of the variability of the dependent variable, cellulase activity, could be explained by the model. The ANOVA showed that sorbitol has a significant effect over enzymatic activity (p-value <0.05). The interaction between sorbitol and benzoic acid as well as with sodium chloride are not significant. Sodium chloride combined with benzoic acid also did not affect cellulase stability. Benzoic acid was,

individually, efficient in accelerated stability experiments, but not in combination with sorbitol.

Based on the analysis of Table 5.4, optimal concentrations of sorbitol are between 25 and 75% and its combination with benzoic acid and sodium chloride was not statistically relevant.

Table 5.4 - Full factorial experimental design 2^3 plus 3 center points for the evaluation of the influence of sorbitol, benzoic acid and sodium chloride (%, m/v) on FPase activity.

Run	Sorbitol (%)	Benzoic acid (%)	Sodium chloride (%)	FPase (U_{FP}/mL)
1	25(-1)	0.01(-1)	0.25(-1)	9.93 ± 1.4
2	75(+1)	0.01(-1)	0.25(-1)	7.21 ± 1.1
3	25(-1)	0.1(+1)	0.25(-1)	11.22 ± 0.6
4	75(+1)	0.1(+1)	0.25(-1)	10.59 ± 0.7
5	25(-1)	0.01(-1)	0.75(+1)	13.36 ± 0.7
6	75(+1)	0.01(-1)	0.75(+1)	7.82 ± 2.9
7	25(-1)	0.1(+1)	0.75(+1)	10.27 ± 2.1
8	75(+1)	0.1(+1)	0.75(+1)	10.39 ± 0.4
9(C)	50(0)	0.05(0)	0.5(0)	11.77 ± 1.1
10(C)	50(0)	0.05(0)	0.5(0)	11.72 ± 1.9
11(C)	50(0)	0.05(0)	0.5(0)	9.34 ± 2.3

A different behavior was observed in the long-term stability experiments, which were conducted with formulations composed of the enzymatic extract with sorbitol and benzoic acid, enzymatic extract with sorbitol and the control (enzymatic extract without additives) maintained at 4ºC or environmental conditions during 30 days that can be observed in Figure 5.7.

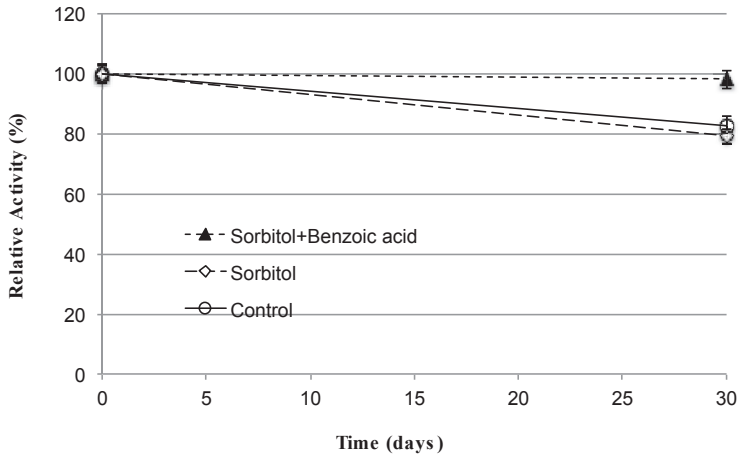

Figure 5.7 – Long-term stability profile of the formulated and non-formulated cellulase during 30 days at 4 ºC.

The presence of benzoic acid combined with sorbitol maintained 100% of the cellulase activity after 30 days at 4 ºC. The biocidal function of the benzoic acid was important, since the control and the sample with only sorbitol presented a reduction of around 20% of cellulase activity. The sample formulated only with sorbitol presented turbidity, probably due to the contamination that was caused, probably, by the presence of sorbitol, which is used as carbon source.

According to the commercial cellulase, Celluclast 1.5L (Novozymes – Denmark) data sheet, sorbitol is used as one of the stabilizers, together with sodium chloride and the preservative potassium sorbate. Other enzymes datasheets such as the one from amylase Thermamyl 120L (Novozymes – Denmark) describes the use of sucrose, sodium chloride and potassium sorbate in its formulation. Sorbitol was already described as a preservative for other enzymes such as lipase (NOEL and COMBES, 2003) hexokinase (TIWARI and BHAT, 2006) amylase (YADAV and PRAKASH, 2011), trypsin (PAZHANG et al., 2016) and xylanase (LEMOS et al., 2000). Benzoic acid has been described as a biocidal agent and was approved for the formulation of technical enzymes (AMFEP, 2018).

5.3.5 Powdered enzymatic formulation

The use of 20% of maltodextrin (MD) and an inlet temperature of 120ºC led to the highest yield, in terms of cellulase activity (40.6%), with a powder moisture of 9,44% (Table 5.5). The highest weight yield (14.3%) was observed using only 5% of MD and higher temperature (180 ºC). This would be related to the fact that higher

temperatures are efficient for the drying process, but are responsible for enzyme denaturation. The same phenomena was observed by Belghith et al. (2001) using 1% MD and 175 ºC, with higher weight yield (85%) and lower cellulase yield (33%). When the MD content was increased to 3%, cellulase yield reached 45%.

Table 5.5 – Mass recovery yield and cellulase activities after spray drying in different conditions.

Sample	MD[1] (%)	Inlet Temperature (ºC)	Weight (g)	Mass recovery (%)	Total cellulase activity (U_{FP})	Cellulase activity yield (%)
A	5	120	2.33	10.67	2440,63	33.9
B	20	120	6.63	8.10	2928,80	40.6
C	5	180	3.13	14.34	1223,31	17.0
D	20	180	0.85	1.03	239,81	3.3
E	12.5	150	1.64	3.16	927,16	12.8
F	12.5	150	3.99	7.69	2130,05	29.6
2% Aerosil®	20	120	5.33	5.23	4112.9	45.70
0% Aerosil®	20	120	7.88	7.73	5211.9	57.91

[1]Maltodextrin

Table 5.6 – Analysis of variance of the tested conditions after spray drying for cellulase recovery yield.

ANOVA; Var.: Activity yield; R-sqr=,89114; Adj:,72786

	SS	df	MS	F	*p*
(1) MD	1211,040	1	1211,040	8,312737	0,102189
(2)Temperature	1171,693	1	1171,693	8,042653	0,105098
1 by 2	2,528	1	2,528	0,017353	0,907253
Error	291,370	2	145,685		
Total SS	2676,631	5			

The analysis of variance is presented in the Table 5.6 where it is possible to verify that the results were not significant for 95% of confidence level. The Pareto chart (data not shown) demonstrated that higher concentrations of maltodextrin and lower temperatures increase the values for activity yield. This result is in accordance to the Table 5.5, where the highest activity yield was achieved using 20% MD in

combined with an inlet temperature of 120ºC. This result may be attributed to the equipment configuration, which affected the reproductibility of the results.

The dry matter content of the cellulase broth is a factor that seems to influence the mass and enzyme recoveries in the drying process. The cellulase broth dry weight obtained after the concentration (MF and UF) process used in this work was 1.82 g, from 400 mL. In a preliminary test, the use of the concentrated broth without additives resulted in absolutely no powder recovery. Other authors (BELGHITH et al., 2001) informed the value of 8 g for the cellulase broth applied in spray drying, achieving a mass recovery and cellulase yields of 62.5% and 52%, respectively, without the use of additives. These results justify the use of additives not only for the microencapsulation process as protection agent, but also to improve dry mass recovery purposes. The filtration, microfiltration and concentration by ultrafiltration of the fermented broth, previously to the spray-drying process, certainly reduced the solids concentration.

The spray drying process as an interesting alternative to maintain enzyme stability by the low water content in the samples. However, the loss of around 60% of enzyme activity, just after the drying process, is a serious disadvantage of this process when compared to liquid formulations. In terms of stability, there is not a considerable difference between liquid and dried formulations. The evaluation of other factors should be carried out to ensure the best way to produce a cellulolytic formulation, such as transportation, the mode of application, the degree of purification, etc.

The highest enzyme stability was maintained (91%) after 60 days for the sample that was dried with 5% of MD at 180 ºC (Figure 5.8). A relative low cellulase activity yield (17%) and higher weight yield (14%) were obtained under this condition. This behavior would be related to better drying results obtained with higher temperatures, but with higher degree of enzyme deactivation. The reduction of water content in the microcapsules is related to better stability.

