ALMAS TAJ AWAN

ORANGE BAGASSE AS BIOMASS FOR 2G-ETHANOL PRODUCTION

BAGAÇO DE LARANJA COMO BIOMASSA PARA PRODUÇÃO DE ETANOL-2G

CAMPINAS
2013

UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE QUÍMICA

ALMAS TAJ AWAN

ORANGE BAGASSE AS BIOMASS FOR 2G-ETHANOL PRODUCTION

Orientadora/ Supervisora: PROFA. DRA. LJUBICA TASIC

BAGAÇO DE LARANJA COMO BIOMASSA PARA PRODUÇÃO DE ETANOL-2G

Tese de Doutorado apresentada ao Instituto de Química da Química da Universidade Estadual de Campinas para obtenção do título de Doutora em Ciências.

Doctoral thesis presented to the Institute of Chemistry, University of Campinas to obtain Ph.D. grade in Sciences.

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA ALMAS TAJ AWAN E ORIENTADA PELA PROFA. DRA. LJUBICA TASIC

_____
Assinatura da Orientadora

CAMPINAS
2013

CATALOGING RECORD PREPARED BY SIMONE LUCAS - CRB8/8144 – LIBRARY, INSTITUTE OF CHEMISTRY, UNIVERSITY OF CAMPINAS

Aw12o

Awan, Almas Taj (1984-).
Orange bagasse as biomass for 2G-ethanol production / Almas Taj Awan. -- Campinas, SP: [s.n.], 2013.

Supervisor: Ljubica Tasic.

Thesis (doctorate) - University of Campinas, Institute of Chemistry.

1. Citrus processing waste from oranges. 2. Submerged co-culture fermentation. 3. *Xanthomonas axonopodis* pv. *citri 306*. 4. Enzymatic hydrolysis. 5. Saccharification. I. Tasic, Ljubica. II. Universidade Estadual de Campinas. Instituto de Química. III. Title.

Information for Digital Library

Portuguese title: Bagaço de laranja como biomassa para produção de etanol-2G

Portuguese keywords:
Resíduos de frutas cítricas
Fermentação submersa
Xanthomonas axonopodis pv. *citri 306*
Hidrólise enzimática
Sacarificação

Subject concentration: Organic Chemistry

Academic degree: PhD in Sciences

Thesis evaluation comission:
Ljubica Tasic [Supervisor]
Mahendra Kumar Rai
Peter Rudolf Seidl
Nelson Eduardo Durán Caballero
José Augusto Rosário Rodrigues

Date: 18/02/2013

Graduate Program: Chemistry

DEDICATION

My Parents,
Malik Taj M. Khan and Zainab K. Awan,
for their love,
support and
encouragement
in every phase of my life.

Don’t Quit!

When things go wrong as they sometimes will,
When the road you're trudging seems all uphill,
When the funds are low, and the debts are high;
And you want to smile, but you have to sigh;
When care is pressing you down a bit

Rest if you must, but don't you quit.

Success is failure turned inside out;
The silver tint of the clouds of doubt,
And you can never tell how close you are;
It may be near when it seems afar.
So, stick to the fight when you're hardest hit

It's when things seem worst that you must not quit.

Anonymous

ACKNOWLEDGEMENTS

First of all, I would like to thank God, the Almighty, for making everything possible by giving me strength, courage, wisdom, health, good resources, loving family, precious friends, caring supervisor and everything that made me accomplish this task.

I would like to express my deepest gratitude to my Ph.D. Supervisor, Prof. Dr. Ljubica Tasic, for her excellent guidance, care, patience, and providing me with an excellent atmosphere for doing research. Buba, I learned a lot from You and I am simply proud of You, being my Ph.D advisor. Also, I would like to thank Dr. Nelson Duran, who always tried to maintain a healthy atmosphere of LQB lab, sharing his life experiences during travelling and also wonderfull ideas in research line. Special thanks for Prof. Carlos Roque to provide me an opportunity to come from Pakistan and start work in his lab on Organic Synthesis. Many many thanks for my thesis evaluation committee (Prof. Dr. Mahendra Kumar Rai, Prof. Dr. Peter Rudolf Seidl, Prof. Dr. Nelson Eduardo Durán Caballero and Prof. Dr. José Augusto Rosário Rodrigues) for considering, encouraging, commenting and figuring out important areas for improving my written work and thesis.

My humble regards to *The World Academy of Science* (TWAS) and *Brazilian National Council for Scientific and Technological Development* (CNPq) for supporting my PhD studies in Brazil. Also, I would like to thank Post-graduate coordinator at Institute of Chemistry, University of Campinas to approve me as a PhD Candidate.

Some people come and leave footprints on our lives, fortunately during my four years of stay in Brazil, there were a number of people. They are so many, that it is difficult for me to write my words for all of them. But I will try to appreciate in particular those who were related to my academic area. First of all, Juliana Fattori, Sebastian Robles and Gabriel Curti for patiently correcting my Portuguese writing for annual reports. Pablo, Marla, Gago, Patricia, Jason, Ilton, Nilton, Airton, Jailton and Angelica from LASSO lab for their help and friendship during my lab hours and PhD qualification exam. Thanks to Junko Tsukamoto for always being there for

guidance in my research just like a co-supervisor. I would also like to thank Ana Paula Espindola for taking interest in my work either its written or practical and putting good suggestions. Special thanks for Fabian for staying late hours in lab and helping out in my thesis. Also, Jennifer for being my cute friend and always offering me to go for volleyball, basketball, kungfu and running (and I was always trying to escape). Chico was my only enemy in Brazil who with anti-pakistani and anti-usama jokes always made me laugh. Thanks to two little angels and my youngest Brazilian friends (kids of Buba) Alex and Aninha for taking me back to my childhood while playing with them. Many many thanks for all LQB lab member, Dani Pott, Ana Paula, Junko, Fabian, Daniela, Lilian, Camila, William, Fabio, Isabella, Alessandra, Dani, Jessica, João, Elias, Alzira, Stephany, Lucas, Priscyla, Ana Maria, Lari (I hope I didn't forget anyone). Thanks for all the technical assistance that was provided by Ricardo in HPLC analyses of my samples, Renata for Infrared analysis, Daniel for SEM analyses, Rita for GC-MS, Anderson and Fabian during my NMR analyses.

My special thanks and love for Prof. Adriana Vitorino Rossi for always taking care of me and Surraya as our Brazilian Mom. My gratitude to Prof. Marcia and Maria Helena for helping me during my painting classes. I will also not forget to mention my friends from IQ Bruna, Tiago, Felipe, Francine, Diana, Carol, Leticia, Daniella, Adriana, Eva, Lilian, Marta, Acacia, Edison, Marcelo, Celio and Paulo Filgueiras. Also, my Pakistani friends Surrayya and Fozia, Indian friends Anamika and Rashmi, Irani friend Pooyii, Colombian friends Monica, Sofia, João, Sebastian and my great sister Iram who were always there to celebrate special occasions with me, not letting me feel lonely without family.

I would like to thank my family including my younger sisters (Iram, Kiran, Masooma and Urooj), my younger brother (Zeeshan), my brother-in-law (Sarosh) and my best friend Nasima. I am very grateful to Dr Shahzad Mughal for his support and encouragement to move ahead for doctoral and post-doctoral research. Finally, I would like to thank my parents Malik Taj and Zainab Awan for always being there to cheer me up and standing by my side through the good times and the bad, I love you both and pray for your healthy and long life, ameen.

Curriculum Vitae

ALMAS TAJ AWAN

ACADEMIC DEGREES

2009-2013: Doctor of Chemistry

Chemistry Institute, University of Campinas, Sao Paulo, Brazil
Project: Orange bagasse as biomass for 2G-ethanol production
Supervisor: Prof. Dr. Ljubica Tasic
Funding agency: CNPq
Fellowship program: TWAS-CNPq post-grad fellowship

2005-2007: Master of Chemistry

Institute of Chemical Sciences
University of Peshawar, Pakistan
Project Supervisor: Prof. Dr. Fazal Mabood
Project: Selection of appropriate analytical techniques in compositional analysis

PUBLICATIONS

- **"Supramolecular catalysis"** (a review)
  Authors: Almas Taj Awan, Suryyia Manzoor, Prof. Vanderlei Inácio de Paula
  Brazilian Journal: Revista Engenho, vol. 6 – september 2012
  Available online at
  http://www.anchieta.br/unianchieta/revistas/engenho/pdf/revista_engenho06.pdf

ORAL PRESENTATIONS IN INTERNATIONAL CONFERENCES

Title: Second generation ethanol from orange bagasse via co-culture fermentations

Global Biofuels & Bioproducts Summit
19th -21st Nov, 2012 Hilton San Antonio,
Texas, USA.

Title: Orange Processing Waste as Biomass in Ethanol Green-Production

The International Conference, Nanotechnology,Biotechnology and Spectroscopy: Tools of success in the coming Era.
ICNBS 1st -3rd March, 2012,
Cataract Pyramid Resort
Cairo, Egypt.

POSTERS PRESENTED IN SCIENTIFIC EVENTS

- **Title: Second generation ethanol from orange bagasse via co-fermentations**
  Global Biofuels & Bioproducts Summit
  19th -21st Nov, 2012 Hilton San Antonio,
  Texas, USA

- **Title: Acidic Hydrolysis of Orange peel for Ethanol Production**
  PIBIC Congress,
  24th Oct, 2012
  State University of Campinas, São Paulo, Brazil

AWARDS AND RECOGNITION

- **TWAS 1st prize winner award in SUSTAINABLE ENERGY PHOTO CONTEST**
  Contest on Project's Photo
  Theme 'Sustainable energy for All'
  International Week of Science, November 2012 (Italy).

- **Inova 2012 UNICAMP Award for Scientific innovation**
  PIBIC Congress; 24th Oct, 2012; Sao Paulo, Brazil.

- **Best Oral presentation Award In an International Conference, Egypt**
  The International Conference, Nanotechnology,Biotechnology &Spectroscopy: Tools of success in the coming Era. ICNBS 1st -3rd March, 2012, Cataract Pyramid Resort, Cairo, Egypt.

- **Winner of All Pakistan Quaid Conference, 2007**
  12-13th August, 2007; Baqai Medical University, Karachi, Pakistan.

- **Best Debater University of Peshawar, 2006-07**
  Recognition by Vice Chancellor, University of Peshawar, Pakistan.

EXTRA CURRICULAR

- 2012-PED-C: Organic Chemistry Laboratory and graduate teaching Assistance; Course Supervisor: Prof. Dr. Fernando Coelho, University of Campinas, Brazil.
- 2007-2008-Pakistan Television Corporation: Anchor TV shows
- 2005-2008-Pakistan Radio Broadcasting: Anchor and Producer Radio programs.

