Universidade do Minho
Escola de Engenharia

Daniel Gonçalves Gomes

Integration of cellulases recycling on 2nd generation bioethanol production from recycled paper sludge

abril de 2018

Integration of cellulases recycling on 2nd generation bioethanol production fromrecycled paper sludge
Daniel Gonçalves Gomes
UMinho | 2018

Universidade do Minho
Escola de Engenharia

Daniel Gonçalves Gomes

Integration of cellulases recycling on 2nd generation bioethanol production from recycled paper sludge

Tese de Doutoramento em Engenharia Química e Biológica

Trabalho efetuado sob a orientação da
Doutora Lucília Maria Alves Ribeiro Domingues
e do
Doutor Francisco Miguel Portela da Gama

abril de 2018

Autor: Daniel Gonçalves Gomes
E-mail: dgg.ebiol@gmail.com

Filiação: Escola de Engenharia – Centro de Engenharia Biológica

Título da tese

Integration of cellulases recycling on 2nd generation bioethanol production from recycled paper sludge

Orientadores

Doutora Lucília Maria Alves Ribeiro Domingues
Doutor Francisco Miguel Portela da Gama

Ano de conclusão: 2018

Doutoramento em Engenharia Química e Biológica

Universidade do Minho, 24 / 04 / 2018

Assinatura: Daniel Gonçalves Gomes

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 24th April, 2018

Full Name: Daniel Gonçalves Gomes

Signature:

Daniel Gonçalves Gomes

Etiam capillus unus habet umbram

Públio Siro

Acknowledgments

Para esta longa e “sinuosa” caminhada, muitos contribuíram para chegar ao momento em que escrevo estas palavras...

A professora **Lucília Domingues**, minha orientadora de longa data, que me deu o tempo, os conselhos, as correcções, as sugestões, a motivação, e a independência...

O professor **Miguel Gama**, que possibilitou esta minha transição para uma nova área de investigação e contribuiu com idéias sempre bastante pertinentes e com discussões altamente interessantes

Sebastián Loaiza, who had a huge pacience and effort during his collaboration with a part of this thesis

The professor **Carlos Cardona and his group** at Universidad Nacional de Colombia, who gave me an tremendous experience in Manizales

Amanda and Yessica Perez, who so kindly received me at their home and made my stay in Manizales so pleasant

Todos os colegas do LF e LBM, que durante estes anos me permitiram trabalhar num ambiente divertido e produtivo ... Obrigado por me deixarem apagar as luzes!!

A **Tatiana** Aguiar, colega e amiga de longa data, que foi uma fonte constante de conselhos, ensinamentos, idéias e um ouvido para todos os desabafos e frustrações

A **Lina, a Sara Silvério e a Carla**, que foram os ouvidos para escutar e ao mesmo tempo a boca para ensinar, motivar e aconselhar

A **Aloia e a Ana C. Rodrigues**, que sempre se disponibilizaram para esclarecer as minhas dúvidas técnico-científicas

My **friends and fellows at ICL**, who are constantly challenging me and keeping me making the right questions and finding unexpected answers

A **Tânia, o Eduardo C e a Márcia**, que foram aquela motivação nesta fase final, mas também ao longo do percurso...todos aqueles cafés e conversas nada científicas

A **Rute, Luis, Lara, Inês e João**, que estiveram lá quando foi preciso e mesmo quando não foi....que chatos pah!!

A minha **mãe**, Conceição, e o meu **pai**, António, são eles que continuam a ser os meus pilares e, de uma forma ou de outra, o meu exemplo.. esta tese também é vossa
Também o meu **irmão, cunhada e o Rodrigo**, que tiveram de “sofrer” com as minhas alienações e falta de tempo, mas onde consegui sempre encontrar serenidade e constância..

Aquelas duas ou três **pessoas que aqui não foram mencionadas**, que significaram muito...

Gostaria de agradecer ao **Centro de Engenharia Biológica** da Universidade do Minho, que me forneceu os meios humanos e materiais necessários para a execução deste trabalho.

Gostaria de agradecer à **RENOVA SA**. pelo fornecimento do material RPS, essencial para a execução deste trabalho, e pela atenção demonstrada desde o primeiro momento.

I would like to acknowledge **Novozymes A/S** and **Dupont** for kindly providing me the samples of enzyme cocktails.

The author would like to acknowledge Portuguese Foundation for Science and Technology (Fundação para a Ciência e Tecnologia) for the research grant SFRH/BD/88623/2012. The author would also like to thank for: the strategic funding of UID/BIO/04469/2013 unit; COMPETE 2020 (POCI-01-0145-FEDER-006684); the MultiBiorefinery project (POCI-01-0145-FEDER-016403); Strategic Project PEst-OE/EQB/LA0023/2013; Project BBioInd-Biotechnology and Bioengineering for improved Industrial and Agro-Food processes, REF. NORTE-07-0124-FEDER-000028 Co-funded by the Programa Operacional Regional do Norte (ON.2–O Novo Norte), QREN, FEDER.Jdkasldjas

Abstract

Finding low-cost cellulosic materials that can provide appropriate amounts of sugars is one of the present challenges for cellulosic ethanol production. The valorization of residues from different sources represent in this context an attractive option. One of these residues is recycled paper sludge (RPS), which is generated in high amounts on the paper recycling process, being usually disposed at landfills. Another critical point concerning the economy of 2G bioethanol is the cost of enzymes. Despite the important cost-reduction achieved lately, enzymes are still very expensive. The recovery and reutilization of enzymes is one of the most promising strategies for a reduction on enzyme cost. The general aim of this thesis is thus to provide relevant insights on the feasibility to integrate enzyme recycling in the process of bioethanol production from RPS.

Despite several studies on enzyme binding to cellulosic materials, no such study exists for RPS. Thus, the first aim of this thesis was to evaluate the hydrolytic performance of cellulases and their adsorption on RPS, since this is very important concerning the definition of a strategy for enzyme recycling. Cellulases efficiently convert RPS, no visible toxic effects being detected. The hydrolysate was also easily fermented by yeast cells, no additional nutrients supplement being required. At the end of the process, a large fraction of Cel7A activity was found soluble on the liquid, the solid-bound fraction being efficiently recovered through alkaline elution. Four rounds of hydrolysis and fermentation were successively conducted, both fractions of the enzymes being recovered after each round, using only 30 % of the original enzyme load used in the first stage. This strategy enabled steady levels of enzyme activity while also allowing important levels of solid conversion.

Targeting the economy of the process, high solid loadings are required for higher ethanol titers to be achieved. Additionally, different enzymes can present distinct performance and binding affinities towards RPS. On a second part of this thesis we aimed to investigate the performance of different enzyme cocktails and process conditions and their impact on the feasibility of enzyme recycling under intensified conditions. Distinct cocktails were assessed for thermostability, hydrolysis performance and activity partition between phases of the solid-liquid system. Celluclast showed an

inferior thermostability, nevertheless, its performance at moderate temperatures was slightly superior to other cocktails (ACCELLERASE®1500 and Cellic®CTec2). Also the enzyme distribution in the solid-liquid medium was more favorable in the case of Celluclast, enabling the recovery of 88 % of the final activity. Using Celluclast, a Central Composite Design was designed to study the influence of solids and enzyme dosage on RPS conversion. Solids loading showed a significant effect on glucose production, no major limitations being found for a concentration under 22 % of solids. Furthermore, an increase on enzyme loading from 20 to 30 FPU/$g_{cellulose}$ showed no significant additional effect on sugars production, thus 22 % solids and 20 FPU/$g_{cellulose}$ were identified as the best operational conditions towards an intensified process. Applying these conditions, a system of multiple rounds of hydrolysis with enzyme recycling was analyzed. Steady levels of activity from one round to another were obtained with only 50 % of fresh enzyme being added at each cycle, enabling interesting levels of solid conversion (70-81 %) in the subsequent rounds.

Finally, an economic study was conducted to analyze the viability of RPS conversion into ethanol, under the intensified conditions and enzyme recycling. Overall, this process was found to be economically viable even though the moderate levels of final ethanol critically affected production costs. On a scenario of enzyme recycling, despite the increase on production costs due to the recycling operations (0.15 Million US$/year), a reduced enzyme consumption and a superior ethanol production enabled a better economic output. The exclusive recycling of the liquid fraction allowed lower production costs; however, total ethanol production decreased leading to an inferior economic output. A sensitivity analysis has further suggested that enzyme cost may represent a critical factor on the economic viability of enzyme recycling, with reductions on its cost above a level of 33 % resulting on a scenario where is economically unattractive.

Summarizing, this work elucidates the important role of the enzyme cocktail and its interaction with the cellulosic material on enzymes recyclability, thus highlighting the high specificity of the presented results. Overall, the technical and economic feasibility of enzyme recycling in the process of bioethanol production from RPS was demonstrated.

Resumo

A identificação de materiais celulósicos de baixo custo com elevado teor de açúcares é um dos desafios atuais para produção de etanol celulósico. A valorização de resíduos de diferentes fontes representa neste contexto uma opção atrativa. Um desses resíduos é o *recycled paper sludge* (RPS), gerado em grandes quantidades no processo de reciclagem de papel, sendo normalmente depositado em aterros. Outro ponto crítico relativo à economia do bioetanol 2G é o custo das enzimas. Apesar da importante redução de custos alcançada ultimamente pelos fabricantes, as enzimas são ainda muito caras. A recuperação e reutilização de enzimas é uma das estratégias mais promissoras para uma redução deste custo. O objetivo geral desta tese é, portanto, fornecer indicações relevantes sobre a viabilidade de integrar reciclagem de enzimas no processo de produção de bioetanol a partir de RPS.

Apesar de existirem vários estudos sobre a ligação de enzimas a materiais celulósicos, nenhum destes incide sobre RPS. Assim, o primeiro objetivo desta tese consistiu na avaliação do desempenho das celulases na conversão do RPS; foi também analisada a adsorção das enzimas no RPS, processo muito importante relativamente à definição de uma estratégia para reciclagem. As celulases convertem eficientemente o RPS, não sendo detetados efeitos tóxicos. O hidrolisado foi também facilmente fermentado por leveduras, não sendo necessário um suplemento adicional de nutrientes. No final do processo, uma grande fração de atividade Cel7A encontra-se na fase líquida, sendo a fração ligada ao sólido eficientemente recuperada por eluição alcalina. Foram conduzidos com sucesso quatro ciclos sucessivos de hidrólise e fermentação, sendo ambas as frações de enzima recuperadas após cada ciclo, utilizando apenas 30 % da carga inicial de enzima usada na etapa inicial. Esta estratégia possibilitou níveis de atividade enzimática estáveis ao longo do processo ao mesmo tempo em que permitiu elevados níveis de conversão de sólido.

Visando a economia do processo é necessário usar altas cargas de sólido para que maiores níveis de etanol sejam alcançados. Adicionalmente, enzimas diferentes podem apresentar um desempenho e afinidade de ligação distintos face ao RPS. Numa segunda parte desta tese pretendeu-se investigar o desempenho de diferentes *cocktails* enzimáticos e condições de processo bem como o seu impacto na viabilidade de reciclagem de enzimas sob condições intensificadas. Foram avaliados *cocktails* distintos quanto à termoestabilidade, desempenho de hidrólise e partição de atividade entre as

fases sólido-líquido do sistema. O Celluclast apresentou uma termoestabilidade inferior; no entanto, o seu desempenho de hidrólise a temperaturas moderadas foi ligeiramente superior a outros cocktails (Accellerase®1500 and Cellic®CTec2). Também a distribuição de enzima no meio sólido-líquido foi mais favorável no caso da Celluclast, permitindo a recuperação de 88 % da atividade final. Usando Celluclast, foi projetado um Desenho de Compósito Central para estudar a influência da carga de sólidos e enzima na conversão de RPS. A carga de sólidos mostrou um efeito significativo na produção de glucose, não sendo encontradas grandes limitações para uma concentração abaixo de 22 % de sólidos. Além disso, um aumento na carga de enzima de 20 para 30 FPU/$g_{cellulose}$ não mostrou qualquer efeito adicional significativo na produção de açúcares, portanto 22 % sólidos e 20 FPU/$g_{cellulose}$ foram identificadas como as melhores condições operacionais para um processo intensificado. Aplicando estas condições, foi analisado um sistema de múltiplos ciclos de hidrólise com reciclagem de enzima. Níveis constantes de atividade foram obtidos de um ciclo para outro com adição de apenas 50 % de nova enzima em cada ciclo, permitindo níveis interessantes de conversão de sólido (70-81 %) nos ciclos subsequentes.

Finalmente, um estudo económico foi conduzido para analisar a viabilidade da conversão de RPS em etanol, sob condições intensificadas e reciclagem de enzimas. No geral, este processo foi considerado economicamente viável, embora os níveis moderados de etanol final tenham afetado de forma crítica os custos de produção. Num cenário de reciclagem de enzimas, apesar do aumento nos custos de produção devido às operações de reciclagem (0.15 Milhões US$/ano), um consumo reduzido de enzimas e uma produção superior de etanol permitiram um melhor resultado económico. A reciclagem exclusiva da fração líquida permitiu menores custos de produção, no entanto, a produção total de etanol diminuiu levando a um resultado económico inferior. Uma análise de sensibilidade sugeriu ainda que o custo da enzima pode representar um fator crítico na viabilidade económica da reciclagem, que pode resultar num cenário onde esta deixa de ser economicamente atrativa (no caso de reduções no seu custo acima de 33 %).

Em suma, este trabalho elucida o papel importante do *cocktail* enzimático e sua interação com o material celulósico na reciclabilidade de enzimas, destacando assim a alta especificidade dos resultados apresentados. No geral, foi demonstrada a viabilidade técnica e económica da reciclagem de enzimas no processo de produção de bioetanol a partir de RPS.

Publications within the thesis

Papers in peer reviewed journals

Published:

Daniel G. Gomes, Ana C. Rodrigues, Lucília Domingues, Miguel Gama. 2015. Cellulase recycling in biorefineries - is it possible? **Applied Microbiology and Biotechnology**, 99:4131-4143.

Daniel G. Gomes, Lucília Domingues, Miguel Gama. 2016. Valorizing recycled paper sludge by a bioethanol production process with cellulase recycling. **Bioresource Technology**, 216: 637-644.

Daniel G. Gomes, Miguel Gama, Lucília Domingues. 2018. Determinants on an efficient cellulase recycling process for the production of bioethanol from recycled paper sludge under high solid loadings. **Biotechnology for Biofuels**, 11:111.

Submitted:

Daniel G. Gomes, Sebastián Serna Loaiza, Carlos Ariel Cardona, Miguel Gama, Lucília Domingues. 2018. Insights into the economic viability of cellulase recycling on bioethanol production from RPS. *Submitted*.

Posters in conferences

Daniel G. Gomes, Miguel Gama, Lucília Domingues. 2014. Integration of cellulases recycling with 2nd generation bioethanol production from waste paper residues. **ESPCA 2014 - São Paulo Advanced School on the Present and Future of BIOENERGY**, Campinas, Brazil.

Daniel G. Gomes, Miguel Gama, Lucília Domingues. 2015. Integration of cellulases recycling with 2nd generation bioethanol production from waste paper residues. **Copenhagen Biosciences Conferences - 7th Conference: Cell Factories and Biosustainability**, Copenhagen, Denmark, 5-5.

Daniel G. Gomes, Lucília Domingues, Miguel Gama. 2016. Valorisation of recycled paper sludge from a biorefinery perspective. **ECO-BIO 2016**, Rotterdam, The Netherlands, P1.18.

Daniel G. Gomes, Lucília Domingues, Miguel Gama. 2016. Implementation of a cellulase recycling system to the hydrolysis of recycled paper sludge. **BIOIBEROAMÉRICA 2016**, Salamanca, Spain, 79.

Contents

List of Figures

Chapter II

Chapter III

Chapter IV

Chapter V

List of Tables

Chapter II

Chapter III

Chapter IV

Chapter V

List of Abbreviations and Acronyms

(v/v)	volume/volume
(w/v)	weigth/volume
(w/w)	weigth/weigth
1G	First Generation
2G	Second Generation
Accellerase	ACCELLERASE®1500
approx.	Approximately
BSA	Bovine Serum Albumin
c-PAM	Cationic polyelectrolyte
C5 sugars	Pentose sugars
Ca-alginate	Calcium-alginate
CBD	Cellulose-binding domain
CBH	Cellobiohydrolase
CBH I	Cellobiohydrolase I
CBH II	Cellobiohydrolase II
CBHs	Cellobiohydrolases
CBP	Consolidated Bioprocessing
CCG	Central Composite Design
CCI	Central Composite Inscribed
CD	Circular Dichroism
Cellic	Cellic®CTec2
Celluclast	Celluclast 1.5 L
cf.	Confer
e.g.	For example
EC	Enzyme Commission
EG	Endoglucanase
EG I	Endoglucanase I
EG II	Endoglucanase II
EG III	Endoglucanase III
EG IV	Endoglucanase IV
EG V	Endoglucanase V
EG VI	Endoglucanases VI
EGs	Endoglucanases
EPLP	Ethanol pretreated Lodgepole pine
etc.	et cetera
FPase	Filter paper activity
FPU	Filter Paper Unit
GRAS	Generally Regarded As Safe
h	hour
HPLC	High-Performance Liquid Chromatography
IPPC	Integrated Pollution Prevention and Control
ITF	Intrinsic Tryptophan Fluorescence
IU/mL	International unit/millilitre
kDa	kilodalton
LCST	Low critical solution temperature

LP steam	Low-Pressure steam
M	Molar
mA	Miliampers
mM	milimolar
MSW	Municipal Solid Waste
MU	4-methylumbelliferone
MUC	4-methylumbelliferyl-β-D-cellobioside
MUC	4-methylumbelliferyl-b-D-cellobioside
MUGlc	4-methylumbelliferyl-b-D-glucopyranoside
MULac	4-methylumbelliferyl-b-D-lactopyranoside
nm	Nanometer
NPV	Net-Present Value
NREL	National Renewable Energy Laboratory
nRPS	Carbonates-neutralized RPS
ºC	Degrees Celsius
PES	Polyethersulfone
pNPG	p-nitrophenyl-beta-D-glucoside
R^2	Coefficient of Determination
RPS	Recycled Paper Sludge
S/L	Solid/Liquid
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SELP	Steam exploded Lodgepole pine
SHCF	Separate Hydrolysis and Co-Fermentation
SHF	Separate Hydrolysis and Fermentation
SSCF	Simultaneous Saccharification and Co-Fermentation
SSF	Simultaneous Saccharification and Fermentation
U/mL	Unit/millilitre
UF	Ultrafiltration
US$	American dollar
USA	United States of America
UV	Ultraviolet
V/m	Volt per meter
VHG	Very High Gravity

Chapter I

Aim and Outline of the thesis

Aim of the thesis

Recycled paper sludge (RPS) residue is abundantly generated world-wide by the paper industry, hence representing an important environmental issue. Despite the relevant amount of sugars found on this material, few studies have explored its potential for biological conversion so far.

A most relevant issue on these processes is the high cost of cellulases. This has increasingly been targeted by distinct enzyme recycling strategies, which gave promising results.

The main aim of this thesis is to provide an insight on the recyclability of cellulases in the process of bioethanol production from RPS. Specifically, this thesis intended to:

- Understand the potential of RPS for bioethanol production
- Evaluate RPS suitability for enzyme recycling, following a strategy of ultrafiltration coupled to an alkaline elution step
- Assess the effect of the enzyme cocktail choice and process conditions on the efficiency of enzyme recycling
- Study the economic viability of enzyme recycling on the process of bioethanol production from RPS

Outline of the thesis

The thesis here presented reports to the main results obtained from research undertaken at CEB - Centre of Biological Engineering, University of Minho, Braga, Portugal, and at the Instituto de Biotecnologia y Agroindustria, Universidad Nacional de Colombia, Colombia, under the supervision of Professor Lucília Domingues and Professor Miguel Gama, and also with the collaboration of Professor Carlos Cardona (Universidad Nacional de Colombia).

This thesis is organized into the following chapters:

Chapter II presents a general introduction describing the most relevant concepts on the lignocellulosic ethanol production process. It also provides an extensive revision in what concerns the utilization of recycled paper sludge (RPS) and the technology of cellulase recycling.

Chapter III addresses a *proof of concept* study aiming to investigate the potential of RPS for bioethanol production and its suitability to be employed under a process of cellulase recycling.

Chapter IV reports to the implementation of cellulase recycling under intensified conditions in the process of bioethanol production from RPS. The selection of the most suitable enzyme cocktail and process conditions was performed.

Chapter V presents an overall study concerning the economic viability of bioethanol production from RPS with special emphasis on the integration of a cellulase recycling system.

Chapter VI refers to the main conclusions of this thesis, with some relevant topics that might be interesting to address on a future work.

Chapter II

General Introdution

Part of this chapter was published in:

Daniel G. Gomes, Ana C. Rodrigues, Lucília Domingues, Miguel Gama. 2015. Cellulase recycling in biorefineries - is it possible? Applied Microbiology and Biotechnology, 99:4131-4143.

2.1 Biofuels: an overview

On the last decades, major changes on the world economic and population picture have dictated important challenges for energy supply. If a significant increase has been registered on world energy demand (International Energy Outlook, 2016), on the other hand, fossil fuels, which for decades had a major role as energy source, face important challenges. In addition to a gradual decrease on oil reserves world-wide (Agarwal, 2007; Vohra *et al.*, 2014), the high geo-political instability at some of its suppliers have caused significant fluctuations on market prices (Ogbonna *et al.*, 2001). Furthermore, a growing environmental concern has recently been observed pointing to their widely-known effects on the depletion of ozone layer (Singh *et al.*, 2010). As a consequence, a higher role must be played by alternative and cleaner technologies such as solar energy, wind, waves and biofuels.

Important political efforts have been made towards a gradual change on the energy picture such as the recent Paris Agreement on Climate Changes. Also, the European Union issued two important directives: the Renewable Energy Directive specifies that 20 % of total energy and 10 % of transport energy should be obtained from renewable sources by 2020 (Directive 2009/28/EC); the Fuel Quality Directive determines an average reduction of 6 % on the lifecycle carbon intensity of transport fuels from 2010 to 2020 (Directive 2009/30/EC).

Biofuels are compounds produced biologically from organic matter which are able to substitute total or partially the traditional fossil fuels (Dragone *et al.*, 2010). Depending on the materials used on its production, they are classified in three main categories. First generation uses mostly energy crops, such as sugarcane in Brazil or corn in USA. Second generation relies on the cellulosic fraction of biological materials or different waste streams (*e.g.* industry residues, municipal waste, etc.). Finally, third generation employs algal biomass (Lee and Lavoie, 2013).

Bioethanol, which is the major biofuel currently produced world-wide, can total or partially substitute gasoline. Comparatively, it has multiple advantages such as higher octanes and flammability limits and inferior SO_2 and CO_2 emissions (Sarkar *et al.*, 2012; Szulczyk *et al.*, 2010), although being 68 % less efficient energetically.

2.2 Lignocellulosic ethanol

Lignocellulosic ethanol is produced by fermentation of the monomer sugars released from lignocellulosic materials, and represent one and possibly the main chemical currently produced using these materials.

2.2.1 The production process at a glance

Usually employing more challenging substrates comparatively to 1G-ethanol, lignocellulosic ethanol requires a process with higher complexity that includes some additional steps to enhance maximum recovery of fermentable sugars (Figure 2.1). The main process usually involves four main stages: pre-treatment(s); hydrolysis; fermentation; final product purification (Maurya *et al.*, 2015).

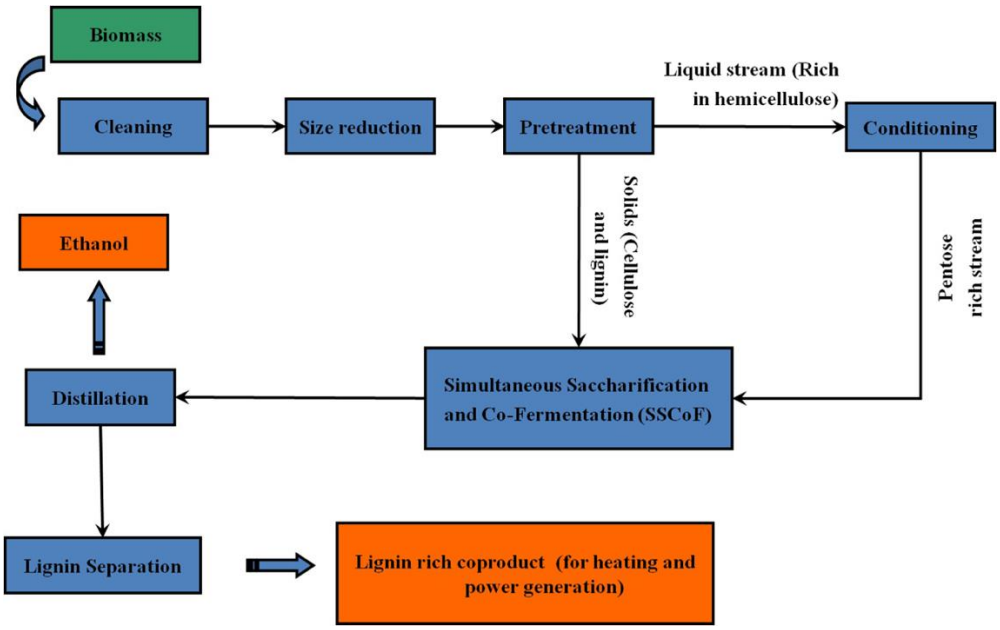

Figure 2.1 Overall schematic of lignocellulosic ethanol production process (reproduced from Kumar and Murthy, 2011).

Initially, according to the used material, one or multiple pre-treatments are applied, which intend to break the rigid structure of lignocellulose matrix and increase enzymes access to cellulose surface (Mosier *et al.*, 2005). After that, a separation step may or may not be applied depending on whether cellulose (and lignin) should be separated from the hemicellulose fraction. One or both of these fractions is then hydrolysed to fermentable sugars. This can either occur chemically or enzymatically, although the

latter one is the most common due to multiple benefits: it presents higher conversion yields, minimal by-product formation, low energy requirements, mild operating conditions and is more environmentally friendly (Wingren *et al.*, 2005). Enzymatic hydrolysis can be achieved either by the addition of enzyme cocktails purchased from *e.g.* Novozymes, DSM, Genencor, etc., or employing cellulase mixtures produced *in-situ*, *e.g.* by culturing *Trichoderma reesei* directly over the cellulosic material. After this process, or simultaneously (depending on process configuration), the monomer sugars released (glucose and xylose) are converted to ethanol by a high-efficiency fermentation organism (*e.g. Saccharomyces cerevisiae*).

2.2.2 Lignocellulosic materials

Lignocellulosic materials represent a wide-variety of available substrates. The most common refers to agro-forest residues, obtained either as a sub-product of agricultural activity and energy crops, or from wastes generated by forest cleaning. Additionally, it can also include several types of wastes generated by industry (*e.g.* brewer's spent grain, paper sludge, etc.) and human activities (*e.g.* municipal solid waste, food, etc.) that also contain a cellulose/hemicellulosic fraction (Hayes, 2013) (Table 2.1).

Table 2.1 Main composition (% w/w) of some lignocellulosic substrates (adapted from Zabed *et al.*, 2016)

Substrate	Cellulose	Hemicellulose	Lignin	Reference(s)
Switch grass	5-20	30-50	10-40	McKendry, 2002
Grass	25-40	25-50	10-30	Saini *et al.*, 2015
Whole sugarcane	25	17	12	Saxena *et al.*, 2009
General MSW	33-49	9-16	10-14	Li *et al.*, 2012
Kraft paper	57	10	21	Schmitt *et al.*, 2012
Food waste	55	7	11	
Newspaper	40-55	25-40	18-30	Howard *et al.*, 2004
Coffee husk	43	7	9	Gouvea *et al.*, 2009
Corn cob	42-45	35-39	14-15	Prasad *et al.*, 2007
Corn stover	38-40	24-26	7-19	Saini *et al.*, 2015
Sugarcane bagasse	42-48	19-25	20-42	Kim and Day, 2011
Wheat straw	33-38	26-32	17-19	Saini *et al.*, 2015
Softwood	27-30	35-40	25-30	McKendry, 2002
Hardwood	20-25	45-50	20-25	
Poplar	48-50	27-29	18-19	Olsson and Hahn-Hägerdal, 1996

These materials contain three main components: cellulose, hemicellulose and lignin. Cellulose consists of long and linear chains of glucose units (usually in the range of several thousands) linked by β(1-4) glycosidic bonds. It presents a high crystallinity degree as a result of the hydrogen bounds established between different layers of cellulose chains. This makes it a robust and hard to digest component in lignocellulosic materials. Hemicellulose, on the other hand, is composed by shorter (usually ranging from hundreds to some thousands of units) and not strictly linear chains of glucose, but also xylose, mannose, galactose, rhamnose and arabinose. Differently from cellulose, it presents an amorphous structure with a frequent presence of ramifications, rendering

a more fragile structure and more suitable for digestion. Finally, lignin is a polymer composed by three main aromatics: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Horn *et al.*, 2012) (Figure 2.2).

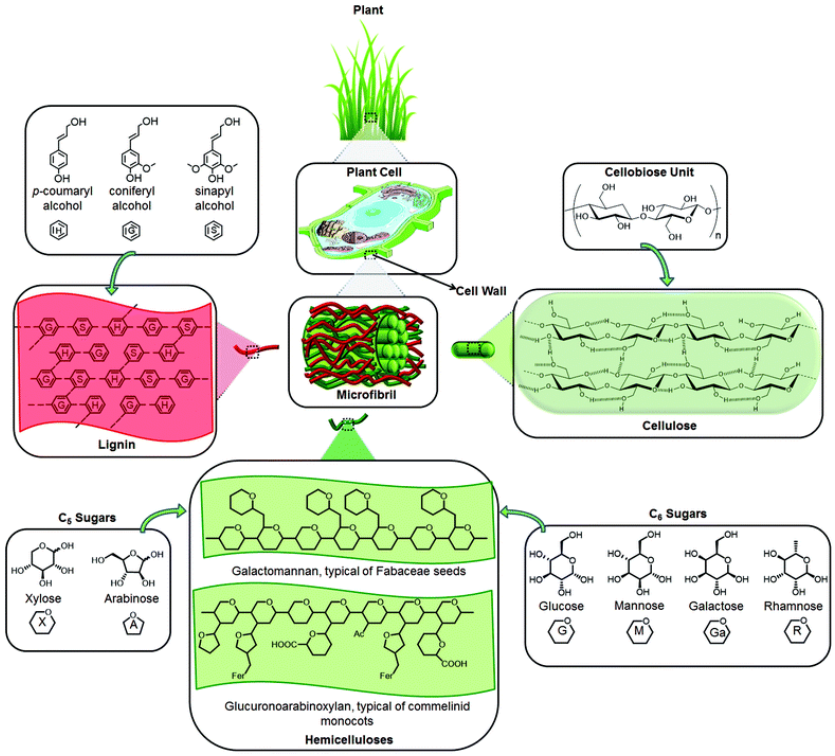

Figure 2.2 Main components on lignocellulosics structure (reproduced from Isikgor and Becer, 2015).

Cellulose holds the larger share of the energetic potential available in this kind of materials, its hydrolysis rendering glucose molecules only, which can be easily used by most of the traditional fermentation microorganisms (*e.g. Saccharomyces cerevisiae*). Hemicellulose, which is present in smaller amounts in most lignocellulosic materials (with some exceptions such as grass), results in different sugars that in some cases cannot be directly used by the traditional fermenting microorganisms (C5 sugars). However, the current existence of some industrial strains able to co-ferment pentoses (*e.g.* DSM, Abengoa), together with recent encouraging results from metabolic engineering strategies towards C5 fermentation (*e.g.* Romaní *et al.* 2015) suggests that, on a near future, hemicellulose may equally hold a high energetic potential. Lignin is usually burned for energy production, although many other applications – largely remaining to be explored - exist for this material, such as the synthesis of different aromatic compounds (Demirbas, 2008).

2.2.3 Pre-treatments

Acting as the skeleton of vegetable materials, lignocellulose presents a very solid and robust structure, hard to digest by cellulolytic microorganisms or directly by enzymes (Himmel *et al.*, 2007). Thus, a pre-treatment is usually required to facilitate saccharification, which in most of the cases means increasing the accessibility of cellulases to the substrate.

These are traditionally classified into four main categories: physical (*e.g.* milling, extrusion, etc.); chemical (acid, alkaline, organosolv, etc.); physico-chemical (*e.g.* steam explosion, ammonia fibre explosion, etc.); and biological (*e.g.* applying white-rot fungi) (Mood *et al.*, 2013). An adequate choice of a pre-treatment (or a combination of multiple ones) is rather complex and will depend on the specific material being used, but also on the final chemicals intended to be produced. Multiple factors must be taken into account such as the good preservation of the hemicellulose fraction, a reduced inhibitory compounds formation, minimal energy requirements and a cost-effective process (Alvira *et al.*, 2010; Yang and Wyman, 2008). Also, a growing interest has been observed on eco-friendly processes, where no chemicals are employed, such as autohydrolysis. Relying exclusively on water at high temperatures, this has been reported to successfully solubilize hemicellulose and recover both cellulose and lignin (Romaní *et al.*, 2010). While facilitating enzyme's action, these processes usually also result in the production of different compounds originated from the degradation of one or multiple components of the lignocellulosic materials. These are inhibitory for the action of enzymes and organisms (Taylor *et al.*, 2012) and should therefore be considered when selecting the applied pre-treatment(s) (Viikari *et al.*, 2012; Cavka and Jönsson, 2013). Inhibitors are commonly classified into three categories: furan derivatives (*e.g.* furfural, hydroxymethyl furfural); weak acids (*e.g.* acetic acid); and phenolic compounds (Almeida *et al.*, 2007, 2009).

Due to its possible harmfull effects over the process, a detoxification step may be considered to partially or totally remove such compounds. This would strongly depend on the toxicity and concentration levels of these compounds but also on the tolerance of enzymes and microrganisms (Zabed *et al.*, 2016). Alternatively, taking into account the associated increase on process complexity and operational costs (von Sivers *et al.*,

1994), the utilization of high tolerant organisms able to cope with these inhibitors is currently pursued (*cf.* section 2.2.8.1).

2.2.4 Enzymatic hydrolysis

After an initial pre-treatment step, which can be optional is some cases, solids slurry originated from the lignocellulosic material is mixed with a cellulases cocktail at specific conditions (temperature and pH) to conduct the enzymatic hydrolysis. This relies on the action of cellulases and hemicellulases, enzymes usually produced by cellulolytic organisms such as *Aspergillus niger*, *Trichoderma reesei*, *Clostridium thermocellum* among others (Kuhad *et al.*, 2016).

Cellulose hydrolysis is a very complex process involving several types of cellulases (Klein-Marcusschamer *et al.*, 2012). In fact, cellulolytic organisms usually code and secret a huge number of different cellulases. The hydrolysis is usually conducted synergistically by two main classes: endoglucanases (EGs) randomly cleave internal β-1,4-glycosidic bonds of cellulose chains; and exoglucanases such as cellobiohydrolases (CBHs), that form cellobiose units by acting either on the reducing or on non-reducing ends of cellulose chains. Finally, one additional class of enzymes, β-glucosidases, hydrolyzes cellobiose into glucose (Segato *et al.*, 2014) (Figure 2.3).

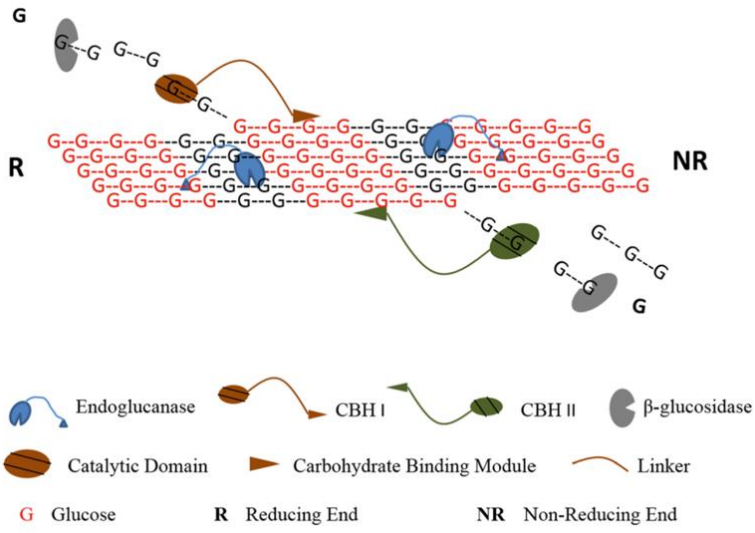

Figure 2.3 Simplified schematic of the enzymatic hydrolysis of lignocellulose (reproduced from Kumar and Murthy (2013)).

The concerted action of endoglucanases and exoglucanases for cellulose hydrolysis implies the following events orchestrated in a well-established order: cellulase adsorption onto the substrate; formation of the complex cellulase-substrate; cleavage of the glycosidic bond and displacement of enzyme to the next cleavage zone; desorption of the enzyme (Figure 2.3).

As in every enzyme-mediated process, the reaction rate is governed by several environmental factors, *e.g.* pH, temperature, presence of surfactants, etc. (Kumar *et al.*, 2008). Also, each cellulase can present very distinct substrate affinity, thermostability, reaction kinetics, etc. (Tu *et al.*, 2007a). Furthermore, the properties of the lignocellulosic material influence the degradation process, namely its structure and composition, cellulose crystallinity and surface area (Bommarius *et al.*, 2008). Altogether, the degradation of cellulose is thus a rather complex process.

Among the several cellulolytic systems reported to date, *Trichoderma reesei* presents probably one of the most studied ones. This fungus is currently the most employed organism in the production of commercial enzymes for biomass hydrolysis (Kumar *et al.*, 2008; Horn *et al.*, 2012). Its cellulolytic system encompasses CBHs (EC 3.2.1.91), EGs (EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21) (Seiboth *et al.*, 2011). According to Suominen *et al.* (1993), approximately 60 % and 20 % of the secreted proteins in *T. reesei* correspond to the CBHs Cel7A (formerly CBH I) and Cel6A (formerly CBH II), respectively, which constitute the only CBHs of this cellulolytic system. They both act processively on cellulose chains, but while Cel6A forms cellobiose from the non-reducing ends, Cel7A acts on the reducing ends. Although their processive way of action, they both present a reversible binding to the substrate, which represents an important feature especially in a context of enzyme recycling. Palonen *et al.* (1999) have observed some differences in this regard: while Cel6A exhibits a binding reversibility between 60 to 70 %, this feature is considerably increased in the case of Cel7A to a minimum of 90 %. Their action is complemented by the synergetic action of several EGs, produced in considerable smaller amounts: Cel5A (formerly EG II), Cel5B, Cel7B (formerly EG I), Cel12A (formerly EG III), Cel45A (formerly EG V), Cel61A (formerly EG IV), Cel61B and Cel75A (formerly EG VI) (Seiboth *et al.*, 2011). Finally, seven β-glucosidases Cel1A (formerly BGL II), Cel1B, Cel3A (formerly BGL I), Cel3B, Cel3C, Cel3D and Cel3E are usually produced in very small

amounts (approx. 0.5 % of total secreted proteins), which in some cases forces the supplementation with β-glucosidases from another organism (*e.g.* Novozymes 188 from *Aspergillus niger*).

2.2.5 Fermentation

Enzymatic hydrolysis of lignocellulosic materials result on the release of two main monomer sugars: glucose and xylose. Differently from cellulose and hemicellulose, glucose can be metabolized directly by a traditional fermentation organism, such as *Saccharomyces cerevisiae*. This represents one of the most employed organisms on industrial ethanol fermentation for multiple reasons: it presents a high ethanol production capacity (Çakar *et al.*, 2012), a high tolerance to ethanol and inhibitors (Almeida *et al.*, 2007; Pereira *et al.*, 2011a) and the GRAS (Generally Regarded as Safe) status (Eksteen *et al.*, 2003).

A different case concerns xylose, which is natively not consumed by most of *S. cerevisae* strains (Aditiya *et al.*, 2016), although being metabolized by other organisms such as *Pichia stipitis* (Nakamura *et al.*, 2001). In specific cases when a significant hemicellulose fraction is present, this could represent an important economic drawback. According to Tomás-Pejó *et al.* (2014), pentose sugars can represent up to 40 % of total sugars present on a lignocellulosic hydrolysate. Consequently, intense efforts have been conducted over the years towards an efficient integration of xylose conversion into *S. cerevisiae* (Romani *et al.*, 2015; Wahlbom *et al.*, 2003; Hahn-Hägerdal and Pamment, 2004; Kötter *et al.*, 1990).