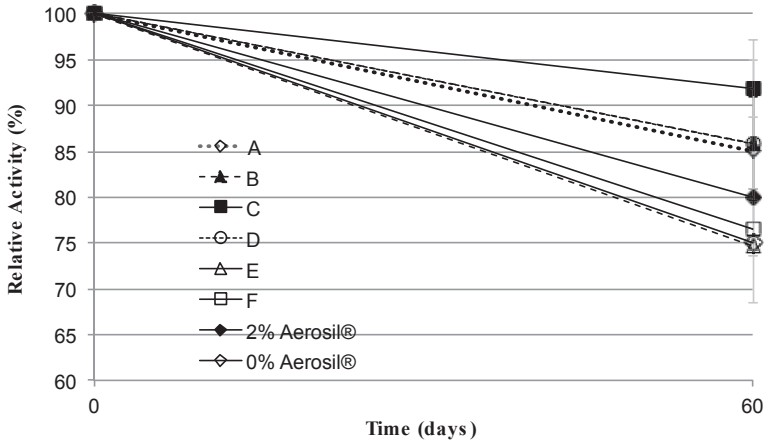

Figure 5.8 – Stability of the cellulase formulations dried variating the temperature, MD% and presence of Aerosil®. (A) MD 5% + 120ºC; (B) MD 20% + 120ºC; (C) MD 5% + 180ºC; (D) MD 20% + 180ºC; (E,F) MD 12.5% + 150ºC.

Colloidal silica (Aerosil®) is a submicroscopic, white, amorphous powder, with neither odor nor flavor. It is known as a phamacotechnical adjuvant used as a desiccant and as a non-stick agent for hygroscopic powders, as well as anti-caking and anti-humectant, dentifrice polishing and flow agent (FERREIRA et al., 2016). According to Figure 5.8, the addition of Aerosil® (80%) did not improve the cellulase powder stability compared to the control (75%) as well as the mass recovery (5.23%) and cellulase activity yield (45.7%) (Table 5.5). However, the addition of Aerosil® promoted less powder adhesion on internal walls of the drying chamber. The moisture content of the powder obtained with the addition of Aerosil® was reduced from 10.02% (control) to 7.25%. Other authors (FERREIRA et al., 2016) reported the positive effect of the addition of colloidal silica, improving the glass transition temperatures when compared to samples without the use of the adjuvant.

According to Figure 5.9, the analysis performed by SEM revealed different powder structure characteristics depending on the applied drying conditions.

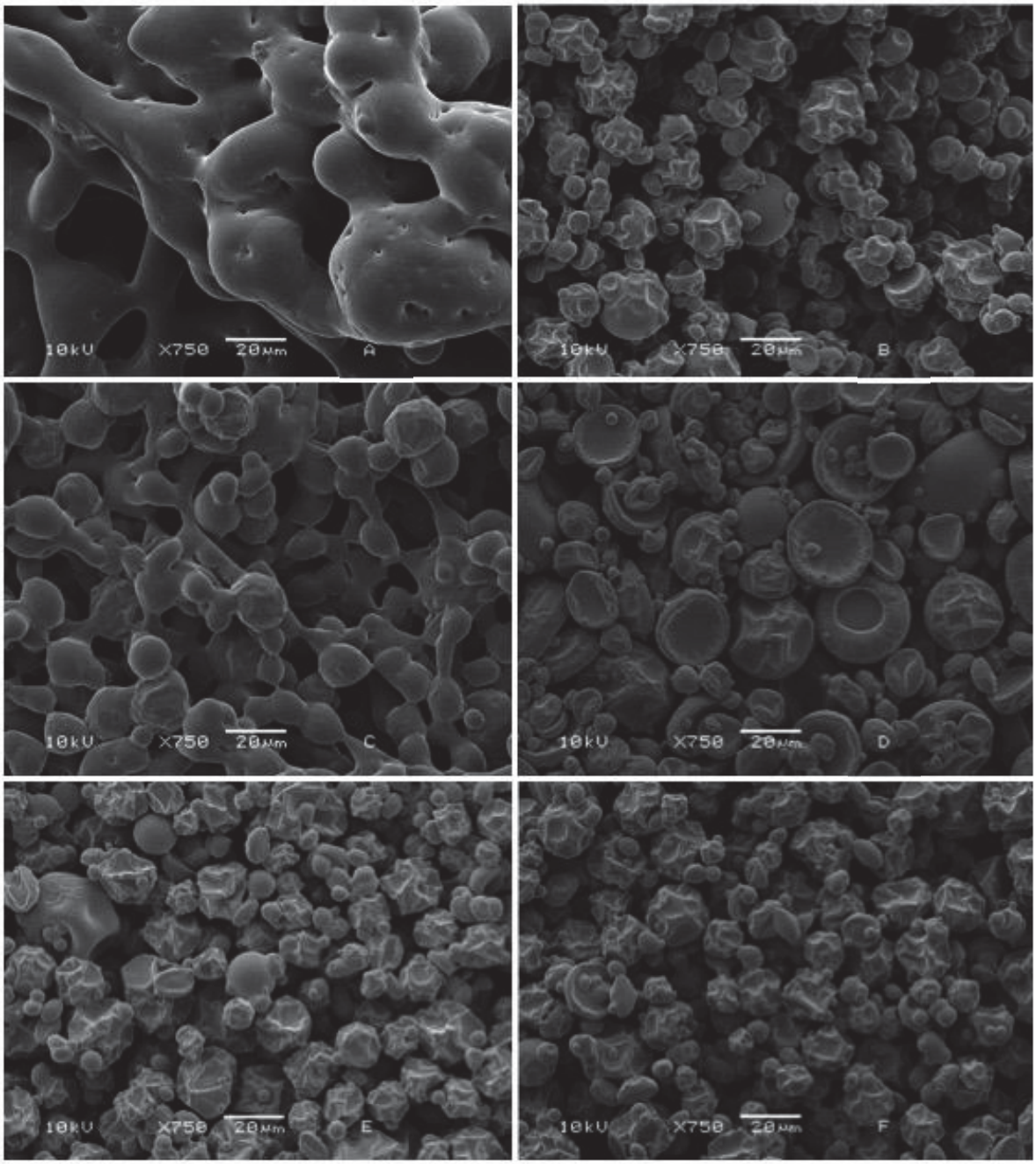

Figure 5.9 - Scanning electron micrograph (SEM) from the dried samples of cellulase extract under different conditions: (A) MD 5% + 120 ºC; (B) MD 20% + 120 ºC; (C) MD 5% + 180 ºC; (D) MD 20% + 180 ºC; (E,F) MD 12.5% + 150 ºC.

According to Figure 5.9, both the MD concentration and the inlet temperature of the spray drying process significatly influenced the morphology of the particles. The use of 5% MD was not sufficient for the formation of spherical particles, using 120ºC or 180ºC inlet temperature. However 20% MD was enough for the microencapsulation of the enzyme complex using both temperatures, with morphological differences, specially using the temperature of 180 ºC. Middle

temperature and MD% (150 ºC, 12.5%) also led to the microencapsulation presenting spherical forms.

These vacuoles are originated as a result of the quick particles expansion during the final stages of drying process. According to Carneiro et al. (2013), the presence of hollow spheres does not mean a fail of the encapsulation since the active material adheres to the internal wall of the spheres matrix, which is a typical behavior of particles obtained by spray drying.

The particle size is another characteristic affected by the drying conditions. According to the particle size distribution analysis (Figure 5.10-A), most of the particles sizes range from 10 to 20 μm.

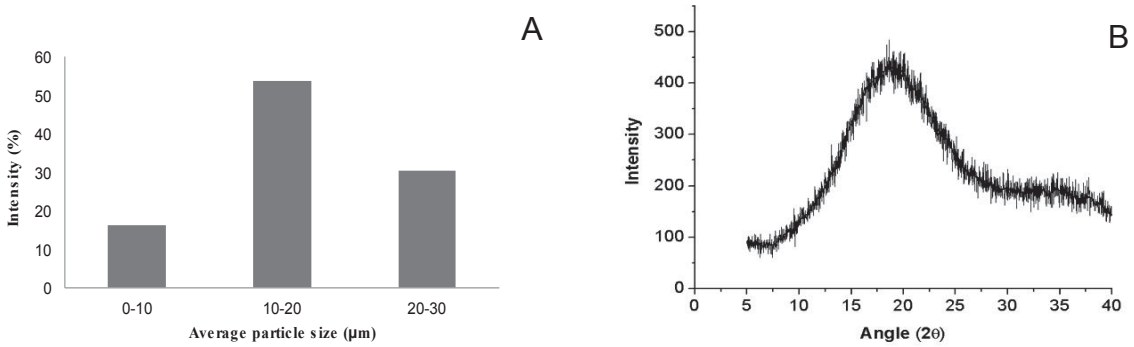

Figure 5.10 – (A) Particle size distribution and (B) X-ray diffraction (XRD) profile of the cellulase powder dried at 120 ºC and 20% MD.

According to Figure 5.10-B, the peak is large and non-defined, indicating an amorphous material that is characteristic of MD. This is in accordance to what was observed by Cano-Chauca et al. (2005), with the use of MD as encapsulating agent for food drying process. According to Chiou and Langrish (2007), amorphous particles are created by the rapid drying, and it is correlated to the sticky properties of the powder. The stickiness and the amorphous nature of the material are correlated to the glass transition temperature of the material. The glass transition causes a drastic decrease in the viscosity and increase in molecular mobility, leading to various time-dependent structural transformations. Addition of high molar mass compounds contributes to an increase in glass transition and to maintaining the glassy state in broader temperatures and relative humidity conditions for powder stability (FERREIRA et al., 2016). Drying conditions above the glass-transition point

lead to amorphous particles that can be sticky. Stickiness of powders is problematic since it is associated to wall depositions and loss of material.