Abstract:

ORANGE BAGASSE AS BIOMASS FOR 2G-ETHANOL PRODUCTION

Second generation biofuels from renewable resources have come forth as a result of energy security coupled with diminishing fossil fuel resources. Lignocellulosic biomass is a renewable resource, which can be converted in to liquid transportation fuels. Utilization of agro-industrial waste for the generation of biofuels makes it a cleaner production (Green Chemistry). Brazil is the world's largest producer of oranges. The current project deals with Citrus Processing Waste from Oranges (CPWO), and obtaining valuable products such as bioethanol, hesperidin, and essential oil. The process of hydrolyzing CPWO was improved and the classical way of biomass saccharification, *i.e.* acid hydrolysis, was compared with the enzyme hydrolysis. In enzyme hydrolysis, apart from applying commercial enzymes, saccharification was also investigated with protein extracts of *Xanthomonas axonopodis* pv. *citri* strain 306 (*Xac* 306), a potent pathogen that causes Citrus canker disease. Later, the obtained reducing sugars were converted into bioethanol by submerged mono- and co-culture fermentations that involved three yeast strains: *Saccharomyces cerevisiae*, *Candida parapsilosis* IFM 48375 and NRRL Y-12969, the last two being isolated from bagasse. Results demonstrated successful hydrolyses by *Xac* enzymes that released high levels of fermentable sugars. Also during co-culture fermentation processes, it was noticed that ethanol yield was improved from 50% to 62% w/w (calculated on the basis of total dry matter contents) and sugars were consumed faster. Thus by employing co-culture fermentation strategy, apart from getting better bioethanol yields, fermentation time is also reduced that makes it a cost effective technique.

Resumo

BAGAÇO DE LARANJA COMO BIOMASSA PARA PRODUÇÃO DE ETANOL-2G

Os biocombustíveis de segunda geração surgiram como fontes energéticas promissoras, podendo ser obtidos a partir de vários tipos de biomassa que não seja utilizada para alimentos. Um tipo de biomassa que apresenta baixo custo além de apresentar níveis elevados de carboidratos, é a biomassa obtida após o processamento da laranja (*Citrus processing waste from oranges*, CPWO). Há um grande interesse na exploração desta biomassa em termos da produção do bioetanol (etanol da 2G). Nosso trabalho visa melhorar os processos de hidrólise do CPWO comparando o rendimento do processo clássico de hidrólise ácida com aplicação de enzimas comerciais ou provenientes do microrganismo *Xanthomonas axonopodis* pv. *citri*, cepa 306 (um fitopatógeno). Os resultados obtidos com a presente investigação evidenciam que ocorreu a conversão bem-sucedida do CPWO em uma mistura de açúcares. A *posteriori*, os açúcares redutores que foram obtidos foram convertidos em bioetanol por meio da fermentação em mono- e co-cultura. Para tanto, foi empregada a espécie *Saccharomyces cerevisiae* e duas cepas de *Candida parapsilosis* IFM 48375 e NRRL Y-12969, sendo que as duas últimas foram isoladas a partir do bagaço da laranja. Os rendimentos em termos de bioetanol obtido nas fermentações aplicando co-culturas estavam ao redor de 50 a 62%, constituindo valores muito maiores comparados com os obtidos por cepas usadas individualmente. Além disso, os açúcares foram consumidos mais rapidamente (6 h), tornando tais processos atraentes em termos de custo e aplicações comerciais.

INDEX

LIST OF ABBREVIATIONS

5-HMF: 5-hydroxymethylfurfural
AD: Acid detergent
ADF: Acid detergent Fiber
ADL: Acid detergent Lignin
AOAC: Association of Official Analytical Chemists
bp: Base pairs
CBD: Cellulose-binding domain
CBH: Cellobiohydrolase
CD: Catalytic domain
C.P IFM: *Candida parapsilosis* IFM48375
C.P NRRL: *Candida parapsilosis* NRRL-12969
CPWO: Citrus Processing Waste from Oranges
CTAB: Cetyl trimethylammonium bromide
CTBE: Brazilian Bioethanol Science and Technology Laboratory
DM: Dry matter
DNS: Dinitrosalicylic acid
DP: Degree of polymerization
DS: Degree of synergism
EC: Enzyme commission/ classification
EG: Endo glucanase
FPU: Filter Paper Unit
GA: Galacturonic acid
GMO: Genetically modified organisms
LB: Luria Bertani
ND: Neutral Detergent
NDF: Neutral Detergent Fiber
NREL: National renewable energy laboratory
PB sheet: parallel β sheet
PNPG: *p*-nitrophenyl-*β*-D-glucopiranoside
PNP: *p*-nitrophenol
pv.: pathovar
RT: Retention time
S.C: *Saccharomyces cerevisiae*
SmF: Submerged Fermentation
SSF: Solid State Fermentation
Str: Stretching
TBI: Triple Resonance Broadband Inverse
USDA: United States Department of Agriculture
Xac 306: *Xanthomonas axonopodis* pv. *citri* (*Xac* 306)
YPD: Yeast peptone dextrose

LIST OF TABLES

LIST OF FIGURES

Figure I-13. Computational model of the structure of the exoglucanase, CBH I; showing cellulose binding module and catalytic domain in the form of a tunel, during enzyme action [figure adapted from Carvalho, 2009].

Figure I-14. Schematic representation of the Cellobiohydrolase I from *Trichoderma reesei* (PDB: 7CEL) catalytic domain with a cellooligomer. Secondary-structure elements are colored as follows: β strands, blue, green and sea green arrows; α helices, orange, yellow and brown spirals; loop regions, orange, red, green, yellow and blue coils. The cellooligomer is shown in blue and red as a ball-and-stick model [Divne *et al.,* 1998].

Figure. I-15. Crystal structure of β-glucosidase (PDB:3CMJ). (A) Overall structure of β-glucosidase. The four loops where the substrate enters are colored in pink. (B) Representation of the surface structure. The large cleft is colored in yellow. This cleft had a cavity of approximately 15 × 10 Å; therefore the related disaccharides substrate could easily enter the active site pocket [Nam *et al*., 2010].

Figure I-16. Mode of cellulolytic enzyme action [adapted from Zhu, 2005; Jørgensen *et al*, 2007].

Figure I-17. The ribbon diagram of *Aspergillus niger* pectin lyase B (PDB: 1QCX). Helices are represented by pink coils and β structure is shown by arrows. The parallel β (PB) sheet, PB1, is yellow; PB2 is blue; and PB3 is red. The antiparallel β structure within the first T3 turn and a short β strand within the third T3 loop are indicated in orange [Vitali *et al.,* 1998].

Figure I-18. (a) Citrus canker lesions caused by *Xanthomonas axonopodis* pv. *citri* on immature fruit with chlorotic halos. (b) SEM of stomata on grapefruit leaf with *Xac* bacteria entering stomatal chamber [Cubero *et al.,* 2001]. (http://www.apsnet.org/publications/apsnetfeatures/Pages/citruscanker.aspx. Web page accessed on January 13th, 2013).

Figure I-19. Time course for the enzymatic hydrolysis of lignocellulosic substrates [adapted from Mansfield *et al*., 1999].

Figure I-20. (a) Large SSF unit at Soufflet's malting plant in Arcis-sur-Aube, France. http://www.allaboutfeed.net/ (Web page accessed on July13th, 2012); (b) Large SmF Wine Unit http://www.wine-searcher.com/m/2012/11/eucalyptus-in-australian-red-wines (Web page accessed on January 12th, 2013).

Figure III-1. (a) CPWO; (b) Food processor; (c) Homogenized CPWO.

Figure IV-1. (a) Gas Chromatogram of the extracted essential oil, (b) mass spectrum of the main peak in the oil extract (D-limonene).

Figure IV-2. Extracted samples of (a) crude and (b) recrystallized hesperidin.

Figure IV-3. Scanning electron micrographs of Hesperidin with (a) 10 µm (b) 5 µm and (iii) 1 µm scales.

LIST OF SCHEMES

*

CHAPTER I- INTRODUCTION

* Figure taken from http://sustainablog.org/2011/12/community-trees-guerrilla-grafters/ (Web page accessed on January10th, 2013).

1. INTRODUCTION

1.1. Motivation

Biofuels generated from renewable resources are currently in high demand related to their prospect for being an alternative to petroleum and diesel. Development of energy from renewable resources can provide domestic energy supplies while reducing net greenhouse gas emissions and providing a more favorable energy balance than traditional petroleum fuel utilization [Farrell *et al*., 2006]. Since 2003, when "flex fuel" vehicles were introduced in the Brazilian market [Cordeiro de Melo, 2012], there was a rapid increase in consumption of bioethanol. Today, in addition to the 80% of Brazilian vehicles that consume bioethanol, small aircraft engines are also being developed that use this technology [Soccol, 2010]. On the other hand, exports of Brazilian bioethanol have increased significantly due to growing worldwide interest related to the safe use of biotechnology to reduce environmental risks [CTBE, 2012].

Biomass is an important renewable energy resource that can be efficiently used for biofuel production. Biomass can be defined as all organic matter of vegetal or animal origin, which is produced in natural or managed ecosystems (agriculture, aquaculture, forestry) [Vandame, 2009]. The most common fuels derived from biomass are ethanol, biodiesel and biogas. Biofuels are theoretically carbon neutral, which means that the carbon dioxide gas emissions after biofuel combustion are captured again by the plants. Biofuels can be classified as first, second and third generation, according to the origin and biomass processing.

Today, most biofuels in use are first generation biofuels that are derived from food crops such as seeds or grains from cereals, sugarcane etc. This situation has triggered growing concerns over future food shortages and increasing food prices [Science business report, 2011]. To solve this problem, second generation biofuels have come forth, from non-food crops or organic waste materials that comes from food wastes, manure and agricultural residues (Figure I-1).

Figure I-1. Cellulosic feedstock for second generation biofuels (figure taken from Bioeconomics of Biofuels by Stephen Polasky. http://faculty.apec.umn.edu/spolasky/. Web accessed on October 23rd, 2012).

Conventionally, sugarcane bagasse is used as a raw material to produce second generation bioethanol [Soccol *et al*, 2010], but other agro-industrial wastes can also be treated, such as citrus processing waste from oranges (CPWO). Citrus fruits are among the most popular fruits grown and consumed all over the world. From the past many years, Brazil has shown significant citrus fruit production (Figure I-2) and Brazilian orange juice

factories generated millions of tons of CPWO annually whose disposal was a big issue.

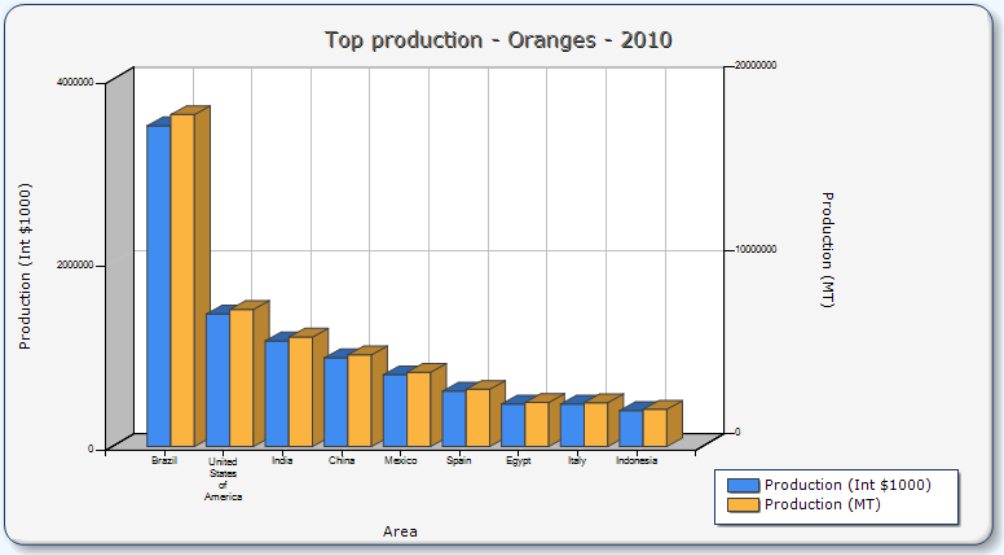


Figure I-2. Top orange fruit country production in 2010 (figure taken from Food and Agriculture Organization of the United Nations, FAOSTAT, http://faostat.fao.org/site/339/default.aspx. Web page accessed on January 10th, 2013).