Another possible critical factor on the fermentation stage is the presence of inhibitors early produced during biomass pre-treatments (*cf.* section 2.2.3) raising the need for more robust organisms able to cope with their toxicity effects. Numerous studies have been conducted on the mechanisms of inhibitors toxicity over the cells, mainly centered on acetic acid and furan derivatives, enabling important steps towards the selection of more robust organisms (Costa *et al.*, 2017; Cunha *et al.*, 2015; Pereira *et al.*, 2014).

2.2.6 Process configurations

Cellulosic ethanol is based on two critical and distinct steps: the enzymatic hydrolysis of complex sugars (cellulose and hemicellulose); the fermentation of monomeric sugars

into ethanol. While an effective integration of both is highly desirable, as to increase overall efficiency and economics of the process, this is rather complex. Optimal performance of cellulolytic enzymes is usually achieved with a temperature in the range of 45-50ºC and a pH of 4-5, while fermentations on the other hand, are usually conducted at lower temperature (Jørgensen *et al.*, 2007).

Two main process configurations are traditionally employed to integrate hydrolysis and fermentation: Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF). On Separate Hydrolysis and Fermentation (SHF) the two stages are conducted sequentially, both being conducted at their respective optimal conditions. This way, hydrolysis can be initially conducted at high temperatures which is then reduced to meet the fermenting organism optimal range. On the other hand, this enables sugars accumulation, leading consequently to end-product inhibition (Chen and Fu, 2016). Usually more prominent for concentrations above 15 g/L (Varga *et al.*, 2002), this cascade mechanism is based on a inhibition effect performed by glucose on the action of β-glucosidases, which can cause an accumulation of cellobiose (Decker *et al.*, 2000); cellobiose itself is an effective inhibitor of cellobiohydrolases, therefore affecting their attack of cellulose chains terminal ends (Holtzapple *et al.*, 1990). In the context of process intensification this represent an important drawback since high sugar concentrations can be generated, therefore causing important reductions on conversion kinetics (Hsieh *et al.*, 2014; Xiao *et al.*, 2004). Opposing to that, on Simultaneous Saccharification and Fermentation (SSF) both processes are conducted simultaneously, hence sugars are converted immediately after their formation, avoiding hydrolysis inhibition. Although requiring the operation under sub-optimal conditions (Balat, 2011), this usually results on higher ethanol yields (Wingren *et al.*, 2003; Alfani *et al.*, 2000) while also allowing a reduction on equipment costs since both stages can be performed on a single vessel (Talebnia *et al.*, 2010). In addition to these processes, some variations emerged aiming an effective conversion of both pentoses and hexoses. Simultaneous Saccharification and Co-Fermentation (SSCF) and Separate Hydrolysis and Co-Fermentation (SHCF) enable the conversion of both sugars by a single organism with consequent reduction on operational costs.

More recently, a growing interest has been observed on CBP (Consolidated BioProcessing), a new strategy based on a full integration of all processes in a single

organism. This would be able to not only secrete the cellulolytic enzymes, but also produce the monomer sugars and then convert them to the final product (Matano *et al.*, 2013). In addition to a process simplification, this strategy would also enable an important reduction on the cost associated to microbial enzymes production (Hasunuma *et al.*, 2015). Since microorganisms usually are not able to both efficiently saccharify and ferment, most of the recent CBP strategies have been based on genetically modifying a high-fermenting organism, as *S. cerevisiae*, enabling it to secret or display cellulolytic enzymes at proper levels (Fujita *et al.*, 2004; Yamada *et al.*, 2011; Matano *et al.*, 2012).

2.2.7 Biorefinery concept

Even though employing less expensive materials, lignocellulose conversion requires a complex process which involves expensive steps such as the pre-treatment and enzymatic hydrolysis. For this reason, it is commonly referred that its economic viability will be strongly dependent of a full residue utilization, with all fractions being used (Tao *et al.*, 2011). Resembling on the similarity to the traditional oil refineries, this concept is usually designated as biorefineries.

Biorefineries are platforms for the production of a wide spectrum of compounds, in some cases currently produced by chemical synthesis, using different types of biomass as feedstock (*e.g.* sugar/starch crops, vegetable oil, micro-algae, etc.). Through different possible conversion techniques, such as fermentation, transesterification, gasification, hydrogenation or anaerobic digestion, biomass can be converted into either energy or chemicals, in a cleaner and sustainable route (Figure 2.4).

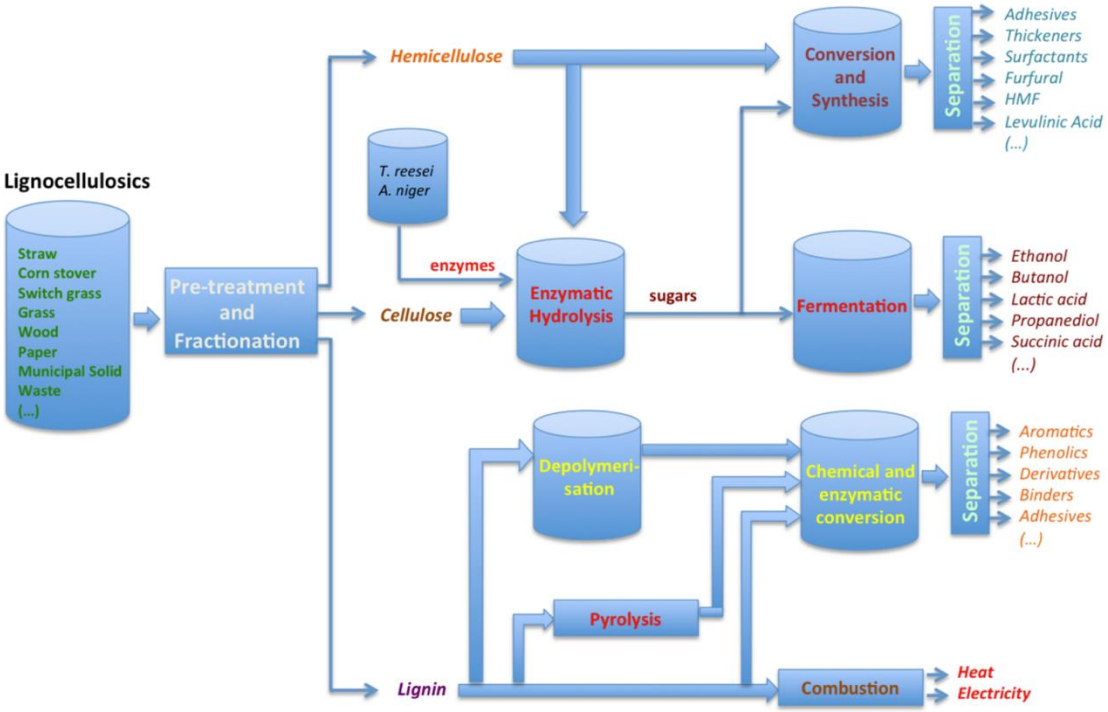

Figure 2.4 Overall schematic of the biorefinery concept.

Numerous studies have recently been published on the application of this concept to different materials, such as spent coffee grounds (Karmee, 2018; Mata *et al.*, 2018) or pulp and paper sludge (Gottumukkala *et al.*, 2016); nevertheless, industrial-scale applications of this concept are still scarce.

2.2.8 Current challenges to 2G bioethanol

Even though lignocellulosic ethanol tackled major bottlenecks of 1G ethanol, while also allowing to valorise a vast amount of, so far, unutilized resources, it still faces several challenges towards an economically viable process (Chen and Fu, 2016).

2.2.8.1 Inhibitors toxicity

Biomass pre-treatment generate varying concentrations of inhibitory compounds, commonly reported to affect both enzymes and microorganisms (Tomás-Pejó *et al.*, 2011). In recent years, this problem has been addressed through three main approaches: reduction of inhibitors formation; development of detoxification technologies; increase of microorganism tolerance (Koppram *et al.*, 2014). Inhibitors formation can in fact be significantly reduced through a carefull selection of the pre-

treatment and its specifications. Here, the option for not using chemicals (Alvira *et al.*, 2010) or an adequate selection of the time-temperature binomy (Bals *et al.*, 2012) have shown to greatly affect inhibitors formation.

In what concerns the detoxification of the solids suspension several methods have been reported: anion exchange, overliming, solvent extraction, use of zeolites or laccases (Tomás-Pejó *et al.*, 2011). However, according to Koppram *et al.* (2014) this option may be controversial as it will lead to increased costs of operation. Opposing to that, alternative process configurations can be employed allowing detoxification by the cells. Operating in a fed-batch design, where multiple substrate addition pulses occur, can enable a continuous conversion of low concentrations of inhibitors by the microorganism without compromising its viability and performance (Hawkins and Doran-Peterson, 2011).

In addition to that, considerable research has been conducted on the development of novel microrganisms able to withstand the presence of significant levels of toxics. One of the followed paths was based on adaptation studies conducted under inhibitory environments, enabling the generation of adapted strains with higher inhibitors tolerance (Hawkins and Doran-Peterson, 2011). Another option may consist on the overexpression of genes commonly associated to inhibitors resistance (*e.g. TKS1*, *ERG2*, *PRS3*, etc.) (Pereira *et al.*, 2011b) as already successfully reported by different authors (Cunha *et al.*, 2015; Hasunuma and Kondo, 2011).

2.2.8.2 Increased solids concentrations – operational challenges

A minimal level of 4 % (v/v) is generally considered as the critical ethanol titre for enabling an economically viable alcoholic fermentation (Hahn-Hägerdal *et al.*, 2006). Differently from 1G-ethanol, where sugar-rich subtrates allow processes of Very-High-Gravity (VHG) fermentation (initial sugars above 300 g/L), lignocellulosic materials represent a much distinct scenario. Considering the average composition of these materials, meeting the referred level of ethanol usually requires operating at high-solid loadings, generally above 20 %. As a result of the hygroscopic nature of these materials, as well as its low density, this commonly lead to significant increases of solids slurry viscosity, which ultimately causes important limitations of heat and mass transfer (Koppram *et al.*, 2014). The former ones may be especially critical as an efficient control

of temperature is hampered, allowing the formation of sub-regions within the reaction vessels where hydrolysis and fermentation could be affected. On the other hand, an efficient mass transfer is commonly regarded as critical for proper enzyme-substrate interactions: it promotes an adequate contact of enzymes and substrate; enables final product diffusion attenuating possible inhibitory effects. On an industrial scale, inefficient mixture can result on considerable problems of homogeneity in the reaction vessel and consequently causing operational problems.

A possibility to address these limitations may be a higher agitation speed, although it will be efficient only up to some values of solid consistency. Furtermore, in addition to an increased energy consumption (Fan and Lynd, 2007), this could represent a physiological stress for some microorganisms and enzymes caused by accentuated shear forces. An operational solution commonly employed is the utilization of a fed-batch feeding strategy where the lignocellulosic material is added through multiple pulses. This enables to operate under critical levels of viscosity as new substrate additions are conducted after the liquefaction of the previous pulse (Jørgensen *et al.*, 2007).

2.2.8.3 Enzymes cost

Cellulases contribute to a significant part of the final bioethanol cost (Aden and Foust 2009), resembling as the second most expensive element (following the raw-material) in the overall process. According to Klein-Marcusschamer *et al.* (2012), the current cost of cellulases on bioethanol production is approximately $ 0.68 per gallon, although the exact cost is truly unknow since it depends on the specific agreements between enzyme producers and industry. In recent years, intense efforts have been made to modify this scenario, a goal pursued by three main strategies: reducing cellulases production cost; creating more efficient cellulases; and reducing the amounts of required cellulases by recycling them over several rounds of hydrolysis (Pribowo *et al.*, 2012). Achieving a significant reduction on the cost of cellulases will allow an important improvement on the economics of second-generation biofuels, facilitating their competition with fossil fuels, but also of other processes based on lignocellulosic materials through the biorefineries concept.

2.3. Recycled paper sludge

Paper manufacturing industry represent one of the sectors with highest growth on global economy, as a result of the notorious growth in the world-wide population and its consequent demand for this product. World production of paper and paperboard is currently estimated in 400 million tons/year and predicted to reach 550 million in 2050 (FAOSTAT, 2015). In a similar way, also a significant increase has been observed on the amounts of different residues generated by this industry. According to Ioelovich (2014) nearly 350 million tons of paper mill sludges are produced every year from pulp and paper-making processes. As direct consequence, their handling costs and effects over economy, society and environment is also expected to increase (Faubert *et al.*, 2016). Nearly 50 % of these residues are being currently disposed on landfills, with an approximate cost of 30 $ per ton (Chen *et al.*, 2014).

2.3.1 Definition and current availability

Depending on the type of process, raw-materials and final product, a wide-variety of waste-water streams are generated by paper industry, which originates different categories of sludge, requiring proper handling (Monte *et al.*, 2009). According to Bajpai (2015) three main types of sludges are produced on paper mills: primary; secondary or biological; and deinking sludge.

Primary sludge is originated from clarification of process water and contains mainly fines and fillers. This residue can either be re-introduced in the process (in some cases) but can also be incinerated, disposed or mixed with other sludges. Secondary sludges are produced from the clarifiers of the biological units of waste water treatment. In some circumstances this residue can also be reintroduced into the process, otherwise it can be incinerated or disposed in landfills. Finally, the deinking sludge refers to the residue formed during paper recycling processes and contains mainly small cellulose fibres (that are not retained by filters), ink particles, deinking agents, adhesive components and fillers (*e.g.* kaolin, clay, calcium carbonate, etc.) (Monte *et al.*, 2009; IPPC, 2001; CANMET, 2005).

According to the configuration of the paper mill, different streams of waste-water can be merged, resulting in a class of solid residue consisting of multiples types of sludge.

On paper recycling mills, usually a common final residue is the recycled paper sludge (RPS), which in some cases can incorporate two or even three of the above mentioned categories of sludges.

According to Balwaik and Raut (2011), approximately 300 Kg of sludge are generated for each ton of recycled paper, although this can significantly vary according to different factors such as the recycling rate, initial materials and final product specifications. It seems clear however that the specific volumes of generated residues will allways be substantial. While primary and secondary sludges can still be potentially re-incorported on the production process, de-inking sludges and RPS can not, requiring proper handling solutions, hence representing an important problem for the sector.

2.3.2 Current handling of Recycled Paper Sludge

Considering its toxic composition, RPS residue is currently handled through a limited number of options. Because of heavy metals occurrence (from inks) RPS cannot be employed as a soil amendment (Marques *et al.*, 2008a), a solution available for other similar residues. The most common handling options currently employed are landfill disposal, incineration and incorporation on the production of cements and other constructions materials (*e.g.* bricks) (Monte *et al.*, 2009).

2.3.3 Conversion of holocellulosic fraction into biofuels and chemicals

Coming from a process with multiple variations, RPS solid composition cannot be defined with proper detail, being affected by several factors. In addition to heavy metals, and many other contaminants, RPS also have a considerable holocellulosic fraction, which can be valorised through biological conversion into different products. Previous studies reported sugar contents ranging from 26 % (Marques *et al.*, 2017) to 63 % (Kang *et al.*, 2010). Although not presenting the highest holocellulosic contents comparatively with other cellulosic materials (*cf.* section 2.2.2), this still represents a considerable amount of potential sugars that can be posteriorly converted into added-value products. Furthermore, in addition to the negative cost commonly associated to this material (Kang *et al.*, 2010), it has the important advantage of not requiring major pre-treatments (Lee *et al.*, 2004), a common requisite of traditional lignocellulosic materials. This may

be due to the high level of processing (both chemical and mechanical) already conducted during paper manufacturing process (Fan and Lynd, 2007; Domke *et al.*, 2004).

The conversion of the holocellulosic fraction from multiple types of paper-derived sludges have been studied for many decades, mainly for the production of cellulosic bioethanol and lactic acid (Table 2.2). Naturally, while this conversion in the particular case of RPS would be economic and environmentally more interesting, it should also correspond to a greater challenge as lower contents of holocellulose are usually found. This will require operating at higher solid loadings, which represent an important operational challenge (*cf.* section 2.2.8.2).

Table 2.2 General overview of distinct paper residues conversion into multiple products employing different process specifications

Reference	Product (maximum produced titer)	Fermentation Organism	Design	Enzime(s)	Substrate
Boshoff *et al.*, 2016	Ethanol (45.5 g/L)	*S. cerevisiae*	Fed-batch SSF	Optiflow RC 2.0 (Genencor); Novozyme 188 (Novozymes)	paper sludge samples
Budhavaram and Fan, 2009	Lactic acid (92 g/L)	*Bacillus coagulan*	Semi-continuous SSCF	cellulases (Genencor)	primary clarifier sludge
Dwiarti *et al.*, 2012	Ethanol (11.8 g/L)	*S. cerevisiae* TJ14	Batch SSF	Produced by *A. cellulolyticus*	paper mill sludge
Gurram *et al.*, 2015	Ethanol (30 g/L)	yeast FermProTM	Batch SHF	Cellic® CTec2 (Novozymes)	paper mill sludge
Kádár *et al.*, 2004	Ethanol (17.8 g/L)	*K. marxianus* Y01070; *Saccharomyces cerevisiae*	Batch SSF	Celluclast and Novozyme 188 (Novozymes); cellulases (Iogen)	paper mill sludge
Kang *et al.*, 2010	Ethanol (45 g/L)	*Escherichia coli* (ATCC-55124); *Saccharomyces cerevisiae* (ATCC-200062)	Fed-batch SSF	Spezyme CP (Genencor)	recycled paper sludge
Lark *et al.*, 1997	Ethanol (35 g/L)	*K. marxianus*	Batch SSF	cellulases (Iogen)	recycled paper sludge
Lin *et al.*, 2012	Ethanol (42.5 g/L)	*Saccharomyces cerevisiae* CICC 1001	Batch SSF	cellulases (Shaihai boao biotechnology co. Ltd)	paper mill sludge
Marques *et al.*, 2008a	Ethanol (19.6 g/L)	*P. stipitis*	Batch SHF	Celluclast and Novozyme 188 (Novozymes)	recycled paper sludge
Marques *et al.*, 2008b	Lactic acid (73 g/L)	*L. rhamnosus* ATCC 7469	Batch SSF	Celluclast and Novozyme 188 (Novozymes)	recycled paper sludge
Marques *et al.*, 2017	Lactic acid (108.2 g/L)	*L. rhamnosus* ATCC 7469	Fed-batch SSF	Celluclast and Novozyme 188 (Novozymes)	recycled paper sludge
Mendes *et al.*, 2016	Ethanol (54.6 g/L)	*S. cerevisiae* ATCC 26602	Batch SSF	enzymatic extract NS 22192 (Novozymes)	paper primary sludge
Min *et al.*, 2015	Sugars	*n.a.*	Batch	commercial cellulases from *T. reesei* ATCC 26921 and *Aspergillus sp.*	fines from recycled paper mill waste rejects
Moreau *et al.*, 2015	Ethanol (0.52 g/L)	*C. thermocellum* DSMZ 1237	Batch SSF	Produced by the fermentation organism	paper primary sludge
Peng and Chen, 2011	Ethanol (9.5 g/L)	*S. cerevisiae*	Batch SHF	Novozyme 342 (Novozymes)	paper mill sludge

(*Continue*)

Table 2.2 (*Continuation*) General overview of distinct paper residues conversion into multiple products employing different process specifications

Reference	Product (maximum produced titer)	Fermentation Organism	Design	Enzime(s)	Substrate
Prasetyo *et al.*, 2010	Sugars (38.4 g/L)	*n.a.*	batch	cellulases from *A. cellulolyticus*; GC220 (Genencor); Cellulosin T2 (HBI Enzymes Inc.)	paper mill sludge
Robus *et al.*, 2016	Ethanol (57.3 g/L)	*S. cerevisiae* MH1000	Fed-batch SSF	Optiflow RC 2.0 (Genencor) and Novozyme 188 (Novozymes)	paper sludge samples
Romaní *et al.*, 2007	Sugars (33.2 g/L)	*n.a.*	Batch	Celluclast and Novozyme 188 (Novozymes)	sludges from Kraft pulp mill
Romaní *et al.*, 2008	Lactic acid (42 g/L)	*L. rhamnosus* CECT-288	Fed-batach	Celluclast and Novozyme 188 (Novozymes)	sludges from Kraft pulp mill
Shen and Agblevor, 2008	Sugars	*n.a.*	Batch	Spezyme AO3117 (Genencor)	RPS mixtured with other material
Thomas, 2000	Lactic acid (21.5 g/L)	*L. delbrueckii* (NRRL B445)	Batch SSF	Cytolase CL (DSM)	pulp mill solid waste
Yamashita *et al.*, 2008	Ethanol (18 g/L)	*Z. mobilis*	SSF	Meicelase (Meiji Seika Co., Ltd.)	paper mill sludge
Yamashita *et al.*, 2010	Ethanol (30.5 g/L)	*S. cerevisiae* AM 12	SSF	Meicelase (Meiji Seika Co., Ltd.)	paper mill sludge
Zhang and Lynd, 2010	Ethanol (45 g/L)	*Z. mobilis* 8b; *S. cerevisiae* RWB222; *S. cerevisiae* D5A	SSCF	Spezyme (Genencor); Novozyme 188 (Novozymes)	paper mill sludge
Zheng *et al.*, 2012	Sugars	*n.a.*	Batch	cellulase XWS-G-1; β-glucosidase and xylanase MJT-G-NIS (Noao Sci&Tec Development Co. Ltd.)	recycled paper sludge

2.3.4 Bioethanol production from RPS

Multiple works have been conducted on the last decades regarding the conversion of pulp and paper sludges into chemicals; however, those referring specifically to sludges originated from paper recycling processes and to the production of ethanol are rather few. All studies on RPS conversion into bioethanol present two mandatory elements: a cellulolytic complex able to convert cellulose and/or hemicellulose fractions to monomeric sugars, usually provided by a cellulase cocktail or produced *in-situ* by a cellulolytic organism; and a fermentation organism. Additionally, due to the traditionally high ash content found on RPS, a de-ashing step may be employed before hydrolysis, as described by some authors (Marques *et al.*, 2008a; Gurram *et al.*, 2015; Robus *et al.*, 2016).

One of the first studies on this topic was reported by Lark *et al.* (1997) who were able to produce a maximum of 35 g/L of ethanol within 72 hours under a process of SSF, using *Kluveromyces marxianus* at 38ºC and an initial solids concentration of 190 g/L. It should be mentioned however that cellulose content on this residue (approx. 50 %) was considerably higher comparatively to other compositions reported for this type of material. Using a slightly different residue (34 % cellulose) Marques *et al.* (2008a) obtained 18.6 g/L ethanol after a 48 hours SSF process employing *Pichia stipites* with 179 g/L of solids. The authors also verified that SHF strategy enabled a slighty superior ethanol concentration (19.6 g/L), however, the required process time was considerably superior (170 hours).

SSF processes have been preferably employed for RPS conversion, which is in agreement with what was referred previously regarding the hydrolysis of lignocellulosic materials under high solid loadings (*cf.* section 2.2.6). As a consequence, this brings additional challenges, namely for the microrganisms, as they are required to operate at moderate-high temperatures while remaining metabolically active. A comparative study was conducted on this regard by Kádár *et al.* (2004) who tested both *K. marxianus*, commonly considered highly-thermotolerant, and *S. cerevisae*, on a SSF process at 40ºC. Unexpectedly, no significant differences were observed between the two species. A possible reason may rely on the very favourable conditions of these tests which only employed 60 g/L of solids (only 8.8 and 9.0 g/L ethanol were produced by *K. marxianus*

and S. cerevisiae, respectively). Additionaly, even though a temperature of 40ºC can be considered high comparatively to the traditional operational range of *S. cerevisae* (25-37ºC), several studies have already been conducted with different strains of *S. cerevisae* at this temperature or even higher (Ruiz *et al.*, 2012; Dwiarti *et al.*, 2012; Costa *et al.*, 2017). Another important aspect for the fermentation organism in this context may be the capacity to metabolize pentose sugars, considering the significant hemicellulose fraction reported by some authors. Surprisingly, only few reports have considered the utilization of this polysaccharidic fraction (Marques *et al.*, 2008a; Kang *et al.*, 2010) which may be attributed to the lack of a microorganism capable of efficiently convert xylose but also to the complexity that would be required in terms of the enzymatic cocktail.

The ability to cope with the toxicity of RPS materials can also be a critical feature in this case. Most of the studies conducted with RPS did not report any major inhibitory effects over the fermentation organisms, excepting for the work reported by Yamashita *et al.* (2008) with *Zymomonas mobilis*. The authors observed that although capable of consuming glucose, *Z. mobilis* ability to produce ethanol was severely affected by the presence of metal ions coming from the solid. Concentrations of Fe^{2+} above 0.4 g/L were found by the authors to critically affect ethanol production by this organism, a value that is significantly inferior to the estimated levels reported on the initial material (5 g/L). Also Ca^{2+} and Mg^{2+} were found to affect ethanol production, but to a much lower extent. In this context, the authors observed that cells immobilization on Ca-alginate was particularly protective against the toxicity of metal ions. Applying this strategy, 18 g/L ethanol were obtained after a 48 hours SSF process, a remarkable improvement considering that no ethanol was produced in the same conditions using non-immobilized cells.

Another important aspect of RPS hydrolsysis, as keenly pointed out by multiple authors, is the absence of an initial pre-treatment usually required to break the lignocellulosic structure and facilitate enzymes action (excepting for the de-ashing steps commonly employed). Indeed, a complete hydrolysis of these materials have already been reported without the application of any pretreatment (*e.g.* Marques *et al.*, 2008a). Distinct pre-treatment strategies were analysed by Yamashita *et al.* (2010) on both the

hydrolysis and fermentation of a 200 g/L suspension of paper sludge. While the single application of mechanical grinding did not result on significant improvements, chemical swelling with phosphoric acid allowed an increase on cellulose conversion from 29.9 % (raw paper sludge) to 96.7 %, and still slightly increasing to 97.1 % when both were combined. Furthermore, when a SSF process was conducted with the untreated material, a maximum of 20.4 g/L ethanol was obtained within 48 hours. Using the pre-treated material, 30.5 g/L ethanol were obtained in only 24 hours, resulting therefore not only in a higher ethanol titre but also in increased procutivity (1.27 vs 0.424 g/L.h).
More recently, Gurram *et al.* (2015) observed that the application of an initial de-ashing step, conducted chemically (with HCl) or mechanically, enabled an ash removal around 77 and 89 %, respectively, leading to significant increases on the carbohydrates concentration of the resulting solid. By removing this critical barrier for enzymes, remarkable improvements were observed on the hydrolysis process. Comparatively to original solid where enzymes only converted 2.2 % of total cellulose, this value increased to 86.3 % when de chemically de-ashed solid was used. In addition to the de-ashing step different chemicals were also tested as possible enhancers of enzymatic hydrolysis: cationic polyelectrolytes (c-PAM) and hydrogen peroxide. According to Haynes *et al.* (2013), cationic polyelectrolytes can increase cellulase binding to cellulose through ‘‘patching mechanism’’ since both, the enzymes and the cellulose fibers, have a negative zeta potential. In fact, as observed by the authors, the application of these compounds enabled slight improvements on cellulose conversion, although this seemed strongly dependent on an initial de-ashing step. Comparatively to the original solid, the application of these compounds only enabled an increase on cellulose conversion to 3 %. When a de-ashing step was initially applied followed by the application of cationic polyelectrolytes, it increased from 86 % to 96 %. Hydrogen peroxide, on the other hand, has been intensively reported as an efficient lignin dissolving agent (Sun *et al.*, 2001; Correia *et al.*, 2013), consequently increasing enzymes access to cellulose. The elimination of ash and lignin may be determinant to the achievement of a complete hydrolysis, as both are considered important barriers to enzymes, namely through non-productive binding. As for the previous case, solid swelling with H_2O_2 after chemicall de-ashing allowed a posterior cellulose conversion of 99.3 %. In a similar way, when the different hydrolysates were fermented with an industrial yeast (FermPro^{TM}) final

ethanol titres have also increased comparatively to the control: from 23.8 g/L to 25.9 g/L, using H_2O_2, and to 28.1 g/L using a cationic polyelectrolyte. It is worth to mention however that in some cases, apparent toxicity against the yeast were observed as longer fermentation periods were required. The most proeminent case was for H_2O_2 which resulted on an ethanol productivity of 1.27 comparatively to 2.33 g/L/h for the control case. It seems clear that, while multiple strategies can be efficiently applied to enhance the initial saccharification process, all their subsequent effects should be carefully considered namely in terms of inhibitors generation and toxics exposure to the cells.

In the scope of an economically viable process, intensification is critical, specially when low cellulose RPS materials are employed. On this context some studies have been conducted more recently towards increasing the solids loading and consequently the final ethanol concentrations.

Early reports by Marques *et al.* (2008a; 2008b) described the production of enzymatic hydrolyzates with approximately 56 g/L of glucose after 120 hours hydrolysis of a RPS suspension with 7.5 % carbohydrates, representing nearly 18 % (w/v) solids. This level of sugar concentration enabled a maximum ethanol production (by SHF) of 19.6 g/L. More recently considerable improvements were achieved by Marques *et al.* (2017) employing a fed-batch feeding strategy. In addition to the initial solid in suspension, corresponding to approximately 75 g/L of carbohydrates, multiple pulses of substrate were added (spaced by 5 h), corresponding nearly to a total of 60 g/L of additional carbohydrates. According to the authors, this strategy enabled a 1.8 and 1.9-fold increase on glucose and xylose production, respectively, achieving glucose concentrations above 80 g/L. Similar findings were observed by Kang *et al.* (2010) using two additional substrate pulses, which enabled a total of 120 g/L of glucans. Even though ethanol production yield decreased to nearly 70 %, clearly inferior to the values obtained on batch process, final ethanol concentration reached 45 g/L.

Employing a distinct material - corrugated recycle paper sludge - with superior glucan contents (40-50 %), Boshoff *et al.* (2016) studied the effect of solid and enzyme loadings on the final yield and ethanol concentrations following a fed-batch SSF strategy. Applying this strategy, a maximum of 33 % (w/w) solids suspension (represents the total amounts after substrate pulses) was used. After conducting a Central Composite Design

(CCG) with these two variables, a Response Surface Methodoly analysis was conducted. Model for ethanol concentration (with a R^2 of 0.946) predicted a maximum value of 53 g/L achieved at the maximum level of solids loading and with an enzyme dosage of 11 FPU/g_{solid}. Superior values of enzyme dosage did not seem to cause relevant improvements for the all range of solids loadings, suggesting a saturation of enzymes positive effect. Additionally, according to the model for ethanol production yield, a maximum value of 80 % is achieved for 28 % (w/w) solids and 13 FPU/g_{solid} indicating that for superior values of solid concentrations a decrease on solid conversion may be observed. The same effect was observed by Gurram *et al.* (2015) although employing a solid with a lower glucan content and smaller solid loadings. Enzymatic hydrolysis of a 10 % (w/v) solids suspension produced 61.8 g/L of glucose, increasing to 76.7 g/L when a loading of 20 % (w/v) was used, which corresponds on the later case to glucan conversions between 55-68 %.

A similar study was conducted by Robus *et al.* (2016) using a different solid (approximately 52 % glucans). After analysis of different sludge samples for their potential on ethanol production, two were selected to evaluate the influence of solid and enzyme loading on the efficiency of ethanol production through a CCD study, also following a fed-batch SSF strategy. For the best material, increased amounts of solid and enzyme had a significant positive effect on ethanol concentration, as expected. Nevertheless, optimum values for ethanol yield were found in the range of 10-15 % (w/w) solids, far from the maximum levels tested (27 %). Considering the levels of desirability, which is a multiresponse factor that takes into account both ethanol yield and final concentrations, a maximum value was found for 21.75 % (w/w) solids and 14.23 FPU/g_{solid}. These were experimentally validated resulting on a final ethanol concentration of 57.31 g/L and a yield of 94.07 %.

2.4. Cellulase recycling

Enzymes recycling has been one of the most pursued routes to reduce cellulase cost on 2G bioethanol, given the high amounts of enzymes that are currently required for efficient hydrolysis (Himmel *et al.*, 2007) but also considering the fact that cellulases have shown remarkable stability (Maheshwari *et al.*, 2000). Over the last 30 years, and especially during the last decade, a suitable recycling process has been investigated in several studies. Nevertheless, more effort is still required for a mature technology to be developed. This process will probably be driven by the biorefineries industry and the Academy, since the manufacturers do not benefit from the development of enzyme´s recycling. However, competition between enzyme producers will eventually make it happen. At this point it must be stated that – technically - enzyme recycling is indeed possible.

An efficient cellulase recycling process would largely depend on 3 major requirements: (1) a highly-stable cellulase; (2) a high hydrolysis efficiency; (3) good control over the substrate adsorption/desorption process.

2.4.1 Cellulase-substrate interactions

During the process of enzymatic hydrolysis three main actors can be distinguished: the cellulases, the lignocellulosic fibers and the liquid phase. The relative amount of free cellulases on the liquid phase is not constant, it rather changes over the extension of hydrolysis. The final composition of the system determines which fraction of the enzyme will be free in solution, and easily available to be re-used, and which fraction will remain bound to the final solid, requiring an additional step of desorption for re-usage. This dynamic process strongly depends on the affinity of each cellulase (*e.g.* Cel7A, Cel5A, Cel7B, β-glucosidase) for cellulose and lignin (Pribowo *et al.*, 2012), on the structure and composition of the substrate (Tu *et al.*, 2007a) and finally on multiple environmental factors (*e.g.* pH, presence of surfactants)(Shang *et al.*, 2014; Seo *et al.*, 2011).

2.4.2 The role of cellulose binding domains (CBDs)

A considerable number of studies have previously demonstrated that different cellulases can present distinct affinities for the substrate. Furthermore, for a given

cellulase, diverse affinities can also be found for different substrates. Cellulose-binding domains (CBDs) play an important role in defining the affinity and specificity of cellulases towards the insoluble fibres (Figure 2.5).

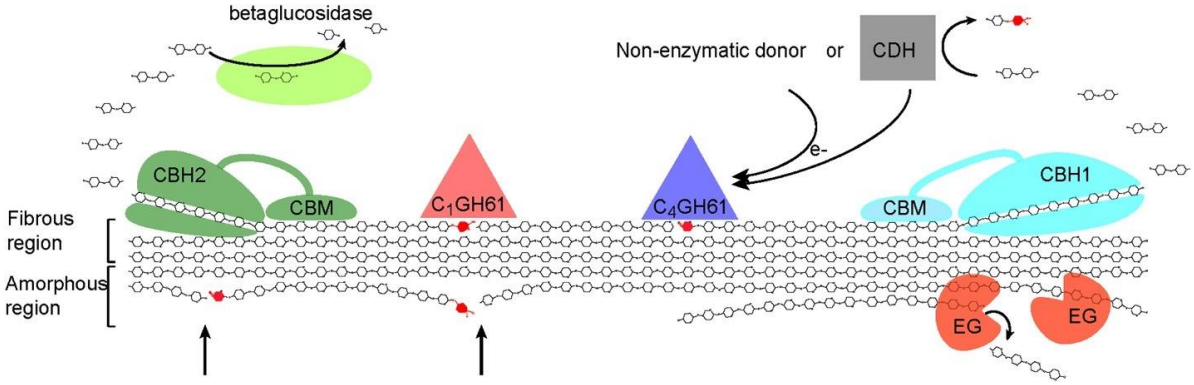

Figure 2.5 Simplified schematic of cellulose enzymatic hydrolysis by a fungal system (reproduced from Horn *et al.*, 2012).

CBDs are part of a wider class of protein components designated carbohydrate-binding modules (CBMs). These are specific amino acid sequences (between 30 and 200 amino acids), present in many carbohydrate-hydrolyzing enzymes, which have a carbohydrate binding activity (Boraston *et al.*, 1998). CBMs are particularly common on cellulases, which usually present a modular structure. In addition to the catalytic module, they also present, at least, one carbohydrate-binding module (Gilkes *et al.*, 1991). These non-catalytic modules can either be linked to the C- or the N- terminal of the protein's structure and seem to facilitate the adsorption to specific carbohydrates. According to Arantes and Saddler (2010), CBMs action on cellulose may occur by three distinct ways: increasing cellulase concentration on the surface of cellulose; promoting substrate selectivity and disruption of crystalline substrate. For the particular case of CBDs, they are reported to enable an efficient adsorption of the enzyme to cellulose, and its processive hydrolysis. After the cleavage of a glycosidic bond, cellulase does not separate from the substrate, but rather slides for the next hydrolysis (Figure 2.5).

In addition to their evident role on substrate hydrolysis, CBDs may also play a critical role on enzyme recycling. As an important element on the process of cellulase adsorption to the substrate, it will significantly dictate the equilibrium between free and bound cellulases, and their desorption from solid residue. With exception of Cel12A, all major EGs and CBHs in *T. reesei* present a CBD (Viikari *et al.*, 2007) suggesting a possible

high affinity towards cellulose. Reverse wise, as β-glucosidases do not present this binding domain, its capacity to bind cellulose will probably be considerably reduced. In fact, on early studies conducted by Ishihara *et al.* (1991) the higher binding affinities for a delignified substrate were observed for CBH, followed by EG, and finally β-glucosidase. Also, using an electrophoretic analysis, Tu *et al.* (2007a) verified that, differently from Cel7A, Cel6A, Cel7B and Cel5A, β-glucosidase levels on the liquid fraction remained constant during hydrolysis, suggesting a low adsorption to substrate. Similar results were also observed by Pribowo *et al.* (2012) and Lindedam *et al.* (2013) who have analyzed the adsorption profiles of the different enzyme components applying a SDS-PAGE analysis. In another example, Tu *et al.* (2007b) observed that *T. reesei* cellulases (Celluclast and Spezyme CP) presented higher substrate affinity than those from Penicillium sp., because the later does not have a CBD (Jørgensen *et al.*, 2003).

2.4.3 The influence of substrate composition on the equilibrium of free versus bound cellulases

Cellulases have a high affinity for both cellulose and lignin. However, while they return into the liquid fraction once cellulose is fully hydrolyzed, lignin-bound cellulases remain adsorbed in a nonproductive way (Yang and Wyman, 2006). Early studies by Desphande and Erikson (1984) showed that after 24 hours of Avicel (almost pure cellulose) hydrolysis, most of the endo-1,4-β-glucanases were free on the liquid fraction (around 85 %). However, when lignin-containing substrates were employed this value decreased to less than 50 %. More recently, Lu *et al.* (2002) analyzed the Langmuir isotherms for the adsorption of cellulases to Avicel and two lignin-containing substrates. While the maximum cellulase adsorption of 80 mg/$g_{substrate}$ was found for Avicel, in the case of a substrate with 46 % of lignin it was nearly 160 mg/$g_{substrate}$. After 48 hours of hydrolysis, the protein content on the liquid fraction was 85 % for the case of Avicel, contrasting with only 30 % for the 46 % lignin substrate. Another example was provided by Qi *et al.* (2011), who obtained around 30 % of the proteins in the supernatant after a 48 hours hydrolysis of a 20 % lignin-substrate, and 65 % when the lignin content decreased to 3.6 %.

The referred results suggest a clear influence of lignin on the final fraction of free enzymes. While the influence of lignin on enzyme adsorption to cellulose, and on its

subsequent hydrolysis, is more or less consensual, the underlying mechanisms for such effects are still not very clear and seems to be case-dependent. According to the most traditional assumptions, lignin can either competitively bind cellulases, reducing the ability for adsorption on cellulose, or block the access of cellulases to cellulose by forming a physical barrier (Kumar *et al.*, 2012). The last one is the most accepted theory, as is supported by the well-known lignin-holocellulose interaction. It is well established that lignin forms a physical hydrophobic barrier to the holocellulose present on the substrate. Nevertheless, the application of different physical or chemical treatments, very common on 2G processes, has been suggested to be able to decrease this lignin barrier (Barsberg *et al.*, 2013). A different case corresponds to the competitive binding of cellulase to lignin, as whether it may really occur or not, seems to be dependent mainly on the chemical structure of lignin, specifically its hydrophobicity, since lignin-cellulase interactions are mostly hydrophobic (Schmaier *et al.*, 1984; Wang *et al.*, 2015). On the other hand, one recent study suggests that cellulose content on the final solid residue may have an even higher role on enzymes desorption. Following wheat straw hydrolysis, solid-bound cellulases were recovered by applying an alkaline wash (at pH 9) and the remaining activity bound to the solid and liquid fractions were quantified (Rodrigues *et al.*, 2012) (Figure 2.6).

