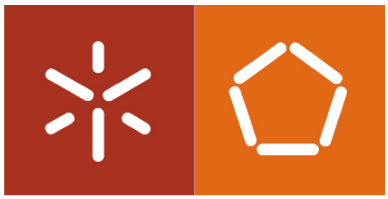

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Process Development for Bioethanol Production Using Wheat Straw Biomass

Doctoral Dissertation for PhD degree in Chemical and Biological Engineering

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"Hasta que el pueblo las canta,
las coplas, coplas no son,
y cuando las canta el pueblo
ya nadie sabe el autor.
Procura tú que tus coplas
vayan al pueblo a parar.
Que, al volcar el corazón
en el alma popular,
lo que se pierde de gloria
se gana de eternidad "

Facundo Cabral

I dedicate this thesis to

Diana Monique

SUMMARY

This thesis is focused on the process development for bioethanol production based in the biorefinery concept. Nowadays, the role of biorefineries is centred on integration of traditional and modern processes for utilization of biological raw material in the production of energy and chemicals.

Lignocellulosic materials such as wheat straw are renewable biomass than can be used as substrate in fermentation processes. The use of wheat straw includes the following steps: milling, pretreatment, enzymatic saccharification and bioconversion of the obtained sugars in bioethanol. In a first stage, autohydrolysis pretreatment was used for solubilization of hemicellulose. In this part, the evaluation of autohydrolysis conditions and determination of their monosaccharide composition in the liquid phase was studied. The results showed the importance of the variation of the particle size distribution on the extraction of total sugars from hemicellulose (glucose, xylose and arabinose), demonstrating that the use of a blend with defined percentages of the various particle sizes is an important parameter to establish before carrying out a pretreatment. In a second stage, the pretreated solids obtained after autohydrolysis were subjected to delignification using organosolv process. A high purity lignin was obtained after the sequential autohydrolysis-organosolv process.

Following the biorefinery philosophy, the autohydrolysis hemicellulose extracted by autohydrolysis optimum conditions was used as reinforcements in a κ-car/ LBG polymeric matrix for biocomposite manufacturing. Biocomposite from extracted hemicellulose showed to be a good material to be used in the reinforcement of edible films.

The susceptibility to enzymatic saccharification of pretreated solids obtained by autohydrolysis and by sequential autohydrolysis-organosolv was studied. According to the obtained results, enzymatic saccharification of the autohydrolysis pretreated solids proved to be more effective than when the organosolv pretreated solids were used. The maximum extent of the enzymatic conversion of cellulose to glucose was 90.88 % and

64.04 %, respectively. These results demonstrate that the autohydrolysis pretreated solids are a good substrate for simultaneous saccharification and fermentation.

The simultaneous saccharification and fermentation (SSF) of lignocellulosic materials requires the utilization of microorganisms capable of working at high temperatures. In this part of the work, a flocculant *Saccharomyces cerevisiae* CA11 was evaluated for its ability to grow and ferment glucose in the temperature range of 40 - 50 ºC. The highest ethanol concentrations obtained were 24.12 and 24.38 g/L at 40°C and 45 °C, respectively, for an initial glucose concentration of 50 g/L. The results indicate that the *S. cerevisiae* CA11 was able to produce ethanol, showing a good performance at high temperatures. In order to evaluate the effects of temperature, substrate concentration (autohydrolysis pretreated wheat straw) and enzyme loading on: 1) ethanol conversion yield, 2) ethanol concentration, and 3) CO_2 concentration, a central composite design (CCD) was used. Results showed that the ethanol conversion yield was mainly affected by enzyme loading, whereas for ethanol and CO_2 concentration, enzyme loading and substrate concentration were found to be the most significant parameters. The maximum ethanol concentrations (14.84 g/L) were obtained at 45 ºC, 3 % (w/v) substrate and 30 FPU of enzyme loading, corresponding to an ethanol yield of 82.4 %. The scale-up of SSF was performed evaluating the effect of stirring on ethanol yield. When the SSF was conducted at higher stirring speed (250 rpm), 15.09 g/L of ethanol was obtained. This corresponds to an overall ethanol yield of 84.49 %.

Overall, results presented in this work, describe the development of an integrated process based on the biorefinery concept for the production of bioethanol from wheat straw. It is shown that wheat straw pretreated with autohydrolysis process can be used as a substrate for bioethanol production using a flocculent *S. cerevisiae* strain. Moreover, extracted hemicellulose is applied as reinforcement for edible films and the obtained lignin is a high purity product.

RESUMO

Esta tese trata do desenvolvimento de um processo para produção de bioetanol com base no conceito de biorefinaria. Hoje em dia o papel das biorefinarias centra-se na integração de processos tradicionais e modernos para utilização de material biológico bruto na produção de energia e reagentes.

Materiais lenhocelulósicos como a palha de trigo são uma biomassa renovável que pode ser usada como substrato em processos de fermentação. Este processo consiste em: moagem da palha de trigo, pré-tratamento da palha de trigo, sacarificação enzimática e bioconversão dos açúcares obtidos em bioetanol. Inicialmente o tratamento de auto-hidrólise foi usado para solubilizar a hemicelulose. Nesta parte do trabalho, avaliaram-se as condições de auto-hidrólise e determinaram-se os monossacáridos presentes na fase líquida. Os resultados demonstraram a importância da variação da distribuição do tamanho das partículas na extração dos açúcares totais a partir da hemicelulose (glucose, xilose e arabinose), e a necessidade de estabelecer a composição da mistura a utilizar antes de proceder a um pré-tratamento. Os sólidos pré-tratados por auto-hidrólise foram recuperados e deslenhificados através do processo organosolv, tendo sido obtida uma lenhina de elevada pureza.

Com base no conceito de biorefinaria, a hemicelulose extraída por auto-hidrólise sob condições óptimas foi incorporada como reforço para uma matriz polimérica de κ-car/ LBG, sendo obtido um biocompósito que demonstrou ser um bom material para ser usado no reforço de filmes edíveis.

A susceptibilidade á sacarificação enzimática dos sólidos pré-tratados obtidos por auto-hidrólise e uma sequência de auto-hidrólise-organosolv foi estudada. Os resultados obtidos demonstram que a sacarificação enzimática é mais eficiente quando se usaram sólidos pré-tratados só por auto-hidrólise. Os valores máximos de conversão enzimática de celulose em glucose foram de 90.88 % e de 64.04 %, para sólidos tratados por auto-hidrólise e pela sequência auto-hidrólise-organosolv, respectivamente.

O processo de sacarificação e fermentação simultânea (SFS) de materiais lenhocelulósicos requer o uso de microrganismos capazes de trabalhar a elevadas temperaturas. Nesta parte do trabalho, uma estirpe floculante de *Saccharomyces cerevisiae* CA11 foi utilizada pela sua capacidade de crescer e fermentar glucose numa gama de temperaturas de 40 - 50 ºC. As produções máximas de etanol obtidas a partir de 50 g/L glucose foram de 24.12 e 24.38 g/L a 40 °C e 45 °C, respectivamente. Os resultados indicam que as células de *S. cerevisiae* CA11 forem capazes de produzir etanol, demonstrando um bom desempenho a altas temperaturas.

Para avaliar os efeitos da temperatura, concentração de substrato (palha de trigo pré-tratada com auto-hidrólise) e carga de enzima em: 1) rendimento de conversão em etanol, 2) concentração de etanol, e 3) concentração de CO_2, usou-se um desenho experimental do tipo "central composite design" (DCC). Os resultados demonstraram que o rendimento de conversão em bioetanol foi principalmente afectado pela carga de enzima, enquanto a concentração de etanol e de CO_2, carga de enzima e concentração de substrato foram considerados os parâmetros mais significativos. A concentraçõe máxima de etanol (14.84 g/L) foi obtida a 45 ºC, 3 % (p/v) de substrato e 30 FPU de carga enzimática, correspondendo a um rendimento em etanol de 82.4 %. O aumento de escala do SFS foi realizado avaliando o efeito da agitação no rendimento de etanol. Quando o SFS foi efectuado a velocidade de agitação mais elevada (250 rpm), obtiveram-se 15.09 g/L de etanol. Este valor corresponde a um rendimento global em etanol de 84.49 %.

Globalmente, os resultados apresentados neste trabalho descrevem o desenvolvimento de um processo integrado baseado no conceito de biorefinaria para a produção de etanol a partir da palha de trigo. Demonstrou-se que a palha de trigo tratada por auto-hidrólise pode ser usada como substrato para a produção de bioetanol utilizando uma estirpe floculante de *Saccharomyces cerevisiae*. Demonstrou-se também a possibilidade de utilizar a hemicelulose extraída como reforço em filmes edíveis e a alta pureza da lenhina obtida.

LIST OF PUBLICATIONS

This thesis is based on the following original articles:

Ruiz HA, Rodríguez-Jasso RM, Fernandes BD, Vicente AA, Teixeira JA. Hydrothermal processing, as an alternative for upgrading agriculture residues and marine biomass according to the biorefinery concept: a review. *Submitted for publication*. Chapter 2

Ruiz HA, Ruzene DS, Silva DP, Quintas MAC, Vicente AA, Teixeira JA. (2011). Evaluation of a hydrothermal process for pretreatment of wheat straw – effect of particle size and process conditions. *Journal Chemical Technology & Biotechnology* 86:88-94. Chapter 3

Ruiz HA, Ruzene DS, Silva DP, da Silva FM, Vicente AA, Teixeira JA. (2011). Development and characterization of an environmentally friendly process sequence (autohydrolysis and organosolv) for wheat straw delignification. *Applied Biochemistry and Biotechnology* 164:629-641. Chapter 4

Ruiz HA, Cerqueira MA, Silva HD, Rodríguez-Jasso RM, Vicente AA, Teixeira JA. Extraction, optimization and characterization of autohydrolysis wheat straw hemicellulose to be used as a biocomposite in the reinforcement of polysaccharide-based films. *Submitted for publication*. Chapter 5

Ruiz HA, Vicente AA, Teixeira. (2012). Kinetic modeling of enzymatic saccharification using wheat straw pretreated under autohydrolysis and organosolv process. *Industrial Crops and Products* 36:100-107. Chapter 6

Ruiz HA, Silva DP, Ruzene DS, Lima LF, Vicente AA, Teixeira JA. Bioethanol production from hydrothermal pretreated wheat straw by a flocculating *Saccharomyces cerevisiae* strain - effect of process conditions. *Fuel* (in press) doi: 10.1016/j.fuel.2011.10.060). Chapter 7

Ruiz HA, Vicente AA, Teixeira. Screening of a flocculant *Saccharomyces cerevisiae* strain and its performance in bioethanol production from wheat straw using simultaneous saccharification and fermentation process. *Submitted for publication*. Chapter 7-8

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LIST OF ABBREVIATIONS

Acl_C	acetyl groups converted into acetic acid
AcO_C	acetyl groups converted into acetyl groups linked to oligosaccharides
AcOS	acetyl groups linked to oligosaccharides
ANOVA	analysis of variance
AOS_C	arabinan converted into arabinooligosaccharides
APS	autohydrolysis pretreated solids
ArOS	arabinooligosaccharides
Ara_C	arabinan converted into arabinose
C_{CO2}	CO_2 concentration
C_{EtOH}	Ethanol concentration
d.f.	degree of freedom
dG/d*t*	initial saccharification rate
DNS	3,5-dinitrosalicylic acid method
DP	degree of polymerization
EB	elongation-at-break
EDTA	ethylenediaminetetra-acetic acid
F_C	xylan converted into furfural
FPU	unit of filter paper cellulase
FTIR	Fourier Transform Infrared
F-value	F distribution
G	guayacyl units
Glu_C	glucan converted into glucose
GO_C	glucan converted into glucooligosaccharides
GOS	glucooligosaccharides
HE	Hemicellulose extracted
HEY	Hemicellulose extraction yield
HMF	hydroxymethylfurfural

HMF_C	glucan converted into HMF
HPLC	High performance liquid chromatography
IU	unit of activity
K	number of independent variables
κ-car	(Gelcarin DX5253)
κ-car/LBG	κ-car and LBG biocomposite
LBG	(Genu gum type RL-200)
LCM	lignocellulose material
MC	moisture content
N	number of experiments
OPS	organosolv pretreated solids
PLA	Polylactic acid
p-value	Probability value
R^2	coefficient of determination
R_0	severity factor
RH	relative humidity
RI	refractive index
S	Syringyl units
SE	Standard Error
SEM	scanning electron microscopy
SSF	simultaneous saccharification and fermentation
TS	tensile strength
Var	variance
WE	Wheat straw after ethanol precipitation
WS	Wheat straw
WVP	water vapor permeability
XOS	xylo-oligosaccharides
XOS_C	xylan converted into xylooligosaccharides
Xyl_C	xylan converted into xylose
Y_{EtOH}	ethanol yield

YPD	Yeast extracted peptone dextrose culture medium

Subscripts

f	Final conditions
0	Initial conditions
max	Maximal conditions
min	Minimal conditions

Greek letters

α	significance level
β	Numerical constant
p	Numerical constant
θ	angle of diffraction
x	Numerical constant

CHAPTER 1

MOTIVATION AND OUTLINE

ABSTRACT

This chapter introduces the thesis motivation for bioethanol production using wheat straw biomass based on the development of a biomass pretreatment process, followed by simultaneous saccharification and fermentation by *Saccharomyces cerevisiae* and adopting the biorefinery concept. Finally, thesis outline is explained.

1.1 THESIS MOTIVATION

Svante Arrhenius in 1896, published a paper entitled "*On the Influence of Carbonic Acid in the Air upon the Temperature of the Ground"*. Arrhenius's paper was the first reference that quantified the contribution of carbon dioxide to the greenhouse effect, starting the speculation about whether variations in the atmospheric concentration of carbon dioxide contribute to long-term variations in clime. Today, Earth must deal with the consequences of global climate change and somehow meet expanding energy needs while limiting greenhouse gas emissions. The Kyoto Protocol to the United Nations Framework Convention on Climate Change (UNFCCC) was agreed upon in December 1997, marked an important turning point in efforts to promote the use of renewable energy worldwide.

Moreover, pressing economic and environmental factors, such as soaring crude oil prices, global warming and diminishing oil reserves, have been driving global interest in searching for renewable energy to replace fossil fuels. The use of biomass as a source of energy was forcefully supported at the Kyoto Conference on the grounds that the CO_2 produced by the combustion or processing of these materials is absorbed by the biomass sources via photosynthesis. The governments of the main countries in the world initiated major programs to fund the development of new energy sources in response to tightening petroleum supplies and skyrocketing energy costs during the "Energy Crises" of the mid- to late-1970s. In reality, these events were actually "Petroleum Crises," because a number of oil-rich countries, particularly in the Middle East, teamed together to form the oil cartel (Organization of the Petroleum Exporting Countries "OPEC") and control the quantity and therefore price of petroleum.

Biomass is seen as an interesting energy source for several reasons. The main reason is that bioenergy can contribute to sustainable development. Ethanol is one form of renewable energy that has been receiving much attention. It is a sustainable energy source and a clean-burning fuel, gives higher thermal efficiency and power than conventional gasoline. Lignocellulose is a complex substrate composed of a mixture of carbohydrate polymers (cellulose and hemicellulose) and lignin. These carbohydrate polymers have to be broken down into fermentable sugars for fermentation.

Autohydrolysis process is an easy, cheap and first step to solubilized hemicellulose while increasing the accessibility of cellulose to enzymes producing sugars for bioethanol production.

Biomass can be converted into useful biofuels, biochemicals, biomaterials that are co-produced via biomass upgrading and biorefinery technologies. In the near future, second generation biofuel facilities are expected to develop towards the biorefinery concept. The biorefinery economy is a vision for a future in which biorenewables replaces fossil fuels.

1.2 THESIS OUTLINE

The main objective of this thesis was a process development for bioethanol production based in the biorefinery concept.

The main focus areas were:

- Evaluation of autohydrolysis process as a first step for biomass treatment.
- Sequential use of autohydrolysis-organosolv processes for delignification.
- Application of autohydrolysis-extracted hemicellulose as reinforcement for κ-car/LBG polymeric matrix for biocomposite manufacture.
- Kinetic modeling of enzymatic saccharification of solids pretreated with either the autohydrolysis process and sequential autohydrolysis-organosolv processes.
- Evaluation of the simultaneous saccharification and bioethanol fermentation process by a flocculent *S. cerevisiae* using as substrate pretreated autohydrolysis wheat straw.

Based on these main objectives, this thesis was organized in nine chapters. Chapter 2 provides an overview of the state of the art on the biorefinery concept, Chapters 3 to 8 contain the main experimental results while Chapter 9 presents the general conclusions and future perspectives.

In Chapter 2, the thesis starts with an overview of ethanol as a fuel in relation to biorefinery concept using autohydrolysis as pretreatment.

In Chapter 3, the evaluation of different operational conditions (temperature, time and particle size) on wheat straw autohydrolysis process was studied. The effects of operational conditions on the formation of fermentation products and inhibitors is presented.

Chapter 4 describes the utilization of a sequential process (autohydrolysis-organosolv) for lignin extraction.

In Chapter 5, the hemicellulose was used as a reinforcement for κ-car/ LBG polymeric matrix for biocomposite manufacture. The optimization and characterization of hemicellulose extraction from autohydrolysis were also considered.

Chapter 6 shows the kinetic modeling of enzymatic saccharification of either autohydrolysis pretreated solids or solids pretreated by a sequence of autohydrolysis-organosolv. Also, untreated wheat straw and two model substrates (Avicel and filter paper) were studied. The yield of saccharification, glucose production, and kinetic model profiles were discussed. The susceptibility to enzymatic saccharification was studied.

The evaluation of simultaneous saccharification and bioethanol fermentation by a flocculent *S. cerevisiae* using an experimental design was evaluated. The effects of enzymes loading, temperature and substrate (autohydrolysis wheat straw) on ethanol yield, ethanol and CO_2 production are presented in Chapter 7.

Chapter 8 shows the scale-up of simultaneous saccharification and bioethanol fermentation, which was performed evaluating the effect of stirring on ethanol yield.

Finally, Chapter 9 present the overall conclusions, recommendations and suggestions for future work.

CHAPTER 2

BIOETHANOL PRODUCTION AND HYDROTHERMAL PROCESSING, AS AN ALTERNATIVE FOR UPGRADING AGRICULTURE RESIDUES ACCORDING TO THE BIOREFINERY CONCEPT

ABSTRACT

The concept of a biorefinery that integrates processes and technologies for biomass conversion demands an efficient utilization of all components. Hydrothermal processing is a potential clean technology to convert raw materials such as lignocellulosic materials into bioenergy (bioethanol) and high added-value chemicals. In this technology, water at high temperatures and pressures is applied for hydrolysis, extraction and structural modification of materials. This chapter is focused on providing an updated overview on the fundamentals of hydrothermal processing and its modelling, as well as separation and applications of the main components of lignocellulosic materials into value-added products.

2.1 INTRODUCTION

Considering the amount of biomass available, there is a clear opportunity to develop commercial processes that could generate products needed at very high volumes and low selling price. Most of such products are now being made from non-renewable resources, mainly through oil refineries. These refineries, starting from a complex mixture (petroleum), use a wide range of unit operations to generate an impressive variety of products that are sold directly or transformed into value-added products such plastics and fibers.

Approximately 17 % of the volume of products derived from petroleum in the US is classified as chemicals. If these chemicals could be obtained from renewable resources (e.g. biomass in a biorefinery), it would reduce petroleum dependence while also having a positive environmental impact. In recent years, the use of different renewable raw materials (lignocellulose material-LCM) has been a growing trend for different applications and products such as energy, fuels, chemical, cosmetics, medical applications, construction materials and high added-value products for food or feed.

The term "biorefinery" borrows its origin from the classical petroleum refinery concept and refers to biomass conversion into fuels and chemicals with high added-value through the integration of clean processes (Octave, 2009; Cherubini et al., 2010). Several technologies have been developed during the last decades that allow this conversion process to occur, the clear objective being now to make this process cost-competitive in today's markets.

Hydrothermal processing is an alternative for the fractionation of these raw materials (LCM). The fractionation refers to the conversion of LCM into its main constituents: (i.e., cellulose, hemicellulose and lignin) (Pronyk et al., 2011; Roesijadi et al., 2010; Chisti, 2010). Bobleter et al. (1976) pioneered in using water for pretreatment to enhance the susceptibility of lignocellulosic material to enzymatic hydrolysis. The processes with liquid water under high temperature and pressure are also called autohydrolysis, hydrothermal treatment, hot compressed water (HCW), hydrothermolysis, liquid hot water (LHW), aquasolve process, aqueous processing and

pressure-cooking in water (Ruiz et al., 2011a; Romaní et al., 2011; Feira et al., 2011; Romaní et al., 2010b; Ruiz et al., 2011b; Díaz et al., 2010; Peterson et al., 2009; Chaogang et al., 2005; Yu and Wu, 2010; Suryawati et al., 2009; Faga et al., 2010; Pérez et al., 2008; Kim and Mosier, 2009; Ingram et al., 2011; Garrote et al., 2008a; Weil et al., 1997; Bobleter et al., 2005).

The objective of this chapter is to present an overview in bioethanol and hydrothermal processing of lignocellulosic materials for the fractionation of their main components. The process fundamentals, mathematical modeling, effects of hydrothermal processing on cellulose, hemicellulose and lignin and their applications are reviewed.

2.2 BIOETHANOL

Ethanol, ethyl alcohol, grain alcohol, CH_3CH_2OH or ETOH is an important organic chemical because of its unique properties, and therefore can be widely used for various purposes. Ethanol is a liquid that under ordinary conditions is a volatile, flammable, clear, colorless liquid, miscible in both water and non-polar solvents. The production of ethanol has two routes: synthetic and biological. The synthetic ethanol production is commonly carried out by a catalytic hydration of ethylene in vapor phase and often is a by-product of certain industrial operations (Logsdon, 2006). The ethanol produced from this process is mostly used as a solvent (60%) and chemical intermediate (40%). The "biological" ethanol is produced from fermentation of sugars extracted mostly from crops. *Saccharomyces cerevisiae* is the most popular microorganism used for ethanol production due to (1) its good fermentative capacity and ethanol tolerance, allowing to produce up to 20% (v/v) ethanol; (2) its GRAS (generally regarded as safe) status; (3) its capacity to grow rapidly under anaerobic conditions (Guimarães et al., 2010).

2.2.1 FUEL PROPERTIES OF BIOETHANOL

Bioethanol can be used directly in cars designed to run on pure ethanol or blended with gasoline to make ''gasohol". As an oxygenated compound, ethanol provides additional

oxygen in combustion, and hence obtains better combustion efficiency. Bioethanol has a higher octane number, broader flammability limits, higher flame speeds and higher heats of vaporization than gasoline. These properties allow for a higher compression ratio, shorter burn time and leaner burn engine, which lead to theoretical efficiency advantages over gasoline in an internal combustion engine (Balat, 2007).

2.2.2 CONVERSION PROCESS OF BIOMASS TO BIOETHANOL

The bioconversion of LCM to ethanol involves a series of processes. Biomass undergoes pretreatment to alter the structure of biomass and enhance accessibility of cellulose to hydrolysis enzymes (see section 2.3). The idea of performing the enzymatic hydrolysis and fermentation simultaneously was put forward by Gauss et al., (1976). To this effect, simultaneous saccharification and fermentation (SSF) of pretreated lignocellulosic feedstock is an excellent choice for process integration. It offers certain advantages over separate hydrolysis and fermentation (SHF) in the production of bioethanol from LCM. It should improve the ethanol yield by eliminating end-product inhibition of cellulose hydrolysis, because the microorganism can utilize the sugars for growth and ethanol production as they are formed (Alfani et al., 2000). The pretreated solid is transferred to a simultaneous saccharification and fermentation vessel where cellulose hydrolysis catalyzed by enzymes and fermentation of glucose by microorganisms takes place at the same time (Figure 2.1). The end product, ethanol, is distilled, purified and used as liquid fuel.

Pretreatment and SSF have become the major challenges in cellulosic ethanol conversion technology to date. For the past decade, extensive studies have been done in these areas to improve their performance. In pretreatment, formation of by-products undesirably inhibits fermentative microorganisms and lowers ethanol yield. There are high production costs associated with the cost of chemicals, chemical neutralization and disposal, and investmet in corrosion resistant equipment. Moreover, incompatible temperatures of hydrolysis and fermentation limit SSF. These challenges create a need to improve pretreatment methods and SSF design to minimize formation of inhibitors and achieve faster hydrolysis and greater ethanol yield in less time. One way to improve SSF is to employ thermotolerant yeast. Thermotolerant yeast enables SSF to be carried

out at temperatures closer to optimum for enzymatic hydrolysis, which improves saccharification and increases ethanol productivity (Alfani et al., 2000; Olofsson et al., 2008; Badal et al., 2011).

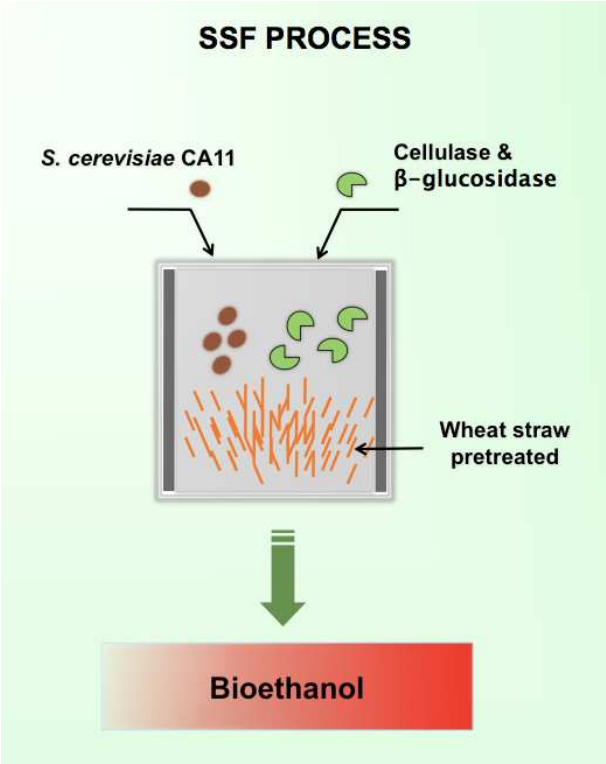

Figure 2.1. Schematic representation of SSF process

2.3 HYDROTHERMAL PROCESSING OF LIGNO-CELLULOSIC MATERIAL

Hydrothermal processing has been considered a cost-effective pretreatment (Yu et al., 2010) and in general, the major advantages that this process offers are: 1) the process does not require the addition and recovery of chemicals different from water, 2) limited equipment corrosion problems, 3) simple and economical operation (Romaní et al., 2010a; Cybulska et al., 2010; Garrote et al., 1999a; Mosier et al., 2005b). For that reason, the hydrothermal processing can be considered an environmentally friendly fractionation process (Garrote et al., 2003a).

Table 2.1. Composition of selected lignocellulosic materials (% dry matter)

Raw Material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Agricultural residues				
Corn cobs	38.8 – 44	33 – 36.4	13.1 – 18	Liu et al. (2010); Wang et al. (2010)
Corn stover	34.32 – 36.5	20.11 – 31.3	11.9 -13.55	Weiss et al. (2010); Liu and Cheng (2010)
Wheat straw	33 – 40	20 – 33.8	15 – 26.8	Ruiz et al. (2011b); Talebnia et al.(2010)
Rice straw	35 – 36.6	16.1 – 22	12 – 14.9	Hsu et al. (2010); Yadav et al. (2011)
Sugar cane bagasse	34.1 – 49	15.79 – 29.6	19.4 – 27.2	Mesa et al. (2010); Maeda et al. (2011)
Barley straw	37.5	25.1 – 37.1	15.8 – 16.9	Sun et al. (2011); García-Aparicio et al. (2011)
Rice husk	33.43	20.99	18.25	Garrote et al (2007); Abbas and Ansumali (2010)
Rye straw	41.1 – 42.1	23.8 – 24.4	19.5 – 22.9	Ingram et al. (2011); Gullón et al. (2010a)
Rapessed straw	36.59- 37	19.6 – 24.22	15.55 – 18	Díaz et al. (2010); Lu et al. (2009)
Sunflower stalks	33.8	20.2 – 24.27	14.6 – 19.9	Ruiz et al. (2008); Caparrós et al. (2008)
Sweet sorghum bagasse	41.33 – 45.3	22.01 – 26.3	15.2 – 16.47	Zhang et al. (2011); Goshadrou et al. (2011)
Herbaceous				
Switchgrass	41.2 – 32.97	25.95 – 31.1	17.34 – 19.1	Keshwani and Cheng (2009); Hu et al.(2011)

Alfalfa stems	24.7	14.7	14.9	Ai and Tschirner (2010)
coastal Bermuda grass	25.59	19.29	19.33	Wang et al. (2010)
Hardwood				
Aspen	43.8	18	20.8	Tian et al. (2011)
Hybrid Poplar	48.95	21.73	23.25	Pan et al. (2006)
Eucalyptus	44.6	21.4	30.1	Gonzalez et al. (2011)
Eucalyptus globulus	44.4	21.8	27.7	Romaní et al. (2011)
Softwood				
Pinus radiata	45.3	22.5	26.8	Araque et al. (2008)
Spruce	43.8	20.8	28.83	Shafiei et al. (2010)
Cellulose wastes				
Newspapers	60.3	16.4	12.4	Lee et al. (2010)
Recycled paper sludge	60.8	14.2	8.4	Peng and Chen (2011)
Industry co-products				
Distiller's grains	12.63	16.9	---	Kim et al. (2010)
Brewer's spent grain	16.8-18.8	28.4 – 32.3	21.7 – 27.8	Carvalheiro et al. (2005); Mussatto et al. (2010)

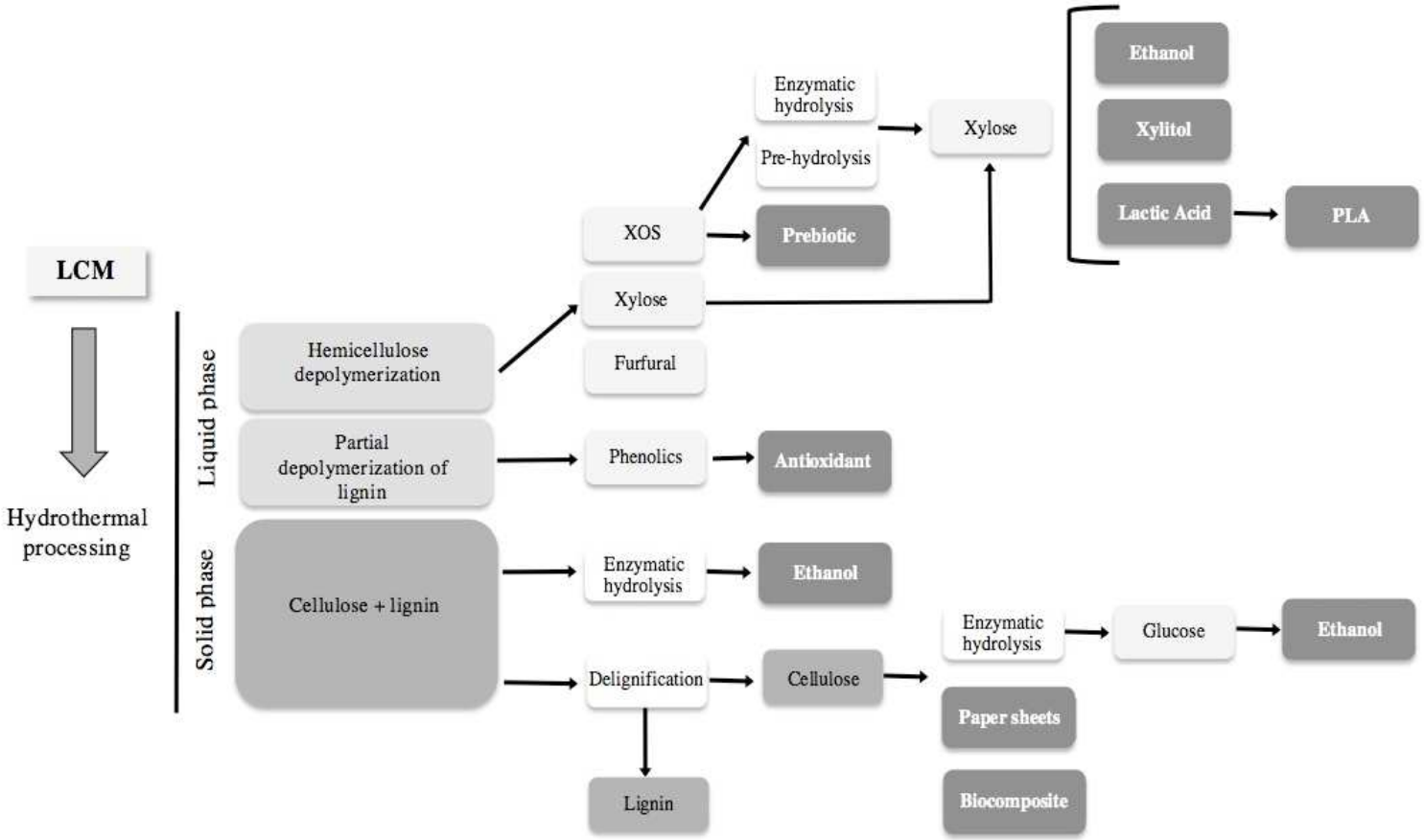

Figure 2.2. Scheme of a biorefinery using hydrothermal processing and LCM as raw material

Lignocellulosic materials (LCM) are the most abundant renewable biomass and its annual production was approximately estimated in 200 billion metric tons worldwide in 2007 (Zhang et al., 2007). LCM are mainly composed of cellulose, hemicellulose and lignin and have great potential as cheap and renewable feedstock for different applications.

In general, LCM includes agricultural residues, herbaceous, hardwood, softwood, cellulose wastes and industry co-products. Table 2.1 shows the composition of different lignocellulose materials. The fractionation of LCM into products derived from their structural components is an attractive possibility leading to the biorefinery concept. However, the main problem of fractionation is the recalcitrant nature of these materials. Fractionation may be achieved through hydrothermal processing, whose first step is hemicellulose solubilization.

Figure 2.2 shows the scheme of a biorefinery using hydrothermal processing. The hydrothermal procesing has been mainly used as a pretreatment for bioethanol production; in recent researches, the use of a sequential process has been applied as an alternative of papermaking production, also as a technology for converting agro-food by-products into useful food ingredients (Ruiz et al., 2011b; Griebl et al., 2006; Gullón et al., 2010b; Gírio et al., 2010; Vila et al., 2011).

2.3.1 FUNDAMENTALS AND OPERATING CONDITIONS OF HYDROTHERMAL PROCESSING

In hydrothermal processing LCM are exposed to water in the liquid state, at elevated temperature and pressures, that penetrates cell's structures, hydrates cellulose, depolymerizes hemicellulose (to oligomers and monomers) being between 40 % and 60 % of the total biomass dissolved in the process (Figure 2.3). In water at high temperatures (150-230 ºC), the H-bonding starts weakening, allowing autoionization of water into acidic hydronium ions (H_3O^+) that act as catalysts and basic hydroxide ions (OH^-).

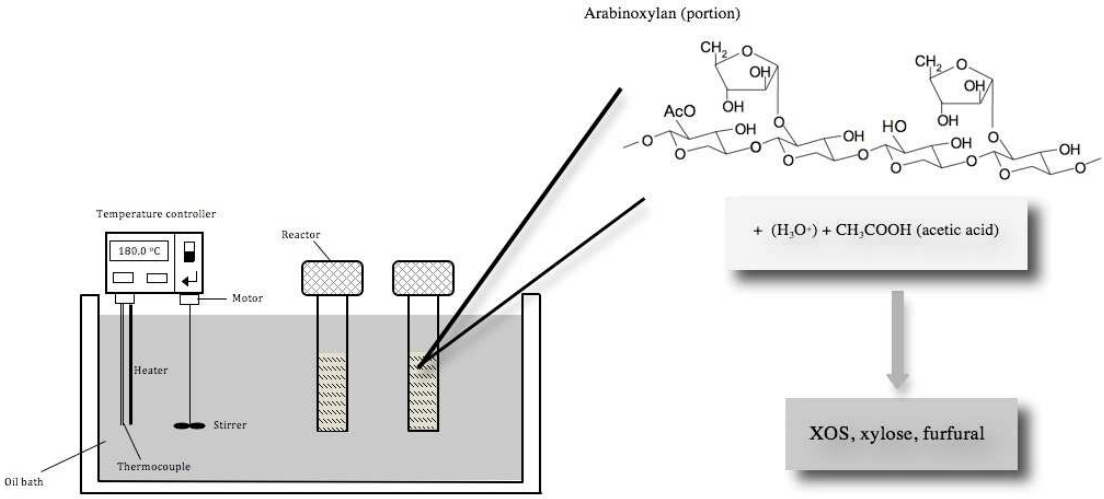

Figure 2.3. Batch reactor systems for hemicellulose depolymerization in hydrothermal processing.

In the subcritical region (100-374 ºC) the ionization constant (Kw) of water increases with temperature. However, when exceeding its critical point (374 ºC and 22.1 MPa), the values of dielectric constant, ionization constant (Kw) and ionic product of water drop drastically. Moreover, hydronium ions are generated from organic acids, mainly acetic acid from acetyl groups and uronic acid (Garrote et al., 1999a; He et al., 2008; Peterson et al., 2008; Loppinet-Serani et al., 2010; Möller et al., 2011). Acetyl groups are present in LCM and as they are associated with hemicellulose, the hydration of the acetyl groups leads to the acidification of the liquor and thus, formation of hydrogen ions. A number of hypotheses have been suggested to explain this phenomenon. According to these considerations, in a recent work, Liu (2010) presented the following model for hemicellulose solubilization in hydrothermal processing.

$$H_2O \leftrightarrow H^+ + OH^- \quad \textbf{(2.1)}$$

$$H^+ + H_2O \leftrightarrow H_3O^+ \quad \textbf{(2.2)}$$

$$R - POA_C + H^+ \leftrightarrow R - POA_C * H^+ \quad \textbf{(2.3)}$$

$$R - POA_C * H^+ + H_2O \leftrightarrow R - POA_C * H^+ + HOA_C \quad \textbf{(2.4)}$$

$$R - POA_C * H^+ + H_2O \leftrightarrow R - OH * H^+ + HOA_C) \quad \textbf{(2.5)}$$

$$HOA_C \leftrightarrow H^+ + OA_C^- \quad \textbf{(2.6)}$$

$$R - POH * H^+ \leftrightarrow R - POH + H^+ \quad \textbf{(2.7)}$$

$$R - OH * H^+ \leftrightarrow R - OH + H^+ \quad \textbf{(2.8)}$$

Reaction steps to solubilize hemicelluloses

$$R - X_nOH + H^+ \leftrightarrow R - X_nOH * H^+ \quad \textbf{(2.9)}$$

$$R - X_nOH * H^+ + H_2O \leftrightarrow R - X_mOH * H^+ + HX_SOH \quad \textbf{(2.10)}$$

Reaction steps to reduce the chain length inside the hydrothermal process liquor

$$HX_nOH + H^+ \leftrightarrow HX_nOH * H^+ \quad \textbf{(2.11)}$$

$$HX_nOH * H^+ + H_2O \leftrightarrow HX_mOH * H^+ + HX_SOH \quad \textbf{(2.12)}$$

where X_n represents an n-xylooligomer middle group, m + s = n, R- denotes the cellulose or lignin bonded to LCM, P represents a segment of hemicellulose, HX_nOH is an *n*-xylooligomer, HOA_C represents the acetic acid molecule and A_C is CH_3CO.

The most important operational variables of hydrothermal processing include temperature, residence time, particle size, moisture content (ratio liquid/solid) and pH influence on the fractionation of LCM and must be taken into consideration to maximize the product yield (i.e. hemicellulose sugar production, accessible surface area for enzymatic saccharification, etc.). The relationship between temperature-time strongly influences the physical-chemical characteristics of LCM in hydrothermal processing. Ballesteros et al. (2002) reported an increase of hemicellulose-sugar degradation at higher temperatures and residence times, concluding that at more severe operational conditions there are more losses of hemicellulosic sugar. For this reason, a strict control is required for high temperature reactions due to thermal degradation. Several works showed that the products (pentose and oil yield) from hydrothermal

processing are favored at lower reaction temperatures and longer residence times (Duff and Murray, 1996; Kálmán et al., 2002; Karaö et al., 2004).

Normally, when larger particle sizes are used, heat transfer problems lead to overcooking of the exterior (with consequent formation of inhibitors) and incomplete autohydrolysis of the interior. This problem can be overcome by reducing particle size as the first pretreatment step. This size reduction process not only changes the particle size and shape, but also increases bulk density, improves flow properties, increases porosity, increases surface area and is usually required to make material handling easier before hydrothermal processing. The higher surface area increases the number of contact points for chemical reaction (Ruiz et al., 2011b; Brownell et al., 1986). Mosier et al. (2005c) reported that size reduction is not needed since the lignocellulose particles break apart when cooked in water. Ballesteros et al. (2000) showed that the utilization of very small chips of softwood in hydrothermal processing would not be desirable to optimize the effectiveness of the process and improve economy, due to the significant energy requirements of particle reduction process. However, in recent work, Hosseini and Shah (2009) reported that it is possible to improve in 50 % the energy efficiency of pretreatment by the optimization of particle size properties.

According to Ruiz et al. (2011b), the use of blends with different particle size distributions has a selective influence over the sugar extraction: thus, the use of a blend with defined percentages of the various particle sizes is recommended before carrying out a hydrothermal processing. Moisture content and ratio liquid/solid may also greatly influence the ability of heat and chemicals (H_3O^+) to penetrate LCM, causing an uneven treatment of material. An uneven treatment can potentially result in the selective degradation of the outer portion of the LCM, while at the same time the interior is less affected by the treatment (Brownell et al., 1986). Cullis et al. (2004) reported that the moisture content has a dramatic effect on the efficacy of the hydrothermal processing as a substantial decrease in the amount of hemicellulose-derived carbohydrates recovered in the water-soluble fraction was observed when increasing the starting moisture content from 12 to 30 %, Rodríguez et al. (2009) showed that it is possible to obtain high glucose, xylose, arabinose and acetic acid concentrations by combining high temperatures with a medium-low treatment time and liquid/solid ratio.

On the other hand, the formation of hydronium ions from water and from organic acids is an important factor during hydrothermal processing, since the LCM and water mixture will reach high temperatures and pressures during the process. These high temperatures and pressures will accelerate the acid-catalyzed hydrolysis of cellulose and hemicellulose as well as the acid-catalyzed degradation of glucose and xylose.