5.4 CONCLUSIONS

The cellulases produced using domestic wastewater as substrate presented kinetic parameters comparable to those found by other authors. The concentrated broth demonstrated EGL, BGL and total cellulases activity. According to the SDS-PAGE a zymogram analysis the EGL molecular weight correspond to 7 different EGLs described in the literature.

The presence of 10 g/L of glucose inhibited around 45% of the BGL activity, indicating that it is a limiting factor for the application of this enzyme for biomass hydrolysis processes.

The drying of the cellulase formulation revealed a loss 60% of the enzyme activity just after the powder formation. Besides, the microencapsulation agents and the drying conditions, the mass recovery and enzyme activity yields seem to be very influenced by the equipment performance. Contrarily, liquid formulations did not present activity loss just after the formulation. According to the stability tests, the liquid formulation presented a better stability performance in comparison to the dried formulations. Probably, for longer storage tests above 60 days the dried formulations could be more stable. Other factors should be evaluated to ensure the best method to produce a cellulolytic formulation, such as the transportation, the mode of application, the degree of purification and the commercial prices.

6 APPLICATION OF A CELLULASES' FORMULATION IN THE HYDROLYSIS OF WASTEWATER TREATMENT PLANT CELLULOSIC RESIDUE

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ABSTRACT

Cellulases from the strain *Trichoderma harzianum* TRIC03-LPBII were previously produced, concentrated and formulated for its application in the hydrolysis of palm oil empty fruit bunches (EFB) and wastewater treatment plant residue (WWTP residue). The cellulosic fraction of the WWTP residue was analyzed (21.3%) and its potential to be recovered and hydrolyzed with an in-house enzymatic blend evaluated. The application of the formulated cellulase for the hydrolysis of EFB and WWTP residue resulted in glucose yields of 37.59 mg/$g_{cellulose}$ and 21.17 mg/$g_{cellulose}$, respectively, as well as 13.12 mg/$g_{residue}$ and 4.52 mg/$g_{residue}$. The WWTP residue has a potential to be used as a source of fermentable sugars by the enzymatic hydrolysis, since huge amounts of such residue are monthly produced in the studied treatment plant (4.07 tons). The hydrolysis of the WWTP residue may reduce costs related to the wastewater treatment process and, simultaneously, be the raw material for the production of biofuels and biochemicals. The produced enzyme Wlase have a potential to be applied in hydrolysis processes for lignocellulosic residues such as EFB and WWTP residues, aiming at the production of biomolecules and the delevolpment of environmentally friendly processes.

Key words: Cellulase application; enzymatic hydrolysis; cellulosic residues

6.1 INTRODUCTION

The increasing demand for green fuels, specially bio-ethanol, pushed the demand for carbon sources other than the well known feedstock such as the corn in USA and the sugarcane in Brazil (LI et al., 2012). Lignocellulosic biomass is widely available and contains significant quantities of cellulose, which could be hydrolyzed into fermentable sugars or biochemicals. The enzymatic saccharification of cellulose has been an acceptable way of obtaining biochemicals. Short-chain polysaccharides that are produced from the hydrolysis process are useful intermediates for diverse biotechnological applications (OBENG et al., 2017).

Although cellulases have been used for the saccharification of cellulosic matter, their cost is still one of the main bottlenecks for the large-scale application. The cellulases off-site production costs ranges from $ 4.4 to $ 8.8 dollars per kilogram of protein. Screening of hyper cellulase secreting mutants, enzyme recycling, selection of potent hydrolytic enzymes and supplementation with other enzymes and additives are some of the methods explored to improve the enzymatic hydrolysis aiming at the reduction of costs (ADSUL et al., 2014). According to Adsul et al. (2014) the blending of on-site enzyme preparations is an interesting choice to get higher hydrolysis yields with lower enzyme costs.

Agricultural wastes, food processing waste, paper industry waste as well as the municipal solid waste have been reported as cellulosic materials to be enzymatically converted to fermentable sugars and/or biochemicals (LI et al., 2012). The concept of biorefinery defines this strategy, centered in the use of agro-waste materials as substrate for valuable biomolecules production.

The enzymatic hydrolysis of oil palm empty palm fruit bunches (EFB) has been widely proposed as an interesting source of cellulose for producing fermentable sugars (HASSAN et al., 2013) as well as other compounds such as lignin (MEDINA et al., 2015). Besides the worldwide yearly disposal of around 37.7 million tons of EFB, causing a serious environmental and economical problem, its high content of cellulose (24-65%) and hemicellulose (21-34%) shows the potentiality of this residue as a source for bioethanol or other biochemicals production after enzymatic hydrolysis. EFB's enzymatic saccharification was performed at mild conditions of temperature (45-50ºC) and pH (4.0-5.0), using less energy and cost comparing to acid conditions (JUNG et al., 2015; SUGIHARTO et al., 2016). In addition, the

enzymatic process does not produce unwanted byproducts as the thermochemical processes, that are inhibitory to microorganisms used in subsequent fermentation (PALAMAE et al., 2017).

The use of EFB in biorefineries has been described as very promising, where other strategies and materials sources have been explored for the development of a bio based economy. In this way, the wastewater biorefinery term was developed, which is characterized by the integration of the wastewater treatment and the production and/or recovery of value-added products (VERSTER et al., 2014).

The recovery of cellulosic substances is thought to be a future trend of sludge treatment and disposal methods at wastewater treatment plants (HONDA et al., 2000). According to Fooij (2015), the toilet paper contributes to 23% of the organic matter in sanitary wastewater. Ruiken et al. (2013) proposed that the cellulose fibers could be a potential source that could be easily recovered from wastewater by, for example, sieving.

Faust et al. (2014) presented that 70% of the material collected by sieving raw sanitary wastewater was composed by cellulose. Ruiken et al. (2013) estimated a contribution of 12,000-15,000 tons of toilet paper per year for a Dutch Wastewater Treatment Plant (WWTP) system, resulting in 17,000 to 21,000 tons of chemical oxygen demand (COD) entering the treatment system. The recovered cellulose-containing material could be used as soil conditioner in agriculture, fuel in biomass based power plants as well as feedstock in the fermentation industry for the production of biofuels such as ethanol. Ruiken et al. (2013) estimated a yearly saving of €125,000 by the use of sieving for the recovery of cellulosic-like material, reducing the costs associated to the sludge treatment and the load entering the WWTP. European consortium between Dutch companies, universities and governmental institutions are already developing a technology for sieving and recovering the cellulosic matter from the WWTP's (CADOS, 2018). The evaluation of the cellulose content from sanitary wastewater is a neglected research area. Until now it was not identified any evaluation regarding the cellulose content in domestic wastewater in Brazil. Although the fact that in Brazil the toilet paper is normally sent to sanitary landfills as solid residue, there is no information about how much of this material is thrown in the toilet and flushed to the wastewater treatment plants.

Most of the literature data reports the hydrolysis processes with the use of commercial enzymatic blends, mainly composed by cellulases. Commercial

enzymatic cellulase cocktails are normally formulated with a balanced proportion of endoglucanases (EGL) and cellobiohydrolases (CBH) and beta-glucosidases (BGL), even produced from different and/or mutant microbial strains, concentrated and even formulated with additives, accessory enzymes and other compounds (SINGHANIA et al., 2017).

This work proposes the application of a cellulase formulation, which was produced from the strain *Trichoderma harzianum* TRIC03-LPBII for the hydrolysis of cellulosic residues such as EFB and the WWTP residue. The potential recovery and use of WWTP residue as a new source of fermentable sugars through enzymatic hydrolysis was also evaluated.

6.2 MATERIAL AND METHODS

6.2.1 Cellulase production and formulation

Cellulase production was performed using *Trichoderma harzianum* (TRIC03) from the Federal University of Paraná Culture Collection, Bioprocess Engineering and Biotechnology Department (LPBII-UFPR). The culture medium was composed of lactose (11.9 g/L), peptone (5.0 g/L), KH_2PO_4 (2.5 g/L), and $MgSO_4.7H_2O$ (0.3 g/L), which were added to the raw domestic wastewater previously collected at the Atuba Municipal Wastewater Treatment Plant from the Sanitation Company of Paraná (SANEPAR), located in Curitiba, Brazil. Previous publication (LIBARDI et al., 2017) proposed the use of sanitary wastewater as basis for the culture medium. A fungal spore suspension of approximately 10^7 spores/mL was used as inoculum. Cellulase production was conducted by submerged fermentation in a 1.5-L Bubble Column Reactor (BCR) with a working volume of 1 L, at 28 ºC with an aeration rate of 0.3 vvm (1 L of filtered air per minute) during 122 h. The fermented broth was filtered through a Whatman paper nº 1, followed by microfiltration and ultrafiltration using 0.2 µm and 30 kDa poliethersulphone (PES) membrane cassettes with a surface area of 200 cm^2. The ultrafiltration was performed in a VivaFlow 200 (Sartorius Stedim, Germany) crossflow filtration system. Sorbitol (50% w/v) and benzoic acid (0.05% w/v) were added to the liquid extract for the composition of the final formulated enzyme. The formulated enzyme was applied for the hydrolysis experiments.