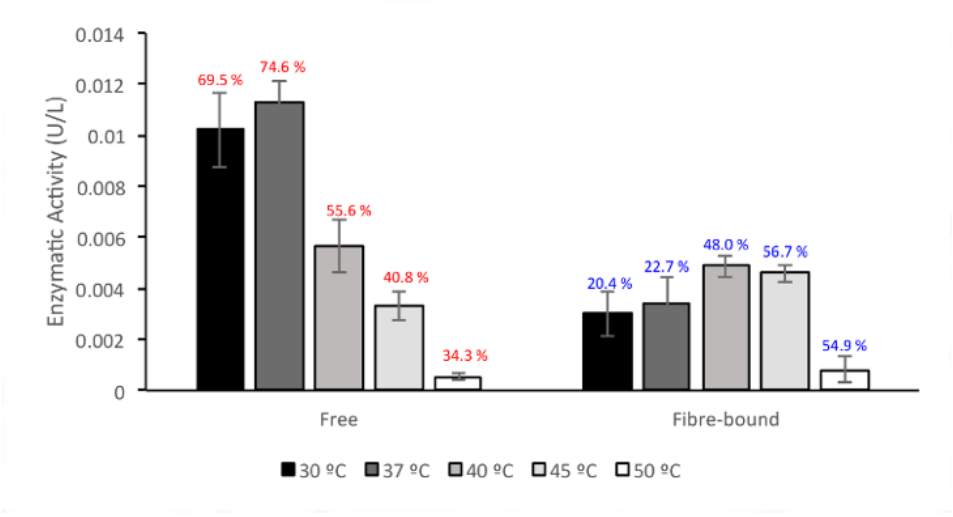

Figure 2.6 Distribution of 4-methylumbelliferyl-β-D-cellobioside (MUC) activity and percentage of enzyme recovered and fibre-bound after an alkaline wash (pH 9) performed over the final solid obtained from the hydrolysis of wheat straw at different temperatures: activity of cellulases released from the solid (Free); activity of cellulases remaining adsorbed to the solid (Fibre-bound) (adapted from Rodrigues *et al.*, 2012).

Interestingly, the percentage of enzyme recovery from the solid was higher for the cases where lower temperatures were employed in the hydrolysis step, such as 30 or 37 °C, rather than higher temperatures, such as 45 or 50 °C (Figure 2.6).

The authors concluded that the lower cellulose conversion that occurred at higher temperatures (a surprising finding that was assigned to the enzyme denaturation at higher temperatures) resulted in a higher amount of residual cellulose, consequently increasing the difficulty to desorb cellulases from the final residue. Indeed, it was also observed that, contrarily to pure lignin-bound cellulases, the cellulose-bound cellulases were not fully desorbed from the final solid when applying an alkaline wash (Rodrigues *et al.*, 2012).

2.4.4 The influence of crystallinity degree of the lignocellulosic substrates

Taking into account how important a complete cellulose conversion seems to be for a proper cellulase recycling, another issue that should be addressed is the substrate crystallinity.

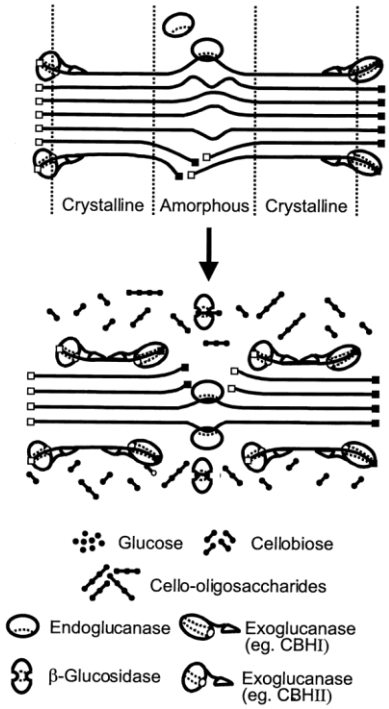

Figure 2.7 Schematic representation of the hydrolysis of different regions on cellulose chains (amorphous and crystalline) by a noncomplex cellulase system (Reprinted from Microbiology and Molecular Biology Reviews, Volume 66, Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS, Microbial Cellulose Utilization: Fundamentals and Biotechnology, pp 506-577, 2012, with permission from American Society for Microbiology).

This property translates the prevalence of crystalline and amorphous domains on cellulose chains as well as the distribution of the different crystalline forms.
Crystalline domains are usually well-structured and organized regions, highly resistant to chemical and enzymatic hydrolysis. These alternate with amorphous regions, which are less ordered and thus, more susceptible to enzymatic or chemical hydrolysis (Adsul *et al.*, 2011) (Figure 2.7). Furthermore, crystalline domains can appear in different forms (allomorphs), with different stabilities and levels of organization. Cellulose I is the most common form found in nature, and thus, on lignocellulosic materials, however Cellulose II is the most resistant form (Segato *et al.*, 2014). Other forms (Cellulose III and IV) can still be obtained when chemical or physical treatments are applied over the main ones. Mittal *et al.* (2011) obtained materials with an improved digestibility by applying a treatment with sodium hydroxide or liquid ammonia and demonstrated that the content of amorphous regions on substrate structure strongly influences its digestibility in the first 24 hours. Furthermore, it was also observed that, while digestibility has a weak correlation with the allomorph type on the first 24 hours, this considerably increases for longer digestion times. These results suggest that the interaction of cellulases with different types of cellulose is not exclusively dependent on the type of cellulose itself, but also relies on other factors such as the current extension of the hydrolysis.

Another important factor refers to the way the degree of crystallinity and the adsorption efficiency are related and to what extent this may affect hydrolysis efficiency. It is a well-known fact that amorphous regions are more easily digested, but the exact reason for that remains unknown. As already observed on several studies, cellulases present a higher adsorption towards less crystalline materials (Klyosov *et al.*, 1986; Lee *et al.*, 1982). This may be partially explained by the fact that different affinities have been reported for a specific CBM towards substrates with distinct crystallinity degrees (McLean *et al.*, 2002). Such assumption was inclusively the base for specific methods employed for measuring crystallinity changes of a material using CBMs (*e.g.* Široký *et al.*, 2012; Gourlay *et al.*, 2012). This would suggest that decreasing crystallinity would increase substrate digestion since cellulase adsorption could be enhanced. However, the results obtained by Hall *et al.* (2010) indicated that this might not be completely true. The authors observed that, reaching a specific enzyme concentration, the substrate

crystallinity continued to influence the initial rate of enzymatic hydrolysis while the amount of enzyme bound to the substrate remained unchanged. This result seems to suggest that the influence of crystallinity on hydrolysis is much broader than its effect on cellulase adsorption.

2.4.5 Recycling strategies

As described above, cellulases can either bind reversibly to the substrate, being posteriorly released to the liquid fraction, or remain adsorbed on the final residue after hydrolysis. An efficient strategy for cellulase recycling will therefore require the recovery of both fractions (Shang *et al.*, 2014).

As mentioned before, the utilization of a particular lignocellulosic substrate and a specific cellulase mixture strongly dictate the distribution of cellulase activity between the solid and liquid fractions. Consequently, these same factors will equally influence the efficiency of cellulase recovery, and ultimately, the adopted method to achieve it.

2.4.5.1 Free cellulases on the liquid fraction

Soluble cellulases on the final hydrolysate have been efficiently recovered using two main methods: i) ultrafiltration of the supernatant collected from the final hydrolysate (Lu *et al.*, 2002; Yang *et al.*, 2010; Qi *et al.*, 2011, 2012; Chen *et al.*, 2013; Rodrigues *et al.*, 2012, 2014); ii) re-adsorption of free cellulases onto fresh substrate (Tu *et al.*, 2007a, 2007b, 2009; Tu and Saddler, 2010; Waeonukul *et al.*, 2013; Ouyang *et al.*, 2013; Eckard *et al.*, 2013; Shang *et al.*, 2014).

Usually, for the first case, the final hydrolysate is initially filtered or centrifuged to separate the solid residue (together with bound cellulases) from the liquid fraction containing free cellulases (Lu *et al.*, 2002). Next, an ultrafiltration unit is employed using a membrane with a cutoff of 10 kDa (Yang *et al.*, 2010; Qi *et al.*, 2011), in order to enable cellulases retention. The final retentate, consisting of cellulases and β-glucosidases, is then added to fresh substrate and buffer to conduct the next hydrolysis round. Using this methodology for three consecutive rounds, Lu *et al.* (2002) observed a decrease on the saccharification efficiency of only 25 % after the 3rd round.

More recently, Chen *et al.* (2013) reported a similar methodology, aiming to enhance ultrafiltration flux through the utilization of electric fields. The authors observed that

the application of an electric field over the membrane caused a decrease on the concentration-polarization, leading consequently to an increased ultrafiltration flux. They observed that the buffer concentration of the hydrolysate, the temperature and the applied current directly affected the strength of the electric field, therefore rising as major determinants on this technology. Using a specific set of conditions, consisting of a current of 150 mA, 5mM buffer concentration and room temperature, an optimum 836 V/m electric field was obtained, which allowed increasing the ultrafiltration flux by a factor of 4.4.

Alternatively, cellulases may also be recovered by simple exposure to fresh substrate, relying on their high capacity to adsorb the solid residue (excepting for β-glucosidases). Fresh substrate (usually the same amount used in the initial round) is added to the free cellulases suspension and the adsorption process is allowed to occur for a period of approximately two hours, under adsorption-promoting conditions (*e.g.* agitation) (Tu *et al.*, 2007b; Ouyang *et al.*, 2013). Afterwards, the overall suspension is either filtered (Shang *et al.*, 2014) or centrifuged (Tu *et al.*, 2009), separating the fresh substrate with bound cellulases from the products of hydrolysis. The solid is finally ressuspended in buffer and supplemented with fresh β-glucosidase, allowing a next round of hydrolysis. The addition of fresh β-glucosidase is a mandatory requirement on this case since, as was described above, they adsorb with a very low efficiency to the solid residue, which hinders their recovery by adsorption with fresh substrate (Lee *et al.*, 1995). Therefore β-glucosidase must be recovered from the liquid phase. With such strategy, Tu *et al.* (2007a) reported a recovery of 88 % of the free cellulases at the end of hydrolysis (51 % of the original load) of mixed softwood. More recently, Shang *et al.* (2014), following the same method, were able to obtain 46.7 % of the glucose yield achieved on the initial round of hydrolysis, suggesting that a significant part of the enzyme was recycled from the liquid. When the hydrolysis efficiencies of these two recovery methods were compared by Qi *et al.* (2011), no significant differences were found. According to the authors, the only difference between these methodologies seems to be the requirement of β-glucosidase supplementation for the case of adsorption into fresh substrate. Although this may constitute a significant economic barrier, the viability and complexity

for an industrial-scale implementation of an ultrafiltration process should also be considered.

Recent efforts have been conducted to surpass the requirement of β-glucosidases for the case of fresh solid adsorption. The utilization of a β-glucosidase secreting yeast by Guo *et al.* (2015) showed to significantly reduce the required levels of supplementation for this enzyme. Following a different approach, Waeonukul *et al.* (2013) were able to efficiently recover β-glucosidase by initially fusing these enzymes with a carbohydrate binding module (CBM3) enabling its binding to the new substrate.

2.4.5.2 Fibre-adsorbed cellulases

Even though most cellulases are found free on liquid fraction at the end of hydrolysis, solid-bound cellulases may also be worth recovering. As already observed in a recent study, this fraction of enzymes is still active and retains its capacity to efficiently adsorb onto fresh substrates (Rodrigues *et al.*, 2012). However, the direct recycling of the final solid with the bound enzyme may not be feasible as it would probably lead to a significant build-up of lignin rich residues that would ultimately have an adverse effect on the hydrolytic ability of the recovered enzyme, in subsequent hydrolysis of fresh substrates (Lee *et al.*, 1995; Tu *et al.*, 2007a, 2007b; Qi *et al.*, 2011). For this reason, and contrasting with the soluble cellulases scenario, the recycling of bound enzymes is complex since it requires desorption from the solid residue, followed by recovery.

Solid-bound cellulases are adsorbed either to residual cellulose or lignin, bearing higher affinity for the former. The interaction with cellulose is driven by specific recognition mediated by the cellulose-binding domains, while the adsorption onto lignin represents an unspecific interaction. The adsorption of proteins in hydrophobic materials, such as lignin, is often associated to denaturation. However, as it has been demonstrated in our group, this is not the case for the cellulase-lignin interaction. Indeed, it was clearly demonstrated that the exposure of an enzyme suspension to 2 % pure-lignin (room temperature, 76 hours) led to no significant alteration in the activity of Cel7A, suggesting therefore that recycling is not compromised by this interaction (Rodrigues *et al.*, 2012). Most desorption methods involve either a pH shift or the addition of chemicals such as alcohols or surfactants, as discussed in the next sections.

2.4.5.3 Effect of pH on the desorption of fibre-bound cellulases

As proteins are composed by amino acids, many of them bearing a side chain with a pH titratable group, their structure, and consequently their interactions with other materials, are strongly influenced by the pH of the medium. The control of pH allows indeed a substantial control over the cellulase adsorption/desorption onto the substrate. Thus, the application of pH shifts becomes an efficient option to desorb bound cellulases.

Early reports by Otter *et al.* (1984; 1989) suggested alkaline wash as a possible method to recover bound cellulases, however, enzymes activity seemed to be affected above specific pH values. Otter *et al.* (1984) observed that Avicelase was significantly desorbed (40-45 %) through an increase on the pH value to 10. A further increase in pH led to an even higher desorption but caused a severe decrease on cellulase activity. Among several methods tested by Zhu *et al.* (2009) for bound-cellulase desorption, a pH shift to an alkaline environment was shown as one of the best options. Increasing the pH from 8 to 13 led to an increase on cellulase desorption efficiency, which reached 85 % and 94 % for Avicel and diluted-acid pre-treated corn stover, respectively; however, no information was provided regarding whether cellulases were able to maintain their activity under such alkaline pH.

In addition to the above-mentioned works, some studies have reported that beyond facilitating cellulase desorption, the alkaline wash also allowed for high cellulase activity recovery. Du *et al.* (2012) reported the maintenance of 97 % of cellulase activity after 2 hours incubation at pH 10. More recently, Shang *et al.* (2014) compared the efficiency of bound-cellulases desorption conducted at different pH values. The amount of desorbed cellulase significantly increased from less than 20 % with an acidic-neutral pH (4.8 and 7) to nearly 85 % with an alkaline pH (10).

Relevant insights into the effects of pH on cellulase structure and stability were provided in the studies by Rodrigues *et al.* (2012). In addition to the fact that an alkaline wash (pH 9 or 10) allowed a considerable desorption of bound cellulases, analysis by Intrinsic Tryptophan Fluorescence (ITF) and Circular Dichroism (CD) revealed significant conformational changes in the structure of Cel7A when the pH was altered from 4.8 to 9 or 10, which were reversed when pH was changed back to 4.8. Furthermore, the

authors also observed that no loss of MUC (4-methylumbelliferyl-β-D-cellobioside) activity arose from the pH alteration.

2.4.5.4 Addition of chemicals

Non-productive and irreversible adsorption of cellulases on lignin residues remains nowadays as one of the mains barriers to an efficient saccharification (Seo *et al.*, 2011). Therefore, a decrease of lignin interference has been intensively pursued either by decreasing its content on the initial lignocellulosic material, applying suitable pre-treatments (Sipponen *et al.*, 2014; Pan *et al.*, 2005), or by trying to control the adsorption and desorption of cellulases. Additionally, as was previously referred here and clearly demonstrated by a previous study, cellulose also represents an important barrier for the recovery of the enzymes, as the affinity of cellulases for cellulose is even higher than for lignin (Rodrigues *et al.*, 2012). Here, the utilization of some types of chemicals showed to significantly decrease the binding of cellulases to both lignin and cellulose, with the consequent improvement of both hydrolysis and cellulases recovery. Otter *et al.* (1989) observed that, among several detergents tested, with exception of sodium dodecyl sulfate (SDS), all caused an increase on Avicelase desorption from Avicel. Tween 80 was found to be the best option, enabling a 67 % enzyme desorption, which supports its wide application in several desorption protocols (*e.g.* Pribowo *et al.*, 2012; Tu *et al.*, 2009). More recently Tu *et al.* (2007b) reported that the utilization of Tween 80 led to a significant increase in the amount of total free enzyme during hydrolysis of EPLP (ethanol pretreated Lodgepole pine) and SELP (steam exploded Lodgepole pine). The authors have also observed that the utilization of 0.2 % (w/v) Tween80 enabled an increase in the fraction of protein released at the end of hydrolysis, from 71 % to 96 % and 46 % to 73 %, for EPLP and SELP, respectively. Furthermore, the application of Triton X-100, Tween 80 or Tween 20 improved the efficiency of a single round of cellulase recycling, using EPLP, by 50 %, while a negative effect was verified for SDS. According to Eriksson *et al.* (2002), it is possible that surfactants (*e.g.* Tween) may compete with cellulases for adsorption sites on lignin-rich residues.

Zhu *et al.* (2009) have also explored a wide range of compounds for this purpose: NaCl; ethylene glycol; glycerol; Tween 80; Triton X-100; sodium dodecyl sulfate. Polyhydric alcohols (ethylene glycol and glycerol) were found to be more efficient in cellulase

desorption compared to surfactants (*e.g.* Tweens, Triton X-100), both from Avicel and diluted-acid pre-treated corn stover, the utilization of 72 % ethylene glycol enabling a 76 % recovery of adsorbed cellulase from pre-treated corn stover. Sipos *et al.* (2010) were also able to increase the recovery of cellulase activity, after hydrolysis of steam-pretreated spruce, when polyethylene glycol was supplemented to the hydrolysis medium. More recently, Eckard *et al.* (2013) have observed that also casein micelles could work as lignin blockers, increasing glucose and ethanol yield by up to 32 % and 34 %, respectively, as well as the final cellulases recycling.

2.4.6 The key relevance of temperature

Temperature is a major determinant of cellulases recycling efficiency as it is related with two critical aspects of this process: the maintenance of good levels of enzyme activity during extensive periods of hydrolysis and its effect on the desorption of solid-bound enzymes.

Several studies have been conducted addressing cellulases stability after exposure to high temperatures. Rosales-Calderon *et al.* (2014) reported that an incubation of cellulases (Celluclast+Novozym 188) for 78 hours at 50 °C caused a decrease by 30 to 45 % (depending on the initial amount of enzyme) on the protein concentration in suspension (suggesting denaturation of proteins). According to Tu *et al.* (2009), the cellulase desorption increased when temperature raised from 25 °C to 45 °C (due to a shift in the thermodynamic equilibrium position), but dropped rapidly in the range of 50 °C to 75 °C (likely due to enzyme denaturation). Also Shang *et al.* (2014) observed higher desorption efficiencies for lower temperatures (4-37 °C), while temperatures above 50 °C rapidly decreased desorption. On a recent study, Lindedam *et al.* (2013) observed that, for a short-period hydrolysis (6 hours), the utilization of a temperature of 40 °C or 50 °C did not significantly compromise the recovery of cellulase activity. However, following incubation for a longer period of 96 hours, cellulase recovery at 50 °C was significantly hampered.

Although higher temperatures may favor a faster reaction rate, it also leads to faster denaturation. Thus, as often observed in enzymology, a long stand enzyme activity (and therefore its recycling) may be achieved by using a moderate temperature that does not

compromise its stability. Such fact was widely demonstrated in some recent studies (Rodrigues *et al.*, 2012, 2014).

Rodrigues *et al.* (2012) observed that Cel7A, the most abundant component on *T. reesei* cellulase cocktails (Pakarinen *et al.*, 2014), did not loose any MUC activity at 30 °C, 37 °C and 40°C over a period of 168 hours, but a considerable decrease occurred for temperatures above 45 °C: only 37.5 % of the original activity was preserved at 50 °C, as compared with 89.7 % for a temperature of 45 °C. Also, the amount of active cellulases bound to the final solid, as suggested by MUC activity measurements, was found to be higher at lower temperatures (30 °C, 37 °C), suggesting that a lower thermal denaturation of cellulases occurred. In a more recent study (Rodrigues *et al.*, 2014) the evolution of enzymes activity was evaluated for three consecutive runs of hydrolysis and fermentation conducted both at 37 °C and 50 °C (Figure 2.8).

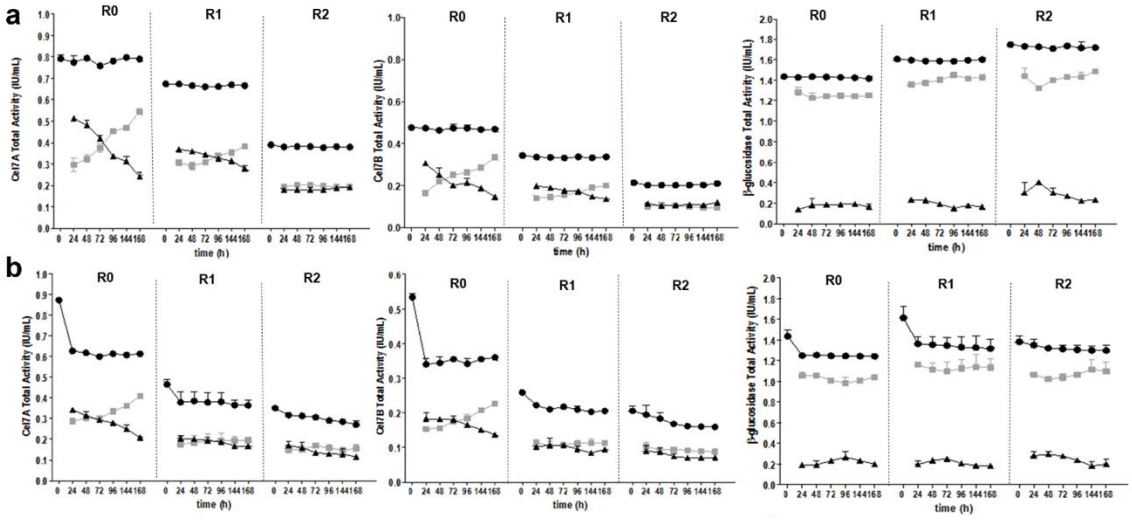

Figure 2.8 Evolution of Cel7A, Cel7B and β-glucosidase activities in solid and liquid fraction during wheat straw hydrolysis and fermentation at (a) 37 °C and (b) 50 °C using 20 FPU/g cellulose: (●) total activity; (■) activity in the liquid fraction; (▲) activity in the solid fraction. R0, R1 and R2 refers to the initial step of hydrolysis and to the 1st and 2nd rounds of enzyme recycling, respectively (Reprinted from Bioresource Technology, Volume 156, Rodrigues AC, Felby C, Gama M, Cellulase stability, adsorption/desorption profiles and recycling during successive cycles of hydrolysis and fermentation of wheat straw, pp 163-169, 2014, with permission from Elsevier).

For a temperature of 37 °C, no considerable changes were observed in the activity of Cel7A, Cel7B and β-glucosidase on each separate run of hydrolysis, although a notorious

reduction could be observed between the different rounds. On the other hand, when a temperature of 50 °C was employed, a clear reduction was observed for all enzyme activities, during the three consecutive rounds. Such reduction was specially observed on the initial 24 hours of each run, and more significantly on the initial one. Considering the particular case of Cel7A and for a temperature of 37 °C, the activity remained constant around 0.8 IU/mL, while for 50 °C the enzymatic activity decreased from approximately 0.87 to 0.62 IU/mL after 24 hours. Following these initial 24 hours, the activity levels continued to decrease, although at a considerably lower rate. This temperature effect is also patent on the efficiencies of enzyme recovery (Table 2.3).

Table 2.3 Activity recovered (% of original load) in each round after an ultrafiltration step compared to the activity recovered in the liquid after fermentation (adapted from Rodrigues *et al.* 2014). R0, R1 and R2 refers to the initial step of hydrolysis and to the 1st and 2nd rounds of enzyme recycling, respectively

Enzyme	Round	37 °C	50 °C
Cel7A	0	55	33
	1	46	28
	2	38	39
Cel7B	0	54	31
	1	47	31
	2	35	39
β-glucosidase	0	77	61
	1	77	67
	2	71	75

As for the levels of enzyme activity, a significant difference was observed for the percentage of cellulase recovery when different temperatures were utilized. However, these differences decreased from the initial to the last round of hydrolysis rendering similar recovery efficiencies on the last round.

In addition to the clear effect of temperature on the maintenance of enzyme activity during the entire process of enzymatic hydrolysis, it should be noted that it can also

directly affect cellulase recycling from the final solid, by influencing the extent of hydrolysis, and consequently the final solid composition (Rodrigues *et al.*, 2012), as already discussed on a previous section. The higher the amount of residual cellulose, the more difficult the recovery of the enzymes will be.

Even with the evident benefits of operating at moderate temperatures (approx. 37 °C), this may not be viable on an industrial scale due to an increased risk of microbial contaminations (Lindedam *et al.*, 2013). Such limitation, together with a wide range of potential advantages as consequence of increasing operating temperature, has recently driven considerable efforts on the development of more thermostable cellulases (Viikari *et al.*, 2007), which will most likely introduce significant improvements on lignocellulosic ethanol.

2.4.7 All enzymes recovery

In addition to the above mentioned strategies, referring to the recovery of either the solid or the liquid fraction, some approaches aim instead the recycling of all enzymes. The simplest way to achieve that is by recycling a fraction of the whole slurry, either after hydrolysis or fermentation. Processually is easy to implement since it only requires pumping part of a process stream (final hydrolyzate or fermentation broth) back into the process with no need for a separation step, enabling its application even with high solids loadings (Jørgensen and Pinelo, 2017). On the other hand, this only enables to recover a fraction of the enzymes, depending on the applied recirculation ratio. Such approach has been recently tested by DONG Energy on a demonstration scale plant with 20 % recirculation (Haven *et al.*, 2015). Employing industrial relevant conditions (20 % initial solids), it was possibe to achieve a 5 % reduction on enzyme consumption. Furthermore, as a fraction of the ethanol produced is also recycled, the authors suggested that initial solid loadings can be reduced without compromising the obtention of high ethanol titres.

In a different approach, enzyme immobilization has been studied as a possible method to reutilize enzymes for several process rounds. Different classes of immobilization supports have been used, which dictate distinct recovery methods. The most common refers to different types of magnetic particles (Jordan *et al.*, 2011; Khoshnevisan *et al.*, 2011; Yuan *et al.*, 2016; Zhang *et al.*, 2015) enabling their recover by simple application

of an electromagnetic field. A common drawback is that immobilized enzymes may suffer in some cases a reduction on their activity to some extent, as reported by Alftrén and Hobley (2014). On the other hand, also positive effects have been observed, possibly as a result of a protection provided by the immobilization. According to Abraham *et al.* (2014), immobilized enzymes presented a higher thermostability at 80 ºC comparatively with the free enzymes. Furthermore, on the hydrolysis of pre-treated hemp hurd biomass a maximum conversion of 93 % was achieved in 48 hours with the immobilized enzyme, reaching 89 % with the free enzymes. Also according to the authors, after 5 consecutive hydrolysis cycles immobilized enzymes still retained 50 % of the inital activity. Similarly, also Qi *et al.* (2015) reported an improvement on cellulase activity when enzymes were immobilized on a magnetic porous terpolymer, where residual activity after 5 rounds was also kept above 50 %.

More recently, a new approach has been reported consisting on the utilization of temperature-responsive compounds that could enable the recovery of cellulases (Mackenzie and Francis, 2013; Limadinata *et al.*, 2015). Using a zwitterionic surfactant, Cai *et al.* (2017) was able to not only reduce the non-productive binding of cellulases but also to directly recover a considerable fraction of enzymes (55.2 %) by simply reducing the temperature after the process, through a co-precipitation mechanism. Following an opposite strategy, Ding *et al.* (2016) initially created a bioconjugate merging the cellulases with a thermo-responsive polymer presenting a low critical solution temperature (LCST) close to 52ºC. After hydrolysis, it was possible to directly recover the bioconjugate (and consequently the enzyme) by precipitation following an increase on temperature to 55ºC. According to the authors, 85.2 % of the bioconjugate initial activity was maintained after 5 repeated cycles of hydrolsyis and recovery.

2.5 References

Abraham RE, Verma ML, Barrow CJ, Puri M. 2014. Suitability of magnetic nanoparticle immobilised cellulases in enhancing enzymatic saccharification of pretreated hemp biomass. *Biotechnol. Biofuels* 7:90.

Aden A, Foust T. 2009. Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. *Cellulose* 16:535-45.

Aditiya HB, Mahlia TMI, Chong WT, Nur H, Sebayang AH. 2016. Second generation bioethanol production: A critical review. *Renew. Sust. Energy Rev.* 66:631-53.

Adsul MG, Singhvi MS, Gaikaiwari SA, Gokhale DV. 2011. Development of biocatalysts for production of commodity chemicals from lignocellulosic biomass. *Bioresour. Technol.* 102(6):4304-12.

Agarwal AK. 2007. Biofuels (alcohols and biodiesel) applications as fuels for internal combustion engines. *Prog. Energy Combust. Sci.* 33:233-71.

Alfani A, Gallifuoco F, Saporosi A, Spera A, Cantarella M. 2000. Comparison of SHF and SSF process for the bioconversion of steam-exploded wheat straw. *J. Ind. Microb. Biotechnol.* 25:184-92.

Alftrén J, Hobley TJ. 2014. Immobilization of cellulase mixtures on magnetic particles for hydrolysis of lignocellulose and ease of recycling. *Biomass Bioenergy* 65:72-8.

Almeida JR, Modig T, Petersson A, Hähn-Hägerdal B, Lidén G, Gorwa-Grauslund MF. 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *J. Chem. Technol. Biotechnol.* 82:340-49.

Almeida JRM, Bertilsson M, Gorwa-Grauslund MF, Gorsich S, Lidén G. 2009. Metabolic effects of furaldehydes and impacts on biotechnological process. *Appl. Microbiol. Biotechnol.* 82:625-38.

Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresour. Technol.* 101:4851-61.

Arantes V, Saddler JN. 2010. Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. *Biotechnol. Biofuels* 3:4.

Bajpai P. 2015. Generation of Waste in Pulp and Paper Mills, In: Bajpai P (Eds.), Management of Pulp and Paper Mill Waste, Springer, Cham, Switzerland, pp. 9-17.

Balat M. 2011. Production of bioethanol from lignocellulosic materials via the biochemical pathway: a review. *Energy Convers. Manag.* 52:858-75.

Bals BD, Teymouri F, Campbell T, Jin M, Dale BE. 2012. Low temperature and long residence time AFEX pretreatment of corn stover. *Bioenergy Res.* 5:372-79.

Balwaik SA, Raut SP. 2011. Utilization of waste paper pulp by partial replacement of cement in concrete. *Int. J. Eng. Res. Appl.* 1(2):300-9.

Barsberg S, Selig MJ, Felby C. 2013. Impact of lignins isolated from pretreated lignocelluloses on enzymatic cellulose saccharification. *Biotechnol. Lett.* 35(2):189-95.

Bommarius AS, Katona A, Cheben SE, Patel AS, Ragauskas AJ, Knudson K, Pu Y. 2008. Cellulase kinetics as a function of cellulose pretreatment. *Metab. Eng.* 6:370-81.

Boraston A, Bray M, Burn E, Creagh AL, Gilkes N, Guarna M, Jervis E, Johnson P, Kormos J, McIntosh L, McLean B, Sandercock L, Tomme P, Haynes C, Warren A, Kilburn D. 1998. The structure and function of cellulose binding domains, In: Claeyssen M, Nerinkx W, Piens K (Eds.), Carbohydrate from *Trichoderma reesei* and other microorganisms, The Royal Society of Chemistry, Cambridge, United Kingdom, pp. 139-46.

Boshoff S, Gottumukkala LD, van Rensburg E, Görgens J. 2016. Paper sludge (PS) to bioethanol: Evaluation of virgin and recycle mill sludge for low enzyme, high-solids fermentation. *Bioresour. Technol.* 203:103-11.

Budhavaram NK, Fan Z. 2009. Production of lactic acid from paper sludge using acid-tolerant, thermophilic *Bacillus coagulan* strains. *Bioresour. Technol.* 100:5966-72.

Cai C, Pang Y, Zhan X, Zeng M, Lou H, Qian Y, Yang D, Qiu X. 2017. Using temperature-responsive zwitterionic surfactant to enhance the enzymatic hydrolysis of lignocelluloses and recover cellulase by cooling. *Bioresour. Technol.* 243:1141-8.

Çakar ZP, Turanli-Yildiz B, Alkim C, Yilmaz Ü. 2012. Evolutionary engineering of *Saccharomyces cerevisiae* for improved industrially important properties. *FEMS Yeast Res.* 12:171-82.

CANMET Energy Technology Centre. 2005. Pulp and Paper Sludge to Energy – Preliminary Assessment of Technologies. Natural Resources Canada.

Cavka A, Jönsson LJ. 2013. Detoxification of lignocellulosic hydrolysates using sodium borohydride. *Bioresour. Technol.* 136:368-76.

Chen G, Song W, Qi B, Lu J, Wan Y. 2013. Recycling cellulase from enzymatic hydrolyzate of acid treated wheat straw by electroultrafiltration. *Bioresour. Technol.* 144:186-93.

Chen H, Fu X. 2016. Industrial technologies for bioethanol production from lignocellulosic biomass. *Renew. Sust. Energy Rev.* 57:468-78.

Chen H, Han Q, Daniel K, Venditti R, Jameel H. 2014. Conversion of industrial paper sludge to ethanol: fractionation of sludge and its impact. *Appl. Biochem. Biotechnol.* 174:2096-2113.

Correia JAC, Júnior JEM, Gonçalves LRB, Rocha MVP. 2013. Alkaline hydrogen peroxide pretreatment of cashew apple bagasse for ethanol production: study of parameters. *Bioresour. Technol.* 139:249-56.

Costa CE, Romaní A, Cunha JT, Johansson B, Domingues L. 2017. Integrated approach for selecting efficient *Saccharomyces cerevisiae* for industrial lignocellulosic fermentations: Importance of yeast chassis linked to process conditions. *Bioresour. Technol.* 227:24-34.

Cunha JT, Aguiar TQ, Romaní A, Oliveira C, Domingues L. 2015. Contribution of *PRS3*, *RPB4* and *ZWF1* to the resistance of industrial *Saccharomyces cerevisiae* CCUG53310 and PE-2 strains to lignocellulosic hydrolysate-derived inhibitors. *Bioresour. Technol.* 191:7-16.

Decker CH, Visser J, Schreier P. 2000. β−glucosidases from five black Aspergillus species: study of their physico-chemical and biocatalytic properties. *J. Agric. Food Chem.* 48:4929-36.

Demirbas A, 2008. Products from Lignocellulosic Materials via Degradation Process. *Energy Sourc. A, Recovery Util. Environ. Effects* 30(1):27-37.

Desphande MV, Erikson K-E. 1984. Reutilization of enzymes for saccharification of lignocellulosic materials. *Enzyme Microb. Technol.* 6:338-40.

Ding Z, Zheng X, Li S, Cao X. 2016. Immobilization of cellulase onto a recyclable thermo-responsive polymer as bioconjugate. *J. Mol. Catal., B Enzym.* 128:39-45.

Domke SB, Aiello-Mazzarri C, Holtzapple MT. 2004. Mixed acid fermentation of paper fines and industrial biosludge. *Bioresour. Technol.* 91:41-51.

Dragone G, Fernandes B, Vicente AA, Teixeira JA. 2010. Third generation biofuels from microalgae, In: Mendez-Vilas A (Eds.), Current research, technology and education topics in applied microbiology and microbial biotechnology. Formatex, Madrid, Spain, pp. 1355-66.

Du R, Su R, Li X, Tantai X, Liu Z, Yang J, Qi W, He Z. 2012. Controlled adsorption of cellulase onto pretreated corncob by pH adjustment. *Cellulose* 19:371-80.

Dwiarti L, Boonchird C, Harashima S, Park EY. 2012. Simultaneous saccharification and fermentation of paper sludge without pretreatment using cellulase from *Acremonium cellulolyticus* and thermotolerant *Saccharomyces cerevisiae*. *Biomass Bioenergy* 42:114-22.

Eckard AD, Muthukumarappan K, Gibbons W. 2013. Enhanced bioethanol production from pretreated corn stover via multi-positive effect of casein micelles. *Bioresour. Technol.* 135:93-102.

Eksteen JM, Van Rensburg P, Cordero Otero RR, Pretorius IS. 2003. Starch fermentation by recombinant *Saccharomyces cerevisiae* strains expressing the α-amylase and glucoamylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*. *Biotechnol. Bioeng.* 84:639-46.

Eriksson T, Börjesson J, Tjerneld F. 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. *Enzyme Microb. Technol.* 31:353-64.

European Parliament, Council of the European Union. 2009. Directive 2009/28/EC of the European Parliament and of the Council of 23 April 2009 on the promotion of the use of energy from renewable sources and amending and subsequently repealing Directives 2001/77/EC and 2003/30/EC. *Off. J. Eur. Union* 52:16-63.

European Parliament, Council of the European Union. 2009. Directive 2009/30/EC of the European Parliament and of the Council of 23 April 2009 amending Directive 98/70/EC as regards the specification of petrol, diesel and gas-oil and introducing a mechanism to monitor and reduce greenhouse gas emissions and amending Council Directive 1999/32/EC as regards the specification of fuel used by inland waterway vessels and repealing Directive 93/12/EEC. *Off. J. Eur. Union* 52:88-114.

Fan Z, Lynd LR. 2007. Conversion of paper sludge to ethanol. I: impact of feeding frequency and mixing energy characterization. *Bioprocess Biosyst. Eng.* 30(1):27-34.

FAOSTAT (Food and Agriculture Organization of the United Nations – Statistics Division). 2015. Domain Forestry, http://faostat3.fao.org/download/F/FO/E (accessed 15.06.15).

Faubert P, Barnabé S, Bouchard S, Côté R, Villeneuve C. 2016. Pulp and paper mill sludge

management practices: What are the challenges to assess the impacts on greenhouse gas emissions? *Resour. Conserv. Recycl.* 108:107-33.

Fujita Y, Ito J, Ueda M, Fukuda H, Kondo A. 2004. Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. *Appl. Environ. Microbiol.* 70:1207-12.

Gilkes NR, Henrissat B, Kilburn DG, Miller RC Jr, Warren RAJ. 1991. Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* 55:303-15.

Gottumukkala LD, Haigh K, Collard F-X, van Rensburg E, Görgens J. 2016. Opportunities and prospects of biorefinery-based valorisation of pulp and paper sludge. *Bioresour. Technol.* 215:37-49.

Gourlay K, Arantes V, Saddler JN. 2012. Use of substructure-specific carbohydrate binding modules to track changes in cellulose accessibility and surface morphology during the amorphogenesis step of enzymatic hydrolysis. *Biotechnol. Biofuels* 5:51.

Gouvea B, Torres C, Franca A, Oliveira L, Oliveira E. 2009. Feasibility of ethanol production from coffee husks. *Biotechnol. Lett.* 31:1315-9.

Guo H, Zou S, Liu B, Su R, Huang R, Qi W, Zhang M, He Z. 2015. Reducing β-glucosidase supplementation during cellulase recovery using engineered strain for successive lignocellulose bioconversion. *Bioresour. Technol.* 187:362-68.

Gurram RN, Al-Shannag M, Lecher NJ, Duncan SM, Singsaas EL, Alkasrawi M. 2015. Bioconversion of paper mill sludge to bioethanol in the presence of accelerants or hydrogen peroxide pretreatment. *Bioresour. Technol.* 192:529-39.

Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Lidén G, Zacchi G. 2006. *Trends Biotechnol.* 24(12):549-56.

Hahn-Hägerdal B, Pamment N. 2004. Special session A microbial pentose metabolism. *Appl. Biochem. Biotechnol.* 116:1207-9.

Hall M, Bansal P, Lee JH, Realff MJ, Bommarius AS. 2010. Cellulose crystallinity - a key predictor of the enzymatic hydrolysis rate. *FEBS J.* 277:1571-82.

Hasunuma T, Ishii J, Kondo A. 2015. Rational design and evolutional fine tuning of *Saccharomyces cerevisiae* for biomass breakdown. *Curr. Opin. Chem. Biol.* 29:1-9.

Hasunuma T, Kondo A. 2011. Development of yeast cell factories for consolidated bioprocessing of lignocellulose to bioethanol through cell surface engineering. *Biotechnol. Adv.* 30(6):1207-18.

Haven MØ, Lindedam J, Jeppesen MD, Elleskov M, Rodrigues AC, Gama M, Jørgensen H, Felby C. 2015. Continuous recycling of enzymes during production of lignocellulosic bioethanol in demonstration scale. *Appl. Energy* 159:188-95.

Hawkins GM, Doran-Peterson JD. 2011. A strain of *Saccharomyces cerevisiae* evolved for fermentation of lignocellulosic biomass displays improved growth and fermentative ability in high solids concentrations and in the presence of inhibitory compounds. *Biotechnol. Biofuels* 4:49.