Monitoring and control of the pH in hydrothermal processing will maximize the solubilization of the hemicellulose fraction as liquid soluble oligosaccharides while minimizing hydronium ions concentration and, more importantly, the degradation of these oligosaccharides and monosaccharides to degradation products (Mosier et al., 2005a,b). Mosier et al. 2005a pretreated corn fiber using pH controlled liquid hot water at 160 °C and a pH value above 4.0 and found that 50 % of the fiber was dissolved in 20 min. The carbohydrates dissolved by the pretreatment were 80 % soluble oligosaccharides and 20 % monosaccharides with < 1 % of the carbohydrates lost to degradation products. Cara et al. (2007) reported a slight pH decrease of hydrothermal processing hydrolyzates, in the range of 3.8 to 3.3, and an increase of degradation product concentrations (furfural) from 0.4 to 1.7 g/L, respectively.

In hydrothermal processing there are different types of reactor configurations. 1) Batch reactor: LCM solid particles are mixed with water in the reactor (Figure 2.4A). The residence time of the reacting solid is long (Ruiz et al., 2011a; Yu and Wu, 2010; Martínez et al., 2010; Hansen et al., 2011; Boussarsar et al., 2009).

In a recent work, Gullón et al. (2010a) reported a conversion of 69.2 % from initial xylan into xylooligosaccharides using a batch reactor configuration at 208 ºC and rye straw as raw material; 2) Semi-continuous reactor (flow-through, partial flow-through): hot water is passed over a stationary bed of LCM and dissolves lignocellulose components while the liquid products are rapidly swept out (Figure 2.4B). The residence time of liquid products is short, compared to a batch reactor (Yu and Wu, 2010; Mosier et al., 2005c; Liu et al., 2005; Ingram et al., 2009; Yu and Wu, 2009; Pronyk and Mazza, 2010). Liu and Wyman (2004) reported that in this type of reactors the fluid velocity in flowthrough has a significant impact on hydrothermal processing. Increasing fluid velocity significantly accelerated solubilization of total mass, hemicellulose and lignin even at the similar liquid residence times; 3) Continuous

reactor (co-current, counter-current): the LCM are passed in one direction while water is passed in the same or opposite direction (Figure 2.4C,D).

A continuous reactor system is also typically required to operate at high temperatures and pressures to achieve a high conversion of the feedstock within a short residence time (Yu and Wu, 2010; Mosier et al., 2005c; Rogalinski et al., 2008; Saito et al., 2009; Kumar et al., 2010). Makishima et al. (2009) reported 82 % conversion of xylan in xylose and xylooligosaccharides using a continuous flow type reactor. Yu and Wu et al. (2010) suggested that the characteristics of liquid products are strongly influenced by the reactor configuration.

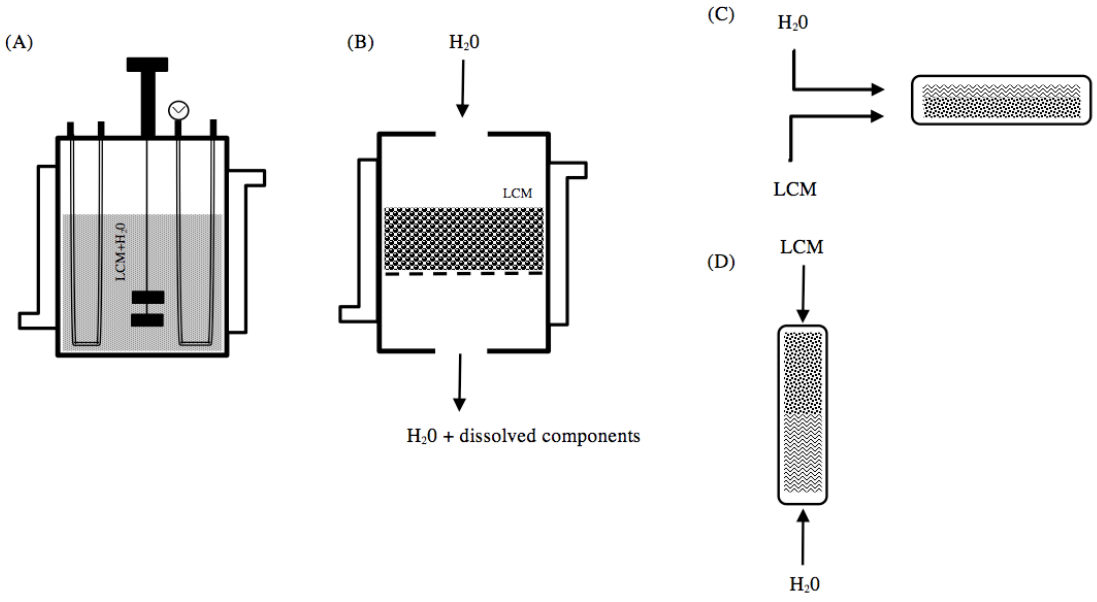

Figure 2.4. Representation of different reactor configurations for hydrothermal processing. (A) batch; (B) semi-continuous (flow-through reactor); (C) continuous (co-current); (D) continuous (counter-current).

2.3.2 MODELING OF HYDROTHERMAL PROCESSING

Modeling in hydrothermal processing provides a way to compare results from experiments carried out at different conditions. Table 2.2 shows the main mathematical models used in both isothermal and non-isothermal hydrothermal processing. An often applied option to model the effects of the main operational variables by pseudo first

order kinetics makes use of the severity factor (R_0) proposed by Overend and Chornet (1987). This empirical model has been generally used to correlate the effects of operational conditions (i.e., temperature, residence time, particle size, pH) on hemicellulose solubilization. It was initially used to control the pulping process in the paper industry, but it was reintroduced for comparison of hydrothermal processing pretreatment severities on LCM (Pedersen and Meyer, 2010).

Hydrothermal processing is appropriate for hemicellulose depolymerization and, for a detailed understanding of the chemical reactions that occur during hydrothermal processing. The development of kinetic models enables a deeper insight on the several phenomena involved and provides mathematical equations suitable for simulation, optimization and design of operational strategies. Mathematical models based on pseudo-first order kinetics have been successfully employed for hydrolysis modeling.

Garrote et al. (1997b) and Gullón et al. (2010a) suggested the kinetic reaction of hydrothermal processing using rye straw as raw material based on the following considerations: 1) a small part of the glucan fraction was degraded into glucooligosacharides, which were partially hydrolyzed to give glucose; 2) hemicelluloses were partially depolymerized along hydrothermal processing; 3) xylan was made up of two fractions (susceptible/non-susceptible to hydrothermal processing; the susceptible xylan fraction was hydrolyzed to give high molecular weight xylooligomers, which can be further decomposed into low molecular weight xylooligomers, subsequent xylose and promote dehydrated of xylose to furfural; 4) arabinosyl and acetyl groups hydrolysis are easily cleaved from xylan; 5) uronic acid fraction was made up of two fractions (susceptible/non-susceptible to hydrothermal processing). Similar considerations of xylan kinetic model were proposed by Garrote et al. (2002) using corncob as raw material as follows:

$$Hemicellulose \xrightarrow{K_i} oligosacharide \xrightarrow{K_{i+1}} monosaccharides \xrightarrow{K_{i+1}} degradation\ product \quad \textbf{(2.13)}$$

Table 2.2. Models used in hydrothermal processing

Effect	Model	Variables	Reference
Severity factor R_0, easy way for comparing results among experiments carried out under different conditions of temperature and time.	$R_0 = \int_0^t exp\left(\frac{T-100}{14.75}\right) dt$	*t* is the reaction time (min), T is temperature (ºC), 100 is the temperature of reference and 14.74 is an empirical parameter related with activation energy, assuming pseudo first order kinetics. The results are usually represented as a function of log (R_0).	Overend and Chornet (1987)
H-factor (*HF*), is a relationship between time and temperature, which is only an approximation of the reaction due to the fact that the proton concentration changes with time and activation energy. The concept of *HF* was development for Kraft/chemical pulping. However, also has been applied for hydrothermal processing Griebl et al (2006).	$HF = \int_0^t exp\left(43.186 - \frac{16115K}{T}\right) dt$	*t* is the time (min), *T* is the temperature in (ºC) and the constants are related with the activation energy.	Liu et al. (2010)

Severity factor R_0, in a non-isothermal hydrothermal processing, which includes the combination of temperature and reaction time along heating and cooling.	$$logR_0 = log\left[R_{0\,Heating} + R_{0\,Cooling}\right]$$ $$logR_0 = \left[\int_0^{t_{MAX}} \frac{T(t)-100}{\omega}dt + \int_{t_{MAX}}^{t_F} \frac{T'(t)-100}{\omega}dt\right]$$	t_{MAX} *(min)* is the time needed to achieve maximum autohydrolysis temperature, t_F (min) is the time needed for the whole heating-cooling period, $T(t)$ and $T'(t)$ stand for the temperature profiles in heating and cooling, respectively and ω is an empirical parameter.	Romaní et al. (2011)
Model that explains the severity factor in function of chip size and processing time taking into account the diffusion of liquid into LCM.	$$R_0 = \frac{t}{\rho r^2\left(\frac{1-0.5ln\varphi}{2M\varphi D\Delta C}\right)} * e^{\frac{T-100}{14.75}10^{-pH}}$$	t is the time of reaction (min), T is the temperature (ºC), D, is the diffusion coefficient, ρ is the density of the fluid, $\boldsymbol{\phi}$ is the void fraction (porosity), r is the particle radius (mm), M is the molecular weight and ΔC is the concentration gradient.	Hosseini et al. (2009)
Relationship between the severity factor and the viscosity of slurries made from sewage sludge during hydrothermal processing.	$$\mu = 2.755x10^5 x R_0^{0.8250}$$	μ is viscosity (Pas·s) and R_0 is the severity parameter.	Yanagida et al. (2010)

Model that explains the time needed for the chips to reach the desired temperature of wood with round or square cross sections (1) and rectangular cross sections (2) in hydrothermal processing.	(1) $t = a(T_{ht})^b(T_{ctr})^c(T_{init})^d D^e M^f G^g$ (2) $t = a(T_{ht})^b(T_{ctr})^c(T_{init})^d(TH)^d W^f M^g G^h$	t is the time estimated (min) for the center reach target temperature, T_{ht} is the heating temperature (ºF), T_{ctr} is the target center temperature, T_{init} is the initial wood temperature (ºF), D is de diameter of round cross section (in), TH is the thickness of rectangular board (in), W is the width of rectangular board (in), M is the moisture content (%), G is the specific gravity, a – h are the regression coefficients.	Simpson (2006)
Model for calculating the time needed for water diffusion into the LCM as a function of the process and LCM characteristics (assuming that LCM have a porous structure) in hydrothermal process.	$t_w = \frac{\rho r^2(1 - 0.5ln\varphi)}{2M\varphi D\Delta C}$	Where ρ is the density, r is the particle, ϕ is the porosity, D is the diffusion coefficient, M is the molecular weight and ΔC is the concentration gradient.	Hosseini et al. (2010)
Model for calculating the temperature needed for different particle sizes. Temperature as a function of severity factor and radius in hydrothermal processing.	$T_2 = T_1 - 14.7ln\left[\frac{R_2}{R_1}\left(\frac{r_1}{r_2}\right)^2\right]$	T_2 is the temperature (ºC) needed for compensate the particle size increase, T_1 is the initial LCM temperature which can be assumed as 20 ºC. r_1 and r_2 are the radius (cm), R_1 and R_2 are de severity factor.	Hosseini et al. (2010)

In order to provide a quantitative interpretation of xylan degradation under non-isothermal conditions, Garrote et al. (2002) proposed a modification, where some degradation of the slow-reacting fraction occurred at high temperatures. Nabarlatz et al. (2004) developed a model for the kinetics of xylan depolymerization. The model assumes that the composition of each of the two xylan fractions in the LCM does not change with the conversion, there is not direct formation of monomers (i.e., xylose, arabinose and acetic acid), the monomers are formed solely by depolymerization of the oligomers and the rates of monomer formation from oligomers are independent of the molar mass, structure and the oligomers. Carvalheiro et al. (2005) proposed two models for xylan and arabinan degradation assuming that furfural was formed from both pentoses. Zhuang et al. (2009) reported the kinetic modeling of xylan, measuring the oligosaccharides and monosaccharides as reducing sugar and the kinetic model was thus adopted as:

$$Xylan \xrightarrow{K_i} reducing\ sugars \xrightarrow{K_2} degradation\ product \quad (2.14)$$

In a recent work Martinez et al. (2010b) proposed kinetic models for arabinan and acetyl groups based on sequential and parallel, irreversible and first-order reactions with Arrhenius-type temperature dependence and reported that the kinetic models provided a good prediction for the data of reaction liquors as function of the operational conditions. An overview about the kinetic modeling of cellulose hydrolysis can be found in Zhao et al. (2009) and Rogalinski et al. (2008) according to the following reactional sequence:

$$Cellulose \xrightarrow{K_i} oligosaccharides \xrightarrow{K_2} hexoses \xrightarrow{K_3} degradation\ product \quad (2.15)$$

2.3.3 EFFECT OF HYDROTHERMAL PROCESSING ON CELLULOSE PROPERTIES

Cellulose is the most abundant biopolymer that can be obtained from numerous LCM resources and there is a clear opportunity to develop commercial processes that could generate products that are needed at very high volumes and low selling price. One

strategy to fractionation of LCM is hydrothermal processing, since hemicelluloses are depolymerized into soluble products, whereas the solids from hydrothermal processing are enriched into cellulose and lignin. A variety of applications can be visualized for this phase from hydrothermal processing of LCM. Currently, the most promising approach for using LCM is enzymatic hydrolysis of the cellulose content after pretreatment for second generation bioethanol production (See Table 2.3). Pretreatment is required to alter the structure and chemical composition due to the robustness of LCM. Hydrothermal processing as pretreatment caused re-localization of lignin on the surface of LCM (Kristensen et al., 2008), thus enzyme accessibility to the LCM structure in the pretreated material is favoured, increasing the potential of cellulose saccharification.

Table 2.3. Production of bioethanol using hydrothermal processing as pretreatment under different operational conditions and raw materials.

Raw material	Temp. (ºC)	Time (min)	Particle size	Ethanol yield (%)	Reference
Wheat straw	195	6 – 12	__	89	Petersen et al. (2009)
Rice straw	180	30	250 – 420 μm	100	Yu et al. (2010)
Switchgrass	210	15	__	72	Suryawati el at. (2009)
Eucalyptus globulus	230	__	__	86.4	Romaní et al. (2010a)
Wheat straw	214	2.7	0.5 – 2 mm	90.6	Pérez et al. (2008)
Corn stover	195	10	__	61.2	Xu et al. (2010)
Sugar cane bagasse	220	2	__	85	Walsum et al. (1996)
Wheat straw	200	40	0.5 – 2 mm	96	Pérez et al. (2007)
Poplar nigra	240	60	2 -5 mm	60	Negro et al. (2003)

Moreover, physical changes that improve enzymatic saccharification include an increase in pore size to enhance enzyme penetration and an increase in accessible area

that has been shown to correlate well with the susceptibility of these substrates to enzyme saccharification using hydrothermal processing as pretreatment (Cybulska et al., 2010; Liu and Wyman, 2005).

Table 2.4 shows the enzymatic saccharification yield using hydrothermal processing as pretreatment at different operational conditions and demonstrate the ability of this technology to make LCM accessible to enzymes. After hydrothermal processing the cellulose shows a small degradation at different temperatures > 230 ºC. In a recent work, Romaní et al. (2010a) reported decreases of 5.29 % and 19.55 % of cellulose present in pretreated solids at 240 ºC and 250 ºC, respectively, defining the operational range where partial cellulose degradation began to take place. According to Sakaki et al. (2002) cellulose started to degrade in hexoses and oligosaccharides above 230 ºC and almost all cellulose was decomposed at 295 ºC. Jin et al. (2004) reported that cellulose hydrolyzes into glucose in 2 min at 300 ºC, but that the glucose decomposes in 30 s under the same conditions.

Other type of solid residues which contain mainly cellulose and lignin after hydrothermal processing are the raw material for pulp and paper marking (Garrote et al., 2003b). Caparrós et al. (2008a) reported similar characteristics of paper sheets obtained from sequencial hydrothermal processing and ethanol pulping to those obtained by soda pulp. Vila et al. (2011) showed the susceptibility of hydrothermally treated solids to kraft processing pulp which provided cellulose pulps with low kappa numbers, highly susceptible to alkaline oxygen bleaching. Romaní et al. (2011) used the solids (cellulose + lignin) obtained after hydrothermal processing for delignification and improved the enzymatic saccharification of cellulose from *Eucalyptus globulus*. According to Alfaro et al. (2010) the cellulose pulp with hydrothermal processing reduces kappa number, viscosity and decreases paper strength. Caparrós et al. (2008b) evaluated the solid phase obtained from hydrothermal processing using the organosolv process for produced paper sheets, analyzing the influence of operational variables on the viscosity, tensile index, burst index, tear index and brightness obtaining suitable characteristics of paper sheets.

Table 2.4. Enzymatic Saccharification of pretreated solids with hydrothermal processing as pretreatment.

Raw material	Temp. (ºC)	Time (min)	Saccharification yield (%)	Reference
Prairie Cord Grass	210	10	94.53	Cybulska et al. (2010)
Oil palm fronds	178	11.1	92.78	Goh et al. (2010)
Coastal Bermuda grass	150	60	67.4	Lee et al. (2009)
Corn stover	190	15	69.6	Zeng et al. (2007)
Wheat straw	195	3	72	Thomsen et al. (2008)
Switchgrass	200	10	74.4	Hu and Ragauskas (2011)
Tamarix ramosissima	200	180	88	Xiao et al. (2011)
Eucalyptus grandis	200	20	96.6	Yu et al. (2010)
Barley husks	212	__	100	Ares-Peón et al. (2011)
Eucalyptus globulus	230	__	97.9	Romaní et al. (2010b)

2.3.4 EFFECT OF HYDROTHERMAL PROCESSING ON HEMICELLULOSE PROPERTIES

The hemicellulose is the second most abundant polysaccharide in nature and is made up of amorphous heteropolysaccharides constituting 14 - 50 % of the raw LCM dry weight. Hemicellulose consists of various structural units, including five-carbon (xylose and arabinose) and six-carbon sugars (mannose, galactose, glucose), which can be substituted with phenolic, uronic or acetyl groups. The most abundant block of hemicellulose in hardwoods and many agricultural residues is xylan (made up mainly of xylose units) (Garrote et al., 2007; Kayserilioglu et al., 2003; Sun et al., 2005).

Hydrothermal processing is a suitable method for hemicellulose depolymerization, as under selected operational conditions hemicellulose can be almost totally removed from LCM (see Figure 2.3) (Vegas et al., 2008), being decomposed into valuable soluble products such as oligosaccharides, monosaccharides, sugar-decomposition products (such as furfural or hydroxymethylfurfural) and acetic acid (from acetyl groups

hydrolysis). Furthermore, when a xylan is subjected to hydrothermal processing under mild temperature, high-molar mass xylo-oligosaccharides (XOS) and xylose are produced, being the major products derived from hemicellulose present in the liquor phase (Garrote et al., 2008a; Gullón et al., 2010a; Carvalheiro et al., 2009).

Under harsh operational conditions, xylose can be dehydrated to furfural, and furfural can be converted into degradation products (Garrote et al., 2002). Xylo-oligosaccharides are bioactive molecules with high-added value and have great prebiotic potential making them useful as ingredients for functional foods. From a nutritional point of view, XOS are usually considered to be nondigestible oligosaccharides (Nabarlatz et al., 2004; Parajó et al., 2004; Gullón et al., 2008; Carvalheiro et al., 2004; Kabel et al., 2002; Gullón et al., 2009).

Recently, solubilization studies of XOS from LCM by hydrothermal processing have shown the efficiency of this technology to improve the yields of extraction. Gullón et al. (2010a) reported a yield of 69.2 % of XOS with respect to the initial xylan at 208 ºC, using rye straw as raw material. Nabarlatz et al. (2007) reported 58.3 % of crude XOS after hydrothermal processing at 179 ºC for 23 min using ultrafiltration for the purification of XOS. Carvalheiro et al. (2004) also reported a similar yield of 61 % of XOS at 190 ºC after 5 min, using brewery's spent grain. According to Garrote et al. (2008b), the hydrothermal processing in nonisothermal reaction conditions produced 23.2 g of oligosaccharides/100 g of oven-dried corncobs at 202 ºC. Boussarsar et al. (2009) showed that it is possible to obtain an acceptable xylose extraction yield and low degradation of sugar monomers for 2 h at 170 ºC. Kabel et al. (2002) and Carvalheiro et al. (2004) in previous studies for the production of XOS by hydrothermal processing of brewery's spent grain reported that several oligosaccharide mixtures of different molecular weight distributions were obtained depending on temperature and reaction time (severity of reaction conditions).

Longer reaction times led to a decreased amount of oligosaccharides and an increase of the concentration of monosaccharides, acetic acid and sugar decomposition products. Montané et al. (2006) used active carbon as an alternative for the purification of XOS produced by hydrothermal processing. Vegas et al. (2006) used ultra- and nano-filtration for the purification of oligosaccharides from rice husk hydrothermal

processing liquors, reporting that it is possible to recover about 90 % of the XOS present in hydrolysis liquors.

Different reactor configurations have been used for improving the recovery of hemicellulose. According to Liu and Wyman (2004), xylose yield improved from 60 to 82 % with an increase in the fluid velocity from 2.8 to 10.7 cm/min on a flowthrough reactor using corn stover as raw material at 200 ºC after 8 min residence time. Makishima et al. (2009) found an effective recovery of hemicellulose using a tubular type reactor at 200 ºC for 10 min, 82 % of xylan fraction recovered as mixture of xylose, XOS and higher XOS with polymerization degree higher than 10. Garrote and Parajó (2002) reported that more than 80 % of the initial xylan can be removed from wood with a conversion in XOS up to 65 % of the initial xylan in a batch reactor. Other important point is the deacetylation of hemicellulose during the hydrothermal processing; Garrote et al. (2001d) studied the time course of acetyl groups' hydrolysis from both xylan and xylan degradation products, and their relationship with the concentration of acetic acid. Xylitol, a pentitol derived from xylose by reduction has technological and biological properties, such as high sweetening power, anticariogenic properties and suitability for consumption by diabetics, that foster its utilization in the food industry.

XOS produced in hydrothermal processing can be used as a source of xylose for the production of xylitol. However, these XOS cannot be directly metabolized by microorganisms. In order to prepare fermentation media for the production of xylitol from hydrothermal processing liquors, XOS must be first converted into monosaccharides by either acid or enzyme catalyzed reactions, this process providing a way to obtain xylose solutions.

Rivas et al. (2002) used the sequence (hydrothermal processing – posthydrolysis) with corncob hydrolysate and observed an increase in the productivity and yield of xylitol in comparison with the results obtained in a fermentation media made by the conventional acid hydrolysis pretreatment. Duarte et al. (2004) produced xylitol and arabitol from brewery's spent grain hydrolysate using the sequence (hydrothermal processing – posthydrolysis) by *Debaryomyces hansenii* without any detoxification treatment. Garrote et al. (2001a,c) used corncobs and *Eucalyptus globulus* as raw material in

hydrothermal processing and concluded that generation of xylose solution to be used as fermentation media through sequential stages of hydrothermal processing – posthydrolysis, shows favourable features in terms of substrate conversion, reaction, selectivity and low inhibitor concentration. Vázquez et al. (2001) reported a concentration of 24 g xylose/L, using the sequence (hydrothermal processing – enzymatic posthydrolysis) with corncobs as raw material at 175 ºC for 20 min, the advantage being that the hydrolysate after this sequence is free of sugar degradation products and the acetic acid concentration could be reduced, thus improving their potential fermentability.

XOS can also be synthesized and used as thermoplastic compounds for biodegradable plastics, water-soluble films, coatings and capsules (Glasser et al., 2009). Glasser et al. (2009) produced a thermoplastic from the chemical modification of pentose-rich oligosaccharides such as xylose. Lindblad et al. (2001) produced hydrogels from hemicellulosic oligosaccharides and 2-hydroxyethylmethacrylate. The need to replace traditional plastics due the negative environmental impact caused, has increased the interest on the development of biodegradable polymers. Polylactic acid (PLA) is a biodegradable polymer with thermoplastic character, produced by polymerization of lactic acid (which can be obtained by fermentation of sugars derived from LCM), that finds applications in such fields as packaging, disposable goods or textile fibres. Vila et al. (2008) evaluated rice husks and *Eucalyptus globulus* in hydrothermal processing for the production of xylose-based culture media for lactic acid production and the application of the fiber contained in the pretreated solids for making PLA-based biodegradable composites. In a recent work González et al. (2011a) produced biodegradable biocomposites from *Citysus scoparius* using hydrothermal processing, obtaining a maximum concentration of oligomers (71 % of the initial xylan) at 215 ºC and a solid phase (cellulose + lignin) suitable as a reinforcement for PLA-based composites, the composites showing better stiffness compared with pure PLA.

2.3.5 EFFECT OF HYDROTHERMAL PROCESSING ON LIGNIN PROPERTIES

Lignin is the most abundant aromatic heterogeneous polymer formed by phenolic compounds and their precursors are three aromatic alcohols namely, 1) *p*-coumaryl, 2)

coniferyl and 3) sinapyl alcohols, which are bonded together with over two-third being ether bonds (C-O-C) and the rest being C-C bonds. The respective aromatic constituents in the polymer are called *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), the structure of lignin suggesting that it can be a valuable source of chemicals, particularly phenolics (Ruiz et al., 2011a; Garrote et al., 2004a; Buranov and Mazza, 2008; Pandey and Kim, 2011; Fang et al., 2008).

Lignin is always associated with hemicelluloses, not only as physical admixtures, but also through covalent bonds. During the hydrothermal processing lignin and lignin-hemicellulose linkages can undergo degradation, partial depolymerization and profound re-localization. Moreover, the fraction of solubilized lignin depends on the operation conditions (severity of reactions conditions) and on the raw material (LCM) (Garrote et al., 1999a; Gullón et al., 2010b; Kristensen et al., 2008).

Lora and Wyman (1978) and Bobleter and Concin (1979) cited by Garrote et al. (1999a) and Zhang et al. (2008) suggested a two-phase mechanism for lignin reaction: 1) a very fast reaction where lignin fragments with low molecular weight and high reactivity are solubilized by breaking lignin-carbohydrate bonds into soluble fragments; 2) a slower reaction where the soluble fragments react with one another by recondensantion and lignin repolimerization, which also occurs in the presence of the organic acids liberated in the hydrothermal processing.

Soluble lignin being aromatic and possessing many chromophoric structural units strongly absorbs ultraviolet (UV) light and the absorbance at either 205 or 280 nm is the basis of several techniques for the quantitative determination of soluble lignin (Chi et al., 2009; Schmidt, 2010). The partial depolymerization of lignin and breaking of lignin-hemicellulose linkages produced part of the phenolics present in the hydrothermal processing liquors (Gullón et al., 2010b; Esteves et al., 2008).

Using hardwood as raw material, Marchessault et al. (1982) reported that the ether linkages of lignin are cleaved during hydrothermal processing causing a decrease in molecular weight and an increase in phenolic content. These phenolics, considered as the by-products of LCM hydrothermal processing are an attractive source for natural

antioxidants and might have potential applications as food additives (Garrote et al., 2004a; Conde et al., 2011).

The extracts and compounds derived from soluble lignin can be selectively extracted from hydrothermal processing liquors with ethyl acetate. This phenolic-rich extract contains a variety of potentially valuable compounds with antioxidant, antimicrobial and biological activities comparable to that of synthetic antioxidants (Conde et al., 2008; Garrote et al., 2004b; Castro et al., 2008). Several lignin-derived products have been identified. Garrote et al. (2008a) reported benzoic, gallic and cinnamic acids in the liquor after hydrothermal processing at 216 ºC using barley husk as raw material. Conde et al. (2009a) found that hydroxytyrosol, homovanillyl alcohol, oleuropein, syringaldehyde, tyrosol, 3,4-dihydroxybenzaldehyde were the major phenolic compounds in the soluble fraction from the hydrolyzates of barley husks obtained by hydrothermal processing.

In a recent work, Tsubaki et al. (2010b) used microwave as an alternative heating source for tea residues autohydrolysis and reported that vanillin, vanillic acid, dihydroconiferyl alcohol and guaiacol are degradation compounds originated from guaiacyl (G) units of lignin. In a similar way, syringaldehyde, syringic acid and sinapaldehyde were considered to be originated from syringyl (S) units of lignin. Conde et al. (2009b) reported that gallic acid, 3,4-dihydroxybenzaldehyde, vanillic acid, syringic acid, vanillin and *p*-coumaric acid were the major low molecular weight phenolics present in the refined media using barley husks as raw material. According to Castro et al. (2008), the major compounds founded in lignin-derived fractions were syringaldehyde, vanillin, 4-formyl benzoic acid methyl ester, desaspidinol, syringol, guaiacol, homosyringic acid and methoxyeugenol using *Olea europea* wood as raw material. Pourali et al. (2010) used subcritical water conditions and reported the production of eleven phenolic compounds (caffeic, ferulic, gallic, gentisic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, sinapic, syringic, vanillic acids and vanillin) and concluded that the content of phenolic compounds increased with the temperature.

Many beneficial effects on human health have been attributed to simple phenolics: oleuropein, hydroxytyrosol, caffeic acids (prevention of cardiovascular diseases); hydroxytyrosol, tyrosol, vanillin, vanillic acid, caffeic acids (prevention of tumoral

diseases); *p*-coumaric acid, caffeic acid, ferulic acid (protection against LDL lipoprotein oxidation); gallic acid (skin protective ability); vanillin, (anti-inflammatory) (Gullón et al., 2010b; Garrote et al., 2004a; Tripoli et al., 2005; Kim, 2007; Jung et al., 2008; Serzer, 2011). In regard to antioxidant activity, Tsubaki et al. (2008) reported the production of polyphenols above 200 ºC, obtaining strong antioxidant activity using tea residues as raw material and microwave heating. Conde et al. (2009b) reported that the hydrothermal-extraction process was suitable for obtaining antioxidants, but the limited mass fraction of phenolics and the values determined for antioxidant activity, suggest that further purification would result in products of improved quality.

Akpinar et al. (2010) used ultrafiltration as an alternative to ethyl acetate extraction and reported that sunflower stalk liquors had higher antioxidant activity than wheat straw, the hydrothermal processing being carried out at 160 ºC for 1 h. Tsubaki et al. (2010a) also studied the extraction of phenolic compounds and reported that antioxidant activity increased in good correlation with the increase in the concentration of phenolic compounds in the extracted hydrothermal processing liquor. Garrote et al. (2003a) reported that the antioxidant activity of ethyl acetate extracts isolated from *Eucalyptus globulus* and corncob hydrothermal processing liquors showed a strong dependence on the hydrothermal conditions. Moreover, Pourali et al. (2010) found that phenolic compounds could be selectively produced by temperature variations. Conde et al. (2011) studied the *in vitro* antioxidant capacities of the ethyl acetate extract from hydrothermal processing using different LCM and reported that the antioxidant capacities were comparable or higher than the ones of synthetic compounds, similar results being obtained by Castro et al. (2008) using *Olea europea* wood at temperatures in the range of 190-240 ºC.

On the other hand, Li and Gellerstedt (2008) reported that the solid residues obtained after hydrothermal processing showed improved susceptibility towards delignification with organic solvents. According to Hongzhang and Liying (2007) the sequential application of hydrothermal processing-organosolv pretreatment is an effective process for the extraction of lignin (delignification) with reasonable yields and purity. Ruiz et al. (2011a) evaluated delignification using the sequence hydrothermal processing-organosolv process and concluded that the temperature and time as well as chemical

structure were variables that showed a strong influence on lignin precipitation. Romaní et al. (2011) used the sequence hydrothermal processing-organosolv process for delignification, resulting in an improved enzymatic saccharification.

2.4 CONCLUSIONS

The conversion of biomass into chemicals and energy is essential in order to sustain our present and future. In general, hydrothermal processing used in LCM is the most promising technology that can be conceived as a first step to the fractionation and obtention of products with high added-value according to the biorefinery concept.

Depending on the operational conditions (temperature, residence time, particle size, moisture and reactor configuration), hydrothermal processing can cause several effects including hemicellulose depolymerization (oligomers, monomers), alteration/degradation of lignin (phenolic compounds) and increased availability of cellulose. Owing to these effects, the products obtained are a valuable source of materials for the chemical, pharmaceutical, food and energy industries.

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CHAPTER 3

EVALUATION OF A HYDROTHERMAL PROCESS FOR PRETREATMENT OF WHEAT STRAW – EFFECT OF PARTICLE SIZE AND PROCESS CONDITIONS

ABSTRACT

Hydrothermal process is an eco-friendly process that provides an interesting alternative for chemical utilization of lignocellulosic materials, in which water and crop residues (LCM) are the only reagents. In this work the effect of process conditions (size distribution of the wheat straw, temperature and time) was evaluated against production of fermentable products. The use of milled wheat straw (WS) fractions as a raw material containing blends of different particle size distribution showed an influence on the final sugars composition in the hydrolysate. The improved values of glucose (21.1%) and xylose yields (49.32%) present in the hydrolysate were obtained with treatment's severity factor of 2.77 and 3.36, respectively. Mathematical models were developed aiming at establishing the effect of process conditions on monosaccharide concentration and its degradation in the liquor. This work shows that the use of wheat straw blends with various sizes has a significant effect on the extraction of fermentable products. The effect of treatment severity, which takes into account both processing time and temperature was also evaluated. These results are of utmost importance for process design.

3.1 INTRODUCTION

Lignocellulosic materials (LCM) are vegetal biomass, mainly made up of cellulose, hemicelluloses and lignin. Such materials are renewable, costless and could be used as a source to produce fuel ethanol (Ruzene et al., 2008). In recent years, there has been an intensive use of lignocellulosic materials such as hardwoods (*Eucalyptus globulus*, white birch, hybrid poplar), softwoods (*Pinus banksian*, fir, red fir) and agricultural residues (corn cobs, wheat straw, rice straw, corn stalks, barley straw) (Garrote et al., 1999). Agriculture and forest product's industries provide a wide range of materials used e.g. as shelter, packaging, clothing, and fuels, meaning that lignocellulosic materials can have different applications. For example, sugar cane bagasse is used as fuel and animal feed, and wheat straw can be used either in construction or in the paper industry. Other forms of biomass are deliberately modified from one energy form into another, for example, wood to charcoal, dung to biogas and fertilizer, and sugar to ethanol. Thus, it may be important to measure processed biomass and the separation of its main components (cellulose, hemicellulose and lignin) as they are an actual and potential energy source (Calle, 2007; Chum, 2011). Actually, this concept is called biorefinery, i.e. co-production of transportation biofuels, bioenergy and marketable chemicals from renewable biomass (Cherubini and Ulgiati, 2010).

Wheat straw (WS), as an agricultural residue, is one of the most abundant biomass sources in the world (Ergudenler and Ghaly, 1992) The straw yield depends on the specific wheat varieties harvested and climatic factors; an average ratio of 1.3 kg of straw per kg of grain is found for the most common varieties (Montane et al, 1998; Peterson, 1987).

The purpose of the pretreatment of LCM to fermentable products is to remove lignin and hemicellulose, disrupt the crystalline structure of cellulose, and increase the porosity of the materials, so that enzymes can easily access and hydrolyse cellulose. Pretreatment of raw material is perhaps the most crucial single step as it has a large impact in an all the other steps in the process, e.g. enzymatic hydrolysis (namely, in terms of digestibility of the cellulose), fermentation (toxicity) and downstream processing (including energy demand). The pretreatment must meet the following

requirements: 1) improve the formation of sugar, 2) avoid the degradation or loss of carbohydrates, 3) avoid the formation of inhibitory by-products and 4) have low capital and operational costs (Kumar et al., 2009; Scheper, 2007; Sun et al., 2002).

Among the existing hydrolysis techniques, hydrothermal process (autohydrolysis) consists of heating LCM with water at high ranges of temperatures (150-230 ºC), undergoing hydrolysis reactions in the presence of the hydronium ions generated by water autoionization, which act as catalyst (Garrote et al., 2001; Targonsky, 1985). Because the bonds of hemicelluloses are the most susceptible ones, autohydrolysis has been considered as a cost-effective method for pretreating LCM. In fact, no chemicals different from water are necessary and hemicelluloses can be converted into hemicellulosic material sugar at good yields with low by-products generation.

Comparing specifically with acid hydrolysis, the autohydrolysis induces a low by-product generation, high pentosan recovery, limited equipment corrosion problems, and reduced operational costs since further neutralization can be omitted. For that reason, the hydrothermal process can be considered as an eco-friendly fractionation technology, leading to the separation of hemicelluloses from the remaining structural components of the feedstock (Carvalheiro et al., 2009; Pérez et al., 2007; Sreenath et al., 1999; Vegas et al., 2008; Walsum et al., 1996; Yu et al., 2010).

The most important variables in hydrothermal processes are residence time, temperature, particle size and moisture content. Normally, when larger chips are used, heat transfer problems lead to overcooking of the exterior (with associated formation of inhibitors) and incomplete autohydrolysis of the interior (Brownell et al., 1984; Drzymala et al., 1993; Duff et al., 1996). This problem can be overcome by reducing particle size before the application of the pretreatment. This size reduction process not only changes the particle size and shape, but also increases bulk density, improves flow properties, increases porosity, and generates new surface area. This higher surface area increases the number of contact points for chemical reaction (Drzymala et al., 1993; Schell et al., 1994) Most of the literature deals with raw materials featuring a unimodal particle size distribution, thus leaving behind all the other fractions resulting from milling.

However, from an operational point of view, raw materials are one of the most expensive incomes in the process; therefore strategies to use of all the disposable materials obtained after milling are needed. During the pretreatment, and depending on the operational conditions of the hydrothermal process, polysaccharides (mainly hemicelluloses) are depolymerized to oligomers and monomers, and the correspondent sugar (pentoses and hexoses) can be dehydrated to furfural and hydroxymethylfurfural (HMF) (Quintas et al., 2007) Such degradation products will impair further fermentation and thus, ethanol production. Hence, thermal degradation of monomers must be minimized during pretreatment.

This chapter aims at evaluating the effect of particle size distribution of milled WS and hydrothermal pretreatment conditions on the fermentable products obtained from it. To achieve this goal, a 3^3 factorial design considering different WS blends, processing time and temperature was applied. The resulting monomeric sugars in the liquor obtained were analyzed.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 RAW MATERIAL

Figure 3.1 shows the whole diagram for the milled material process. Wheat straw was supplied by a local farmer (Elvas, Portugal). The straw was cut into small chips (1-5 mm) and milled using a laboratory knife mill (Cutting Mill SM 2000, Retsch, Germany). Aliquots from the homogenized WS lot were subjected to moisture determination (drying at 105 ºC to constant weight). Approximately, 2 g of ground WS were treated with 10 mL of 72% (w/w) H_2SO_4, stirring for 7 min at 45 °C. The reaction was interrupted by adding 50 mL of distilled water and the mixture was then transferred to a 500 mL Erlenmeyer flask and the volume brought to 275 mL. The flask was autoclaved for 30 min at 121 °C for complete hydrolysis of oligomers. The mixture was filtered and the hydrolysate brought to 500 mL.

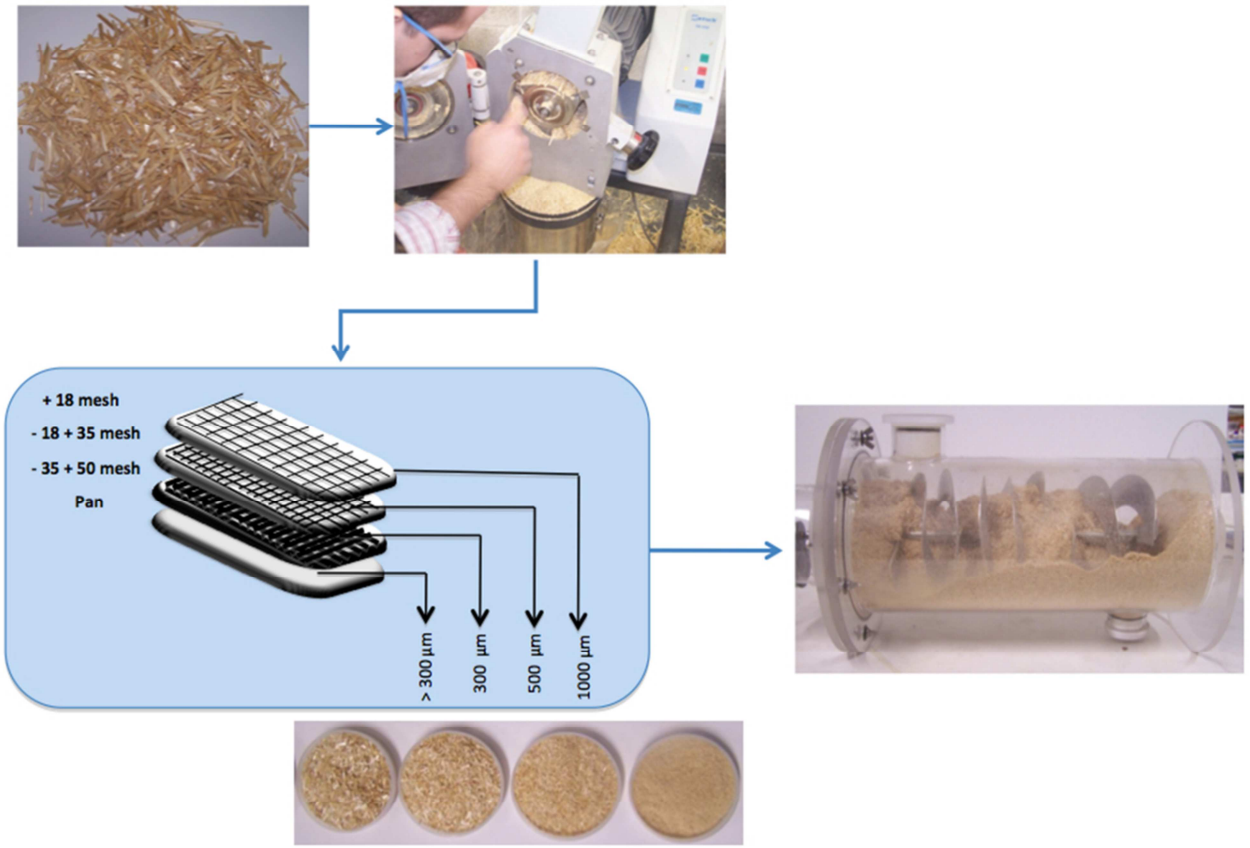


Figure 3.1. Schematic diagram for preparation of milled wheat straw and blends of different particle sizes.