Citrus fruits (oranges, tangerines, grapefruits and lemons) and their juices are the most widely consumed. According to the U.S. Department of Agriculture (United States Department of Agriculture), Brazil is the largest producer of oranges in the world, while the United States ranks second [USDA, 2012], both of them contribute to 90% of the world's orange juice production [USDA, 2012; Zvaigzne, 2009]. In 2011, about 19 million tons of oranges were produced in Brazil, of which 15 million were generated only in the State of Sao Paulo [IBGE, 2011]. As almost 99% of the fruit from this region is processed for export, it is the overwhelming giant in worldwide orange juice production. Orange juice is traded internationally in the form of frozen concentrated orange juice to reduce the volume used, so that storage

and transportation costs are lower [Citrus production report Wikipedia, 2013]. After extraction of the juice, about 50-60% of the fruit is left as residue, which consists of: membranes, seeds and peel [Grohman and Baldwin, 1992; Wilkins *et al*., 2005; Wilkins *et al*., 2007a]. This type of lignocellulosic waste raises a number of issues for the orange juice industries in Brazil [Bansal *et al*., 2011]. These wastes are sometimes used in the production of cattle feed in the form of citrus pulp pellets, but this is not economically attractive in an industrial set up [Wilkins *et al*., 2007a]. CPWO is rich in soluble and insoluble carbohydrates, with a small proportion of lignin [Grohmann *et al*., 1995]. There is a great need to explore this residue in a more attractive, inexpensive process without any environmental hazards [Edwards and Doran-Peterson, 2012], such as production of second generation bioethanol. The composition of orange peel shows that it is rich in fermentable sugars, that is, glucose, fructose, and sucrose, along with insoluble polysaccharide cellulose and pectin [Grohmann *et al*., 1995]. The presence of low lignin levels in comparison to other lignocellulosic biomass makes such substrates ideal for fermentation-based products, such as ethanol production; however, the presence of pectin requires either harsh pretreatment or application of enzymes for the fermentable sugar release [Grohmann *et al*., 1995].

Acidic and enzymatic hydrolysis of CPWO which enables the breakdown of complex carbohydrate polymers is known in the literature [Wilkins *et al*., 2005]. But the composition of CPWO varies significantly depending on the nature of the substrate (where it is grown) and hence changes enzyme response to substrate and also the composition of hydrolyzates. In the present work, Brazilian CPWO is explored, first by

applying classical acid hydrolysis and later comparing it with enzyme hydrolysis. Previous studies conducted by our research group focused on the microorganism *Xanthomonas axonopodis* pv. *citri* (*Xac* 306), a potent gram-negative bacterial pathogen, responsible for Citrus canker disease [Tasic *et al*., 2007; Khater *et al*., 2007; Fattori *et al*., 2011]. This bacterium is able to penetrate the stomata, and provoke visible and circular spotted wounds [Brunings and Gabriel, 2003]. *Xac* 306 genome was completely sequenced in 2002 by da Silva *et al*. and it shows presence of genes corresponding to several hydrolytic enzymes that can help in biomass degradation.

Literature studies show that yeast strains, other than *Saccharomyces cerevisiae*, contribute to the enhanced flavor and aroma of wine because of the greater quantity of secondary metabolites produced [Garde-Cerdán and Ancín-Azpilicueta, 2006, Moreira *et al*., 2008]. Although *Saccharomyces cerevisiae* strain is more effective for the fermentation, it yields lower levels of aromatic compounds [Andorrà *et al*., 2012]. That is why it is important to explore the non-*Saccharomyces* strains not only to check their ability to ferment, but also to test the strains that might be tested further in wine production.

1.2. Orange Fruit

Orange trees are widely cultivated in tropical and subtropical climates for their delicious sweet fruit. The orange fruit is composed of the following fundamental parts (Figure. I-3):

i) the exocarp called flavedo (external colored part of the peel),

ii) the mesocarp called albedo (white internal part of the peel),

iii) the endocarp pulp, subdivided into segments containing vesicles filled with juice and seeds

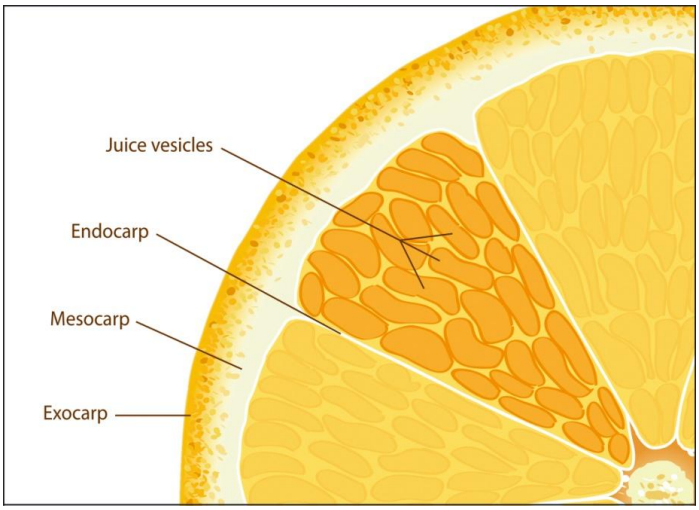


Figure I-3. Anatomy of Orange (figure taken from sig biz website, http://www.sig.biz/site/en/medien/medien_/medienarchiv/news_archive_combibloc/news_detail_archive_combibloc_2313.jsp. Web page accessed on January 5th, 2013).

1.3. CPWO Composition

In order to harness the maximum value from CPWO, it is essential to have knowledge regarding its chemical composition [Rivas *et al*., 2008; Ali *et al*., 2010; Bermejo *et al*., 2011; Asikin *et al*., 2012]. Briefly CPWO contains 75-82% moisture contents, soluble sugars, fibers (including cellulose, hemicellulose, lignin and pectin) along with ash, fats and proteins [Ali *et al*., 2010]. The quantitative composition determined by Rivas *et al.* (2008) is detailed in Table I-1:

The small quantities not identified (about 4.35% of the peel) include organic acids such as citric acid, malic acid, malonic acid, oxalic acid (which collectively represent about 1%) and vitamins such as Vitamin C (ascorbic acid).

Table I-1. Chemical composition of orange peel in percentage on dry matter (DM) basis [Rivas *et al*., 2008]

Soluble sugars (%)	Starch (%)	Cellulose (%)	Hemi-cellulose (%)	Lignin (%)	Pectin (%)	Ash (%)	Fat (%)	Protein (%)	Other components (%)
16.90	3.75	9.21	10.50	0.84	42.50	3.50	1.95	6.50	4.35

Bampidis and Robinson (2006) also investigated the composition of orange peel, and reported that the dry matter (DM) content of orange peel is mainly organic, containing proteins and other short-chain (no more than four carbons) organic acids (Table I-2).

Table I-2. Chemical composition of orange peel (g/kg DM) [Bampidis and Robinson, 2006]

Organic matter (g/kg)	Crude protein (g/kg)	Neutral detergent fibre (g/kg)	Acid detergent fibre (g/kg)	Lactic acid (g/kg)	Acetic acid (g/kg)	Propionic acid (g/kg)	Isobutyric acid (g/kg)	Calcium (g/kg)	Phosphate (g/kg)	pH
975	58	200	129	23	20	0.3	0.6	7.3	1.7	3.64

One kilogram of orange peel analyzed in these analyses had 233g DM.

It is important to note that just like all other plants products, the chemical composition of oranges varies. It is affected by various factors such as growing conditions, fruit maturity, rootstock, variety, and climate

[Kale and Adsule, 1995]. The pH of citrus peel is also variable and it can be as low as 3.64. It should be tested before any application as neutralization might be required.

Bermejo *et al*. (2011) did analysis of bioactive agents in citrus varieties. They observed that rind contents of different varieties showed similar tendencies for the most of compounds like the flavanone glycosides hesperidin and narirutin, carotenoid and β-cryptoxanthin. Limonene was the most abundant terpene found in peel essential oil in all cultivars studied, followed by myrcene. Calcium and potassium were the dominant macronutrients.

CPWO composition (as shown in Tables I-1 and I-2), show CPWO potential to be employed in different applications. Extraction of highly valuable natural products, limonene and hesperidin for example, could transform what is typically considered to be a problematic substrate to a high value commodity. Among these, pectin is also very attractive, as well as, fermentable sugars that can be used to produce bioethanol.

1.3.1. Essential oil

Citrus fruit contains essential oil, located in oil sacs or glands that range in diameter from 0.4 to 0.6 mm. They are present in irregular depths in the flavedo located at the outer rind of the fruit. Merle *et al*. (2004) studied different varieties of citrus and identified that 97% of the oil content is limonene. D-limonene ($C_{10}H_{16}$) is a hydrocarbon, classified as a cyclic

terpene. It is colorless liquid at room temperature, with an extremely strong orange odor.

The extraordinarily high amount of limonene that accumulates in orange oil glands suggests an important biological role for this terpene compound in fruit aroma and in the plant's interactions with the environment. It has a defensive function to makes fruit resistant not only to fungal and bacterial citrus pathogens, but it is also known to function as an insect repellent [Ibrahim *et al.,* 2001], repellent of some birds like Starlings [Clark, 1997] and mammals such as deer [Vourc'h *et al.,* 2002]. D-limonene is employed in chemical industry, cosmetics, domestic household products and as a flavoring agent in food and medicine [Smyth and Lambert, 1998].

1.3.2. Hesperidin

The flavonoids are secondary plant metabolites that belong to the class of plant phenolics. Currently, there is a great interest in these compounds because of interesting biological activities [Mazzaferro and Breccia, 2012]. Moreover, they are abundant in the by-products, mostly in peels (albedo + flavedo), accounting for 4–12% dry weight of fruit not being used [Marín *et al*., 2007]. In citrus varieties, the most abundant flavonoid is hesperidin ($C_{28}H_{34}O_{15}$). Its aglycon form is called hesperetin.

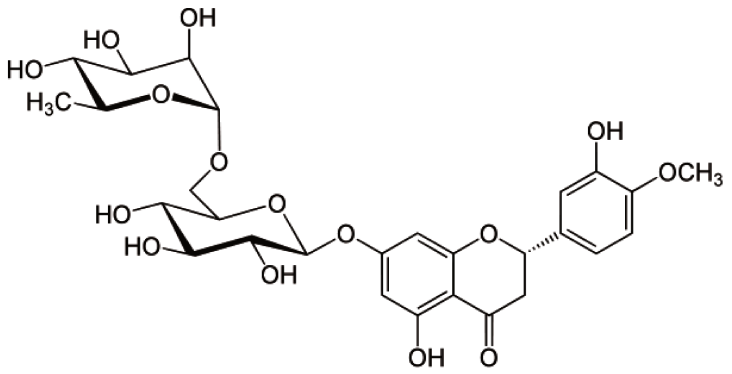


Figure I-4. Structure of hesperidin.

Hesperidin (Figure I-4) is believed to play a role in plant defense. Researchers have shown that there is a close connection between fruit growth and content of hesperidin. Also harvest time effect hesperidin and other flavonoid contents of fruit peel. Del Rio *et al.* (1995) showed that highest level of hesperidin accumulated in very young tissues inside the fruit, which indicates the protective function of flavonoids.

Hesperidin is of great biological and medicinal interest as it is associated with lowered risk of certain cancers, reduced cholesterol and stroke. Moreover, antioxidant, anti-inflammatory, antimicrobial and antiviral properties have also been demonstrated both '*in vitro'* and '*in vivo'* [Cano *et al.,* 2008; Mazzaferro and Breccia, 2012].