Hayes DJM. 2013. Second-generation biofuels: why they are taking so long. *WIREs Energy Environ.* 2:304-34.

Haynes RD, Banerjee S, Mora S. 2013. Application of enzyme accelerant technology for cellulosic sludge in pulp and paper mills. *J-FOR* 3:42-9.

Himmel ME, Ding S, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD. 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* 5813:804-7.

Holtzapple M, Cognata M, Shu Y, Hendrickson C. 1990. Inhibition of *Trichoderma reesei* cellulase by sugars and solvents. *Biotechnol. Bioeng.* 36:275-87.

Horn SJ, Vaaje-Kolstad G, Westereng B, Eijsink VG. 2012. Novel enzymes for the degradation of cellulose. *Biotechnol. Biofuels* 5(1):45.

Howard R, Abotsi E, Van Rensburg EJ, Howard S. 2004. Lignocellulose biotechnology: issues of bioconversion and enzyme production. *Afr. J. Biotechnol.* 2:602-19.

Hsieh CW, Cannella D, Jørgensen H, Felby C, Thygesen LG. 2014. Cellulase inhibition by high concentrations of monosaccharides. *J. Agric. Food Chem.* 62:3800-5.

International Energy Outlook. 2016. Report Number: DOE/EIA-0484.

Ioelovich M. 2014. Waste paper as promising feedstock for production of biofuel. *J. Sci. Res. Rep.* 3:905-16.

IPPC (Integrated Pollution Prevention and Control). 2001. Reference Document on Best Available Techniques in the Pulp and Paper Industry. European Commission, 475.

Ishihara M, Uemura S, Hayashi N, Jellison J, Shimizu K. 1991. Adsorption and Desorption of Cellulase Components during Enzymatic Hydrolysis of Steamed Shirakamba (*Betula platyphylla Skatchev*) Wood. *J. Ferment. Bioeng.* 72:96-100.

Isikgor FH, Becer CR. 2015. Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. *Polym. Chem.* 6:4497.

Jordan J, Kumar CSSR, Theegala C. 2011. Preparation and characterization of cellulase-bound magnetite nanoparticles. *J. Mol. Catal., B Enzym.* 68:139-46.

Jorgensen H, Eriksson T, Borjesson J, Tjerneld F, Olsson L. 2003. Purification and characterization of five cellulases and one xylanase from *Penicillium brasilianum* IBT 20888. *Enzyme Microb. Technol.* 32:851-861.

Jørgensen H, Kristensen JB, Felby C. 2007. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels Bioprod. Bior.* 1:119-34.

Jørgensen H, Pinelo M. 2017. Enzyme recycling in lignocellulosic biorefineries. *Biofuels Bioprod. Bior.* 11:150-67.

Kádár Z, Szengyel Z, Réczey K. 2004. Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. *Ind. Crops Prod.* 20:103-10.

Kang L, Wang W, Lee YY. 2010. Bioconversion of Kraft Paper Mill Sludges to Ethanol by SSF and SSCF. *Appl. Biochem. Biotechnol.* 161:53-66.

Karmee SK. 2018. A spent coffee grounds based biorefinery for the production of biofuels,

biopolymers, antioxidants and biocomposites. *Waste Manag.* 72:240-54.

Khoshnevisana K, Bordbarb A-K, Zarec D, Davoodic D, Noruzic M, Barkhic M, Tabatabaeid M. 2011. Immobilization of cellulase enzyme on superparamagnetic nanoparticles and determination of its activity and stability. *Chem. Eng. J.* 171:669-73.

Kim M, Day DF. 2011. Composition of sugar cane, energy cane, and sweet sorghum suitable for ethanol production at Louisiana sugar mills. *J. Ind. Microbiol. Biotechnol.* 38:803-7.

Klein-Marcusschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch WH. 2012. The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnol. Bioeng.* 109:1083-89.

Klyosov AA, Mitkevich OV, Sinitsyn AP. 1986. Role of the activity and adsorption of cellulases in the efficiency of the enzymatic hydrolysis of amorphous and crystalline cellulose. *Biochemistry* 25(3):540-2.

Koppram R, Thomás-Péjo E, Xiros C, Olsson L. 2014. Lignocellulosic ethanol production at high-gravity: challenges and perspectives. *Trends Biotechnol.* 32:46-52.

Kötter P, Amore R, Hollenberg CP, Ciriacy M. 1990. Isolation and characterization of the *Pichia stipitis* xylitol dehydrogenase gene, XYL2, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant. *Curr. Genet.* 18:493-500.

Kuhad RC, Deswal D, Sharma S, Bhattacharya A, Jain KK, Kaur A, Pletschke BI, Singh A, Karp M. 2016. Revisiting cellulase production and redefining current strategies based on major challenges. *Renew. Sust. Energy Rev.* 55:249-72.

Kumar D, Murthy GS. 2011. Impact of pretreatment and downstream processing technologies on economics and energy in cellulosic ethanol production. *Biotechnol. Biofuels* 4:27.

Kumar D, Murthy GS. 2013. Stochastic molecular model of enzymatic hydrolysis of cellulose for ethanol production. *Biotechnol. Biofuels* 6(1):63.

Kumar L, Arantes V, Chandra R, Saddler J. 2012. The lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. *Bioresour. Technol.* 103:201-8.

Kumar R, Singh S, Singh OV. 2008. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J. Ind. Microbiol. Biotechnol. 35(5):377-91.

Lark N, Xia Y, Qin C-G, Gong CS, Tsao GT. 1997. Production of ethanol from recycled paper sludge using cellulase and yeast, *Kluveromyces marxianus*. *Biomass Bioenergy* 12(2):135-143.

Lee D, Yu AHC, Saddler JN. 1995. Evaluation of cellulase recycling strategies for the hydrolysis of lignocellulosic substrates. *Biotechnol. Bioeng.* 45:328-36.

Lee RA, Lavoie JM. 2013. From first-to-third-generation biofuels: Challenges of producing a commodity from a biomass of increasing complexicity. *Animal Frontiers* 3(2):6-11.

Lee SB, Shin HS, Ryu DD, Mandels M. 1982. Adsorption of cellulase on cellulose: Effect of physicochemical properties of cellulose on adsorption and rate of hydrolysis. *Biotechnol. Bioeng.* 24(10):2137-53.

Lee SM, Koo YM, Lin J. 2004. Production of lactic acid from paper sludge by simultaneous saccharification and fermentation. *Adv. Biochem. Eng. Biotechnol.* 87:173-94.

Li S, Zhang X, Andresen JM. 2012. Production of fermentable sugars from enzymatic hydrolysis of pretreated municipal solid waste after autoclave process. *Fuel* 92:84-8.

Limadinata PA, Li A, Li Z. 2015. Temperature-responsive nanobiocatalysts with an upper critical solution temperature for high performance biotransformation and easy catalyst recycling: efficient hydrolysis of cellulose to glucose. *Green Chem.* 17:1194-1203.

Lin Y, Wang D, Wang T. 2012. Ethanol production from pulp & paper sludge and monosodium glutamate waste liquor by simultaneous saccharification and fermentation in batch condition. *Chem. Eng. J.* 191:31-7.

Lindedam J, Haven M, Chylenski P, Jørgensen H, Felby C. 2013. Recycling cellulases for cellulosic ethanol production at industrial relevant conditions: potential and temperature dependency at high solid processes. *Bioresour. Technol.* 148:180-8.

Lu YP, Yang B, Gregg D, Saddler JN, Mansfield SD. 2002. Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues. *Appl. Biochem. Biotechnol.* 98:641-654.

Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. 2002. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* 66(3):506-77.

Mackenzie KJ, Francis MB. 2013. Recyclable Thermoresponsive Polymer−Cellulase Bioconjugates for Biomass Depolymerization. *J. Am. Chem. Soc.* 135:293-300.

Maheshwari R, Bharadwaj G, Bhat MK. 2000. Thermophilic fungi: their physiology and enzymes. *Microbiol. Mol. Biol. Rev.* 64(3):461-88.

Marques S, Alves L, Roseiro JC, Gírio FM. 2008a. Conversion of recycled paper sludge to ethanol by SHF and SSF using *Pichia stipites*. *Biomass Bioenergy* 32:400-6.

Marques S, Gírio FM, Santos JAL, Roseiro JC. 2017. Pulsed fed-batch strategy towards intensified process for lactic acid production using recycled paper sludge. *Biomass Convers. Biorefin.* 7:127-37.

Marques S, Santos JAL, Gírio FM, Roseiro JC. 2008b. Lactic acid production from recycled paper sludge by simultaneous saccharification and fermentation. *Biochem. Eng. J.* 41:210-16.

Mata TM, Martins AA, Caetano NS. 2018. Bio-refinery approach for spent coffee grounds valorization. *Bioresour. Technol.* 247:1077-84.

Matano Y, Hasunuma T, Kondo A. 2012. Display of cellulases on the cell surface of *Saccharomyces cerevisiae* for high yield ethanol production from high-solid lignocellulosic biomass. *Bioresour. Technol.* 108:128-33.

Matano Y, Hasunuma T, Kondo A. 2013. Cell recycle batch fermentation of high-solid lignocellulose using a recombinant cellulase-displaying yeast strain for high yield ethanol production in consolidated bioprocessing. *Bioresour. Technol.* 135:403-9.

Maurya DP, Singla A, Negi S. 2015. An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol. *3 Biotech* 5(5):597-609.

McKendry P. 2002. Energy production from biomass (part 1): overview of biomass. *Bioresour. Technol.* 83:37-46.

McLean BW, Boraston AB, Brouwer D, Sanaie N, Fyfe CA, Warren RA, Kilburn DG, Haynes CA. 2002. Carbohydrate-binding modules recognize fine substructures of cellulose. *J. Biol. Chem.* 277:50245-54.

Mendes CVT, Cruz CHG, Reis DFN, Carvalho MGVS, Rocha JMS. 2016. Integrated bioconversion of pulp and paper primary sludge to second generation bioethanol using *Saccharomyces cerevisiae* ATCC 26602. *Bioresour. Technol.* 220:161-7.

Min BC, Bhayani BV, Jampana VS, Ramarao BV. 2015. Enhancement of the enzymatic hydrolysis of fines from recycled paper mill waste rejects. *Bioresour. Bioprocess.* 2:40.

Mittal A, Katahira R, Himmel ME, Johnson DK. 2011. Effects of alkaline or liquid-ammonia treatment on crystalline cellulose: changes in crystalline structure and effects on enzymatic digestibility. *Biotechnol. Biofuels* 4:41.

Monte MC, Fuente E, Blanco A, Negro C. 2009. Waste management from pulp and paper production in the European Union. *Waste Manag.* 29(1):293-308.

Mood SH, Hossein Golfeshan A, Tabatabaei M, Salehi Jouzani G, Najafi GH, Gholami M, Ardjmand M. 2013. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. *Renew. Sust. Energy Rev.* 27:77-93.

Moreau A, Montplaisir D, Sparling R, Barnabe S. 2015. Hydrogen, ethanol and cellulase production from pulp and paper primary sludge by fermentation with *Clostridium thermocellum*. *Biomass Bioenergy* 72:256-62.

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* 96:673-86.

Nakamura Y, Sawada T, Inoue E. 2001. Mathematical model for ethanol production from mixed sugars by *Pichia stipitis*. *J. Chem. Technol. Biotechnol.* 76:586-92.

Ogbonna JC, Mashima H, Tanaka H. 2001. Scale up of fuel ethanol production from sugar beet juice using loofa sponge immobilized bioreactor. *Bioresour. Technol.* 76:1-8.

Olsson L, Hahn-Hägerdal B. 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme Microb. Technol.* 18:312-31.

Otter DE, Munro PA, Scott GK, Geddes R. 1984. Elution of *Trichoderma reesei* cellulase from cellulose by pH adjustment with sodium hydroxide. *Biotechnol. Lett.* 6:369-374.

Otter DE, Munro PA, Scott GK, Geddes R. 1989. Desorption of *Trichoderma reesei* cellulose from cellulose by a range of desorbents. *Biotechnol. Bioeng.* 34: 291-298.

Ouyang J, Liu B, Zhang M, Zheng Z, Yu H. 2013. Enzymatic hydrolysis, adsorption, and recycling during hydrolysis of bagasse sulfite pulp. *Bioresour. Technol.* 146:288-93.

Pakarinen A, Haven MØ, Djajadi DT, Várnai A, Puranen T, Viikari L. 2014. Cellulases without carbohydrate-binding modules in high consistency ethanol production process. *Biotechnol. Biofuels* 7:27.

Palonen H, Tenkanen M, Linder M. 1999. Dynamic interaction of *Trichoderma reesei* cellobiohydrolases Cel6A and Cel7A and cellulose at equilibrium and during hydrolysis. *Appl. Environ. Microbiol.* 65:5229-33.

Pan X, Xie D, Gilkes N, Gregg DJ, Saddler JN. 2005. Strategies to enhance the enzymatic hydrolysis of pretreated softwood with high residual lignin content. *Appl. Biochem. Biotechnol.* 124:1069-1079.

Peng L, Chen Y. 2011. Conversion of paper sludge to ethanol by separate hydrolysis and fermentation (SHF) using *Saccharomyces cerevisiae*. *Biomass Bioenergy* 35:1600-6.

Pereira FB, Guimarães PMR, Gomes DG, Mira NP, Teixeira MC, Sá-Correia I, Domingues L. 2011b. Identification of candidate genes for yeast engineering to improve bioethanol production in very gravity and lignocellulosic biomass industrial fermentations. *Biotechnol. Biofuels* 4:57.

Pereira FB, Guimarães PMR, Teixeira JA, Domingues L. 2011a. Robust industrial *Saccharomyces cerevisiae* strains for very high gravity bio-ethanol fermentations. *J. Biosci. Bioeng.* 112:130-36.

Pereira FB, Romaní A, Ruiz HA, Teixeira JA, Domingues L. 2014. Industrial robust yeast isolates with great potential for fermentation of lignocellulosic biomass. *Bioresour. Technol.* 161:192-99.

Prasad S, Singh A, Joshi H. 2007. Ethanol as an alternative fuel from agricultural, industrial and urban residues. *Resour. Conserv. Recycl.* 50:1-39.

Prasetyo J, Kato T, Park EY. 2010. Efficient cellulase-catalyzed saccharification of untreated paper sludge targeting for biorefinery. *Biomass Bioenergy* 34:1906-13.

Pribowo A, Arantes V, Saddler JN. 2012. The adsorption and enzyme activity profiles of specific *Trichoderma reesei* cellulose/xylanase components when hydrolyzing steam pretreated corn stover. *Enzyme Microb. Technol.* 50:195-203.

Qi B, Chen X, Su Y, Wan Y. 2011. Enzyme adsorption and recycling during hydrolysis of wheat straw lignocelluloses. *Bioresour. Technol.* 102:2881-89.

Qi B, Luo J, Chen G, Chen X, Wan Y. 2012. Application of ultrafiltration and nanofiltration for recycling cellulase and concentrating glucose from enzymatic hydrolyzate of steam exploded wheat straw. *Bioresour. Technol.* 104:466-72.

Qi H, Duan H, Wang X, Meng X, Yin X, Ma L. 2015. Preparation of Magnetic Porous Terpolymer and Its Application in Cellulase Immobilization, *Polym. Eng. Sci.* 55(5):1039-45.

Robus CLL, Gottumukkala LD, van Rensburg E, Görgens JF. 2016. Feasible process development and techno-economic evaluation of paper sludge to bioethanol conversion: South African paper mills scenario. *Renew. Energy* 92:333-45.

Rodrigues AC, Felby C, Gama M. 2014. Cellulase stability, adsorption/desorption profiles and recycling during successive cycles of hydrolysis and fermentation of wheat straw. *Bioresour. Technol.* 156:163-9.

Rodrigues AC, Leitão AF, Moreira S, Felby C, Gama M. 2012. Recycling of cellulases in lignocellulosic hydrolysates using alkaline elution. *Bioresour. Technol.* 110:526-33.

Romaní A, Garrote G, Alonso JL, Parajó JC. 2010. Experimental assessment on the enzymatic hydrolysis of hydrothermally pretreated eucalyptus globulus wood. *Ind. Eng. Chem. Res.* 49:4653-63.

Romaní A, Pereira F, Johansson B, Domingues L. 2015. Metabolic Engineering of *Saccharomyces cerevisiae* ethanol strains PE-2 and CAT-1 for efficient lignocellulosic fermentation. *Bioresour. Technol.* 179:150-8.

Romaní A, Yáñez R, Garrote G, Alonso JL, Parajó JC. 2007. Sugar production from cellulosic biosludges generated in a water treatment plant of a Kraft pulp mil. *Biochem. Eng. J.* 37:319-27.

Romaní A, Yáñez R, Garrote G, Alonso JL. 2008. SSF production of lactic acid from cellulosic biosludges. *Bioresour. Technol.* 99:4247-54.

Rosales-Calderon O, Trajano HL, Duff SJB. 2014. Stability of commercial glucanase and β-glucosidase preparations under hydrolysis conditions. *Peer J.* 2:e402.

Ruiz HA, Silva DP, Ruzene DS, Lima LF, Vicente AA, Teixeira JA. 2012. Bioethanol production from hydrothermal pretreated wheat straw by a flocculating *Saccharomyces cerevisiae* strain – Effect of process conditions. *Fuel* 95:528-36.

Saini JK, Saini R, Tewari L. 2015. Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. *3 Biotech.* 5:337-53.

Sarkar N, Ghosh SK, Bannerjee S, Aikat K. 2012. Bioethanol production from agricultural wastes: an overview. *Renew. Energy* 37:19-27.

Saxena R, Adhikari D, Goyal H. 2009. Biomass-based energy fuel through biochemical routes: a review. *Renew. Sust. Energy Rev.* 13:167-78.

Schmaier AH, Silver L, Adams AL, Fischer GC, Munoz PC, Vroman L, Colman RW. 1984. The effect of high molecular weight kininogen on surface-adsorbed fibrinogen. *Thromb. Res.* 33:51-67.

Schmitt E, Bura R, Gustafson R, Cooper J, Vajzovic A. 2012. Converting lignocellulosic solid waste into ethanol for the State of Washington: an investigation of treatment technologies and environmental impacts. *Bioresour. Technol.* 104:400-9.

Segato F, Damásio ARL, de Lucas RC, Squina FM, Prade RA. 2014. Genomics Review of Holocellulose Deconstruction by Aspergilli. *Microbiol. Mol. Biol. Rev.* 78(4):588-613.

Seiboth B, Ivanova C, Seidl-Seiboth V. 2011. *Trichoderma reesei*: A Fungal Enzyme Producer for Cellulosic Biofuels, In: Bernardes MAS (Eds.), Biofuel Production - Recent Developments and Prospects. InTech, pp. 309-40.

Seo D-J, Fujita H, Sakoda A. 2011. Effects of a non-ionic surfactant, Tween 20, on adsorption/desorption of saccharification enzymes onto/from lignocelluloses and saccharification rate. *Adsorption* 17:813-22.

Shang Y, Su R, Huang R, Yang Y, Qi W, Li Q, He Z. 2014. Recycling cellulases by pH-triggered adsorption-desorption during the enzymatic hydrolysis of lignocellulosic biomass. *Appl. Microbiol. Biotechnol.* 98(12):5765-74.

Shen J, Agblevor FA. 2008. Optimization of enzyme loading and hydrolytic time in the hydrolysis

of mixtures of cotton gin waste and recycled paper sludge for the maximum profit rate. *Biochem. Eng. J.* 41:241-50.

Singh A, Pant D, Korres NE, Nizami A-S, Prasad S, Murphy JD. 2010. Key issues in life cycle assessment of ethanol production from lignocellulosic biomass: challenges and perspectives. *Bioresour. Technol.* 101:5003-12.

Sipos B, Dienes D, Schleicher Á, Perazzini R, Crestini C, Siika-aho M, Réczey K. 2010. Hydrolysis efficiency and enzyme adsorption on steam-pretreated spruce in the presence of poly(ethylene glycol). *Enzyme Microb. Technol.* 47:84-90.

Sipponen MH, Pihlajaniemi V, Pastinen O, Laakso S. 2014. Reduction of surface area of lignin improves enzymatic hydrolysis of cellulose from hydrothermally pretreated wheat straw. *RSC Adv.* 4:36591-6.

Široký J, Benians TAS, Russell SJ, Bechtold T, Knox JP, Blackburn RS. 2012. Analysis of crystallinity changes in cellulose II polymers using carbohydrate-binding modules. *Carbohydr. Polym.* 89:213-21.

Sun R, Tomkinson J, Mao FC, Sun XF. 2001. Physicochemical characterization of lignins from rice straw by hydrogen peroxide treatment. *J. Appl. Polym. Sci.* 79:719-32.

Suominen PL, Mantyla AL, Karhunen T, Hakola S, Nevalainen KMH. 1993. High frequency one-step gene replacement in *Trichoderma reesei*. II. Effects of deletions of individual cellulase genes. *Mol. Gen. Genet.* 241:523-30.

Szulczyk KR, Mccarl BA, Cornforth G. 2010. Market penetration of ethanol. *Renew. Sust. Energy Rev.* 14:394-403.

Talebnia F, Karakashev D, Angelidaki I. 2010. Production of bioethanol from wheat straw: an overview on pretreatment, hydrolysis and fermentation. *Bioresour. Technol.* 101:4744-53.

Tao L, Aden A, Elander RT, Pallapolu VR, Lee YY, Garlock RJ, Balan V, Dale BE, Kim Y, Mosier NS, Ladisch MR, Falls M, Holtzapple MT, Sierra R, Shi J, Ebrik MA, Redmond T, Yang B, Wyman CE, Hames B, Thomas S, Warner RE. 2011. Process and technoeconomic analysis of leading pretreatment technologies for lignocellulosic ethanol production using switchgrass. *Bioresour. Technol.* 102:11105-14.

Taylor MP, Mulako I, Tuffin M, Cowan D. 2012. Understanding physiological responses to pre-treatment inhibitors in ethanologenic fermentations. *Biotechnol. J.* 7:1169-81.

Thomas S. 2000. Production of Lactic Acid from Pulp Mill Solid Waste and Xylose Using *Lactobacillus delbrueckii* (NRRL B445). *Appl. Biochem. Biotechnol.* 84:455-468.

Tomás-Pejó E, Alvira P, Ballesteros M, Negro MJ. 2011. Pretreatment Technologies for lignocellulose-to-bioethanol conversion, In: Pandey A, Larroche C, Ricke SC, Dussap C-G, Gnansounou E (Eds.), Biofuels. Alternative Feedstocks and Conversion Processes. Academic Press, pp. 149–76.

Tomás-Pejó E, Bonander N, Olsson L. 2014. Industrial yeasts strains for biorefinery solutions: Constructing and selecting efficient barcoded xylose fermenting strains for ethanol. *Biofuels Bioprod. Bior.* 8:626-634.

Tu M, Chandra RP, Saddler JN. 2007a. Evaluating the distribution of cellulases and the recycling of free cellulases during the hydrolysis of lignocellulosic substrates. *Biotechnol. Prog.* 23:398-406.

Tu M, Chandra RP, Saddler JN. 2007b. Recycling cellulases during the hydrolysis of steam exploded and ethanol pretreated Lodgepole pine. *Biotechnol. Prog.* 23:1130-7.

Tu M, Saddler JN. 2010. Potential enzyme cost reduction with the addition of surfactant during the hydrolysis of pretreated softwood. *Appl. Biochem. Biotechnol.* 161:274-87.

Tu M, Zhang X, Paice M, MacFarlane P, Saddler JN. 2009. The potential of enzyme recycling during the hydrolysis of a mixed softwood feedstock. *Bioresour. Technol.* 100:6407-15.

Varga E, Szengyel Z, Reczey K. 2002. Chemical pretreatments of corn stover for enhancing enzymatic digestibility. *Appl. Biochem. Biotechnol.* 98:73–87.

Viikari L, Alapuranen M, Puranen T, Vehmaanperä J, Siika-Aho M. 2007. Thermostable enzymes in lignocellulose hydrolysis. *Adv. Biochem. Eng. Biotechnol.* 108:121-45.

Viikari L, Vehmaanpera J, Koivula A. 2012. Lignocellulosic ethanol: From science to industry. *Biomass Bioenergy* 46:13-24.

Vohra M, Manwar J, Manmode R, Padgilwar S, Patil S. 2014. Bioethanol production: feedstock and current technologies. *J. Environ. Chem. Eng.* 2:573-84.

von Sivers M, Zacchi G, Olsson L, Hahn-Haegerdal B. 1994. Cost Analysis of Ethanol Production from Willow Using Recombinant *Escherichia coli*. *Biotechnol. Prog.* 10:555-60.

Waeonukul R, Kosugi A, Prawitwong P, Deng L, Tachaapaikoon C, Pason P, Ratanakhanokchai K, Saito M, Mori Y. 2013. Novel cellulase recycling method using a combination of *Clostridium thermocellum* cellulosomes and *Thermoanaerobacter brockii* β-glucosidase. *Bioresour. Technol.* 130:424-30.

Wahlbom CF, van Zyl WH, Jönsson LJ, Hahn-Hägerdal B, Otero RRC. 2003. Generation of the improved recombinant xylose-utilizing *Saccharomyces cerevisiae* TMB 3400 by random mutagenesis and physiological comparison with *Pichia stipitis* CBS 6054. *FEMS Yeast Res.* 3:319-26.

Wang W, Zhu Y, Du J, Yang Y, Jin Y. 2015. Influence of lignin addition on the enzymatic digestibility of pretreated lignocellulosic biomasses. *Bioresour. Technol.* 181:7-12.

Wingren A, Galbe M, Roslander C, Rudolf A, Zacchi G. 2005. Effect of reduction in yeast and enzyme concentrations in a simultaneous-saccharification-and-fermentation-based bioethanol process: technical and economic evaluation. *Appl. Biochem. Biotechnol.* 122:485-99.

Wingren A, Galbe M, Zacchi G. 2003. Techno-economic evaluation of producing ethanol from softwood: comparison of SSF and SHF and identification of Bottlenecks. *Biotechnol. Prog.* 19:1109-17.

Xiao ZZ, Zhang X, Gregg DJ, Saddler JN. 2004. Effects of sugar inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. *Appl. Biochem. Biotechnol.* 115:1115–1126.

Yamada R, Taniguchi N, Tanaka T, Ogino C, Fukuda H, Kondo A. 2011. Direct ethanol production

from cellulosic materials using a diploid strain of *Saccharomyces cerevisiae* with optimized cellulase expression. *Biotechnol. Biofuels* 4:8.

Yamashita Y, Kurosumi A, Sasaki C, Nakamura Y. 2008. Ethanol production from paper sludge by immobilized *Zymomonas mobilis. Biochem. Eng. J.* 42:314-19.

Yamashita Y, Sasaki C, Nakamura Y. 2010. Development of efficient system for ethanol production from paper sludge pretreated by ball milling and phosphoric acid. *Carbohydr. Polym.* 79:250-54.

Yang B, Wyman CE. 2006. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. *Biotechnol. Bioeng.* 94:611-17.

Yang B, Wyman CE. 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels Bioprod. Bior.* 2:26-40.

Yang J, Zhang X, Yong Q, Yu S. 2010. Three-stage hydrolysis to enhance enzymatic saccharification of steam-exploded corn stover. *Bioresour. Technol.* 101:4930-5.

Yuan B, Yang X, Xue L, Feng Y, Jiang J. 2016. A novel recycling system for nano-magnetic molecular imprinting immobilised cellulases: Synergistic recovery of anthocyanin from fruit and vegetable waste. *Bioresour. Technol.* 222:14-23.

Zabed H, Sahu JN, Boyce AN, Faruq G. 2016. Fuel ethanol production from lignocellulosic biomass: An overview on feedstocks and technological approaches. *Renew. Sust. Energy Rev.* 66:751-74.

Zhang J, Lynd LR. 2010. Ethanol Production From Paper Sludge by Simultaneous Saccharification and Co-Fermentation Using Recombinant Xylose-Fermenting Microorganisms. *Biotechnol. Bioeng.* 107:235-244.

Zhang W, Qiu J, Feng H, Zang L, Sakai E. 2015. Increase in stability of cellulase immobilized on functionalized magnetic nanospheres. *J. Magn. Magn. Mater.* 375:117-23.

Zheng H, Liu Y, Liu X, Han Y, Wang J, Lu F. 2012. Overexpression of a *Paenibacillus campinasensis* xylanase in *Bacillus megaterium* and its applications to biobleaching of cotton stalk pulp and saccharification of recycled paper sludge. *Bioresour. Technol.* 125:182-87.

Zhu Z, Sathitsuksanoh N, Zhang Y-HP. 2009. Direct quantitative determination of adsorbed cellulase on lignocellulosic biomass with its application to study cellulose desorption for potential recycling. *Analyst* 134:2267-72.

Chapter III

Valorizing recycled paper sludge by a bioethanol production process with cellulase recycling

The work reported on this chapter was published in:
Daniel G. Gomes, Lucília Domingues, Miguel Gama. 2016. Valorizing recycled paper sludge by a bioethanol production process with cellulase recycling. Bioresource Technology, 216: 637-644.

Abstract

Despite the considerable efforts developed on cellulosic ethanol production in the last decades, these processes are still not economically competitive with 1st generation or the traditional fossil fuels. Among others, the high cost of enzymes has been a major obstacle for this technology. Multiple strategies have been pursued to reduce this cost, namely recycling the enzymes.

The overall feasibility of cellulase recycling in the scope of bioethanol production from recycled paper sludge (RPS), an inexpensive byproduct from paper industry with around 39 % of carbohydrates, was analyzed. RPS was easily converted by a cellulases cocktail with no visible inhibition from the toxics on both enzymes and cells. Final enzyme partition between solid and liquid phases indicated that a considerable fraction of the enzymes stay free after hydrolsysis with the solid-bound enzymes being efficiently recovered by alkaline elution.

RPS hydrolysis and fermentation was then conducted over four rounds, recycling the cellulases present in both fractions. A great overall enzyme stability was observed towards the entire process: 71, 64 and 100 % of the initial Cel7A, Cel7B and β-glucosidase activities, respectively, were recovered. Even with only 30 % of fresh enzymes added on the subsequent rounds, solid conversions of 92, 83 and 71 % were achieved for the round 2, 3 and 4, respectively.

This work demonstrates the suitability of RPS to be used on cellulosic ethanol production. Furthermore, the partition and recovery efficiency of final enzyme suggests that RPS may be suitable for enzyme recycling. The implementation of such system would thus enable considerable savings on enzymes cost (50-60 %) while equally contributing to a 40 % reduction in RPS disposal costs.

3.1 Background

The economic feasibility of second-generation bioethanol relies on two major cost factors: the substrate and the enzymes. The identification of a cheaper, abundant and easily hydrolysable material has assumed a critical role for a more economic production of fermentable sugars. Recently, an increased utilization of different kinds of residues came as an interesting alternative to the traditional lignocellulosic substrates, enabling a considerable reduction on substrate cost, and also an additional valorization for some of these otherwise useless materials.

Recycled paper sludge (RPS) is a residue originated from the paper recycling process, more specifically, from the treatment of the liquid effluents generated in that process. It is mostly composed of small fibers with approximately 40 % of carbohydrates that cannot anymore be incorporated on recycled paper (Marques *et al.*, 2008a). Also, due to the chemical contamination, namely with ink particles, this residue has high environmental impact being usually disposed on landfills, which represents a considerable expenditure for these companies. Considering an approximate production of this waste around 300 kg per ton of recycled paper (Balwaik and Raut, 2011) and taking into account an estimated 47 million tons of recycled paper produced only in Europe by the year of 2005 (Monte *et al.*, 2009), this corresponds to around 14 million tons of RPS that need to be discarded. In spite of the notable potential of this material, coupled with a high worldwide availability, only few studies have been conducted so far exploring its further valorization (Prasetyo and Park, 2013). Some examples refer to Lark *et al.* (1997) who have studied RPS hydrolysis and subsequent fermentation to ethanol by *Kluyveromyces marxianus*. Also Marques *et al.* studied its potential for bio-ethanol production by *Pichia stipitis* (2008a) and lactic acid production by *Lactobacillus rhamnosus* ATCC 7469 (2008b).

In addition to the substrate cost, the cost of the enzymes required to hydrolyze lignocellulosic materials (cellulases and/or hemicellulases) represents one of the biggest obstacles for their economically viable conversion, due the competition from the less expensive fossil fuels. Great debate has been established concerning the exact cost of cellulases, with distinct values being pointed out by different authors. Klein-

Marcusschamer *et al.* (2012) estimated a cost on ethanol production around $ 0.68 per gallon, close to $ 0.5 per gallon recently suggested by Novozymes (http://novozymes.com/en/news/news-archive/Pages/45713.aspx). However, Aden and Foust (2009) have also already reported a value around $ 0.1 per gallon, close to $ 0.3 reported by Lynd *et al.* (2008) and $ 0.32 reported by Dutta *et al.* (2010). Independently of the exact figure, it is consensually recognized that the enzymes cost is a major determinant of the cellulosic ethanol competitiveness, driving in the last years intense efforts to reduce the loading employed in the process. The reduction of the cost associated to enzymes has been commonly pursued following three main strategies: increasing the efficiency of enzymes; reducing enzymes production cost; and reutilizing the enzymes (Pribowo *et al.*, 2012). Over the last years (even decades), most of the attention has been given to the first two strategies, through intense and constant research operated by both industry (*e.g.* Novozymes; DSM; Genencor) and academia. Through a close collaboration with Novozymes and Genencor, NREL (USA) conducted a joint project that resulted in a reduction of cellulase cost up to 10 fold (http://www.nrel.gov/docs/fy13osti/59013.pdf). Nevertheless, some authors have already admitted that such strategies will not allow pushing down cellulases cost much further. In this context, the recovery (and posterior reutilization) of cellulases has recently emerged as a very promising concept, as using enzymes multiple times will allow a natural reduction on its consumption.

Numerous studies have been conducted for some years now in what concerns the mechanisms of enzyme adsorption/desorption (Lindedam *et al.*, 2013; Pribowo *et al.*, 2012; Rodrigues *et al.*, 2014; Tu *et al.*, 2007), addressing the complexity associated to different enzymes and substrates. In a similar way, possible strategies to facilitate and/or conduct the recovery of these enzymes have already been individually studied. According to Gomes *et al.* (2015), enzymes remaining in the liquid fraction are usually recovered either by ultrafiltration or by addition to fresh substrate (and posterior separation), while solid-bound enzymes normally require a change of pH or the addition of specific chemical compounds (that interfere with solid-enzyme interaction). Nevertheless, very few studies were conducted so far presenting an integrated approach of such strategies to the hydrolysis of a specific lignocellulosic material over multiple rounds.

Here, an overall study on the feasibility of using RPS as substrate for 2G-bioethanol production in a system of multiple rounds of hydrolysis with cellulase recycling was conducted. The conservation of enzymatic activity and its final partition between solid and liquid fractions is initially accessed followed by an evaluation regarding the recovery efficiency of solid-bound enzymes. Afterwards, a process with multiple rounds of hydrolysis and enzymes recycling was implemented, monitoring the activity levels and the degree of solids conversion over the entire process.

3.2 Materials and Methods

3.2.1 Enzymes and substrate

Enzymatic hydrolysis were conducted through the combined action of the commercial cocktail Celluclast (Sigma-Aldrich, C2730), complemented with the commercial β-glucosidase preparation Novozyme 188 (Novozymes). The activities of these preparations were determined to be 45 FPU/mL and 611 IU/mL, respectively.

The recycled paper sludge (RPS) was kindly provided by RENOVA (Torres Novas, Portugal). This refers to a solid (with approx. 53 % (w/v) water) obtained from the wastewater treatment of paper recycling effluents generated by this company. This material contains high carbonates content, which results on an alkaline solid. Similarly to Marques *et al.* (2008a), prior to its utilization RPS material was treated with hydrochloric acid 37 % and then washed, first with water and then with buffer (0.1 M acetic acid/sodium acetate). This process rendered a neutralized RPS (*n*RPS), which was used in all tests of the current work.

3.2.2 Hydrolysis and Fermentation

Enzymatic hydrolysis of *n*RPS material were conducted under a standardized system with variable times and temperatures according to the purpose of each study. After RPS neutralization (and washing), the wet neutralized solid (with approx. 85 % (w/v) water) was resuspended in 0.1 M acetic acid/sodium acetate buffer (pH 4.8) to a consistency of 5 % (w/v) (dry weight basis). After sterilization and cooling to room temperature, enzymes were added on a small volume of the abovementioned buffer, being filter-sterilized (sterile Polyethersulfone (PES) syringe filters; 0.22 µm) into the mixture of solids. Unless otherwise stated, enzymes were added in a dosage of 20 FPU/$g_{cellulose}$ of Celluclast and with a β-glucosidase/Celluclast activity ratio of 5. This ratio was defined aiming to attenuate any limitation of beta-glucosidase activity in order to ensure that cellulase action would be the limiting element. Solid suspension was then incubated at 200 rpm on an orbital shaker at variable times and temperatures (35/50 ºC).

When a further fermentation was conducted, this mixture was inoculated with cells of *Saccharomyces cerevisiae* PE-2 strain (Basso *et al.*, 2008; Pereira *et al.*, 2014; collected on the beginning of the stationary phase) and the temperature reduced to 30ºC. After

harvested from the culture medium, yeast cells were resuspended on ice-cold 0.9 % (w/v) NaCl and then added to the solids suspension in a ration of 8 g/L (fresh biomass). Periodic sampling was conducted accordingly with the purpose of each study. A minimum of 2 independent replicates was always conducted for every test of this work.

3.2.3 Recovery of solid-bound enzymes

Enzymes adsorbed to the solid were recovered by a process of alkaline elution, as described previously by Rodrigues *et al.* (2012, 2014). Briefly, the final hydrolysate was centrifuged for 15 minutes at 2710 g, after which the supernatant was collected (discarded or stored). The harvested solid was resuspended on an equal volume of freshly prepared 0.1 M Tris-HCl buffer (pH 9-10) and mixed for 2 hours on a turning wheel (Rotator SB3-Stuart) at room temperature. At the end, the solids mixture was once again centrifuged and the supernatant (containing the eluted enzymes) collected and stored accordingly (at 4ºC) for future use.

3.2.4 Multiple rounds of hydrolysis with enzyme recycling

Enzymatic hydrolysis in the context of cellulase recycling were conducted on a similar way comparatively to the single-round experiments. Some modifications were however introduced concerning the solid preparation as described below.

For the first round, hydrolysis was performed according to the common procedure employed so far. The solids suspension (5 % (w/v)) was mixed with 20 FPU/$g_{cellulose}$ of Celluclast (complemented with *β*-glucosidase) and incubated for 48 or 72 hours at 35 ºC. Afterwards, this mixture was inoculated with yeast cells and incubated for 6 hours at 30 ºC.

At the end of the round, and after samples collection, final broth was centrifuged (2710 g for 15 minutes) to separate liquid and solid fractions. Supernatant, containing free enzymes (in the liquid fraction), was filtered through a 0.2 µm pore PES filter to remove major impurities, being posteriorly stored at 4 ºC until further use. The solid was subjected to an alkaline wash, as previously described on this section, after which phases were once again separated. Similarly to the liquid phase, the elution liquid, containing the desorbed enzymes, was filtered to remove impurities and stored until further use. Prior to its storage the alkaline pH of this liquid was adjusted to the common

operational pH (4.8) through the addition of 1 M acetic acid/sodium acetate buffer (pH 4.8). Final solid was repeatedly washed with distilled water, oven dried (at 45 ºC) until an estimated water content below 10 %, and finally stored until further use.

For cellulase recycling, both fractions (stored at 4 ºC) were mixed and concentrated using a tangential ultrafiltration system Pellicon XL membrane with a 10 kDa cut-off PES membrane (Millipore, Billerica, MA, USA). The two fractions were initially concentrated by diafiltration and, at the end, adjusted to a final fixed volume. Considering the results of exploratory ultrafiltration tests, where the enzyme concentration on the final liquid seemed to significantly influence the level of activity loss, the final ultrafiltration volume was set on this case to the maximum value allowed, considering the amount of liquid on the sterilized solid. To enable a maximum final ultrafiltration volume, the fresh new solid was centrifuged in sterile conditions after the sterilization process, contrarily to its utilization in the entire suspension. For a new round of hydrolysis, the sterilized solid was resuspended and transferred to a new sterilized Erlenmeyer flask using the enzyme suspension obtained from the previous ultrafiltration procedure, posteriorly filter-sterilized with 0.2 µm PES syringe filters. For each recycling stage, a portion of fresh enzymes was added to this suspension, corresponding to 20/30 % of the original enzyme dosage. The new solids suspension was then subjected to the same conditions of hydrolysis and fermentation, as previously described.