6.2.2 WWTP cellulosic-residue characterization

The cellulose content of the sieved material from the Atuba WWTP (Curitiba, Brazil) was determined by the enzymatic degradation of its cellulosic fraction. A total of 100 L of raw sanitary wastewater was collected from the Parshall flume and sieved with a fine-mesh sieve (0.35 mm). The solid material was dried, and the dry weight was measured. Cellulose quantification was carried out through enzymatic hydrolysis of cellulose (FAUST et al., 2014) that was performed in 50 mL Falcon tubes with a working volume of 20 mL of 50-mM citrate buffer at pH 4.8, 120 rpm and 50ºC for 72 h. The commercial enzyme Cellic CTec 2 (Novozymes, Denmark) was used at a concentration of 15 FPU per gram of WWTP residue. Residual solids were washed twice with distilled water and the dry weight measured. The experiment was performed in triplicate.

Cellulose fraction from WWTP was also analyzed using a Leco TGA-601 analyzer. The collected residue was dried in an oven at 105ºC and 0.3 - 1 g was transferred to the TGA analyzer

The morphological characteristics of WWTP were observed in a scanning electron microscope (SEM) (JEOL, JSM-6360LV). Samples were fixed on the SEM stubs of 12 mm diameter and then subjected to metallization with a thin layer of gold. Samples were observed at magnifications of 300x, 750x and 1500x. An acceleration potential of 10 kV and backscattering electrons (BSE) mode was used during micrograph. Samples were also analyzed through optical microscopy. The SEM analysis as well as the optical microscopy was performed with enzymatically hydrolysed and non-hydrolysed residues samples for the evaluation of morphological changes.

6.2.3 Enzymatic hydrolysis of cellulosic substrates by produced cellulase (Wlase)

The previously produced, concentrated and formulated cellulase (Wlase) was employed in the enzymatic hydrolysis of three different cellulosic materials. Experiments were performed with 5% (w/v) Avicel® (pure cellulose) (Sigma-Aldrich, Germany), 5% (w/v) empty palm fruit bunches (EFB) (34.9% cellulose; SOBRAL, 2016) and 5% (w/v) WWTP residue (21.3 % of cellulose). The EFB (Vale S.A., Brazil) was grinded and sieved in 43 mesh (35 mm) and previously pre-treated with NaOH

5% (w/v) at a concentration of 10% (w/v) for 1 hour in autoclave (121ºC). The solid material, from the Atuba WWTP, here called as “WWTP residue”, was manually collected from the raw sanitary wastewater passing through the Parshall flume with a fine-mesh sieve (0.35 mm) and dried. The WWTP residue was autoclaved before the hydrolysis experiments.

Enzymatic hydrolysis was performed in 50 mL Falcon tubes with a working volume of 20 mL of 50-mM citrate buffer at pH 4.8, 120 rpm and 50ºC for 72 h. The enzyme load of 15 FPU per gram of substrate was used (OUYANG et al., 2010). The in-house and concentrated cellulase named as “Wlase”, with activities of 7.3 U_{FP}/mL; 7.4 U_{CMC}/mL and 4.4 U_{BGL}/mL was employed for the enzymatic hydrolysis. A comparison of Wlase’s results was performed with the commercial cellulase Cellic CTec 2 (Novozymes, Denmark), with measured activities of 380 U_{FP}/mL, 143.7 U_{CMC}/mL and 5100 U_{BGL}/mL, in the same enzyme load (15 U_{FP}) and dilutions. Each 24 h, aliquots were withdrawn, centrifuged for 10 min at 3000 g and the supernatant used for reducing sugars analysis. HPLC analyses were carried out with the supernatant that was centrifuged and filtered through 0.22 µm syringe filters. All hydrolysis experiments were performed in triplicate.

6.2.4 Enzyme and hydrolyzate analyses

Total cellulase activity was measured according to filter paper assay (FPase) standard method (MANDELS and REESE, 1957; GHOSE, 1987), adapted for deep-well microplates according to Camassola and Dillon (2012). Filter paper stripes (0.6 x 1.0 cm) were employed as the substrate, and 50 µL of samples were diluted in sodium citrate buffer (50 mM; pH 4.8) and incubated for 60 min at 50ºC. For the evaluation of endoglucanases (EGL), the carboximethylcellulase assay (CMCase) was used (GHOSE, 1987), also adapted for deep-well microplates. Aliquots of 20 µL of carboxymethylcellulose 2% (CMCase) were used as substrate and 20 µL of the samples were appropriately diluted in the same buffer and incubated for 30 min at 50ºC. Reducing sugars were measured by the dinitrosalicylic acid (DNS) method (MILLER, 1959). One unit of cellulase activity was defined as the amount of enzyme producing 1 µmol of reducing sugars per minute.

Beta-glucosidase (BGL) activity determination was performed by the use of 10 mM *ρ*-nitrophenol-β-glucoside (*ρ*NPG). After 15 minutes of incubation at 40ºC, the

enzyme activity was stopped with sodium carbonate and the absorbance red at 405 nm. One unit of BGL is defined as the amount of enzyme that liberates 1 µmol of *ρ*-nitrohpenol per minute (SINGHANIA et al., 2017).

The filtered samples and calibration standards were analyzed by HPLC (Agilent 1260, Germany) equipped with a refractive index detector (G1362A XR RI). An Aminex® HPX-87H column (Bio-Rad, USA) was employed. The column temperature was fixed at 50°C and 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/min.

The glucose yield was calculated according to equation 1 and the reducing sugars yield according to equation 2

$$Glucose\ yield = \frac{Gluc \times Vol}{Mass/_{Cellu}} \quad (1)$$

$$Reducing\ sugars\ yield = \frac{Reduc \times Vol}{Mass/_{Cellu}} \quad (2)$$

Where: Gluc (mg/mL) represents the concentration of glucose and Reduc the concentration of reducing sugars obtained after the hydrolysis; Vol corresponds to the hydrolysis working volume (20 mL); Mass represents the mass of cellulosic substrate (1g); Cellu corresponds to the cellulosic fraction of each tested substrate (Avicel 100%; EFB 34.9% and WWTP residue 21.3%).

6.3 RESULTS AND DISCUSSION

6.3.1 Cellulases' production and formulation

The stable cellulase formulation, stored at 4 ºC, presented the enzymatic activities of 7.3 U_{FP}/mL; 7.4 U_{CMC}/mL and 4.4 U_{BGL}/mL. Wlase was applied in the hydrolysis of Avicel®, pretreated EFB and WWTP residue. The commercial Novozymes' cellulase Cellic CTec 2 was also employed in the comparison with Wlase's performance. Previously to the hydrolysis experiments, the cellulose content of WWTP residue was evaluated. Cellulose composition of Avicel and EFB was already known.

6.3.2 WWTP residue's cellulose estimation

Since the WWTP residue has been related as a potential source of cellulosic matter and the published data about this material is very scarce, a partial characterization was performed, focusing on its cellulosic content.

The enzymatic hydrolysis method for the estimation of cellulose content of the WWTP residue revealed that 21.3% of the total mass was composed by cellulose-like material that was hydrolyzed and measured as reducing sugars. The thermal analysis of the WWTP residue indicates that 27.76% of the material analyzed is composed by cellulose-like material. As a comparison, the Avicel® hydrolysis was also carried out, under the same conditions, revealing a 71% mass loss or almost 71 % cellulose composition. Other authors (RUIKEN et al., 2013) presented that 79% of the total mass collected from sieved raw wastewater could be considered cellulose. Cellulose concentrations in the sanitary wastewater are greatly influenced by the region and the consumption standards. While Ruiken et al. (2013) propose that toilet paper is the major cellulose source in WWTPs in Europe, this is not the same for other regions. While in countries like Netherlands and Germany the toilet paper is thrown into the toilet and flushed to the WWTP's, in Brazil it is classified as a solid residue and sent to sanitary landfills.

The residues collected with a sieve in the WWTP present diversity in morphology and sizes (Figure 6.1-B), showing a certain variability of their macroscopic structures, which were observed as feather, seeds, hair, wood and grease grains. Most of the particles are bigger than 500 μm, but some elongated fibers have diameters lower than 50 μm, and are similar to vegetal structures as well as to hair or synthetic fibers. According to Ruiken et al. (2013) the toilet paper has cellulose fibers in the range of 1 to 1.2 mm, whereas a 0.35 mm mesh size sieve is enough to retain most of the fibers and the particulate fraction passes through the sieve.

Figure 6.1 – (A) Wastewater sieving for WWTP residue collection, (B) Dried sieved residue, (C,D) SEM of the WWTP residue samples.

6.3.3 Enzymatic hydrolysis of cellulosic materials

The cellulosic materials Avicel®, EFB and WWTP residue were hydrolyzed with the enzyme Wlase during 72 hours. The released reducing sugars were monitored each 24 hours and presented in Table 6.1 and Figure 6.2.