Hesperidin can be isolated by variety of ways using both analytical and preparative techniques. Its extraction from CPWO is very important as it can convert an agricultural waste into a valuable commodity.

1.3.3. Lignocellulosic fibers

Cell wall is a characteristic feature of all plant cells and is rich in polysaccharides. The structural material in the cell wall is known as lignocelluloses, which provides support, strength and shape to the plant. It mainly contains: pectin, cellulose, hemicelluloses and lignin [Wyman, 1996]. Different parts of the plant have different proportion of these components. The outer wall is mainly composed of lignin that is called primary cell wall. It surrounds growing and dividing plant cells. Next is the much thicker and stronger secondary cell wall (Figure I-5), which accounts for most of the carbohydrate part in biomass [Carpita *et al*., 2001]. The secondary wall usually consists of three sub-layers, which are termed as S1 (outer), S2 (middle), and S3 (inner), respectively. The formation of these three distinctive layers in secondary cell walls is a result of changes in the orientation of cellulose microfibrils during their deposition. The S1 and S3 layers are typically thin and have cellulose microfibrils oriented in a flat helix relative to the elongation axis of the cell [Zhong and Ye, 2009]. The S2 layer is thick and has cellulose micro-fibrils that accounts for 75% to 85% of the total thickness of the cell wall [Plomion *et al*., 2001]. It is the S2 layer that largely determines the mechanical strength of fibres in wood. Hemicelluloses and lignin are also present in each of these layers [Plomion *et al*., 2001; Zhao *et al*., 2012].

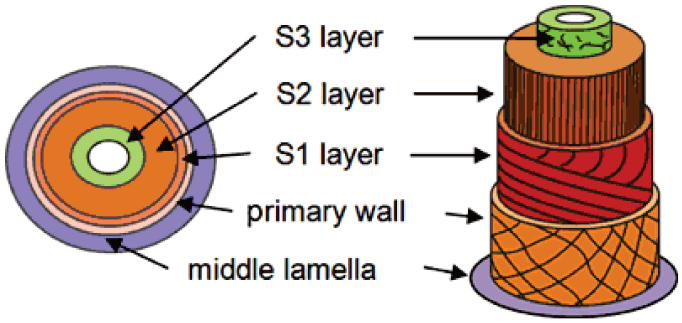


Figure I-5. Three dimentional structure of plant cell wall with its primary and secondary layers (http://www.accessscience.com/search.aspx?rootID=791275. Web page accessed on January 14th, 2013).

Pectin is an important component of cell wall and is a polysaccharide rich in galacturonic acid and galacturonic acid methyl ester units (Figure I-6). As a soluble fiber and combined with proteins and other polysaccharides, pectin forms skeletal tissue that makes plant chemically stable and physically strong [Willats *et al*. 2006].

Cellulose (Figure I-6) is a high molecular weight linear insoluble polymer of D-glucose units connected via β (1,4) glycosidic bonds. Cellulose is usually found in close association with hemicellulose and lignin. In most cases cellulose does not occur alone in a free threadlike chain, but is present in a bundle of fibrillar units with a supramolecular structure consisting of crystalline and amorphous regions [Mansfield *et al*., 1999].

Typically, the crystalline cellulosic region consists of several sheets of long chain fibers arranged by both intra- and intermolecular hydrogen bonds as shown in Figure I-7 [Mansfield *et al*., 1999].

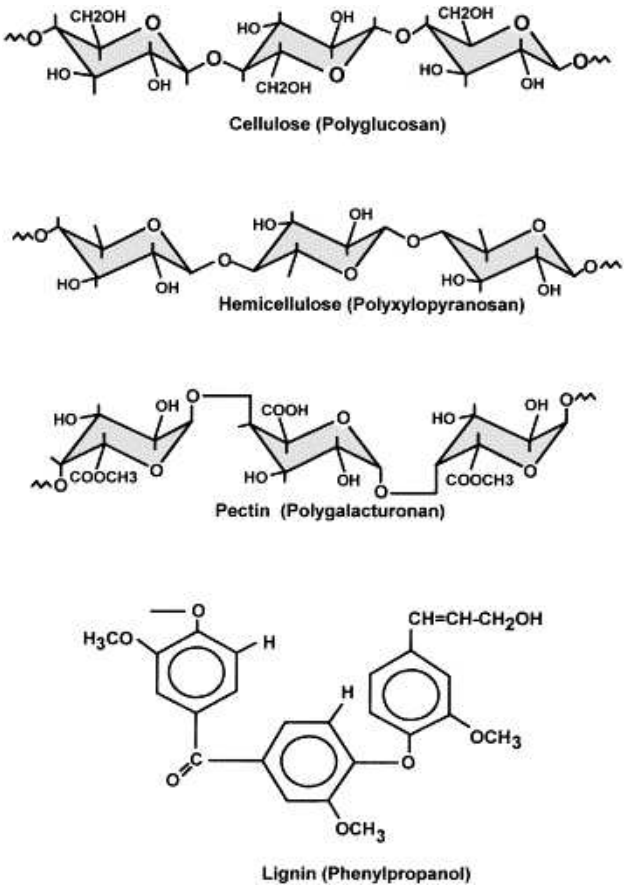


Figure I-6. Basic molecular structures of the major biopolymers forming the cell wall of biomass: cellulose, hemicellulose, pectin and lignin [Greil, 2001].

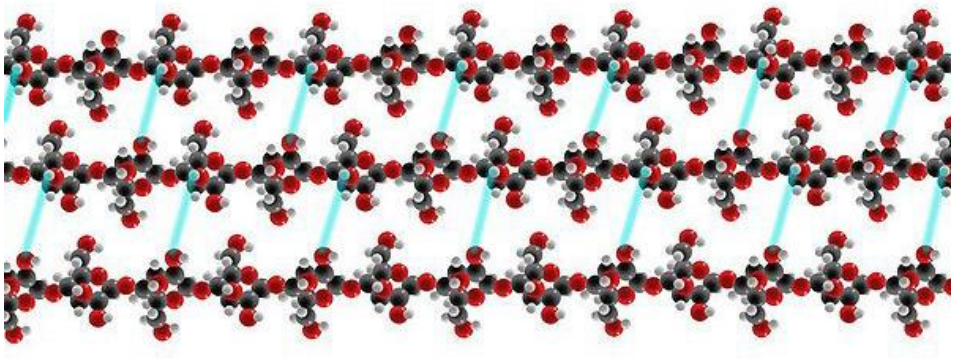


Figure I-7. Illustration of three cellulose strands. White balls are hydrogen atoms, black balls are carbon atoms, red balls are oxygen atoms, and turquoise lines are electrostatic hydrogen bonds.
(http://en.wikipedia.org/wiki/File:Cellulose_spacefilling_model.jpg. Web page accessed on January 12th, 2013).

The structure of cellulose can be cleaved by hydrolysis of β (1→4) glycosidic bond catalyzed with acids or cellulase. However, the hydrogen bonds present in long chains of cellulose in a crystalline structure (Figure I-7), prevents hydrolysis and thus harsh pre-treatment conditions are sometimes required [Gardner and Blackwel, 1974]. It is widely accepted that the higher the crystalline content of cellulose, the more difficult is the enzymatic attack to hydrolyze this polymer. The amorphous regions are more accessible to enzymes and are therefore more easily hydrolyzed, while in the crystalline areas enzyme contact efficiency is decreased [Chang *et al.,* 2000; Carvalho, 2009].

Hemicellulose (Figure I-6) is a heteropolysaccharide of arabinose, galactose, glucose, mannose and xylose, and can also contain minor amounts of other compounds, such as acetic acid. Unlike cellulose, hemicellulose polymers are chemically heterogeneous, have lower degrees of polymerization and are mostly amorphous. That is why, hemicellulose chains are more easily hydrolyzed into monomeric units as compared to cellulose chains [Wyman, 1999]. There are many suitable pretreatments for removing hemicellulose, which greatly facilitates subsequent cellulose digestion [Saha *et al*., 2005].

Lignin (Figure I-6) is a cross linked macromolecular material based on a phenylpropanoid monomer structure [Doherty *et al.,* 2011]. Lignin adds strength and rigidity to cell walls and is more resistant to enzyme attack than cellulose and other structural polysaccharides [Kirk, 1971; Baurhoo *et al*., 2008]. Lignin is able to form covalent bonds with hemicellulose (Figure I-8). Covalent bonds between lignin and carbohydrates mostly consist of benzyl

esters, benzyl ethers and phenyl glycosides [Smook, 2002]. In this way, lignin provides integrity, structural rigidity and prevention of swelling of lignocelluloses. It is commonly accepted as one of the major factors responsible for biomass recalcitrance to enzymatic hydrolysis. It implies steric hindrance and prevention of fiber swelling, the later being an important factor to increase internal surface area [Mooney *et al*., 1998; Carvalho, 2009].

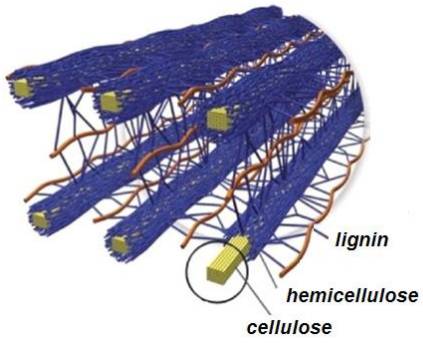


Figure I-8. Lignin (orange) and hemicellulose (blue) binding with cellulose (yellow) strands (Department of Energy Genomic, http://genomics.energy.gov. Web page accessed on January12th, 2013).

1.4. Pretreatment of Biomass

It is known that porosity (accessible surface area) of the waste materials, cellulose with high crystallinity, along with high lignin and hemicellulose content affect the hydrolysis of cellulose [McMillan, 1994]. Thus, to achieve high yields of glucose, lignocellulose must be pretreated first. The effect of pretreatment of lignocellulosic materials has been recognized for a long time [McMillan, 1994; Sun and Cheng, 2002]. A simplified representation of lignocellulose pretreatment is shown in the Figure I-9.

The main objectives of pretreatment are:

i) to reduce cellulose crystallinity,

ii) to remove lignin,

iii) to increase the porosity of the materials, and

iv) to make substrate significantly more susceptible to enzyme action.

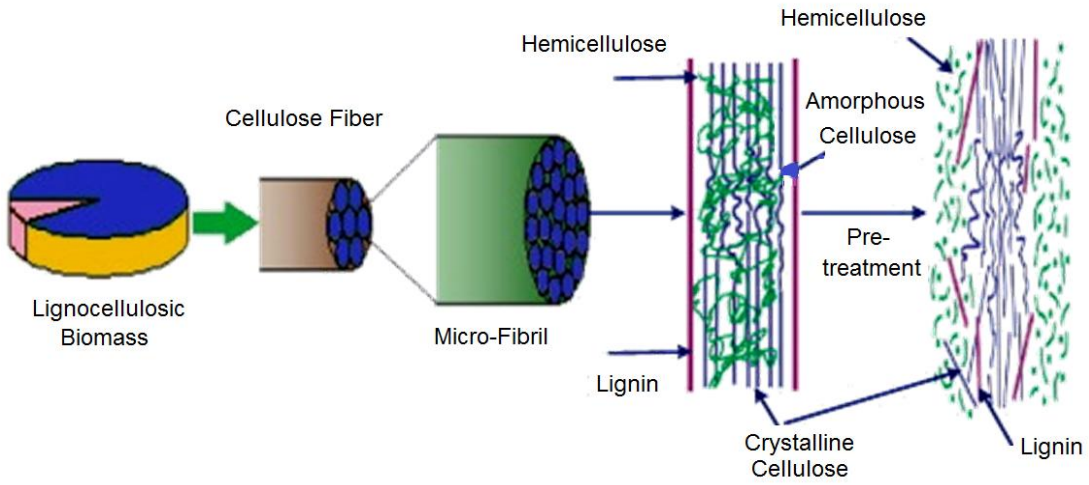


Figure I-9. Scheme showing lignocellulosic biomass fibril structure. The lignin and hemicellulose surrounds cellulose. Pretreatment of biomass removes hemicellulose and lignin from the cellulose polymer chains before hydrolysis [adapted from Chandra *et al.*, 2012].