This procedure was applied over a total of 4 rounds of hydrolysis and fermentation, as illustrated on Figure 3.1.

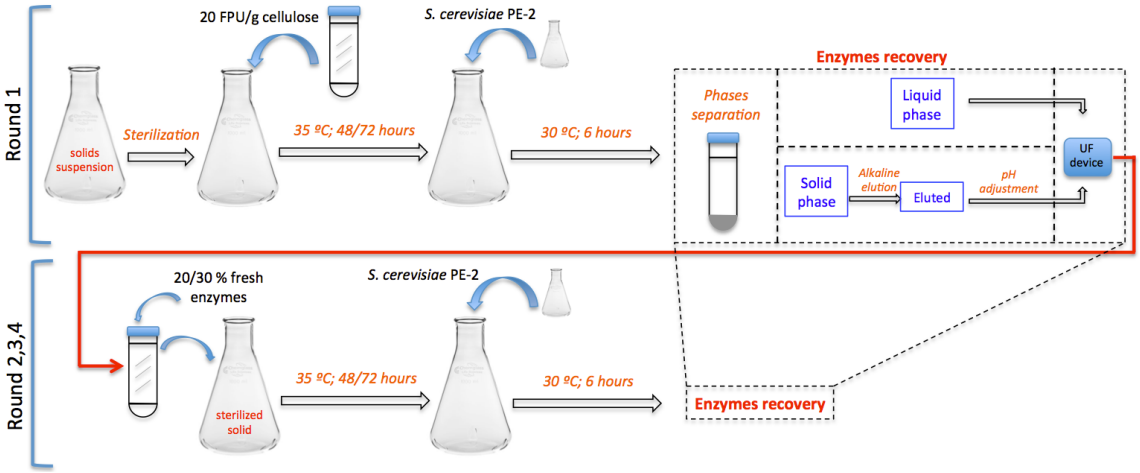

Figure 3.1 Overall representation of the procedure for *n*RPS hydrolysis during four consecutive rounds.

3.2.5 Analytical procedures

Sugars and ethanol quantification

After thawing, aliquots from hydrolysis and fermentation experiments were diluted, filtered and then analyzed by HPLC (High-Performance Liquid Chromatography) for glucose and ethanol quantification. Samples were eluted on a Varian MetaCarb 87H column at 60 ºC, with 0.005 M H_2SO_4 at a flow rate of 0.7 mL/min, and a refractive-index detector.

Measurement of enzymatic activity

Samples collected for quantification of enzymatic activity were stored at 4 ºC until further utilization. Cel7A, Cel7B and β-glucosidase activities were quantified by fluorescence spectroscopy with slight differences according to the specific cellulolytic component, following a modified version of the protocol previously published by Bailey and Tähtiharju (2003). For Cel7A, Cel7B and β-glucosidase quantification, 400 µL of a freshly prepared solution of 1 mM 4-methylumbelliferyl-b-D-cellobioside (MUC, Sigma–Aldrich, M6018), 4-methylumbelliferyl-b-D-lactopyranoside (MULac, Sigma–Aldrich, M2405) and 4-methylumbelliferyl-b-D-glucopyranoside (MUGlc, Sigma–Aldrich, M3633), respectively, were mixed with 50 µL of enzyme sample (properly diluted on buffer considering the range of the method) and then incubated for 15 minutes at 50 ºC. After that, the reaction was stopped by the addition of 550 µL of 1 M Na_2CO_3 and measured on a black bottom 96-well UV fluorescence microplate using a Biotech Synergy HT Elisa plate reader. For Cel7B quantification, the addition of 50 µL of a mixture containing 1 M glucose and 50 mM cellobiose is still required, in order to inhibit Cel7A and β-glucosidase activities. Cel7A, Cel7B and β-glucosidase act on their specific substrates releasing free 4-methylumbelliferone (MU, Sigma– Aldrich, M1508), which results on a change of the fluorescence spectra that is quantified for an excitation and emission wavelengths of 360 and 460 nm, respectively.

Determination of solid composition

The solids main composition, either corresponding to the initial material or after enzymatic hydrolysis, was determined by quantitative acid hydrolysis. After oven drying

(at 45 ºC) to a water content inferior to 10 %, approximately 0.5 g of solid was mixed with 5 mL of 72 % (w/v) H_2SO_4 for 1 hour at 30 ºC. Afterwards, this mixture was subjected to a dilute hydrolysis by raising the volume to a total mass of 148.67 g and posteriorly autoclaving for 1 hour at 121 ºC. After this, the solid residue was recovered by filtration (crisol Gooch nº3) and posterior drying (at 105 ºC) until constant weight. Different sugar monomers formed during hydrolysis were quantified by HPLC analysis of the liquid fraction.

3.3 Results and Discussion

3.3.1 RPS composition on the main lignocellulosic components

The feasibility of using *n*RPS as substrate for 2G-bioethanol must be assessed. This depends on the presence of a meaningful amount of carbohydrates that can be later converted, and on its susceptibility to hydrolysis by cellulases.

As a residue derived from a production process that uses materials with some degree of heterogeneity (different types of paper residues), RPS composition is equally expected to present some variations from different production batches (Chen *et al.*, 2014). Additionally, its composition may also vary depending on the specific context of its production as some variations on the paper residues used may be expected from one industry to another and also from different countries.

Table 3.1 Percentages of the main components in RPS composition

	Original material	**After neutralization**	**Marques *et al.* (2008a)**
Cellulose	16.25 ± 0.36	30.98 ± 0.95	34.1
Xylan	4.74 ± 0.00	7.02 ± 0.19	7.90
Klason lignin	*Not determined*	*Not determined*	20.4
Ash	*Not determined*	*Not determined*	29.3
Protein			4.80
Fat			3.50
Acid-insoluble solids	41.58 ± 0.49	62.55 ± 1.87	*Not presented by authors*

The *n*RPS used in this work was kindly provided by RENOVA (Torres Novas, Portugal) and was initially analyzed for its main components (Table 3.1). From this analysis, only 21 % carbohydrates were estimated for this material, which makes it a relatively poor material as compared to most of the traditional lignocellulosic materials. Just to refer few examples, corncobs and wheat straw have approximately 80 % of carbohydrates (Sun and Cheng, 2002). Considering the high carbonates content of this material, already reported by other authors (Marques *et al.*, 2008a), a neutralization with hydrochloric acid was conducted for carbonates removal allowing to concentrate the carbohydrate

fraction. As a matter of fact, cellulose content increased by around 2 fold to approximately 31 % (w/w) and xylans to 7 % (Table 3.1). A similar composition was reported by Marques *et al.* (2008a) for a RPS sample obtained from the same industry (RENOVA, Portugal). This is a more suitable composition considering its economic conversion to fermentable sugars.

3.3.2 RPS digestibility and fermentation

Considering that *n*RPS is originated from the treatment of effluents with high amounts of contaminating chemicals, being inclusively associated to a considerable environmental impact (Park *et al.*, 2001), it would be most relevant to investigate a possible toxicity effect over cellulases and cells, which would ultimately affect its conversion and fermentation. For that purpose, the profile of hydrolysis and posterior fermentation of a 5 % (w/v) solids suspension was investigated using a enzyme loading of 20 FPU/$g_{cellulose}$ (*data not shown*).

Glucose and ethanol profiles suggested that both cellulases and cells acted quickly and efficiently over the *n*RPS material. After 48 hours of hydrolysis approximately 92 % of solid conversion was already achieved.

In what concerns glucose fermentation into ethanol, a fast conversion was equally achieved, being completed within a total of 6 hours. No indication of *n*RPS toxicity was evident, yielding an ethanol productivity around 1.16 g/L h^{-1}. Smaller values (0.6 g/L h^{-1}) were obtained by Marques *et al.* (2008a) with *Pichia stipitis,* although using different conditions. This seems to support the high robustness and tolerance of PE-2 strain, as already reported by several reports (Gomes *et al.*, 2012; Pereira *et al.*, 2010, 2011, 2012, 2014). Additionally, further improvements may still be achieved in the current context with the possible utilization of MEC1121 strain (Romani *et al.*, 2015), which resulted from the introduction of xylose fermentation pathway on the industrial robust *S. cerevisiae* PE-2 strain.

3.3.3 Cellulase stability and final solid-liquid partition

In the particular context of a cellulase recycling system, a specific set of factors gets special relevance. In addition to an effective substrate hydrolysis, cellulases must be

stable enough as to allow multiple stages of hydrolysis, without compromising substrate conversion.

Besides a possible negative effect caused by the toxic nature of *n*RPS, some other factors must also be accounted for, such as the temperature. Even though the optimal temperature of hydrolysis for fungal cellulases is commonly reported to be around 50ºC, an extensive exposure to this range of temperatures is usually associated to significant losses of activity (Chylenski *et al.*, 2012; Rodrigues *et al.*, 2014). For this reason, a system of multiple rounds of hydrolysis will require a careful choice of the temperature. The cellulase cocktail employed on this work was Celluclast, a widely used and studied commercial product from Novozymes, which has been reported to have great stability towards different environmental factors. In a previous study Rodrigues *et al.* (2012) accessed the thermal stability of Celluclast towards different possible operational temperatures. On a week long experiment the cellulase activity was not significantly affected in the range of 30-45 ºC, while more than 50 % of the activity was lost for a temperature of 50 ºC. Also, Chylenski *et al.* (2012) observed considerable increases on protein precipitation of *T. reesei* preparations for temperatures above 40 ºC. To access possible toxicity effects from the substrate, a study was performed incubating *n*RPS material with the cellulases at 35ºC (to avoid thermal denaturation), employing two distinct enzyme dosages (Figure 3.2), and the loss of activity was monitored.

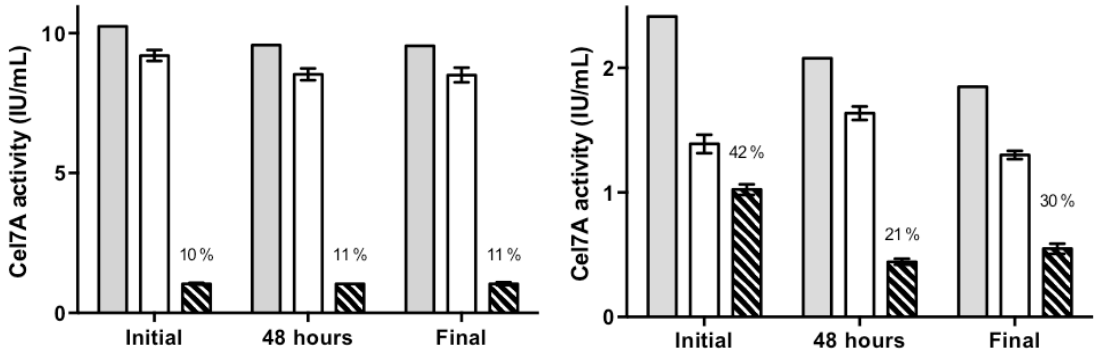

Figure 3.2 Distribution of Cel7A activity between fractions (Total activity Liquid phase Solid phase) over a process of SHF of *n*RPS: (A) using 80 FPU/$g_{cellulose}$ and (B) 20 FPU/$g_{cellulose}$ (percentages of the solid-bound enzymes are presented for the different stages of the process; yeast cells were added at 48 hours of hydrolysis and fermentation occurred for additional 24 hours); (variation of enzyme activity was statistically different at a significant level of 95 % (a) or 90 % (b)).

From the levels of Cel7A activity during the process (the most abundant cellulase component secreted by *T. reesei* and hence considered here as an indicator of overall cellulase activity) it was possible to observe that some loss of activity occurred, being more prominent for the lower enzyme dosage. Using 80 FPU/$g_{cellulose}$, which is considerably high when compared to the traditional range of values employed on literature (10-40 FPU), approximately 6.8 % of Cel7A activity was lost over the entire process (Figure 3.2A). Most of this loss occurred on the first stage of the process, corresponding to a 48 hours period of exclusive hydrolysis, and after which the activity seemed to stabilize with no significant changes during the additional 24 hours of fermentation. As opposing to that, Cel7A activity decreased nearly 23.4 % when a dosage of 20 FPU/$g_{cellulose}$ was applied, with 13.8 % occurring on the first phase and the remaining 9.5 % in the second one (Figure 3.2B). It is most relevant to refer that in terms of absolute values, the activity losses were actually not very different (0.698 and 0.564 FPU/$g_{cellulose}$, respectively), which somehow suggests that this loss of activity might be dependent on the amount of substrate (perhaps related to enzyme inactivation by adsorption to the solid).

In addition to the overall variation of activity during the process of hydrolysis, one other factor that may critically affect the efficiency of cellulase recycling refers to its distribution among the liquid and solid fractions (Gomes *et al.*, 2015). This will strongly determine the strategies required for their recovery, and ultimately, the process efficiency.

Most of final Cel7A activity was present on the liquid fraction for both enzyme dosages, which is in agreement with the common mechanism of cellulose hydrolysis, after which enzymes are released to the liquid fraction. Similarly to what was observed for the activity loss, even though the fraction of cellulase activity adsorbed to the final solid was considerably higher at lower enzyme dosage (30 % for 20 FPU/$g_{cellulose}$; 11 % for 80 FPU/$g_{cellulose}$), the amount of adsorbed activity in both cases (1.045 IU/mL; 0.548 IU/mL) was not so different, suggesting as expected that enzyme adsorption relies on the substrate availability and its respective contact area. In this way, for a given amount of solid it seems to exist a maximum enzyme retention capability at the end of the process.

In what concerns the most realistic scenario, using 20 FPU/$g_{cellulose}$, it is worth noting that despite most of the final activity being located in the liquid fraction, the amount that remained adsorbed to the solid was still relevant (30 %), which gets special significance in this context of cellulase recycling since it cannot be directly recovered, as occurs for the liquid fraction enzymes.

3.3.4 Recovery of solid-bound cellulases

The interaction of cellulases with lignocellulosic materials is a complex process. Not only different enzymes present distinct affinities for a specific solid (Ishihara *et al.*, 1991; Pribowo *et al*., 2012), but also a specific enzyme seems to behave differently towards diverse materials (Tu *et al.*, 2007). In a previous work Rodrigues *et al.* (2012) have clearly demonstrated that the final solid composition plays an essential role determining the efficiency of cellulase recovery, showing that Cel7A has apparently higher affinity for cellulose rather than lignin, hence being harder to recover when adsorbed to the former. Aiming to clarify whether cellulases can be recovered from the solid fraction, an alkaline elution of the enzymes adsorbed on the solid residue was tested.

Table 3.2 Cel7A recovery by alkaline elution of the *n*RPS hydrolysis residue

Enzyme activity		**Cel7A activity (IU/mL)**
Solid-bound activity at the end of hydrolysis		0.789 ± 0.014
After alkaline elution	- total	0.764 ± 0.060
	- in the supernatant	0.646 ± 0.035
	- bound to the solid	0.118 ± 0.009
	Fraction recovered from the solid (%)	81.8 ± 4.71

Taking into account the levels of Cel7A activity over the entire process, one can observe that near 82 % of the solid-bound enzymes were recovered to the elution liquid, and thus, can equally be reused on a new hydrolysis (Table 3.2). Furthermore, no significant loss of activity occurred as a result of the elution process, suggesting that this specific procedure, by which the solid with the adsorbed cellulases is incubated with Tris-HCl

(pH 9), does not compromise the enzyme functionality. Employing similar strategies, equally based on a shift to alkaline pH, similar results were reported on other studies. With a pH change from 8 to 13, Zhu *et al.* (2009) were able to desorb approximately 94 % of the cellulases adsorbed on diluted acid pre-treated corn stover. More recently, Shang *et al.* (2014) were able to recover approximately 85 % of cellulases adsorbed to corncob by increasing the pH to 10.

3.3.5 *n*RPS hydrolysis with cellulase recycling over multiple rounds

The previous results gave promising indications regarding the possibility to recover cellulases after hydrolysis, to be later reused. Following these results, it comes the question of whether a system developed to recover and reuse these enzymes can be applied without compromising the efficiency of solid conversion over the several rounds of hydrolysis.

To answer that question, four consecutive rounds of *n*RPS hydrolysis and fermentation (SHF) were conducted, with enzyme recycling complemented with only 20 % of fresh enzyme (4 FPU/$g_{cellulose}$) added in the beginning of each new round. At the end of each round, enzymes (from both fractions) were recovered and separated from the final products (by ultra-filtration), and then mixed with fresh enzymes and incubated with fresh solid. The activities of three important cellulolytic components (Cel7A, Cel7B and β-glucosidase) were monitored to access possible variations during the entire process (Figure 3.3).

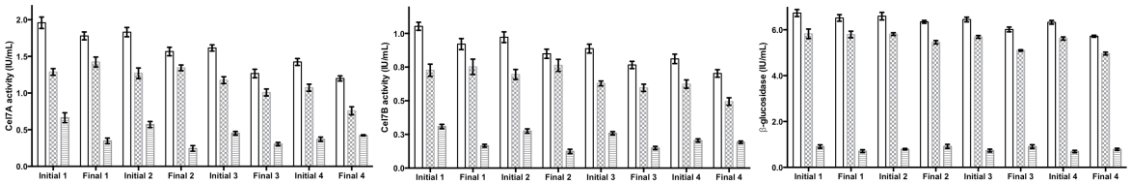

Figure 3.3 Variation of Cel7A, Cel7B and β-glucosidase activities (▌▌ Total activity ▒ Liquid phase ▤ Solid phase) over four rounds of *n*RPS hydrolysis (48 hours hydrolysis [35 ºC]->6 hours SSF [30 ºC]) with cellulase recycling. 20 FPU/$g_{cellulose}$ were initially employed with a posterior supplementation of 20 % fresh enzymes on each recycling stage; total activity was statistically different over the distinct rounds at a significant level ($p < 0.05$).

From an overall analysis of Figure 3.3, it seems clear that the variation on the activity levels for the different cellulase components presented some differences, mostly between the profiles of Cel7A and Cel7B, and the one of β-glucosidase.

As it was seen before, some of the Cel7A activity was lost during each round, which seemed to occur on a slightly higher degree for the two last rounds. Nevertheless, approximately 61 % of Cel7A activity was conserved over the four consecutive rounds of hydrolysis (and fermentation): the activity decreased from an initial 1.96 IU/mL (beginning of 1st round) to 1.20 IU/mL. The supplementation of 20 % of fresh cellulases (conducted on each recycling stage) was apparently not enough to fully compensate the losses occurred on each round. Apparently, a slightly superior supplementation of enzyme (25-30%) would suffice to keep constant the initial levels of activity, leading to significant savings of enzyme. Exploratory tests previously conducted (*results not shown*) showed the occurrence of some loss of activity during the ultrafiltration process, which can change according to the range of the working enzyme dosage. Employing a similar enzyme recovery method (based on an ultrafiltration device, equipped with a 10kDa membrane), and the same cellulase preparation, Rodrigues *et al.* (2014) reported activity losses between 11 and 29 %.

In what concerns Cel7B activity, a similar behavior was observed. Total activity levels decreased from an initial 1.05 IU/mL to 0.7 IU/mL, which corresponds to an activity maintenance around 67 %. Once again, the supplementation of fresh enzyme enabled the satisfactory maintenance of activity along the different rounds, although not being sufficient to restore it completely. As for the case of Cel7A, the activity levels also suggested the occurrence of some enzyme loss during the steps of enzyme recovery and concentration.

The enzyme distribution between the solid residue and the supernatant occurred accordingly to what was observed previously. Most of Cel7A and Cel7B were free on the liquid fraction but a significant portion, ranging from 15 to 36 % for Cel7A and 14 to 28 % for Cel7B, remained adsorbed to the final solid, which somehow justifies our option to also recover solid-bound enzymes. Worth noting is that, for both Cel7A and Cel7B, a gradual increase could be observed for this parameter from the second to the last round. This seems to meet an equally visible reduction produced on solid conversion for each round, which will be discussed below (Table 3.3).

Differently from Cel7A (a cellobiohydrolase) and Cel7B (an endoglucanase), β-glucosidase presented a considerably distinct behavior. Even though a loss of activity also occurred on each round, these were observed to a much smaller extent comparatively to the other cases, resulting in a cellulase activity equivalent to 85 % of the initial level after the four rounds of hydrolysis. In this case there was almost no variation on the levels of β-glucosidase activity adsorbed to the final solid over the different rounds (Figure 3.3). In addition to that, the fraction of enzymes remaining adsorbed to the final solid were around 14 %, which is clearly inferior comparatively to the other enzymes. This suggests a lower solid-adsorption efficiency for these enzymes (Gomes *et al.*, 2015; Ishihara *et al.*, 1991; Lindedam *et al.*, 2013), which is explained by the lack of a cellulose-binding module (CBM) in this class of enzymes.

Table 3.3 Multiple rounds of *n*RPS hydrolysis (48 hours hydrolysis [35 ºC]->6 hours SSF [30 ºC]) with cellulase recycling (20 FPU/$g_{cellulose}$; 20 % fresh enzymes)

Round	Glucose* (g/L)[a]	Ethanol (g/L)[b]	Glucans in final solid (%)[a]	Glucans conversion (%)[a]
1	14.87 ± 0.06	7.06 ± 0.07	2.52 ± 0.06	91.88 ± 0.21
2 (recycling 1)	13.89 ± 0.05	6.21 ± 0.36	4.33 ± 0.48	86.02 ± 1.55
3 (recycling 2)	12.19 ± 0.03	5.69 ± 0.04	8.74 ± 0.35	71.79 ± 1.14
4 (recycling 3)	9.82 ± 0.41	4.63 ± 0.08	13.25 ± 0.49	57.23 ± 1.60

* before yeast inoculation
a – differences between the values obtained in the distinct rounds are statistically significant ($p < 0.05$)
b – differences between the values obtained in the distinct rounds are statistically significant ($p < 0.1$)

Previous results suggested some heterogeneity in what concerns the stability and adsorption behavior of different cellulolytic components over the entire recycling experiment. Although providing important indications, the viability of this process is ultimately accessed based on how it allows high solid conversions. Glucans conversion decreased from 92 %, on the first round, to 57 % in the last one (Table 3.3), probably translating the gradual decrease in the Cel7A and Cel7B activities from one round to another (Table 3.3). Still, it is worth noting that the decrease observed on glucans conversion appeared to occur on a higher rate comparatively to the depletion of enzymes activities. As example, while the relative decrease on glucans conversion was around 20.3 % from round 3 to round 4, final Cel7A activity only decreased 5.3 %. A

possible explanation may rely on the fact that the reported values of activity are associated to the utilization of specific substrates, which may not entirely simulate the hydrolysis of *n*RPS material. As commonly known, the lignocellulosics hydrolysis relies on the simultaneous action of several enzymes, being therefore limited by the availability of all of these enzymes. It is possible that, even conserving the ability to act on the low-molecular weight substrates (MUC; MULAC), a small part of these enzyme's ability to bind and/or convert *n*RPS material is lost during the several rounds of hydrolysis. Such fact inclusively supports the importance of adding some portion of fresh enzymes at the beginning of each new round, which can attenuate this effect.

Even with the abovementioned decreases on solid conversion, it should be referred that by using only 20 % of fresh enzyme on the second round, 86 % of glucans were converted, dropping to 72 % on a third round. From a technical-economic overall analysis, considering a four-round system, this strategy enables a 60 % saving on enzyme utilization. Process analysis should be carefully conducted in this context, as a proper balance must be achieved considering the decrease on solid conversion and the costs of strategies aiming further improvements. On this specific case, it should be considered that after a specific critical point, which can be assumed as the end of round 2, or ultimately the round 3, the values of glucans conversion decreased to a level that compromise the overall viability of this process. In the scope of an optimization of this system, some strategies may be explored to surpass this limitation. Increasing the hydrolysis time or the supplementation of fresh enzyme may result on valuable improvements, as was observed on this work.

Table 3.4 Multiple rounds of *n*RPS hydrolysis (72 hours hydrolysis [35 ºC]->6 hours SSF [30 ºC]) with cellulase recycling (20 FPU/$g_{cellulose}$; 30 % fresh enzymes)

Round	Glucose* (g/L)[a]	Ethanol (g/L)[a]	Glucans in final solid (%)[a]	Glucans conversion (%)[a]
1	16.15 ± 0.11	7.70 ± 0.02	1.78 ± 0.05	94.27 ± 0.16
2 (recycling 1)	15.79 ± 0.18	7.59 ± 0.06	2.50 ± 0.25	91.95 ± 0.80
3 (recycling 2)	14.21 ± 0.11	6.63 ± 0.03	5.27 ± 0.09	83.01 ± 0.30
4 (recycling 3)	11.99 ± 0.14	5.65 ± 0.08	9.08 ± 0.28	70.69 ± 0.91

* before yeast inoculation

a – differences between the values obtained in the distinct rounds are statistically significant ($p < 0.05$)

When the current study was conducted in similar conditions but with a hydrolysis time of 72 hours and 30 % fresh enzymes supplementation, considerable improvements were observed (Table 3.4; Figure 3.4). Comparatively to the previous scenario, the depletion on solid conversion between rounds occurred considerably slower as 83 % of glucans were still converted on round 3, opposing to 72 % observed in the first case. For the last round, 71 % of glucans were still converted, opposing to a prohibitive 57 % achieved in the first case.

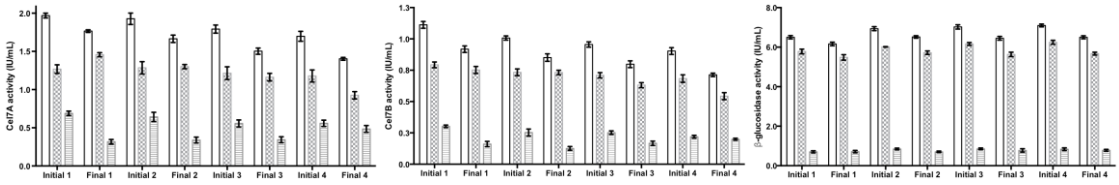

Figure 3.4 Variation of Cel7A, Cel7B and β-glucosidase activities (▌▌ Total activity Liquid phase Solid phase) over four rounds of *n*RPS hydrolysis (72 hours hydrolysis [35 ºC]->6 hours SSF [30 ºC]) with cellulase recycling. 20 FPU/$g_{cellulose}$ were initially employed with a posterior supplementation of 30 % fresh enzymes on each recycling stage; total activity was statistically different over the distinct rounds at a significant level ($p < 0.05$).

Similarly to what has been observed for glucans conversion, improvements were also detected in the activity profiles of the several cellulases (Figure 3.4), ultimately supporting the previous findings. Although similar enzyme distributions among different fractions were obtained, considerable improvements were observed in terms of enzyme stability along the different rounds. Specifically for the cases of Cel7A and Cel7B, the addition of a higher amount of fresh enzyme allowed not only to compensate the losses occurred during enzyme recycling (also observed on the previous scenario) but also to slightly exceed the final values of the previous round (before recycling). As a result, approximately 71.2 and 64.1 % of enzymatic activity was conserved over the four rounds for Cel7A and Cel7B, respectively. Higher improvements were even obtained for the case of β-glucosidase, as activity levels did not change over the entire process, although a small loss occurred during each round. For this particular component it was even possible to exceed the levels of enzymatic activity of the initial round, which together with previous findings could suggest that a lower amount of this enzyme can be initially employed.

Assuming that similar solid conversions are obtained in the different rounds, which may require some process adjustments (*e.g.* higher hydrolysis time), considerable savings on enzyme consumption can be expected through the implementation of this enzyme recycling system. In a four-rounds scenario, by decreasing the enzyme loading to only 30 %, a reduction of approximately 53 % can be expected on enzymes consumption. When applied on an industrial scale, tremendous economic gains can be achieved. For an industrial facility with an estimated annual production of 30 million gallons per year (DuPont have recently opened a similar facility in Nevada, IA, USA), and assuming an enzyme cost of approximately 0.5 $/gal$_{ethanol}$ (http://novozymes.com/en/news/news-archive/Pages/45713.aspx), the application of the above described strategy would enable an approximate saving around $8 million in enzymes. Several factors still need to be accounted for, such as the fact that enzymes can present a different cost (lower and higher values have been reported on the literature for this parameter) and that the recycling process also has a cost, but it seems very clear that, on a large scale production, this strategy of cellulase recycling can enable considerable economic gains. It should be considered, however, that these results were obtained for the particular case of *n*RPS material and different results may be expected for other materials. Therefore, this work shows first and mostly that *n*RPS is a suitable material to be employed in the scope of cellulase recycling, although giving some indications that similar lignocellulosic materials may also be employed, after proper testing.

3.4 Conclusions

This study demonstrated the feasibility of cellulase recycling following hydrolysis/fermentation of RPS. This system may be highly interesting economically, as it exploits a substrate with significant costs of disposal. A strategy of cellulase recycling was efficiently applied over 4 rounds of hydrolysis. The addition of only 30 % of fresh enzymes enabled an efficient conservation of activity levels and high solid conversions through the process. Additional improvements may still be achieved considering *e.g.* different times of hydrolysis or different fractions of fresh enzymes. Using this system, it was possible to reach enzyme savings in the range of 53-60 %.

3.5 References

Aden A, Foust T. 2009. Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. *Cellulose* 16:535-45.

Bailey MJ, Tähtiharju J. 2003. Efficient cellulose production by *Trichoderma reesei* in continuous cultivation on lactose medium with a computer-controlled feeding strategy. *Appl. Microbiol. Biotechnol.* 62:156-62.

Balwaik SA, Raut SP. 2011. Utilization of waste paper pulp by partial replacement of cement in concrete. *Int. J. Eng. Res. Appl.* 1:300-9.

Basso LC, Amorim HV, Oliveira AJ, Lopes ML. 2008. Yeast selection for fuel ethanol production in Brazil. *FEMS Yeast Res.* 8:1155-63.

Chen H, Venditti R, Gonzalez R, Phillips R, Jameel H, Park S. 2014. Economic evaluation of the conversion of industrial paper sludge to ethanol. *Energy Econ.* 44:281-90.

Chylenski P, Felby C, Haven MØ, Gama M, Selig MJ. 2012. Precipitation of *Trichoderma reesei* commercial cellulose preparations under standard enzymatic hydrolysis conditions for lignocelluloses. *Biotechnol. Lett.* 34:1475-82.

Dutta A, Dowe N, Ibsen KN, Schell DJ, Aden A. 2010. An economic comparison of different fermentation configurations to convert corn stover to ethanol using *Z. mobilis* and *Saccharomyces*. *Biotechnol. Prog.* 26:64-72.

Gomes D, Rodrigues AC, Domingues L, Gama FM. 2015. Cellulase recycling in biorefineries: is it possible?. *Appl. Microbiol. Biotechnol.* 99:4131-43.

Gomes DG, Guimarães PMR, Pereira FB, Teixeira JA, Domingues L. 2012. Plasmid-mediate transfer of *FLO1* into industrial *Saccharomyces cerevisiae* PE-2 strain creates a strain useful for repeat-batch fermentations involving flocculation–sedimentation. *Bioresour. Technol.* 108:162-8.

Ishihara M, Uemura S, Hayashi N, Jellison J, Shimizu K. 1991. Adsorption and desorption of cellulase components during enzymatic hydrolysis of steamed shirakamba (*Betula platyphylla* Skatchev) wood. *J. Ferment. Bioeng.* 72:96-100.

Klein-Marcusschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch WH. 2012. The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnol. Bioeng.* 109:1083-9.

Lark N, Xia YK, Qin CG, Gong CS, Tsao GT. 1997. Production of ethanol from recycled paper sludge using cellulase and yeast, *Kluveromyces marxianus*. *Biomass Bioenergy* 12:135-43.

Lindedam J, Haven M, Chylenski P, Jørgensen H, Felby C. 2013. Recycling cellulases for cellulosic ethanol production at industrial relevant conditions: potential and temperature dependency at high solid processes. *Bioresour. Technol.* 148:180-8.

Lynd LR, Laser MS, Bransby D, Dale BE, Davison B, Hamilton R, Himmel M, Keller M, McMillan JD, Sheehan J, Wyman CE. 2008. How biotech can transform biofuels. *Nat. Biotech.* 26:169-72.

Marques S, Alves L, Roseiro JC, Gírio FM. 2008a. Conversion of recycled paper sludge to ethanol by SHF and SSF using *Pichia stipites*. *Biomass Bioenergy* 32:400-6.

Marques S, Santos JAL, Gírio FM, Roseiro JC. 2008b. Lactic acid production from recycled paper sludge by simultaneous saccharification and fermentation. *Biochem. Eng. J.* 41:210-16.

Monte MC, Fuente E, Blanco A, Negro C. 2009. Waste management from pulp and paper production in the European Union. *Waste Manage.* 29:293-308.

Park EY, Michinaka A, Okuda N. 2001. Enzymatic hydrolysis of waste office paper using viscosity as operating parameter. *Biotechnol. Prog.* 17:379-82.

Pereira FB, Gomes DG, Guimarães PMR, Teixeira JA, Domingues L. 2012. Cell recycling during repeated very high gravity bio-ethanol fermentations using the industrial *Saccharomyces cerevisiae* strain PE-2. *Biotechnol. Lett.* 34:45-53.

Pereira FB, Guimarães PMR, Teixeira JA, Domingues L. 2011. Robust industrial *Saccharomyces cerevisiae* strains for very high gravity bio-ethanol fermentations. *J. Biosci. Bioeng.* 112:130-6.

Pereira FB, Guimarães PMR, Teixeira JA, Domingues L. 2010. Selection of *Saccharomyces cerevisiae* strains for efficient very high gravity bio-ethanol fermentation processes. *Biotechnol. Lett.* 32:1655-61.

Pereira FB, Romaní A, Ruiz HA, Teixeira JA, Domingues L. 2014. Industrial robust yeast isolates with great potential for fermentation of lignocellulosic biomass. *Bioresour. Technol.* 161:192-9.

Presetyo J, Park EY. 2013. Waste paper sludge as a potential biomass for bio-ethanol production. *Korean J. Chem. Eng.* 30:253-61.

Pribowo A, Arantes V, Saddler JN. 2012. The adsorption and enzyme activity profiles of specific *Trichoderma reesei* cellulose/xylanase components when hydrolyzing steam pretreated corn stover. *Enzyme Microb. Technol.* 50:195-203.

Rodrigues AC, Felby C, Gama M. 2014. Cellulase stability, adsorption/desorption profiles and recycling during successive cycles of hydrolysis and fermentation of wheat straw. *Bioresour. Technol.* 156:163-9.

Rodrigues AC, Leitão AF, Moreira S, Felby C, Gama M. 2012. Recycling of cellulases in lignocellulosic hydrolysates using alkaline elution. *Bioresour. Technol.* 110:526-33.

Romani A, Pereira F, Johansson B, Domingues L. 2015. Metabolic engineering of *Saccharomyces cerevisiae* ethanol strains PE-2 and CAT-1 for efficient lignocellulosic fermentation. *Bioresour. Technol.* 179:150-8.

Shang Y, Su R, Huang R, Yang Y, Qi W, Li Q, He Z. 2014. Recycling cellulases by pH-triggered adsorption-desorption during the enzymatic hydrolysis of lignocellulosic biomass. *Appl. Microbiol. Biotechnol.* 98:5765-74.

Sun Y, Cheng J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour. Technol.* 83:1-11.

Tu M, Chandra RP, Saddler JN. 2007. Evaluating the distribution of cellulases and the recycling of free cellulases during the hydrolysis of lignocellulosic substrates. *Biotechnol. Progr.* 23:398-406.

Zhu Z, Sathitsuksanoh N, Zhang Y-HP. 2009. Direct quantitative determination of adsorbed cellulase on lignocellulosic biomass with its application to study cellulose desorption for potential recycling. *Analyst* 134:2267-72.

Web references

1. http://novozymes.com/en/news/news-archive/Pages/45713.aspx (last accessed in 15-04-2016)

2. http://www.nrel.gov/docs/fy13osti/59013.pdf (last accessed in 15-04-2016)

Chapter IV

Determinants on an efficient cellulase recycling process for the production of bio-ethanol from recycled paper sludge under high solid loadings

The work reported on this chapter was published in:
Daniel G. Gomes, Miguel Gama, Lucília Domingues. 2018. Determinants on an efficient cellulase recycling process for the production of bioethanol from recycled paper sludge under high solid loadings. Biotechnology for Biofuels, 11:111.

Abstract

In spite of the continuous efforts and investments in the last decades, lignocellulosic ethanol is still not economically competitive with fossil fuels. Optimization is still required in different parts of the process. Namely, the cost effective usage of enzymes has been pursued by different strategies, one of them being recycling.

Cellulase recycling was analyzed on Recycled Paper Sludge (RPS) conversion into bioethanol under intensified conditions. Different cocktails were studied regarding thermostability, hydrolysis efficiency, distribution in the multiphasic system and recovery from solid. Celluclast showed inferior stability at higher temperatures (45-55 ºC), nevertheless its performance at moderate temperatures (40ºC) was slightly superior to other cocktails (ACCELLERASE®1500 and Cellic®CTec2). Celluclast distribution in the solid-liquid medium was also more favorable, enabling to recover 88 % of final activity at the end of the process.

A Central Composite Design studied the influence of solids concentration and enzyme dosage on RPS conversion by Celluclast. Solids concentration showed a significant positive effect on glucose production, no major limitations being found from utilizing high amounts of solids under the studied conditions. Increasing enzyme loading from 20 to 30 FPU/$g_{cellulose}$ had no significant effect on sugars production, suggesting that 22 % solids and 20 FPU/$g_{cellulose}$ are the best operational conditions towards an intensified process. Applying these, a system of multiple rounds of hydrolysis with enzyme recycling was implemented, allowing to maintain steady levels of enzyme activity with only 50 % of enzyme on each recycling stage. Additionally, interesting levels of solid conversion (70-81 %) were also achieved, leading to considerable improvements on glucose and ethanol production comparatively with the reports available so far (3.4 and 3.8 *fold*, respectively).

Enzyme recycling viability depends on enzyme distribution between the solid and liquid phases at the end of hydrolysis, as well as enzymes thermostability. Both are critical features to be observed for a judicious choice of enzyme cocktail. This work demonstrates that enzyme recycling in intensified biomass degradation can be achieved through simple means. The process is possibly much more effective at larger scale,

hence novel enzyme formulations favoring this possibility should be developed for industrial usage.

4.1 Background

Over the last decades, lignocellulosic ethanol assumed a major role on the definitive affirmation of biofuels in the new global energy picture. Relying on cheaper raw-materials, such as agro-forestry wastes, it can represent an important boost for the economy of small and local communities (Abban-Mensah *et al*., 2014). Additionally, it may also encompass the utilization of industrial/municipal wastes, enabling some value recovery from a negative-cost material and a reduction on its environmental impact.

Despite the notorious progresses made, the development of suitable hydrolytic enzymes still faces challenges, such as the high cost and sensitivity to process conditions. Distinct estimations for the cost of cellulases have been pointed out by different studies. According to Klein-Marcusschamer *et al*. (2012), the cellulase cost on ethanol production is approximately $ 0.68 per gallon, close to $ 0.5 per gallon suggested by Novozymes (http://novozymes.com/en/news/news-archive/Pages/45713.aspx). Aden and Foust (2009), however, already reported a value around $ 0.1 per gallon, similar to $ 0.3 reported by Lynd *et al*. (2008) and $ 0.32 reported by Dutta *et al*. (2010). Even though important reductions have been achieved on their production cost, operated by intense research from both industry and academia, some authors already admitted these strategies will not allow much further reductions. Independently of the current cost of enzymes, it is widely recognized as a critical determinant for cellulosic ethanol competitiveness.

A reduction on cellulase cost has been intensively pursued through different strategies, being one of them the reutilization of enzymes (Pribowo *et al*., 2012). This has been achieved by distinct ways: recovering enzymes by ultra-filtration (Gomes *et al*., 2016; Rodrigues *et al*., 2014; Chen *et al*., 2013; Yang *et al*., 2010); re-adsorption of free enzymes into fresh solid (Huang *et al*., 2017; Gomes *et al*., 2015; Shang *et al*., 2014; Eckard *et al*., 2013; Tu and Saddler, 2010); finally, partial recycling of whole final medium, and consequently, of the enzymes (Haven *et al*., 2015). While less complex, the two later options present limitations that can severely hamper an efficient recovery process. Re-adsorption into fresh solid requires that a significant fraction will efficiently adsorb over the process of solids separation. Also, low cellulose-binding enzymes, such as β-glucosidase, would require to be supplemented (Haven *et al*., 2015; Tu *et al*., 2007;

Lee *et al*., 1995). On the other hand, partial/total whole medium (solids and liquid) recycling will always be restricted by lignin build-up constraints and the consequent increase of non-productive enzyme binding (Jørgensen and Pinelo, 2017). As an alternative, ultra-filtration can allow an efficient separation of enzymes, that can then be directly applied on a new hydrolysis process. In addition to being potentially more expensive, the late approach requires the enzymes to be freely available in the liquid phase, *i.e.*, they should have low affinity towards the final solid residue. Hence, a critical role is attributed to the composition and structure of the raw-material but also to the selected cellulases. Both have shown to significantly affect the specific distribution of free (soluble) and solid-bound (adsorbed) enzymes as well as the effectiveness of their recovery (Pribowo *et al*., 2012; Rodrigues *et al*., 2014). Enzymes adsorbed to the solid can still be recovered by pH switch (Shang *et al*., 2014; Rodrigues *et al*., 2012; Du *et al*., 2012) or by using different chemicals (Eckard *et al*., 2013; Sipos *et al*., 2010). Therefore, it seems clear that the binomial substrate-enzyme will determine the most suitable recycling strategy for each case.