The hydrolysate was analyzed by HPLC with a MetaCarb 87H (300 x 7.8 mm) column at 45 °C using a Jasco chromatograph with refraction-index detector; the mobile phase was 0.005 mol/L H_2SO_4 at a flow rate of 0.6 mL/min. The samples were analyzed for glucan, xylan, arabinan and acetyl groups. Sugar concentrations reported as xylan and glucan were determined using calibration curves of pure compounds. The solid obtained in the filtration after hydrolysis was oven-dried and weighted. The obtained mass corresponded to the residual lignin (Klason lignin) and the soluble lignin was determined by spectroscopy at 280 nm (Rocha, 2000; Ruzene et al., 2008).

The milled material, under conditions usually applied in experiments of hydrolysis (0.9 mm), and its residues were initially separated into fraction: > 1.0 mm (mesh 18), 1.0-0.5 mm (mesh 35), 0.5-0.3 mm (mesh 50) and < 0.3 mm (basis) using a portable sieve shaker (Model Analysette, Fritsch, Germany). These fractions were mixed in different proportions to achieve mixtures with different mean particle sizes (Table 3.1 and Figure 3.1).

Table 3.1. Size distribution blends (B1, B2 and B3) of wheat straw (w/w %)

Wheat Straw Size Distribution (Frequency)			
Particle Size (mm)	B1	B2	B3
> 1	0.25	0.1	0.1
1 - 0.5	0.25	0.1	0.4
0.5 - 0.3	0.25	0.4	0.4
< 0.3	0.25	0.4	0.1
Mean Size (mm)	0.488	0.330	0.435
Size Variance (mm^2)	0.103	0.061	0.049

3.2.2 HYDROTHERMAL PROCESS

Hydrothermal processing was performed in 160 mL total volume stainless steel cylinders reactors, submerged in an oil bath open heating circulator (JULABO

Labortechnik GmbH, Seelbach, Germany) with PID temperature control under different operating conditions. Blends with different size distribution (Table 1) were processed.

Temperature and residence time were chosen based on values normally used for hydrolysis of other lignocellulosic materials. For this, water was added to samples of WS blends in a solid:liquid ratio of 1:10 (g:mL) in a closed and pressurized vessel. The moisture content of WS was considered as water in the mass balances.

The reactor was heated following the different operation conditions established by the factorial design (see Statistical Procedures section). At the end of the desired reaction time, the reactor was removed from oil bath and immediately immersed in an ice bath to quench the reaction. The solid and liquid were separated via vacuum filtration. The effects of time and temperature on WS were interpreted based on the severity parameter log R_0 (Overend et al., 1987). Two true replicates of each experiment were carried out.

$$R_0 = \int_0^t exp\left(\frac{T-100}{14.75}\right) dt \qquad \textbf{(3.1)}$$

3.2.3 CHEMICAL CHARACTERIZATION OF LIQUORS FROM HYDROTHERMAL PROCESS

Hydrolysate samples were analyzed by HPLC using a Jasco chromatograph 880-PU intelligent pump (Jasco, Tokyo, Japan). Glucose, xylose, arabinose and acetic acid concentrations were determined with a refractive index (RI) detector and a MetaCarb 87H (300 x 7.8 mm) column at 60 ºC, using 0.005 mol/L H_2SO_4 as eluent at 0.7 mL/min flow rate. Furfural and hydroxymethylfurfural were determined using a Jasco chromatograph 2080-PU intelligent pump (Jasco, Tokyo, Japan) equipped with a Jasco 2070-UV intelligent UV-VIS detector (Jasco, Tokyo, Japan) at 276 nm and a Jasco AS-2057 Plus intelligent auto sampler (Jasco, Tokyo, Japan) with a CC 250/4 6 Nucleosil 120-5 C_{18} column (Macherey-Nagel, Düren, Germany) at room temperature, using acetonitrile-water in a ratio 1:8 (v/v) containing 10 g/L acetic acid as the eluent, at a

flow rate of 0.9 mL/min. All samples were filtered through 0.45 μm membranes before analysis (Mussatto and Roberto, 2006).

3.2.4 STATISTICAL PROCEDURES

Experiments were conducted following a 3^3 design (Box et al., 1978) at three residence times (10, 30 and 60 min), three processing temperatures (160, 180 and 200 ºC) using three blends with different particle size distributions (see size distribution characterization in Table 3.1. The experimental design can be seen on Table 3.3, together with results. Results were analysed using Experimental Design package of STATISTICA™ v 6.0 (Statsoft®, Tulsa, OK, USA). Models were proposed based on ANOVA for selecting significant variables and interactions ($p < 0.05$). The coefficient of determination (R^2), residuals' histogram and the 95% Standard Error of the parameters (SE) were obtained directly from the software and used as statistical indicators for the models.

3.3 RESULTS & DISCUSSION

3.3.1 RAW MATERIAL COMPOSITION

WS composition varies depending on plant variety and culture conditions. The chemical composition of the WS samples tested in this work is presented in Table 3.2

WS moisture content was 8%. The lignocellulosic materials present in higher amounts were cellulose with 37.4% (estimated as glucan content) followed by hemicellulose with 33.8% and lignin representing 26.8% of dry weight. Hemicellulose fraction was mainly composed of xylose (29.4%), arabinose (1.9%) and acetyl groups (2.5%). This chemical composition is in good agreement with other values found in the literature for this feedstock material (Bjerre et al., 2000; Linde et al., 2008; Zimbardi et al., 1999).

Table 3.2 Composition of wheat straw (% of dry weight).

Components	Composition (%)
Cellulose *	37.4
Hemicellulose	33.8
Xylan	29.4
Arabinan	1.9
Acetyl group	2.5
Total Lignin	26.8
Soluble Lignin	7.4
Klason Lignin	19.4
Ash	1.6

*Measured as glucan

3.3.2 EFFECT OF THE HYDROTHERMAL PROCESS ON LIQUOR COMPOSITION

The different blends of WS (Table 3.1) were subjected to different conditions of pretreatment and sugars obtained for each hydrolysate are presented in Table 3.3. It can be observed that the formation of these compounds varied for each set of hydrolysis conditions employed and for the different WS blends tested. The highest glucose yield found was 21.1% corresponding to a severity factor R_0 = 2.77 (160 ºC, 10 min), for B3 blend. For xylose, the highest yield was 49.32% corresponding to a hydrolysis treatment with a severity factor R_0 of 3.36 (180 ºC, 10 min) for blend B2. For arabinose the highest yield observed was 14.48% corresponding to a severity factor R_0 = 3.94 (200 ºC, 10 min) for B1. The effect of hydrothermal treatment can be evaluated by the total sugar present in the obtained liquor and the solid yield of each treatment (Figure 3.2 A and B, respectively).

Table 3.3 Composition (% molar) of the liquors obtained from hydrothermal process of wheat straw.

Temperature (ºC)	time (min)	Log (R_0)	Blend	Molar percentages		
				Glucose	Xylose	Arabinose
			B1	19.07±0.57	47.36±0.90	8.5±0.66
	10	2.77	B2	17.27±0.59	46.24±0.90	6.47±0.02
			B3	21.10±0.46	48.02±0.55	6.88±0.80
			B1	16.66±0.39	41.25±0.44	4.96±0.11
160	30	3.24	B2	17.76±0.25	41.61±1.96	6.14±1.15
			B3	16.06±0.19	39.43±0.59	5.83±0.73
			B1	11.90±1.01	36.12±5.5	7.21±4.19
	60	3.54	B2	11.32±0.28	24.71±1.45	11.71±0.60
			B3	11.17±0.25	24.11±0.79	10.16±0.10
			B1	20.65±0.66	46.57±2.61	7.99±0.94
	10	3.36	B2	20.40±0.66	49.32±1.58	7.20±2.46
			B3	18.5±0.28	43.47±2.11	7.23±0.27
			B1	12.5±1.16	32.15±3.56	8.25±0.34
180	30	3.83	B2	9.33±0.21	24.94±0.40	14.45±1.03
			B3	11.27±1.63	30.65±3.51	10.09±0.21
			B1	3.56±0.34	12.87±0.49	11.53±0.43
	60	4.13	B2	3.78±0.47	13.02±0.40	13.65±1.5
			B3	4.99±0.40	15.23±1.17	13.4±2.58
			B1	12.96±0.13	6.87±0.21	14.48±0.04
	10	3.94	B2	8.61±1.09	25.49±0.56	3.13±0.21
			B3	17.88±2.32	38.84±1.81	5.8±0.28
			B1	2.6±0.0.3	2.25±0.08	3.42±0.05
200	30	4.42	B2	3.43±0.22	17.04±0.35	13.19±0.06
			B3	3.62±0.85	15.92±0.24	9.77±0.97
			B1	0.61±0.00	1.45±0.07	0.93±0.05
	60	4.72	B2	0.84±0.17	10.06±0.29	0.33±0.00
			B3	0.62±0.07	10.30±0.19	0.28±0.00

Regarding total sugar present in the liquor, results show that severity treatments higher than 4.13 led to an increased sugar extraction (0.04-0.26 mol/L). On the contrary, lower severities led to poor release of sugars. Similar behavior has been reported by (Liavoga et al., 2007), where highest sugar production was obtained with higher severity treatments. As for solid residue yield, Figure 3.2B shows that for the less severe conditions (R_0 < 3.94), the solid solubilization that occurred was very low. For higher severity treatments, a lower solid yield was obtained – e.g. around 55 % of original material was solubilized as a consequence of pretreatment with the severity factor of log R_0 = 4.72. This decrease could be correlated to the solubilization of sugars, principally hemicellulose. These results are in a good agreement with previous reports. (Carvalheiro et al., 1999; Perez et al., 2008).

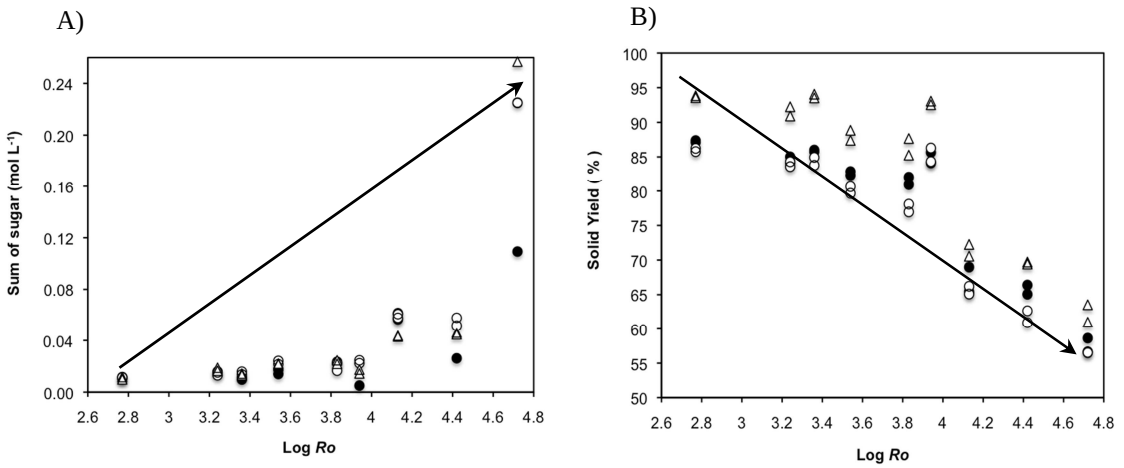


Figure 3.2. Effect of hydrothermal process in the sum of sugar contents and solid yield present in the liquor. A) Sum of sugar contents; B) Solid Yield: B1 (●); B2 (○) and B3 (△). The arrows represent main trends.

For better evaluating the effect of treatment severity and type of straw blend, molar percentages of xylose, arabinose and glucose in the liquors were also estimated. Moreover, the molar percentages of these sugars thermal degradation products (acetic acid, hydroxymethylfurfural (HMF), and furfural) were quantified (Figure 3.3). From these results, it can be observed that different severity factors lead to different compositions of the liquor.

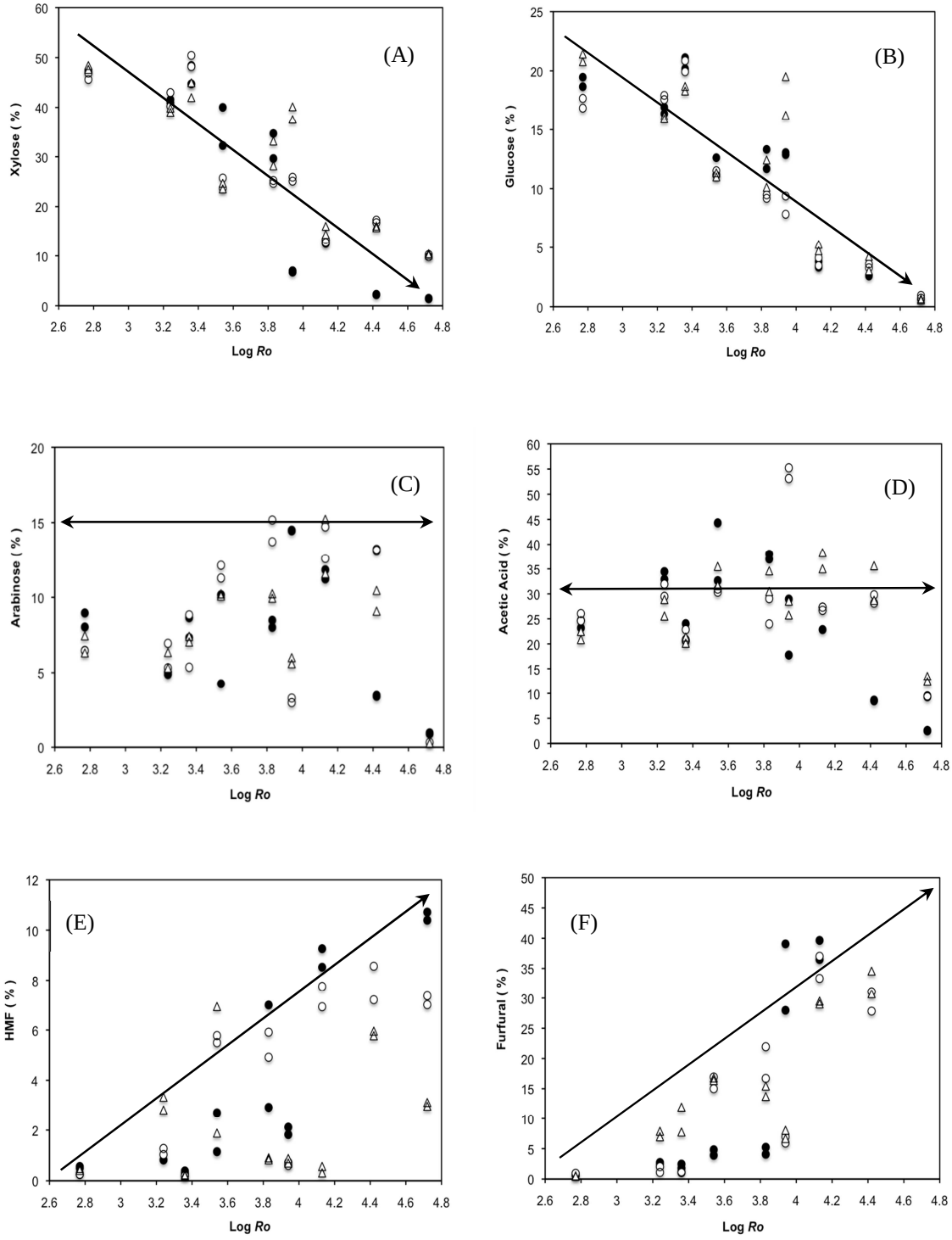


Figure 3.3 Relationship between severity parameters and molar percentage of hydrolysed sugars and the respective degradation products: A) Xylose; B) Arabinose; C) Glucose; D) Acetic acid, E) Hydroxymethylfurfural (HMF), and F) Furfural. Wheat straw blends: B1 (●); B2 (○) and B3 (△). The arrows represent main trends.

In terms of sugars composition, it can be seen that xylose and glucose are degraded for higher severity treatments (Figure 3.3A and B), whereas arabinose seems to be more resistant to heat degradation (Figure 3.3C). As for the degradation products, the intensity of the heat treatment has a more important role on HMF and furfural production (Figure 3.3E and F) than on the formation of weak acids (here assessed in terms of acetic acid (%)), (Figure 3.3D). The formation of HMF was enhanced at higher process severity (log R_0 = 4.72) and was largely consistent with the decomposition of glucose. Furfural production achieved the maximum at 200 ºC for 60 min. A high temperature and/or prolonged heating resulted in a loss of xylose or arabinose, probably because it was converted to furfural or it reacted with peptide amino groups to give Maillard browning products that resulted in a dark color and a burnt-sugar odor (Liavoga et al., 2007). A similar effect of temperature on HMF and furfural formation has been reported (Carvalheiro et al., 2009). The production of acetic acid was not affected by treatments severity, this can be partially explained by the lower acetyl groups content of WS.

For every tested treatment condition it can also be observed that the main sugar fraction in the liquor is xylose and lower quantities of glucose are observed; this is because the glucan remained in the solid phase and only a small part of it was depolymerized to glucose (Carvalheiro et al., 2009). When using autohydrolysis, the hydrolysate reported by (Carvalheiro et al., 2009) contained 0.255, 0.1, 0.066, 0.584, 0.017, and 0.157 in molar % for xylose, arabinose, glucose, acetic acid, HMF and furfural. The severity factors used in that work varied between 3.96 and 4.68. These results are in agreement with the sugars contents obtained in the present work (see Table **3.3**). These results contrast with the obtained for the other materials also using hot-compressed water (Yu et al., 2010).

3.3.3 EVALUATION OF PROCESS CONDITIONS: PARTICLE SIZE, TIME AND TEMPERATURE

Hydrothermal process of WS aims at two different and competing objectives: maximizing the extraction of sugar monomers (which can be assessed by the release of

glucose, xylose and arabinose into the liquor) and minimizing the degradation of these monosaccharides. These response variables where studied for different operation conditions (temperature and time) in WS blends with different particle size distributions (evaluated by the variance of the distribution,Table 3.1).

The extraction of sugars, evaluated by the sum of glucose, xylose and arabinose molar concentrations in the liquor, was significantly affected by processing time and temperature and by the variance of WS particle size (5% significance level). These effects can be described by the following model:

$$Sum\ of\ sugars = a + (b \cdot Var) + (c \cdot t) + (d \cdot Var \cdot T) + (e \cdot Var \cdot t) + (f \cdot T \cdot t) \quad \textbf{(3.2)}$$

Where *Var* is the variance of size distribution of the blends (mm^2); *t* is residence time (min); *T* is temperature (ºC); *a, b, c, d, e and f* are estimated parameters.

Table 3.4 shows the estimated parameters (± 95% confidence level error) and regression evaluation on the basis of adjusted R^2 and histogram of residuals. The positive value of estimate of the *b* parameter may indicate that blends with higher size distribution variance, i.e. higher heterogeneity of particle sizes, favour sugars extraction. As for processing time, it has a negative effect on the *sum of sugars* ($c < 0$). However, this is probably due to the fact that longer treatments lead to further sugar degradation and is not directly related with hemicellulose extraction. The same hypothesis can be supported for the estimates of time and temperature interactions with the type of blend used (*d* and *e* parameters).

Percentage of glucose and xylose in the liquor are important parameters since higher degradation of these sugars may impair the use of liquor for fermentation purposes.

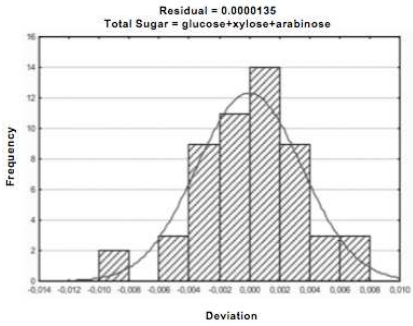
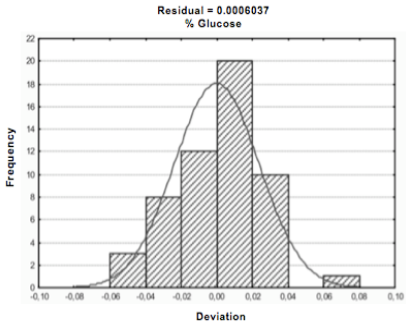
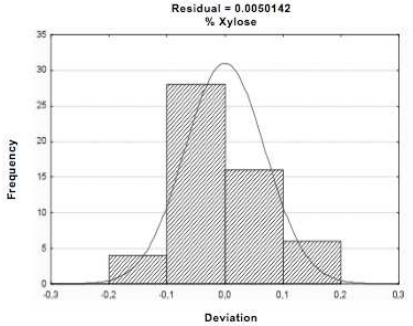
Results show that both % glucose and % xylose were significantly affected by both temperature and time of processing and not by the distribution of particle size.

$$\%glucose = a' + (b' \cdot T) + (c' \cdot t) + (d' \cdot t^2) \quad \textbf{(3.3)}$$

$$\%xylose = a'' + (b'' \cdot T) + (c'' \cdot t) \quad \textbf{(3.4)}$$

Where *t* is residence time (min); *T* is temperature (ºC); *a'*, *b'*, *c'*, *d'*, *a''*, *b''* *and* *c''* are estimated parameters.

Table 3.4. Estimated parameters and regression evaluation on the basis of adjusted R2 and histogram residuals of the hydrothermal process optimization.

Dependent Variable	R^2_{adj}	Histogram of residuals	Estimated parameter		± 95% Confidence Error
Sum of total sugars	0.63		a	0.009151	0.006191
			b	0.511205	0.265915
			c	-0.001130	0.000507
			d	-0.003031	0.001404
			e	-0.002140	0.002117
			f	0.000008	0.000003
% Glucose	0.87		a'	0.675711	0.077842
			b'	-0.002531	0.000411
			c'	-0.005004	0.001742
			d'	0.000037	0.000024
% Xylose	0.79		a"	1.528142	0.216403
			b"	-0.006129	0.001185
			c"	-0.004466	0.000941

Results show that the amount of glucose in the liquor was greatly affected by heat treatment, making the type of blend used non-significant. This observation is supported by the negative values of *b'* and *c'* parameters estimates (Table 3.4). The percentage of xylose was the main component in the liquor obtained for every tested treatment (Table 3.3). However, processing time and temperature also significantly affected this sugar, while no effect of the type of blend was observed (Table 3.4).

These results show that although the type of blend has an influence on sugar extraction from the WS, it has no effect on the thermal degradation of these compounds once they are free on the syrup. Also, such results are indicative that thermal degradation occurs when these sugars are in the liquor and not during the extraction process. Such fact makes the use of milled WS blends with high heterogeneity of particle sizes even more interesting, since it may lead to higher sugar extraction, thus compensating the unavoidable sugar degradation due to the heat treatment.

3.4 CONCLUSIONS

In this work, the effect of using milled WS blends of different particle size distributions on sugar monomers extraction and obtainment of fermentable products was studied. Results showed that although variance of the size distribution does not affect sugar degradation in the liquor, it has a selective influence over the extraction of the sum of total sugar (glucose, xylose and arabinose). Thus, it can be established that the use of a blend with defined percentages of the various sizes of particles (preferably with high size variability) is an important parameter to be defined before carrying out a pretreatment. These results can contribute to improving total monosaccharide recovery for treatments of the same severity.

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CHAPTER 4

DEVELOPMENT AND CHARACTERIZATION OF AN ENVIRONMENTALLY FRIENDLY PROCESS SEQUENCE (AUTOHYDROLYSIS AND ORGANOSOLV) FOR WHEAT STRAW DELIGNIFICATION

ABSTRACT

The present work describes the delignification of wheat straw through an environmentally friendly process resulting from sequential application of autohydrolysis and organosolv processes. Wheat straw autohydrolysis was performed at 180 ºC during 30 min with a liquid-solid ratio of 10 (v/w); under these conditions, a solubilization of 44 % of the original xylan obtained, with 78% of sugars as xylooligosaccharides of the sum of sugars solubilized in the autohydrolysis liquors generated by the hemicellulose fraction hydrolysis. The corresponding solid fraction with 63.7 % of glucan and 7.55 % of residual xylan was treated with a 40 % ethanol and 0.1 % NaOH aqueous solution at a liquid-solid ratio of 10 (v/w), being the best results obtained at 180 ºC during 20 min. The highest lignin recovery, measured by acid precipitation of the extracted lignin, was 3.25 g/100 mL. The lignin obtained by precipitation was characterized by FTIR, and the crystallinity indexes from the native cellulose, the cellulose recovered after autohydrolysis and the cellulose obtained after applying the organosolv process were obtained by X-ray diffraction, values of 21.32, 55.17 and 53.59 % being obtained. Visualization of the fibers was done for all the processing steps using Scanning Electron Microscopy.

4.1 INTRODUCTION

In recent years, lignocellulosic materials (LCM) have become an interesting raw material for ethanol production, for chemical, paper, pharmaceutical and biomaterials industries. The efficient separation of LCM mains components is one of the major obstacles for its utilization as renewable resource. Conversion of LCM into chemicals requires the development of commercially viable technologies that allow efficient and clean fractionation into its components (Hongzhang and Liying, 2007; Turley, 2008) that will be further transformed into high value added products according to the so-called "biorefinery" concept. In order to become a viable alternative to e.g. a petrochemical refinery, a biorefinery must be competitive and cost effective (Pan et al., 2006).

Wheat straw is one of the most abundant agricultural residue which is generated in huge quantities around the world every year. Although straw yield depends on the specific varieties harvested and is widely affected by agronomic and climatic factors, an average ratio of 1.3 kg of straw per kg of grain is found for the most common varieties, representing around 611 millions tons of wheat worldwide in 2007 (Montane et al., 1998; Peterson, 1988) these amounts are significant enough to consider wheat straw as a complementary source of raw material.

Lignin is the most abundant aromatic heterogeneous polymer, formed by phenolic compounds, and its characteristics structure depends to the extractive method and the plant source. Lignin is made up of three precursors: trans-coniferyl, trans-sinapyl and trans-pcoumaryl alcohols. Gymnosperm lignins show predominance of guaiacyl groups, angiosperms lignins contain guaiacyl-syringyl groups and lignins from grasses contain guaiacyl-syringyl-p-hydroxyphenyl groups (Garrote et al., 2004). Lignin is an essential component of higher plants and has a very complex structure whose function is to provide rigidity and cohesion to the material cell wall, to confer water-impermeability to xylem vessels, and resistance physic-chemical resistance against microbial attack (Brunow, 2006; Fengel and Wegener, 1989)

The chemical separation of lignin from cellulose has been termed "delignification" and is a complex processes. Industrially, lignin is found during the process of papermaking from wood and commercial lignin is divided into two categories. The first category comprises conventional or sulfur-containing lignins, which include Kraft lignin and lignosulfonates; these products have been available for many years. Nowadays, lignin in amounts larger than 70 million tonnes per year is produced by kraft process worldwide. However, the major disadvantages of the kraft process include the low pulp yield, the high consumption of bleaching chemicals to obtain bright pulp, formation of odorous gases (Sjödahl, 2006). From that amount 99 % are burnt in chemical recovery furnaces and, consequently, not recovered for industrial applications (Brunow, 2006; Mansouri and Salvado, 2006).

The second category comprises non-sulfur lignins obtained from many different processes, most of which are not yet commercially implemented: organosolv lignins, soda lignins, steam explosion lignins, hydrolysis lignins (mainly from biofuel production), and oxygen delignification lignins. Almost all lignins extracted from lignocellulosic materials from the pulp and paper industry are burned to generate energy, only a small amount (1–2 %) is commercially used in a wide range of products.

On the other hand, an alternative for the pulping processes utilizing aqueous organic solvents, known as organosolv, has been studied in the last 30 years as an alternative to conventional chemical processes of pulping (Azis, 1989; Garrote et al., 2003; Gonçalves and Ruzene, 2001; Gonçalves and Ruzene, 2003; Kleinert, 1974; Mansouri and Salvado, 2006; Pan et al., 2005). The organosolv process was developed by General Electric Company in the 1970s to make a clean biofuel for turbine generators. Consequently the Canadian pulp and paper industry did modifications to the process resulting in the Alcell pulping process and different demonstration plants have been developed. Actually, the organosolv process has been developed as part of a commercial lignocelluloses biorefinery technology known as the Lignol process. Lignol is a pilot plant that allows for the obtention of several high-value products with a cost-effective process in which solvents were recovered and recycled at the end of the process (Arato et al., 2005; Muurinen, 2000; Pan et al., 2005).

Organosolv (organic solvent-based) pulping is an environmentally friendly chemical pulping method compared with the kraft and sulphite processes, in which delignification of biomass (usually wood) is performed by an organic solvent (frequently ethanol) or solvent plus water system (Azis and Sarkanen, 1989; Young and Akhtar, 1998; Ziaie and Mohammadi, 2007) which involves treating the raw LCM at temperatures in the range of 180 – 200 ºC. During the organosolv process, the lignin structure is broken into smaller parts and dissolved from the raw material and separated in the form of a liquor rich in phenolic compounds that represent the process effluent (Young and Akhtar, 1998; Ziaie and Mohammadi, 2007).

This lignin can be isolated and has the advantage of being a product relatively pure with excellent properties which may be used as precursor in the production of various commercial products such as: vanillin, syringaldehyde, phenol, benzene and substituted phenols, dispersants, emulsifiers and chelanting agents, antioxidants, pesticides, fertilizers, vegetal charcoal, polymers, adhesives, concrete additives, components for resins, animal vitamin supplements, among others (Arato et al., 2005; Fengel and Wegener, 1989; Pan et al., 2005; Young and Akhtar, 1998; Ziaie and Mohammadi, 2007).

The main advantages reported for organosolv process are: a) can be economically operated at a smaller scale than the kraft process, b) significantly lower environmental impact (no sulfur-containing substances), c) can be used with any type of woody and nonwoody raw material, d) the properties of the resulting pulp are similar to those of kraft pulp, with a higher extraction yield although with a lower lignin content, e) the production of by-products of potentially significant commercial interest, f) the pulp is whiter and more readily bleached than kraft pulp, which saves bleaching reagents, g) the pulp is easier to beat, h) the presence of organic compounds in the liquors reduces their viscosity, thus, improving both their handling and the penetration of the impregnation chemicals into the chips, i) the solvents can be efficiently recovered, j) the process uses less water, energy and reagents than traditional alternatives, k) raw materials are more efficiently used (virtually the whole mass can be used for some purpose) (Gaegulak and Lebo, 2000; Garrote et al., 2003; Gilarrahz et al., 1998; Jimenez et al., 2001; Young and Akhtar, 1998).

Additionally, the presence of lignin is known to reduce the efficiency and is one of the major barriers in enzymatic hydrolysis; the other phenomena is the adsorption of cellulases onto lignin is believed to be due to hydrophobic and different interactions lignin-enzyme. Thus, the increased removal of lignin could be advantageous compared with other pretreatments and it has been shown that the solid fiber remaining with residual lignin is highly susceptible to cellulose hydrolysis, the extent of cellulose saccharification being greater than 90 % of theoretical maximum and decreasing enzyme losses, when the organosolv process is used (Chum et al., 1990; Jørgensen et al., 2007; Lora, 1992; Zhao et al., 2009).

The aim of this section was to develop and characterize an environmentally friendly process sequence, involving autohydrolysis and organosolv process for delignification of wheat straw. Extracted lignin was further characterized following acid precipitation

4.2 EXPERIMENTAL PROCEDURES

4.2.1 RAW MATERIAL

Wheat straw was kindly provided by a local farmer (Elvas, Portugal). The wheat straw was cut into small pieces (1-3 cm). Samples were milled to pass a 0.5 mm screen. Aliquots from the homogenized wheat straw lot were subjected to moisture determination (drying at 105 ºC to constant weight) and quantitative acid hydrolysis with 5 mL of 72% (w/w) sulfuric acid for an hour followed by quantitative posthydrolysis with 4 % sulfuric acid (adding water until 148.67 g) at 121 ºC during 60 min. Before HPLC analysis, the solid residue from posthydrolysis process was recovered by filtration and considered as Klason lignin (Browning, 1967). The monosaccharides sugars and acetic acid contained in hydrolysates were determined by HPLC in order to estimate the contents of samples in cellulose (as glucan), xylan, arabinan and acetyl groups. The moisture of wheat straw was considered as water in the material balances. Chromatographic separation was performed using a Metacarb 87H column (300 x 7.8 mm, Varian, USA) under the following conditions: mobile phase

0.005 mol/1 sulfuric acid, flow rate 0.7 mL/min, and column temperature 60 ºC using a Jasco chromatograph 880-PU intelligent pump (Jasco, Tokyo, Japan) equipped with a Jasco 830-IR intelligent refraction-index detector (Jasco, Tokyo, Japan) and a Jasco AS-2057 Plus intelligent auto sampler (Jasco, Tokyo, Japan). The volume injected was 20 µl.

4.2.2 AUTOHYDROLYSIS PROCESS

Milled wheat straw samples with a particle size distribution (w/w %) was (> 1 mm, 10 %; between 1 and 0.5 mm, 40 %, between 0.5 and 0.3 mm, 40 %; < 0.3 mm, 10 %) and water were mixed in order to obtain a ratio 10:1 liquid/solid and treated in a 3.75 L total volume stainless steel reactors (Parr Instruments Company, Moline, Illinois, USA) with PID temperature control. The moisture content of wheat straw was considered as water in the material balances. The reactor was filled and heated to 180 ºC at a heating rate of 3 ºC/min until to reaching the desired temperature, the reaction time was 30 min, these conditions being previously evaluated by Ruiz et al. (2011). After completing the reaction time, the reactor was cooled down at a rate of about 3.2 ºC/min. The agitation speed was set at 135 rpm. At the end of treatment, the liquid and solid phases were separated by centrifugation and the solid residues were washed with distilled water.

The liquid was analyzed by HPLC with a MetaCarb 87H (300 x 7.8 mm, Varian, USA) column at 45 °C using a Jasco chromatograph equipped with refraction-index detector (Jasco, Tokyo, Japan); the mobile phase was 0.005 M sulfuric acid at a flow rate of 0.6 mL/min. The samples were analyzed for glucose, xylose, arabinose, 5-hydroxymethylfurfural (HMF), furfural and acetic acid. Sugars and compounds concentration were determined using calibration curves of these pure compounds.

A second aliquot of liquors (20 mL) was subjected to quantitative posthydrolysis (with 4 % H_2SO_4 at 121 ºC during 60 min) before HPLC analysis. The increase in the concentrations of monosaccharides and acetic acid caused by posthydrolysis measured the concentrations of oligomers and acetyl groups bound to oligosaccharides (Ruiz et al., 2011). Equations 4.1 to 4.10 were used to calculate: a) the percentage of feedstock xylan converted into concentrations of xylooligosaccharides (XOS_C), xylose (Xyl_C) and

furfural (F_C); b) the percentage of feedstock arabinan converted into arabinooligosaccharides (AOS_C) and arabinose (Ara_C); c) the percentage of feedstock acetyl groups converted into acetyl groups linked to oligosaccharides (AcO_C) and acetic acid (Acl_C); and d) the percentage of feedstock glucan converted into glucooligosaccharides (GO_C), glucose (Glu_C) and HMF (HMF_C).

$$XOS_{\mathrm{C}} = \frac{132}{150} \cdot \frac{XOS}{\mathrm{X}_{\mathrm{n_{FS}}}} \cdot \frac{\mathrm{W_L}}{\mathrm{W_{FS}}} \cdot 10 \quad \textbf{(4.1)}$$

$$Xyl_{\mathrm{C}} = \frac{132}{150} \cdot \frac{Xyl}{\mathrm{X}_{\mathrm{n_{FS}}}} \cdot \frac{\mathrm{W_L}}{\mathrm{W_{FS}}} \cdot 10 \quad \textbf{(4.2)}$$

$$AOS_{\mathrm{C}} = \frac{132}{150} \cdot \frac{AOS}{\mathrm{A}_{\mathrm{rn_{FS}}}} \cdot \frac{\mathrm{W_L}}{\mathrm{W_{FS}}} \cdot 10 \quad \textbf{(4.3)}$$

$$Ara_{\mathrm{C}} = \frac{132}{150} \cdot \frac{Ara}{\mathrm{A}_{\mathrm{rn_{FS}}}} \cdot \frac{\mathrm{W_L}}{\mathrm{W_{FS}}} \cdot 10 \quad \textbf{(4.4)}$$

$$F_{\mathrm{C}} = \frac{132}{96} \cdot \frac{F}{\mathrm{X}_{\mathrm{n_{FS}}}} \cdot \frac{\mathrm{W_L}}{\mathrm{W_{FS}}} \cdot 10 \quad \textbf{(4.5)}$$

$$A_{\mathrm{C}}O_{\mathrm{C}} = \frac{43}{60} \cdot \frac{A_{\mathrm{C}}\mathrm{OS}}{A_{\mathrm{C}}l_{\mathrm{FS}}} \cdot \frac{\mathrm{W_L}}{\mathrm{W_{FS}}} \cdot 10 \quad \textbf{(4.6)}$$

$$A_{\mathrm{C}}l_{\mathrm{C}} = \frac{43}{60} \cdot \frac{A_{\mathrm{Ce}}}{A_{\mathrm{C}}l_{\mathrm{FS}}} \cdot \frac{\mathrm{W_L}}{\mathrm{W_{FS}}} \cdot 10 \quad \textbf{(4.7)}$$

$$GO_{\mathrm{C}} = \frac{162}{180} \cdot \frac{GOS}{Gl_{\mathrm{n_{FS}}}} \cdot \frac{\mathrm{W_L}}{\mathrm{W_{FS}}} \cdot 10 \quad \textbf{(4.8)}$$

$$Glu_{\mathrm{C}} = \frac{162}{180} \cdot \frac{Glu}{Gl_{\mathrm{n_{FS}}}} \cdot \frac{\mathrm{W_L}}{\mathrm{W_{FS}}} \cdot 10 \quad \textbf{(4.9)}$$

$$HMF_{\mathrm{C}} = \frac{162}{180} \cdot \frac{HMF}{Gl_{\mathrm{n_{FS}}}} \cdot \frac{\mathrm{W_L}}{\mathrm{W_{FS}}} \cdot 10 \quad \textbf{(4.10)}$$

The following terms correspond to the stoichiometric factor for the conversion of xylose into xylan or arabinose into arabinan (132/150), furfural into xylan (132/96), acetyl groups into acid acetic (43/60), glucose into glucan (162/180) and HMF into glucan (162/126) (Carvalheiro et al., 2005; Carvalheiro et al., 2009; Garrote et al., 2001). The solids residues were washed, air dried and milled to a particle size < 0.5 mm in order to make certain quantitative polysaccharide hydrolysis. Milled samples were assayed for glucan, xylan, arabinan and acetyl groups, the acid insoluble residue was considered as klason lignin using same methods as for raw material analysis.

4.2.3 DELIGNIFICATION AND LIGNIN RECOVERY

The solid residue obtained from autohydrolysis, was delignified with an organosolv process by a solvent consisting of an aqueous solution of 40 % (v/v) ethanol and 0.1 % (w/v) NaOH conditions (Table 4.1). The solid/liquid ratio was fixed at 1:10 w/v. These experiments were carried out in 160 mL total volume stainless steel cylinders reactors. The reactor was closed and mounted vertically and then submerged in a oil bath open heating circulator (JULABO Labortechnik GmbH, Seelbach, Germany) with PID temperature control, previously heated to the desired reaction temperature (see Table 4.1). At the end of the desired reaction time (Table 4.1), the reactor was removed from oil bath and cooled down by soaking in an ice-water bath for 5 min. The solid and liquid were separated via vacuum filtration. The filtrate was distilled to recover the ethanol at low temperature. Recovered ethanol could be reused.

In order to recover lignin, the pH of concentrated liquor was lowered with 0.3 mol/L hydrochloric acid to a point where the lignin was no longer soluble and precipitation took place (pH = 1.6). The precipitated lignin was recovered by centrifugation at 3500 rpm for 15 min, water washed, air-dried at 60 ºC (Hongzhang, and Liying, 2007; Uloth and Wearing, 1989).

Table 4.1. Experimental conditions for delignification and precipitated lignin mass (liquid/solid ratio 1:10, aqueous solution of 40 % ethanol and 0.1 % NaOH).

Sample	Temperature (°C)	Time (min)	Precipitated lignin mass (g)	Yield [a] (g/100 mL)
1	180	20	0.65	3.25
2	180	40	0.51	2.55
3	190	30	0.44	2.2
4	200	20	0.42	2.1
5	200	40	0.38	1.9

[a] Lignin recovery yield from 100 mL of liquor

4.2.4 CHARACTERIZATION OF LIQUORS AND LIGNIN RECOVERY FROM ORGANSOLOV PROCESS

The concentration of the phenolic acids - vanillic, ferulic, syringic, gallic and *p*-hydroxybenzoic – in the liquors was determined by HPLC using a Jasco chromatograph 2080-PU intelligent pump (Jasco, Tokyo, Japan) equipped with a Jasco 2070-UV intelligent UV-VIS detector (Jasco, Tokyo, Japan) at 276 nm and a Jasco AS-2057 Plus intelligent auto sampler (Jasco, Tokyo, Japan) with a Nucleosil 120-5 C_{18} (5 μm particle size, Macherey-Nagel, Düren, Germany) column. The samples were filtered in 0.45 μm Millipore membrane and injected in the chromatograph under the following conditions: column at room temperature; acetonitrile/water 1:8 containing 10 g/L acetic acid and pH adjusted to 2.5 by H_3PO_4 addition) as mobile phase; a flow rate of 0.9 mL/min. (Ruiz et al., 2011).

4.2.4.1 FTIR analysis

Fourier Transform Infrared (FTIR) spectra were obtained on an FTIR spectrophotometer, (FTLA 2000 series, ABB Bomem Inc., Quebec, Canada). The conditions of analysis were as follows: resolution 4 cm^{-1}, co-adding 20 scans and

frequency range of 400-4000 cm^{-1} using a KBr disc containing 1% finely ground samples.

4.2.4.2 X-ray diffraction analysis and crystallinity

Cellulose crystallinity of wheat straw treated with autohydrolysis and organosolv process were analyzed an X-ray diffractometry (Bruker D8 Discover, USA). The operating voltage and current were 40 kV and 60 mA, respectively. The crystallinity index (CI) was defined using the equation $CI = (I_{002} - I_{am})/I_{002x} \times 100$, where I_{002} is the maximum intensity (2θ, 22.6º) of the (002) lattice diffraction and I_{am} is the intensity of the amorphous diffraction (2θ, 18.7º) respectively (Fengel and Wegener, 1984; Yoshida et al., 2008).