Pretreatment must meet the following requirements [Sun and Cheng, 2002]:

i) improve the formation of fermentable sugars,

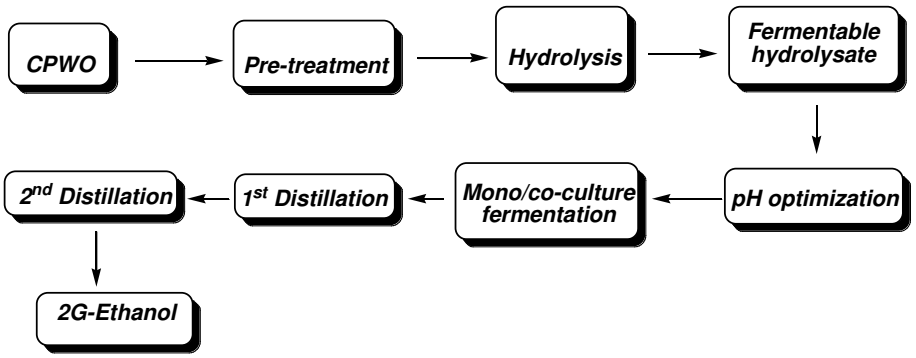
ii) avoid the degradation or loss of carbohydrate,

iii) retain nearly all of the cellulose present in the original material,

iv) avoid the formation of byproducts that are inhibitory to the subsequent hydrolysis and fermentation processes, and

v) be cost-effective.

Physical, physico-chemical, chemical, and biological processes have been used in pretreatments of lignocellulosic materials. Typical processes include hot water, dilute acids, steam explosion, ammonia fiber explosion (AFEX), strong alkali process, as well as mechanical treatment such as hammer and ball milling are conventionally used to treat lignocellulosic biomass. In this work, autohydrolysis and limonene pretreatments were executed before hydrolysis step. The main steps in the overall process are shown in scheme I-1:



Scheme I-1: Representation of overall process showing different steps involved in this work.

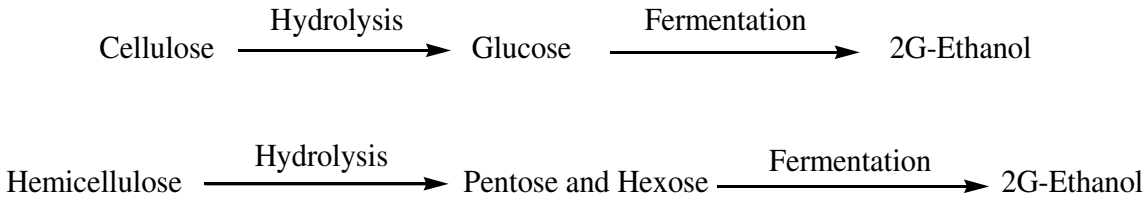
1.5. Hydrolysis

After pretreatment, the carbohydrate polymers in the lignocellulosic materials need to be converted further into simple sugars before fermentation, through a process called hydrolysis. The hydrolysis reaction for cellulose conversion is given in Reaction I-1.

$$(C_6H_{10}O_5)_n + nH_2O \longrightarrow nC_6H_{12}O_6$$

(Reaction I-1)

Cellulose and hemicelluloses are converted first into simple sugars and then to 2G-ethanol by fermentation, while lignin remains as a by-product (Scheme I-2):



Scheme I-2. Schematic representation showing cellulose and hemicelluloses conversion into 2G-ethanol.

There are several possible methods to hydrolyze lignocelluloses. The most commonly applied methods are acidic and enzymatic hydrolyses. In addition, there are some other hydrolyses methods in which no chemicals or enzymes are applied. For instance, lignocelluloses may be hydrolyzed using gamma-ray or electron-beam irradiation, or microwave irradiation. However, these processes are far from being commercially applicable [Taherzadeh and Karimi, 2007].

1.5.1.Brief history of biomass hydrolysis

Acid hydrolysis of plant lignocellulosic biomass has been known since 1819 [Galbe and Zacchi, 2002]. However, enzymes for biomass hydrolysis can barely account for 80 years of employment and research. The search for biological causes of cellulose hydrolysis did not begin until World War II. The U.S. Army started a basic research program to understand the

causes of deterioration of military clothing and equipment in the jungles. Out of this effort to screen thousands of samples collected from the jungle came the identification of one of the most important organisms in the development of cellulase enzymes, called *Trichoderma reesei*. The research was started to find out the causes of deterioration of cellulosic materials but later in early 1970's transformed to look at cellulases as means for converting solid waste into food and energy products [US department of energy website, 2013].

1.5.2. Acid Hydrolysis

Acid Hydrolysis is a type of chemical hydrolysis that involves exposure of lignocellulosic materials to an acid for a specific period of time at specific conditions such as temperatures and pressures [Deejing *et al*., 2009], that results in the release of monosaccharides from cellulose and hemicellulose [Panagiotopoulos *et al*., 2012]. Acid hydrolysis of plant lignocellulosic biomass has been known for almost 200 years [Galbe and Zacchi, 2002]. Later, commercial scale plants were also started as for example, the modified Bergius process (40% HCl for 1 hour at 35^0C) operated during World War II in Germany, and more recently, modified Scholler processes (0.4% H_2SO_4) in the former Soviet Union, Japan and Brazil [Keller, 1996]. Acid hydrolysis can be performed with several types of acids, including sulfurous, sulfuric, hydrochloric, hydrofluoric, phosphoric, nitric and formic. These acids may be either concentrated or diluted [Galbe and Zacchi, 2002; Lenihan *et al*., 2010]. Literature shows that

most commonly used acid is Sulfuric acid [Harris *et al*., 1945; Oberoi *et al*., 2010; Panagiotopoulos *et al*., 2012].

The mechanism of acid hydrolysis is shown in Figure I-10 [Verendel *et al.,* 2011]. Acid hydrolysis of cellulose chain begins with the protonation of the glycosidic oxygen with subsequent cleavage of C1-O bond [Mendgen and Deising, 1993; Daniel, 1994]. Delocalization of existing pair of electrons on the oxygen of the glycosidic ring occurs adjacent to C1. In the next step, the nucleophilic attack of water on C1 results in acid regeneration that terminates the depolymerization step (when hydrolysis occurs within the cellulose chain to generate new terminals) or glucose production (when hydrolysis occurs directly at the terminals) [Ogeda and Petri, 2010].

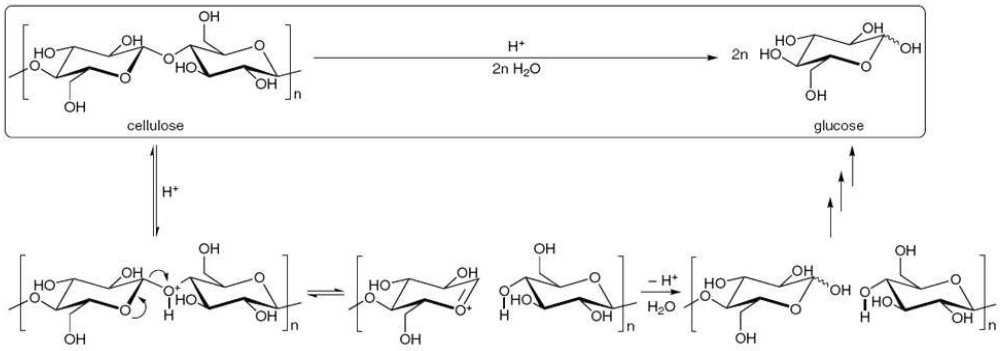


Figure I-10. Mechanism of acid hydrolysis [Verendel *et al*., 2011].

Acid Hydrolysis can be performed using concentrated or dilute acid. A comparison between these two cases is given in Table I-3:

In economical aspects, concentrated acid hydrolysis is comparatively expensive process. Due to large amount of acids used, problems are

associated with equipment corrosion and energy-demanding acid recovery. So, there is no remarkable on-going development of concentrated acid hydrolysis of softwood.

Table I-3. Comparison between Concentrated- and Dilute-Acid Hydrolysis [adapted from Galbe and Zacchi, 2002; Taherzadeh and Karimi, 2007]

Hydrolysis Method	Advantages	Disadvantages
Concentrated-acid process	i) Operate at low temperature ii) High sugar yield	i) High acid consumption ii) Equipment corrosion iii) High energy consumption for acid recovery iv) Longer reaction time (2-6 h)
Dilute-acid process	i) Low acid consumption ii) Short residence time	i) Operated at high temperature ii) Low sugar yield iii) Equipment corrosion iv) Formation of undesirable by-products v) Sugar degradation vi) Inhibition of fermentation because of degradation products

1.5.3.Enzyme Hydrolysis

Over the past few years scientists from all over the world have been shown their interest and efforts in the area of biomass enzymatic hydrolysis. Enzymes application to biomass has shown distinct advantages over acid based hydrolysis methods, for example, it occurs at a very mild process conditions, give potentially higher yields and has no corrosion problems. Therefore, enzyme hydrolysis is considered most suitable one for future 2G-ethanol production from biomass [Duff and Murray, 1996; Hsu, 1996]. In this process, the long chained polymers of cellulose and hemicellulose are consumed by enzymes and monomers, hexoses and pentoses are released [Stewart *et al*., 2006; Stewart *et al*., 2008; Oberoi *et al.*, 2010]. The CPWO can be hydrolyzed to monosaccharides by applying a combination of enzymes: pectinase, cellulase and beta-glucosidase.

Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic material. Compared with fungi, cellulolytic bacteria produce low amounts of cellulolytically active enzymes [Sternberg, 1976; Fan *et al*., 1987; Duff and Murray, 1996]. Of all the fungal genera, *Trichoderma* has been most extensively studied for cellulase production [Sternberg, 1976]. It produces a complex mixture of cellulase enzymes with high specificity towards β-1,4 -glucosidic bonds. Cellulases are usually a mixture of various cellobiohydrolases and endoglucanases supplemented with beta-glucosidase [Sun and Cheng, 2002]:

Endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4.) attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends. Figure I-11 shows endoglucanase that is hydrolyzing internal bonds in the cellulose chain and break intermolecular bonds between adjacent cellulose chains. They act mainly on the amorphous parts and cleave glucosidic bonds randomly, generating soluble carbohydrate chains with low degrees of polymerization [Sun and Cheng, 2002; Kumar *et al*, 2008].