In the scope of a more economic process, also intensification has been pursued from multiple angles, namely through an increase on solid loadings (Cunha *et al*., 2018; Romaní *et al*., 2016) or through an optimized integration of hydrolysis and fermentation (Kelbert *et al*., 2016; Kelbert *et al*., 2015; Romaní *et al*., 2014). For high-water retention materials, such RPS (recycled paper sludge), converting high-solid loadings represents however a serious challenge as enzymes have a reduced mobility due to a lower free liquid in suspension. In fact, Marques *et al.* (2008) reported 17.9 % RPS as the maximum solid concentration that enabled hydrolysis. Considering the moderate levels of cellulose and hemicellulose in this material, maximizing sugars concentration on the final hydrolysate is critical for a sustainable process. On the other hand, this should also be taken into account when selecting and designing a cellulase recycling strategy. High solid loadings and/or materials with a high lignin content could be a serious challenge, particularly when solid is recycled.

Here, a structured and sequential study was performed on the implementation of cellulase recycling in the process of bioethanol production from recycled paper sludge under high solid loadings. The performance of different cellulase cocktails is addressed in terms of hydrolytic performance, stability and final enzyme recovery. Aiming at

process intensification, the effect of higher amounts of solid and enzyme on the hydrolysis efficiency is studied, in order to find the best operational conditions. Those were then considered on the implementation of a system of multiple rounds with cellulase recycling where the levels of enzyme activity and solid conversion were evaluated.

4.2 Materials and Methods

4.2.1 Enzymes, substrate and microorganisms

Enzymatic hydrolysis assays were conducted separately with different cellulase cocktails: Celluclast 1.5 L (from Novozymes A/S); ACCELLERASE® 1500[b,c] (from DuPont); Cellic® CTec2 (from Novozymes A/S). FPase activity of these preparations were determined to be 60, 40 and 120 FPU/mL, respectively. Also, pNPG β-glucosidase activities were determined as 42, 499 and 3609 U/g, respectively. The protein content assessed by Bradford assay (using BSA as standard) were 30, 20 and 58 mg/g, respectively.

Due to the low level of β-glucosidase activity found on Celluclast, this cocktail was always supplemented with the β-glucosidase preparation Novozyme 188 (from Novozymes A/S) on a β-glucosidase/FPase ratio of 3.

Recycled paper sludge (RPS) was kindly provided by RENOVA (Torres Novas, Portugal) and refers to the residue obtained from the wastewater treatment of paper recycling effluents generated by this company. Due to its high carbonates content, which results on an alkaline solid with a reduced holocellulose fraction, prior to its utilization RPS was treated with hydrochloric acid 37 % and then washed, first with water and then with buffer (0.1 M acetic acid/sodium acetate) (Gomes *et al*., 2016). This resulted on a neutralized RPS (*n*RPS), which was used in the current work, with an increased holocellulose fraction: 27.1 % cellulose, 7.3 % xylan and 65.7 % acid-insoluble solid.

Fermentations were conducted with *Saccharomyces cerevisiae* CA11, a strain which was recently reported to have a good fermentation performance at high temperatures (Costa *et al*., 2017; Ruiz *et al*., 2012).

4.2.2 Thermostability assays

In order to assess which cellulase mixture is more stable towards thermal deactivation, the efficiency of *n*RPS (carbonates-neutralized RPS) solid conversion was quantified after enzymes exposure to increasing periods of incubation at different temperatures (45 ºC, 50 ºC and 55 ºC). Then, after the pre-incubation period, *n*RPS hydrolysis for 18 hours, with 5 % (w/v) solids at 50 ºC, was performed to evaluate the remaining activity.

4.2.3 Comparative hydrolysis efficiency and enzyme activity phase distribution of different cellulase mixtures

To enable a direct comparison of the performance of the three cellulase mixtures, their profiles of glucose production was studied using two distinct solids concentrations (10 and 18 % (w/v)). For that purpose, the solid suspension was incubated with a volume of enzyme equivalent to 20 FPU/$g_{cellulose}$ in 0.1 M sodium acetate/acetic acid buffer (pH of 4.8) and incubated at 40 ºC for 96 hours.

To evaluate activity distribution of the three cellulase mixtures in the multiphasic system, Cel7A (major cellulase component of *Trichoderma reesei* cocktails) levels were quantified in both the solid and liquid fractions, both after hydrolysis and alkaline washing (Rodrigues *et al*., 2012).

4.2.4 Effect of solids concentration and enzyme loading on the efficiency of *n*RPS hydrolysis

The effect of both solids concentration and enzyme loading on the efficiency of *n*RPS hydrolysis was studied conducting a central composite inscribed (CCI) design. Each factor was tested for 5 levels for the nominal values of -1, -0.7, 0, +0.7 and +1. Solids concentration was tested in the range of 14-22 % (w/v), defined according to preliminary tests on the mixing efficiency as a function of *n*RPS consistency. Enzyme loading was set to the range of 20-30 FPU/$g_{cellulose}$. The lower level is within the usual values employed on the literature (Pribowo *et al*., 2012; Gomes *et al*., 2016; Domínguez *et al*., 2017; Rodrigues *et al*., 2015). The upper level is slightly superior to evaluate potential improvements on enzyme hydrolysis efficiency. In the context of enzyme recycling, the overall enzyme load is actually reduced, as only a fraction of the initial load is used in the subsequent cycles.

The matrix of the CCI design with both the nominal and the real values is presented in Table 4.1.

Table 4.1 CCI design matrix presenting the normalized and the real values for each run

	Normalized value		Real value	
Run	**X_1**	**X_2**	**X_1 [%(w/v)]**	**X_2 [FPU/$g_{cellulose}$]**
1	-1	-1	14	20
2	-1	0	14	25
3	-1	+1	14	30
4	0	+1	18	30
5	+1	+1	22	30
6	+1	0	22	25
7	+1	-1	22	20
8	0	-1	18	20
9	0	0	18	25
10	0	0	18	25
11	0	0	18	25
12	0	0	18	25
13	-0.7	-0.7	15.2	21.5
14	-0.7	+0.7	15.2	28.5
15	+0.7	+0.7	20.8	28.5
16	+0.7	-0.7	20.8	21.5

*X_1 n*RPS solids concentration, *X_2* enzyme dosage

4.2.5 Multiple rounds of hydrolysis with enzyme recycling

Enzymatic hydrolysis in the context of cellulase recycling were conducted similarly to the single-round experiments. For the first round, the sterilized solids suspension (22 % (w/v)) was mixed with 20 FPU/$g_{cellulose}$ of Celluclast (complemented with β-glucosidase) and incubated for 120 hours (40 ºC; 200 rpm). Afterwards, this mixture was inoculated with 8 g/L (fresh biomass) CA11 yeast cells and incubated for 24 hours at 35 ºC.

At the end of the round, final broth was centrifuged (9000 rpm for 20 minutes) to separate fractions. Supernatant, containing free enzymes (in the liquid fraction), was filtered through a 0.22 µm Polyethersulfone (PES) filter to remove impurities and stored (4 ºC) until further use. The solid was subjected to an alkaline washing, as previously described on Chapter III. The elution liquid, containing the desorbed enzymes, was filtered to remove major impurities and stored until use. Prior to its storage the pH of this liquid was adjusted to the common operational pH (4.8) through the addition of 1 M acetic acid/sodium acetate buffer (pH 4.8). Final solid was repeatedly washed, oven dried (at 45 ºC) until an estimated water content below 10 % was reached, and finally stored until final analysis.

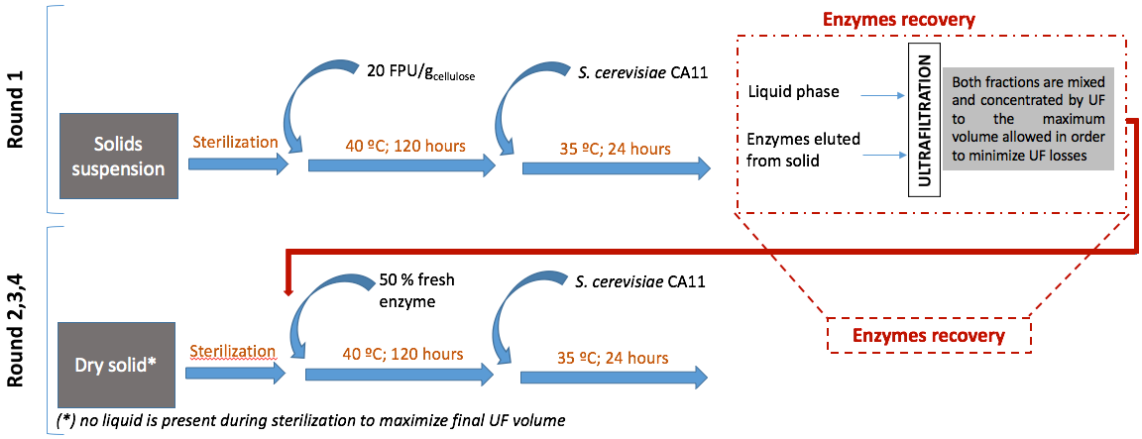

Figure 4.1 Schematic representation for the system of multiple rounds of hydrolysis (and fermentation) with cellulase recycling.

For cellulase recycling, both enzyme containing fractions (stored at 4 ºC) were mixed and concentrated using a tangential ultrafiltration system Pellicon XL membrane with a 10 kDa cut-off PES membrane (Millipore, Billerica, MA, USA). The two fractions were initially concentrated by diafiltration and, at the end, adjusted to a final fixed volume. For a new round of hydrolysis, the freshly sterilized solid was resuspended on the enzyme suspension obtained from the previous ultrafiltration procedure, filter-sterilized with 0.2 µm PES syringe filters. For each recycling stage, a portion of fresh enzyme was added to this suspension, corresponding to 50 % of the original enzyme dosage (maintaining the β-glucosidase/FPase activity ratio). The new solids suspension was then subjected to the same conditions of hydrolysis and fermentation, as previously described. This procedure was applied over a total of 4 rounds of hydrolysis and fermentation as schematically described on Figure 4.1.

4.2.6 Analytical procedures

Sugars and ethanol quantification

After thawing, aliquots from hydrolysis and fermentation experiments were diluted, filtered and then analyzed by HPLC (High-Performance Liquid Chromatography) for glucose and ethanol quantification. Samples were eluted on a Varian MetaCarb 87H column at 60 ºC, with 0.005 M H_2SO_4 at a flow rate of 0.7 mL/min, and a refractive-index detector.

Measurement of enzymatic activity

Samples collected for quantification of enzymatic activity were stored at 4 ºC until further utilization. Cel7A, Cel7B and β-glucosidase activities were quantified by fluorescence spectroscopy with slight differences according to the specific cellulolytic component, following a modified version of the protocol previously published by Bailey and Tähtiharju (2003). For Cel7A, Cel7B and β-glucosidase quantification, 400 µL of a freshly prepared solution of 1 mM 4-methylumbelliferyl-b-D-cellobioside (MUC, Sigma–Aldrich, M6018), 4-methylumbelliferyl-b-D-lactopyranoside (MULac, Sigma–Aldrich, M2405) and 4-methylumbelliferyl-b-D-glucopyranoside (MUGlc, Sigma–Aldrich, M3633), respectively, were mixed with 50 µL of enzyme sample (properly diluted on buffer considering the linearity range of the method) and then incubated for 15 minutes at 50 ºC. After that, the reaction was stopped by the addition of 550 µL of 1 M Na_2CO_3 and measured on a black bottom 96-well UV fluorescence microplate using a Biotech Synergy HT Elisa plate reader. For Cel7B quantification, the addition of 50 µL of a mixture containing 1 M glucose and 50 mM cellobiose is still required, in order to inhibit Cel7A and β-glucosidase activities. Cel7A, Cel7B and β-glucosidase act on their specific substrates releasing free 4-methylumbelliferone (MU, Sigma– Aldrich, M1508), which results on a change of the fluorescence spectra that is quantified for an excitation and emission wavelengths of 360 and 460 nm, respectively.

Determination of solid composition

The solids main composition, either corresponding to the initial material or after enzymatic hydrolysis, was determined by quantitative acid hydrolysis (Sluiter *et al*., 2008). After oven drying (at 45 ºC) to a water content inferior to 10 %, approximately 0.5 g of solid was mixed with 5 mL of 72 % (w/v) H_2SO_4 for 1 hour at 30 ºC. Afterwards, this mixture was subjected to a dilute hydrolysis by raising the volume with water to a total mass of 148.67 g and subsequently autoclaved for 1 hour at 121 ºC. Next, the solid residue was recovered by filtration (cresol Gooch nº3) and dried (at 105 ºC) until constant weight. Different sugar monomers formed during hydrolysis were quantified by HPLC analysis of the liquid fraction.

Estimation of hydrolysis and fermentation yields

For an overall assessment of hydrolysis and fermentation processes, glucose and ethanol production yields (GY_{120} and EY_{23}, respectively) were estimated according to the following equations:

$$GY_{120}(\%) = \frac{[Glucose]_{120} + 1.053[Cellobiose]_{120}}{1.111[Solids]_i \times F_{cel}} \times 100$$

$$EY_{23}(\%) = \frac{[Ethanol]_{23}}{0.51(1.111[Solids]_i \times F_{cel} \times 0.963)} \times 100$$

where $[Glucose]_{120}$ and $[Cellobiose]_{120}$ are the concentrations of glucose and cellobiose, respectively, at 120 of hydrolysis and $[Ethanol]_{23}$ is the ethanol concentration at 23 hours of fermentation. $[Solids]_i$ refers to the initial concentration of dry solid and F_{cel} is the fraction of cellulose on a dry solid base. 1.111 consists on the glucan to glucose conversion ratio, 0.51 is the maximum theoretical conversion of glucose into ethanol and 0.963 was the dilution factor imposed by cells inoculation.

4.3 Results and Discussion

On the previous chapter of this thesis it was demonstrated that *n*RPS can be used for bioethanol production, and additionally, is suitable for the implementation of a cellulase recycling system (Gomes *et al*., 2016). As a proof-of-concept approach, these tests were however conducted under non-intensified conditions (5 % (w/v) solids; hydrolysis temperature of 35 ºC).

Here two important factors were addressed targeting the scalability and the economic feasibility of the process, either in terms of *n*RPS solid conversion but also on the integration of an enzyme recycling system: the selection of the cellulase cocktail and the intensification of solid conversion.

4.3.1 Thermostability of different cellulase mixtures

Considering that optimal enzymatic hydrolysis occurs around 50 ºC, increased thermostabilities represents an important feature in the context of enzymes re-utilization. Figure 4.2 presents the variation of *n*RPS solid conversion after incubation of the cellulase suspension at 45 ºC, 50 ºC and 55 ºC, for different time periods.

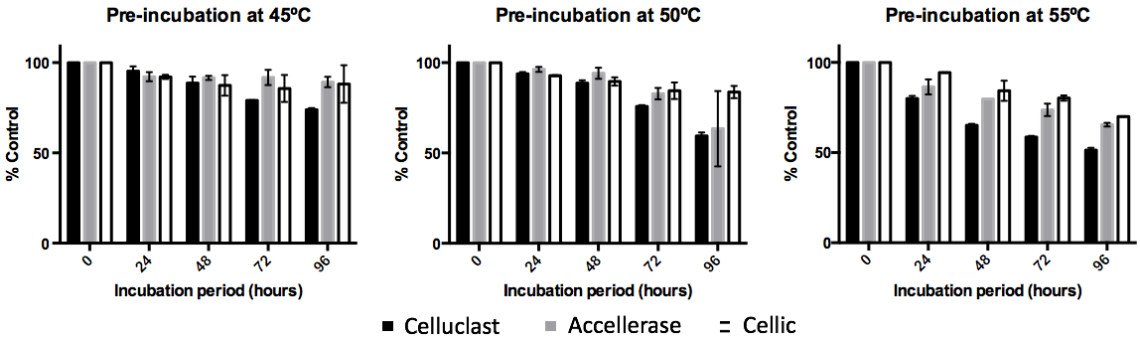

Figure 4.2 Variation of solid conversion by different cellulase mixtures after increasing periods of pre-incubation at different temperatures.

As expected, all cocktails presented an increasing loss of hydrolysis capacity with cumulative periods of incubation, being this behavior more prominent for higher temperatures. As an example, for an incubation at 45 ºC, after 72 hours of incubation the conversion degree still remained above 78 % for all cocktails comparatively to the control levels. On the other hand, for a temperature of 55 ºC the conversion dropped to 59, 74 and 80 % for Celluclast 1.5 L (Celluclast), ACCELLERASE® 1500 (Accellerase) and

Cellic® CTec2 (Cellic), respectively. Differences on thermal deactivation between cocktails were minor for the smallest periods of incubation, excepting for the study at 55 ºC, where some differences are already found on an early stage. Considering an incubation period equal or higher to 48 hours, significant differences are visible. The hydrolysis efficiency of Celluclast was significantly more affected comparing to Accellerase or Cellic. It is worth noting, however, that the absolute values of glucose production were 4-21 % higher for the case of Celluclast, as described in more detail in the next section.

4.3.2 Hydrolysis efficiency of different cellulase cocktails

Thermal deactivation assays were not enough to clearly identify the most suitable cellulase cocktail to be employed at moderate-high temperatures. Although Celluclast present an inferior resistance to thermal denaturation, it enabled higher values of solid conversion. Therefore, and considering the notorious reduction of activity observed in the range of 45-55 ºC, which may be especially critical on a cellulase recycling context, the profiles of glucose production obtained by the three cocktails were evaluated for a temperature of 40 ºC at different solid concentrations (Figure 4.3). Thermal denaturation tests conducted with Celluclast at 40 ºC on a week-long experiment provided indications of no activity loss under these conditions.

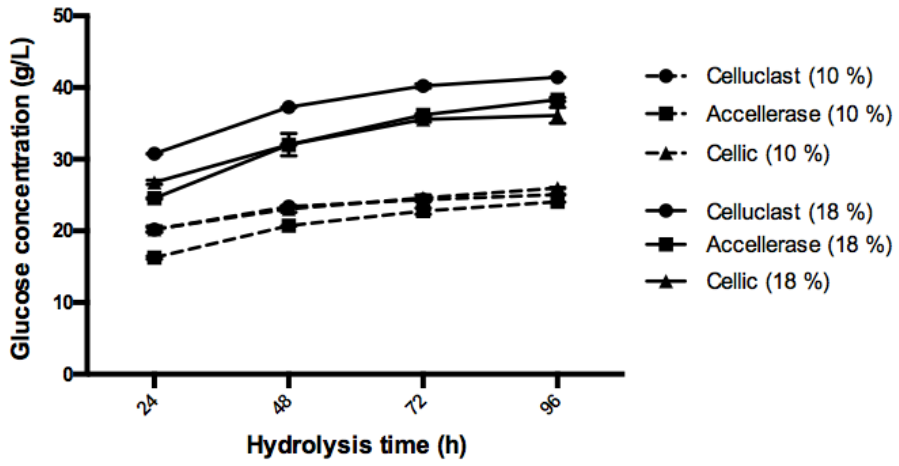

Figure 4.3 Profiles of glucose production using distinct enzyme mixtures under different solid concentrations, at 40ºC.

For a solid concentration of 10 % there was not a significant difference on solid conversion between cocktails, although Accellerase presented a slightly inferior

performance on the first 48-72 hours. On the other hand, for 18 % solids Celluclast enabled an average 15 % higher glucose production over the entire hydrolysis period, comparatively to the other cocktails. These results suggest that at moderate temperatures (40ºC) where thermal denaturation is low or absent, both Accellerase and Cellic could not surpass Celluclast. It is worth to mention that even supplemented with Novozyme 188, β-glucosidase levels on Celluclast assays are considerably inferior comparatively to the other cocktails: 4.11 U/mL for Celluclast; 13.53 and 37.41 U/mL for Accellerase and Cellic, respectively. This seems to confirm that on this set of conditions (enzyme and solid loadings) the levels of β-glucosidase are not limiting the hydrolysis, as suggested by the absence of cellobiose accumulation (*data not shown*), hence it does not represent a relevant factor for the different performances.

On these particular conditions, Celluclast seemed to present a slight advantage over the other cocktails regarding hydrolysis performance, nevertheless, enzyme distribution between phases still needed to be assessed.

4.3.3 Phase activity distribution and efficiency of alkaline washing

The final activity distribution among solid and liquid fractions is critical for enzyme recycling and process complexity. Even though lignin represents nearly 20 % of RPS composition (Marques *et al*., 2008), being commonly reported as an efficient enzyme adsorbent (by non-productive binding), it was recently observed that 70 % of final Cel7A activity is found on the liquid fraction after hydrolysis of RPS with Celluclast under 5 % (w/v) solids (Chapter III; Gomes *et al*., 2016). This represents a good scenario for enzyme reutilization, as a significant part of the activity is easily recovered.

As reported by other authors (Pribowo *et al*., 2012; Rodrigues *et al*., 2015), different cellulase mixtures may display diverse solid-liquid distributions. To enable the evaluation of the different cellulase mixtures behavior in this regard, Cel7A levels were quantified on both liquid and solid fractions after hydrolysis and alkaline washing, used to extract the adsorbed enzyme (Table 4.2).

Table 4.2 Final distribution of Cel7A activity after hydrolysis of *n*RPS and alkaline washing using different cellulase mixtures

		Celluclast		**Accellerase**		**Cellic**	
Initial activity (IU/mL)		7.837 ± 0.341		18.107 ± 0.102		15.003 ± 0.411	
		Activity level (IU/mL)	Fraction (%)**	Activity level (IU/mL)	Fraction (%)**	Activity level (IU/mL)	Fraction (%)**
Activity after hydrolysis	**Liquid**	**4.566 ± 0.508**	**61.3**	**12.646 ± 0.361**	**62.9**	**6.031 ± 0.100**	**40.1**
	Solid	**2.077 ± 0.121**	**38.7**	**5.391 ± 0.056**	**37.1**	**6.519 ± 0.596**	**59.9**
Alkaline washing	**Liquid**	**1.381 ± 0.077**	**60.2**	**4.281 ± 0.038**	**52.5**	**4.194 ± 0.081**	**41.2**
	Solid	**0.651 ± 0.071**	**39.8**	**2.791 ± 0.020**	**47.5**	**4.331 ± 0.125**	**58.8**
Overall recovery (%)*		**87.9**		**80.8**		**60.2**	

Hydrolysis were conducted for 96 hours with 18 % solids and 20 FPU/$g_{cellulose}$ at 40ºC
(*) – refers to the sum of the free enzymes on the liquid phase after hydrolysis and alkaline elution
(**) – refers to the fraction of the total number of IUs found on each fraction

It is worth of note firstly that significant differences were observed regarding the initial levels of Cel7A for the different cocktails even though the same FPU activity was applied on every case. This suggests differences on the composition of each cocktail and on its synergetic mechanisms of enzymatic hydrolysis. Taking into account the values of Cel7A activity one can observe that Celluclast and Accellerase distribute similarly among fractions, with 61.3 and 62.9 % of total final activity being found on the liquid fraction, respectively. A significant part still remains adsorbed to the final solid, hampering a more efficient enzyme re-utilization. In what concerns Cellic mixture, the enzyme levels on solid fraction were even higher, close to 60 % of the final activity. Similarly, different efficiencies were also attained for alkaline washing: 60 %, 53 % and 41 % of the enzymes were recovered for Celluclast, Accellerase and Cellic, respectively. As the performances of the different cocktails did not vary considerably (and consequently the final solid composition), no major differences on enzyme fractionation are expected due specifically to distinct binding affinities to cellulose and lignin (Rodrigues *et al*., 2014). On the other hand, these results seem to suggest that different cellulase preparations can, in fact, present very distinct enzyme fractionation profiles for the same material, possibly due to different binding affinities associated to enzymes from different sources.

A similar difference was observed by Rodrigues *et al*. (2015) for Celluclast and Cellic binding during the hydrolysis of wheat straw: 26-28 % of original Cel7A activity was found soluble on the final liquid fraction on Celluclast; final soluble Cel7A for Cellic was only around 6 %. Also, a recent study conducted by Strobel *et al*. (2015) have demonstrated that specific mutations on the *T. reesei* Cel7A *CBM* can cause significant differences on the binding affinity to both cellulose and lignin, confirming the determinant role of enzyme properties on its binding mechanism to distinct fractions of the solid.

As it can be seen from Table 4.2, it was possible to achieve an overall recovery of final activity in the range of 60 %, for Cellic, 81% for Accellerase and 88 %, for Celluclast. Thus, the two later cocktails may be recycled to larger extent, potentially enabling important savings.

4.3.4 Effect of *n*RPS concentration and cellulase loading

Even though *n*RPS is a residue currently with a negative price associated to disposal costs, maximization of solids concentration should still be pursued, as more concentrated hydrolysates allow higher productivities and lower process costs (*e.g.* distillation). Preliminary studies indicated that a maximum level of 22 % (w/v) in solids consistency can be used, still enabling the "liquefaction" of fibres through enzymes action. For higher amounts of solid a very-high viscosity suspension is obtained which enzymes are unable to process.

Considering the results from previous sections - thermostability, hydrolysis efficiency and distribution in the heterogeneous system (recyclability) - Celluclast was chosen for a CCI design studying the influence of enzyme loading and solids concentration on the *n*RPS hydrolysis (Table 4.3).

From the results of the CCI design four distinct variables of response were fitted to the experimental data through a second-order polynomial model: glucose concentration (Glu_{120}) and production yield (GY_{120}) after 120 of hydrolysis; ethanol concentration (Eth_{23}) and production yield (EY_{23}) after 23 hours of fermentation (Eth_{23}). The models representing the variables of response as a function of the normalized values of solids concentration (X_1) and enzyme loading (X_2) are presented on the equations 1-4.

Table 4.3 Experimental values obtained from a CCI design testing different levels of solid concentration and enzyme loadings

Run	g_{solids}/mL_{liquid} (%)	$FPU/g_{cellulose}$	Glu_{120}(g/L)	Eth_{23}(g/L)	GY_{120} (%)	EY_{23}(%)
1	14.0	20.0	34.8	15.7	84.7	78.0
2	14.0	25.0	36.3	17.4	88.4	86.2
3	14.0	30.0	38.0	18.8	92.6	93.1
4	18.0	30.0	46.7	24.1	88.4	92.9
5	22.0	30.0	58.9	29.4	91.3	92.8
6	22.0	25.0	56.6	28.7	87.6	90.7
7	22.0	20.0	52.4	26.8	81.2	84.5
8	18.0	20.0	44.0	21.2	83.3	81.6
9	18.0	25.0	47.1	21.5	89.1	83.0
10	18.0	25.0	44.8	22.1	84.8	85.2
11	18.0	25.0	46.3	22.4	87.7	86.3
12	18.0	25.0	46.3	22.3	87.7	85.9
13	15.2	21.5	38.3	18.6	85.8	85.0
14	15.2	28.5	40.6	19.9	91.0	90.9
15	20.8	28.5	52.6	26.9	86.2	89.6
16	20.8	21.5	51.1	24.6	83.7	82.2

$$Glu_{120} = 45.955 + 9.560X_1 + 1.891X_2 + 0.515X_1^2 - 0.584X_2^2 + 0.573X_1X_2 \quad \text{(Equation 1)}$$

$$GY_{120} = 87.025 - 1.322X_1 + 3.557X_2 + 1.153X_1^2 - 1.017X_2^2 + 0.165X_1X_2 \quad \text{(Equation 2)}$$

$$Eth_{23} = 22.212 + 5.285X_1 + 1.391X_2 + 0.512X_1^2 - 0.087X_2^2 + 0.012X_1X_2 \quad \text{(Equation 3)}$$

$$EY_{23} = 87.762 + 0.975X_1 + 5.533X_2 + 1.551X_1^2 + 0.414X_2^2 - 1.207X_1X_2 \quad \text{(Equation 4)}$$

From ANOVA analysis it was verified that these models adequately represent the values of Glu_{120}, GY_{120}, Eth_{23} and EY_{23}, with an estimated determination coefficient (R^2) of 0.989, 0.824, 0.989 and 0.877, respectively. *F*-value was higher than the tabular F (3.33)

for all the models, indicating that they are statistically significant for a confidence level of 95 %. Additionally, the non-significant values of lack of fit also suggest an adequate fitting of the different models (Table 4.4). For each model the correspondent response surface was constructed, in order to better visualize the influence of each variable on the different responses (Figure 4.4).

Table 4.4 Regression indicators and analysis of variance (ANOVA) for the different models

Indicator		Glu_{120}	Eth_{23}	GY_{120}	EY_{23}
p-value	X_1	4.79E-11	4.91E-11	0.04175	0.18523
	X_2	0.00017	1.60E-11	9.122E-5	1.090E-5
	X_1^2	0.38003	0.13104	0.26593	0.21956
	X_2^2	0.32261	0.78432	0.32293	0.73420
	X_1X_2	0.19371	0.96022	0.82280	0.19477
F-value (model)		180.660	184.940	9.36420	14.3010
Significance F		1.82E-9	1.62E-9	0.00156	0.00028
F-value (lack of fit)		0.89019	2.00101	0.68907	1.93724
R^2		0.98905	0.98930	0.82401	0.87731
R^2_{adj}		0.98358	0.98395	0.73601	0.81596

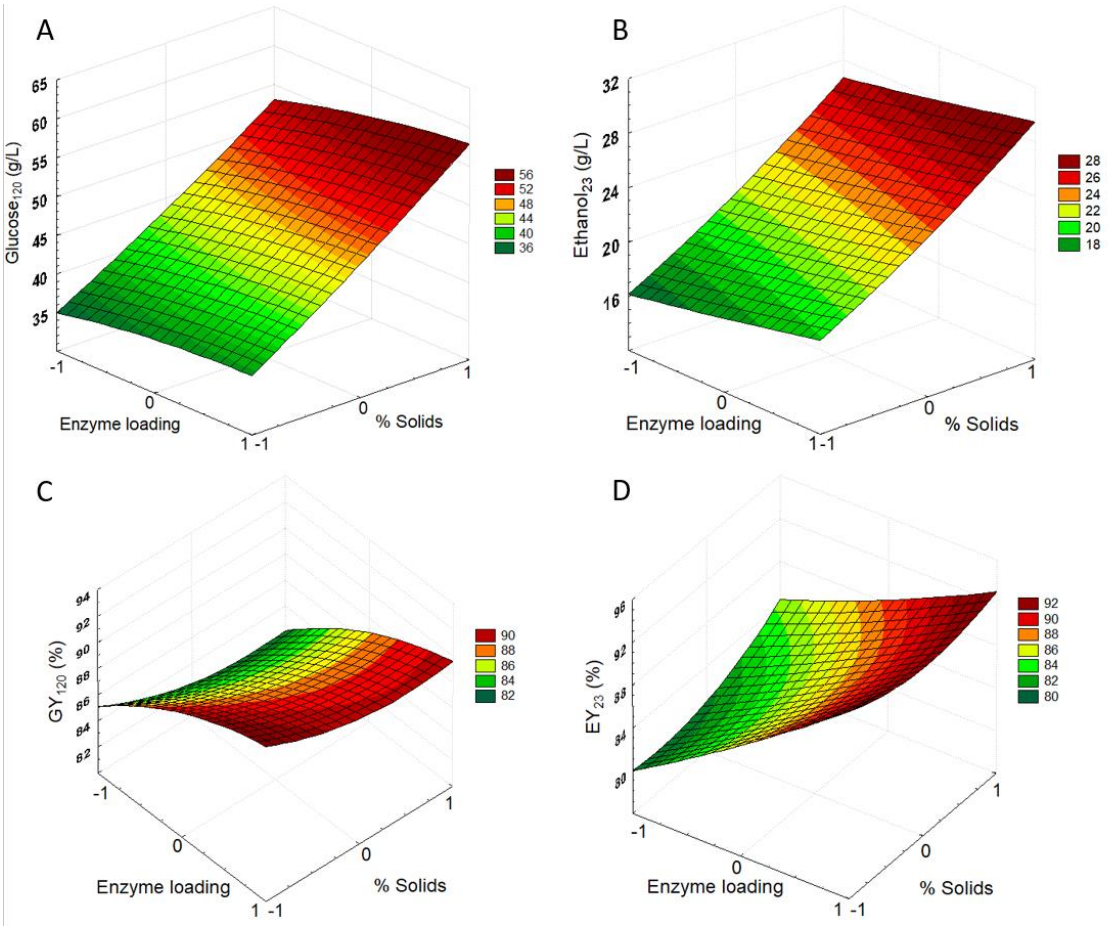

Figure 4.4 Response surfaces for Glu_{120} (A), Eth_{23} (B), GY_{120} (C), and EY_{23} (D) as a function of solids concentration (X_1) and enzyme loading (X_2).

Considering firstly the concentration of solids (X_1), as expected, a significant positive (linear) effect was observed on both glucose and ethanol concentration (*p*-value of $4.8x10^{-11}$ and $4.9x10^{-11}$, respectively), justified by an increased availability of cellulose and fermentable sugars, respectively. Furthermore, there were no evidences of critical limitations caused by the high amounts of solids, namely mass transference related or end-product inhibition. That could be also observed from the model of glucose production yield (Figure 4.4C), where no clear negative effect is visible; indeed, the glucose yield varies around values of 84-91%, a clear trend associated to solids content being unnoticeable. Very high solids concentrations are reported to have a significant negative impact on glucose yield, an effect that is not observed in this case since the range of solids concentration used was selected in exploratory assays. Also, it is worth of note that the hydrolysis was conducted for 120 hours, which is the time required for satisfactory yields to be reached under the highest solids loadings, attenuating therefore time-dependent limitations. In a similar way, also the utilization of this specific range of enzyme loadings, may have contributed to attenuate limitations resulting from increased solid loadings such as non-productive binding of enzymes to the solid. These results suggest that further intensification may still be achievable at industrial scale, using better mixing conditions than the ones available at lab scale in this study.
Finally, it still should be highlighted that, as the solid has a negative cost on this case, more important than the production yield is the productivity, equally critical for lowering operational costs. 22 % solids can therefore be considered as the most adequate option under the lab scale setup available, as it leads to satisfactory glucose yield, enabling the maximum glucose concentration.

Reporting now to the influence of enzyme loading, although a slight increase is visible for all response variables, it is not expressive. Additionally, it seems to impact similarly in the entire range of solids concentration, while a superior effect would be expected for the highest consistency where possible enzyme limitations would be more likely. Thus, it seems that for this range of solid and enzyme loadings there is indeed no significant limitation of enzyme availability. On the previous chapter it was verified that this specific cellulase cocktail is particularly efficient on the hydrolysis of *n*RPS (Gomes *et al*., 2016).

Maximum values of glucose concentration were achieved for the highest level of enzyme dosage, as expected (Table 4.3). However, when enzyme dosage was increased in 50 % (from 20 to 30 FPU/$g_{cellulose}$) for the highest solid concentration, glucose concentration only increased approximately 12 % (from 52.4 to 58.9 g/L). Considering the high cost of enzymes and negative cost of the substrate, a lower enzyme dosage may be a sensible choice in this scenario.

4.3.5 *n*RPS hydrolysis with cellulase recycling under high solid loadings

Taking in account the results from CCI design, it was envisaged the *n*RPS conversion to high ethanol concentrations while enabling cellulase recycling. Hence, a system of multiples rounds of hydrolysis was implemented with Celluclast, applying the pre-determined conditions of solid and enzyme loadings (Figure 4.5).

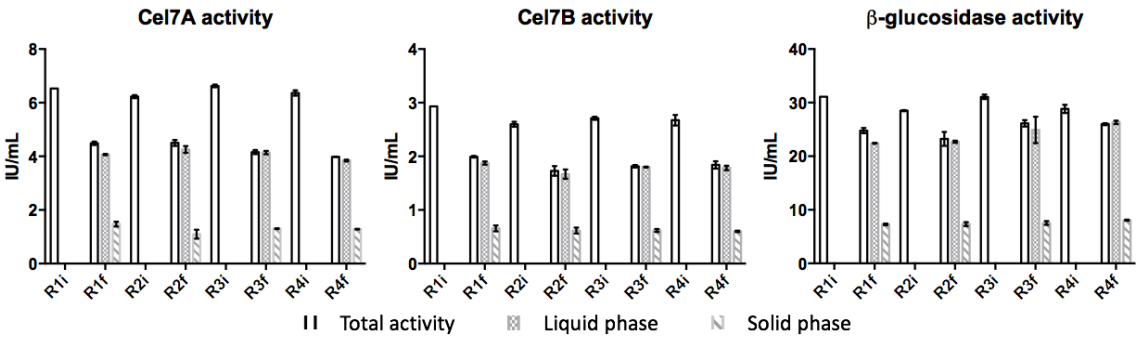

Figure 4.5 Variation of Cel7A, Cel7B and β-glucosidase activities over four rounds of *n*RPS hydrolysis (120 h hydrolysis [40 ºC] → 24 h SSF [35 ºC]) with cellulase recycling. 20 FPU/$g_{cellulose}$ were initially employed with a posterior supplementation of 50% fresh enzymes on each recycling stage (*R**x**i and R**x**f refers to the initial and final activity of round **x**, respectively*).

From the analysis of Figure 4.5, it may be observed that the initial levels of the three cellulases analyzed (Cel7A, Cel7B and β-glucosidase) were similar over the four rounds of hydrolysis and fermentation, an outcome that could be achieved using a 50 % supplementation with fresh enzymes in each round. As a matter of fact, for each round there is a considerable decrease on the activity levels, an average reduction of 33.4, 32.4 and 16.1 % being observed for Cel7A, Cel7B and β-glucosidase, respectively. A lower reduction observed for β-glucosidase may be attributed to its well known lack of cellulose-binding domain. Also, the fact that β-glucosidase levels may have been used in excess enables an inferior relative variation. Referring to the previous chapter, the levels

of activity variations for this case were considerably higher comparing to averages decreases of 14.3, 17.6 and 7.0 % obtained for Cel7A, Cel7B and β-glucosidase, respectively (Gomes *et al*., 2016). Considering that there was no thermal deactivation, it may be possible that the higher concentrations of ethanol achieved on this case may have caused some loss of enzyme activity (Chen and Jin, 2006) since the intensification strategy followed in the present study allowed a 3.8-fold increase in ethanol concentration.

Referring to the enzyme distribution at the end of each cycle, the results demonstrate that a considerable fraction of activity remained solid-bound: an average of 30.4, 32.6 and 30.3 % for Cel7A, Cel7B and β-glucosidase, respectively. This result highlights the need to recover both fractions in spite of increasing process complexity.

Table 4.5 Multiple rounds of *n*RPS hydrolysis with cellulase recycling (20 FPU/$g_{cellulose}$; 50 % fresh enzymes)

Round	$Glucose_{120}$ (g/L)*	$Ethanol_{23}$ (g/L)**	Glucans conversion (%)
1	50.14 ± 0.55	25.86 ± 0.67	80.68 ± 0.44
2 (recycling 1)	41.86 ± 1.06	20.94 ± 0.85	70.55 ± 1.34
3 (recycling 2)	42.31 ± 0.76	21.39 ± 0.08	70.26 ± 0.13
4 (recycling 3)	40.74 ± 0.36	20.28 ± 0.15	70.18 ± 0.35

Hydrolysis were conducted for 120 h (40ºC) followed 24 h fermentation (35ºC)
(*) - Glucose produced at 120 hours of hydrolysis
(**) - Ethanol produced at 23 hours of fermentation

From the steady levels of initial activity for the different cellulases along the different cycles, one could expect the applied strategy of cellulase recycling to achieve equal levels of solid conversion along the process. Nevertheless, it was verified that hydrolysis efficiency had an average decrease of 12.5 % in the rounds with recycled enzyme comparatively to the initial one (Table 4.5). A major part of this reduction may possibly arise from a different sterilization process used. While the first *n*RPS batch was sterilized after being suspended in the liquid (approx. 22% solids), the following ones were processed at high consistency (approx. 95% solids), which leads to a decrease on solid conversion by around 14 %. This was required to enable a higher volume of concentrate

after ultrafiltration since high final enzyme concentrations have shown before to cause higher losses during this process. On an industrial scale however, the utilization of different sterilization processes or UF devices with lower limitations may enable to overcome in some degree this reduction. In addition, this decrease may equally be attributed to the fact that on rounds 2, 3 and 4, 50 % of the enzymes have already undertaken at least one cycle of hydrolysis and fermentation, which can cause to some extent a reduction on their efficiency.