4.2.4.3 Scanning Electron Microscopy

The scanning electron microscopy (SEM) surface of wheat straw treated with autohydrolysis and organosolv processes were visualized by a scanning electron microscope (Nova NanoSEM 200, Netherlands) and photographed. Samples were coated with a layer of gold by sputtering with an accelerating voltage varying to 15 kV.

4.3 RESULTS & DISCUSSION

4.3.1 COMPOSITION OF RAW MATERIAL

The wheat straw used had the following composition (% dry weight, w/w): 37.4 % cellulose (glucan), 29.4 % xylan, 1.9 % arabinan, 2.5 acetyl group, 23.6 % lignin and 2.1 % ashes. The amounts of xylan, arabinan and acetyl groups correspond to the content of hemicellulose. This chemical composition is in good agreement with other values found in the literature for this material (Bjerre et al., 1996; Hongzhang and Liying, 2007; Ruzene et al., 2008).

4.3.2 EFFECT OF AUTOHYDROLYSIS ON THE COMPOSITION ON THE LIQUID PHASE

The conditions of time, temperature and particle size distribution used during the process of autohydrolysis for wheat straw sugars solubilization were established in a previous work (Ruiz et al., 2011). Autohydrolysis can be conceived as an environmentally friendly process that caused a substantial fractionation into non-volatile components including monosaccharides (xylose, arabinose and glucose), oligosaccharides (xylooligosaccharides – XOS, arabinooligosaccharides – ArOS, glucooligosaccharides – GOS, and acetyl groups linked to oligosaccharides – AcOS); and volatile components mainly acetic acid, furfural and HMF (Garrote et al., 2007).

The concentrations (g/L) of the liquid phase components derived principally from wheat straw hemicellulose fractions by autohydrolysis at 180 ºC and 30 min were 14.72 ± 0.095, 0.86 ±0.059, 1.25 ± 0.074, 1.94 ± 0.003, 1.25 ± 0.006, 2.04 ± 0.030 and 0.636 ± 0.066 for XOS, ArOS, AcOS, xylose, arabinose, acetic acid and furfural, respectively. The yields of soluble sugars (XOS and xylose) of the original xylan were 44 % and 5.8 %, respectively. The XOS yield is in agreement with previous studies reported by Carvalheiro et al. (2009) and Nabarlatz et al. (2007) with feedstock yields of 45% and 58%, respectively. The recovered oligomers, mainly XOS, represented 78% of the sum of sugars solubilized in the autohydrolysis liquors generated by the hemicellulose fraction hydrolysis. As can be observed, the hemicellulose can be selectively removed from wheat straw by the autohydrolysis. Arabinose rapidly released from hemicelluloses demonstrating that this fraction showed high susceptibility to hydrolysis reactions, the yields of soluble sugars (ArOS and arabinose) were 39.83 % and 57.89 % respectively, Gullón et al. (2010) reported a value of 28.2 % for rye straw. Xylose and arabinose yields obtained in the liquid product were not high because they readily decomposed into furfural at the reaction conditions. Yields for AcOS and acetic acid were 35.83 % and 58.58 %, respectively, these results are in agreement with other reported data (Gullón et al., 2010; Vegas et al., 2004); the low content of acetyl groups can be partially explained by the wheat straw composition, related with the low recovery production of degradation products as furfural yield (2.94 %).

In contrast to hemicellulose components, the effect of the autohydrolysis reaction on the cellulose (glucan) basically remained in the solid phase and only a small part was depolymerized to oligomers and glucose. The concentrations (g/L) were 1.76 ± 0.085, 0.51 ± 0.009 and 0.122 ± 0.002 for GOS, glucose and HMF, respectively. The glucooligosaccharides and glucose yields were 4.23 % and 1.22 %, respectively and the HMF as degradation product was 0.41 %, demonstrating that only a minor part of the glucose present in the liquor was degraded by the autohydrolysis process.

4.3.3 EFFECT OF AUTOHYDROLYSIS ON THE COMPOSITION OF THE SOLID PHASE

The cellulose (glucan) content in the solid residue was of 63.7 %, xylan content was 7.55 %, arabinan 0.29 %, acetyl groups 1.51 % and klason lignin 26.91%, revealing that the glucan was almost not affected by the autohydrolysis process and a solid residue with increased glucan was obtained. This decrease could be correlated to the solubilization of hemicellulose components and these results are in agreement with the data reported for the similar feedstock; (Gullón et al., 2010) observed a similar behavior for rye straw. The klason lignin content follows a similar pattern to glucan and the majority of the lignin content is retained in the solid phase.

4.3.4 CHARACTERIZATION OF LIQUOR AND LIGNIN RECOVERY

The obtained solid phase after autohydrolysis process is treated using the organosolv process and extracted/solubilized lignin is recovered by precipitation with hydrochloric acid. In the organosolv process using ethanol pulping, removal of lignin depends not only on cleavage of ether bonds in lignin macromolecules, but also on the ability of aqueous ethanol solution to dissolve lignin fragments.

The results describing the effect of operation variables (time and temperature) on the amount of extracted lignin (quantified by acid precipitation) by ethanol pulping are shown in Table 4.1. Although it has been previously shown that temperature, time and pH were the dominant factors in lignin extraction (Garcia et al., 2009; Hongzhang and

Liying, 2007), our results indicate that a temperature increase up to 190 ºC did not influence lignin precipitation and that the highest yield of precipitated mass occurred at 180 ºC for processing times between 20 and 40 min. This can be explained by the variation of the phenolic acids in the liquors. Five phenolic acids were quantified in the liquors obtained after of application of the organosolv process. The choice for these compounds was based on the fact that they are the main acids present in the wheat straw (Lawther et al., 1996).

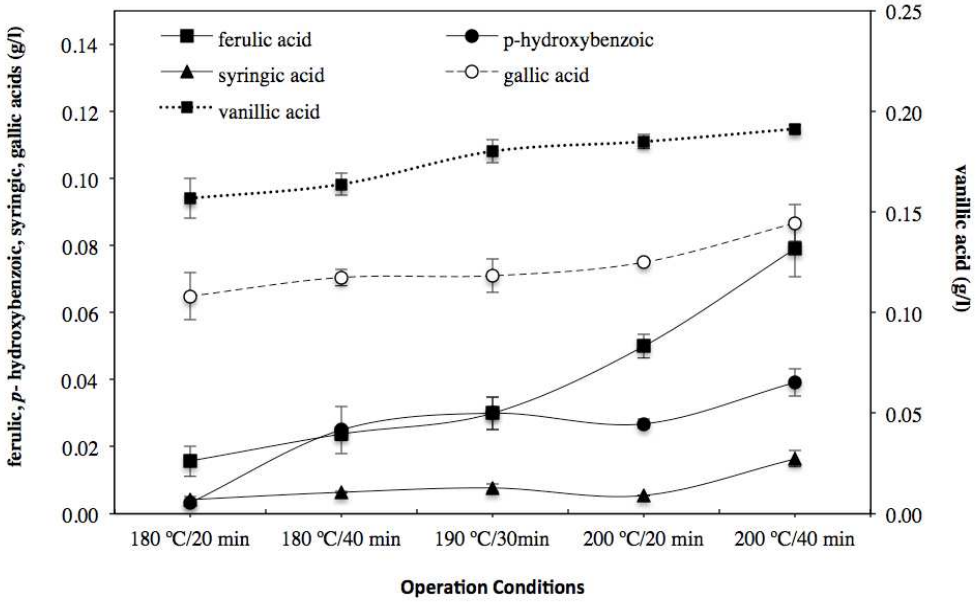

Figure 4.1. Phenolic acids content (removal profiles) in the liquor obtained by organosolv process.

The removal profiles obtained (Figure 4.1) allow evaluating the effect of increasing time and temperature on the concentration of lignin-derived compounds in the liquor. Figure 4.1 shows that temperature is not the only factor influencing lignin extraction (Nimz, 1974) as the chemical structure of the compounds being extracted is also relevant. The highest removal was observed for vanillic acid (0.19 g/L) and the lowest for syringic acid (0.004 g/L). In the case of ferulic acid, the removal profile was different from those observed for the other acids, as a significant increase in its extraction was observed when time was increased from 20 to 40 min, at 200 ºC. The extracted amounts of *p*-hydroxybenzoic and gallic acids showed an increment of 0.003 to 0.039 g/L and 0.065 to 0.087 g/L, respectively. The values obtained for the

extraction of phenolic acids as a function of temperature and time are similar to those obtained using steam treatment and alkaline peroxide post-treatment, with higher recovery yields for vanilic acid and lower for ferulic acids (Sun, 2004).

4.3.5 FTIR SPECTRA OF LIGNIN RECOVERY

Infrared spectroscopy is an effective way to identify the presence of certain functional groups in a molecule. Also, one can use the unique collection of absorption bands to confirm the identity of a pure compound or to detect the presence of specific impurities (Faix, 1991). FTIR spectra of the obtained different lignin precipitates at different operation conditions were obtained between 3500 and 500 cm^{-1} (Figure 4.2).

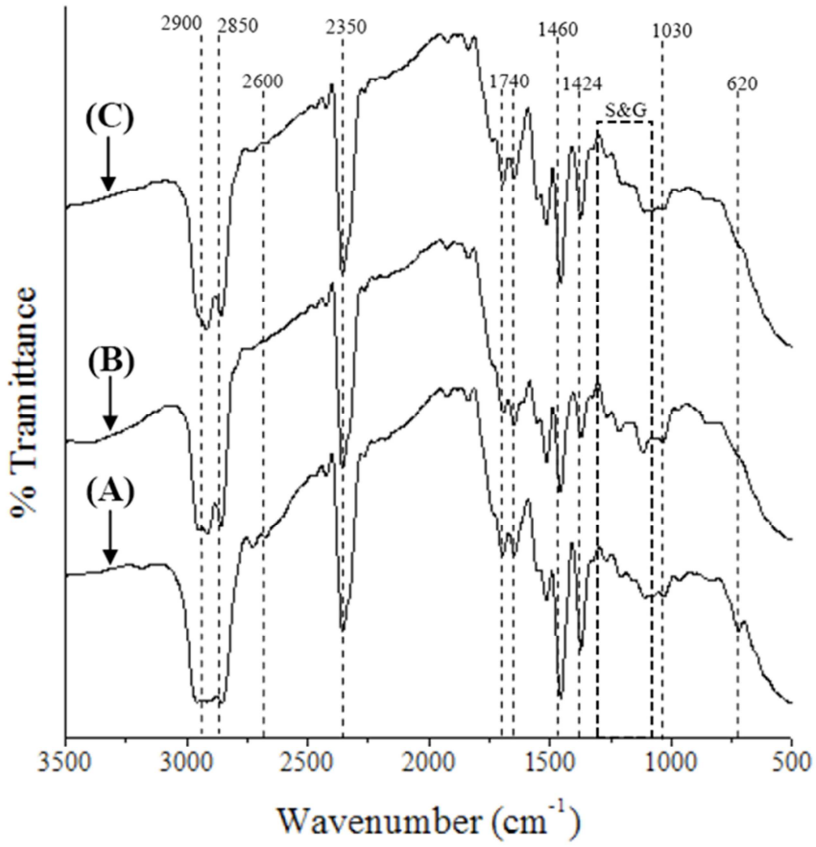

Figure 4.2. FTIR spectra of lignin recovered by precipitation at different conditions: (A) 180 °C/20 min; (B) 190 °C/30 min; (C) 200 °C/40 min.

FTIR spectra showed characteristic vibrations of typical LCM groups; carboxylic acids group is identified by the broad band centered in the region 3300-2700 cm^{-1}, caused by the presence of the O-H group that overlaps with the C-H stretch band which appears near 3000 cm^{-1}. Aldehyde type C-H absorption bands are identified with two weak absorptions to the right of the C-H stretch, near 2850 and 2600 cm^{-1}.

The three spectra of lignin extraction showed a similar broad band of the characteristic vibration of typical lignocellulosic materials at 2350 and 1740 cm^{-1}, assigned to the aromatic ring C=C and C-O non conjugated bond, respectively. Some special absorption signals of lignin were observed in the 1460 cm^{-1} region, corresponding to C-H deformations of methyl and methylene, showing the incidence of an intensive band that indicates the highest methoxy content in the treatments at low time and temperature. Moreover, a decrease in the signals absorption associated to syringyl (S) groups in the lignin (1325 cm^{-1}) is observed, indicating a cleavage of the ether linkage and a certain degree of lignin demethoxylation during the most intense conditions of organosolv process. On the contrary, there was no deformation of the guayacyl (G) units (1265–1130 cm^{-1}) at the various conditions tested. Bands at 1120 cm^{-1} were assigned to C-O and C-C stretching vibrations.

4.3.6 X-RAY DIFFRACTION

Cellulose from wheat straw has crystalline and amorphous regions, these regions are affected by the different treatments applied to wheat straw. Figure 4.3 shows X-ray curves of cellulose samples from different treatments. The diffraction peaks around 15-16º and 21-22º (2θ) are characteristics in the cellulose from wheat straw and their occurrence is in agreement with other works (Gümüfikaya and Usta, 2002; Liu et al., 2005). The crystallinity index of 21.3 % obtained for untreated wheat straw and its increase to 55.17 % after autohydrolysis indicates significant structural changes.

Removal of amorphous materials (lignin, hemicellulose) from lignocellulosic fractions and rearrangement of the remaining components have been associated with improved crystallinity (Öztürk et al., 2009). According to other works, increases of the crystallinity index values were observed following steam explosion and microwave

digestion of sorghum bagasse (Carrasco et al., 2994; Dogaris et al., 2009). The crystallinity index of 53.59 %, obtained after the organosolv process indicates damages on the fiber structure and a more exposed cellulosic surface. Similar results have been reported in a previous work (Ouajai and Shanks, 2005).

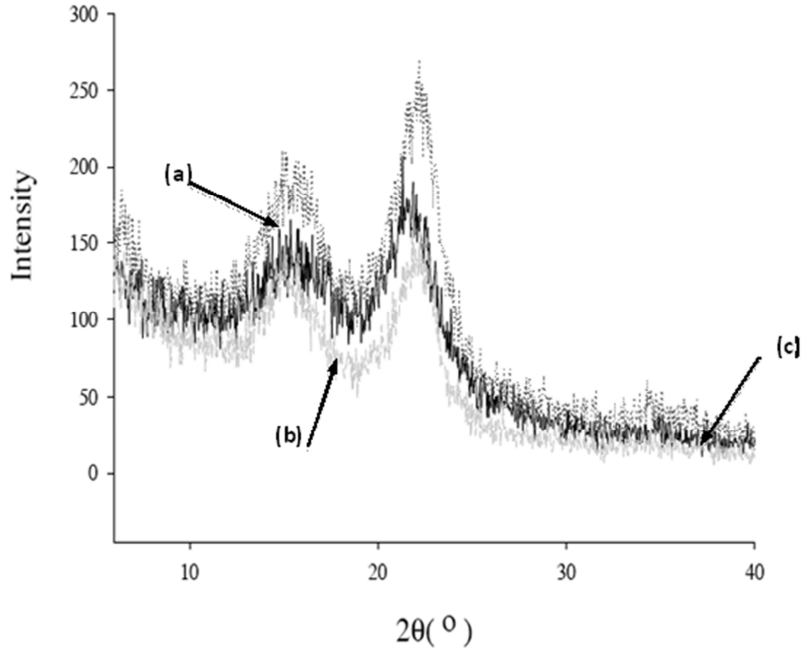

Figure 4.3. X-ray diffraction curves of cellulose from wheat straw: (a) untreated wheat straw, (b) autohydrolysis at 180 ºC/30 min, (c) organosolv process at 180 ºC/20 min.

4.3.7 SCANNING ELECTRON MICROSCOPY (SEM)

Scanning electron microscopy (SEM) images presented in Figure 4.4 were used to complete the analysis on the effect of the autohydrolysis and organosolv process on the structure. The untreated sample exhibited a rigid and highly ordered fibers (Figure 4.4A). In contrast, the fibers obtained after autohydrolysis appeared to be distorted (Figure 4.4B) because illustrated partition from the initial connected structure, increasing the external surface area and the porosity. In Figure 4.4C, the fibers after the organosolv process are shown. Sample treated with organosolv process demonstrated a high effect in fiber matrix separation and materials exposition, allowing the material to be more susceptible to enzymatic attack for subsequential use in fermentation processes.

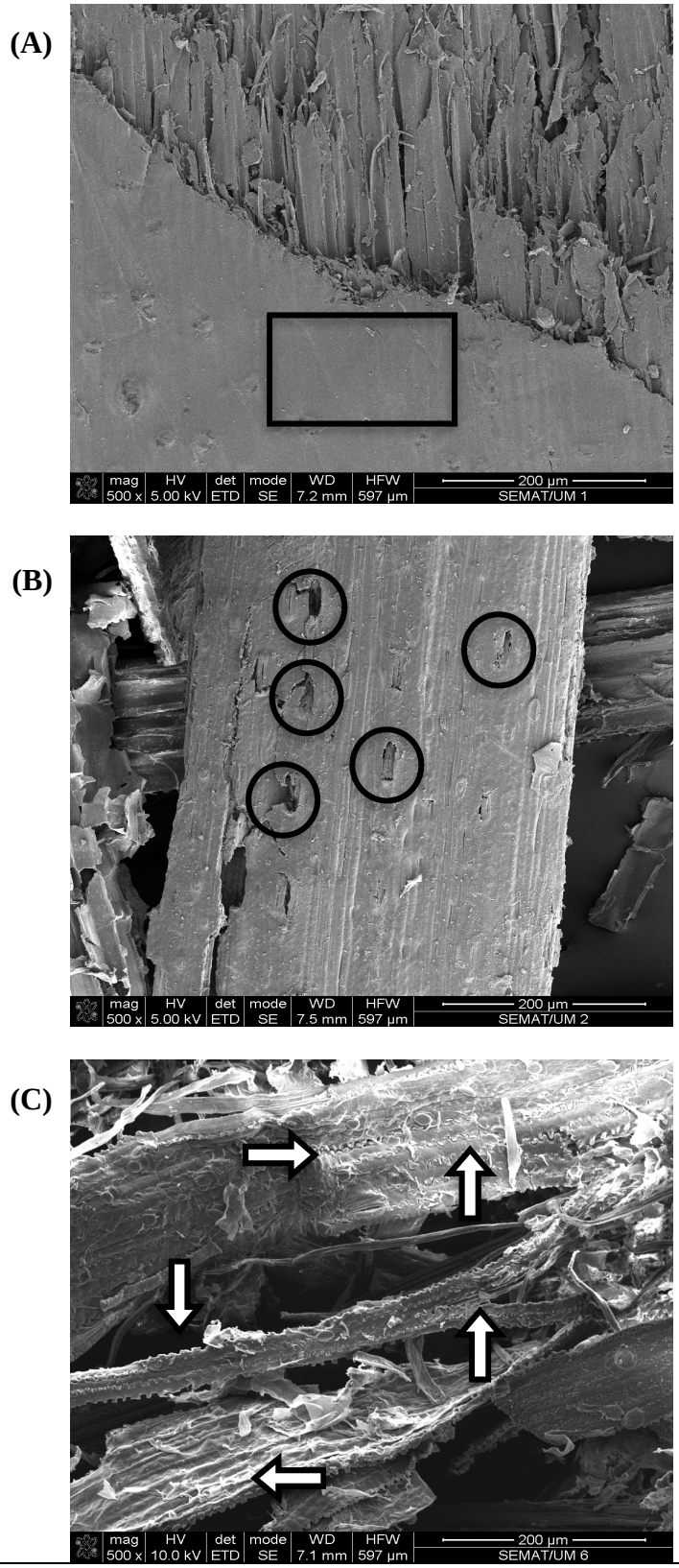

Figure 4.4. Scanning electron microscopy images of treated and untreated wheat straw: (A) wheat straw, (B) autohydrolysis, (C) organosolv process: Ordered fibers (□), high porosity area (○), fiber matrix separation and exposition (↑).

4.4 CONCLUSIONS

Results presented in this chapter show that a sequential autohydrolysis and organosolv process is a suitable technology for hemicellulose sugars recovery mainly as XOS and the generation of cellulose-enriched solids as well as the precipitation of lignin like a higher value product. Samples treated at 180 ºC and 30 min under autohydrolysis process showed a 44% of xylan conversion into xylooligosaccharides; application of the organosolv process at 180 ºC for 20 min allowed the highest lignin recovery by acid precipitation (3.25 g/ 100 mL). Temperature and time as well as chemical structure were variables that showed a strong influence on lignin precipitation in the organosolv liquors. This research evidenced that the combination of autohydrolysis and organosolv process is an interesting alternative for adding value to wheat straw compounds in accordance with the "biorefinery" concept.

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CHAPTER 5

EXTRACTION, OPTIMIZATION AND CHARACTERIZATION OF AUTOHYDROLYSIS WHEAT STRAW HEMICELLULOSE TO BE USED AS A BIOCOMPOSITE IN THE REINFORCEMENT OF POLYSACCHARIDE-BASED FILMS

ABSTRACT

The aims of this work were the extraction of hemicellulose to be used as a biocomposite in the reinforcement of polysaccharide-based films. The optimization and characterization of hemicellulose extraction from autohydrolysis were also considered. The hemicellulose extracted obtained under optimum conditions was used as reinforcement for κ-car/ LBG polymeric matrix for biocomposite manufacture. Edible films were prepared by adding the biocomposite (ranging from 0 to 0.4 %) into the κ-car/LBG film-forming solutions. Barrier properties (through the determination of water vapor permeability, WVP), mechanical properties (tensile strength, TS and elongation-at-break, EB), moisture content and opacity of the resulting films were determined and related with the incorporation of the biocomposite. The incorporation of the biocomposite in concentrations of 0.4 % causes a decrease of WVP and an increase of the TS, showing an improvement of the physical properties of the films. Moreover, the opacity of the films increased with the incorporation of the biocomposite. Biocomposite from hemicellulose extraction showed to be a good material to be used in the reinforcement of edible films, in this case in κ-car/LBG edible films, and a biodegradable alternative to be applied in the packaging industry.

5.1 INTRODUCTION

Due to environmental considerations concerning sustainable development in the last years, the renewable resources currently attract increasing interest as raw material for industry. The term "biorefinery" or fractionation of lignocellulosic materials (LCM) from forest, agricultural residues, set-aside lands, industry or urban solid wastes borrows its origin from the classical petroleum refinery concept and refers to biomass conversion into fuels and chemicals with high added value as well as biocomposites production through the integration of clean processes (Cherubini and Ulgiati, 2010).

Biocomposites may be understood as the combination of two or more materials, for example, reinforcement elements or filler involved by a polymeric matrix. In recent years, studies about the utilization of LCM as reinforcement in polymeric composites are increasing due to the improvements that natural fibers can provide to the product, such as low density and biodegradability, excellent mechanical and physical properties, besides the fact that these materials are from renewable and less expensive sources (Dogan and McHugh, 2007; Liu et al., 2010; González et al., 2011; Vicente et al., 2011). For these reasons, material components such as natural fibres and biodegradable polymers can be considered as interesting environmentally safe alternatives for the development of new biodegradable composites (biocomposites) (Avérous and Digabel, 2006).

Wheat straw (WS) is one of the most abundant agricultural by-products in the world. According to Food and Agriculture Organization of the United Nations (FAO, 2011) statistics reported a world annual wheat production in 2009 of 682 million tons. WS presents many interesting characteristics that facilitate its biotechnology upgrade in a biorefinery philosophy and consist mainly of cellulose (30-40 %), hemicellulose (20-35 %) and lignin (15-25 %).

Hemicelluloses are heteropolysaccharides made up of pentoses (xylose, arabinose), hexoses (mannose, glucose and galactose), uronic acid units and the most abundant hemicellulosic polymers are xylans. The composition of hemicelluloses depends on the LCM considered. WS hemicelluloses are arabinoglucuronoxylans and consist of a linear

β-D-(1,4)- linked xylopyranosyl backbone, substituted with arabinofuranose, 4-*O*-methylglucuronic acid, xylose and acetic, ferulic or coumaric acids (Carvalheiro et al., 2009; Gírio et al., 2010). The hemicelluloses can be separated by various extraction and isolation methods, and then utilized in a number of ways. Moreover, the process of xylan polymerization (including the conversion of xylan into high-molecular weight xylooligomers, low-molecular weight xylooligomers, xylose and dehydration of xylose into furfural). In polymeric (xylan) form they can be used as hydrogels and biodegradable barrier films for food packaging, in oligomeric form they can be used as functional food ingredients and the monomeric form can be fermented to ethanol or xylitol. (Hansen and Plackett, 2008).

Hemicelluloses from wheat straw have been studied after alkaline extraction (Sun et al. 2000); however, hemicellulose extraction using eco-friendly and benign extraction processes has received little attention. Autohydrolysis or hydrothermal processing is an environmentally friendly process in which LCM is pretreated with compressed hot water, only and that can be used to extract hemicelluloses into the water phase. The acidic groups bound to the hemicelluloses are released at elevated temperatures. These acids, mainly acetic acid and hydronium ions that come from water autoionization, participate in the hydrolysis of the solid LCM to soluble polysaccharides and oligo-saccharides (Ruiz et al., 2011a).

Recently, it has been shown that the mixture of polysaccharides could be used to improve physical properties of edible films (Nieto, 2009; Martins et al., 2011a). The mixture of κ-carrageenan and Locust Bean Gum (κ-car/LBG) has been studied and it has been shown that their "synergistic" effects gives rise to enhanced properties that allow their application as edible films (Martins et al., 2011a). The incorporation of biocomposites in this κ-car/LBG system can be very attractive leading to the improvement of their physical properties. The main objective of this chapter was the extraction of hemicellulose to be used as a biocomposite in the reinforcement of polysaccharide-based films. The optimization and characterization of hemicellulose extraction from autohydrolysis were also considered. The hemicellulose extracted obtained under optimum condition was used as reinforcement for κ-car/ LBG polymeric matrix for biocomposite manufacture.

5.2 EXPERIMENTAL PROCEDURES

5.2.1 RAW MATERIAL

Wheat straw used in this study was kindly provided by a local farmer (Elvas, Portugal). Wheat straw was cut into small pieces (1-3 cm) and milled using a laboratory knife mill (Cutting Mill SM 2000, Retsch, Germany). The material composition was previously analyzed by Ruiz et al. (2011a), containing 37.4 % glucan, 29.4 % xylan, 26.8 % total lignin, and 1.6 % ash. κ-car (Gelcarin DX5253) and LBG (Genu gum type RL-200) were supplied by FMC Biopolymer (Norway) and CP Kelco (USA), respectively. A blend of these polymers was chosen as the polymeric matrix for composite manufacture (Martins et al., 2011a). All standard chemicals used, such as sugars were of analytical grade. Birchwood xylan was used as a representative of the hemicellulose component and was purchased from Sigma-Aldrich (Steinheim, Germany).

5.2.2 AUTOHYDROLYSIS EXTRACTION OF HEMICELLULOSE FROM WHEAT STRAW

Autohydrolysis extraction of hemicellulose experiments was carried out in 160 mL total volume stainless steel cylinders reactors. The reactor was closed and mounted vertically and then submerged in an oil bath open heating circulator (JULABO Labortechnik GmbH, Seelbach, Germany) with PID temperature control. For the extraction reactions, milled wheat straw with a particle size distribution of 10 % > 1 mm; 40 % between 1-0.5 mm; 40 % between 0.5-0.3 mm; and 10 % to < 0.3 mm was efficiently mixed in a acrylic cylinder blender and suspended in the desired amount of distilled water in order to obtain a 1:10 solid/liquid mass ratio. Conditions of temperature and time used in each experiment are shown in Table 5.1. At the end of the desired reaction time, the reactor was removed from oil bath and cooled down by soaking in an ice-water bath for 5 min. The solid and liquid were separated via vacuum filtration. To the filtrate was added three volumes of 95% ethanol absolute (99.8%, Carlo Erba, France) in order to precipitated high-molecular weight hemicellulose. The liquid phase was separated from

the precipitated hemicellulose by centrifugation (7885 x *g*, 10 min, 4 ºC). Distilled water was added to the precipitated hemicellulose in a proportion of 1:2 (precipitated hemicellulose: water) and the hemicellulose extracted (HE) was lyophilized and kept in a dry place until further use (Cerqueira et al., 2009). Hemicellulose extraction yield (% HEY) was calculated according to the following equation:

$$\%\ \mathrm{HEY} = \frac{\mathrm{WE}}{\mathrm{WS}} \tag{5.1}$$

where, WE is the dry mass weight obtained after ethanol precipitation, WS is the wheat straw dry mass weight used in each experiment.

Table 5.1. Experimental conditions used for hemicellulose extraction yield according to experimental design. Real and (normalized) values of the operational variable temperature (X_1), times (X_2), and results obtained for the hemicellulose extraction yield (% HEY) response.

Assay	Variables				Responses	
	X_1		X_2		Y^a (% HEY)	Y^b (% HEY)
1	160	(-1)	10	(-1)	5.7	3.05
2	160	(-1)	50	(1)	10.3	8.80
3	160	(-1)	30	(0)	7.8	11.94
4	200	(1)	10	(-1)	8.3	8.82
5	200	(1)	50	(1)	2.99	4.66
6	200	(1)	30	(0)	14.96	12.65
7	180	(0)	10	(-1)	11.7	13.82
8	180	(0)	50	(1)	14.8	14.61
9	180	(0)	30	(0)	21.55	20.22
10	180	(0)	30	(0)	19.69	20.22
11	180	(0)	30	(0)	23.47	20.22
12	180	(0)	30	(0)	18.14	20.22

[a] Experimental value; [b] Model predicted value

5.2.3 EXPERIMENTAL DESIGN AND OPTIMIZATION OF HEMICELLULOSE EXTRACTION

For optimization of the hemicellulose extraction yield (% HEY) a response surface methodology (RSM) was applied. The independent variables temperature (X_1, ºC), time (X_2, min) at three variation levels on the extraction of hemicellulose is shown in Table 5.1. The total number of observations required for two independent variables (*N*) was calculated using the following equation:

$$N = 2^K + 2 \text{ x } K + 1 \tag{5.2}$$

where *K* is the number of independent variables. *N* is found to be 8 with four replicates at the centre point leading to a total number of 12 experiments for the evaluation of extraction process. Four assays at the centre point of the design were carried out to estimate the random error needed for the analysis of variance. The values of the independent variables were normalized from -1 to +1 using Eq. (5.3) to provide the comparison of the coefficients and visualisation of the individual independent variables on the response variable.

$$X_n = 2\frac{X - \bar{X}}{X_{max} - X_{min}} \tag{5.3}$$

where X is the absolute value of the independent variable concerned $\bar{X}$ is the average value of the variable and X_{max} and X_{min} are its maximum and minimum value, respectively. The second-order polynomial was calculated with MATLAB® Version 7.6.0, R2008a software (MathWorks, Inc., Natick, Massachusetts, USA) to estimate the response of the dependent variables. The mathematical model corresponding to the experimental design is:

$$Y_i = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=2}^{2} \sum_{j=i+1}^{3} \beta_{ij}\, x_i x_j \tag{5.4}$$

where *Y*i is the predicted value, x_i and x_j are the normalized values of the factors (time, temperature), β_0 is a constant coefficient, β_i are the linear coefficients, β_{ij} (i and j) are the interaction coefficients and β_{ii} are the quadratic coefficients. The quality of the fit of the polynomial model equation was evaluated by the coefficient of determination R^2 and the statistical significance was evaluated by the Fisher's *F*-test for analysis of variance (ANOVA) with a 95 % confidence level. The effect of each independent variable and also their interaction effects were determined. The experimental design package STATISTICA™ v 7.0 (Statsoft ®, Tulsa, OK, USA) was the software used for data analysis.

5.2.4 CHARACTERIZATION OF AUTOHYDROLYSIS HEMICELLULOSE EXTRACTED

5.2.4.1 Total sugar analysis of autohydrolysis liquid fraction

After the autohydrolysis extraction, the total sugar content in the liquid fraction was determined by the Dreywood anthrone method (using glucose as standard) and prepared as described by Rodriguez-Jasso et al. (2011) by dissolving 0.05 g of anthrone (VWR Prolabo) in 25 mL of concentrated H_2SO_4. The reagent was allowed to stand for 30-40 min with occasional shaking until it was perfectly clear. The reagent was freshly prepared and used within 12 hr. Water content was eliminated of the standard sugar by drying at 35 ºC for 6-8 h. Sample solution (1 mL) was layered into walled Pyrex test tubes and mixed with anthrone solution and cooled for 5 min. The tubes were fitted with a cotton wool stopper, heated as required in a vigorously at 80 ºC for 15 min and then cooled with ice water for 5 min. The measurements of test solutions and of reagent blanks were made spectrophotometrically at 630 nm against distilled water as a reference. Sugar concentration of samples was calculated using the calibration curves.

5.2.4.2 Acid hydrolysis of extracted hemicellulose for sugar composition determination

In order to determine the main sugar components, the HE was hydrolyzed according to Akpinar et al. (2009) with a slight modification. In order to compare the hydrolysis profiles of HE, a commercial birchwood xylan was used as reference material. HE (20 mg) was suspended in 5 mL of 0.25 M H_2SO_4, and this suspension was incubated in a boiling water bath for 3 h. The resulting supernatant was filtered through a 0.2 μm sterile membrane filter. The reaction products xylose, glucose, arabinose and acetic acid were quantified by HPLC in a Jasco 880-PU intelligent pump (Tokyo, Japan) chromatograph equipped with a refractive index detector and a Metacarb 87 H (300 x 7.8 mm, Varian, USA) column. Chromatographic separation was performed under the following conditions: mobile phase 0.005 M H_2SO_4, flow rate 0.7 mL/min, and column temperature 60 ºC. The volume injected was 20 μl per sample.

5.2.4.3 Fourier-transform infrared spectroscopy

Fourier-transform Infrared (FTIR) spectra of HE and xylan birchwood were obtained on an Perkin-Elmer 16 PC spectrometer (Perkin-Elmer, Boston, USA) using 16 scans and frequency range of 400–4000 cm^{-1}. Signal averages were obtained at a resolution of 4 cm^{-1}. For FTIR measurement, the polysaccharide was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into 1 mm pellets. The FTIR spectra of the films were recorded using Attenuated Total Reflectance mode (ATR). The vibration transition frequencies of each spectrum were baseline corrected and the absorbance was normalized between 0 and 1.

5.2.4.4 Preparation of edible films

Edible films were prepared using the method described by Martins et al. (2011a). Briefly, the polysaccharides κ-car and LBG mixture was prepared by mixing the solutions at 25 ºC prior to heating at 70 ºC under stirring during 30 min. Biocomposite was added to the solutions in concentrations of 0.2 and 0.4 % (w/w) when the two solution were mixed. Film-forming solutions were then degassed under vacuum to

remove air bubbles and dissolved air as much as possible. Then, 28 mL of the film-forming solution was cast into polystyrene Petri dishes, and dried at 35 ºC during 16 h. Films were conditioned at 54 ± 1 % relative humidity (RH) and 20 ± 1 ºC by placing them in a desiccator containing a saturated solution of $Mg(NO_3)_2.6H_2O$.

5.2.5 FILM CHARACTERIZATION

5.2.5.1 Fourier-transform infrared spectroscopy of the films

The FTIR spectra characterization of edible films was analyzed as described above (see the point 5.2.4.3).

5.2.5.2 Thickness

A digital micrometer (No. 293-5, Mitutoyo, Japan) was used to measure the film thickness. Five thickness measurements were randomly taken on each film sample and the average was used to calculate water vapour permeability and tensile strength.

5.2.5.3 Water vapor permeability (WVP)

WVP was determined gravimetrically using the ASTM E96-92 method described by Casariego et al. (2009). Three samples were cut from each film. Each sample was sealed on a permeation cell (cup containing distilled water at 100 % RH; 2.337 x 10^3 Pa vapor pressure at 20 ºC), and placed in a desiccator containing silica gel (0 % RH; 20 ºC). The water transferred through the test films was determined from cup weight loss over time. The cups were weighed with a 0.1 mg precision at 2 h intervals. The steady state of weight loss was reached after 10 h. The experiments were performed in triplicate for each film formulation.

5.2.5.4 Moisture content

The moisture content (MC) was expressed as the percentage of water removed from the initial mass sample. MC was determined gravimetrically by drying film samples (of about 20 mg) at 105 ºC in an oven with forced air circulation for 24 h. The experiments were performed on each film sample in triplicate.

5.2.5.5 Tensile strength and elongation-at-break

Tensile strength (TS) and elongation-at-break (EB) were determined with an Instron Universal Testing Machine (Model 4500, Instron Corporation) using ASTM Standard Method D 882-91. Film specimens (45 × 20 mm strips) were cut from each preconditioned (54 % RH) film and placed between the tensile grips. The initial grip separation and crosshead speed were set to 30 mm and 5 mm min^{-1}, respectively. Five samples for each type of films were replicated.

5.2.5.6 Opacity

The opacity of the samples was determined according to the Hunterlab method, as the relationship between the opacity of each sample on a black standard and the opacity of each sample on a white standard. For each type of film, optical measurements were performed six times.

5.2.5.7 Statistical Analysis

The statistical analysis of the physical and mechanics properties on the film was carried out with the single-factor analysis of variance (ANOVA), the multiple comparison tests was used to determine the statistical significance with a 95 % confidence level. For the data analyses, MATLAB software was used.

5.3 RESULTS & DISCUSSION

5.3.1 EFFECT OF AUTOHYDROLYSIS ON HEMICELLULOSE EXTRACTION

Table 5.1 shows the values of hemicellulose extraction yield. The degree of solubilization of hemicellulose increased for higher heating temperatures and ranged between 18.14 and 21.55 % at 180 ºC for 30 min, decreasing to 2.99 % for a heating temperature of 200 ºC for 50 min, probably due to the degradation of hemicellulose into furfural and other derivatives (Ruiz et al., 2011a). Yoshida et al. (2010) reported a similar effect on the extraction of carbohydrates from corn starch using microwave-assisted extraction. Recently, Ruiz et al. (2011a) reported the effect of temperature, time and particle size on the composition of monosaccharides (xylose, glucose and arabinose) and degradation production (hydroxymethylfurfural (HMF) and furfural from wheat straw hemicellulose, showing that glucose and xylose were degraded into HMF and furfural for higher severity treatment (200 ºC for 60 min). Overall, the behaviour of xylose solubilization and its degradation production strongly corresponds with hemicellulose extraction yield showed in this study.

On the other hand, it is known that when the hemicellulose is subjected to autohydrolysis under specific operational conditions, xylooligosaccharides (XOS) are the major products. According to Boussarsar et al. (2009), the soluble fraction shows the presence of xylan oligomers and polymers with large distribution of degree of polymerization (DP). In a previous work (Ruiz et al., 2011b), it was reported that XOS represented 78 % of the sum of sugars solubilized in the autohydrolysis liquid phase generated by the hemicellulose fraction hydrolysis. Makishima et al. (2009) used a continuous flow type autohydrolysis reactor for hemicellulose fraction from corncob and reported that during the purification of solubilized fraction, higher-XOS was recovered as the precipitate, presenting a DP ranging from 11 to 21. Garrote et al. (2002), proposed a reaction model for xylan formation under autohydrolysis conditions, which the ratio of the higher-XOS decreased with reaction time and the concentration of monomer sugars such as xylose increased.

5.3.2 STATISTICAL ANALYSIS AND OPTIMIZATION OF HEMICELLULOSE EXTRACTION

Table 5.1 shows the process variables, experimental data and the values predicted by the model. The obtained second-order polynomial model in terms of normalized values are expressed in Eq. (5.5), that represent the hemicellulose extraction yield, $Y_{(\%\ HEY)}$, as function of temperature (x_1) and time (x_2), respectively.

$$Y_{(\%\ HEY)} = 20.22 + 0.4x_1 - 7.88{x^2}_2 + 0.39x_2 - 6.01x_2^2 - 2.47x_1x_2 \quad (5.5)$$

The ANOVA results listed in Table 5.2 revealed that second-order polynomial model was found to be adequate for prediction of hemicellulose extraction yield response within the range of experimental variables.

Table 5.2. Analysis of variance (ANOVA) for hemicellulose extraction yield model as a function of temperature (x_1) and time (x_2).

Source	Sum of Squares	d.f	Mean Square	F-value	p-value
Model	415.87	5	83.17	8.95	0.0094 *
x_1	1.00	1	1.00	0.10	0.7539
x_2	0.95	1	0.95	0.10	0.7597
x_1x_2	24.55	1	24.55	2.64	0.1501
${x_1}^2$	165.58	1	156.58	17.82	0.0055 *
${x_2}^2$	96.32	1	96.32	10.36	0.0181 *
Error	55.75	6	9.29		
Total	417.61	11			

d.f., degree of freedom, * significant.

The determination coefficient of the second-order polynomial model was R^2 = 0.8817, indicating that 11.83 % of the total variations were not explained by the model. As shown in Table 5.2, the model F-value of 8.95 is high compared to the tabular $F_{5,6}$ value

of 4.39 indicating that the model was significant. The statistical analysis revealed that the autohydrolysis variables had a significant influence on the hemicellulose extraction yield.

As can be deduced from Table 5.1, the Pareto chart shows the effect and significance of square of the variables, x^2_1, x^2_2, on hemicellulose extraction yield with *p*-value under a significance level of $\alpha = 0.05$. The others terms were not significant ($p > 0.05$). The negative sign shows that the hemicellulose extraction yield decreases as these variables increase. Zou et al. (2011) reported a similar effect of time on the extraction of polysaccharides using an ultrasonic process.

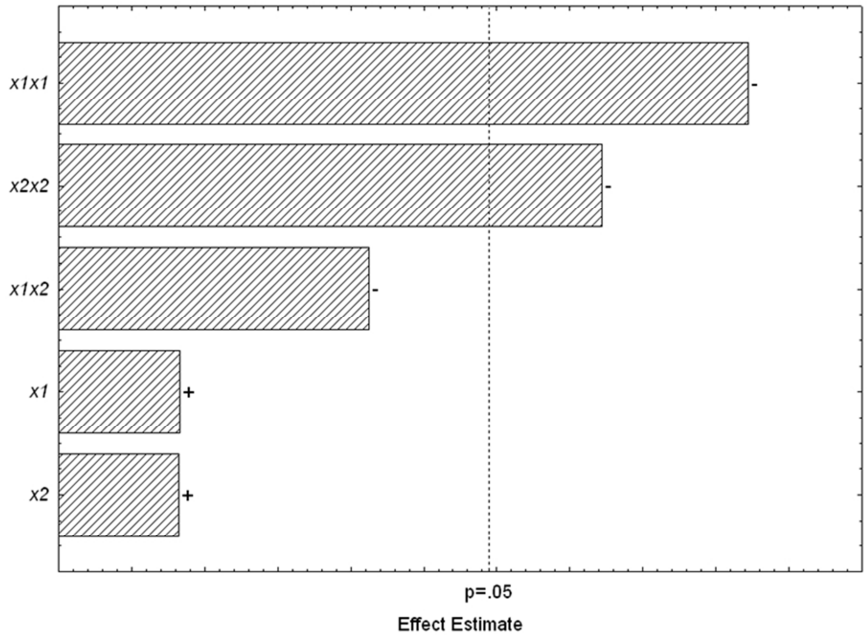

Figure 5.1. Pareto chart for standardized effects on hemicellulose extraction yield.