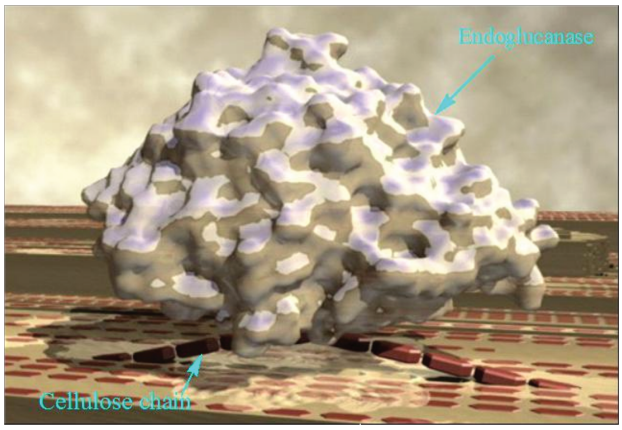


Figure I-11. Computer model of the structure of the endoglucanase showing catalytic domain during hydrolyses of internal bonds in the cellulose chain [Sarita, 2010].

Figure I-12 shows an endoglucanase enzyme from *Trichoderma reesei* (PDB: 1EG1). The arrows indicate beta-strands while the twisted ribbons are alpha-helices.

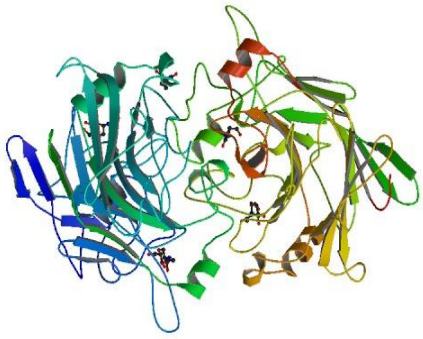


Figure I-12. Endoglucanase enzyme from *Trichoderma reesei* (PDB: 1EG1). The arrows indicate beta-strands while the twisted ribbons are alpha-helices. http://www.rcsb.org/pdb/images/1eg1_asr_r_500.jpg. Web page accessed on January 12th, 2013).

Exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase, or EC 3.2.1.91.) degrades the molecule further by removing cellobiose units from the free chain-ends [Sun and Cheng, 2002]. These enzymes release mainly cellobiose, but also glucose and small cellodextrins (cellotriose). One exoglucanase can either act on the reducing or the non-reducing ends or the chains, but microorganisms often produce more than one type of exoglucanase, degrading cellulose chains from both directions [Zhang *et al.*, 2004]. Figure I-13 shows the computational model of the structure of the exoglucanase enzyme and its action at the free ends of cellulose polymer chain. In exoglucanase enzyme, the catalytic domain is represented by a tunel of approximately 50 Å in length [Sarita, 2010] The X-ray studies by Divne *et al.* (1998) revealed some details about three dimentional enzyme structure of exoglucanases (Cellobiohydrolase I) (Figure I-14).

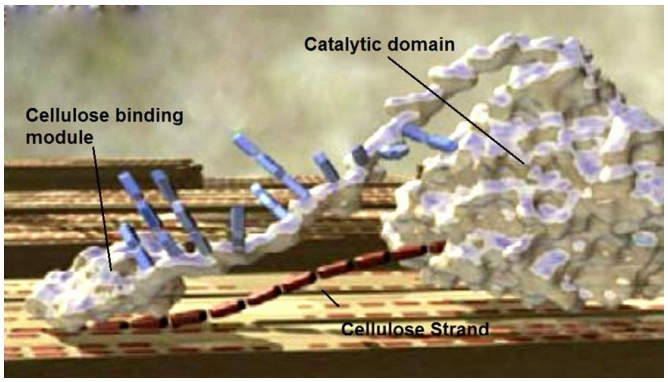


Figure I-13. Computational model of the structure of the exoglucanase, CBH I; showing cellulose binding module and catalytic domain in the form of a tunel, during enzyme action [figure adapted from Carvalho, 2009].

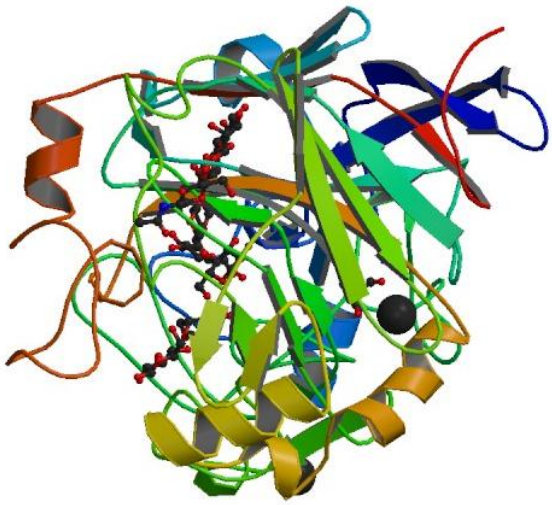


Figure I-14. Schematic representation of the Cellobiohydrolase I from *Trichoderma reesei* (PDB: 7CEL) catalytic domain with a cellooligomer. Secondary-structure elements are colored as follows: β strands, blue, green and sea green arrows; α helices, orange, yellow and brown spirals; loop regions, orange, red, green, yellow and blue coils. The cellooligomer is shown in blue and red as a ball-and-stick model [Divne *et al.,* 1998].

Beta-glucosidase (EC 3.2.1.21) hydrolyzes cellobiose that is an end-product inhibitor of many cellulases and produces glucose monomers [Sun and Cheng, 2002]. Endo- and exoglucanases beak down larger oligosaccharides into cellobiose by primary hydrolysis, but the most important step is the action of β-glucosidases, that completes hydrolysis in a form that resulting sugar monomer (glucose) can be used further in the process of fermentation. Nam *et al*. (2010) explained crystal structure of beta-glucosidase (PDB:3CMJ), and proposed four loops where substrate (cellobiose) molecule is entered/captured for hydrolysis (Figure I-15).

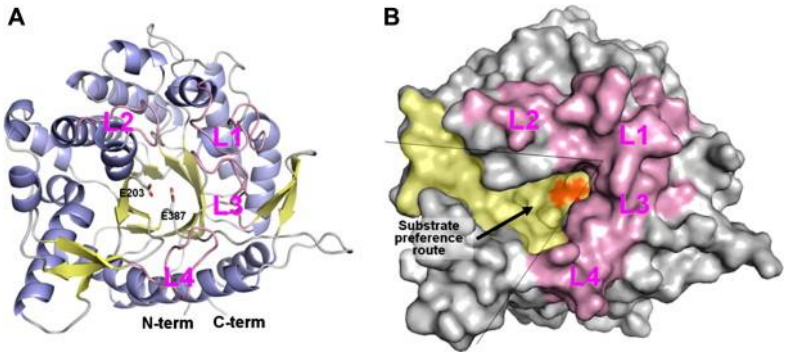


Figure. I-15. Crystal structure of β-glucosidase (PDB:3CMJ). (A) Overall structure of β-glucosidase. The four loops where the substrate enters are colored in pink. (B) Representation of the surface structure. The large cleft is colored in yellow. This cleft had a cavity of approximately 15 × 10 Å; therefore the related disaccharides substrate could easily enter the active site pocket [Nam *et al*., 2010].

Overall, the mode of cellulolytic enzyme action for cellulose hydrolysis can be summarized as demonstrated in Figure I-16:

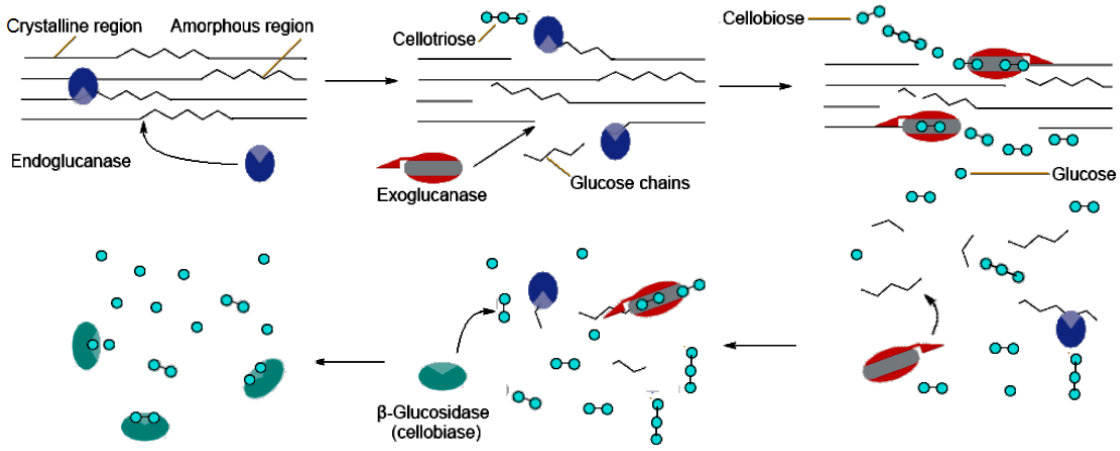


Figure I-16. Mode of cellulolytic enzyme action [adapted from Zhu, 2005; Jørgensen *et al*, 2007].

In addition to the three major groups of cellulase enzymes, there are also a number of enzymes that attack hemicelluloses, such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase [Duff and Murray, 1996]. Hemicellulases are produced by many species of bacteria and fungi, as well as by several plants. Today, most commercial hemicellulase preparations are produced by genetically modified *Trichoderma* or *Aspergillus* strains [Mussatto and Teixeira, 2010].

Apart from cellulose and hemicelluloses, pectins are abundant in the soft tissues of citrus fruits, sugar beet pulp and apple [Numan and Bhosle, 2006]. Pectinases are one of the most widely distributed enzymes in bacteria, fungi and plants. Protopectinases, polygalacturonases, lyases and pectin esterases are among the extensively studied pectinolytic enzymes [Jayani *et al*., 2005].

Figure I-17 shows the three-dimensional structure of *Aspergillus niger* pectin lyase B (PDB: 1QCX). The ribbon diagram is illustrating the secondary structure.

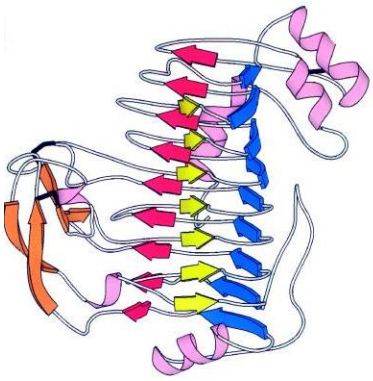


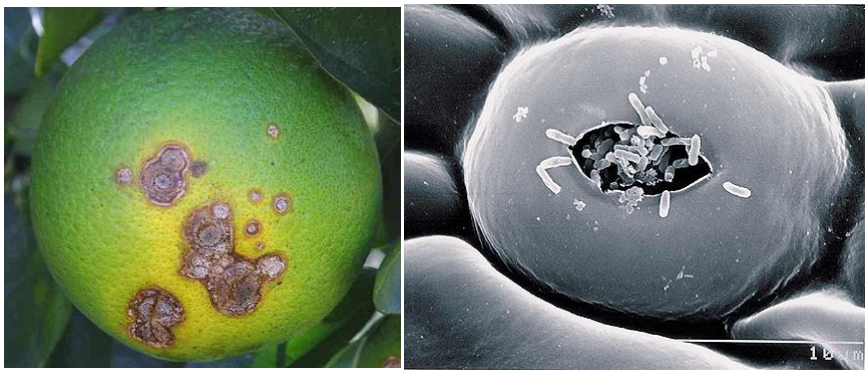
Figure I-17. The ribbon diagram of *Aspergillus niger* pectin lyase B (PDB: 1QCX). Helices are represented by pink coils and β structure is shown by arrows. The parallel β (PB) sheet, PB1, is yellow; PB2 is blue; and PB3 is red. The antiparallel β structure within the first T3 turn and a short β strand within the third T3 loop are indicated in orange [Vitali *et al*., 1998].