In spite of this decrease on hydrolysis efficiency, it should be highlighted that it was still possible to reach important improvements in both glucose and ethanol production comparatively to the existing literature. Using a similar substrate (although with slightly superior cellulose content), the maximum ethanol concentration obtained by Marques *et al*. (2008) was 19.6 g/L. Also, Marques *et al*. (2017) were able to achieve nearly 80 g/L of glucose, nevertheless, this was obtained through a fed-batch strategy with multiple pulses of substrate addition and not a single addition as for the current work. Comparing specifically to a previous work also applying cellulases recycling on RPS conversion (Gomes *et al*., 2016), it was verified an increase of 3.4 and 3.8 fold on glucose and ethanol production, respectively. Even employing a set of much more challenging conditions to the process, namely a higher temperature of hydrolysis and fermentation and a considerable increase on solids loading, it was still possible to successfully implement the recycling of cellulases enabling an approximate enzyme saving of 50 %, to nearly 10 FPU/$g_{cellulose}$. It should be referred that when hydrolysis was conducted in the same conditions as for the cycles with recycled enzyme but using instead only 10 FPU/$g_{cellulose}$ (simulating the estimated enzyme saving) glucose production decreased approximately 35 % (from 41.6 to 27.0 g/L).

4.4 Conclusions

This work provides critical insights from the perspective of a future industrial implementation of enzyme recycling in the specific case of bioethanol production from RPS. It demonstrates that this material can be efficiently converted by different commercial cocktails currently available even under intensified conditions. Also, it elucidates the important role of enzyme cocktail selection on determining the final distribution of enzymatic activity between phases and its overall recovery after the process, a critical factor on the establishment of a simple recycling strategy. In this scope, Celluclast showed a more favorable scenario comparatively to other cocktails, enabling as well a slight advantage on the hydrolysis efficiency.

Even employing intensified operational conditions, cellulase recycling was successfully implemented on RPS conversion with the addition of only 50 % of enzymes on each recycling stage, suggesting that process intensification may be combined with enzyme recycling.

4.5 References

Abban-Mensah I, Vis M, van Sleen P. 2014. Socio-Economic Impacts of a Lignocellulosic Ethanol Refinery in Canada, In: Rutz D, Janssen R (Eds.), Socio-Economic Impacts of Bioenergy Production. *Springer*, pp. 233-51.

Aden A, Foust T. 2009. Techno-economic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. *Cellulose* 16:535-45.

Bailey MJ, Tähtiharju J. 2003. Efficient cellulose production by *Trichoderma reesei* in continuous cultivation on lactose medium with a computer-controlled feeding strategy. *Appl. Microbiol. Biotechnol.* 62:156-62.

Chen G, Song W, Qi B, Lu J, Wan Y. 2013. Recycling cellulase from enzymatic hydrolyzate of acid treated wheat straw by electroultrafiltration. *Biores. Technol.* 144:186-93.

Chen H, Jin S. 2006. Effect of ethanol and yeast on cellulase activity and hydrolysis of crystalline cellulose. *Enzyme Microb. Technol.* 39:1430-2.

Costa CE, Romaní A, Cunha JT, Johansson B, Domingues L. 2017. Integrated approach for selecting efficient *Saccharomyces cerevisiae* for industrial lignocellulosic fermentations: importance of yeast chassis linked to process conditions. *Biores. Technol.* 227:24-34.

Cunha M, Romaní A, Carvalho M, Domingues L. 2018. Boosting bioethanol production from Eucalyptus wood by whey incorporation. *Biores. Technol.* 250:256-64.

Domínguez E, Romaní A, Domingues L, Garrote G. 2017. Evaluation of strategies for second generation bioethanol production from fast growing biomass Paulownia within a biorefinery scheme. *Appl. Energ.* 187:777-89.

Du R, Su R, Li X, Tantai X, Liu Z, Yang J, Qi W, He Z. 2012. Controlled adsorption of cellulase onto pretreated corncob by pH adjustment. *Cellulose* 19:371-80.

Dutta A, Dowe N, Ibsen KN, Schell DJ, Aden A. 2010. An economic comparison of different fermentation configurations to convert corn stover to ethanol using *Z. mobilis* and *Saccharomyces*. *Biotechnol. Prog.* 26:64-72.

Eckard AD, Muthukumarappan K, Gibbons W. 2013. Enhanced bioethanol production from pretreated corn stover via multi-positive effect of casein micelles. *Biores. Technol.* 135:93-102.

Gomes D, Domingues L, Gama M. 2016. Valorizing recycled paper sludge by a bioethanol production process with cellulase recycling. *Biores. Technol.* 216:637-44.

Gomes D, Rodrigues AC, Domingues L, Gama M. 2015. Cellulase recycling in biorefineries--is it possible?. *Appl. Microbiol. Biotechnol.* 99:4131-43.

Haven MØ, Lindedam J, Jeppesen MD, Elleskov M, Rodrigues AC, Gama M, Jørgensen H, Felby C. 2015. Continuous recycling of enzymes during production of lignocellulosic bioethanol in demonstration scale. *Appl. Energ.* 159:188-95.

Huang R, Guo H, Su R, Qi W, He Z. 2017. Enhanced cellulase recovery without β-glucosidase supplementation for cellulosic ethanol production using an engineered strain and surfactant. *Biotechnol. Bioeng.* 114:543-51.

Jørgensen H, Pinelo M. 2017. Enzyme recycling in lignocellulosic biorefineries. *Biofuels Biopro. Bioref.* 11:150-67.

Kelbert M, Romaní A, Coelho E, Pereira FB, Teixeira JA, Domingues L. 2016. Simultaneous Saccharification and Fermentation of Hydrothermal Pretreated Lignocellulosic Biomass: Evaluation of Process Performance Under Multiple Stress Conditions. *BioEnergy Res.* 9:750-62.

Kelbert M, Romaní A, Coelho E, Pereira FB, Teixeira JA, Domingues L. 2015. Lignocellulosic bioethanol production with revalorization of low-cost agroindustrial by-products as nutritional supplements. *Ind. Crops Prod.* 64:16-24.

Klein-Marcusschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch WH. 2012. The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnol. Bioeng.* 109:1083-9.

Lee D, Yu AHC, Saddler JN. 1995. Evaluation of cellulase recycling strategies for the hydrolysis of lignocellulosic substrates. *Biotechnol. Bioeng.* 45:328-36.

Lynd LR, Laser MS, Bransby D, Dale BE, Davison B, Hamilton R, Himmel M, Keller M, McMillan JD, Sheehan J, Wyman CE. 2008. How biotech can transform biofuels. *Nat. Biotech.* 26:169-72.

Marques S, Alves L, Roseiro JC, Gírio FM. 2008. Conversion of recycled paper sludge to ethanol by SHF and SSF using *Pichia stipites*. *Biomass Bioenerg.* 32:400-6.

Marques S, Gírio FM, Santos JAL, Roseiro JC. 2017. Pulsed fed-batch strategy towards intensified process for lactic acid production using recycled paper sludge. *Biomass Conv. Bioref.* 7:127-37.

Pribowo A, Arantes V, Saddler JN. 2012. The adsorption and enzyme activity profiles of specific *Trichoderma reesei* cellulose/xylanase components when hydrolyzing steam pretreated corn stover. *Enzyme Microb. Technol.* 50:195-203.

Rodrigues AC, Felby C, Gama M. 2014. Cellulase stability, adsorption/desorption profiles and recycling during successive cycles of hydrolysis and fermentation of wheat straw. *Biores. Technol.* 156:163-9.

Rodrigues AC, Haven MØ, Lindedam J, Felby C, Gama M. 2015. Celluclast and Cellic® CTec2: saccharification/fermentation of wheat straw, solid-liquid partition and potential of enzyme recycling by alkaline washing. *Enzyme Microb. Technol.* 79-80:70-7.

Rodrigues AC, Leitão AF, Moreira S, Felby C, Gama M. 2012. Recycling of cellulases in lignocellulosic hydrolysates using alkaline elution. *Biores. Technol.* 110:526-33.

Romaní A, Ruiz HA, Pereira FB, Teixeira JA, Domingues L. 2014. Integrated approach for effective bioethanol production using whole slurry from autohydrolyzed *Eucalyptus globulus* wood at high-solid loadings. *Fuel* 135:482-91.

Romaní A, Ruiz HA, Teixeira JA, Domingues L. 2016. Valorization of Eucalyptus wood by glycerol-organosolv pretreatment within the biorefinery concept: An integrated and intensified approach. *Renew. Energy.* 95:1-9.

Ruiz HA, Silva DP, Ruzene DS, Lima LF, Vicente AA, Teixeira JA. 2012. Bioethanol production from hydrothermal pretreated wheat straw by a flocculating *Saccharomyces cerevisiae* strain-effect of process conditions. *Fuel* 95:528-36.

Shang Y, Su R, Huang R, Yang Y, Qi W, Li Q, He Z. 2014. Recycling cellulases by pH-triggered adsorption-desorption during the enzymatic hydrolysis of lignocellulosic biomass. *Appl. Microbiol. Biotechnol.* 98:5765-74.

Sipos B, Dienes D, Schleicher Á, Perazzini R, Crestini C, Siika-aho M, Réczey K. 2010. Hydrolysis efficiency and enzyme adsorption on steam-pretreated spruce in the presence of poly(ethylene glycol). *Enzyme Microb. Technol.* 47:84-90.

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. 2008. Determination of structural carbohydrates and lignin in biomass. NREL chemical analysis and testing laboratory analytical procedures. NREL/ TP-510-42618.

Strobel KL, Pfeiffer KA, Blanch HW, Clark DS. 2015. Engineering Cel7A carbohydrate binding module and linker for reduced lignin inhibition. *Biotechnol. Bioeng.* 113:1369-74.

Tu M, Chandra RP, Saddler JN. 2007. Evaluating the distribution of cellulases and the recycling of free cellulases during the hydrolysis of lignocellulosic substrates. *Biotechnol. Prog.* 23:398-406.

Tu M, Saddler JN. 2010. Potential enzyme cost reduction with the addition of surfactant during the hydrolysis of pretreated softwood. *Appl. Biochem. Biotechnol.* 161:274-87.

Yang J, Zhang X, Yong Q, Yu S. 2010. Three-stage hydrolysis to enhance enzymatic saccharification of steam-exploded corn stover. *Biores. Technol.* 101:4930-5.

Web references

1. http://novozymes.com/en/news/news-archive/Pages/45713.aspx (last accessed in 15-04-2016)

Chapter V

Insights into the economic viability of cellulase recycling on bioethanol production from RPS

The work reported on this chapter has been submitted for publication:

Daniel G. Gomes, Sebastián Serna Loaiza, Carlos Ariel Cardona, Miguel Gama, Lucília Domingues. 2018. Insights into the economic viability of cellulase recycling on bioethanol production from RPS. *Submitted*.

Abstract

Enzyme manufacturers have been reducing consistently the cellulases market price, making processes such as the production of cellulosic ethanol more competitive. Even though current strategies towards making the enzymes cheaper seems to be based on the reduction of its production cost, recycling may further boost the enzyme-based biotechnological processes. It is therefore relevant to assess the economic impact of recycling, a subject scarcely addressed so far.

Initially, the economic viability of bioethanol production from RPS under standard conditions was evaluated. The process presented a positive economic output (pay-back period of 7.11 years; accumulated NPV of 13.4 Million US$) even though low final ethanol titers critically increased distillation costs. The implementation of enzyme recycling, considering the recovery of both liquid and solid fractions, resulted in considerable savings on enzyme cost. Nevertheless, it also resulted on a visible increase on annual costs (1.4 %) due to a higher requirement of equipment and utilities. Due to the recovery of solid fraction, part of the ethanol retained on the solid was also recovered, enabling a slight increase (2.5 %) in total ethanol production. Overall, enzyme recycling enabled a superior economic output in comparison to the base case: pay-back period decreased to 6.65 years and the accumulated NPV increased to 17.01 Million US$. A hypothetic scenario with only the liquid fraction recovered allowed a clear reduction on equipment cost, however, there was also a decrease on total ethanol production, attenuating the abovementioned benefits. Targeting higher ethanol concentrations, a recycling scenario with superior solids consistency was evaluated: despite a clear reduction on production costs, total ethanol production decreased due to an increased ethanol retention on the solid. In a final approach, a sensitivity analysis has detailed the importance of the cost of enzyme and UF membrane in the economic feasibility of enzyme recycling.

This work suggests that under specific assumptions, which were based on experimental data, the implementation of enzyme recycling in the process of bioethanol production from RPS is economically feasible. Nevertheless, a progressive reduction on enzymes cost in the following years may revert this result on a near future, hence, similar reductions in the cost of enzyme recycling processes may be required.

5.1 Background

Over the last years, cellulosic ethanol is increasingly establishing itself as a feasible alternative to fossil fuels. This can have an important boost of small and local economies, while at the same time eliminating the potential competition with food crops.

Both the feedstock and the enzymes have been central elements here regarding the economic performance of these processes. Materials with high sugar contents and high accessibility have been pursued in order to increase the economics of their conversion. On the other hand, more attention has been given to residues (from forest, agriculture, industry, etc.) rather than energy crops: they usually have a negative cost and can, to some extent, significantly impact the environment.

RPS (recycled paper sludge) is a substrate generated in great amounts worldwide by paper-manufacturing industry (Monte *et al.*, 2009). More specifically, it results from the treatment of the effluents generated on paper recycling processes (Marques *et al.*, 2008a). Due to its toxic composition, it has a limited number of handling options, being usually disposed in landfills, which represents a considerable economic drawback for the sector. Nevertheless, this material also contains a considerable amount of sugars that can be valorized through multiple products, namely bioethanol. In fact, several studies have already demonstrated the practical feasibility of RPS for the production of bioethanol (Marques *et al.*, 2008a; Gomes *et al.*, 2016, 2018) or lactic acid (Marques *et al.*, 2008b). Chen *et al.* (2014) have recently demonstrated that from an economic point of view, the conversion of different paper manufacturing residues is in fact viable to implement on an industrial scale.

Despite all the progresses made by enzyme manufactures, cellulases still harbor a significant fraction of ethanol final cost (Gomes *et al.*, 2015). For this reason, numerous strategies have been studied towards a reduction on enzyme cost. In the scope of cellulase recycling, considerable research has been conducted on the mechanisms of enzyme adsorption/desorption to the substrate, nevertheless, the number of studies demonstrating their practical feasibility are rather small. Furthermore, to the better of our knowledge, no work currently exists reporting to the economic viability of any of these strategies. In the particular case of enzyme recycling through an ultrafiltration

strategy, this aspect gains even more relevance as these processes are usually associated with a very high cost.

Here, an overall analysis on the economic viability of cellulase recycling in the specific context of bioethanol production from RPS was performed. In a first stage, the economics of the conversion of RPS following the traditional route was analyzed, which also represents the first study of this kind for this particular material. Afterwards, based on experimental data gathered in this thesis, it was evaluated the impact of implementing a specific cellulase recycling system on different economic aspects of the process.

5.2 Methods

5.2.1 General assumptions

The processes and correspondent scenarios described in the following sections take into account some major assumptions. The feedstock is *n*RPS, which refers to the RPS residue after carbonates neutralization, with the following composition (dry mass basis): 34.1 % cellulose; 7.9 % xylan; 4.8 % protein; 3.5 % fat; 29.3 % ash; 20.4 % lignin (Marques *et al.*, 2008a). The process of *n*RPS conversion will be integrated on an existing facility that generates this residue, or within a very short range; hence, no transportation costs are considered. For economic evaluation purposes, a null cost is associated to *n*RPS. This way, the economic gain from avoiding disposal costs were not considered here. For all cases, it was assumed an average uptake of 200 dry tons/day of *n*RPS and a total annual operating period of 8766 hours.

5.2.2 Process description

Different scenarios were considered to evaluate the impact of cellulase recycling on the economic viability of *n*RPS conversion into ethanol (Table 5.1).

Table 5.1 Overall description of the different simulation scenarios

Designation	Description
BaseSc	Base case – 22 % solids
BaseScComb	Base case + solids combustion – 22 % solids
Rec2Frac	Enzyme recycling 2 Fractions – 22 % solids
Rec1Frac	Enzyme recycling 1 Fraction – 22 % solids
Rec2Frac28%	Enzyme recycling 2 Fractions – 28 % solids

The base case scenario (BaseSc) refers to the process where enzymes are not recycled. Based on results detailed in the previous chapter, a recycling scenario was elaborated which considers the recovery of both liquid and solid fraction of enzymes (Rec2Frac). A simpler process, with only liquid fraction recycling was also elaborated (Rec1Frac). The later was not tested experimentally and thus is based on some assumptions.

Considering the low final ethanol titers achieved using a solid content of 22 %, a scenario homologous to Rec2Frac was also considered for an initial solid loading of 28 %. Although the experimental data available obtained at lab scale correspond to a maximum solids loading of 22 %, it is considered that on an industrial scale the existence of much more efficient mixing solutions would enable operating at this higher range of solids consistency.

Finally, the final solid valorization through a combustion-gasification process was also analyzed (BaseScComb).

BaseSc - RPS conversion into ethanol without cellulase recycling

The base case approach was based on the process described on Chapter IV, section 4.2.5 (Figure 5.1). Briefly, *n*RPS is mixed with water in a liquid to solid ratio of 4.55 (22 % (w/v) consistency) for BaseSc, or 3.57 (28 % (w/v) consistency) for Rec2Frac28%. Then, a cellulase cocktail is added at an enzyme loading of 20 FPU/$g_{cellulose}$. This mixture is incubated at 40ºC until a cellulose conversion of 86 % is achieved. Xylan is not hydrolyzed under the experimental conditions used. Afterwards, the sugars syrup is inoculated with yeast cells and the temperature set to 35 ºC, enabling glucose conversion into ethanol, carbon dioxide and biomass.

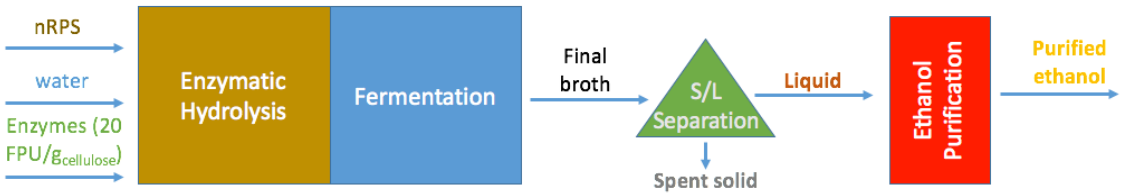

Figure 5.1 Overall scheme of the traditional *n*RPS conversion into ethanol.

After hydrolysis and fermentation, the liquid (containing ethanol, residual glucose and soluble enzymes) and the final solid residue are separated. The liquid stream then proceeds to an ethanol purification stage enabling a final purified stream with 99.5 % (w/w) ethanol.

Rec2Frac and Rec1Frac - RPS conversion into ethanol with enzyme recycling

For *n*RPS conversion with enzyme recycling, some modifications are introduced after hydrolysis (Figure 5.2).

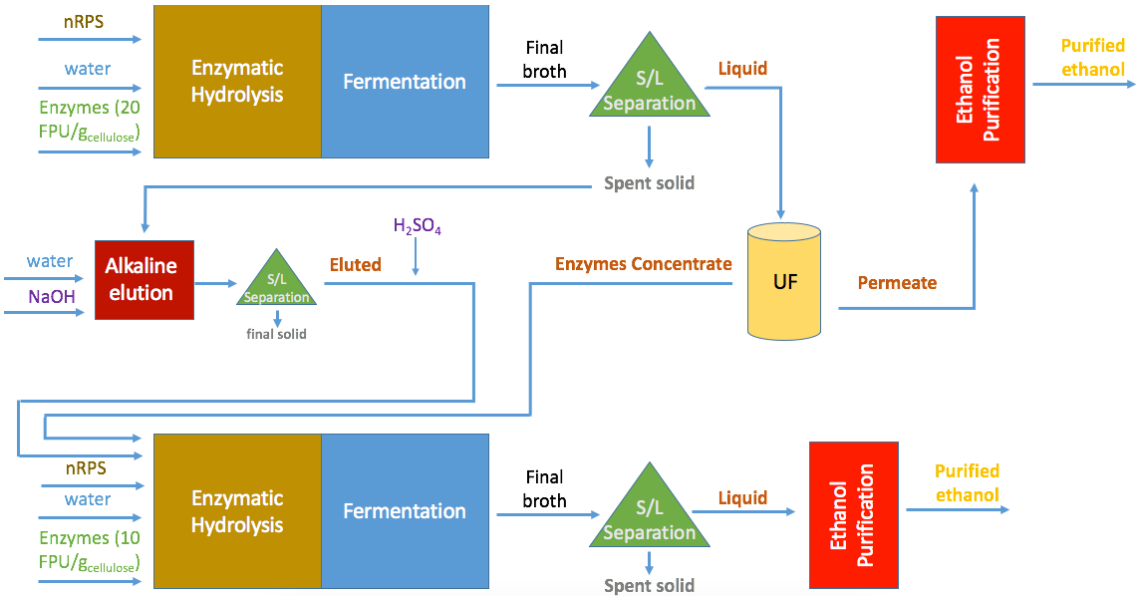

Figure 5.2 Overall scheme of *n*RPS conversion with enzymes recycling.

Following the main solid-liquid separation process, as performed in the base case, the liquid fraction is concentrated by ultrafiltration (UF) enabling the separation of cellulases (on the concentrate) from water and ethanol (and other low-molecular weight solutes). The solid is resuspended in water and the pH increased to 9 (with NaOH) to allow enzyme desorption from the solid. Then, the mixture is centrifuged and the pH of the obtained liquid fraction adjusted to 5 (with H_2SO_4). This liquid stream containing desorbed enzymes is mixed with the enzyme concentrate obtained from ultrafiltration and used to process a new batch of fresh substrate.

For the new hydrolysis run, 50 % of the initial enzyme loading is added to compensate activity loss which occurs during the overall process. As experimentally tested, this strategy is applied during 4 consecutive rounds, after which 100 % of enzyme is once again employed.

Enzyme recycling concerning only the liquid fraction (Rec1Frac) is conducted in a similar way, omitting the enzyme recovery from the final solid residue; only the enzyme ultrafiltrated from the liquid fraction is thus reutilized in this scenario on the subsequent round.

BaseScComb - Valorization of final solid residue by a combustion-gasification process

Final solid residue obtained after hydrolysis and fermentation contain some components that can be further converted into energy through a thermo-chemical process. Specifically, the final dry biomass undergoes initially a devolatilization (pyrolysis) where is decomposed into carbon, hydrogen, oxygen and ash, according to its elemental analysis. All these components go into a combustion chamber where they react with oxygen to produce CO_2, CO, H_2O and heat. The char obtained on pyrolysis and combustion passes to a reduction zone where gasification occurs producing CO_2, CO, H_2 and CH_4. Ash and remaining char are separated from the syngas using a cyclone. The generated synthesis gas from gasification has a high energy content which can be used directly as fuel in a gas engine to produce electricity. An internal combustion engine burns the gaseous fuel to produce electricity by means of a generator. A more detailed description can be found on García *et al.* (2017a, 2017b).

5.2.3 Process Modeling and Simulation

A simulation model was constructed for each scenario using the modeling tool Aspen Plus v8.6 (Aspen Technology, Inc., USA). These models will then be used to generate the correspondent mass and energy balances, providing the data for the economic analysis. Process simulation assumed a continuous approach based on a fixed value of substrate uptake, in this case 200 tons/day, and conversion rates experimentally obtained.

As to simulate the abovementioned enzyme recycling system, the models assumed four production lines, each with a RPS uptake capacity of 50 tons/day. Each stream of recovered enzymes is subsequently re-introduced in another production line, which properly simulate also the recovery of other products, such as ethanol.

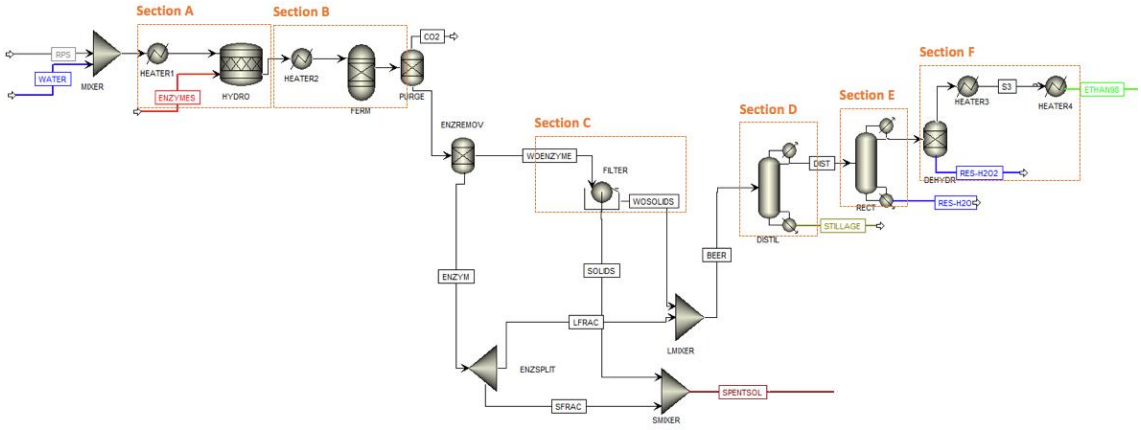

Figure 5.3 Overall scheme of process modeling for main process operations.

Relevant specifications on the main process for the different scenarios are detailed next in accordance with Figure 5.3.

Section A – Enzymatic hydrolysis

The stream of water and RPS are heated to a temperature of 40 ºC and mixed with a proper amount of enzyme. Inside a stoichiometric reactor (*HYDRO*), Reaction1 occurs considering a cellulose conversion of 86 %. Excepting for water, which is also used for cellulose hydrolysis, all other components are inert.

$$1\ \text{Cellulose} + 1\ H_2O \rightarrow n\ \text{Glucose} \qquad \text{(Reaction 1)}$$

Section B – Fermentation

Stream from Section A is temperature-changed to 35 ºC entering then a yield reactor (*FERM*) where glucose is converted to ethanol, CO_2, and yeast cells according to specific ratios (Table 5.2).

Table 5.2 Yield of conversion of glucose into multiple products

Product	Yield ($g/g_{glucose}$)
Ethanol	0.493
CO_2	0.430
Yeast cells	0.065
Unconverted glucose	0.012

Section C – Solid-liquid separation

Final broth from hydrolysis and fermentation is fed into a filtration system (rotary drum filter) enabling a total separation of solid components (hemicellulose, remaining

cellulose, lignin, ashes, yeast cells) from the liquid fraction. Considering the experimental values of solid humidity after solid-liquid separation, a value of 0.5 was set for the mass fraction of liquid in the final solid.

Sections D, E and F – Ethanol purification stage

Liquid stream obtained from solid-liquid separation is processed by an ethanol purification stage composed by three steps. Distillation (*DISTL*) initially allows to increase ethanol concentration from 3-5 % to 75 % (w/w). Posteriorly, a rectification column (*RECT*) enables an increase to nearly 95 %. A final step of dehydration removes a considerable amount of the remaining water, using in this case a molecular sieve, resulting on a final stream of purified ethanol with a concentration of 99.5 % (w/w).

Alkaline washing

On a mixing vessel, the solid from the primary solid-liquid separation is resuspended in water, at the same consistency as initially adopted for hydrolysis. This is then mixed with a given amount of NaOH, which was calculated considering a pH shift of 4.8 to 9 for a total volume equal to the solid suspension. Afterwards, this suspension passes through another process of solid-liquid separation (similar to the initial one) being the liquid fraction pH reduced to 4.8 by the addition of H_2SO_4 in a mixing vessel, where it is stored until further use.

Enzyme concentration

Liquid fraction from the primary solid-liquid separation is fed into a UF system which produces two main streams: a permeate, containing mostly water and ethanol; the retentate, referring to a cellulases concentrate, containing a large majority of the enzymes, but also some residual levels of ethanol, water, and other components. Permeate and retentate volumes are set assuming a concentration factor of 20, as considered in some case studies available within SuperPro Designer (Intelligen Inc.) platform.

5.2.4 Economic Assessment

The capital, operating costs and revenues were estimated. The ethanol selling price was set as 0.986 US$/kg. Cost of equipment was calculated with Aspen Economic Analyzer v8.6 considering the specifications obtained from process simulation. Fix capital investment was estimated based on the purchasing cost of the equipment and other

additional factors accounting for delivery (10% of purchasing costs), direct costs (% of purchasing costs: installation 47%, instrumentation and control 36%, piping 68%, electrical systems 11%, buildings 18%, yard improvements 10%, service facilities 70%) and indirect costs (% of purchasing costs: engineering and supervision 33%, construction expenses 41%, legal expenses 4%, contractor's fee 22%, contingency 44%) (Peters *et al.*, 2003).

The costs were estimated on an annual basis, taking in account the following categories: raw materials, utilities, maintenance, labor, fixed & general, overhead and capital depreciation. Costs of the raw materials were estimated according to the mass balances, and the correspondent market prices. Utilities were estimated based on the energy balances and after energy integration internally conducted by Aspen. Maintenance costs refers to maintenance (6% of fix capital investment) and operating supplies (15% of maintenance). Fixed & general refer to taxes (2% of fix capital investment), insurance (1% of fix capital investment), and general cost (20% of labor, supervision and maintenance). Plant overhead was estimated as 60% of labor, supervision and maintenance (Peters *et al.*, 2003).

Cost of raw-material, chemicals and enzymes

As RPS is commonly associated with a negative (disposal) cost, and considering the integration of this process on an existing facility that generates this residue, hence with no transportation costs, a null price was considered for this material. Also, the neutralized RPS (after carbonates removal) was considered as the initial raw-material.

A cost of 4 US$/$kg_{enzyme}$ was assumed on this study considering different sources providing values on this range (Chen *et al.*, 2014; NREL 2013, 2015). It is worth of note however, that this represents an indicative value. For H_2SO_4 and NaOH an indicative price of 0.085 and 0.4 US$/kg was assumed, respectively, considering multiple estimations from different suppliers (*e.g.* MS SANSA Co., Ltd; Wuhan Kangzheng Science and Technology Co., Ltd).

Cost of utilities, labor and consumables

The cost of the main utilities was considered as follows: LP (low-pressure) steam, 7.56 x 10^{-3} US$/kg; electricity, 0.14 US$/kWh; cooling water price was defined according to

Ulrich and Vasudevan (2006); UF membranes, which require regular replacement, were given a cost of 400 US$/m^2. This was based on several case studies available within SuperPro Designer software. Total labor costs were calculated as the sum of operating labor, operation supervision (15 % of operating labor) and laboratory charges (15 % of operating labor), assuming 3 shifts of 8 hours per day and an operator pay rate of 2.72 US$/hour. The number of operators per shift was calculated according to the method reported by Peters *et al.* (1991) which considers mainly the plant capacity and the complexity of their processes.

Estimation of UF costs

SuperPro Designer was used to estimate the cost of the equipment and the consumption of electricity and membrane used on the UF process. For that purpose, it was considered a Feed/Retentate ratio of 20, a filtrate flux of 200 L/(m^{2}h) and a membrane replacement periodicity of 1000 operating hours. Electricity consumption was calculated considering an average level of 0.00065 kWh/L/hour, as employed by Alriols *et al.* (2014).

Main economic parameters

To assess the overall economic performance of the different scenarios, the Net Present Value (NPV) accumulated after the project life-time (15 years) was estimated. For this it was considered a total project life-time of 15 years, an annual interest rate of 8 % and an income tax rate of 21%. Capital depreciation was computed following the straight line method. Total project life-time was established based on previous studies referring to similar processes (Chen *et al.*, 2014; NREL, 2013,2015). Annual interest rate was defined in accordance to the average values reported by the European Central Bank for Europe in 2018.

5.3 Results and Discussion

5.3.1 Overall analysis of production indicators

The following table (5.3) presents an overall mass balance for the base case scenario (BaseSc) regarding the consumption and production of the main elements of the process.

Table 5.3 Mass flows for the main components of base case scenario

Type	Designation	Flow (kg/h)
Consumption	*n*RPS	8333
	Cellulose	*2842*
	Xylan	*658*
	Lignin	*1700*
	Ash	*2442*
	Protein	*400*
	Fat	*292*
	Water	37660
	Enzymes	100
	Cooling water	516142
	LP Steam	9205
	Electricity	*n.a.*
Production	Purified ethanol	1163
	Ethanol	*1156*
	Water	*6*
	CO_2	1168
Disposal	Water	1869
	Ethanol	187
	Glucose	27
	Protein	400
	Fat	292
	Cellulase	99
	Ash	2442
	Cellulose	398
	Hemicellulose	658
	Lignin	1700
	Cells	177

n.a. – not applicable

From Table 5.3 analysis, one can observe that most of the process components, corresponding to a significant mass fraction of raw-materials, can be classified as inert materials in terms of their net balance along the process. In the particular case of *n*RPS, with exception of cellulose, which is largely converted, all the remaining components stay unaltered towards the end of the process. Consequently, although cellulose conversion on *n*RPS enables an important reduction of the total amount of solid to be

disposed, an important fraction of the initial solid (approx. 66 %) still remains after the process.

The main products of the process are CO_2, yeast biomass and ethanol. CO_2 is released to the atmosphere, while yeast cells are discarded with the final residue. Ethanol represents, therefore, the only valuable product of the current process, hence, the only source of revenue.

In what regards the overall levels of ethanol production, it can be observed that there is a significant loss during its production. Taking into account the levels of ethanol on the purified final stream – 1156 kg/hour – and the initial cellulose uptake, an overall conversion rate of 0.407 $kg_{ethanol}kg_{cellulose}^{-1}$ can be estimated, which it is not in agreement with the specifications of production efficiency previously detailed on section 5.2.3 (86 % cellulose conversion; 95 % of maximum theoretical fermentation yield; this would lead to 0.471 $kg_{ethanol}kg_{cellulose}^{-1}$). Indeed, a significant fraction still remains retained on the solid during the processes of liquid-solid separation (Figure 5.4), hence a lower conversion rate is obtained.

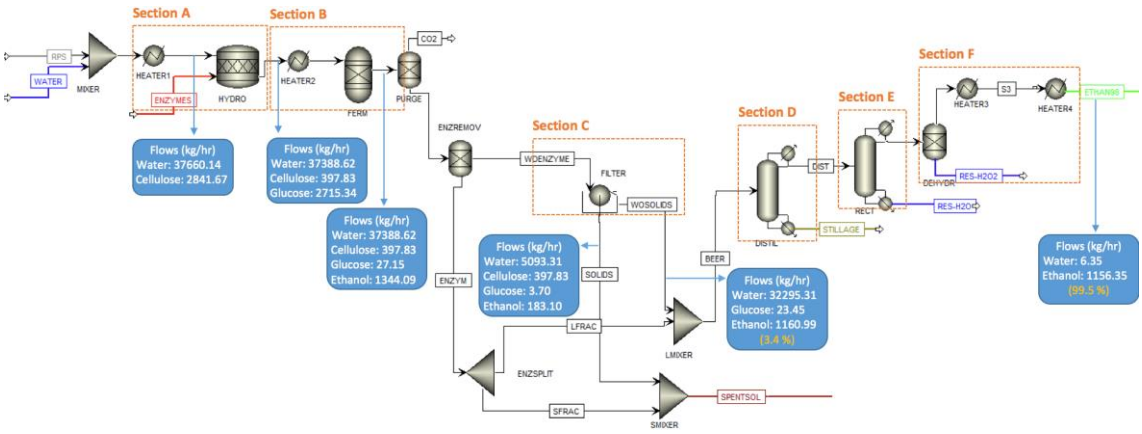

Figure 5.4 Overall scheme of the base case scenario and mass flows for the main components.

This results from a high water-retention capacity commonly associated to the final solid residue. As it can be seen from Table 5.3, due to both solid retention and the distillation process, a total of 186.58 kg/hour of ethanol is redirected to disposal, representing nearly 13.9 % of total production.

Referring to another aspect of ethanol production, as Figure 5.4 suggests, the ethanol concentration entering the purification stage is rather low. This can be mainly attributed to 1) a moderate level of solids concentration and 2) a low level of available sugars in

the raw-material. Even though the value of 22 % solids was established based on an intensification study previously conducted, considering the moderate levels of cellulose on the initial substrate, superior values of solids loading should be required by industry to enable an economically feasible process. After solid-liquid separation, ethanol concentration in the liquid stream was 3.4 % (w/w), below the critical level of 4 % commonly reported for an economically viable fermentation process (Hahn-Hägerdal *et al.*, 2006). As it will be detailed in the next section, this will have a considerable impact on the economics of the overall process, both in terms of capital investment and also operational cost.

5.3.2 Economic viability of RPS conversion under the base case scenario

To assess whether the described process of ethanol production will be economically viable, both the production costs and the putative income needs to be analyzed.
Figure 5.5 presents the estimated annual production costs distributed among the main categories.

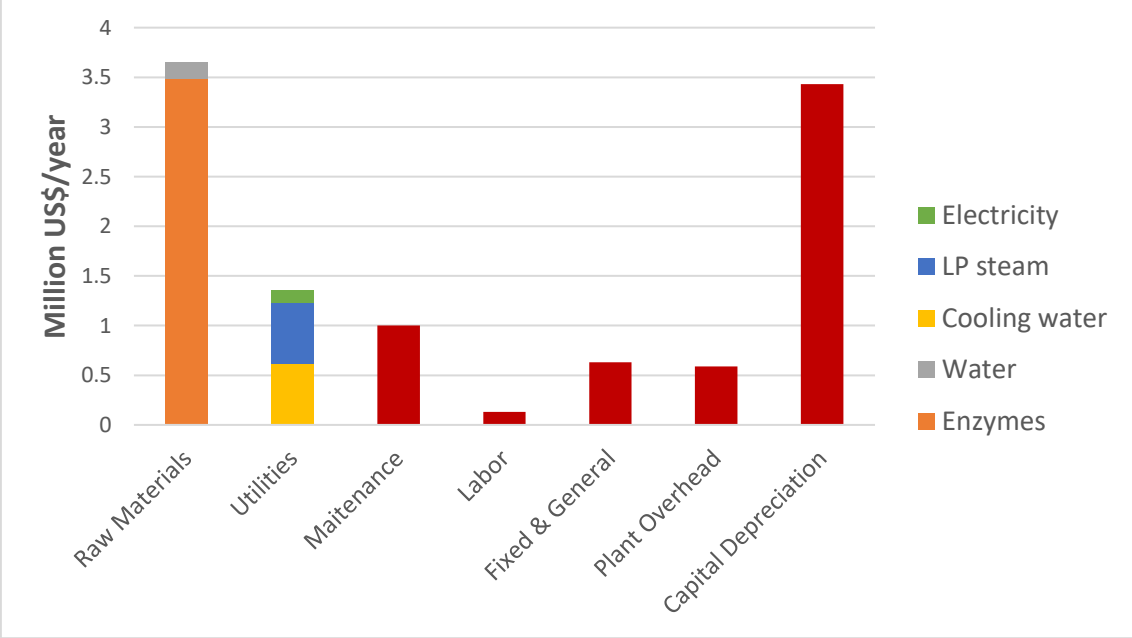

Figure 5.5 Distribution of annual operational costs into main categories for base case scenario.

As Figure 5.5 suggests, Raw-Materials, more specifically the enzymes, represent one of the main contributors for total costs, accounting for nearly 32 % of total costs, being only exceeded by Capital Depreciation. This can be partially explained by the fact that *n*RPS have an associated null cost and no other major chemicals are required, usually

employed for instance on pre-treatments stages. Capital depreciation, on the other hand, is more complex as is mostly determined by the initial capital investment but also by the assumed economic parameters of the study, namely the Annual Interest Rate, Income Taxes and the Time Period of the project.

Table 5.4 Total equipment cost for the different sections of the process facility

Section	Total Equipment Cost (US$)
A – Enzymatic Hydrolysis	1 013 600
B – Fermentation	1 069 600
C – Solid-Liquid Separation	530 800
D – Distillation	7 806 500
E - Rectification	3 576 300
F – Dehydration and product storage	124 100
Wastewater Treatment Plant	368 619

The total cost of equipment (Table 5.4) was approximately 2.98 Million US$, not considering the ethanol purification stage, which can be considered as quite acceptable taking into account the total capacity of the facility (200 tons RPS/day). On the other hand, the specific cost associated to ethanol purification was remarkably high, representing nearly 79.5 % of the total cost. This was most likely due to the low levels of ethanol concentration present in the beginning of ethanol purification stage (3.4 %), which will result on a critical increase on equipment dimensions. Similarly, this also resulted on a considerable increase on utilities consumption.