Table 6.1 – Results of enzymatic hydrolysis of cellulosic materials by Wlase

Substrate	Time (h)	Reducing Sugars Yield ($mg/g_{cellulose}$)	Reducing Sugars Yield ($mg/g_{residue}$)	Glucose yield ($mg/g_{cellulose}$)	Glucose yield ($mg/g_{residue}$)	Reducing sugars productivities ($mg/g_{cellulose}h^{-1}$)
Avicel®	24	2.76	9.06	-	-	0.53
	48	20.0	14.2	-	-	0.41
	72	26.39	18.74	20.88	14.83	0.36
EFB	24	9.97	3.48	-	-	0.41
	48	25.73	8.98	-	-	0.47
	72	48.0	16.76	37.59	13.12	0.66
WWTP residue	24	21.69	4.62	-	-	0.90
	48	31.92	6.8	-	-	0.66
	72	34.64	7.38	21.17	4.51	0.48

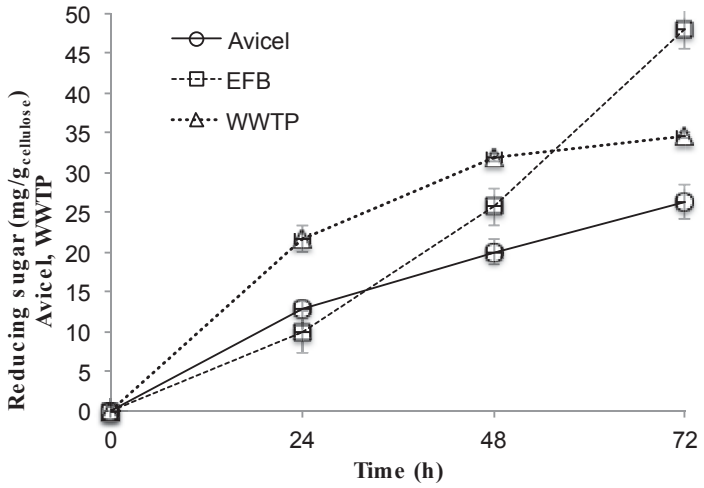

Figure 6.2 – Reducing sugars released during cellulosic substrates (Avicel, EFB and WWTP residues) hydrolysis with Wlase enzyme.

It is possible to observe in Figure 6.2 the different behavior of Wlase in the hydrolysis of Avicel, pretreated EFB and WWTP residue. The higher reducing sugars' yields were achieved with EFB (48.0 mg/g), followed by WWTP residue (34.64 mg/g) and Avicel® (26.39 mg/g). The same pattern was observed for glucose yield, according to Table 6.1. For the Avicel® and EFB the reducing sugars' concentration was still increasing after 72 h. Although for WWTP residue, 72 hours seemed to be enough for the hydrolysis process since, after this time, a decrease in the reducing sugars productivities was observed. The yields related to the residues mass showed

a quite diferent result, whereas the Avicel® presented the higest results (18.74 $mg/g_{residue}$ and 14.83 $mg/g_{residue}$), in terms of reducing sugars yields and glucose yield, respectively, followed by EFB (16.76 $mg/g_{residue}$ and 13.12 $mg/g_{residue}$) and WWTP residue (7.38 $mg/g_{residue}$ and 4.51 $mg/g_{residue}$). These diferences were probably associated to the cellulosic composition of the residues. The results presented in Table 2 are related to the glucose yields in residue mass basis. According to Palamae et al. (2017), the cellulosic composition of EFB could range from 24 to 65%. This implies that the their yield in cellulose basis ranges from 150 to 408 $mg/g_{cellulose}$, whereas the glucose yield in terms of residue mass is 629 $mg/g_{residue}$. The cellulosic composition of EFB usied in this work was 34.9%, according to previuos analysis performed by Sobral (2016).

The hydrolysis of EFB presented higher glucose and reducing sugars yields compared to that observed for Avicel®, indicating that Wlase posses an interesting combination of cellulases for the hydrolysis of different pretreated lignocellulosic residues. The pretreatment of EFB obviously influenced the hydrolysis when compared to the other two residues. The type of pretreatment has a strong influence on enzymatic hydrolysis and the resultant reducing sugars yield. Among biomass chemical pretreatments, the acid and alkaline hydrolysis, are the most used to access the cellulose fraction. The alkaline pretreatment remove the majority of lignin, breaking down the structural bounds and improving the cellulose porosity, and polysaccharides reactivity (HASSAN et al., 2013). In addition to EFB pretreatment, the use of commercial cellulases and/or the combination of them, as well as the presence of additives has been proposed as a strategy for the improvement of hydrolysis process (OUYANG et al., 2010; HASSAN et al., 2013; ADSUL et al., 2014). Obeng et al. (2017) applied the commercial enzyme Celluclast 1.5 L (Sigma-Aldrich, Germany) over thermal pretreated EFB, resulting in 70 mg of reducing sugars per gram of residue after 48h of hydrolysis. The authors did not reveal the value in residue weight basis. Other authors (Table 6.2) revealed different results in terms of glucose yield and glucose concentration, which were strongly influenced by the pretreatment, the employed enzymatic complex and the EFB hydrolysis conditions such as temperature, time and pressure.

Table 6.2 – Glucose yields from EFB hydrolysis with different pretreatment conditions and enzymes.

Pretreatment	Enzyme	Glucose yield ($mg/g_{residue}$)	Glucose yield ($mg/g_{cellulose}$)	References
NaOH 10% + steam explosion; 1 h	Wlase	13.12	37.59	This work
Diluted sulfuric acid	Celluclast 1.5L® + Novozymes 188®	140	54.88	(JUNG et al., 2013)
Untreated EFB	Accelerase® 1500 + XC	3.0	0.72-1.95	(PALAMAE et al., 2017)
Peracetic acid + alkaline peroxide	Accelerase® 1500 + XC	629.8	150-408	(PALAMAE et al., 2017)
Alkali + steam explosion	Celli Ctec® 2	382.2	259.7	(SUGIHARTO et al., 2016)

Most of the published data are related to the application of commercial enzyme cocktails produced from various microbial sources and only few authors tested the application of in-house enzyme formulations. The enzymatic complex produced and formulated in this work shows a good balance between EGL, CBH and BGL's, represented by the measured activities (7.3 U_{FP}/mL; 7.4 U_{CMC}/mL and 4.4 U_{BGL}/mL). Another point is the fact that Wlase was not produced by a genetically modified *Trichoderma* strain and, even so, was able to produce low concentrations of BGL. Generally, the addition of BGL, from its well-known producer *A. niger*, to EGL and CBH extracts, is widely reported in the literature as a solution for the low capacity of *Trichoderma* in producing BGL. However, the work with two different strains is surely more complex. Adsul et al. (2014) cited the importance of the synergy of combined enzymes, from different microbial sources, is even more important than higher cellulase activities obtained from a unique source. The formulation of commercial enzymes may contain accessory enzymes or additives for high cellulase activity stability. The addition of salts, surfactants and chelating agents, such as $CaCl_2$ and Tween 20 has been reported (OBENG et al., 2017), as improving the synergistic activity of cellulases resulting in higher reducing sugar yields.

The hydrolysis of WWTP residue by Wlase showed lower glucose and reducing sugar yields of 21.17 and 34,64 mg/g, respectively, when compared to pretreated EFB, probably due to the heterogeneity of the residue, the presence of non-cellulosic materials. It should also be taken into account the smaller fraction of

cellulose in the WWTP residue (21.3%) in comparison to the raw EFB (34.9%). Although the WWTP residue was not pretreated, the cellulosic particles would be more susceptible to the enzymatic hydrolysis due to the mechanical stress and microbial degradation of the material in the wastewater. In addition, a fraction of the cellulosic particles was previously processed, like toilet paper. These characteristics may be related to the higher glucose and reducing sugars yields obtained in comparison to Avicel®. Other authors presented the cellulosic composition of EFB with different pretreatments ranging from 34.4 to 85% (HASSAN et al., 2013; PALAMAE et al., 2017). Champagne and Li (2009) suggested that the metal content in this kind of feedstock would impact on enzyme activity and proposed some pre-treatments to avoid this phenomena. Publications reporting the hydrolysis of the cellulosic fraction of the raw sanitary wastewater are scarce; however, the hydrolysis of the primary sludge from WWTP's has also been cited. According to Champagne and Li (2009), the hydrolysis of the primary sludge from a WWTP located in Canada resulted in 31.1% of reducing sugars conversion (% of dry mass), using a commercial cellulase preparation from *T. reesei* (Sigma-Aldrich, Germany).

Li et al. (2012) proposed the potential of municipal solid wastes as feedstock for the production of fermentable sugars by enzymatic hydrolysis. Municipal solid wastes have a very similar composition to the WWTP residues, consisting of food residues, fibers, garden waste, metal, glass, plastics and textile and require a previous autoclaving for sanitization and pretreatment of the cellulosic fibers. The authors achieved a maximum reducing sugars yield of 385 mg per gram of fiber recovered from the municipal solid waste, resulting in 53% of hydrolysis conversion. However the authors did not reveal the fiber content that was recovered from the municipal solid waste.