1.6. *Xanthomonas axonopodis* pv. *citri* (*Xac* 306)

Xanthomonas is an important genus of plant pathogenic Gram-negative bacteria [Khater *et al*., 2007; Tasic *et al*., 2007; Jalan *et al.,* 2011; Fattori *et al*., 2011; Liu *et al*., 2012]. A great number of citrus diseases are caused by distinct pathovars (pv.) of *Xanthomonas* species [Vauterin *et al*., 1995; Schaad *et al.,* 2006]. Citrus canker is caused by several pathogenic variants of *Xanthomonas axonopodis* pv. *citri (Xac). Xac* strain 306 with a suspected origin in southeastern Asia causes Asiatic type canker and is the most widespread and virulent bacteria [Jalan *et al.*, 2011].

Like many other bacterial diseases, the pathogen enters host plant tissues through stomatas [Brunings and Gabriel, 2003; Cubero *et al.,* 2001] and wounds [Koizumi and Kuhara, 1982]. The optimum temperature for infection falls between 20 and 30°C [Koizumi, 1977]. Bacteria multiply 3-4 log units per lesion under optimum conditions and cells may emerge from stomatal openings in as little as 5 days to provide inoculums for further disease development [Jalan *et al*., 2011]. The earliest symptoms on leaves appear as tiny, slightly raised blister-like lesions beginning around 9 days post-infection (Figure I-18). It produces hyperplasic and hypertrophic (corky) lesions surrounded by oily or water-soaked margins and a yellow halo on leaves, stems, and fruits [Jalan *et al*., 2011].

These bacterial attacks on the host tissue involves impressive arsenal of proteins, including pectinases and celullases for their hydrolytic activity. *Xanthomonas axonopodis* pv. *citri* (strain 306) genome was completely sequenced in 2002 by da Silva *et al*., and showed that it has one circular chromosome comprising 5,175,554 base pairs (bp), and two plasmids: pXAC33 (33,699 bp) and pXAC64 (64,920 bp). The transfer of macromolecules in to the host body by *Xac* is the main cause of infection. Two completely distinct and highly complex multiprotein systems are considered to mediate this transfer: the type III and the type IV secretion system [Alegria *et al.,* 2005]. Degradative enzymes and toxins are secreted by two clusters of Type II secretion system (T2SS) that cause degradation of biomass [da Silva *et al.,* 2002; Moreira *et al.,* 2004].



(a) (b)

Figure I-18. (a) Citrus canker lesions caused by *Xanthomonas axonopodis* pv. *citri* on immature fruit with chlorotic halos. (b) SEM of stomata on grapefruit leaf with *Xac* bacteria entering stomatal chamber [Cubero *et al.,* 2001]. (http://www.apsnet.org/publications/apsnetfeatures/Pages/citruscanker.aspx. Web page accessed on January 13th, 2013).

The genome of *Xac* 306 allows the biosynthesis of enzymes with cellulolytic, hemicellulolytic and pectinolytic activities [da Silva *et al*., 2002]. The complete genomic sequence shows the presence of gene related enzymes: three pectate lyase with gene (pel, degenerated pel, and pelB) and two polygalacturonase with genes (peh-1 and pglA) [da Silva *et al*., 2002]. Lin *et al.* (2010) showed that *Xac* proteins play a vital role in expressing pectinolytic activity. The presence of 12 copies of genes for cellulolitic and hemicellulolytic enzymes has also been reported [Moreira *et al*., 2004].

This impressive arsenal of hydrolytic proteins triggered the possibility to test *Xac* hydrolytic activity in biomass degradation. This is a completely new idea that has not been tested before and was explored in this project.

1.7. Factors affecting the enzymatic hydrolysis

Various characteristics within the lignocellulosic substrates can limit both the rate and degree of hydrolysis [Converse, 1993; Mansfield *et al*., 1999; Lynd *et al*., 2002; Zhang and Lynd, 2004; Jafari *et al*., 2011]. However, the enzyme action also alters the inherent characteristics of lignocellulosic substrates as hydrolysis proceeds. Researchers have shown that the enzyme-substrate interactions are influenced by various physiochemical properties of the substrate at different levels [Mansfield *et al*., 1999], such as:

i) microfibril (crystallinity and degree of polymerization),

ii) fibril (lignin content and distribution in substrate),

iii) fiber (pore size and distribution, available surface area, and degree of swelling).

1.7.1 Substrate related characteristics

Several substrate characteristics have been suggested to play key roles in determining both the rates and the degrees of hydrolysis.

1.7.1.1. Cellulose Crystallinity

Cellulose crystallinity is thought to play a major role in limiting hydrolysis, because the hydrolysis rate of amorphous cellulose is reported to be 3-30 times faster when compared to high crystalline cellulose [Fan *et al*., 1987; Lynd *et al*., 2002]. It would be expected that crystallinity should increase over the course of cellulose hydrolysis as a result of preferential

reaction of amorphous cellulose. For example, the initial rapid rate of hydrolysis followed by slower and sometimes incomplete hydrolysis (Figure I-19) suggests rapid consumption of the amorphous constituents of the cellulosic substrates in the start of hydrolysis. The resistance after certain time period is thought to be due to higher crystallinity of the residual substrate [Mansfield *et al*., 1999].

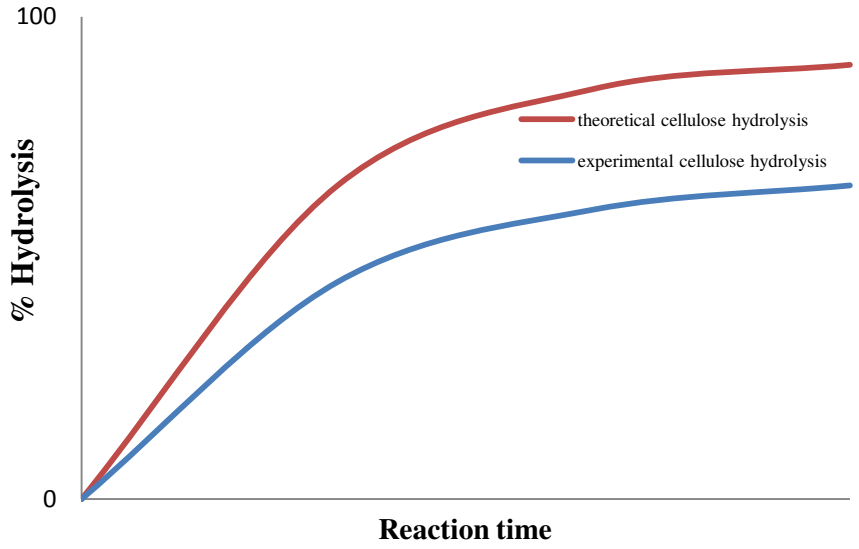


Figure I-19. Time course for the enzymatic hydrolysis of lignocellulosic substrates [adapted from Mansfield *et al*., 1999]

Comparing the hydrolysis rates on various sources of model cellulosic substrates, Fierobe *et al*. (2002) concluded that accessibility of cellulose is a more important factor than crystallinity index in determining the hydrolysis rate [Maeda *et al*., 2011].

1.7.1.2. Lignin Content

Lignin is one of the strong impacting factors on enzymatic conversion of biomass since it is responsible for the maintenance of fiber integrity and structure rigidity [Mooney *et al*., 1998; Del Rio *et al*., 2011; Li *et al.*, 2012]. It impedes enzyme access to glucan chains by its protective sheathing and also reduce cellulase effectiveness as a result of unproductive binding and steric hindrance [Mansfield *et al*., 1999; Chang and Holtzapple, 2000; Li *et. al.,* 2010; Del Rio *et al*., 2011; Li *et al*., 2012].

Li *et al.* (2010) performed hydrolysis of a typical raw material i.e. rice straw, by enzymes. They observed that for pretreated biomass, the experimental yield was more close to calculated yield than the case of un-treated sample since the influence of lignin in biomass was diminished by alkaline solution pretreatment. Similarly, Samayam and collaborators while working on ionic liquid pretreatments of lignocellulosic materials, used high temperatures as lignin was restricting the swelling of cellulose at low temperatures [Samayam *et al*., 2011].

1.7.1.3. Specific surface Area

Surface area of the lignocellulosic biomass can be divided into exterior and interior surface areas [Chandra *et al*., 2007]:

i) exterior surface area (depends maily upon the fiber length and width), or

ii) interior surface area (depends upon size of the lumen and number of fiber pores)

The efficacy of cellulose hydrolysis is enhanced when the pores of the substrate or interior surface are large enough to accommodate enzyme components to maintain the synergistic action of the cellulase enzyme system [Tanaka *et al.*, 1988; Chandra *et al*., 2007]. Therefore, drying of biomass prior to hydrolysis is not recommended as it reduces the pore volumes of pulps [Laivins and Scallan, 1993; Chandra *et al*., 2007]. Although rehydration of biomass may increase the surface area, pores are not restored [Van Dyk and Pletschke, 2012]

Literature studies reveal that the rates and extents of hydrolysis have also been directly correlated to the initial specific surface area of biomass. That is why, prior to hydrolysis step, particle size is reduced to increase the accessible surface area for enzyme action [Zeng *et al*., 2007; Jeoh *et al*., 2007]. Prior to enzyme hydrolysis, giving a strong pretreatment (like steam pretreatment) also decreases the average particle size and in turn the hydrolysis yield could be increased [Tanahashi, 1990; Sawada *et al*., 1995; Mansfield *et al*., 1999].

1.7.1.4. Degree of polymerization

The degree of polymerization (DP) of cellulosic substrates determines the relative abundance of terminal and interior β-glucosidic bonds for exo-acting and endo-acting enzymes, respectively [Zhang and Lynd, 2004]. It varies greatly, depending on substrate origin and preparation.

The change in DP over the course of hydrolysis for cellulosic substrates is determined by the relative proportion of exo- and endo-acting activities and cellulose properties. Exoglucanases act on chain ends, and thus

decrease DP only incrementally while endoglucanases act on interior portions of the chain and thus rapidly decrease DP [Zhang and Lynd, 2004]. Exoglucanase has been found to have a marked preference for substrates with lower DP [Wood, 1975], as would be expected to encounter greater availability of chain ends in substrate with decreasing DP. It is well known that endoglucanase activity leads to an increase in chain ends [Reverbel-Leroy *et al*., 1997; Zhang and Lynd, 2004].

1.7.2 Enzyme-related factor

End-product inhibition of the cellulase complex, enzyme inactivation, irreversible adsorption of the enzymes and enzyme synergism are the main factors that affect the enzymatic hydrolysis process.

1.7.2.1. End Product Inhibition

End-product inhibition is a major enzymatic factor that limits cellulase hydrolysis [Xiao *et al*., 2004; Van Dyk and Pletschke, 2012]. Cellulase activity is inhibited by cellobiose and to a lesser extent by glucose [Sun and Cheng, 2002] while beta-glucosidase is inhibited by glucose [Andric *et al*., 2010]. The inhibition performance by the major lignocelluloses degradation products, including organic acids, furan derivatives, lignin derivatives, and ethanol has also been known from past many years.

Adding extra β-glucosidase, which hydrolyzes cellobiose to glucose, thereby preventing inhibition of cellobiohydrolases by cellobiose [Breuil *et*

al., 1992], increasing cellulase loading, removing sugars during hydrolysis by filtration [Gan *et al*., 2005] or using simultaneous saccharification and fermentation [Vinzant *et al*.,1994] are strategies designed to resolve this problem.