Three dimensional response surface of the second-order polynomial Eq. (5.5) was plotted in order to obtain the optimum point in the hemicellulose extraction yield (Table 5.2). Hemicellulose extraction yield was estimated to be 20.22 % at the optimum point (temperature; 180.42 ºC, time; 30.56 min). This optimum point was confirmed using the Hessian matrix analytical method, evaluating the values of the second order partial derivatives from second-order polynomial. The HE obtained in the optimum point was used as material in the reinforcement of κ-car and LBG matrix blend.

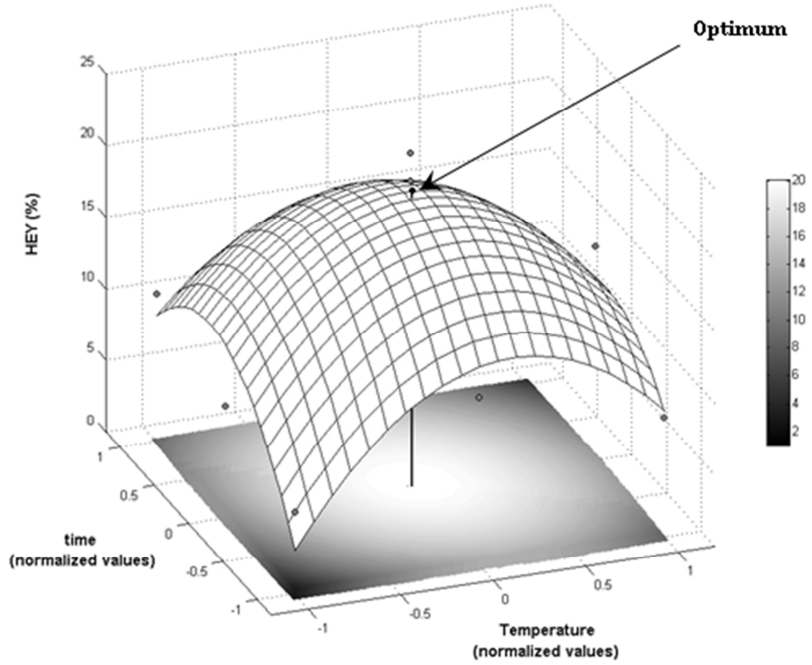

Figure 5.2. Response surface and contour plot showing the effects of temperature (x_1) and time (x_2) on the hemicellulose extraction yield. (ǀ) optimum point; (●) experimental conditions.

5.3.3 EXTRACTED HEMICELLULOSE CHARACTERIZATION

5.3.3.1 Total sugar recovery in the liquid fraction

Figure 5.3 shows total sugar content in the liquid fraction obtained from each extraction condition. The total sugars in the soluble fraction are derived mainly from hemicellulose extracted from WS raw material. The treatment at 160 ºC showed a minimal extraction of total sugar and slow solubilization in function of treatment time. Moreover, the results showed that the highest total sugar is found when the WS is treated at 180 ºC for 30 min, whereas the total sugar content in the liquid fraction decreases at 200 ºC for 50 min. The decreasing trend of total sugar can be ascribed to a balance between the depolymerization of the oligomers sugars and the secondary degradation of the product monomers and by Maillard reaction in the autohydrolysis treatment (Ruiz et al., 2011a). These results are in good agreement with Sato et al. (2010), who reported that total sugar increased with both temperature and residence time.

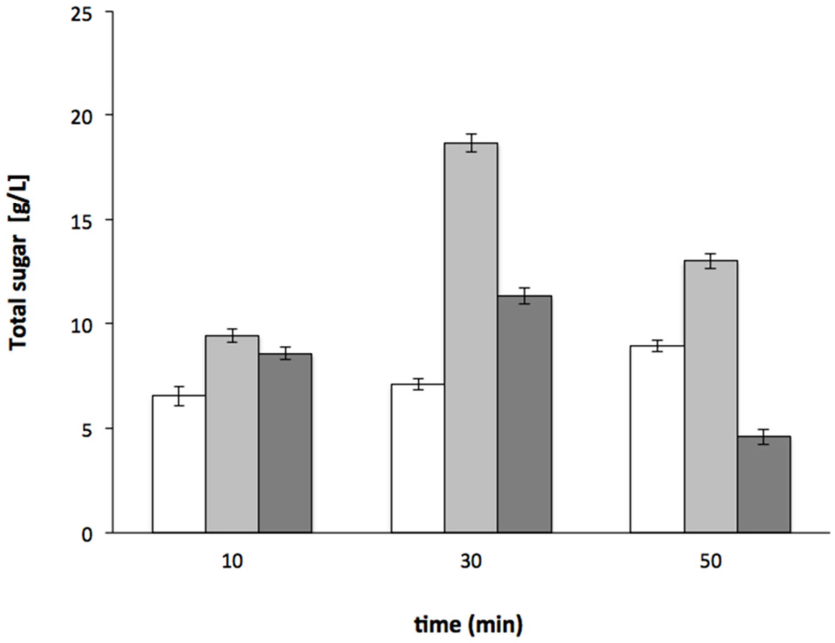

Figure 5.3. Total sugar content in the liquid fraction: (□) 160 ºC; (□) 180 ºC; (■) 200 ºC.

5.3.3.2 Acid hydrolysis of extracted hemicellulose

Figure 5.4 shows the chromatogram profiles of HE and birchwood xylan after acid hydrolysis, respectively. Chromatogram profiles revealed the existence of xylose as the major sugar constituent in the HE using autohydrolysis treatment at 180 ºC for 30 min and indicating the presence of a polysaccharide of xylan. Arabinose, glucose and acetic acid were present in small amounts for HE. Birchwood xylan, which contains > 90 % of sugars in the polysaccharides form of xylose, was confirmed with the chromatogram profile. HE and commercial birchwood xylan, were found to be slightly similar, both composed predominantly by xylose.

5.3.3.3 Fourier-transform infrared spectroscopy

Infrared spectroscopy is an effective way to identify the presence of certain functional groups in a molecule. Also, one can use the unique collection of absorption bands to confirm the identity of a pure compound or to detect the presence of specific impurities.

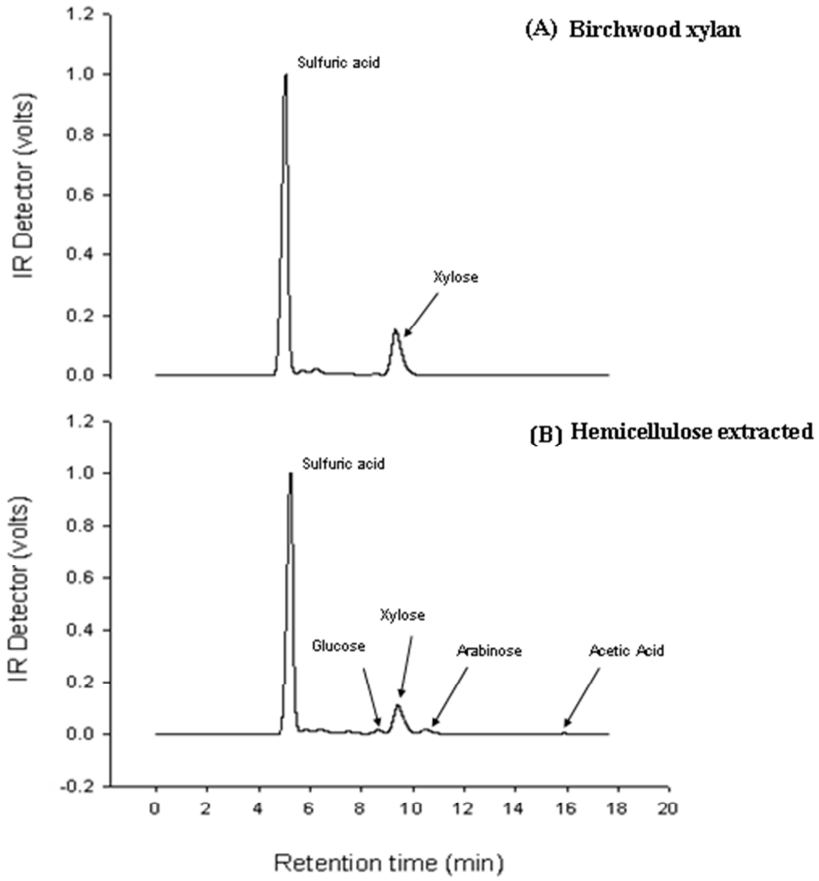

Figure 5.4. Chromatographic profiles: (A) birchwood xylan and (B) extracted hemicellulose, after acid hydrolysis.

The HE infrared spectrums and birchwood xylan (Figure 5.5) showed the typical signal pattern expected for hemicellulose fractions. Broad absorption band at 3405 cm^{-1} is attributed to hydroxyl groups that normally occur as a result of the association between the polymers being its intensity influenced by the analyzed sample concentration (Sun et al., 2005). In addition, a band at 2920 cm^{-1} was detected and is indicative of C-H stretching vibrations due to CH_2 and CH_3 groups; also, the signal at 1375 cm^{-1} is due to the C-H bending vibration present in cellulose and hemicelluloses chemical structures (Peng et al., 2009). The sharp absorption band near to 1613 cm^{-1} is principally attributed to the absorbed water in xylan-type polysaccharides (Sun et al., 2000; Oliveira et al., 2010). The prominent band at 1043 cm^{-1} is the wavenumber characteristic for typical xylans, assigned to the C-O and C-C stretching and the glycosidic linkage contributions.

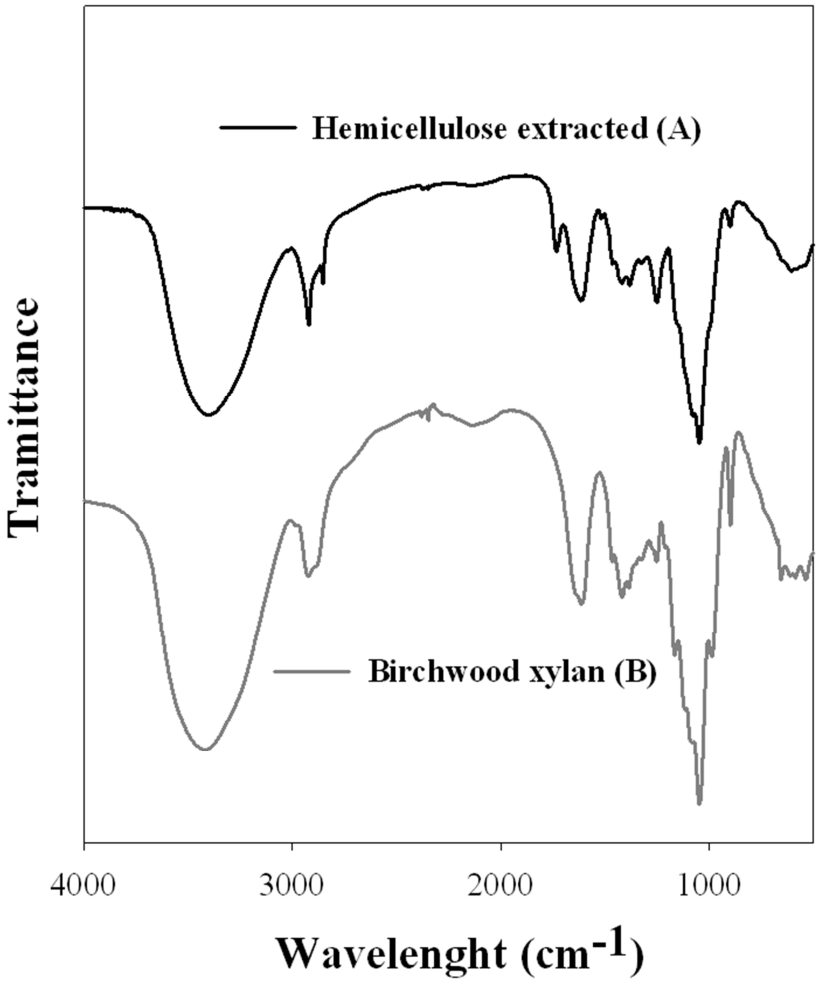

Figure 5.5. FTIR spectra of extracted hemicellulose obtained by autohydrolysis at 180 ºC for 30 min (A) and birchwood xylan (B).

Moreover a sharp band at 890 cm^{-1}, corresponding to the C_1 group frequency or ring frequency, is attributed to β-glycosidic linkages (1→4) between xylose units in hemicelluloses (Sun et al., 2000; Sun et al., 2005). Low-intensity shoulders at 1161 and 983 cm^{-1} were only detected at birchwood fraction showing the association with arabinosyl side chains (Sun et al., 2005). On the contrary, extracted hemicellulose showed a signal at 1733 cm^{-1} that implies that the hemicellulosic fraction, solubilized during the water treatment, contains small amounts of the acetyl, uronic, and ester groups or the ester binds of the carboxylic groups of ferulic and/or *p*-coumaric acids (Peng et al., 2009). Furthermore, the weaker absorbances at 1507 and 831 cm^{-1} observed

in this fraction are originated from aromatic skeletal vibrations in associated lignin, indicating that HE was slightly contaminated with minimal amounts of bound lignin (Sun et al., 2005).

5.3.4 FILMS CHARACTERIZATION

5.3.4.1 Fourier-transform infrared spectroscopy of the films

FTIR spectroscopy has been used to characterize different polysaccharides and their structures (e.g. edible films) (Turquois et al., 1996). When chemical groups interact at the molecular level, changes in FTIR spectra such as the shifting of absorption bands could happen. These changes can be an indication of good miscibility of polymers, and can be useful to evaluate the interaction between the biocomposite and κ-car/LBG films (Xu et al., 2007).

Figure 5.6 shows the FTIR spectra of edible films with increasing biocomposite concentrations. The broad band ranging between 3500 and 3100 cm^{-1} was attributed to O-H stretching vibration formed by the hydroxyl group being the broad band around 2800-3000 cm^{-1} attributed to C-H stretching vibration (Cerqueira et al., 2011). FTIR spectra of the samples also shows a band in the region of 750-1300 cm^{-1} that is characteristic of the carbohydrate region. These wavenumbers are within the so-called fingerprint region, where the bands are specific for each polysaccharide, allowing its possible detection (Şen and Erboz, 2010). Martins et al. (2011b) showed that the mixture of the two polysaccharides lead to the presence of the peaks characteristic of the κ-carrageenan (at 1220 cm^{-1} corresponding to the ester sulfate groups, a peak at 922 cm^{-1} attributed to the 3,6-anhydrogalactose group, a peak at 846 cm^{-1} corresponding to galactose-4-sulfate and a peak at 805 cm^{-1} corresponding to 3,6-anydro-D-galactose-2-sulphate) and from the Locust Bean Gum (LBG) (that shows absorption bands at 817 cm^{-1} and 873 cm^{-1} indicating the presence of α-linked D-galactopyranose units and β-linked D-mannopyranose units). It has been shown that the interaction between the two polysaccharides lead to changes in the peak position of their main groups (Martins et al., 2011b).

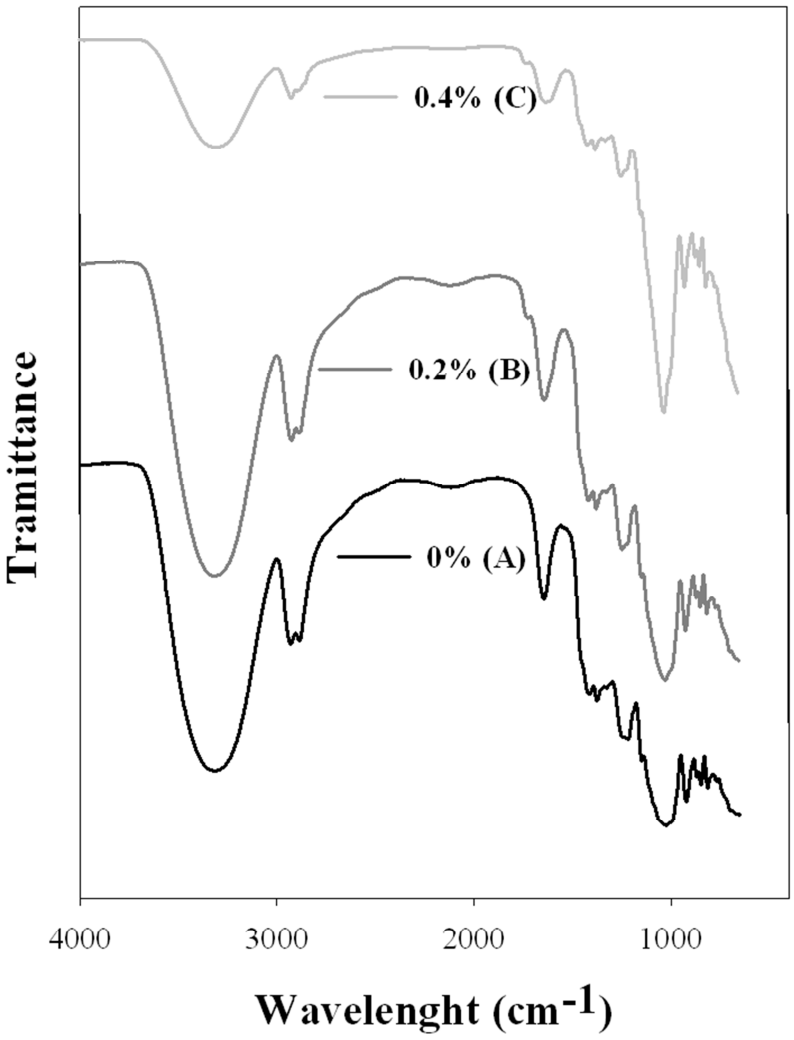

Figure 5.6. FTIR spectra of edible films for increasing biocomposite concentrations: (A) 0 %; (B) 0.2 % and (C) 0.4 %.

In the spectra of the films is possible to observe shifts in characteristic peaks when the biocomposite is added. Such as those corresponding to C-H stretching vibrations that shift from 2934 to 2930 and 2923 cm^{-1} and from 2891 to 2896 and 2902 cm^{-1} for the films with 0.0, 0.2 and 0.4 % of biocomposite, respectively. The presence of a new peak is also clear in the films when the biocomposite is added. The peak at 1733 cm^{-1} corresponds to the presence of the hemicellulosic fraction in the biocomposite samples, as explained before. The shifts of the peaks at 1184 to 1190 and 1195 cm^{-1} and the shift from 973 to 952 and 953 cm^{-1} when 0.2 and 0.4 % of biocomposite is added to the

films, respectively, indicates a change in the polysaccharides fingerprint region, that can be related with the composition of the biocomposite.

The modifications in the stretching frequencies of some groups that are involved in interactions can be a indication of the interaction between the films constituents and the added biocomposite, being the change (shift) of peak position dependent of the strength of the interaction (Wanchoo and Sharma, 2003). These interactions may lead to changes in films properties (e.g. transport and mechanical properties).

5.3.4.2 Water vapour permeability

The utilization of edible films as packaging presents has as one of the major purposes to block the moisture transfer between the food and the surrounding atmosphere. The water vapour permeability (WVP) depends on many factors, such as the integrity of the film, the hydrophilic–hydrophobic ratio, the ratio between crystalline and amorphous zones, the polymeric chain mobility and the tortuosity of the film matrix (Miller and Krochta, 1997; Souza et al., 2010).

Table 5.3 reports the WVP values of κ-car/LBG films with different biocomposite concentrations. The incorporation of 0.4 % of biocomposite leads to lower WVP values, being statistically different ($p < 0.05$) from the films without and with 0.2 % of biocomposite. The presence of biocomposite in the film structure decrease the water permeability of the films, that can be related with the increase of the matrix crystallinity but also with the tortuosity of the films. The biocomposite dispersion in the film increased the tortuosity that leads to a lower diffusion of water molecules through the matrix. This possibility was confirmed by the moisture content of the films (Table 5.3), where no statistically significant differences were observed for different biocomposite concentrations. Being so, the biocomposite do not change the hydrophilicity of the film, but in some way affect the tortuosity of the matrix, changing the available pathways for water molecules diffuse through the matrix. Similar results were obtained by Martins et al. (2011) with the incorporation of Cloisite 30 B content in the same film composition. The presence of 1 % of Cloisite 30B (the closer value for the used in the present work) in κ-car/LBG films leads to a decrease of 6 % of the WVP values, in the same range of

the value obtained with the addition of 0.4 % of biocomposite (1.6 %). The obtained values are in agreement with reported results for edible films of galactomannan, and where biocomposites and other biopolymers were added (Casariego et al., 2009; Martins et al., 2011b).

Table 5.3. Water vapour permeability (WVP), moisture content (MC), opacity, tensile strength (TS) and elongation-at-break (EB) for the edible films with different biocomposite concentrations.

Biocomposite concentration	WVP (10^{11}g (m s Pa)$^{-1}$)	MC (%)	Opacity (%)	TS (MPa)	EB (%)
0 %	6.29 ± 0.02[a]	24.08 ± 1.96[a]	5.36 ± 0.07[a]	16.49 ± 0.30[a]	24.29 ± 1.13[a]
0.2 %	7.01 ± 0.26[a]	21.71 ± 1.81[a]	9.77 ± 0.14[b]	17.54 ± 0.80[a]	24.15 ± 0.90[a]
0.4 %	6.19 ± 0.12[b]	21.21 ± 0.58[a]	13.48 ± 0.35[c]	19.71 ± 0.16[b]	24.95 ± 0.33[a]

5.3.4.3 Moisture content

The moisture content of the films gives information about how the biocomposite incorporation in κ-car/LBG-based films can affect the water sensitivity of edible films. Table 5.3 shows the water content of κ-car/LBG films with different biocomposite concentrations. Results show that the addition of biocomposite do not lead to a significant statistically decrease of water content ($p > 0.05$), allowing to conclude that the biocomposite addition does not have significantly influence the hydrophobicity of the films. These obtained values of MC are in the range of those obtained for other polysaccharide-based films (Garcia et al., 2006).

5.3.4.4 Mechanical properties – Tensile strength (TS) and elongation-at-break (EB)

The mechanical strength and extensibility (given by TS and EB, respectively) are required for a film to resist external stress and maintain its integrity when applied as packaging for food products. The effect of the incorporation of biocomposite in κ-car/LBG-based films on the mechanical properties of the films is shown in Table 5.3. The incorporation of biocomposite in κ-car/LBG-based films leads to higher TS values

($p < 0.05$). However, no statistically significant differences ($p > 0.05$) are observed when the EB values are compared. The biocomposite incorporation changes the TS of the edible films, indicating a network modification. FTIR analyses showed that the biocomposite presence lead to some modifications in the chemical structure of the films, that can be related with the improved TS. Similar results are shown for wheat gluten based films, where the presence of a high ratio of xylan lead to the increase of TS values (Kayserilioğlu et al., 2003). An increase of TS has been also reported when microcrystalline cellulose was added to hydroxypropylmethylcellulose-based films (Dogan et al., 2007). The obtained values are in agreement with reported results for other polyssacharide-based films (Martins et al., 2010; Mikkonen et al., 2007).

5.3.4.5 Opacity

Opacity is a valuable property in films once they can interfere with consumers' choice, but also with the capacity of the films to be a barrier to light. Table 5.3 shows the values of the opacity for the films, that in general present low values of opacity (higher transparency). The transparency can be reported as a way to relate the good or lower miscibility of the biopolymers (Li et al., 2006). The presence and the increase of biocomposite concentrations in the films lead to a increase of the opacity of the films. This increase can be related with the biocomposite structure and the modification of the film strucutre after the incorporation in the films.

5.4 CONCLUSIONS

The role of biorefineries in the production of chemicals (as biocomposite) and energy from lignocellulosic materials has received special attention with the fractionation of these raw materials. In this work, autohydrolysis under selected conditions caused the selective extraction of hemicellulose. Moreover, the characteristics of the hemicellulose extracted indicate that it is a good candidate for application in the upgrading of biocomposites. Results showed that the presence of the biocomposite in κ-car/LBG-based films could be used to tailor its physical properties, being one of the benefits of

the biocomposite incorporation the increase of the barrier to water vapour, TS and opacity of κ-car/LBG-based films.

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CHAPTER 6

KINETIC MODELING OF ENZYMATIC SACCHARIFICATION USING WHEAT STRAW PRETREATED UNDER AUTOHYDROLYSIS AND ORGANOSOLV PROCESS

ABSTRACT

The enzymatic saccharification kinetics of untreated wheat straw, pretreated solids obtained by a sequence of autohydrolysis (solubilization of hemicellulose) and organosolv (solubilization of lignin) were studied together with two pure cellulose model substrates, filter paper and Avicel. Two kinetic models for glucose production were compared and its kinetic constants calculated. According to the obtained results, enzymatic saccharification of the autohydrolysis pretreated solids (APS) proved to be more effective than when the organosolv pretreated solids (OPS) were used. The maximum extent of the enzymatic conversion of cellulose to glucose was 90.88 % and 64.04 %, for APS and OPS respectively, at 96 h. This result was probably due to an increase in accessible area for APS and a possible inhibition by phenolic acids deposited on the surface of OPS, acting as a barrier for enzymatic saccharification. Initial saccharification rate for APS and OPS was 0.47 g/(L•h) and 0.34 g/(L•h), respectively. Models based on first and second order cellulase deactivation kinetics satisfactory predicted the behavior of glucose production, however the second order model had a higher accuracy than the first order one. Visualization of structural modification induced by enzymatic saccharification at 12 h for the pretreated solids was done using scanning electron microscopy.

6.1 INTRODUCTION

Agricultural residues represent a major source of lignocellulosic material (LCM) with considerable potential for use in biomass conversion to ethanol. Wheat straw is one of the most abundant agricultural by-products in the world, has a low commercial value and a large part is applied as cattle feed and the rest as waste. In terms of total production, wheat is the second most important grain crop in the world. According to Food and Agriculture Organization of the United Nations (FAO, 2011) statistics reported a world annual wheat production in 2009 of 682 million tons and in average the production of 1.3 kg of the harvested grain is accompanied by the production of 1 kg of straw; this gives an estimation of about 524 million tons of wheat straw in 2009; these amounts are significant enough to consider wheat straw as a complementary source of raw material in the production of bioethanol (Petersen, 1988).

One promising technology is to convert this abundant and renewable LCM to monomer sugars using enzymes that are to be applied after a pretreatment process and the microbes convert the sugars to ethanol. Enzymatic saccharification of cellulose is generally described as a heterogeneous reaction system in which cellulases in an aqueous environment react with the insoluble and structured cellulose. According to the traditional enzyme classification system the cellulolytic enzymes are divided into three classes; 1) exo-1,4-β-D-glucanases or cellobiohydrolases (EC 3.2.1.91), which move processively along the cellulose chain and cleave off cellobiose units from the ends; 2) endo1,4-β-D-glucanases (EC 3.2.1.4), which hydrolyse internal β-1,4-glucosidic bonds randomly in the cellulose chain; and 3) 1,4-β-D-glucosidases (EC 3.2.1.21), which hydrolyse cellobiose to glucose and cleave glucose units from cellooligosacharides.

All these enzymes work synergistically to hydrolyse cellulose by creating new accessible sites for each other, removing obstacles and relieving product inhibition (Eriksson et al., 2002; Valjamae et al., 2003; Bansal et al., 2009). Consequently, these reactions can be affected by different obstacles as the use of high substrate concentrations, the presence of lignin, which shields the cellulose chains and adsorbed enzymes, crystallinity of cellulose, surface area, pore size, degree of polymerization

(DP) and hemicellulose content (Chang and Holtzapple, 2000; Arantes and Saddler, 2010).

In a recent work, Rollin et al. (2011) showed that improving the surface area accessible to cellulase is a more important factor than the removal of lignin for achieving a high sugar yield. However, due to the robust structure of LCM a pretreatment is required to alter the structural and chemical composition, improving the accessibility of the cellulose component to the action of hydrolytic enzymes so that an efficient hydrolysis of carbohydrates to fermentable sugars occurs (Zeng et al., 2007; Kim et al., 2008; Cybulska et al., 2010).

Autohydrolysis or hydrothermal processing is an environmentally friendly process in which LCM is pretreated with just compressed hot water; it is based on the selective depolymerization of hemicellulose, which is catalyzed by hydronium ions generated in situ by water autoionization and by acetic acid from acetyl groups. Moreover, autohydrolysis pretreatment caused re-localization of lignin on the surface of LCM (Kristensen et al., 2008). This process avoids difficult steps in chemicals handling and recovery (e.g., sulfuric or hydrochloric acid) compared with dilute sulfuric acid or bases pretreatment (Garrote et al., 2008; Gullón et al., 2009; Díaz et al., 2010; Ruiz et al., 2011a).

On the other hand, industrially, lignin is found during the process of papermaking from wood through conventional or sulfur-containing lignins, which include kraft lignin and lignosulfonates. However, the major disadvantages of the kraft process include the low pulp yield, the high consumption of bleaching chemicals to obtain bright pulp, and formation of odorous gases (Ruiz et al., 2011a). In a previous work Ruiz et al. (2011b), isolated lignin using the organosolv process, having as result a production of partially pure and high quality lignin. This pretreatment, in which delignification of LCM is performed using an organic solvent (frequently ethanol) plus water, is an environmentally friendly chemical process compared with the kraft and sulfite processes (Young and Akhtar, 1997; Shirkolaee et al., 2008).

For the all mentioned above, the objective of present chapter was to investigate the susceptibility to enzymatic saccharification of pretreated solids after autohydrolysis and

the sequential autohydrolysis-organosolv processes compared with untreated wheat straw and two model substrates (filter paper and Avicel) and to find an adequate model, using first and second order kinetics, to describe the enzymatic saccharification of the different substrates.

6.2 EXPERIMENTAL PROCEDURES

6.2.1 RAW MATERIAL

The wheat straw used in this study was kindly provided by a local farmer (Elvas, Portugal). Wheat straw was cut into small pieces (1-3 cm) and milled using a laboratory knife mill (Cutting Mill SM 2000, Retsch, Germany). The material composition was previously analyzed by Ruiz et al. (2011a) and their particle size distribution (w/w %) was as follows: 10 % > 1 mm; 40 % between 1-0.5 mm; 40 % between 0.5-0.3 mm; and 10 % to < 0.3 mm.

6.2.2 WHEAT STRAW PRETREATMENT BY AUTOHYDROLYSIS PROCESS

Milled wheat straw samples were mixed with water in order to obtain a 1:10 solid/liquid mass ratio and treated in a 3.75 l total volume stainless steel reactor (Parr Instruments Company, Moline, Illinois, USA) with PID temperature control. The moisture content of wheat straw was considered as water in the material balances. The reactor was filled and heated to 180 ºC at a heating rate of 3 ºC/min until reaching the desired temperature, the reaction time was 30 min, these conditions having been previously evaluated by Ruiz et al. (2011a). After completing the reaction time, the reactor was cooled down at a rate of about 3.2 ºC/min and the agitation speed was set at 135 rpm. At the end of the treatment, the liquid and solid phases were separated by centrifugation and the solid residues were washed several times with distilled water. The characterization and quantification of structural carbohydrates, sugars and degradation products in both solid and liquid phases has been previously reported by Ruiz et al.

(2011b). Autohydrolysis was performed in order to achieve fractionation, mainly related to the selective removal of hemicelluloses and also to help solubilize the extract and facilitate the access by delignification reagents. The autohydrolysis pretreated solids (APS) were used as substrate for enzymatic saccharification and for delignification by organosolv process (see Figure 6.1).

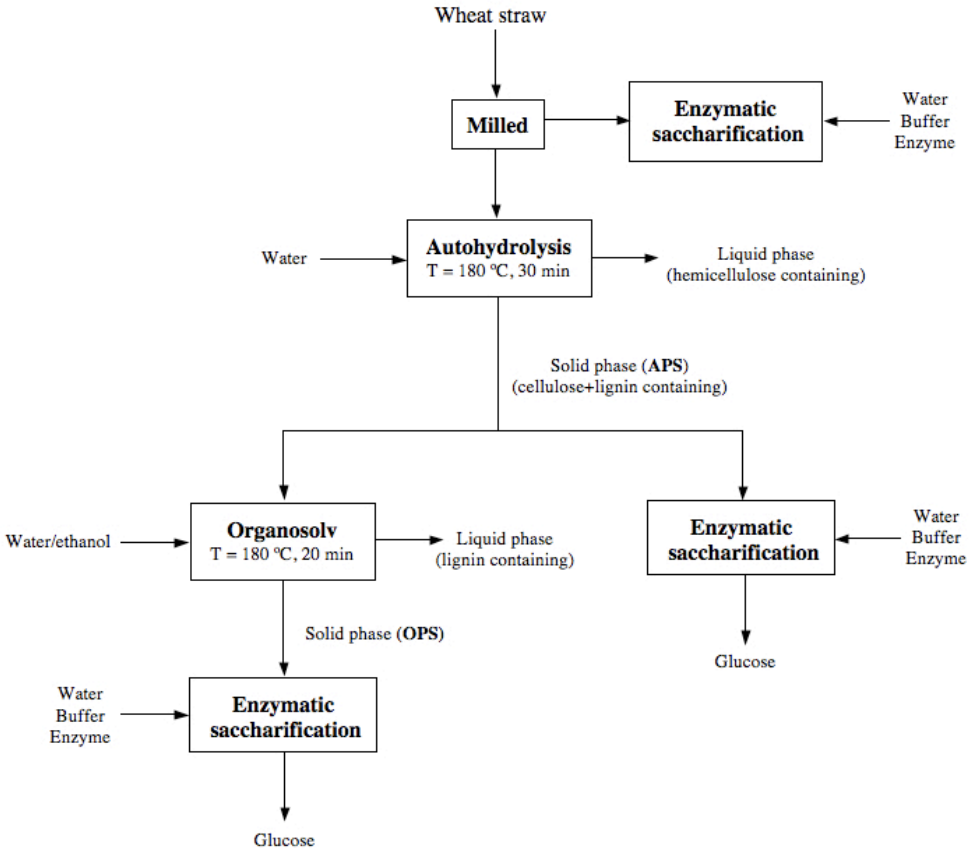


Figure 6.1. Schematic representation of the sequence autohydrolysis and organosolv process.

6.2.3 DELIGNIFICATION BY ORGANOSOLV PROCESS

The solid residue obtained after autohydrolysis pretreatment was delignified by the organosolv process using an aqueous solution of 40 % (v/v) ethanol and 0.1 % (w/v) NaOH. The used conditions, previously defined (Ruiz et al., 2011b) for an efficient lignin removal from wheat straw were solid/liquid ratio: 1:10 w/v, temperature: 180 ºC

and time: 20 min. These experiments were carried out in batch cylinder reactors fabricated from 316 stainless steel having a length of 12.5 cm, an outside diameter of 4.93 cm, wall thickness 0.91 cm and a nominal volume of 160 mL (Figure 6.2). The reactor was closed and mounted vertically and then submerged in an oil bath open heating circulator (JULABO Labortechnik GmbH, Seelbach, Germany) with temperature control, previously heated to the desired reaction temperature. At the end of the reaction, the reactor was removed from the oil bath and immediately cooled down by soaking in an ice-water bath for 5 min. The residual solid material was separated via vacuum filtration and washed with distilled water. Quantification of structural carbohydrates of solid residue was performed according to the procedure reported by Ruiz et al. (2011b). The organosolv pretreated solids (OPS) were used as substrate for enzymatic saccharification

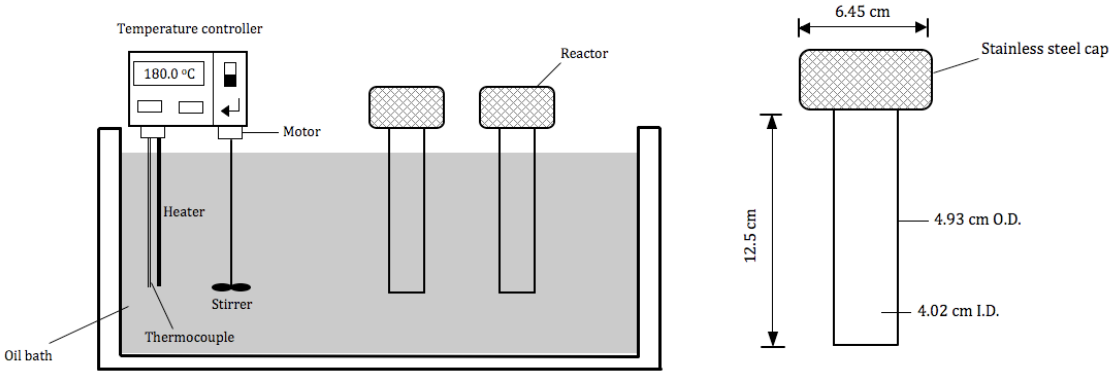


Figure 6.2. Schematic of batch system for organosolv pretreatment.

6.2.4 ENZYME

Commercially available enzyme solutions, cellulase from *Trichoderma reesei* (Celluclast 1.5L) and β-glucosidase from *Aspergillus niger* (Novozym 188), were kindly supplied by Novozymes A/S (Bagsvaerd, Denmark). The cellulase activity from Celluclast 1.5 L was analyzed in terms of FPU in accordance with the standard analytical methods established by the National Renewable Energy Laboratory (Adney and Baker, 1996). One unit of filter paper cellulase (FPU) was defined as the amount of enzyme which produces 2.0 mg of reducing sugar from 50 mg of filter paper within 1h. The experiment was carried out in a reaction mixture containing 0.5 mL of diluted

enzyme solution, 1.0 mL of 50 mM citrate buffer (pH 4.8), and 50 mg of a 1 x 6 cm strip of a Whatman No. 1 filter paper. The reaction solution was incubated at 50 ºC for 1h. Then the concentration of the released reducing sugar was measured using an adaption of the 3,5-dinitrosalicylic acid (DNS) method (Gonçalves et al., 2010). The β-glucosidase activity was determined for Novozym 188. The β-glucosidase activity was measured by incubating the enzyme solution with 4 mM *p*-nitrophenyl-β-D-glucopyranoside and 50 mM sodium citrate buffer (pH 4.8) at 30 ºC for 15 min. The reaction was stopped by addition of 60 µl of 1 M Na2CO3 and the amount of liberated p-nitrophenol measured spectrophotometrically at 400 nm. One unit of activity (IU) was defined as the release of 1 µmol of nitrophenol per minute. The enzyme activities of commercial concentrates were 43.05 FPU/mL for Celluclast 1.5 L and 576.39 UI/mL for Novozym 188.

6.2.5 ENZYMATIC SACCHARIFICATION

The APS, OPS and untreated wheat straw were used as a substrate (Figure 6.1) and two pure cellulose model substrates were also used for comparison (a microcrystalline cellulose Avicel PH-101 and Whatman No. 1 filter paper). Enzymatic saccharification were performed in a jacketed glass reactor with a working volume of 50 mL (total volume of 75 mL) at 50 ºC by duplicate, using cellulase (Celluclast 1.5 L) and β-glucosidase (Novozyme 188) with a loading of 40 FPU/g and 60 IU/g of cellulose, respectively, in 50 mM citrate buffer (pH 4.8) with 2 % (w/v) sodium azide to inhibit microbial contamination and a final cellulose concentration of 1 % (w/v) as described by Dowe and McMillan, (2001) and Selig et al. (2008).

The necessary amount of deionized water was calculated and added to make the total volume of 50 mL. Novozyme 188 was supplemented in order to eliminate the inhibition effect of cellobiose (Coward-kelly et al., 2003). Agitation was carried out using a magnetic stirrer (150 rpm) and samples were taken at 3 h intervals for the first 12 h and at 24 h intervals until a total time of 96 h. The samples were kept in boiling water for 5 min to inactive enzymatic activity, and then centrifuged at 8260 x g for 10 min to remove insoluble substrate; the supernatant was filtered through a 0.2 µm sterile membrane filter and analyzed for soluble sugars as described below.

The saccharification yield (%) was calculated according to Equation (6.1) (Dowe and McMillan, 2001).

$$Saccharification\ Yield\ (\%) = \frac{[Glucose] + 1.053[Cellobiose]}{1.111f[Biomass]} x100\% \quad (6.1)$$

where [*Glucose*] is glucose concentration (g/L), [*Cellobiose*] is cellobiose concentration (g/L), [*Biomass*] is dry biomass concentration at the beginning of the enzymatic saccharification (g/L), f is cellulose fraction in dry biomass (g/g), 1.111 is the factor that converts cellulose to equivalent glucose and the factor 1.053 converts cellobiose to equivalent glucose.

6.2.6 ANALYSIS OF SUGARS BY HPLC

Glucose and cellobiose were quantified by HPLC in a Jasco 880-PU intelligent pump (Tokyo, Japan) chromatograph equipped with a refractive index detector and a Metacarb 87 H (300 x 7.8 mm, Varian, USA) column. Chromatographic separation was performed under the following conditions: mobile phase 0.005 M H_2SO_4, flow rate 0.7 mL/min, and column temperature 60 ºC. The volume injected was 20 µl per sample. Sugars concentrations were determined using calibration curves obtained from standard solutions (Ruiz et al., 20011b).

6.2.7 MODELING THE KINETICS OF GLUCOSE PRODUCTION

The kinetic model used in this work was proposed by Shen and Agblevor (2008a,b) and modified by Zhang et al. (2010). The models are function of three-constants that directly express the relationship between glucose production and two hydrolytic conditions (time and initial enzyme concentration). The assumptions of the model are: 1) cellulase enzyme containing endo-β-1,4-glucanase, exo-β-1,4-cellobiohydrolase and glycosidase and assuming a single combined effect in the hydrolysis of insoluble substrate; 2) surface structure of insoluble substrate was considered homogeneous.