The direct comparison between results from different authors should be made with care since different conditions for the enzymatic hydrolysis are applied, i.e. the enzyme load and buffer used, temperature, agitation, residue pre-treatments and cellulose content.

In the present work, reducing sugars concentration reached by the use of commercial enzymes was 10 to 15 mg/mL, in all the tested substrates. This behavior is obviously related to the hydrolysis conditions such as enzyme load, agitation, temperature, cellulose accessibility, but could be also related to the inhibitory effect

of the released glucose over BGL, since the glucose remains in contact with the enzymes during the hydrolysis.

The hydrolysis of the substrates Avicel®, EFB and WWTP residue by the commercial enzyme Cellic Ctec 2 (Novozymes, Denmark) resulted in glucose yields of 964.45 mg/$g_{residue}$, 336.77 mg/$g_{residue}$ and 242.06 mg/$g_{residue}$, respectively. The higher hydrolysis conversions presented by the commercial enzyme for the three substrates would be attributed to the higher BGL activities (5100 U_{BGL}/mL) in comparison to Wlase (4.4 U_{BGL}/mL). The higher enzyme activities reported for commercial enzymes were taken into consideration since the hydrolysis experiments were performed in the same enzyme load, with interference only over the enzyme volumes.

Saini et al. (2015) tested the 24 h hydrolysis of Avicel® using the commercial cellulase Cellic CTec 3 (Novozymes, Denmark) and SaccharISEB C6 (Advanced Enzymes, India) and in-house produced cellulase from *T. reesei* Rut C-30 and *Penicillium oxalicum*. The released reducing sugars concentrations were 615.2 mg/$g_{residue}$, 700 mg/$g_{residue}$, 800 mg/$g_{residue}$, and 600 mg/$g_{residue}$, respectively. Ouyang et al. (2010) achieved 28.3% of conversion yield and 23.2% of glucose yield, with the release of 2.5 g/L of cellobiose in the hydrolyzate, after 48 h of the hydrolysis of Avicel®. The author found that the addition of external BGL in the form of the commercial cellulase Celluclast 1.5L (Novozymes, Denmark) increased the conversion and glucose yield to 41.1% and 39.5%. Prévot et al., (2013) reached glucose and cellobiose concentrations of 6.78 and 1.10 g/L in the hydrolysis of Avicel® after a 72 h treatment using in-house produced *T. reesei* Rut C-30 cellulases. The use of Celluclast 1.5L led to 5.63 g/L of glucose and 1.85 g/L of cellobiose. The hydrolysis of wheat bran in the same conditions using the produced enzyme led to 1.8 g/L of glucose, 0.69 g/L of cellobiose, 2.09 g/L of xylose and 0.23 g/L of arabinose. Adsul et al. (2014) achieved 32% of Avicel hydrolysis and 27% of wheat straw hydrolysis (in terms of mass) using an enzyme load of 10 U of *T. reesei* Rut-C30 cellulase (0.45 U_{FP}/mL, 3.3 U_{CMC}/mL and 0.12 U_{BGL}/mL). The authors blended the cellulases from *Penicillium janthinellum* (2 U_{FP}/mL, 10.5 U_{CMC}/mL and 0.11 U_{BGL}/mL) and *Aspergillus tubingenesis* (0.4 U_{FP}/mL, 2.1 U_{CMC}/mL and 3.3 U_{BGL}/mL) increasing the hydrolysis rate to 70% and 50%, in terms of residue mass, presenting their synergic effect.

6.3.4 SEM of the hydrolyzed residues

The Figure 6.3 A and B revealed the heterogeneity of the WWTP residue, showing that the enzymatic hydrolysis promoted the fragmentation of the cell wall, exposing the cellulosic fibres. This residue heterogeneity suggests the use of an enzymatic blend composed of various enzymes to promote the complete hydrolysis.

The Figure 6.3 revealed that the surface of the EFB fibres suffered the attack of the cellulases, since the structure presented alterations (Figure 6.3-D) compared to the fibres without enzymatic treatment. The holes observed in the surface of the fibres in Figure 6.3-C are related to the silica corps removed by the alkali pretreatment. According to Hassan et al. (2013), the pretreatments remove the silica bodies and destroy the recalcitrance of the fiber surface to ease the enzyme penetration and action. The lignin coating on the fiber surface is a natural barrier preventing the enzyme attack on cellulose and hemicellulose.

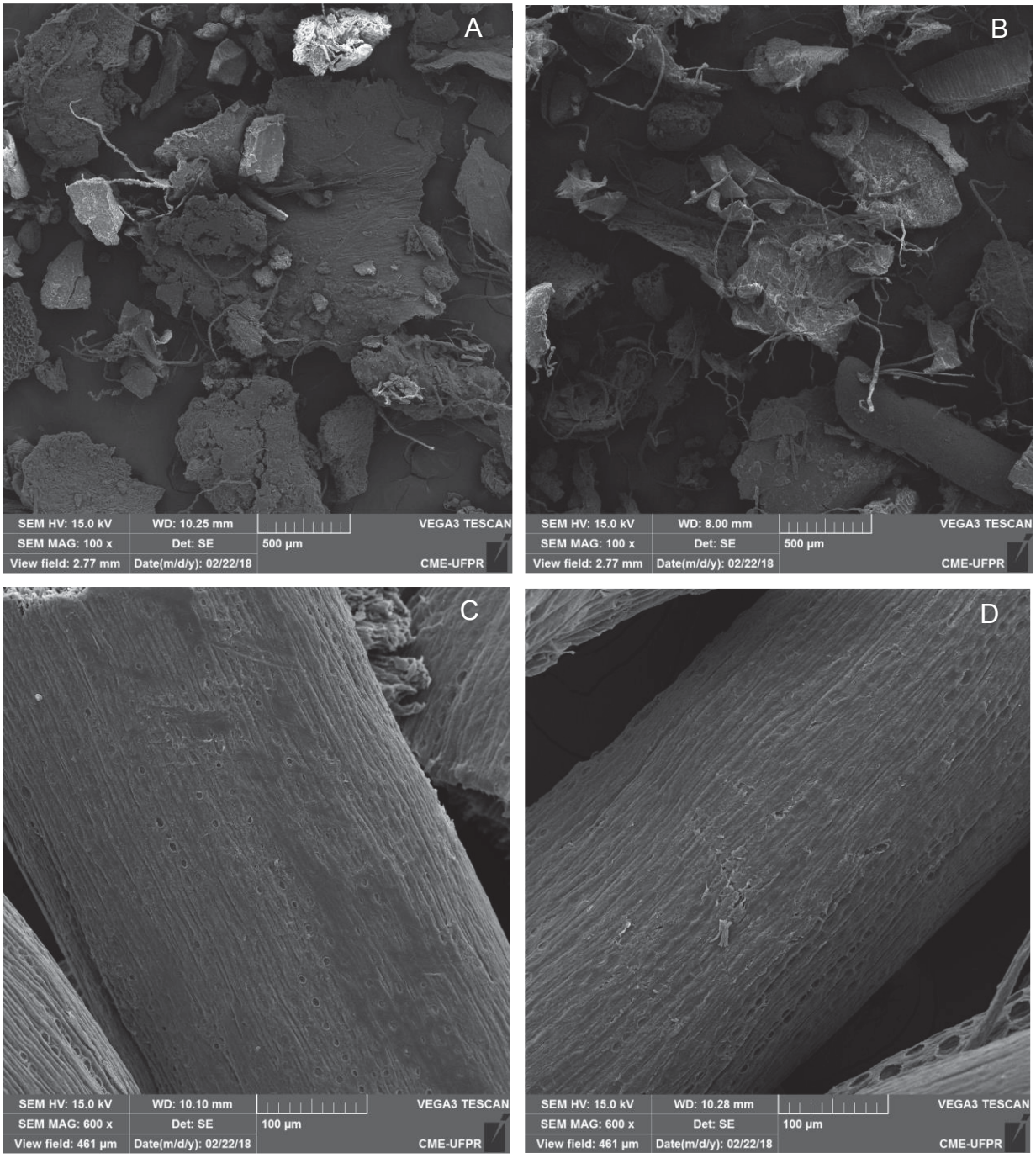

Figure 6.3 – SEM (600x) of WWTP residue and EFB before and after enzymatic hydrolysis with Wlase. (A) WWTP residue; (B) hydrolyzed WWTP residue; (C) EFB; (D) hydrolyzed EFB 600x.