Table 5.5 Overall economic indicators of ethanol production for base case scenario

Product	Gross Income (Million US$/year)	Allocation Factor (Economic)	Allocated Cost (Million US$/year)	Production Cost (US$/$kg_{ethanol}$)
Ethanol	10.05	1.00	10.79	1.06

Taking into account the annual ethanol production - 10.19 x 10^6 kg - and a base selling price of 0.986 US$/kg, the estimated annual ethanol income is approximately 10.05 Million US$ (Table 5.5). On the other hand, considering the total annual operational cost

of 10.79 Million US$ and the total ethanol production, it can be estimated an approximate ethanol production cost of 1.06 US$/kg.

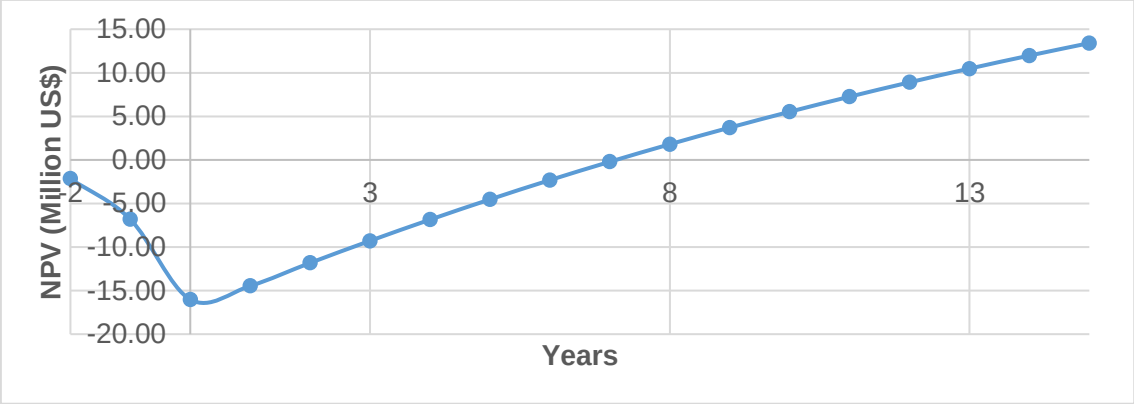

Figure 5.6 Variation of the accumulated NPV over the project life-time for base case scenario.

Considering both the expected annual income and total costs, it can be observed that the present scenario has a positive economic output. As shown in Figure 5.6, the pay-back period of the process is 7.11 years and after project life-time (15 years) there is a positive NPV accumulated of 13.4 Million US$. The Pay-back period for the investment can be considered slightly high when compared to similar industrial processes. This is, somehow, unexpected considering the null cost of the cellulosic material and the absence of pre-treatments, elements which usually represent a considerable fraction of total costs on cellulosic ethanol production. For this, it most likely contributed the significant costs of ethanol purification derived from the modest ethanol titer achieved, as already detailed above.

5.3.3 Impact of the solids gasification process

Taking into account the considerable amounts of solid residue obtained and its potential for energy generation, the impact of the integration of a combined combustion-gasification system on the process was assessed.

Table 5.6 Effect of the integration of solids combustion over different categories of operational costs

Item	Base case (BaseSc) (Million US$/year)	With solids combustion (BaseScComb) (Million US$/year)
Raw Materials	3.65	3.65
Utilities	1.35	1.64
Maintenance	1.00	1.50
Labor	0.13	0.13
Fixed and General	0.63	0.94
Plant Overhead	0.59	0.85
Capital Depreciation	3.43	5.14

As oppose to what one could expect, there was actually an increase in the overall cost of utilities, resulting from a higher requirement in electricity (Table 5.6). Even though solids combustion allowed the generation of approximately 1302 kWh, on the other hand, it caused a critical increase on total electricity requirement in the process from 100.7 kWh (in the base case) to 1650 kWh. Consequently, the net energy balance for electricity on BaseScComb was a negative 347.8 kWh, hampering any economic gain from this strategy. Furthermore, due to the additional equipment required for this particular process there was also a critical increase on equipment cost in the range of 6.7 Million US$. This unexpected result can be partially explained by the high humidity content of the residue (50 % (w/w)), which would require a considerable amount of energy initially required to reduce it to appropriate levels for this process (Pellegrini and de Oliveira, 2007). According to different authors, solid humidity should be below 20 % (Zainal *et al.*, 2001; Lv *et al.*, 2004). Another possible explanation may be the high ash content of the final residue – approx. 38 % $(w/w)_{dry\ basis}$ – which is not converted on this process and thus a considerable mass of the solid will not be converted into energy (García *et al.*, 2017b; Munalula and Meincken, 2009).

5.3.4 Integration of cellulase recycling – solid and liquid fractions

Considering the results and process specifications from Chapter III and IV, a recycling strategy scenario was envisioned. Similar to the experimental setup, this scenario took into account the recovery and reutilization of both liquid and solid fractions, with only 50 % of the initial enzyme loading being utilized on each recycling stage. As one could expect, there was a direct impact on different categories of operational costs as a result of the changes introduced on the process. Table 5.7 shows the distribution of operational costs under this scenario and the variation comparatively to the base case.

Table 5.7 Effect of the integration of enzyme recycling over different categories of operational costs

Item	Base case (BaseSc) (Million US$/year)	Enzyme Recycling (Rec2Frac) (Million US$/year)	Variation (%)
Raw Materials	3.65	2.33	- 36.2
Enzymes	3.49	2.18	
Others	0.16	0.15	
Utilities	1.35	2.12	+ 57.0
Electricity	0.123	0.28	
Heat transfer	1.227	1.42	
UF	--	0.42	
Maintenance	1.00	1.13	+ 13.0
Labor	0.13	0.13	--
Fixed and General	0.63	0.71	+ 12.7
Plant Overhead	0.59	0.66	+ 11.9
Capital Depreciation	3.43	3.86	+ 12.5
Total	10.79	10.94	+ 1.39

As expected, there was a clear reduction in the total cost of enzyme – 38 % - resulting from employing only a fraction of fresh enzyme on each recycling stage. Consequently, the estimated contribution of enzyme on total costs decrease from 32.3 % on the base case to 19.9 %. Even though some chemicals were required for enzyme recovery by alkaline wash (NaOH and H_2SO_4), the cost of other materials other than enzymes actually decreased from 0.16 to 0.15 Million US$. This most likely occurred due to the almost insignificant cost attributed to these chemicals, but also to a decreased water

requirement on this case, which possibly resulted from a slight improvement on the efficiency of process water re-utilization.

Opposing to this, the cost attributed to utilities increased 57 %. For this it greatly contributed the cost of UF membrane, here representing 0.42 Million US$, a critical element required for the continuous operation of the UF equipment, employed here to separate enzymes from the final ethanol. Also electricity consumption increased, justified by the higher number of operations required such as solid-liquid separation, alkaline washing and the UF procedure. More interesting is the increase on heat transfer utilities, *e.g.* steam and cooling water, which possibly comes as a result of some changes on ethanol purification requirements.

As a result of an additional number of equipments, specifically required for the recycling stage, there was also an evident increase in different categories of costs direct or indirectly associated to them. The cost of equipments increased approximately 1.46 Million US$: 1.29 Million US$ directly associated to enzyme recovery from the solid (alkaline washing) and 0.166 Million US$ to UF equipment. Consequently, the annual cost due to Capital Depreciation also increased in a similar rate – 12.5 % – but also Maintenance, Fixed and General and Plant Overhead, all strictly related to the facility complexity (Table 5.7).

Table 5.8 Overall economic indicators of ethanol production for the base case scenario (BaseSc) and integration of cellulase recycling – solid and liquid fractions (Rec2Frac)

Indicator	BaseSc	Rec2Frac
Ethanol Production (kg/year)	10.19×10^6	10.44×10^6
Gross Income (Million US$/year)	10.05	10.30
Allocated Cost (Million US$/year)	10.79	10.94
Ethanol Production Cost (US$/kg)	1.06	1.05

Although total annual costs increased from 10.79 (base case) to 10.94 Million US$ (approx. 1.4 %), on the other hand there was also an increase on annual income of nearly 2.5 %, from 10.05 to 10.30 Million US$ (Table 5.8). This resulted from a superior annual ethanol production, which increased from 10.19 to 10.44×10^6 kg. Adding to the enzyme

saving, this system also enabled the recovery of an important fraction of the ethanol that otherwise stays with the final solid, due to its high water retention capacity. This was possible as a result of the alkaline elution step, which not only removed part of the solid-bound enzymes but also "washed" some of the ethanol present inside the solid. Opposing to the base case scenario, where a constant ethanol production flow of 290.2 kg/hour was observed, on the present case this value gradually increased from 274.6 to 297, 298.8 and 314.7 kg/hour on the 2nd, 3rd and 4th round, respectively (*data not shown*).

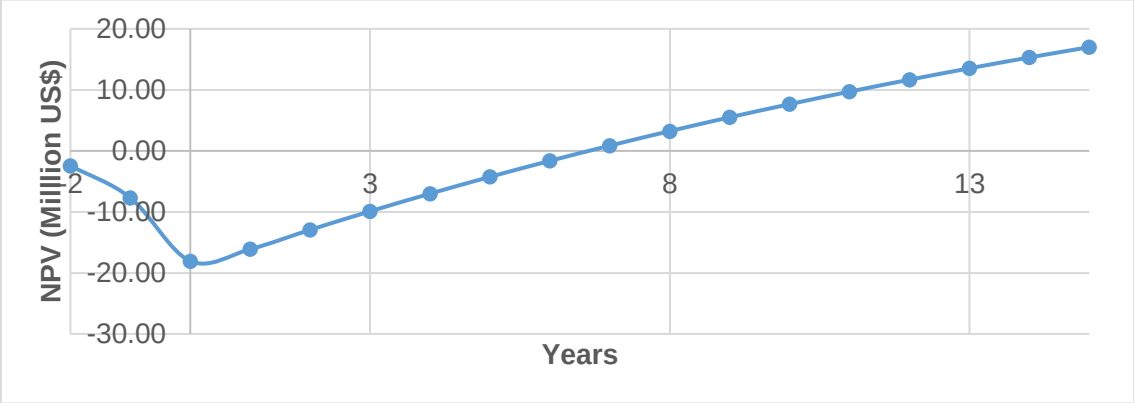

Figure 5.7 Variation of the accumulated NPV over the project life-time for Rec2Frac.

Considering the new values for annual operational costs and annual income, there was a notorious economic gain in the process performance comparatively to the base case: the pay-back period decreased from 7.11 to 6.65 years; accumulated NPV after project lifetime increased from 13.4 to 17.01 Million US$ (Figure 5.7). This suggest that cellulase recycling implementation under similar conditions may in fact be advantageous from an economic point of view. It is worth mentioning that although these results may provide some economic insights into overall systems of enzyme recycling, these are highly specific for RPS material. As it was shown on the previous chapter, the viability of cellulase recycling is strongly dependent on the final enzyme fractionation and recovery at the end of the process. As some authors have already reported (Rodrigues *et al.*, 2014, 2015), this can strongly vary according to multiple factors, one of the most important being the employed material and its composition.

5.3.5 Cellulase recycling – exclusively liquid fraction

Taking into account the variation on annual operational costs observed as a result of cellulase recycling, and more particularly due to solid enzymes recovery, it was hypothesized a scenario with exclusive recovery of soluble (non-adsorbed) enzymes. For that purpose, it was assumed an "ideal" enzyme recovery process from liquid fraction, which would mean that the levels of activity loss during this process would be reduced to an extent that the initial enzyme activity on the following rounds would be the same as for the previous scenario, where solid bound enzymes were also recovered. The main differences on annual operational costs and production outcomes are presented on Table 5.9.

Table 5.9 Effect of the elimination of solid fraction recycling over the operational costs and production outcomes

Item	Recycling 2 Fractions (Rec2Frac)	Recycling 1 Fraction (Rec1Frac)
Electricity Cost (Million US$/year)	0.281	0.235
Maintenance (Million US$/year)	1.130	1.040
Fixed & General (Million US$/year)	0.710	0.660
Plant Overhead (Million US$/year)	0.660	0.640
Capital Depreciation (Million US$/year)	3.860	3.560
Total Costs (Million US$/year)	10.94	10.50
Ethanol Production (kg/year)	10.44×10^6	9.98×10^6
Annual Income (Million US$/year)	10.30	9.840

As expected, there was a small reduction on electricity consumption, possibly due to a less complex enzyme recycling process, where enzymes recovery from the solid was no longer considered. On the other hand, a reduction on equipment requirements seemed to cause a much greater impact. Totalizing the effects on Maintenance, Fixed and General, Plan Overhead and Capital Depreciation, there was an annual decrease on the costs of approximately 0.46 Million US$. Overall, total annual costs decrease nearly 4 %, from 10.94 to 10.50 Million US$/year (Table 5.9).

Although total costs are slightly lower in this simpler process, there is an important drawback on total ethanol production. As solid-bound enzymes are no longer recovered, part of the ethanol inside solid matrix that was being recovered alongside alkaline washing is no longer being recovered. Comparatively to the previous scenario, with an annual ethanol production of 10.44 x 10^6 kg, in this case this value decreases to 9.98 x 10^6. Additionally, it is worth mentioning that this value is still slightly inferior comparatively to the base case, nearly 2.1 %, which could be explained by the fact that differently from the base case, where ethanol-containing stream goes directly to ethanol purification stage, on this case it passes through the UF process. As this fraction is recirculated into the process, more ethanol is lost in this case.

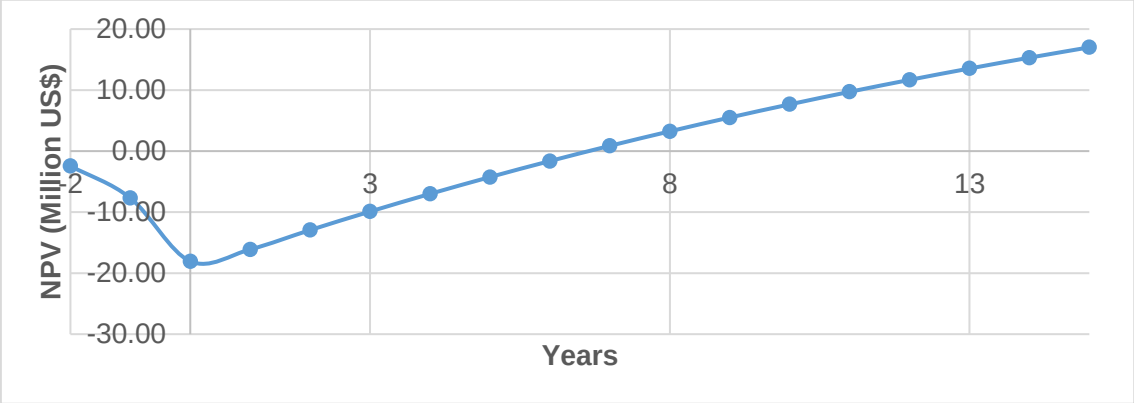

Figure 5.8 Variation of the accumulated NPV over the project life-time for Rec1Frac.

Accordingly, simplifying the cellulase recycling system yields an inferior economic performance, as compared to the former process with integral enzyme recycling: pay-back period increased to 6.81 years and the accumulated NPV decreased to 15.04 Million US$ (Figure 5.8). Nevertheless, and spite the existence of the referred reduction on total ethanol production comparatively to the base case, this recycling scenario is still economically more attractive than the base case: pay-back period decreases from 7.11 to 6.81 years and the accumulate NPV increases from 13.4 to 15.04 Million US$. It is worth mentioning that the assumptions made for solid-liquid separation calculations were based on experimental data and represent a direct consequence of the intrinsic properties of RPS residue regarding water-retention capacity. Thus, the ethanol recovery is not expected to vary significantly at industrial scale.

5.3.6 Cellulase recycling using increased solids loading

Reporting to the base case scenario, it was clearly demonstrated that ethanol purification stage represents a large majority of total equipment costs, which may be explained on a large extent by low final ethanol titers. It was thus envisioned a hypothetic scenario where solids concentration is 28 %, instead of 22 %, and under these conditions the recycling of both fractions was also considered, similarly to Rec2Frac.

Table 5.10 Effect of the increase of solids concentrations on operational costs (Million US$/year)

Item	Rec2Frac	Rec2Frac28%	Variation (%)
Raw Materials	2.33	2.30	- 1.3
Utilities	2.12	2.02	- 4.7
Maintenance	1.13	1.09	- 3.5
Labor	0.13	0.13	--
Fixed & General	0.71	0.69	- 2.8
Plant Overhead	0.66	0.64	- 3.0
Capital Depreciation	3.86	3.74	- 3.1
Total	10.94	10.62	- 2.9

Assuming a constant uptake of RPS material for all scenarios, an increased solids concentration would represent an inferior volume of liquid where solids are suspended. Indeed, this was observed by a small reduction on Raw-Materials, most likely due to an inferior water consumption (Table 5.10). Consequently, due to a reduction on total working volume, a decrease of equipment size, and thus the equipment cost, may also be expected. There was in fact a clear reduction on equipment related costs, which was observed on an average level of 3 %. Referring specifically to the ethanol purification stage, no differences were detected on rectification and dehydration steps, but clear improvements were observed on the initial step: distillation equipment cost decreased approximately 5.8 %, from 8.2 to 7.7 Million US$. This reduction on the working volume enabled a slight increase on final ethanol titers, (Figure 5.9), directly impacting the specifications and requirements of the distillation process.

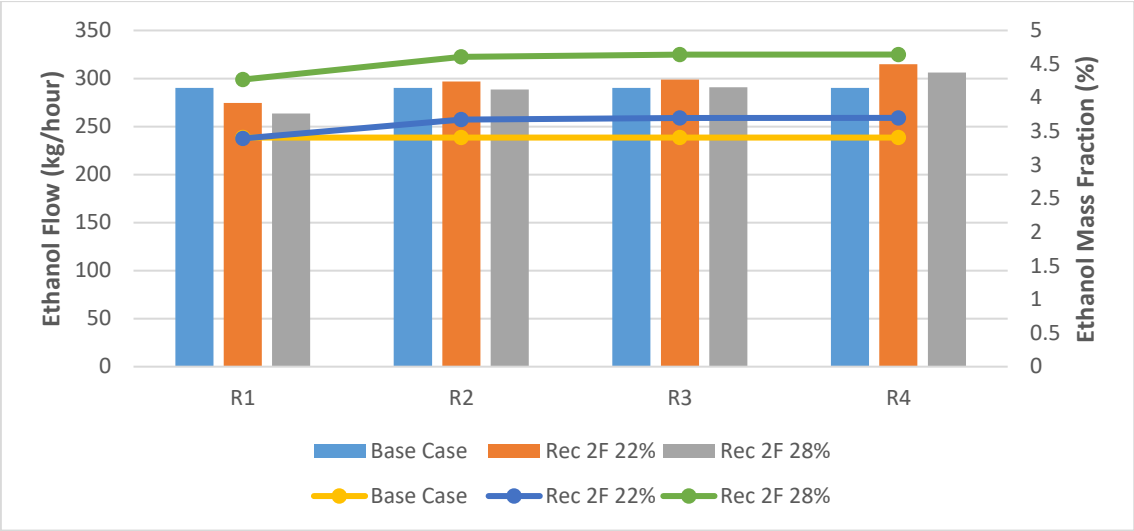

Figure 5.9 Variation of ethanol flow and its mass fraction for different scenarios along the different rounds.

As shown in Figure 5.9, there is a clear increase of the final ethanol titers, from around 3.5 % (w/w) (BaseSc and Rec2Frac), to 4.6 % (Rec2Frac28%). Additionally, the utilities requirements are expected to decrease, which occurs on this case at levels around 4.7 %. Part of this reduction may be assigned to a small decrease on UF membrane consumption as the total volume of filtration is also reduced in this case.

Even though final ethanol titers slightly increased with the % solids used, the actual total mass flow of ethanol entering the ethanol purification stage has decreased from 1185.15 to 1149.03 kg/hour. Since the ethanol that is produced on this case is diluted on a smaller volume of water, hence being more concentrated, the volume of liquid that naturally is retained by the solid after solid-liquid separation will also contain a higher amount of ethanol. Consequently, the gross annual income from ethanol selling decreased from 10.30 to 9.97 Million US$, directly affecting the overall economic output achieved with this strategy: the payback-period increased from 6.65 to 6.71 years, while the accumulated NPV decreased from 17.01 to 16.21 Million US$.

It should be referred that, for both the scenario with only liquid fraction recycling and the one with 28 % solids (solid and liquid fraction recycling), there was a clear reduction on operational costs, which theoretically would enable a more favorable economic output. Nevertheless, for both cases this improvement was total or partially nullified due to the high water-retention (and thus ethanol retention) capacity of the solid.

Current values for the dimensioning of solid-liquid separation were largely based on experimental specifications. It can be hypothesized, however, that on an industrial real context, a lower liquid content retained by the solid may allow a more interesting economic output for these scenarios.

5.3.7 Sensitivity analysis

The abovementioned results are based on the assumption of a specific set of standard values for nuclear variables. These were defined considering current values employed on similar processes and/or based on literature reports. While for some cases it may not be expected relevant variations, some are considerably susceptible to fluctuations. To understand the validity of the results referred above, it is necessary to assess the impact of such possible fluctuations. Aiming that, it was conducted a sensitivity analysis based on the accumulated NPV for varying values of different variables (Figure 5.10).

The accumulated NPV showed to be highly sensitive to both ethanol selling price and enzyme cost, presenting a relative total amplitude above 1000 and 260 %, respectively (*due to considerable scale differences, these are not plotted on the graphs*).

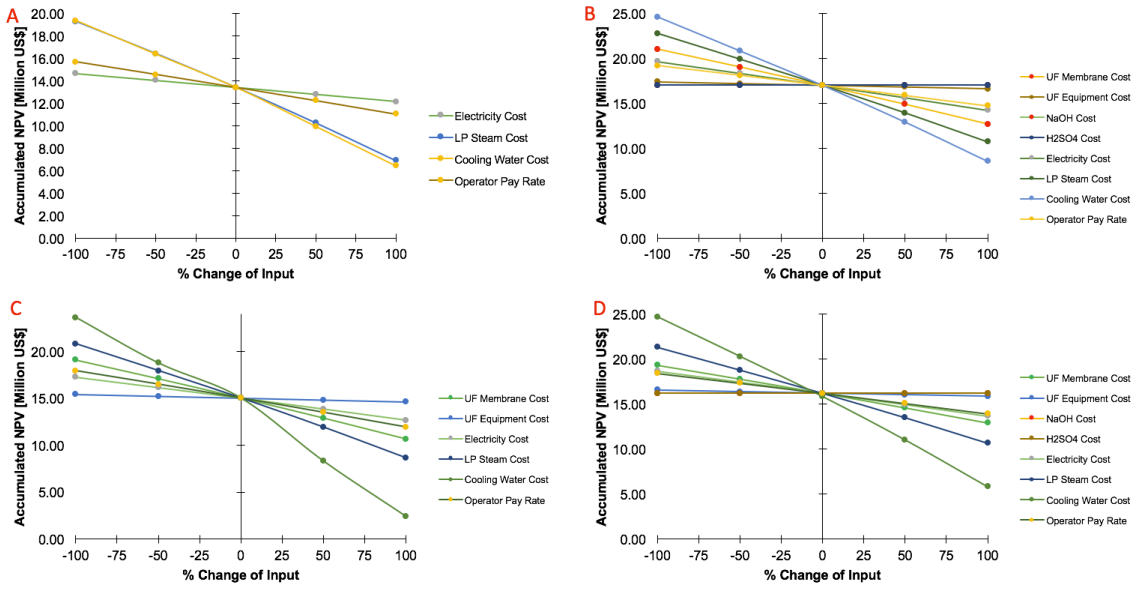

Figure 5.10 Accumulated NPV after project lifetime for varying levels of different variables for BaseSc (A), Rec2Frac(B), Rec1Frac(C) and Rec2Frac28%(D).

Additionally, from the analysis of Figure 5.10, it can be observed that also the cost of UF membrane and heat transfer utilities (cooling water and LP steam) showed a considerable influence on NPV, hence, on the overall economic performance. UF

membrane base price is considerably high, 400 US$/m^2, which justifies in part its impact. On the other hand, the role of heat transfers utilities can be explained by this particular context of low ethanol titres and consequent higher consumption of this item.

It was further aimed to investigate in which conditions the main conclusions from previous sections are valid. Specifically, to understand for which exact range of relevant variables cellulase recycling is economically viable, *i.e.* has a superior economic output comparatively to the base case. Figure 5.11 shows a comparison of the accumulated NPV variation for different scenarios as a function of ranging values of Ethanol Selling Price, Enzyme Cost and UF membrane cost. These were chosen under the assumption that they are the most relevant variables under a scenario of enzyme recycling.

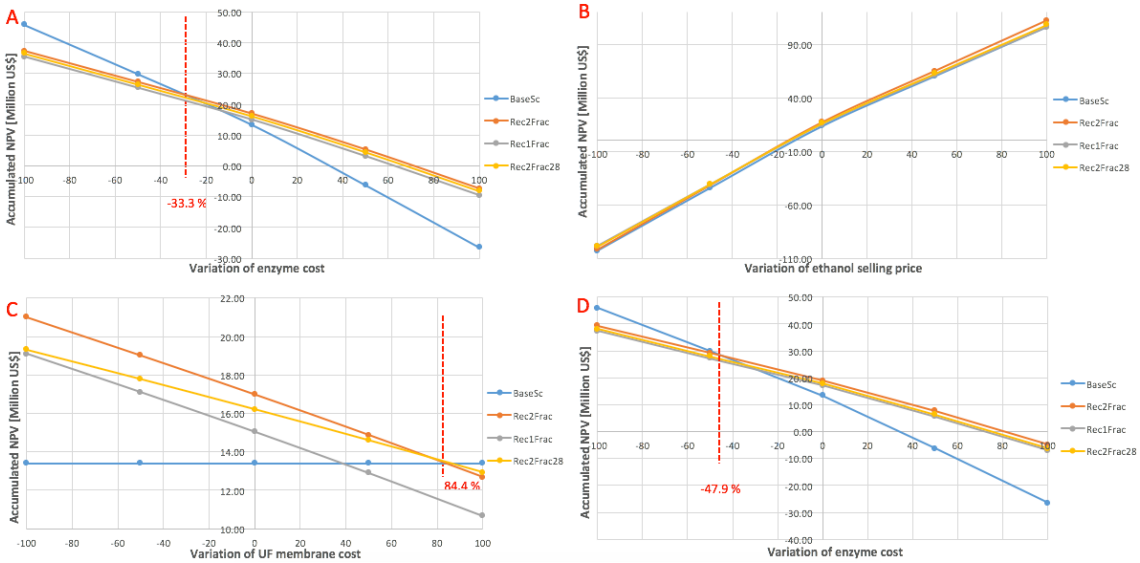

Figure 5.11 Variation of the accumulated NPV for varying levels of (A) enzyme cost, (B) ethanol selling price, (C) UF membrane cost and (D) enzyme cost (assuming 200 US$/m^2 of UF membrane).

Ethanol selling price has a clearly significant effect in the economics of the process for all scenarios, however, it seems to show no apparent influence on the decision of whether cellulase recycling is viable or not. Differently, as expected, enzyme cost evidenced an important role on this decision. As observed on Figure 5.11(A), the accumulated NPV is superior for the scenario of cellulase recycling (comparatively to the base case) for a wide range of enzyme cost. Furthermore, it can be seen that for cellulases recycling becoming economically unviable enzymes price has to decrease at

least 33 % of its base price (to a value of 2.68 US$/kg); beyond this barrier, accumulated NPV for the base case becomes superior to the recycling scenario. Considering the recent achievements on enzyme cost obtained by industry, one may expect that this scenario would be achieved in the near future. On the other hand, this is also dependent on other assumptions. For instance, if a base cost for UF membrane of 200 US$/$m^2$ is considered instead of 400 US$/$m^2$ (D) enzyme recycling consequently gains more relevance and the range for its economic viability becomes wider.

Thus, the cost of UF membrane seems to importantly affect the possible benefit of enzyme recycling. Accumulated NPV for the recycling scenarios is superior to the base case in a wide range of membrane cost. Specifically considering the scenarios with recycling of both fractions, cellulase recycling only becomes unviable when membrane cost increases above 84 % of its base cost. If it is rather considered Rec1Frac, as a direct result of an inferior ethanol revenue, this margin considerably decreases to a level of 40 %. Opposing to enzyme cost, such variations, however, are not expected to occur in the near future.

5.4 Conclusions

This work provides relevant insights into the economic aspects associated to the implementation of enzyme recycling in the industrial process of bioethanol production from RPS. Firstly, it demonstrates that bioethanol production from this specific type of RPS is economic viable even though modest final ethanol titres significantly prejudiced equipment costs for ethanol purification. Furthermore, under specific conditions already experimentally validated, the recycling of enzymes considering both fractions showed a superior economic performance when compared to the base case. A hypothetic scenario referring to the elimination of solid fraction recovery showed an important impact in the reduction of total annuals costs, however, overall economic output was less attractive due to a slight reduction in total ethanol production. Finally, a sensitive analysis suggested that enzymes recycling economic viability is particular susceptible to variations on the cost of enzyme and UF membrane, hence, significant changes on these variables may dictate a scenario where enzyme recycling becomes unviable.

5.5 References

Alriols MG, García A, Muñoz V, Serrano L, Labidi J. 2014. Analysis of the Performance of Membrane Ultrafiltration in the Biorefinery by Aspen Plus ® Simulation. *Chemical Engineering Transactions* 39:1711.

Chen H, Venditti R, Gonzalez R, Phillips R, Jameel H, Park S. 2014. Economic evaluation of the conversion of industrial paper sludge to ethanol. *Energy Economics* 44:281-90.

Davis R, Tao L, Scarlata C, Tan ECD, Ross J, Lukas J, Sexton D. 2015. Process Design and Economics for the Conversion of Lignocellulosic Biomass to Hydrocarbons: Dilute-Acid and Enzymatic Deconstruction of Biomass to Sugars and Catalytic Conversion of Sugars to Hydrocarbons. Technical Report NREL/TP-5100-62498.

Davis R, Tao L, Tan ECD, Biddy MJ, Beckham GT, Scarlata C, Jacobson J, Cafferty K, Ross J, Lukas J, Knorr D, Schoen P. 2013. Process Design and Economics for the Conversion of Lignocellulosic Biomass to Hydrocarbons: Dilute-Acid and Enzymatic Deconstruction of Biomass to Sugars and Biological Conversion of Sugars to Hydrocarbons. Technical Report NREL/TP-5100-60223.

García CA, Betancourt R, Cardona CA. 2017a. Stand-alone and biorefinery pathways to produce hydrogen through gasification and dark fermentation using Pinus Patula. *Journal of Environmental Management* 203:695-703.

García CA, Moncada J, Aristizábal V, Cardona CA. 2017b. Techno-economic and energetic assessment of hydrogen production through gasification in the Colombian context: Coffee Cut-Stems case. International Journal of Hydrogen Energy 42:5849-5864.

Gomes D, Domingues L, Gama M. 2016. Valorizing recycled paper sludge by a bioethanol production process with cellulase recycling. *Biores. Technol.* 216:637-44.

Gomes D, Rodrigues AC, Domingues L, Gama M. 2015. Cellulase recycling in biorefineries--is it possible?. *Appl. Microbiol. Biotechnol.* 99:4131-43.

Gomes DG, Gama FM, Domingues L. 2018. Determinants on an efficient cellulase recycling process for the production of bioethanol from recycled paper sludge under high solid loadings. *Biotechnol Biofuels*, In press.

Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Lidén G, Zacchi G. 2006. *Trends Biotechnol*. 24(12):549-56.

Lv PM, Xiong ZH, Chang J, Wu CZ, Chen Y, Zhu JX. 2004. An experimental study on biomass air-steam gasification in a fluidized bed. *Bioresour. Technol*. 95:95-101.

Marques S, Alves L, Roseiro JC, Gírio FM. 2008. Conversion of recycled paper sludge to ethanol by SHF and SSF using *Pichia stipites*. *Biomass Bioenerg.* 32:400-6.

Marques S, Santos JAL, Gírio FM, Roseiro JC. 2008b. Lactic acid production from recycled paper sludge by simultaneous saccharification and fermentation. *Biochem. Eng. J.* 41:210-16.

Monte MC, Fuente E, Blanco A, Negro C. 2009. Waste management from pulp and paper production in the European Union. *Waste Manag.* 29(1):293-308.

Munalula F, Meincken M. 2009. An evaluation of South African fuelwood with regards to calorific value and environmental impact. *Biomass Bioenergy* 33:415-20.

Pellegrini LF, de Oliveira S. 2007. Exergy analysis of sugarcane bagasse gasification. *Energy* 32:314-27.

Peters M, Timmerhaus K, West R. 2003. Plant Design and Economics for Chemical Engineers. McGraw Hill, New York.

Peters MS, Timmerhaus KD, West RE. 1991. Chaper 6: Cost Estimation, In: Plant Design and Economics for Chemical Engineers, p. 923.

Rodrigues AC, Felby C, Gama M. 2014. Cellulase stability, adsorption/desorption profiles and recycling during successive cycles of hydrolysis and fermentation of wheat straw. *Bioresour. Technol.* 156:163-9.

Rodrigues AC, Haven MØ, Lindedam J, Felby C, Gama M. 2015. Celluclast and Cellic® CTec2: saccharification/fermentation of wheat straw, solid-liquid partition and potential of enzyme recycling by alkaline washing. *Enzyme Microb. Technol.* 79-80:70-7.

Ulrich GD, Vasudevan PT. 2006. How to estimate utility costs. *Chem. Eng*. 113:66-9.

Zainal ZA, Ali R, Lean CH, Seetharamu KN. 2001. Prediction of performance of a downdraft gasifier using equilibrium modeling for different biomass materials. *Energy Conversion and Management* 42:1499-1515.

Chapter VI

General conclusions and future perspectives

Lignocellulosic ethanol has been studied for several decades now and yet many technical obstacles remain. Lignocellulosic materials traditionally require complex pre-treatments, in some cases very expensive, which increases production costs but can also originate considerable amounts of toxics. Despite the recent advances on cells tolerance to these compounds, they still constitute a relevant obstacle to enzymatic hydrolysis.
On the other hand, even though enzyme manufactures have achieved remarkable reductions on enzyme prices, mostly through developments on their production process and strain developments, cellulases are still considered one of the main determinants on ethanol final cost.

One of the first aims of this thesis was to explore the potential of Recycled Paper Sludge for bioethanol production. As opposing to the traditional lignocellulosic materials, no pre-treatment is usually required due to previous intense processing during paper manufacturing. Additionally, RPS is generated in high amounts world-wide, usually being disposed on landfills. Even though some studies already exist describing the utilization of similar materials, reports specifically referring to their utilization for ethanol production are surprisingly scarce.
Although the small levels of cellulose found on RPS on its raw form (around 15 % (w/w)), these significantly increased to nearly 30 % (w/w) after a simple chemical (HCl) neutralization (due to the removal of inorganic load in the material), which already represent an interesting substrate concentration for hydrolysis. Furthermore, despite the putative toxicity commonly associated to this material (mostly originated from ink particles and solvents), there was no visible indication of such effects over the enzymes, which were able to promptly hydrolyse this material with high conversions yields. On the other hand, in the scope of process intensification, RPS presented some operational challenges. As a result of its high water retention capacity, the solid suspensions were critically limited by the difficulty in achieving an effective mixing. On a lab scale, 22 % solids was found to be the maximum operational consistency enabling the liquefaction of solids suspension. Future studies should explore more efficient mixing systems, such as stirred tanks, or alternative process configurations such as the fed-batch operation.

This could be critical for the economic viability of the process, considering that a level of 22 % (w/w) solids enabled final ethanol titres of 3.7 % (v/v).

In the same context, also the possibility to convert the hemicellulosic fraction on RPS, corresponding to nearly 8 % (w/w) of the initial material, should be addressed. For this purpose, proper levels of hemicellulase activity are required, in addition to suitable yeast platforms able to efficiently utilize pentose sugars. Another possibility that might be interesting to explore is an enrichment on sugar content through a multi-waste valorization approach such as the one reported recently for *Eucalyptus* wood ethanol production by cheese whey incorporation (Cunha et al. 2018 Bioresour Technol 250:256-264).

A second aim of this thesis was to integrate the recycling of enzymes on the process of RPS conversion. One of the possibilities to recover the enzymes from the final product consists on the ultrafiltration of the liquid phase and its direct use on fresh substrate. Comparatively to the few alternatives available this is possibly the most easy to implement on an industrial scale. Enzyme recyclability on this case would thus be strongly dependent on a favourable enzyme adsorption/desorption behaviour. This will dictate the levels of enzyme easily available in the liquid phase for recovery at the end of the process.

The analysis of enzyme partition among the solid/liquid fractions suggest that both the enzyme cocktail and the lignocellulosic material may be critical for enzyme recovery. Different enzyme cocktails enabled very distinct levels of soluble enzyme after hydrolysis, the same being observed for the recovery of the solid-bound fraction by alkaline elution. Celluclast utilization allowed to recover a remarkable 88 % of the final enzyme activity, a promising indicator for its suitability for enzyme recycling. This is even more surprising considering that the levels of non-adsorbed enzyme reported for other materials using the same cocktail were considerably lower. As enzyme-substrate interaction are intimately related to hydrophobic interactions, the chemical composition of the RPS seems to play an important role.

For an efficient recycling, cellulases must remain stable over the process, as to enable their reutilization for several times. On a traditional enzymatic hydrolysis this is mostly affected by thermal denaturation, but also by enzyme interaction with components of

the solid. When the stability of the different cocktails was analysed in the range of temperatures between 45-55 ºC important differences were observed, especially for incubation periods above 72 hours. However, for all cocktails important reductions were detected, which would significantly hamper their reutilization. Opposing to that, for a temperature of 40 ºC no thermal denaturation was observed. However, the enzyme activity decreased approximately by 22 % after RPS hydrolysis (over 120 hours) at this temperature. This may be assigned to some enzyme inactivation resulting from the interaction with specific components of the solid (lignin or ash). For this reason, and despite the efficiency of final enzyme recovery, a fraction of fresh enzyme is required to compensate multiple activity losses. An option that may be interesting to test on the future it to attempt a reduction on the ash and lignin content of RPS through simple processes and assess its effects on the levels of enzyme loss during hydrolysis.

Both enzyme stability and final partition on the multiphasic system enabled to successfully conduct four consecutive rounds of hydrolysis with the addition of only a small amount of fresh enzyme. A similar scenario was observed when this strategy was tested under more intensified conditions, despite the higher levels of lignin and ash available on this case. This suggest that process intensification may not be a critical obstacle for the feasibility of enzyme recycling following this strategy. From this scope, it may be interesting to test even higher levels of solid, as long as enzyme partition is not compromised. Additionally, the evaluation of the current strategy on a superior scale may be essential as would allow to reduce some enzyme activity losses, traditionally more likely on small scales, hence allowing to refine and optimize some important operational variables, namely the fraction of fresh enzyme required.

Despite the obvious benefits of this strategy on enzyme consumption, a most relevant factor to consider is the cost of its industrial implementation. According to an economic study here conducted, enzyme recovery corresponds in fact to an expensive process, especially due to the solid-bound fraction. Nevertheless, in addition to a reduction on total enzyme cost it also allowed to recover an important fraction of ethanol which stays retained on the final solid, thus contributing to a superior ethanol production. Although overall enzyme recycling showed to be economically viable, it was found to be highly sensitive to the cost of enzyme, thus, future reductions on this element may change this

scenario. Opposing to that, this could be compensated if an equal reduction could be achieved on the cost of enzyme recycling process, namely in the ultrafiltration step.

As final remarks, this work demonstrates the technical and economic feasibility of cellulases recycling in the process of bioethanol production from RPS. Additionally, it clarifies the critical role of enzyme cocktail and process conditions on enzyme recyclability.

In the sequence of these results it may be especially relevant to test this strategy on a higher scale to assess possible limitations on a future scale-up process (*e.g.* problems of enzyme stability). The assessment of alternative recycling strategies, such as adsorption on fresh solid or enzyme immobilization, and their corresponding cost could also be a valuable information for this discussion.

Another question raised by this work is the possibility to apply this system to other materials, which would require to be extensively assessed due to the specificity of these results. This would enable a wider range of possible applications, going beyond the traditional bioethanol to other added-values products. Ultimately, this may represent a great contribution for the economic valorisation of lignocellulosic materials.