When cellulase deactivation is considered as a first order reaction, the model is as follows:

$$[P] = [S_0]x\left\{1 - \left[1 - \frac{1 - exp(-k_{de1}t)}{1 + {K_e}/{[E_0]}}\right]^{{k_2}/{k_{de1}}}\right\} \quad (6.2)$$

and when cellulase deactivation is considered as a second order reaction, the mathematical model for sugars production is:

$$[P] = [S_0]x\left\{1 - \left[1 + \frac{K_e[E_0]}{K_e + [E_0]}k_{de2}t\right]^{\frac{-k_2}{K_e k_{de2}}}\right\} \quad (6.3)$$

where [P] is the glucose concentration (g/L), t is the hydrolysis time, [S_0] and [E_0] represent initial substrate and enzyme (g/L), respectively, K_e is the equilibrium constant (g/L), k_2 is the constant of product formation (h^{-1}), k_{de1} and k_{de2} are the first order (h^{-1}) and second order (L/(h*g)) rate constants of cellulase deactivation. The glucose concentration profiles for first and second order reaction were fitted using the nonlinear regression analysis of POLYMATH 6.10 software (Polymath Software, Willimantic, CT, USA).

6.2.8 SCANNING ELECTRON MICROSCOPY

The scanning electron microscopy (SEM) analysis was carried out to show morphological changes of enzymatic saccharification of pretreated solids. The images were visualized by a scanning electron microscope (Nova NanoSEM 200, Netherlands) and photographed. Samples were coated with a layer of gold by sputtering with an accelerating voltage varying to 5 kV.

6.3 RESULTS & DISCUSSION

6.3.1 EFFECT OF AUTOHYDROLYSIS AND ORGANOSOLV PRETREATMENT ON THE COMPOSITION OF THE SOLID PHASE

The chemical composition of APS, OPS, untreated wheat straw and two model substrates Avicel and filter paper are presented in Table 6.1. The initial composition of the wheat straw (untreated) showed that the cellulose (glucan) was the most abundant fraction (37.4 %), followed by hemicellulose (33.8 %) and Klason lignin (19.4 %), these values being in the range reported for wheat straw (Zabihi et al., 2010). The solid phase composition of APS showed that the hemicellulose content was reduced from 33.8 to 7.84 % confirming the extensive removal caused by pretreatment; cellulose content was 63.7 % and Klason lignin 26.91 %, revealing that the cellulose was almost not affected by the autohydrolysis process and a solid residue with increased cellulose and lignin concentration was obtained. These results are in agreement with Gullón et al. (2010) that observed a similar behavior for rye straw. The Klason lignin content follows a similar pattern to cellulose and the majority of the lignin content remained in the solid phase.

Table 6.1. Chemical composition, expressed as % of dry matter, of untreated wheat straw and solid residues after autohydrolysis and organosolv treatment.

Component	Untreated [a]	Autohydrolysis [b]	Organosolv	Avicel [c]	Filter paper [d]
Cellulose	37.4 ± 0.9	63.7 ± 0.6	75.86 ± 0.93	> 97	> 98
Hemicellulose	33.80 ± 0.5	7.84 ± 0.13	6.63 ± 0.25		
Klason Lignin	19.4 ± 0.9	26.91 ± 0.39	16.18 ± 0.4		
Others [e]	9.4 ± 0.5	1.55 ± 0.86	1.33 ± 0.29		

[a] Data published in Ruiz et al. (2011b).

[b] Data published in Ruiz et al. (2011a).

[c] Data published in Qing et al. (2010).

[d] Data published in Geng et al. (2010).

[e] Others components may include ash, protein and extractives.

The solid material obtained after autohydrolysis was delignified using the organosolv process, and a solid phase with cellulose content of 75.86 % and 16.18 % of the lignin was obtained, while the hemicellulose content was further reduced to 6.63 %. The partial delignification resulted in the removal of 38 % of the Klason lignin. Moreover, the composition of Avicel and filter paper is showed in Table 6.1, containing more than 97 and 98 % of cellulose, respectively. These model substrates have the advantage of not containing lignin (Kristensen et al., 2009). In recent studies Alfaro et al. (2009) reported that the susceptibility of different LCM to delignification depends on the content and structural characteristics of lignin (syringyl and guayacyl units); also Hallac et al. (2010) reported a reduction of Klason lignin from Buddleja davidii of 37.19 % at similar conditions of the described organosolv pretreatment using sulfuric acid as catalyst.

6.3.2 ENZYMATIC SACCHARIFICATION OF AUTOHYDROLYSIS AND ORGANOSOLV PRETREATED SOLIDS

The profile of enzymatic saccharification yield (cellulose to glucose) is shown in Figure 6.3A. The maximum saccharification yields were 90.88 %, 64.04 % and 30.36 %, for APS, OPS and untreated straw respectively, and were reached at 96 h. For filter paper and Avicel, the obtained yields were 93.98 % and 79.59 % correspondingly. The saccharification yield of APS and OPS showed significant differences compared to the untreated wheat straw. The data indicated that the enzymatic saccharification yield was improved by autohydrolysis with similar values of saccharification yield reported (Lee et al., 2009) using the same pretreatment.

Díaz et al. (2010) reported a saccharification yield of 94.85 % using autohydrolysis pretreatment at severity conditions (218.3 ºC/ 30 min) and rapeseed straw as raw material. According to Zhao et al. (2009), the use of hydrothermal pretreatment caused the hemicellulose solubilization, allowing an improved enzymatic saccharification yield, as enzyme accessibility to the LCM structure in the pretreated material is favored, increasing the potential of cellulose saccharification.

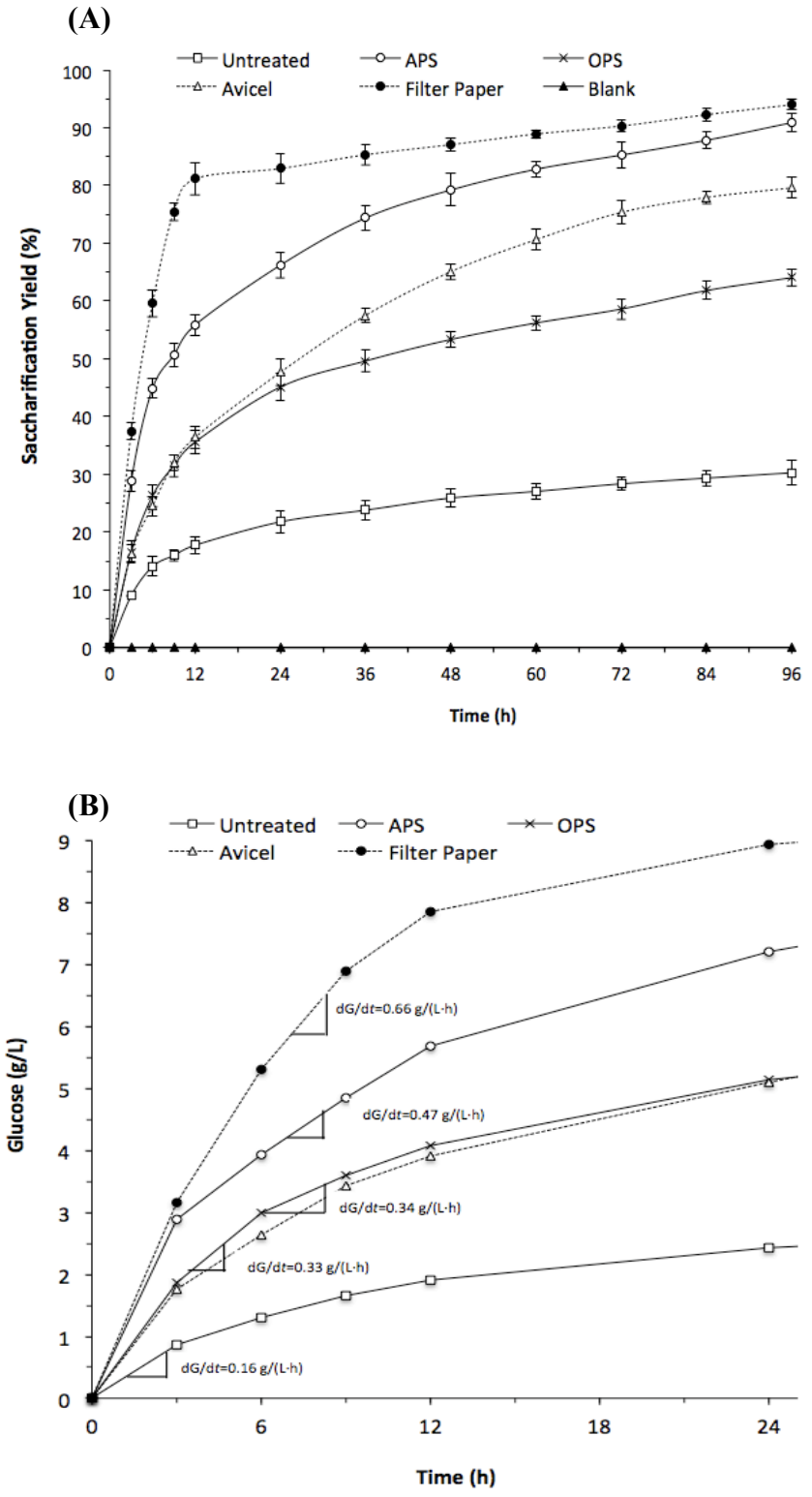


Figure 6.3. Kinetic profile of enzymatic saccharification. (A) Saccharification yield of each substrate; (B) Initial saccharification rate of each substrate. dG/d*t* represents the initial saccharification rate calculated from the saccharification rate at 0-12 h.

Moreover, physical changes by autohydrolysis pretreatment that improve the enzymatic saccharification include an increase in pore size to enhance enzyme penetration and an increase in accessible area that has been shown to correlate well with the susceptibility of these substrates to enzyme saccharification (Liu and Wyman, 2005; Cybulska et al., 2010). Rollin et al. (2011) showed that improving the surface area accessible to cellulase is a more important factor for achieving a high sugar yield, suggesting that the amount of lignin remaining in the autohydrolysis solids is not a main obstacle for enzymatic saccharification.

On the other hand, the saccharification yield obtained from OPS (Figure 6.3A) was lower compared with the obtained from APS. The delignification through organosolv pretreatment did not increase the saccharification. Similar values of saccharification yield (52.6 %) have been reported by Cara et al. (2006) using peroxide delignification after steam pretreatment. These results are in agreement with the data showed by Liao et al. (2005) for enzymatic saccharification of delignified manure.

To explain this behavior it has been suggested that during solid delignification using a physicochemical pretreatment as organosolv, lignin is often modified or degraded, resulting in demethylation and solubilization or formation of simple and oligomeric phenolics (Zhao et al., 2009). These phenolic acids are re-deposited on the surface of the pretreated solid acting as a barrier for enzymatic saccharification (Anderson et al., 2008). In a previous work Ruiz et al. (2011b) reported the production of these phenolic acids compounds after applying the organosolv process in the delignification of wheat straw. Tejirian and Xu (2011) suggested that simple and oligomeric phenolics were the most inhibitory to enzymatic saccharification, being formed at inhibitory levels when biomass pretreatments are carried out at high solid level. According to Kim and Mosier (2009), the solubilization of lignin and lignin derived phenolic compounds during the pretreatment might have a negative impact on the action of enzymes during the hydrolysis due to unspecific binding of enzyme. Hodge et al. (2008) showed that the primary causes of inhibition of the enzyme action are the soluble compounds (acetic acid, phenolic compounds).

In respect to untreated wheat straw, a cellulose conversion yield of 30.36 % was obtained. Thus, the sequence of autohydrolysis and organosolv pretreatments increased

saccharification compared to untreated wheat straw, which is in general agreement with literature data (Rémond et al., 2010). However, this sequential treatment proved to be less effective than the autohydrolysis with no further processing.

Complementary, the saccharification of APS and OPS was compared with the action of the enzymes in two model substrates, filter paper and Avicel, under the same conditions. These substrates are of course not identical to the LCM. However, these profiles allow observing the performance of the enzymes on pure cellulose substrate. Saccharification profiles of these model substrates are shown in Figure 6.3A. Cellulose saccharification yield of filter paper was 93.98 %, while for Avicel was 79.59 % at 96 h. Therefore, knowing that the degree of polymerization (DP) of filter paper is much higher than the obtained with Avicel, Gupta and Lee (2009) showed that the DP of these substrates plays a significant role in enzymatic saccharification. The filter paper is more accessible to saccharification than APS, OPS, untreated wheat straw and Avicel. Moreover, the catalytic activity of the enzyme is controlled by the chemical characteristics of cellulose, for the case of Avicel with less DP and high crystallinity these being the main reasons for the sluggish saccharification (Gupta and Lee, 2009).

Results obtained in the present study are comparing favorably to those described in previous works (Bak et al., 2009; Kristensen et al., 2009). This in contrast with results published in our previous work (Ruiz et al., 2011b) reporting the crystallinity index for untreated wheat straw, APS and OPS. Crystallinity index of wheat straw increased after autohydrolysis pretreatment from 21.3 % to 55.15 % and a crystallinity index of 53.59 % for organosolv pretreated solids was obtained. However, the increased crystallinity did not negatively affect the enzymatic saccharification of APS. Dogaris et al. (2009) reported an increased crystallinity of 48.16 % to 58.38 % for sorghum bagasse treated with hydrothermal processing. No significant correlation between crystallinity of pretreated solids from autohydrolysis and enzymatic saccharification was reported by Lee et al. (2010).

On the other hand, the initial saccharification rate (dG/dt) is calculated from the saccharification that occurs in the first 12 h (the slope of glucose concentration vs time) and is shown in Figure 6.3B. The initial saccharification rate of APS and OPS were 0.47 g/(L•h) and 0.34 g/(L•h), respectively. Untreated wheat straw had a low initial

saccharification rate of 0.16 g/(L•h). With respect to the model cellulosic substrates filter paper and Avicel, the initial saccharification rate is 0.66 g/(L•h) and 0.33 g/(L•h), respectively. The APS significantly increases the saccharification rate, 56 % of conversion being achieved at 12 h, while the saccharification of OPS and Avicel had a similar performance achieving 35.59 % and 36.42 % of conversion, respectively, at the same 12 hours. Untreated wheat straw showed a conversion of 17.73 % at 12h. Obviously, the filter paper achieves an 81.41 % conversion, as might be expected from the comments mentioned above.

6.3.3 MODELING OF ENZYMATIC SACCHARIFICATION

The experimental glucose data concentration profiles of the enzymatic hydrolysis of different substrates, were fitted using the first and second order kinetic models, and results are shown in the Figure 6.4A-E. The fitted constants K_e, k_2, k_{de1} and k_{de2} and the correlation coefficients R^2 are listed in Table 6.2.

In all cases, a good agreement with the experimental results was obtained (correlation coefficient, $R^2 > 0.97$). However, the correlation coefficient value R^2 for the second order kinetics is better than for the first order; the equilibrium constant K_e and the constant of product formation k_2 for the second order model were smaller than for the first order, the first order K_e value deviating greatly from the second order one; the constants of deactivation k_{de1} and k_{de2} were not significantly different. Therefore, the second order reaction models based on the correlation coefficient values R^2 for all substrates allow a satisfactory prediction of kinetic saccharification behavior. Zhang et al. (2010) also reported a better performance for the second order kinetic model than for the first order one on enzymatic saccharification

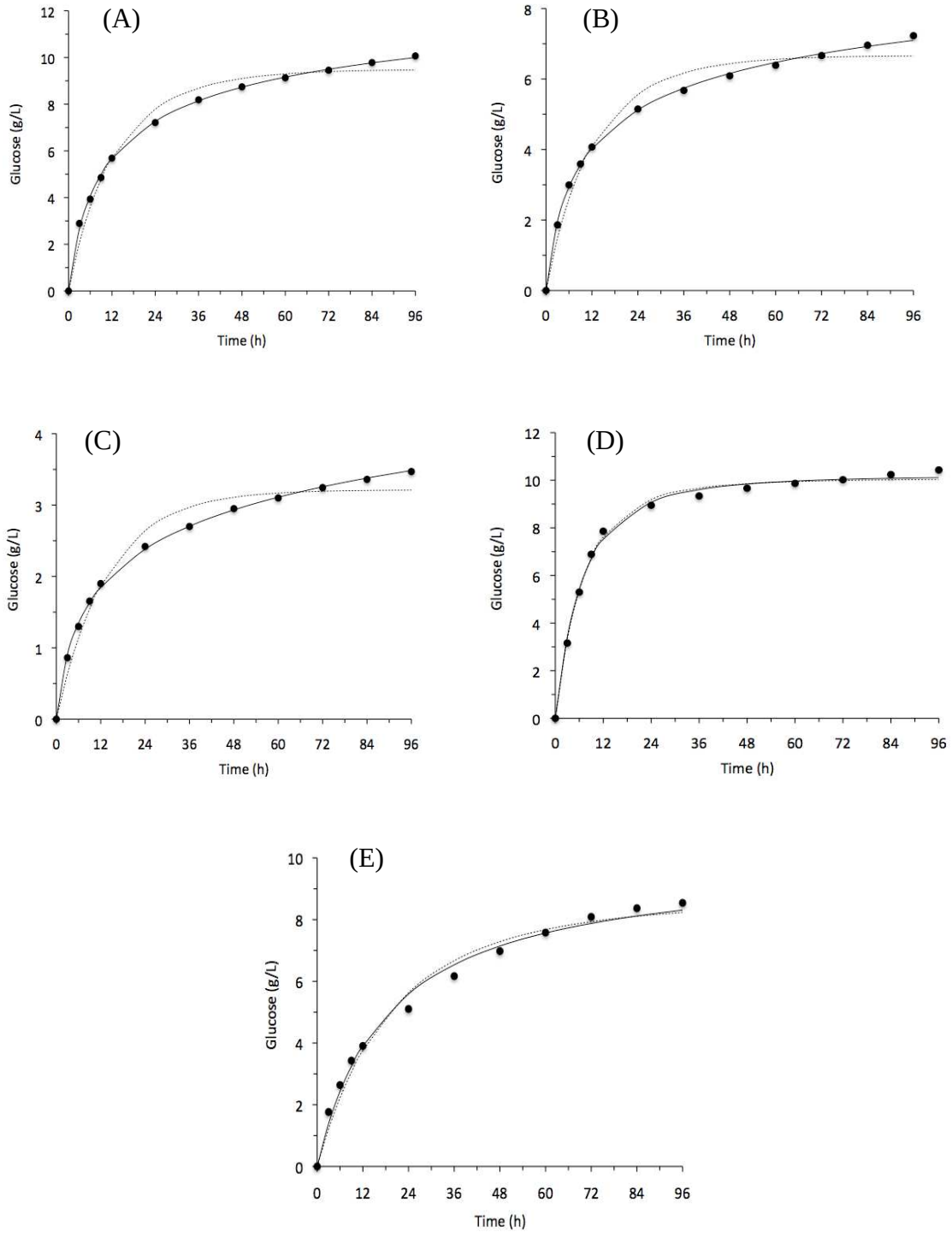


Figure 6.4. Kinetic modeling for glucose concentration by enzymatic saccharification. (A) APS; (B) OPS; (C) untreated wheat straw; (D) filter paper; (E) Avicel. Experimental values (●); modeling based on 1st order reaction (· · · ·); modeling based on 2nd order reaction (—).

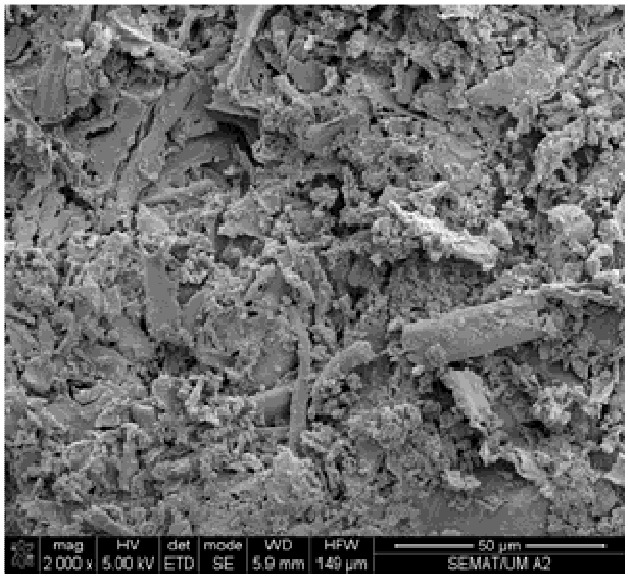
Table 6.2. Model parameters from Eq. (6.2) and (6.3) for the different kinetics of autohydrolysis and organosolv pretreated solids, untreated wheat straw, filter paper and Avicel.

	Autohydrolysis		Organosolv		Untreated		Filter paper		Avicel	
Parameters	Eq. (6.2)	Eq. (6.3)	Eq. (6.2)	Eq. (6.3)	Eq. (6.2)	Eq. (6.3)	Eq. (6.2)	Eq. (6.3)	Eq. (6.2)	Eq. (6.3)
K_e	101.99	4.91	1010.99	9.93	238.79	97.63	273.69	0.25	808.99	0.904
k_2	0.45	0.11	3.50	0.14	0.20	0.17	3.11	0.59	2.92	0.05
k_{de1}	0.05	-----	0.063	-----	0.07	-----	0.32	-----	0.02	-----
k_{de2}	-----	0.079	-----	0.068	-----	0.05	-----	0.23	-----	0.06
R^2	0.981	0.999	0.976	0.999	0.975	0.999	0.995	0.996	0.983	0.991
R^2adj	0.977	0.998	0.971	0.998	0.969	0.999	0.994	0.995	0.980	0.989

6.3.4 SCANNING ELECTRON MICROSCOPY

Differences in fiber structure between APS and OPS substrates are presented in Figure 6.5A-B. SEM image of autohydrolysis (Figure 6.5A) clearly shows a destruction of the fibers at 12 h of saccharification (56 % of saccharification yield and 0.47 g/(L•h) initial saccharification rate), demonstrating that the structure fiber was destroyed, explaining the behavior of the saccharification yield and glucose concentration.

(A)



(B)

Figure 6.5. SEM images of enzymatic saccharification at 12 h. (A) APS; (B) OPS.

In the case of OPS, the saccharification efficiency and initial saccharification rate: 35.59 % and 0.34 g/(L*h), respectively, were lower than the values presented for APS. These results together with images presented in Figure 6.5A-B, where significant structural changes are observed, indicate once again the important role played by the above mentioned inhibitory products formed during the organosolv treatment on the saccharification reaction.

6.4 CONCLUSIONS

This work demonstrates the suitability of wheat straw treated by autohydrolysis or by autohydrolysis followed by organosolv process to enzymatic saccharification. Enzymatic saccharification conversion yield and reaction rate was higher for the autohydrolysis pretreated solids than for the solids subjected to a sequential autohydrolysis/organosolv treatment. The model based on the second order kinetics for cellulase deactivation was accurate on the description of the enzymatic saccharification process of all the studied substrates. This model can provide useful indications for the optimization of the kinetics of enzymatic saccharification of insoluble substrate for industrial applications.

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CHAPTER 7

BIOETHANOL PRODUCTION FROM AUTOHYDROLYSIS PRETREATED WHEAT STRAW- EFFECT OF PROCESS CONDITIONS

ABSTRACT

The simultaneous saccharification and fermentation (SSF) process of lignocellulosic materials requires the utilization of microorganisms capable of working at high temperatures. The selection of *Saccharomyces cerevisiae* strains able to ferment sugars obtained from lignocellulosic material at temperatures above 37 ºC with high ethanol yield has become a necessity. In this chapter, a flocculant *S. cerevisiae* CA11 was evaluated for their ability to grow and ferment glucose in a temperature range of 40 - 50 ºC. Maximum ethanol production obtained from 50 g/L glucose were 24.12 and 24.38 g/L at 40°C and 45 °C, respectively. Growth and ethanol production decreased at 50 °C. Autohydrolysis pretreated wheat straw with high cellulose content (> 60 %) at 180 ºC for 30 min was used as substrate in simultaneous saccharification and fermentation (SSF) process for bioethanol production by *S. cerevisiae* CA11. In order to evaluate the effects of temperature, substrate concentration (as effective cellulose) and enzyme loading on: 1) ethanol conversion yield, 2) ethanol concentration, and 3) CO_2 concentration a central composite design (CCD) was used. Results showed that the ethanol conversion yield was mainly affected by enzyme loading, whereas for ethanol and CO_2 concentration, enzyme loading and substrate concentration were found to be the most significant parameters. The highest ethanol conversion yield of 85.71 % was obtained at 30 ºC, 2 % substrate and 30 FPU of enzyme loading, whereas the maximum ethanol and CO_2 concentrations (14.84 and 14.27 g/L, respectively) were obtained at 45 ºC, 3 % substrate and 30 FPU of enzyme loading, corresponding to an ethanol yield of 82.4 %, demonstrating a low enzyme inhibition and a good yeast performance during SSF process.

7.1 INTRODUCTION

Bioethanol is an increasingly important alternative fuel for the replacement of gasoline, with a world production in 2009 of 19,535 millions of gallons and an estimate, only for USA in 2022, of 36,000 millions of gallons. It is thus expected that the production of bioethanol will keep on increasing in the next ten years (Figure 7.1) (RFA, 2009). Second-generation bioethanol obtained from lignocellulosic materials (LCM) has received major attention due to their abundance and immense potential for conversion into sugars and fuels. However, there are relevant obstacles such as production costs, technology and environmental problems that need to be overcome in the production of second-generation bioethanol (Linde et al., 2007; Goh et al., 2010).

Wheat straw is one of the most abundant agricultural by-products, presents a low commercial value and most of it is being used as cattle feed and waste. In terms of total production, wheat is the second most important grain crop in the world. FAO statistics (2011) reported a world annual wheat production in 2009 of 685 million tons and, in average, the harvesting of 1.3 kg of grain is accompanied by the production of 1 kg of straw; this gives an estimation of about 527 million tons of wheat straw in 2009, an amount that clearly justifies the need to consider wheat straw as a complementary source of raw material for the production of bioethanol (Petersen, 1987).

The process for the production of second-generation bioethanol includes three main steps: pretreatment, enzymatic hydrolysis and fermentation. Autohydrolysis (hydrothermal processing), a pretreatment based in the use hot compressed water, presents several advantages: no chemicals other than water are needed, no problems derived from equipment corrosion occur, toxic compounds formation is minimized, a high recovery of valuable hemicellulose derived products is obtained and cellulose structure is made susceptible to enzymatic hydrolysis (Romaní et al., 2010; Ruiz et al., 2011a).

Simultaneous saccharification and fermentation (SSF) processes, firstly described by Takagi et al. (1977), combine enzymatic hydrolysis of cellulose with simultaneous fermentation of the obtained sugars to ethanol and are one the most promising process

option for bioethanol production from LCM (Olofsson et al., 2008). SSF process has shown to be superior to separate hydrolysis and fermentation (SHF) in terms of overall ethanol yield. Furthermore, SSF reduces the processing time, which in turn leads to increases in ethanol productivity as a consequence of the fast conversion of glucose to ethanol by the fermenting microorganisms, that reduces the enzymes inhibition due the presence of sugars. Reduction in equipment costs is also obtained by performing the hydrolysis and fermentation in a single reactor. However, differences between the optimal temperatures for cellulose activity and yeast growth is an issue that needs to be solved for an efficient SSF. The optimal temperature for cellulase enzymes (about of 50 ºC) is higher than the tolerance range reported by the most used yeast for industrial ethanol production (about 30-37 ºC) (Faga et al., 2010; Krishna et al., 2001). This requires the matching of the temperature conditions required for the optimum performance of the enzymatic and the fermenting microorganism.

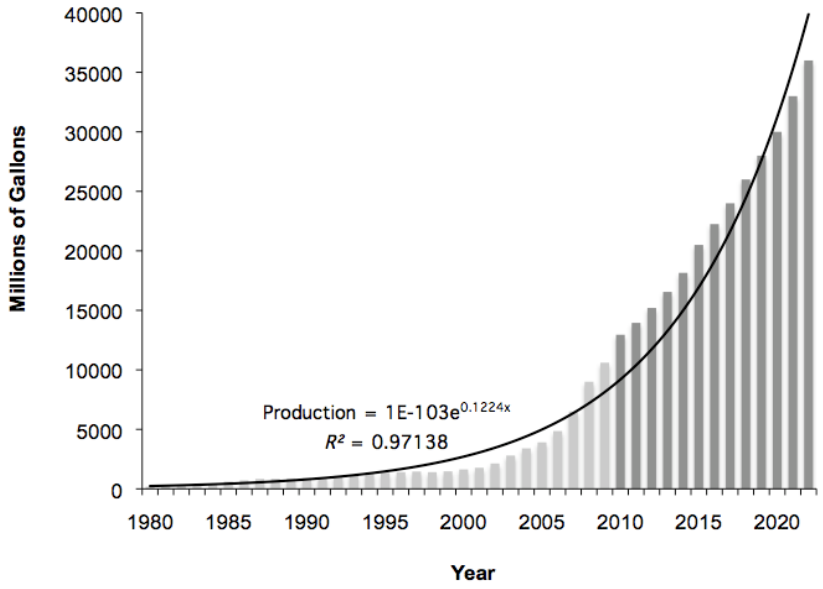


Figure 7.1. Trends in U.S fuel ethanol production; (▒) real production; (■) estimate production. The points represent the trend model.

On the other hand, yeasts isolated from extreme environments, exhibit the capability of growth at high temperatures while producing ethanol; a proof of this is the industrial ethanol fermentation in some tropical countries as Brazil where fermentation takes place

at ambient temperatures and during the process the temperature can reach 41 ºC as, due to its expensive costs (Vianna et al., 2008), no cooling systems are used. This requires the use of yeast strains able to produce high ethanol yields at such high temperatures. Abdel-Banat et al. (2010) demonstrated that if the fermentation step could be performed at higher temperatures, for instance within a 40-50 ºC range, significant cost reductions in fuel ethanol production could be obtained. Advantages of processing at higher temperatures include a more-efficient simultaneous saccharification and fermentation, significant reduction of contamination and a continuous shift from fermentation to distillation (Shi et al., 2009).

Additionally, the use of flocculating yeasts is one of the most interesting ways to provide an increase of the efficiency of bioethanol production processes as a significant reduction of capital costs is achieved with the elimination of centrifugation (or at least a substantial reduction of the demand for such an expensive operation), making the process more competitive. The flocculation of yeast cells is a reversible, asexual and calcium-dependent process in which cells adhere to form flocs consisting of thousands of cells, the use of high cell density systems being investigated and used for separating yeast cells from beer in the brewing industry. In fact, these systems present several advantages as reduced downstream processing costs, reuse of the biomass for extended periods of time, higher productivity, protection against ethanol stress and resistance to contamination by other microorganism (Vicente et al., 1998; Domingues et al., 2000; Verstrepen et al., 2003; Soares et al., 2011; Zhao and Bai, 2009). Overall, improved efficiency of the SSF will be obtained by using a yeast strain that can work at higher temperatures and has flocculant properties.

The first objective of this chapter was to investigate the performance of *Saccharomyces cerevisiae* CA11 in the production of ethanol at elevated temperatures. Moreover, the effect of SSF operating conditions (temperature, substrate and enzyme loading) of autohydrolysis pretreated wheat straw on ethanol yield, ethanol and CO_2 production with a flocculating strain *Saccharomyces cerevisiae* CA11, using a central composite experimental design, was evaluated.

7.2 EXPERIMENTAL PROCEDURES

7.2.1 YEAST STRAIN CULTIVATION

The flocculating *S. cerevisiae* CA11 was obtained from the microbial collection at the Microbial Physiology Laboratory/Department of Biology from the Federal University of Lavras (UFLA), Brazil. The flocculating yeast strain was isolated from a ‘‘cachaça’’ distillery in the state of Minas Gerais, Brazil and used in all the fermentation experiments. The strain was kept on agar plates made of yeast extract 10 g/L, peptone 20 g/L, agar 20 g/L and D-glucose 20 g/L as an additional carbon source at 4 ºC.

7.2.2 EVALUATION OF A FLOCCULANT *SACCHAROMYCES CEREVISIAE* AT DIFFERENT TEMPERATURES

Fermentations were performed in 100 mL Erlenmeyer flasks with 50 mL of sterile culture medium YPD (10 g/L yeast extracted, 10 g/L peptone, 50 g/L glucose); glucose was sterilized (121 ºC/15 min) separately from the other components to prevent damage to the nutritional qualities of the medium. One loop of *S. cerevisiae* CA11 from agar plate was aseptically transferred to the fermentation media. Flasks were stoppered with cotton plugs and incubated in an orbital incubator at 40, 45 and 50 ºC, 150 rpm for 24 h. Samples of 1 mL were withdrawn aseptically with sterile pipette tips at 3 h intervals for the first 9 h and at 24 h. Samples were immediately centrifuged at 8260 x *g* for 10 min. Supernatant samples were filtered through 0.2 μm sterile membrane filter. Ethanol, glucose, glycerol were measured directly by HPLC, (see below). The theoretical ethanol yield was calculated as:

$$\text{EtOH yield (\%)} = \frac{[\text{EtOH}]}{[\text{Ig}] \cdot 0.511} \cdot 100 \qquad \textbf{(7.1)}$$

where [EtOH] is the final ethanol concentration (g/L); [Ig] is the initial glucose concentration (g/L) and 0.511 is the conversion factor for glucose to ethanol based on the stoichiometry of the reaction.

Yeast (biomass) growth was monitored by cell couning using hemacytometer chamber (Neubauer chamber) and viability was obtained using the methylene blue method after appropriate dilution of the samples in NaCl solution (15 g/L) to prevent cell aggregation after deflocculation (Mills, 1941). The yeast viability was calculated as the ratio between viable (non-stained) and total cell counts. For the flocculent strain, cells were harvested by centrifugation; the biomass was deflocculated according to the procedure described by Vicente (1997), pellets were washed 2 times with 50 mM of ethylenediaminetetra-acetic acid (EDTA) solution. In order to remove the EDTA, a final wash was performed with ultrapure water and the pellet resuspended in 15 g/L of NaCl pH 4.0. All determinations were performed in duplicate.

7.2.3 RAW MATERIAL

The wheat straw used in this study was kindly provided by a local farmer (Elvas, Portugal). Wheat straw was cut into small pieces (1-3 cm) and milled using a laboratory knife mill (Cutting Mill SM 2000, Retsch, Germany). The material composition was previously analyzed by Ruiz et al. (2011a) and their particle size distribution (w/w %) was as follows: 10 % > 1 mm; 40 % between 1-0.5 mm; 40 % between 0.5-0.3 mm; and 10 % to < 0.3 mm. The same batch of raw material was used for all experiments.

7.2.4 WHEAT STRAW PRETREATMENT BY AUTOHYDROLYSIS PROCESS

Milled wheat straw samples were mixed with water in order to obtain a 10:1 liquid/solid mass ratio and treated in a 3.75 l total volume stainless steel reactor (Parr Instruments Company, Moline, Illinois, USA) with PID temperature control. The moisture content of wheat straw was considered as water in the material balances. The reactor was filled and heated to 180 ºC at a heating rate of 3 ºC/min until reaching the desired temperature, the reaction time was 30 min, these conditions having been previously evaluated by Ruiz et al. (2011a). After completing the reaction time, the reactor was cooled down at a rate about of 3.2 ºC/min and the agitation speed was set at 135 rpm. Figure 7.2 shows a typical heating and cooling temperature profile obtained for a experiment done in triplicate at 180 ºC, confirming the excellent reproducibility of the

process. At the end of the treatment, the liquid and solid phases were separated by centrifugation and the solid residues were washed with distilled water. Quantification of structural carbohydrates, sugars and degradation products in both solid and liquid phases has been previously reported by Ruiz et al. (2011b). The solid residue was used as substrate for simultaneous saccharification and fermentation.

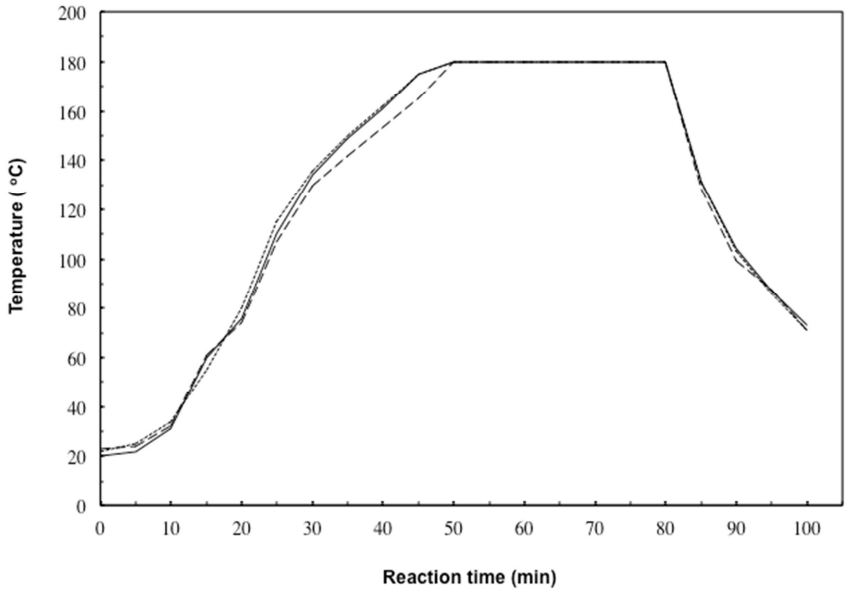


Figure 7.2. Heating profile for autohydrolysis pretreatment at 180 ºC for 30 min.

7.2.5 YEAST INOCULUM PREPARATION

Yeast for inoculation was grown in 250 mL Erlenmeyer flasks with 125 mL of sterile culture medium containing 50 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, 0.5 g/L $(NH_4)_2HPO_4$ and 0.5 g/L $MgSO_4.7H_2O$; glucose was sterilized separately from the other components to prevent damage to the nutritional qualities of the medium. Yeast growth was carried out at 30 ºC and 150 rpm in an orbital shaker for 10–12 h. The cell suspension was aseptically collected by centrifugation (15 min at 7885 x *g*, 4 ºC) and suspended in sterile 0.9 % NaCl to a concentration of 200 mg fresh yeast/mL. The yeast cells were inoculated at about 8 mg fresh yeast/ mL into 50 mL of culture medium to start the fermentation (Pereira et al., 2010).

7.2.6 ENZYME

Commercially available enzyme solutions, cellulase from *Trichoderma reesei* (Celluclast 1.5 L) and β-glucosidase from *Aspergillus niger* (Novozym 188), were kindly supplied by Novozymes A/S (Bagsvaerd, Denmark). The cellulase activity from Celluclast 1.5 L was analyzed in terms of FPU in accordance with the standard analytical methods established by the National Renewable Energy Laboratory (Adney and Baker, 1996). One filter paper unit (FPU) of cellulase was defined as the amount of enzyme which produces 2.0 mg of reducing sugar from 50 mg of filter paper within 1 h. The experiment was carried out in a reaction mixture containing 0.5 mL of diluted enzyme solution, 1.0 mL of 50 mM citrate buffer (pH 4.8), and 50 mg of a 1 x 6 cm strip of a Whatman No. 1 filter paper. The reaction solution was incubated at 50 ºC for 1h. Then the concentration of the released reducing sugar was measured using an adaptation of the 3,5-dinitrosalicylic acid (DNS) method (Gonçalves et al., 2010). β-glucosidase activity was determined for Novozym 188. The β-glucosidase activity was measured by incubating the enzyme solution with 4 mM *p*-nitrophenyl-β-D-glucopyranoside and 50 mM sodium citrate buffer (pH 4.8) at 30 ºC for 15 min. The reaction was stopped by addition of 60 µl of 1 M Na_2CO_3 and the amount of liberated *p*-nitrophenol was measured spectrophotometrically at 400 nm. One unit of activity (IU) was defined as the release of 1 µmol of nitrophenol per minute (Ruiz et al., 2012). The enzyme activities of commercial concentrates were 43.05 FPU/mL for Celluclast 1.5 L and 576.39 UI/mL for Novozym 188.

7.2.7 SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

The SSF experiment was conducted in accordance with NREL standard procedure (Dowe and McMillan, 2001). The fermentations were performed in 100 ml Erlenmeyer flasks and each one was equipped with a thick rubber stopper, through which one stainless-steel capillary had been inserted; the exterior tip was submerged in glycerol. Glycerol was used as a lock to prevent oxygen back-diffusion to the medium, while permitting evolved CO_2 to leave the flask and to maintain anaerobic conditions (Figure 7.3).

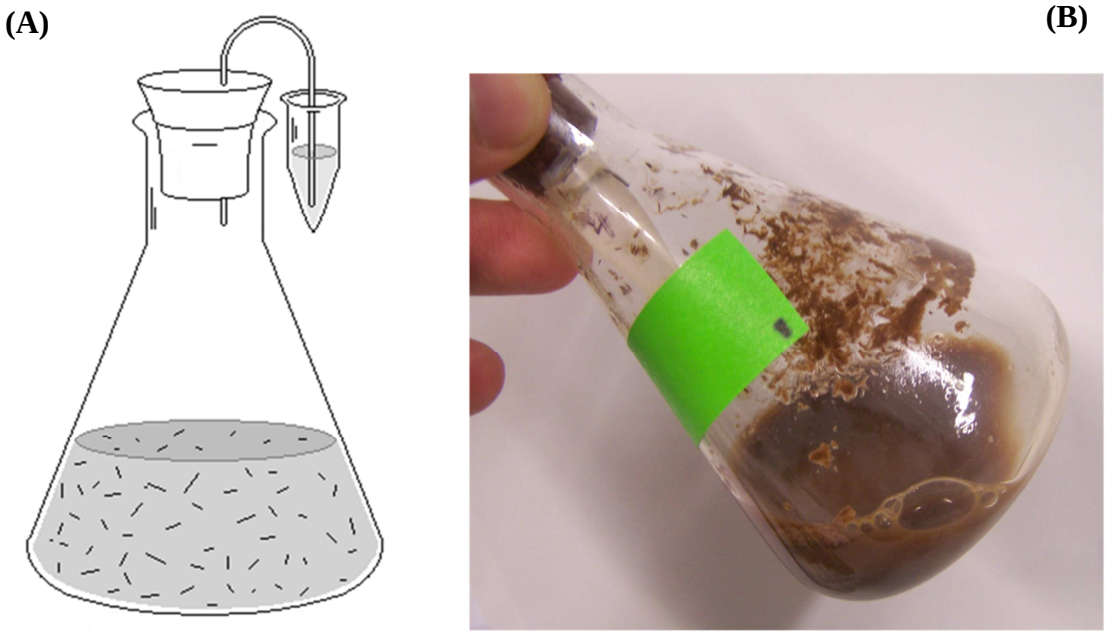


Figure 7.3. Conical system for batch cultivation used in SSF process.
A) Flask diagram assembly for SSF process; B) Autohydrolysis wheat straw after 24 h.