6.3.5 Potentialities for the hydrolysis of WWTP residue

Since 21.3% of the sieved material from WWTP is cellulose-like material and the estimated WWTP residue generation is 18.5 ton per month in the Atuba WWTP (1700 L/s flow), it is expected the production of 4.07 ton per month of cellulosic-like material. According to the reducing sugars yields for the WWTP residue hydrolysis (7.38 mg/g), there is a potential of producing 7.38 kg of reducing sugars per ton of residue or a monthly reducing sugars production of 30 kg. A complete study should be conducted in order to obtain more reliable data on the concentration of cellulose in Brazilian WWTPs, considering the fluctuations of wastewater flow during the year, consumption standards, and other factors, which surely influence the concentration of solids and cellulosic materials in sanitary wastewater. Li et al. (2012) presented that 1-ton cellulosic fibers recovered from municipal solid wastes have the potential to produce, after pretreatment and commercial enzymatic hydrolysis, 400 kg of fermentable sugars. The authors did not publish the amount of solid waste necessary to recover 1 ton of cellulosic fibers.

The hydrolysis of the WWTP residue or other residues from WWTP process such as the sludge may aggregate value to the wastewater treatment process, reducing the costs associated to the treatment and producing fermentable sugars for the production of other media to high value biomolecules.

The presence of the cellulosic fraction in primary sludge and activated sludge would justify its previous retention with sieving avoiding the increasing costs associated to the sludge treatment and disposal. The annual sludge production in Brazil is estimated between 150 to 220 thousand of tons of dry matter (SOARES, 2006). The Belem WWTP, located in the city of Curitiba – Brazil, generates around 9.6 tons of sludge per day (PEGORINI, 2003). The high amounts of sludge generated in Brazil reveals the potential of recovering its cellulosic fraction for the production of added-value biomolecules as well as for the costs reduction of sludge treatment and disposal.

An efficient enzymatic hydrolysis of heterogeneous residues like the sieved WWTP residue or even the resulting sludge from activated sludge systems could be achieved blending different enzymes. A blend of cellulases, kitinases, keratinases, amylases, proteases and lipases would be able to hydrolyze the materials found in the WWTP sieved residue, such as paper and wood fibers, synthetic fibers, keratin

from animal and vegetal sources as feather, hair, starch materials, proteins, oils and grease (SONG and FENG, 2011). Although the heterogeneity of the WWTP sieved material seems to be an obstacle for the recovery of the organic fraction, some initiatives show that the enzyme technology is a central issue for the resources recovery from this kind of residue. In England, the world's largest waste-to-biogas conversion plant (Renescience) use enzymes for the liquefaction of the organic fraction of unsorted domestic wastes, producing a high quality "bioliquid" for generation of biogas (ORSTED, 2018). Besides being a well-known cellulase producer, fungi from the *Trichoderma* genus are able to produce a very complete enzyme cocktail, composed by proteases and kitinases, being an interesting candidate for the formulation of a complete enzymatic blend (VALE et al., 2012).

6.4 CONCLUSIONS

The hydrolysis of EFB and WWTP residue by Wlase, an enzymatic formulation produced by *Trichoderma harzianum* TRIC03-LPBII released different reducing sugars concentrations, what demonstrates that it has specificity for the substrates. In addition, the efficiency of hydrolysis is dependent on the residue nature, whereas the WWTP residue heterogeneity and inhibitors may have a strong impact in the hydrolysis rate. With appropriate separations and pretreatments the WWTP residue is an interesting candidate as cellulose source for the production of fermentable sugars and biochemicals. The lower costs of an in-house enzyme preparation would positively impact in the hydrolysis processes, since the enzyme cost is still one of the main bottlenecks for the production of biofuels and biochemicals from cellulosic residues. The double benefit of WWTP residue recovery for the reduction of wastewater treatment process's costs and its use as feedstock for biomolecules production is certainly comprised in the wastewater biorefinery concept. In this case, the wastewater treatment is coupled with the recovery and production of added-value biomolecules. The WWTP residue certainly has the potential to be better explored, and cellulases formulations play a central role for the development of wastewater treatment plant based biorefineries.

CONCLUSIONS

The low cost cellulases' production is still a matter for discussion. The commercial production of cellulases is already well established in the market and the application for biofuel production is reality. The on-site small-scale cellulase production is one strategy to turn the industrial consumers independent from the international suppliers, what brings the new challenge in developing enzyme cocktails that could efficiently replace the established commercial products in an economical and sustainable way.

The use of sanitary wastewater as a constituent of the culture medium for *Trichoderma* cellulases' production was the central issue of this work. It is possible to conclude the viability of the use of sanitary wastewater as an alternative substrate for cellulase production, thereby contributing to the development of sustainable processes for enzyme production. The use of sanitary wastewater did not cause any inhibition to the fungal growth or cellulase production and it was possible to scale-up the process to a bubble column-reactor (BCR), achieving productivities of 64 U/Lh. The replacement of microcrystalline cellulose by lactose as carbon source and inducer in the culture medium improved about 10 times the maximum enzyme productivity (645.4 U/Lh). The BCR reactor achieved the highest productivity, when compared to the stirred tank reactor (STR) (236.5 U/Lh) and the immobilized bed BCR (80.4 U/Lh). The high cellulases' productivities were compared to the published data and demonstrated the potential for this production process in a pilot-scale.

The use of residues as alternative source of carbon and nutrients for lowering the culture medium costs brings concerns about the downstream process. The behavior of microfiltration (MF) and ultrafiltration (UF) processes for recovering and concentrating the produced cellulases were evaluated. The use of MF followed by the 30 kDa UF membranes resulted in recovery percentages above 70% and fouling of 19.8%, which are in accordance to some of the best results found in the literature.

The use of wastewater as part of the culture medium for cellulases production allowed the simultaneous production of cellulases and the reduction of the pollution charge of the effluent/culture medium, through its consumption as carbon and nutrient sources for microbial growth and energy production. The bioreactor fermentation process resulted in a maximum chemical oxygen demand (COD) and

nitrogen removal efficiencies of 98 and 78%, whereas the membrane concentration allowed 81 and 52.9% removal efficiencies, respectively. At the final of the fermentation process, high titers of cellulases were produced and the final effluent presented organic matter concentration in accordance to the legislation, reducing the costs associated to wastewater treatment processes. This double benefit is related to the wastewater biorefinery concept that combines the treatment of wastewater to the desired water quality and the simultaneous production of value-added products, as either commodity or energy products.

The simultaneous cellulase production using sanitary wastewater and the reduction of the effluent pollution potential is a very interesting strategy for the development of a sustainable process. Although, the in-house cellulases' production brings the challenge for the development of stable cellulase cocktails that could replace the established commercial products in an economical and sustainable way. The liquid formulations maintained 100% of their stability after 30 days of incubation at 4ºC. The dried formulation was not efficient since presented 60% of enzyme activity loss just after the drying process.

The characterization of the produced cellulases revealed the production of a cellulolytic complex composed of endoglucanases, cellobiohydrolases and beta-glucosidases, with optimum pH and temperature values between 4 and 5 and 50 and 70ºC. SDS-PAGE and zymogram revealed molecular weights of 48, 43 and 25 kDa. The concentration of 10 g/L of glucose inhibited around 45% of the BGL activity, which is impacting for the application of the cellulases in hydrolysis processes.

The cellulases' liquid formulation was applied for the hydrolysis of pretreated empty palm fruit bunches (EFB) and the solid fraction of the sieved sanitary wastewater, named WWTP residue. The highest glucose yields (37.59 $mg/g_{cellulose}$) were achived in the hydrolysis of EFB. The WWTP residue may needs a pretreatment to increase its potential for the production of fermentable sugars. The monthly production of around 4 tons of cellulosic-matter from the WWTP residue shows its importance as a resource to be recovered.

FUTURE PROSPECTS

The view of wastes, including wastewater, as a potential raw material resource is a key issue for the development of sustainable processes. This approach can be used through the wastewater biorefinery concept that combines the treatment of wastewater to the desired water quality and the simultaneous production of value-added products, as either commodity or energy products. The use of WWTP residues for the production of biomolecules such as enzymes is still a challenge. Although the reviewed authors have proposed the use of sanitary wastewater for biomolecules production, some concerns about its diluted nature as well as the presence of microbial inhibitors could affect the enzyme production. The use of residues as alternative source of carbon and nutrients for lowering the culture medium costs for biomolecules production is an interesting strategy, however it brings other problems associated to the presence of undesired compounds. One certain thing is that the use of membrane technologies for the recovery and concentration of high added value biomolecules from waste streams coupled with the filtration of the wastewater is a future technology to be developed.

Although the production of cellulases has been already extensively reviewed, the low cost production is still a matter for discussion. The commercial production of cellulases is already well established in the market and the application for biofuel production is reality. The on-site small-scale cellulase production is one strategy to turn the industrial consumers independent from the international suppliers, what brings the new challenge in developing enzyme cocktails that could efficiently replace the established commercial products in an economical and sustainable way.

The production of cellulolytic enzymes is reported to be performed using single microbial strains, fungi or bacteria, or, a combination in co-cultures. There is still a lack of processes for the production of these enzymes in microbial communities such as those that are found in WWTP's. The production of methane is an example of a well-established non-sterile mixed culture process. The more in depth understanding of the microbial community involved in the production of the biomolecules in wastewater, as well as the modulation of the microbial community metabolism by the better control of the environmental parameters, could provide in the future the possibility of producing cellulases in non-sterile bioreactors with mixed microbial communities

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ATTACHMENT A – PUBLICATION OF CHAPTER 3

Process Biochemistry 57 (2017) 190–199

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Domestic wastewater as substrate for cellulase production by *Trichoderma harzianum*

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ARTICLE INFO

Article history:
Received 24 November 2016
Received in revised form 25 February 2017
Accepted 6 March 2017
Available online 10 March 2017

Keywords:
Cellulase production
Domestic wastewater
Trichoderma harzianum

ABSTRACT

Cellulase production using residues as substrate has been well described, as it is an interesting method of reducing the costs of processes, one of the main bottlenecks for the production of enzymes. This research describes for the first time the use of raw domestic wastewater, which is largely and continuously generated, as a culture base medium for cellulase production. The strain *Trichoderma harzianum* HBA03 was selected according to the highest activity produced for FPase (5.4 U/mL) and CMCase (8.2 U/mL). Peptone was selected as a nitrogen source and microcrystalline cellulose as the inducer for cellulase production, resulting in FPase activities of 5.6 and 5.0 U/mL and CMCase activities of 12.0 and 14.4 U/mL. The use of domestic wastewater as the culture medium led to an increase of 1.41 and 1.14 fold of FPase and CMCase production, respectively, compared to the synthetic medium. Production was also carried out in a bubble column reactor in which the maximum productivities achieved 10.2 U/L.h (FPase) and 64.6 U/L.h (CMCase). The presented results demonstrate the feasibility of the use of domestic wastewater for cellulases production, thereby contributing to the development of a sustainable process for reusing wastewater with a significant reduction in environmental impact.

1. Introduction

Cellulases production using alternative substrates has been well studied, as it is an interesting way to reduce enzyme production costs, which is still one of the major bottlenecks for its application, i.e., second-generation biofuel. The composition of the culture medium significantly affects product concentration, yield, and volumetric productivities, which is of central importance for economically viable production. The use of agro-industrial residues is an interesting strategy for the reduction of costs associated with the culture medium formulation of cellulase production. There has been an increasing trend toward efficient utilization of agro-industrial residues in cellulase production by means of submerged and solid-state fermentation, such as wheat straw [1], sugarcane bagasse [2,3], palm oil extraction residues [4], corn cob [5], sorghum bran [6], paper pulp [7], and elephant grass [8].

In addition to the importance of productivity, the sustainability of the cellulase production process or any bioprocess should also be taken into account. Besides the use of agro industrial residues, another approach is the use of effluents in such processes. Wastewaters are an interesting source of nutrients and products to be recovered such as carbon, nitrogen, phosphorous, organic fertilizers, methane and others. Its composition can vary according to the place, the period of the year, and the pluviometric index. In addition, cellulose, which is the main cellulase inducer, is also present in the raw sanitary wastewater [9]. The presence of these macronutrients greatly contributes to the costs associated with the wastewater treatment plants (WWTP). The nutrient loads in municipal wastewaters are diluted but still add up to significant daily loads because of the massive volumes generated in urban populations [10]. Verstraete et al. [11] presents that from 1 m^3 of sanitary wastewater it is possible to recover 0.05 kg of Nitrogen, 0.14 m^3 of Methane, 0.1 kg of organic fertilizer and 0.01 kg of phosphorous. According to Verster et al. [10], the Athlone Municipal Treatment Plant, one of the 23 unities of the City of Cape Town, generates an estimated 70 tons of carbon, 6 tons of nitrogen, and 1 ton of phosphorous per day, and treats 120,000 m^3 of effluent per day. The Atuba WWTP, a water-treatment unity of Curitiba, Brazil, also treats around 120,000 m^3 of effluent per day. The treatment capacity of a WWTP is directly related to the contributing population. According to Henze and

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http://dx.doi.org/10.1016/j.procbio.2017.03.006
1359-5113/

ATTACHMENT B – PATENT DEPOSIT

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Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: Universidade Federal do Paraná

1.2 Qualificação: Autarquia Federal

1.3 CNPJ/CPF: 75095679/0001-49

1.4 Endereço Completo: Rua João Negrão, 280 2°andar Curitiba/PR

1.5 CEP: 80010-200

1.6 Telefone: 41-33607441 1.7 Fax: 41-33607416

1.8 E-mail: inovacao@ufpr.br

☐ continua em folha anexa

2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

PROCESSO DE PRODUÇÃO DE CELULASES UTILIZANDO EFLUENTE SANITÁRIO BRUTO

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº **Data de Depósito:**

5. Prioridade: ☐ Interna (66) ☐ Unionista (30)

O depositante reivindica a(s) seguinte(s):

País ou Organização do depósito	Número do depósito (se disponível)	Data de depósito

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6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seus nome(s), neste caso não preencher os campos abaixo.

6.1 Nome: Carlos Ricardo Soccol

6.2 Qualificação: Professor Doutor

6.3 CPF: 275.584.799-91

6.4 Endereço Completo: Av. Cel. Francisco H. dos Santos, 100, Jardim das Américas

6.5 CEP: 81531-980

6.6 Telefone: (41) 33613555 6.7 FAX:

6.8 E-mail: soccol@ufpr.br

☒ continua em folha anexa

7. Declaração de divulgação anterior não prejudicial. ☐
Artigo 12 da LPI – período de graça.
Informe no item 11.13 os documentos anexados, se houver.

8. Declaração na forma do item 3.2 da Instrução Normativa PR nº 17/2013:

☐ Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

9. Procurador (74):

9.1 Nome:

9.2 CNPJ/CPF: 9.3 API/OAB:

9.4 Endereço Completo:

9.5 CEP:

9.6 Telefone: 9.7 FAX:

9.8 E-mail:

☐ continua em folha anexa

10. Listagem de sequências biológicas.
Informe nos itens 11.9 ao 11.12 os documentos anexados, se houver. ☐

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Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

11. Documentos Anexados:

(Assinale e indique também o número de folhas):
(Deverá ser indicado o número total de somente uma das vias de cada documento).

		Documentos Anexados	folhas
☒	11.1	Guia de Recolhimento da União (GRU).	02
☐	11.2	Procuração.	
☐	11.3	Documentos de Prioridade.	
☐	11.4	Documento de contrato de trabalho.	
☒	11.5	Relatório descritivo.	19
☒	11.6	Reivindicações.	02
☒	11.7	Desenho(s) (se houver). Sugestão de figura a ser publicada com o resumo: nº, _____ por melhor representar a invenção (sujeito à avaliação do INPI).	01
☒	11.8	Resumo.	01
☐	11.9	Listagem de sequências em arquivo eletrônico: _____ nº de CDs ou DVDs (original e cópia).	
☐	11.10	Código de controle alfanumérico no formato de código de barras referente às listagem de sequências.	
☐	11.11	Listagem de sequências em formato impresso.	
☐	11.12	Declaração relativa à Listagem de sequências.	
☒	11.13	Outros (especificar) DOU e Anexo de Continuação - Formulário FQ001	02

12. Total de folhas anexadas: 27 **fls.**

13. Declaro, sob as penas da Lei que todas as informações acima prestadas são completas e verdadeiras.

Curitiba, 17 de maio de 2016
Local e Data

Assinatura e Carimbo
Prof. Dr. ZAKI AKEL SOBRINHO
Reitor

ATTACHMENT C – BOOK CHAPTER

AGRO-INDUSTRIAL WASTES AS FEEDSTOCK FOR ENZYME PRODUCTION

Apply and Exploit the Emerging and Valuable Use Options of Waste Biomass

Edited by

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CONTENTS

"Microbial enzyme factories: current trends in production processes and commercial aspects"

Luciana Porto de Souza Vandenberghe[1], Júlio Cesar de Carvalho[1],Nelson Libardi[1], Cristine Rodrigues[1],Carlos Ricardo Soccol[1]

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ABSTRACT

Microorganisms are the main sources of the most demanded commercial enzymes. They are produced in large scale with the employment of different strains, which can be parental strainsor molecularly modified. The capacity of a strain to synthetize higher concentrations of the protein of interest depends not only on the selected microorganism, but on the condition of cultivation and media composition. In this way, some agro-industrialbyproducts started to be consideredas an interesting possibility to solve environmental problems and add value to these substrates. Some aspects of enzyme synthesis are described in this chapter, including the main factors that affect the productivity. Some aspects of theenzyme's process development, mainly hydrolases such as cellulases, xylanases, phytases, mannanases, amylases, lipases and others,from batch to industrial scaleare reported. Besides, general downstream operations for the separation, recovery and purification of these enzymes are also presented. Finally, the most important hydrolases produced and commercialized arealsolisted.

INTRODUCTION

Enzymes are special proteins, which catalyze chemical reactions with great specificity and rate enhancements. These reactions are the basis of the metabolism of all living organisms, and provide tremendous and economical biocatalyst conversions. First half of the last century saw a rapid development in enzyme chemistry. The Enzyme Commission, set up by the International Union of Biochemists (1965), has published a