The fermentation medium contained pretreated wheat straw, 1% w/v yeast extract, 2% w/v peptone, 50 mM citrate buffer (pH 4.8), cellulase and β-glucosidase enzyme. The β-glucosidase enzyme was added at a ratio of 2:1 IU of β-glucosidase to FPU of cellulase. Spindler et al. (1989) reported that the β-glucosidase supplementation is necessary to achieve efficient cellulose conversion. The necessary amount of deionized water was calculated and added to make the total weight of 50 g. The pretreated wheat straw was used as the control. SSF was started by adding enzymes and flocculating *S. cerevisiae* CA 11 and then incubated at different conditions (Table 7.1) in an orbital shaker at 150 rpm. All the experiments were carried out under sterile conditions. Samples of 1 mL were withdrawn aseptically with sterile pipette tips at 3 h intervals for the first 12 h and at 24 h intervals until a total of 96 h. Samples were immediately cooled on ice and centrifuged at 8260 x *g* for 10 min. Ethanol concentration and that of remaining sugars were determined by means of HPLC (see below). As an estimate, the CO_2 was kinetically monitored by weight loss of the Erlenmeyer flasks at regular intervals (the influence of sample withdrawal was taken into account). Fermentation monitoring based

on CO_2 production is a common practice in wine fermentation (Bely et al., 1990; Varga et al., 2004; Pereira et al., 2010). All determinations were performed in duplicate.

7.2.8 ANALYTICAL METHODS

All samples taken from SSF were filtered through a 0.2 μm sterile membrane filter and analyzed for cellobiose, glucose and ethanol by HPLC. Chromatographic separation was performed using a Metacarb 87 H column (300 x 7.8 mm, Varian, USA) under the following conditions: mobile phase 0.005 M H_2SO_4, flow rate 0.7 mL/min, and column temperature 60 ºC. The system was comprised of a Jasco chromatograph 880-PU intelligent pump (Jasco, Tokyo, Japan) equipped with a Jasco 830-IR intelligent refraction-index detector (Jasco, Tokyo, Japan) and a Jasco AS-2057 Plus intelligent auto sampler (Jasco, Tokyo, Japan). The volume injected was 20 μl per sample. Sugars and ethanol concentrations were determined based on calibration curves of these pure compounds (Ruiz et al., 2011b).

7.2.9 ETHANOL YIELD CALCULATIONS

The ethanol yield was calculated according to the NERL standard procedure (Dowe and McMillan, 2001).

$$\text{Ethanol yield} = \frac{[EtOH]_f}{0.51f[Biomass]\text{x}1.111}\text{x}100\% \quad \textbf{(7.2)}$$

where $[EtOH]_f$ is the ethanol concentration at the end of the fermentation (g/L). The term "0.51 × f × [*Biomass*] × 1.111" corresponds to the theoretical ethanol concentration, where [*Biomass*] is the dry biomass weight concentration at the beginning of the fermentation (g/L); *f* is the cellulose fraction of dry biomass (g/g); 0.51 is the conversion factor for glucose to ethanol based on the stoichiometry of the reaction and 1.111 is the conversion factor for cellulose to equivalent glucose.

The ethanol concentration in the liquid phase was analyzed using HPLC as described above. Then the accurate ethanol yield could be obtained using Equation 7.2.

Table 7.1. Statistical analysis of experimental design arrangement, responses and predicted values for ethanol yield, ethanol and CO_2 concentration.

Run	Normalized variables[a]			Real value			Ethanol Yield (%)		Ethanol (g/L)		CO_2 (g/L)	
	X_1	X_2	X_3	x_1	x_2	x_3	Exp[b] (Y_{EtOH})	Pre[c] (Y_{EtOH})	Exp[b] (C_{EtOH})	Pre[c] (C_{EtOH})	Exp[b] (C_{CO2})	Pre[c] (C_{CO2})
1	-1	-1	-1	30	2	5	49.51	48.42	6.22	5.86	6.57	5.90
2	-1	-1	+1	30	2	30	85.71	83.45	10.09	9.86	9.96	9.63
3	-1	+1	+1	30	3	30	72.88	71.26	13.20	12.67	12.96	12.15
4	-1	+1	-1	30	3	5	32.10	37.56	5.78	6.59	5.08	5.97
5	+1	-1	-1	45	2	5	45.13	47.00	5.31	5.63	4.74	5.27
6	+1	-1	+1	45	2	30	79.05	73.84	9.51	8.49	8.94	7.78
7	+1	+1	+1	45	3	30	82.40	83.74	14.85	15.00	14.28	14.68
8	+1	+1	-1	45	3	5	55.74	58.25	10.04	10.06	9.65	9.71

9	-1	0	0	30	2.5	17.5	52.00	51.51	7.73	8.04	6.68	7.60
10	+1	0	0	45	2.5	17.5	57.56	57.05	8.56	9.09	8.39	8.55
11	0	-1	0	37.5	2	17.5	46.01	52.69	5.42	6.71	4.64	6.27
12	0	+1	0	37.5	3	17.5	59.90	52.21	10.79	10.33	10.29	9.75
13	0	0	-1	37.5	2.5	5	41.06	32.31	6.10	5.31	5.78	4.97
14	0	0	+1	37.5	2.5	30	54.83	62.58	8.15	9.78	7.42	9.32
15	0	0	0	37.5	2.5	17.5	45.35	45.62	8.00	7.68	7.50	7.17
16	0	0	0	37.5	2.5	17.5	44.89	45.62	7.88	7.68	7.26	7.17
17	0	0	0	37.5	2.5	7.5	45.24	45.62	8.20	7.68	7.98	7.17
18	0	0	0	37.5	2.5	17.5	44.98	45.62	8.31	7.68	8.12	7.17

[a] X_1 : temperature, X_2 : substrate, X_3 : enzyme loading; [b] Experimental value; [c] Model predicted value

7.2.10 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

In order to relate the dependent variables ethanol yield (Y_{EtOH}, %), ethanol (C_{EtOH}, g/L) and CO_2 (C_{CO2}, g/L) concentration and independent variables temperature (X_1, ºC), substrate (X_2, %) and enzyme loading (X_3, FPU/g of cellulose) in the process of SSF with the minimum possible number of experiments, a 2^n central composite experimental design (CCD) for three factors that enabled the construction of second-order polynomials in the independent variables and the identification of statistical significance in the variables was used (Montgomery, 1997). The total number of observations required for three independent variables (*N*) was calculated using the following equation:

$$N = 2^K + 2 \text{ x } K + 1 \quad \textbf{(7.3)}$$

where *K* is the number of independent variables. *N* is found to be 15 with four replicates at the center point leading to a total number of 18 experiments for the evaluation of SSF process. The values of the independent variables were normalized from -1 to +1 using Eq. (7.4) to provide the comparison of the coefficients and visualisation of the individual independent variables on the response variable. The lowest and the highest levels of variables are given in Table 7.2.

$$X_n = 2\frac{X - \bar{X}}{X_{max} - X_{min}} \quad \textbf{(7.4)}$$

where *X* is the absolute value of the independent variable concerned, $\bar{X}$ is the average value of the variable and X_{max} and X_{min} are its maximum and minimum value, respectively. The second-order polynomials and calculation of predicted responses were calculated with MATLAB® Version 7.6.0, R2008a software (MathWorks, Inc., Natick, Massachusetts, USA) to estimate the response of the dependent variables. The mathematical model corresponding to the experimental design is:

$$Y_i = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=2}^{2} \sum_{j=i+1}^{3} \beta_{ij}\, x_i x_j + \varepsilon \quad \textbf{(7.5)}$$

where Y_i is the predicted value, x_i and x_j are the normalized values of the factors, β_0 is a constant coefficient, βi are the linear coefficients, βij (i and j) are the interaction coefficients and βii are the quadratic coefficients and ε is the random error.

The quality of the fit of the polynomial model equation was evaluated by the coefficient of determination R^2 and the statistical significance was evaluated by the Fisher's F-test for analysis of variance (ANOVA) with a 95 % confidence level. The effect of each independent variable and also their interaction effects were determined. ANOVA results generated the Pareto charts of interactions and effects. The experimental design package STATISTICA™ v 7.0 (Statsoft ®, Tulsa, OK, USA) was the software used for data analysis.

Table 7.2. Variables and levels used in the central composite design.

Independent variable	Symbol	Range and levels		
		-1	0	+1
Temperature (º C)	X_1	30	37.5	45
Substrate (%) [a]	X_2	2	2.5	3
Enzyme loading [b]	X_3	5	17.5	30

7.3 RESULTS & DISCUSSION

7.3.1 EVALUATION OF A FLOCCULANT *SACCHAROMYCES CEREVISIAE* AT DIFFERENT TEMPERATURES

S. cerevisiae is a well-known thermotolerant yeast species. Its ability to produce ethanol by fermentation at high temperature and its use of a wide range of carbon sources make

this species attractive for industrial applications (Edgardo et al., 2008; Pereira et al., 2010).

The effect of temperature on growth kinetics of *S. cerevisiae* CA11 is shown in Figure 7.4A. Maximum growth was obtained at 45 ºC in 3 h, and with increasing the temperature to 50 ºC, the values decreased. Temperature is accepted as one of the most important physical parameters influencing yeast growth. This factor can affect the sensitivity of yeasts to alcohol concentration, growth rate, rate of fermentation, viability, length of lag phase, enzyme and membrane function, etc. Growth of yeast cells at high temperatures produced dramatic differences in heat sensitivity. *S. cerevisiae* yeast, capable of fermenting at 40 ºC and 45 ºC, have been obtained using progressive cultures. Additionally, thermotolerant yeasts have been obtained by selecting survivors after a shock process at relatively high temperatures. In a recent work, Mehdikhani et al., (2011) reported a good performance growth of three different *S. cerevisiae* at 40 ºC and two strains showed growth at 42 ºC. Lu et al., (2011) used a recombinant *S. cerevisiae* yeast that showed to grow at 45 ºC. Balakumar et al., (2001) reported that the thermotolerant *S. cerevisiae* survived at 45 ºC for 15 h.

In order to determine the extent of the heat resistance of *S. cerevisiae* CA11, the cells were grown at 40 ºC, 45 ºC and 50 ºC. The number of dead cells at 40 ºC and 45 ºC did not exceed 15 % and 10 %, respectively. More rapid losses of viability were observed at the highest temperature (Figure 7.4B), the cell viability at 50 ºC was 66 % in 24 h. According to Ohta et al. (1988), high temperatures are thought to cause increased fluidity in members and yeast respond to this physical change by changing their fatty acid composition. Walton and Pringle (1980) suggested that the mechanism by which heat-induced membrane disruption leads to cell death may be more complex than a simple lysis of the cell. Taylor and Orton (1975) cited by Soares (2011) reported that the temperature can also affect yeast flocculation by acting on cell-cell interaction, promoting the reversible dispersion of the flocs. *S. cerevisiae* have been extensively studied by exposing cells to various stresses such as high temperatures, ethanol inhibition and osmotic pressure. Vianna et al., (2008) showed that the major yeast stress protection mechanisms is the trehalose accumulation. Lu et al., (2011) reported

that the yeast cells are changed by various environmental stresses during the industrial process of ethanol production.

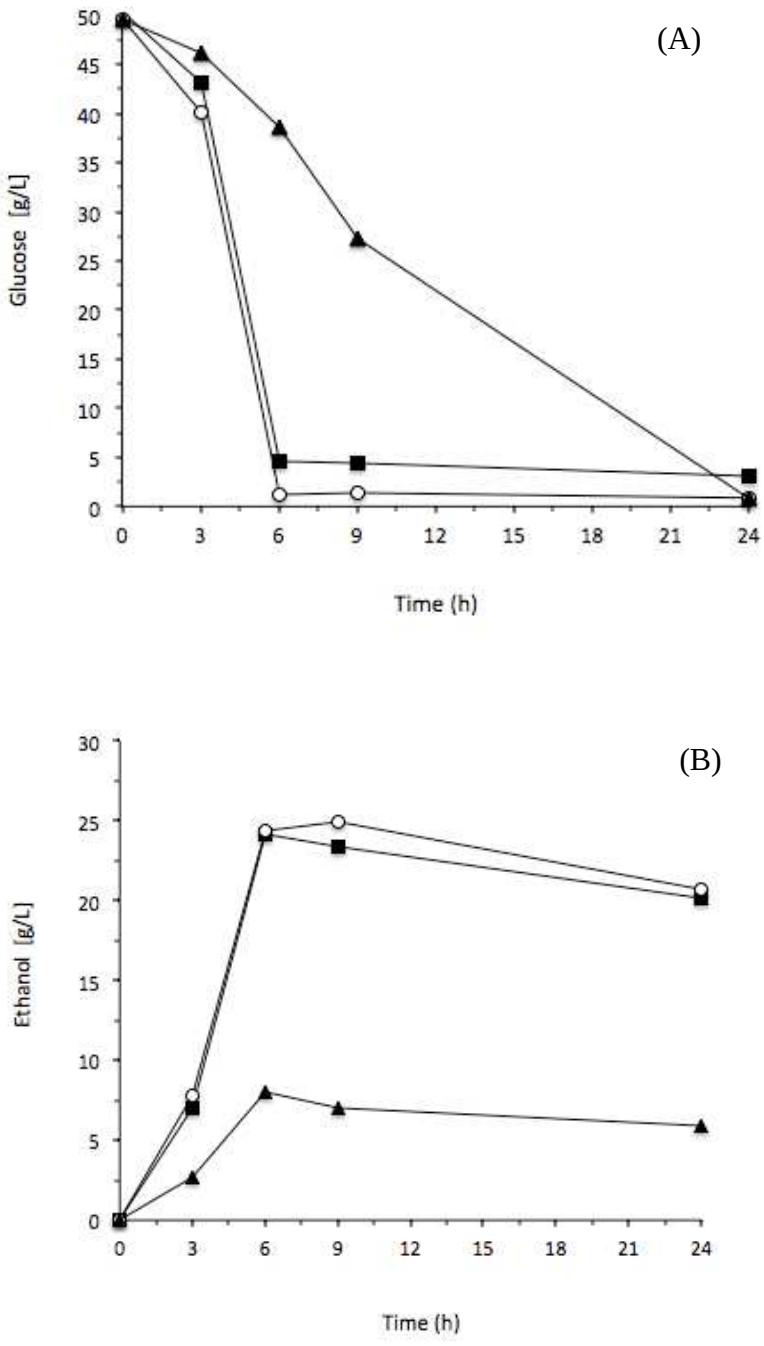


Figure 7.4. Kinetics of growth (A), viability (B) at different temperatures: (■) 40 ºC; (○) 45 ºC; (▲) 50 ºC.

Glucose consumption and ethanol production were also examined at 40 ºC, 45 ºC and 50 ºC (Figure 7.5A-B). *S. cerevisiae* CA11 exhibited similar levels of ethanol production and glucose consumption at 40 ºC and 45 ºC. However, when the ethanol fermentation was carried out at 50 ºC, *S. cerevisiae* CA11 showed a lower production than at others temperatures. From these results, it was conclude that *S. cerevisiae* CA11 had the fastest ethanol production at temperatures between 40 ºC and 45 ºC.

Glucose to ethanol conversion yield was between 94 % and 96 % of the theoretical value at 40 ºC and 45 ºC, respectively in 6 h, whereas at 50 ºC the ethanol yield was 32 % (Figure 7.5C). Abdel-Banat et al., (2010) observed a similar behaviour on the ethanol evaporation at 28 ºC, 35 ºC, 40 ºC and 50 ºC. Balakumar et al. (2001) reported the ethanol production from 100 g/L of glucose by *S. cerevisiae* S1 was 46 g/L, 38 g/L and 26 g/L at 40, 43 and 45 ºC, respectively. Edgardo et al., (2008) reported an ethanol yield of 50 % using *S. cerevisiae* isolated from wine cultures at 48 h. Balakumar et al. (2001) showed that the ethanol conversion yield by *S. cerevisiae* was 90 % at 40 ºC. The yields of ethanol and other fermentation by-products are also related to temperature (Torija et al., 2002).

Glycerol is formed as a by-product in the production of ethanol during fermentation of *S. cerevisiae* under anaerobic and aerobic growth conditions (Nissen et al., 2000). Glycerol production by yeast is influenced by the growth and many environmental factors. Several studies have shown that an increase in temperature resulted in higher glycerol production. According to Wang et al., (2001) the optimum temperature for glycerol production by yeast is apparently similar to the optimum growth temperature of the organism. Glycerol production of wine yeast is also known as one of the most important criteria in strain selection (Scanes et al., 1998; Duarte et al., 2010). In this work the maximum levels for glycerol concentration were 1.03 g/L and 0.95 g/L obtained at 40 ºC and 45 ºC, respectively. At 50 ºC the production of glycerol was 0.45 g/L (Figure 7.5D).

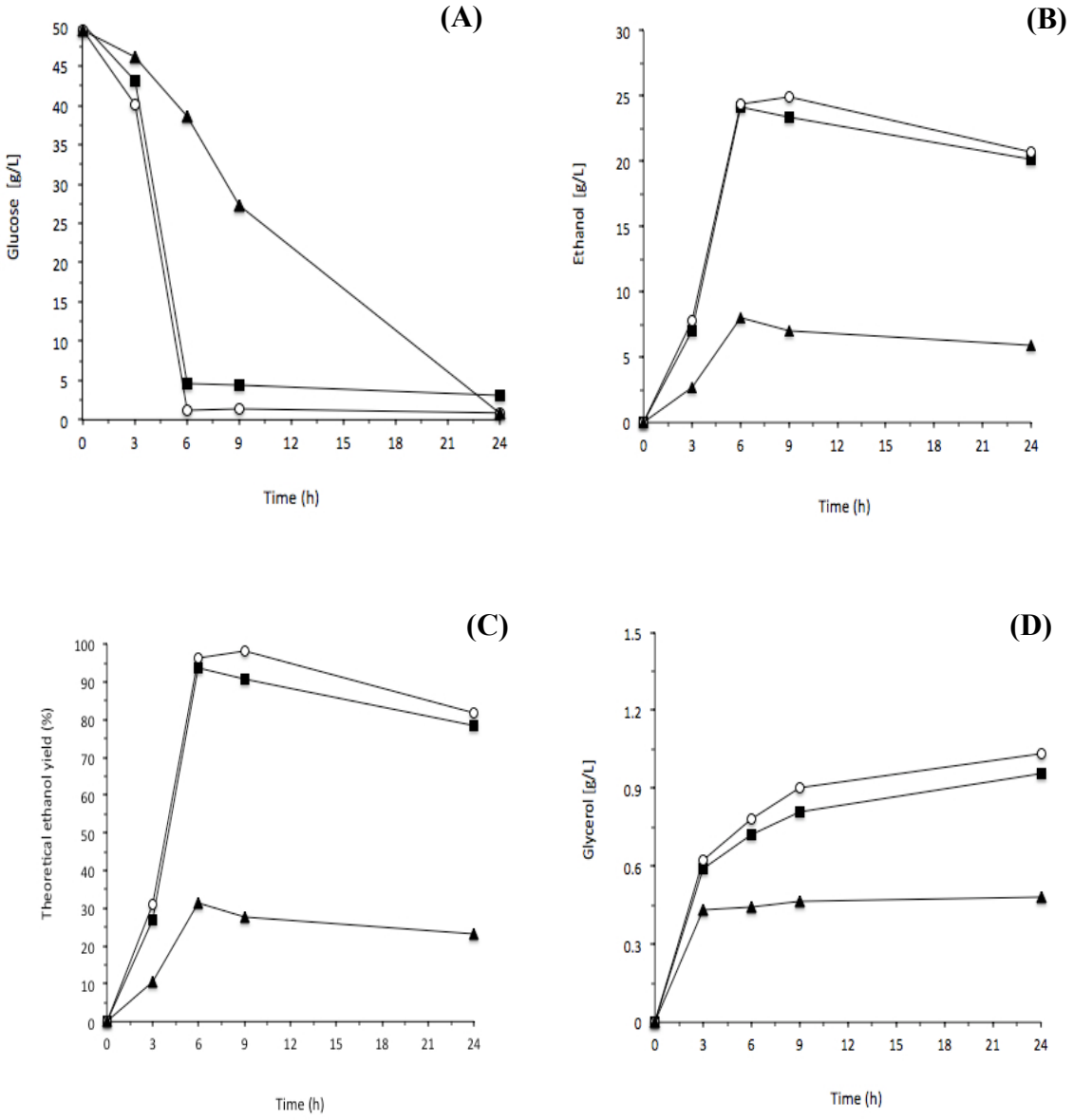


Figure 7.5. Effect of temperatures on the process kinetics: glucose consumption (A), ethanol production (B), theoretical ethanol yield (C), glycerol production (D). (■) 40 ºC; (○) 45 ºC; (▲) 50 ºC.

This behaviour was probably due to the stress conditions and decrease in biomass production. Ortiz-Muñiz et al. (2010) suggested that the glycerol production was not stimulated by the presence of ethanol in the culture medium, probably because glycerol does not carry out a physiological function when the yeast strain is submitted to the stress caused by the presence of ethanol in the fermentation medium. Nissen et al. (2000) reported that during aerobic growth, glycerol is formed at high glucose

concentration, where respiration is repressed, reducing the capability of the organism. Sevda et al. (2011) showed that the maximum glycerol production was achieved when the fermentation condition changed from 20 ºC to 38 ºC using *S. cerevisiae*.

7.3.2 EFFECT OF AUTOHYDROLYSIS ON THE COMPOSITION OF THE SOLID PHASE

The cellulose (glucan) content in the solid residue was of 63.7 %, xylan 7.55 %, arabinan 0.29 %, acetyl groups 1.51 % and klason lignin 26.91 %, revealing that the glucan was almost not affected by the autohydrolysis process and a solid residue with increased glucan percentage was obtained (Ruiz et al., 2011b). This increase of glucan could be correlated to the solubilization of hemicellulose components, because the hemicellulose is more amorphous and less stable than cellulose and, as expected, results show a substantial removal of hemicelluloses during autohydrolysis process (Mok and Antal, 1992). These results are in agreement with the data reported for a similar feedstock; Gullón et al. (2010) observed a similar behaviour for rye straw and using a similar pretreatment process with switchgrass, Suryawati et al. (2008) reported an increase in glucan content from 36.6 % to 56.6 % and a decrease in xylan content from 21.0 % to 2.4 %. The klason lignin content follows a similar pattern to glucan and the majority of the lignin content remains in the solid phase. Kristensen et al. (2008) showed that hydrothermal pretreatment caused re-localization of lignin on the surface of LCM and subsequently the materials are more susceptible to enzymatic hydrolysis.

7.3.3 KINETICS OF SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF)

The experimental design matrix is given in Table 7.1. Eighteen experiments were performed to evaluate the ethanol yield, ethanol and CO_2 concentration using SSF process (performed in duplicate) by a flocculating *S. cerevisiae* CA11 using as substrate the autohydrolysis pretreated wheat straw. During all SSF assays, continuous increases in ethanol content occurred whereas glucose content remained very low. These results

indicate that glucose from enzymatic hydrolysis could be fermented to ethanol by *S. cerevisiae* CA11, thus showing a good fermentation performance by the yeast.

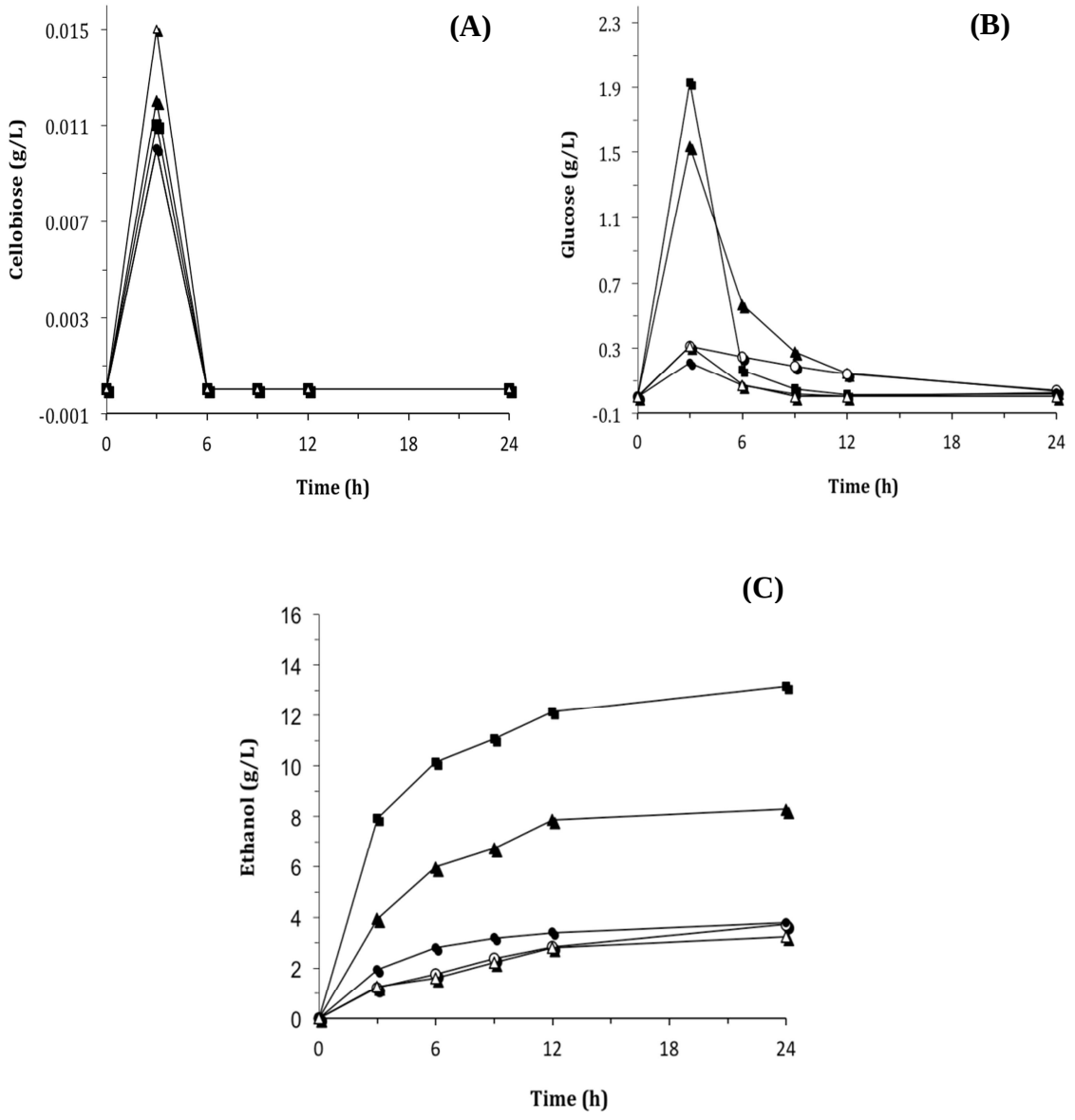


Figure 7.6. Cellobiose (A), glucose (B) and ethanol (C) concentrations obtained in SSF assays carried out under the condition of experiments 2, 4, 5,7 and 11 of Table 7.1. 45 ºC, 3 % of cellulose, 30 FPU of enzyme (■); 30 ºC, 2 % of cellulose, 30 FPU of enzyme (▲); 45 ºC, 2 % of cellulose, 5 FPU of enzyme (●); 30 ºC, 3 % of cellulose, 5 FPU of enzyme (○); 37.5 ºC, 2 % of cellulose, 17.5 FPU of enzyme (△).

Enzymatic hydrolysis of cellulose to glucose first produced a disaccharide, cellobiose, which was then hydrolyzed to a monosaccharide, glucose. The experiments had a similar pattern for cellobiose and glucose concentration during the initial 12 h (Figure 7.6A and B). No cellobiose accumulation was observed throughout fermentations as it was hydrolyzed to glucose continuously, indicating sufficient β-glucosidase activity in the cellulase preparation and low inhibition by glucose. Figure 7.6B and C show the concentration profiles determined for glucose and ethanol in the experiments 2, 4, 5, 7 and 11 of Table 7.1. Glucose from enzymatic hydrolysis was rapidly consumed by *S. cerevisiae* as indicated by a decrease in glucose from 0 to 12 h and, in other cases, from 0 to 24 h (Figure 7.6B). Ethanol concentration remained relatively constant, indicating cessation of fermentation. The SSF process was completed after 48 h. The highest ethanol concentration on the pretreated wheat straw was 14.85 g/L at 45 ºC for an enzyme loading of 30 FPU/g cellulose with 3 % of effective cellulose (experiment no. 7), corresponding to an ethanol yield of 82.40 % and 14.28 g/L of CO_2. The greatest ethanol yield observed was 85.71 % (experiment no. 2), and the lowest ethanol yield observed was 32.10 % at 30 ºC (experiment no. 4), with enzyme loadings of 30 and 5 FPU/ g cellulose, respectively.

The greatest CO_2 concentration was 14.27 g/L (experiment no. 7) and the lowest was 4.64 g/L (experiment no. 11). Table 7.1 shows the theoretical ethanol yield, ethanol and CO_2 concentration for each temperature/substrate/enzyme loading combination; larger ethanol yields suggest greater hydrolysis of cellulose to glucose. Walsum et al. (1996) reported an ethanol production of 18 g/L with 90 % of final conversion in 75 h using the same pretreatment and hard wood flour as raw material and Negro et al. (2003) achieved an ethanol concentration of 20 g/L in 72 h using poplar biomass in SSF process using steam explosion pretreatment.

7.3.4 STATISTICAL ANALYSIS OF SSF

Multiple regression analysis and analysis of variance (ANOVA) of the experimental data were performed for the mathematical model fitting. The models in terms of

normalized values (Table 7.2) expressed in Eq. (7.6), (7.7) and (7.8) represent the ethanol yield, $Y_{EtOH(\%)}$, ethanol concentration, C_{EtOH}, and C_{CO2} concentration, as function of temperature (X_1), substrate (X_2) and enzyme loading (X_3), respectively. Table 7.1 shows the values predicted by the obtained models.

$$Y_{EtOH(\%)} = 45.61 + 2.76X_1 - 0.23X_2 + 15.13X_3 + 5.52X_1X_2 - 2.05X_1X_3 - 0.33X_2X_3 + 8.66X_1^2 + 6.83X_2^2 + 1.82X_3^2 \quad (R^2 = 0.92) \quad \textbf{(7.6)}$$

$$C_{EtOH} = 7.67 + 0.52X_1 + 1.81X_2 + 2.23X_3 + 0.92X_1X_2 - 0.28X_1X_3 + 0.52X_2X_3 + 0.88X_1^2 + 0.84X_2^2 - 0.13X_3^2 \quad (R^2 = 0.92) \quad \textbf{(7.7)}$$

$$C_{CO_2} = 7.17 + 0.47X_1 + 1.74X_2 + 2.17X_3 + 1.09X_1X_2 - 0.30X_1X_3 + 0.61X_2X_3 + 0.90X_1^2 + 0.83X_2^2 - 0.028X_3^2 \quad (R^2 = 0.89) \quad \textbf{(7.8)}$$

The ANOVA results are listed in Table 7.3., 7.4. and 7.5.revealed that second-order polynomial models adequately represent responses of ethanol yield, ethanol and CO_2 concentration as shown by coefficients of determination R^2, indicating that 92.4, 92.3 and 89.2 % of the variability in the responses could be explained by the models. These values were in reasonable agreement with the adjusted determination coefficient R^2adj = 0.838, 0.84 and 0.771 respectively. As shown in Table 7.3, for ethanol yield, the model *F*-value of 10.81 is high compared to the tabular $F_{9,8}$ value of 3.39 indicating that the model was significant. The same situation occurred with the models for ethanol and CO_2 concentration, where the model *F*-value is 10.74 and 7.37 respectively.

According to ANOVA results for ethanol yield (Table 7.3), all the linear X_1, X_2, X_3, square X_1^2, X_2^2, X_3^2 and interactions X_1X_2, X_1X_3, X_1X_2 terms have significant effect on ethanol yield responses with *p*-value under a significance level of α = 0.05. These effects can be visualized in the standardized Pareto charts (Figure 7.7A). It can be observed that enzyme loading, temperature-substrate interactions and temperature are important variables at the 95% confidence level on the ethanol yield and that the effect of enzyme loading, when raised from the lowest to the highest level, is positive (Figure 7.7A). This positive effect of X_3 shows that ethanol yield is improved at higher enzyme loadings.

Li et al. (2009) observed that during the SSF process, ethanol yield was 94.7 % using similar conditions and bermudagrass as raw material. Linde et al. (2008) evaluated the influence of low enzyme loading on SSF using the same raw material obtaining an ethanol yield of 67 %. Varga et al. (2004) reported an ethanol yield of 75 % using high solids concentration in SSF and concluded that increased enzyme loadings accelerated the enzyme reaction, resulting in higher ethanol yield. When observed in terms of temperature, yields increased with increases in temperature up to 45 °C. However, at low substrate concentrations, an increase in cellulase concentration of 30 FPU/g of cellulose showed higher yields. This could be due to the low quantity of substrate and the presence of excess enzyme. Also, the high ethanol yields indicate the absence, or the presence at non-inhibitory concentrations, of inhibitors of the fermentation process.

Table 7.3. Analysis of variance (ANOVA) for ethanol yield (Y_{EtOH}) model as a function of temperature (X_1), substrate (X_2), enzyme (X_3).

Source	Sum of Squares	d.f	Mean Square	*F*-value	*p*-value
Model	3676.62	9	408.51	10.81	0.0013 *
X_1	76.60	1	76.60	1653.49	<0.0001 **
X_2	0.56	1	0.56	12.18	0.0397 *
X_3	2321.05	1	2321.05	50103.78	<0.0001 **
X_1X_2	244.07	1	244.07	5268.65	<0.0001 **
X_1X_3	33.58	1	33.58	724.95	0.0001 **
X_2X_3	0.90	1	0.90	19.35	0.0217 *
X_1^2	212.33	1	212.33	4583.54	<0.0001 **
X_2^2	133.80	1	133.80	2888.37	<0.0001 **
X_3^2	6.24	1	6.24	134.66	0.00137 *
Residual	302.39	8	37.80		
Total	3979.02	17			
R^2	0.924				
R^2adj	0.838				

d.f. degree of freedom, * significant, ** highly significant

Table 7.4. Analysis of variance (ANOVA) for ethanol concentration (C_{EtOH}) model as a function of temperature (X_1), substrate (X_2), enzyme (X_3).

Source	Sum of Squares	d.f	Mean Square	*F*-value	*p*-value
Model	104.33	9	11.59	10.74	0.0014 *
X_1	2.75	1	2.75	73.30	0.0033 *
X_2	32.81	1	32.81	875.11	<0.0001 **
X_3	49.90	1	49.90	1331.01	<0.0001 **
X_1X_2	6.83	1	6.83	182.28	0.0008 *
X_1X_3	0.66	1	0.66	17.49	0.0249 *
X_2X_3	2.16	1	2.16	57.49	0.0047 *
X_1^2	2.11	1	2.11	56.26	0.0049 *
X_2^2	1.93	1	1.93	51.50	0.0055 *
X_3^2	0.05	1	0.05	1.29	0.3378
Residual	8.63	8	1.08		
Total	112.95	17			
R^2	0.923				
R^2adj	0.84				

d.f. degree of freedom, * significant, ** highly significant

Table 7.5. Analysis of variance (ANOVA) for CO_2 concentration (C_{CO2}) model as a function of temperature (X_1), substrate (X_2), enzyme (X_3).

Source	Sum of Squares	d.f	Mean Square	*F*-value	*p*-value
Model	103.85	9	11.54	7.37	0.0049 *
X_1	2.26	1	2.26	13.90	0.0336 *
X_2	30.30	1	30.30	186.47	0.0008 *
X_3	47.27	1	47.27	290.92	0.0004 *
X_1X_2	9.56	1	9.56	58.83	0.0046 *
X_1X_3	0.75	1	0.75	4.60	0.1213
X_2X_3	3.02	1	3.02	18.61	0.0229 *
X_1^2	2.22	1	2.22	13.64	0.0344 *
X_2^2	1.91	1	1.91	11.75	0.0416 *
X_3^2	0.00	1	0.00	0.01	0.9112
Residual	12.53	8	1.57		
Total	116.92	17			
R^2	0.892				
R^2adj	0.771				

d.f. degree of freedom, * significant, ** highly significant

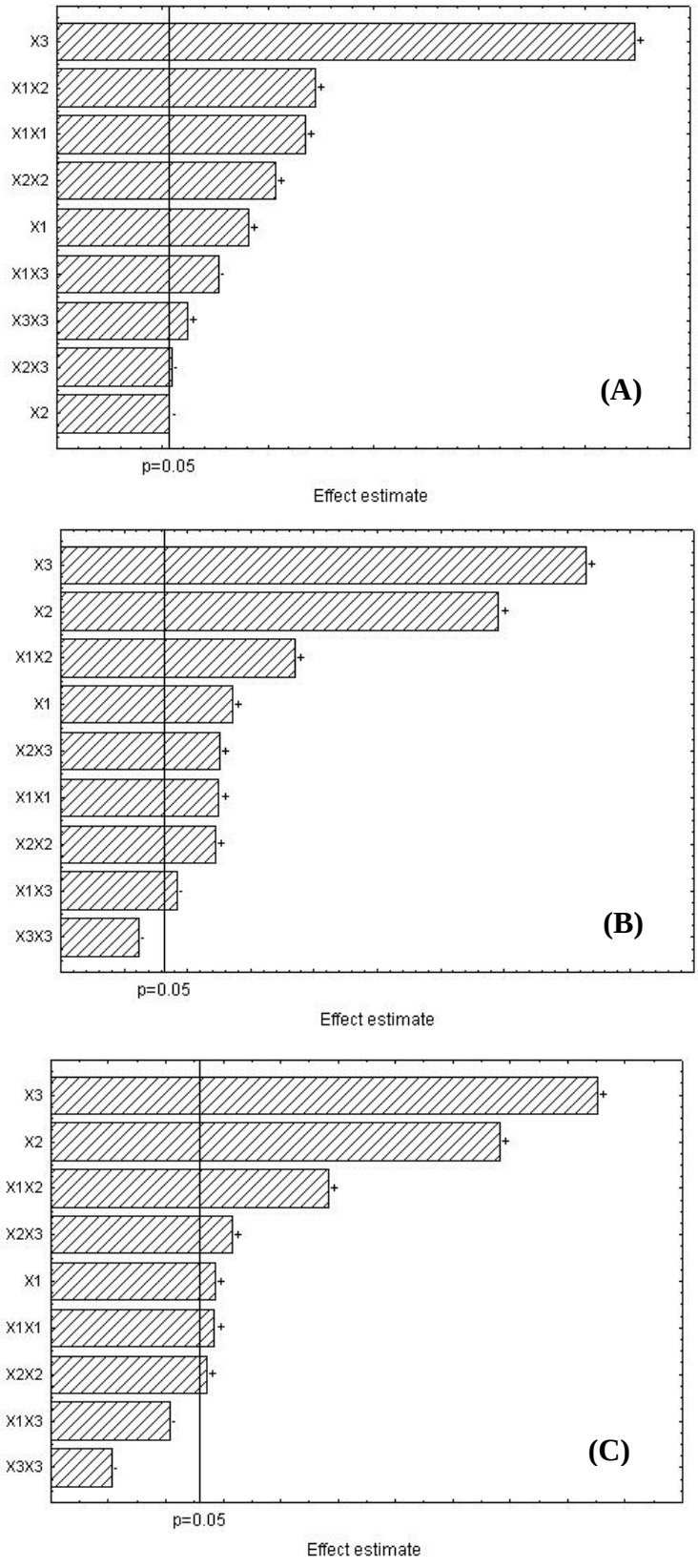
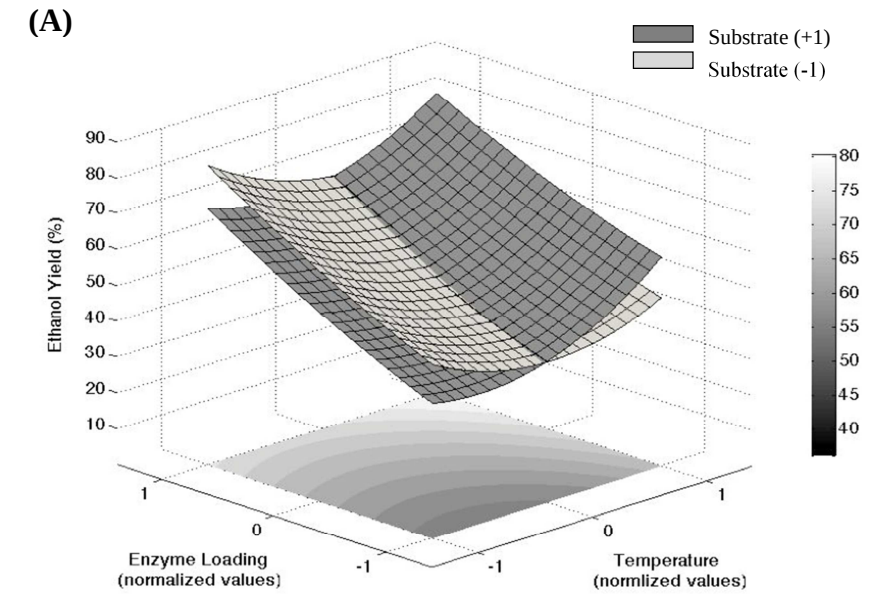
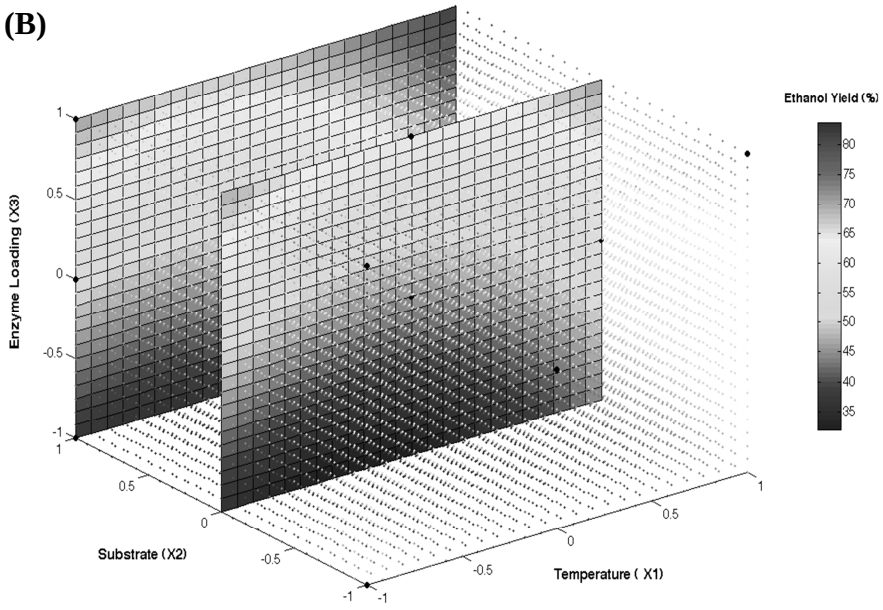


Figure 7.7. Pareto charts for standardized effects of ethanol yield (A), ethanol (B) and CO_2 (C).

Table **7.4** and Table 7.5 also show the ANOVA analyses for ethanol and CO_2 concentration. For ethanol concentration all the variables except X_3^2 have a significant effect on ethanol concentration at the 95 % confidence level and ANOVA shows that enzyme loading (X_3) is the most effective variable, followed by substrate concentration (X_2) and interaction between temperature and substrate (X_1X_2), suggesting a positive effect of enzyme loading on ethanol concentration (Figure 7.7B). This is likely to occur due to the dependence of the SSF rate on the hydrolysis rate as enzyme loading is considered one of the most important factors in ethanol production from LCM. This becomes more evident as the effects between the variables (X_1X_2, X_1^2, X_2^2, X_1X_3) on ethanol concentration were less significant. Linde et al. (2008) reported an ethanol concentration of 11.7 g/L using the same raw material and Luo et al. (2008), when evaluating the influence of enzyme concentration, reported an optimal ethanol concentration of 22.7 g/L using the same enzyme loading at 35 ºC. Faga et al. (2010) studied the effect of reduced enzyme loading on SSF process and reported a reduction of glucan hydrolysis and lowered ethanol production.

The synergetic enzymatic hydrolysis by cellulase supplemented with β-glucosidase greatly reduced the inhibition caused by cellobiose accumulation, thereby effectively improving SSF performance. In terms of CO_2 concentration, ANOVA (Table 7.5) showed that the linear X_1, X_2, X_3, square X_1^2, X_2^2 and interaction X_1X_2, X_2X_3 terms have a significant effect on CO_2 concentration responses except X_3^2 and X_1X_3. The Pareto chart presented in Figure 7.7C shows that the parameters that most strongly affect CO_2 production are enzyme loading, substrate concentration and the interaction between temperature and substrate concentration at the 95 % confidence level. The positive effect of X_3, X_2 and X_1X_2, when raised from the lowest to the highest level is positive. By comparing Figure 7.7B and Figure 7.7C, it can be observed that operating variables had similar effects on ethanol and CO_2 concentration. This should be expected having in mind the stoichiometry of the conversion of glucose into ethanol and CO_2 in the alcoholic fermentation process.


(A)
Substrate (+1)
Substrate (-1)
Ethanol Yield (%)
90
80
70
60
50
40
30
20
10
80
75
70
65
60
55
50
45
40
1
0
-1
Enzyme Loading
(normalized values)
Temperature
(normlized values)


(B)
Ethanol Yield (%)
Enzyme Loading (X3)
Substrate (X2)
Temperature (X1)

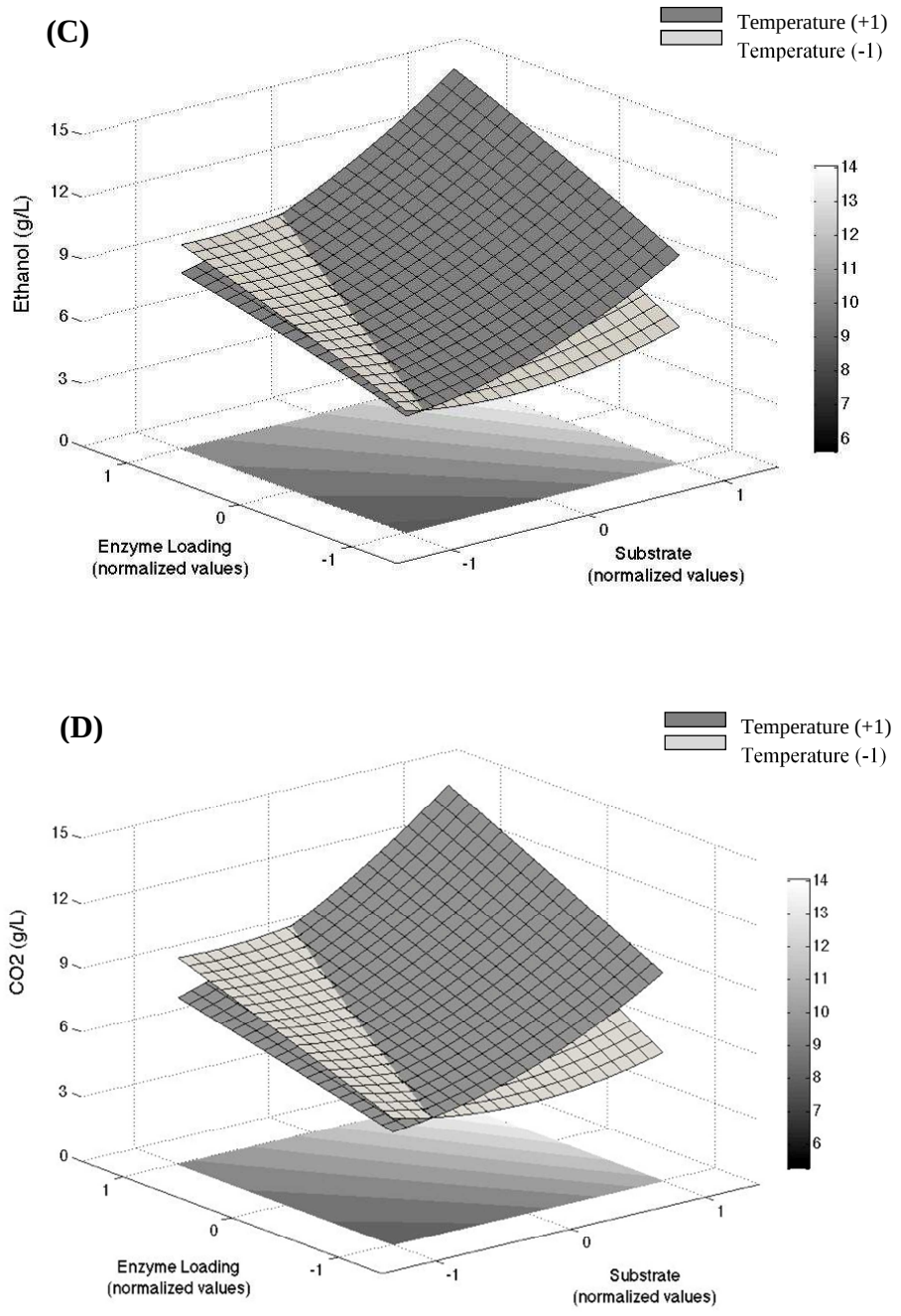


Figure 7.8. Response surface and contour plot for SSF process. (A) Ethanol yield variation as a function of enzyme loading and temperature at two substrate levels; (B) Ethanol yield in function of high substrate; (C) Ethanol concentration variation as a function of enzyme loading and substrate at two temperature levels; (D) CO_2 concentration variation as a function of enzyme loading and substrate at two temperature levels.

Response surfaces were drawn as three-dimensional plots of the second-order polynomial models (Eq. 7.6, 7.7, and 7.8) as a function of the two most strongly influencing variables. Ethanol yield was plotted as a function of enzyme loading and temperature, for substrate values kept constant both at the highest (+1) and at the lowest level (-1) (Figure 7.8A). Figure 7.8B shows that a high ethanol yield could be obtained at a high enzyme loading and high temperature at high level of substrate concentration. This could be due to the depletion of the substrate and the presence of excess enzyme. At low enzyme loadings, increases in substrate concentration showed improvement in terms of conversion. However, at high enzyme loadings, increases in substrate concentration resulted in improved yield. In the case of ethanol and CO_2 production, the most important variables were enzyme loading and substrate concentration and response surfaces were plotted as a function of these variables, for temperatures kept constant both at the highest (+1) and at the lowest level (-1) (Figure 7.8C and Figure 7.8D).

Figure 7.8C and Figure 7.8D show that in order to obtain high ethanol and CO_2 concentrations high enzyme loadings and substrate concentrations could be used at high temperatures. An increase in substrate concentration resulted in better ethanol production, which is due to the consumption of sugars by *S. cerevisiae* CA11. Temperature is a crucial factor for SSF process, so a compromise between the optimal temperatures for the actions of cellulolytic enzymes and yeast is needed. However, it must be pointed out that these results were obtained with a thermolerant yeast strain that made possible to perform the SSF at a temperature close to the optimal for the enzyme action, which was a real advantage.

7.4 CONCLUSIONS

The results of yeast strains indicate that the flocculant *Saccharomyces cerevisiae* CA11 was able to produced ethanol, showing a good performance at high temperatures. Moreover, autohydrolysis is an effective pretreatment that increased the cellulose concentration of wheat straw raw material, making it a good substrate for SSF. The results of this chapter show that the flocculating, thermotolerant *S. cerevisiae* CA11 strain has potential to be used for ethanol production in SSF process at 45º C and

contributes to avoid one of the main disadvantages of SSF, while providing SSF yields comparable to those obtained with other fermenting yeasts. The experimental design used in the present study established efficient second-order polynomial models describing the effects of temperature, substrate concentration and enzyme loading on ethanol yield, ethanol and CO_2 production by a SSF process using a thermotolerant flocculating yeast strain.

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CHAPTER 8

SIMULTANEOUS SACCHARIFICATION AND BIOETHANOL FERMENTATION FROM AUTOHYDROLYSIS WHEAT STRAW – EFFECT OF STIRRING SPEED

ABSTRACT

Wheat straw is nowadays being considered a potential lignocellulose raw material for fuel ethanol production of second generation and as an alternative to conventional fuel ethanol production from cereal crops. In the present study, autohydrolysis wheat straw was used as substrate in simultaneous saccharification and fermentation (SSF) process for bioethanol production *Saccharomyces cerevisiae* CA11. In order to evaluate the effect of stirring on ethanol yield four stirring speeds were studied. When the SSF was conducted at 150 rpm, 11.33 g/L was obtained after 96 h. When the SSF was conducted at higher stirring speed (250 rpm), 15.09 g/L of ethanol was obtained. This corresponds to an overall ethanol yield of 63.5 % and 84.49 %, respectively. In terms of ethanol yield there were statistical significant ($p < 0.05$) between the different stirring speeds. The results show that combining high temperatures and stirring speed can be an alternative to achieve high levels of ethanol yield in SSF

8.1 INTRODUCTION

Industrial ethanol production in the US has grown vastly over the past decade. The RFA (Renewable Fuels Association) reported US ethanol production increased from 175 to 13,230 millions of gallons from 1980 to 2010 (RFA, 2011). One of the most promising processes to convert cellulose from lignocellulose materials (LCM) into ethanol is the simultaneous saccharification and fermentation (SSF) process. In this process, the solid portion of pretreated LCM is gradually hydrolyzed into the liquid slurry containing monosaccharides sugars (glucose), oligomers sugars and insoluble lignin/ashes under the catalysis of enzymes and this glucose is converted quickly to ethanol by the fermenting microorganism in the same reactor, thus minimizing end-product inhibition to cellulase caused by glucose and cellobiose accumulation (Chen and Wang, 2010). Other advantage of this process is the shorter fermentation time and a reduced risk of contamination, due to the high temperature of the process and the presence of ethanol in the medium (Olofsson et al., 2008).

One disadvantage of SSF, however, is that the optimum conditions for the cellulases and the microorganisms differ. The difference in pH optimum is small, but the optimum temperature differs more. One approach to improve SSF is by using thermotolerant strain, producing ethanol in SSF at higher temperature. On the other hand, in practice, when the fermentation starts and carbon dioxide generates, a complex gas-liquid-solid multiphase system forms. In a recent work, Zhang et al. (2010) suggested that the mixing is the central barrier on the use of a SSF bioreactor with LCM solids loading, to achieve high ethanol. The sufficient mixing capacity, the low energy consumption, and the low stress to the enzyme and microbial cell should be considered as a key factor for designing mixing processes in LCM.

Yeasts isolated from extreme environments exhibit the capability of growing at high temperatures while producing ethanol; a proof of this is the industrial ethanol fermentation in some tropical countries as Brazil where fermentation takes place at ambient temperatures and during the process the temperature can reach 41 ºC as, due to its expensive costs (Vianna et al., 2008), no cooling systems are used. This requires the use of yeast strains able to produce high ethanol yields at such high temperatures.

Abdel-Banat et al. (2010) demonstrated that if the fermentation step could be performed at higher temperatures, for instance within a 40-50 ºC range, significant cost reductions in fuel ethanol production could be obtained. Advantages of processing at higher temperatures include a more-efficient simultaneous saccharification and fermentation, significant reduction of contamination and a continuous shift from fermentation to distillation (Nonklang et al., 2008; Shi et al., 2009). Additionally, the use of flocculating yeasts is one of the most interesting ways to provide an increase of the efficiency of bioethanol production processes as a significant reduction of capital costs is achieved with the elimination of centrifugation (or at least a substantial reduction of the demand for such an expensive operation), making the process more competitive (Vicente et al., 1998; Domingues et al., 1999).

The flocculation of yeast cells is a reversible, asexual and calcium-dependent process in which cells adheres to form flocs consisting of thousands of cells, the use of high cell density systems being investigated and used for separating yeast cells from beer in the brewing industry. In fact, these systems present several advantages as reduced downstream processing costs, reuse of the biomass for extended periods of time, higher productivity, protection against ethanol stress, and resistance to contamination by other microorganism (Verstrepen et al., 2003; Soares, 2011; Zhao and Bai, 2009; Domingues et al., 2000). Overall, improved efficiency of the SSF will be obtained by using a yeast strain that can work at higher temperatures and has flocculant properties. The objective of this chapter was to perform SSF using autohydrolysis wheat straw pretreated as substrate in order to evaluate the influence of stirring speed on ethanol yield by *Saccharomyces cerevisiae* CA11.

8.2 EXPERIMENTAL PROCEDURES

8.2.1 YEAST STRAIN CULTIVATION

The flocculating *S. cerevisiae* CA11 was obtained from the microbial collection at the Microbial Physiology Laboratory/Department of Biology from the Federal University of

Lavras (UFLA), Brazil. The flocculating yeast strain was isolated from a ‘‘cachaça’’ distillery in the state of Minas Gerais, Brazil and used in all the fermentation experiments. The strain was kept on agar plates made of yeast extract 10 g/L, peptone 20 g/L, agar 20 g/L and D-glucose 20 g/L as an additional carbon source at 4 ºC.

8.2.2 SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

In order to evaluate the performance of flocculant *Saccharomyces cerevisiae* CA11 in SSF, wheat straw pretreated with autohydrolysis at 180 ºC for 30 min was used. The chemical composition was as follows: cellulose (as glucan) 63.7 %, xylan 7.55 % and Klason lignin 26.91 %, previously evaluated by Ruiz et al. (2011). Two commercially available enzyme solutions, Celluclast 1.5 L and β-glucosidase were used. These enzymes were kindly supplied by Novozymes A/S (Bagsvaerd, Denmark). The enzyme activities of commercial concentrates were 43.05 FPU/mL for Celluclast 1.5 L and 576.39 UI/mL for Novozym 188 (Ruiz et al., 2012). In the chapter 6, the susceptibility of autohydrolysis pretreated wheat straw to enzymatic saccharification was studied. The results showed that the enzymatic saccharification conversion of cellulose to glucose was 90.88 %.

8.2.2.1 Yeast inoculum preparation

Yeast for inoculation was grown in 250 mL Erlenmeyer flasks with 125 mL of sterile culture medium containing 50 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, 0.5 g/L $(NH_4)_2HPO4$ and 0.5 g/L $MgSO_4.7H_2O$; glucose was sterilized separately from the other components to prevent damage to the nutritional qualities of the medium. Yeast growth was carried out at 30 ºC and 150 rpm in an orbital shaker for 10–12 h. The cell suspension was aseptically collected by centrifugation (15 min at 7885 x *g*, 4 ºC) and suspended in sterile 0.9 % NaCl to a concentration of 200 mg fresh yeast/mL. The yeast cells were inoculated at about 8 mg fresh yeast/ mL into 700 mL of culture medium to start the fermentation (Pereira et al., 2010).

8.2.2.2 SSF bioreactor and batch operation

SSF experiments were performed on autohydrolysis pretreated solids from wheat straw in a 2-L stirred tank bioreactor (Autoclavable Benchtop Fermenter Type R'ALF, Bioengineering AG, Wald, Switzerland), equipped with a Rushton flat blade impeller (Figure 8.1) in batch mode with a working weight of 0.7 kg. The bioreactor contained 4.7 - 5 % of autohydrolysis pretreated solids, 50 mM citrate buffer solution (pH 4.8 – 5). The fermentation medium was supplemented with 1% w/v yeast extract and 2% w/v peptone.

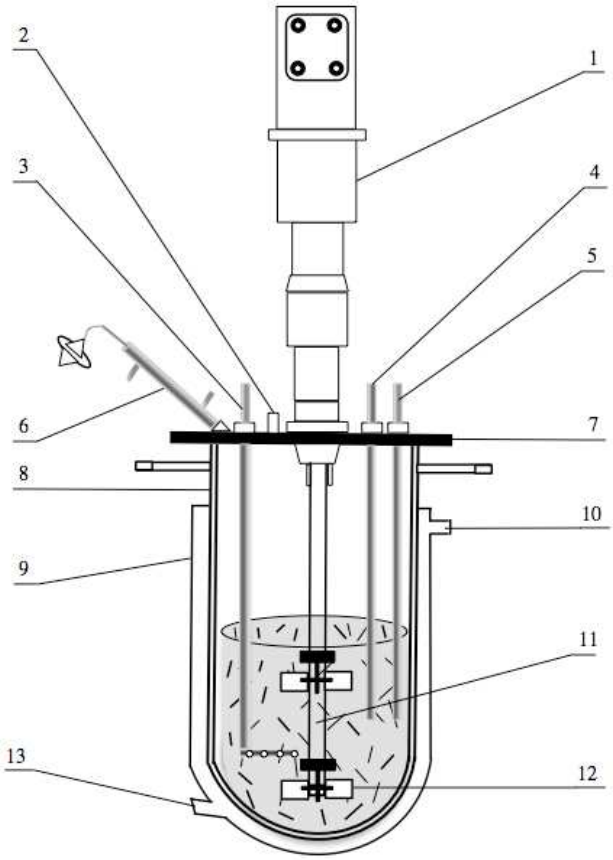


Figure 8.1. Diagram of bioreactor for simultaneous saccharification and fermentation process: (1) motor, (2) sample port, (3) gas disperser, (4) pH-meter port, (5) thermometer port, (6) condenser, (7) lid, (8) tank wall, (9), water-bath jacket, (10) water–bath jacket outlet, (11) drive shaft, (12) Rushton flat blade impeller (13) water-bath inlet.

The moisture content of autohydrolysis pretreated solids was considered as water in the material balances and the necessary amount of deionized water was calculated and added to make the total weight of 0.7 kg. The bioreactor (containing autohydrolysis pretreated solids and yeast nutrients) was sterilized in an autoclave at 121 ◦C for 20 min. SSF was started by adding enzymes and flocculating *S. cerevisiae* CA11. The amount of Celluclast 1.5 L added corresponded to a cellulase activity of 30 FPU/g cellulose, and the amount of Novozyme 188 added corresponded to a β-glucosidase activity of 60 IU/g cellulose. Spindler et al. (1989) reported that the β-glucosidase supplementation is necessary to achieve efficient cellulose conversion. The SSF experiments were performed at pH 4.8 ± 0.3 and 45 ºC (Figure 8.2). Zhang et al. (2010) reported that the mixing of solid lignocellulose in SSF is crucially important to increase the ethanol production, for this reason also, the influence of stirring on glucose bioconversion to ethanol by *S. cerevisiae* CA11 were investigated using four different stirring speeds (150, 180, 200 and 250 rpm, respectively). These parameters were monitored on the control panel. All the experiments were carried out under sterile conditions and samples of 2 -3 mL were withdrawn aseptically with serological pipette at 3 h intervals for the first 12 h and at 24 h intervals until a total of 96 h. Samples were immediately cooled on ice and centrifuged at 8260 x g for 10 min. Ethanol concentration and the remaining sugars (glucose and cellobiose) were determined by means using HPLC.

The ethanol yield was calculated assuming that all the potential glucose in autohydrolysis pretreated solids is available for fermentation and that 1 g of glucose theoretically gives 0.51 g of ethanol and 1 g of glucan gives 1.111 g of glucose, according to Dowe and McMillan (2001). Therefore, the theoretical ethanol concentration in the SSF studies was 17.82 g/L.

The ethanol yield was calculated according to the NERL standard procedure (2001).

$$\text{Ethanol yield (\%)} = \frac{[EtOH]_f}{0.51f[Biomass]\text{x}1.111}\text{x}100 \qquad (8.1)$$

where $[EtOH]_f$ is the ethanol concentration at the end of the fermentation (g/L). The term "0.51 × *f* × [*Biomass*] × 1.111" corresponds to the theoretical ethanol concentration, where [*Biomass*] is the dry biomass weight concentration at the beginning of the fermentation (g/L); *f* is the cellulose fraction of dry biomass (g/g); 0.51 is the conversion factor for glucose to ethanol based on the stoichiometry of the reaction and 1.111 is the conversion factor for cellulose to equivalent glucose.

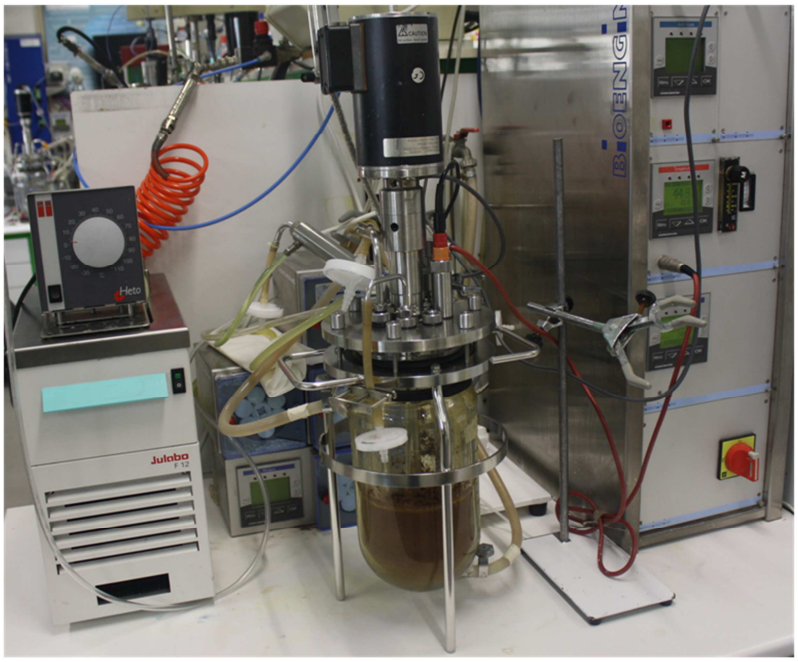


Figure 8.2. SSF bioreactor operated with autohydrolysis pretreated wheat straw.

8.2.3 STATISTICAL ANALYSIS.

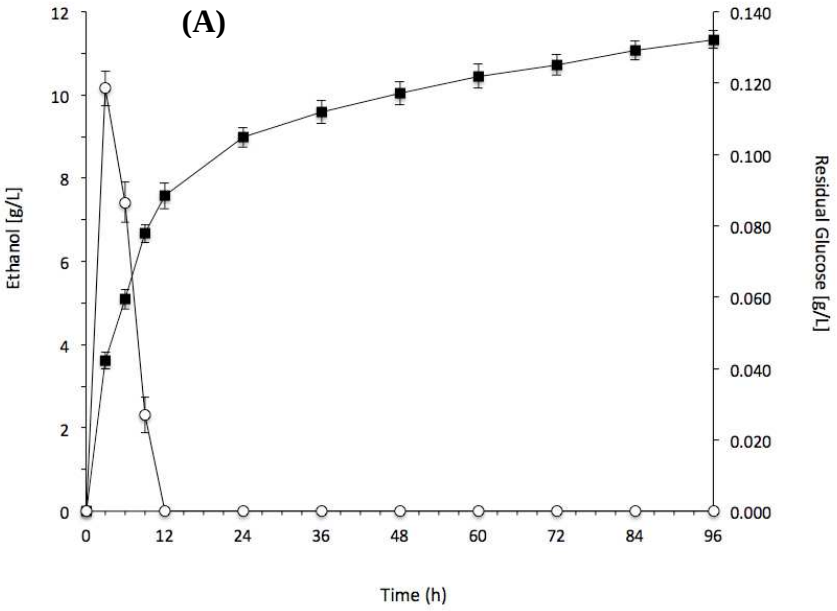
The statistical analysis of the stirring speeds on ethanol yield was carried out with the single-factor analysis of variance (ANOVA), the multiple comparison tests and graphic representation by using the box plot was used to determine the statistical significance with a 95 % confidence level. For the data analyses, MATLAB® Version 7.8.0, R2009a software (MathWorks, Inc., Natick, Massachusetts, USA) was used.

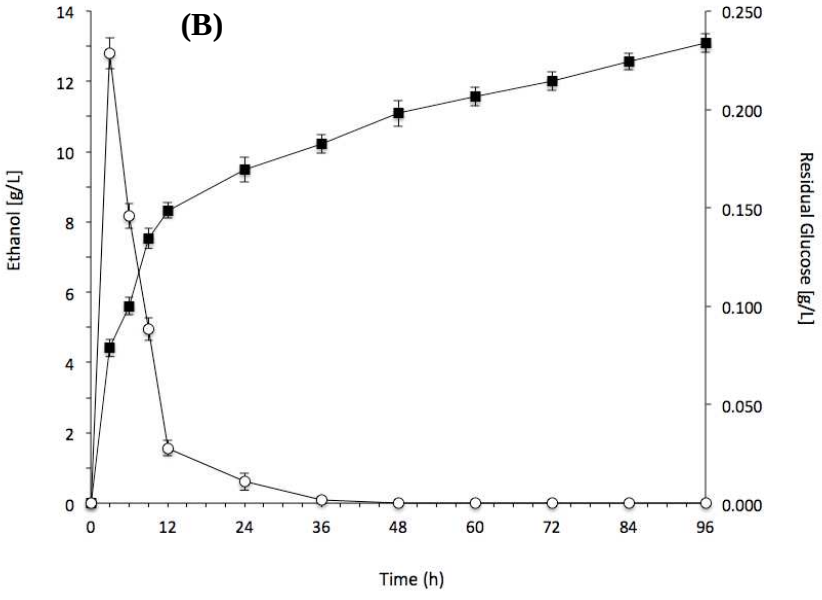
8.3 RESULTS & DISCUSSION

8.3.1 SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF)

SSF was run at four different stirring speeds 150, 180, 200 and 250 rpm. Figure 8.3A-D, showed the time evolution of ethanol concentration and residual glucose. During all SSF assays, continuous increases in ethanol content occurred whereas glucose content remained very low. No cellobiose accumulation was observed throughout fermentations as it was hydrolyzed to glucose continuously, indicating sufficient β-glucosidase activity in the cellulase preparation and low inhibition by glucose. The residual glucose concentration in all SSFs, shown in Figure 8.3A-D, increased during the first 3 h indicating that hydrolysis of glucan from pretreated wheat straw to glucose was occurring faster than ethanol production. After 3 h, ethanol production occurred faster than hydrolysis, resulting in a decrease in glucose concentration. At 24 h, the residual glucose had been reduced to < 0.1 g/L remaining at these low values till the end of the experiment. The *S. cerevisiae* CA11 produced more than 8 g/L of ethanol at 24 h for all SSF assays (Figure 8.3A-D). The highest ethanol concentration achieved by *S. cerevisiae* CA11 was 15.09 g/L at 96 h and 250 rpm. The concentration of ethanol (11.33 g/L) at 150 rpm was lower than the other conditions. Previous research has found similar ethanol production results at elevated temperatures to those of the *S. cerevisiae* strain used in this study. Edgardo et al. (2008) performed SSF on organosolv-pretreated substrate using a strain of *S. cerevisiae* at 40 ºC and reported an ethanol concentration of 24 g/L after 60 h. Kádar et al. (2004) achieved ethanol concentration of 14.2 g/L using industrial wastes (old corrugated cardboard) at 40 ºC.

Krishna et al. (2001) reported an ethanol concentration of 21 g/L from sugar cane leaves as raw material at 40 ºC. In a recent work, Romaní et al. (2010) reported a similar behaviour of ethanol production using hydrothermal pretreated *Eucalyptus globulus* wood as raw material. Zhu et al. (2006) obtained the optimal point of SSF process, achieving an ethanol concentration of 34.3 g/L with *S. cerevisiae* YC-097 using microwave-assisted alkali pretreated wheat straw at 40 ºC in 72 h.


(A)
Ethanol [g/L]
Residual Glucose [g/L]
Time (h)
0
2
4
6
8
10
12
0.000
0.020
0.040
0.060
0.080
0.100
0.120
0.140
0
12
24
36
48
60
72
84
96


(B)
Ethanol [g/L]
Residual Glucose [g/L]
Time (h)
0
2
4
6
8
10
12
14
0.000
0.050
0.100
0.150
0.200
0.250
0
12
24
36
48
60
72
84
96

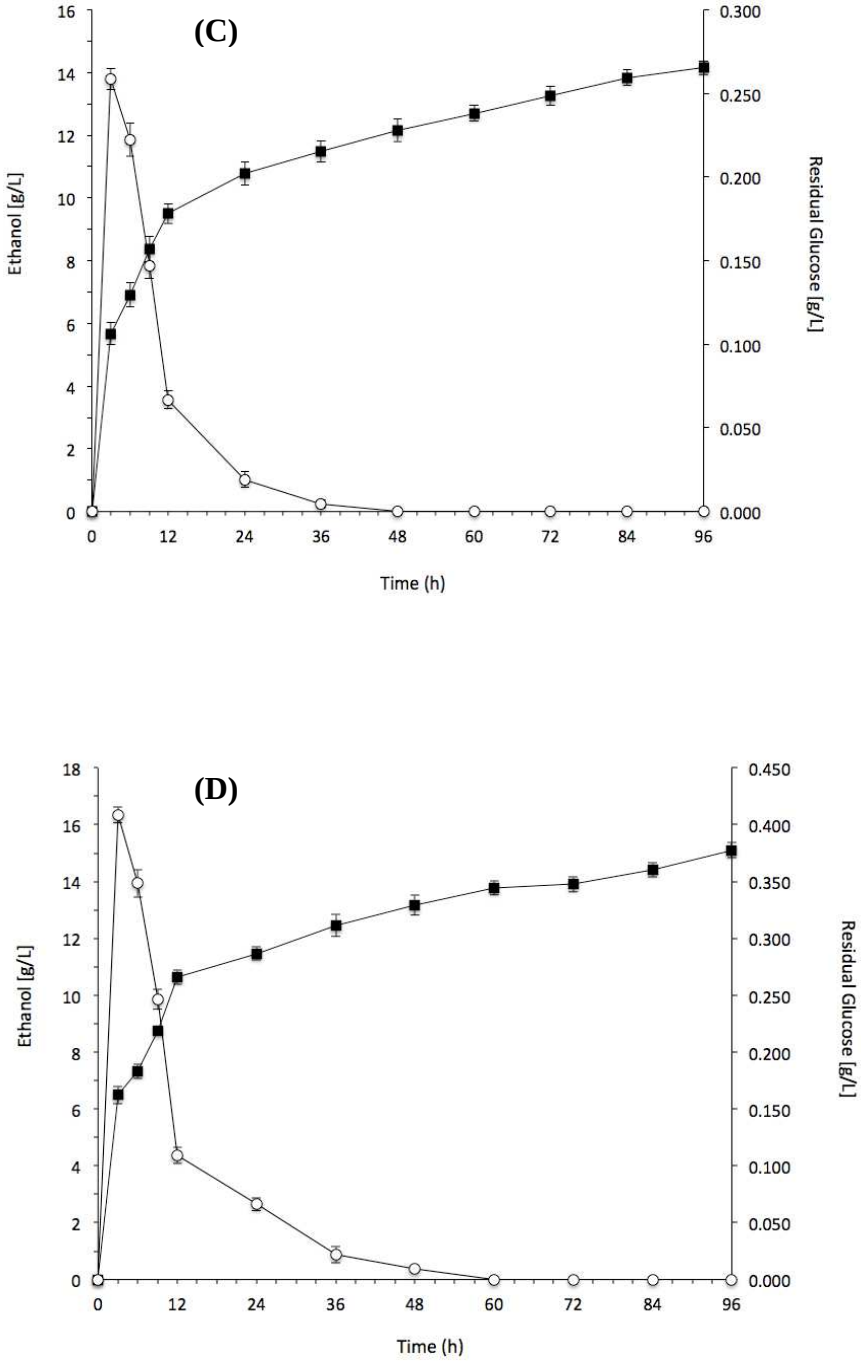


Figure 8.3. Theorical ethanol yield at different stirrer speeds: (■) 150 rpm; (●) 180 rpm; (□) 200 rpm; (▲) 250 rpm.

An important advantage of SSF over separate hydrolysis and fermentation (SHF) was founded by Alfani et al. (2000), which reported that the SSF process required a much shorter overall process time (30 h) than the SHF process (96 h) and resulted in a large increase in ethanol productivity (0.837 g/L*h for SFF compared to 0.313 g/L*h for SHF).

During SSF with *S. cerevisiae* CA11, the experimental ethanol yield was lower than the theoretical yield of ethanol. Ethanol yields from autohydrolysis pretreated wheat straw using *S. cerevisiae* CA11 at different stirring speeds are shown in the Figure 8.4. A maximum ethanol yield of 84.49 % was observed at 250 rpm, whereas the lowest value was 63.50 % at 150 rpm in 96 h. The high ethanol yield for 250 rpm and 200 rpm (80.29 %) indicate that the stirring speed had a positive influence on ethanol yield. The ethanol yield obtained in the present work can be well compared to others values achieved using SSF process. Hoyer et al. (2009) studied the effect of mass transfer on the ethanol yield, which increasing the stirred speed from 200 rpm to 700 rpm led to an increase in ethanol yield from 84.6 % to 95.8 %. Zhang et al. (2010) concluded that the mixing between the pretreated solids and liquid enzyme during the SSF process is crucially important, due to the increase in the apparent viscosity of the slurry when all the solid feedstock is fed into the bioreactor. Moreover, with the beginning of fermentation and generation of carbon dioxide, a complicated gas-liquid-solid multiphase system is formed and this multi-phase system may lead to the reduction in mass and heat transfer efficiency, low sugar yield and low ethanol yield. Lee et al. (2009) suggested that the ethanol yield depended of the pretreatment conditions on the substrate. Suryawati et al. (2008) reported a similar behavior of ethanol yield using *S. cerevisiae* D_5A at 37 ºC and switch-grass pretreated with autohydrolysis as substrate in SSF.

Box plots display the ethanol yield as a function of the different stirring speeds (Figure 8.5). Boxes represent the 50 % interquartile range, solid line within the boxes represents the sample median. According to the multiple comparison test, ethanol yield for 150 rpm, 180 rpm, 200 rpm and 250 rpm showed statistical significance. At 250 rpm the ethanol yield was greater than 150 rpm, 180 rpm and 200 rpm ($p < 0.05$). This statistical

significance was observed in all SSF assays, demonstrating that increasing the stirring speed in SSF process leads to an increase in the ethanol yield.

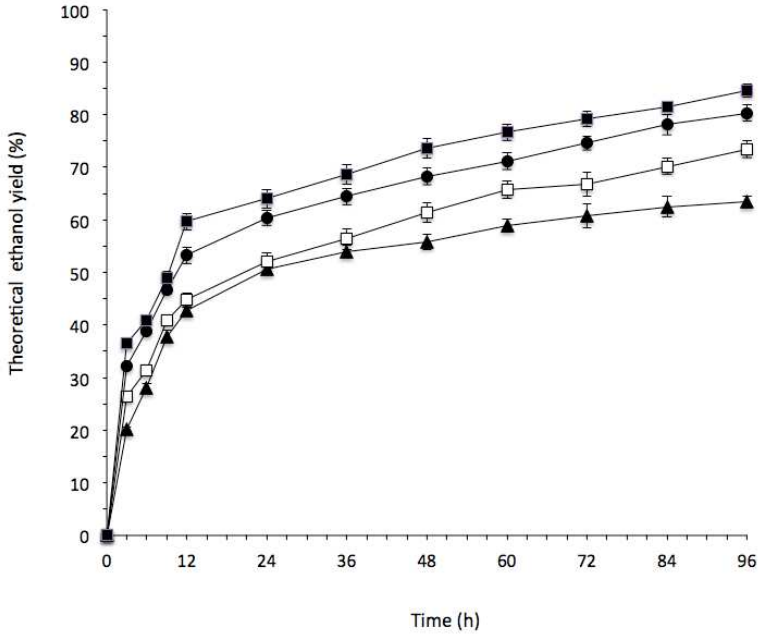


Figure 8.4. Theoretical ethanol yield at different stirrer speeds:(■) 150 rpm; (●) 180 rpm; (□) 200 rpm; (▲) 250 rpm.

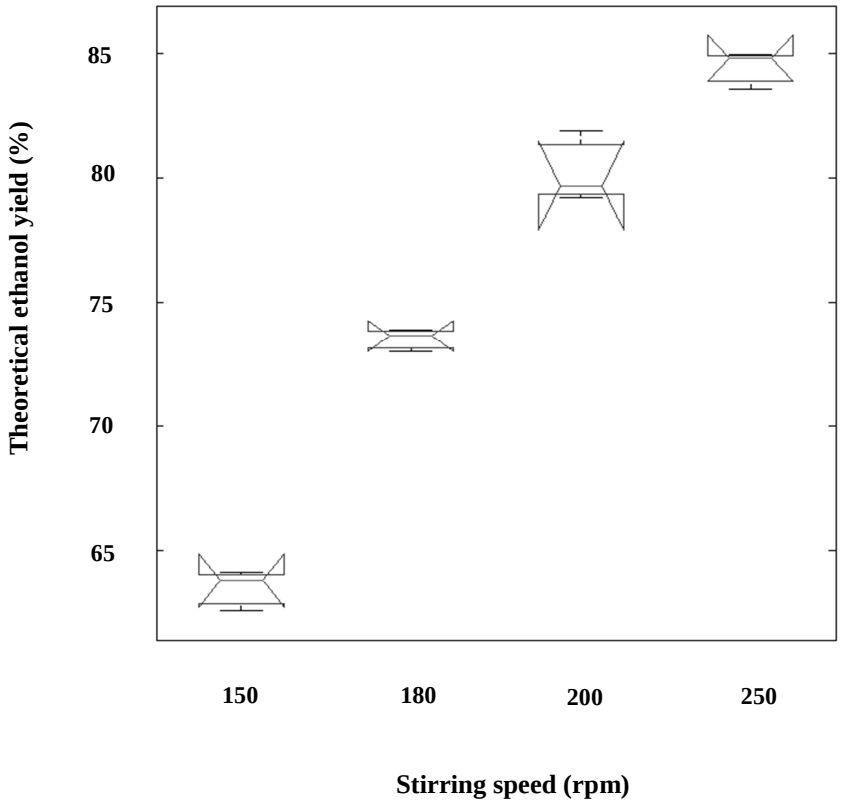


Figure 8.5. Box-plot representing the significant differences, for each theoretical ethanol yield at different stirrer speeds.

8.4 CONCLUSIONS

Bioethanol can be successfully produced by *Saccharomyces cerevisiae* CA11 using simultaneous saccharification and fermentation and autohydrolysis wheat straw pretreated as substrate; also was demonstrated that the stirring speed is an important factor that affected the efficiency of this process. Therefore, the combination of high temperatures and stirring in batch operational conditions allowed to achieve high levels of ethanol yield making the SSF process more efficient.

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CHAPTER 9

GENERAL CONCLUSIONS AND SUGGESTION FOR FUTURE WORK

9.1 GENERAL CONCLUSIONS

Bioethanol production from wheat straw using an environmentally friendly process such as autohydrolysis is an attractive alternative to the biorefinery concept. Amongst the advantages of autohydrolysis are the possibility of solubilizing the hemicellulose in the liquid fraction and obtaining a pretreated solid more susceptible to simultaneous saccharification and fermentation and, also, a delignificated solid obtained through an organosolv process.

The main conclusions that can be withdrawn from the results obtained are listed below.

The evaluation of the autohydrolysis condition led to the conclusion that the variation of the particle size distribution has a selective influence on the extraction of the total sugar from hemicellulose and that the use of a blend with defined percentages of the various particle sizes must be established before carrying out a pretreatment. The sequential autohydrolysis and organosolv process is an adequate method for the extraction of high purity lignin. The results in Chapter 5, showed that autohydrolysis is a suitable technology for the extraction of hemicellulose with suitable physicochemical characteristics for its application as reinforcement in polymeric matrix for biocomposites manufacture.

The results of the enzymatic saccharification showed that autohydrolysis pretreated solids are more effective for enzymatic saccharification.

The experimental design used in the Chapter 7 established efficient second-order model describing the effects of temperature, substrate and enzyme loading on ethanol yield, ethanol concentration. The results indicate that the *Saccharomyces cerevisiae* CA11 was able to produce ethanol and showed a good performance at high temperatures.

The scale-up of SSF was performed evaluating the effect of stirring on the ethanol yield. When the SSF was conducted at higher stirring speed (250 rpm), 15.09 g/L of ethanol was obtained. This corresponds to an overall ethanol yield of 84.49 %, concluding that the stirring speed is an important factor that affected the efficiency of the process.

In broad terms, the autohydrolysis process is an effective pretreatment that increases the cellulose content of wheat straw, making it a good substrate for SSF. Additionally, the flocculating *S. cerevisiae* CA11 strain has potential to be used for bioethanol production in SSF processes at 45º C. The fractionation and use of hemicellulose as biocomposite is according to the biorefinery concept.

9.2 GUIDELINES FOR FUTURE WORK

a) The extraction of high purity lignin through an organosolv process could be used to prepare phenolic-type resins.
b) A cost reduction of the cellulase is, therefore, necessary to make the process more economically attractive. One alternative could be the development of strategies for cellulase recovery after the SSF process.
c) In order to improve the ethanol concentration it is important to use the high dry matter content possible in the SSF process.
d) Other operational strategies should be carried out as fed-batch or continuos, in order to improve the ethanol concentration required for an economically viable industrial-scale ethanol distillation.

The results at laboratory scale showed insight into the operational strategies applicable to the production of bioethanol using autohydrolysis pretreated solids by *Saccharomyces cerevisiae*. However, the bioreactor size and the operational conditions do not allow a full evaluation of the process. An interesting next step would be to upscale to pilot plant and consider the results to further evaluate the process.