1.7.2.2 Adsorption Reaction

The adsorption reaction between lignocellulosic substrates and cellulase is important for an efficient hydrolysis process. It has been shown that cellulases interact with the cellulose surface with the cellulose-binding domain (CBD) and the catalytic domain (CD). The overall adsorption binding efficiency of cellulase is markedly enhanced by the presence of CBDs [Mansfield *et al*., 1999; Maurer *et al.*, 2012]. Structural differences, for example, in the hydrophobicity of the surface of these enzymes may have an effect on the general adsorption affinity [Gusakov *et al*., 2000]. It has been observed in the studies that the different pre-treatment conditions affect the adsorption of the lignocelluloses [Ooshima *et al*., 1990].

1.7.2.3. Enzyme inactivation

Enzymes are optimally active at a specific pH and temperature [Van Dyk and Pletschke, 2012]. These conditions must be optimized to achieve suitable hydrolysis of substrates. Increasing temperature augments the frequency of collision between substrates and active sites, resulting in higher reaction speeds [Ovissipour *et al*., 2009]. However, too high temperatures cause enzyme denaturation, a phenomenon in which biocatalyst‘s internal structure is changes. There is an optimal temperature at which reaction rates are the fastest and enzymes retain activity for a long period of time. pH can also affect activity, since at certain pH values the shape of the active site can

get changed, possibly denaturing the enzyme [Ortega *et al*., 2001; Carvalho, 2009].

1.7.2.4. Synergism

Synergism is defined as when the activity exhibited by mixtures of components is greater than the sum of the activity of these components evaluated separately [Zhang and Lynd, 2004; Han and Chen, 2011]. 'Degree of synergism' (DS) is equal to the ratio of the activity exhibited by mixtures of components divided by the sum of the activities of separate components [Zhang and Lynd, 2004; Han and Chen, 2011].

Synergism seems to be particularly important for crystalline cellulose hydrolysis. Amorphous cellulose can be hydrolyzed by both endoglucanases and cellobiohydrolases, while crystalline cellulose is largely hydrolyzed by cellobiohydrolases. Thus, crystallinity probably influences hydrolysis when synergism is lacking [Mansfield *et al*., 1999]. Jalak *et al.* (2012) recently gave a mechanism of how endoglucanase increased the rate constant of cellobiohydrolase-catalyzed cellulose hydrolysis. In addition to substrate nature, experimental conditions also affect the extent of synergy observed [Hu *et al*., 2011; Asztalos *et al*., 2012].

1.8. Fermentation of Hydrolyzates

After hydrolysis of lignocellulosic substrate, the monosaccharides are converted into bioethanol via process of fermentation [Chaudhary and Qazi, 2011; Liu *et al.*, 2012]. Approximately 80% of the ethanol produced in the world is still obtained from fermentation while the remainder comes largely

through synthesis from the petroleum products [Lin and Tanaka, 2006; Qin, 2010]. During combustion ethanol reacts with oxygen to produce carbon dioxide, water, and heat (used to drive the engine). The overall reaction can be represented as Reaction I-2.

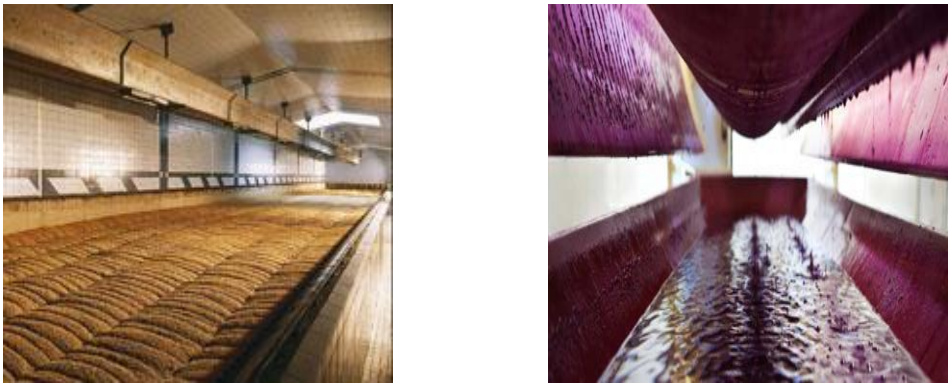
$$C_2H_5OH + 3O_2 \rightarrow 2CO_2 + 3H_2O + \text{heat}$$

(Reaction I-2)

There are a variety of microorganisms, generally bacteria, fungi or yeast, which has been used in fermentation. They do so to obtain energy and to grow [Lynd, 1990; Lin and Tanaka, 2006]. Historically, yeasts are the most commonly used microbe, among which *Saccharomyces cerevisiae* is the most preferred microorganism for most ethanolic fermentations [Joshi *et al*., 2011]. This yeast can ferment monosaccharides and is generally recognized as safe because of its consumption as a food additive in human diet. Literature studies show many other microorganisms such as *Zymomonas mobilis* and *Kluyveromyces marxianus* that can also ferment glucose to ethanol with higher yields, but is less common [Rogers *et al*., 1979; Lawford and Rousseau, 1998; Wilkins *et al*., 2007b]. Important to note is that these microorganisms are not able to ferment xylose (a 5-carbon hemicelluloses). Since lignocellulosic hydrolyzates contain pentoses, several attempts to genetically engineer *Saccharomyces cerevisiae* [Walfridsson, 1996; Tonn *et al*., 1997], *Zymomonas mobilis* [Lawford *et al*., 1997] and the bacteria *Escherichia coli* have been performed [Galbe and Zacchi, 2002]. Fermentation using immobilized yeast has also been shown to increase fermentation efficiency by almost 20% [Nikolic *et al*., 2009].

1.8.1. Submerged-Fermentation System

Most commonly used fermentations are classified as solid-state and submerged-fermentations. Solid-state fermentation (SSF, Figure I-20a) can be defined as any fermentation process allowing the growth of microorganisms on moist solid materials in the absence of free-flowing water [Pandey *et al*., 2000]. Submerged fermentation (SmF, Figure I-20b) systems can be defined as the cultivation of microorganisms in a liquid medium containing soluble carbon source and nutrients, maintained or not under agitation [Singhania *et al*., 2010].



(a) (b)

Figure I-20. (a) Large SSF unit at Soufflet's malting plant in Arcis-sur-Aube, France. http://www.allaboutfeed.net/ (Web page accessed on July13th, 2012); (b) Large SmF Wine Unit http://www.wine-searcher.com/m/2012/11/eucalyptus-in-australian-red-wines (Web page accessed on January 12th, 2013).

In SSF, the low moisture content means that fermentation can only be carried out with a limited number of microorganisms [Santos *et al*., 2004]. The nature of the solid substrate is one of the most important factor affecting SSF processes [Zhu *et al*., 2011; Vastrad and Neelagund, 2011; Giraldo *et al*., 2012].

On the other hand, SmF systems have several characteristics that make these systems attractive for the microorganism cultivation which include:

i) the liquid medium allows uniform conditions for the microorganisms growth;
ii) the modification of cultivation conditions like pH, dissolved oxygen, temperature, agitation, and nutrient concentration are easy and fast; and
iii) the temperature control is favored by the high specific heat and thermal conductivity [Mussatto and Teixeira, 2010].

Fermentation media used in these systems may be synthetically formulated or produced through hydrolysis of lignocellulose. A number of microorganism strains have been employed in fermentation processes using submerged cultivation, including bacteria, yeasts, fungi and algae [Taherzadeh and Karimi, 2007]. Shrestha (2008) showed biomass decomposing abilities of wood rot fungi in fermentation media. Oberoi *et al*. (2010) evaluated yeast cells in the submerged fermentation of orange peel feed stock after acid hydrolysis. As a result, ethanol yields of 0.25 g g^{-1} on a biomass basis and 0.46 g g^{-1} on a substrate-consumed basis were obtained. Dhillon *et al*. (2011) conducted submerged fermentations using apple (*Malus domestica*) pomace ultrafiltration sludge as an inexpensive substrate for Citric acid bioproduction, using *Aspergillus niger* NRRL567. Wilkins (2009) studied the effect of limonene oil of CPWO in ethanol production using the ethanologenic bacterium *Zymomonas mobilis* that presented a better performance.

Most fermentation studies concerning biomass, are based upon executing fermentation with a single microorganism, called mono culture fermentation. But it is also known that the use of non-*Saccharomyces* yeasts together with *Saccharomyces cerevisiae* may improve wine quality and flavor due to the production of variety of volatile compounds [Andorra *et al.,* 2010]. Therefore, an increasing interest in the use of non-*Saccharomyces* yeasts in co-culture with *Saccharomyces cerevisiae* is observed [Sadoudi *et al*., 2012]. The secondary metabolites produced by non-*Saccharomyces* species brings change in the chemical composition of wine that in turn changes the flavor and aroma profiles [Garde-Cerdán and Ancín-Azpilicueta, 2006; Moreira *et al.*, 2008]. Sadoudi *et al.* (2012) analyzed the volatile profiles obtained with several mono and co-culture fermentations. The main strains chosen for this study were *Candida zemplinina*, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* in mono or co-culture with *Saccharomyces cerevisiae*. Their results showed that the aromatic compounds formed during fermentation suffered alterations due to yeast-yeast interactions in co-cultures.

Lee *et al.* (2012) studied the biotransformation of durian pulp using mono- and co-cultures of *Saccharomyces cerevisiae* and *Williopsis saturnus* and got better results in the co-culture medium. During the production of wines, Andorra *et al.* (2010) also observed an increase in the production of alcohols when mixed cultures were used, in contrast to results for pure cultures of *Saccharomyces cerevisiae*. However, some studies have indicated opposite results, in which the mixed culture fermentations produced lower quantities of alcohol in comparison with fermentation performed with mono-culture of *Saccharomyces cerevisiae* [Moreira *et al*.,

2008; Lee *et al*., 2010; Kumar and Mishra, 2010]. This difference in results is may be a consequence of applying yeast strain that is not compatible with *Saccharomyces* [Lee *et al.,* 2012]. There are several factors that can influence the adaptation of the fermenting microorganism to the environment, such as temperature that directly affects the growth rate [Charonchai *et al*., 1998; Rakin *et al*., 2009]. Other significant variables are: the concentration of fermentable sugars in the raw material [Fleet and Heard, 1993] and pH which can vary from 2.75 to 4.25, and is considered an important factor for growth and survival of the yeasts [Fleet and Heard, 1993; Arroyo-López *et al*., 2009].

Usually hydrolytic processes do not just release sugars from lignocellulosic biomass but also a variety of compounds (toxins / inhibitors) which are derived from lignin or degradation of sugars [Liu *et al*., 2012]. The furfural and 5-hydroxymethylfurfural (HMF) are the furans that are normally found in significant amounts in hydrolyzates [Taherzadeh *et al.,* 1997]. The HMF corresponds to the main product of degradation of hexoses [Harris *et al.,* 1984], and it remains for much longer time in the hydrolyzate than the furfural. Therefore, the effects of HMF last longer and it is important to apply procedures for HMF and other inhibitors removal before performing the fermentation processes [Liu *et al*., 2012].

*

CHAPTER II: OBJECTIVES

* Image taken from Forestsareforever website. http://forestsareforever.org/Objectives.php (Web page accessed on 13th January, 2013).

2. OBJECTIVES

The main objective of this work was recycling of a raw material, Citrus Processing Waste from Oranges (CPWO), and obtaining valuable products, mainly 2-G bioethanol along with other biologically/industrially important constituents like hesperidin, pectin and essential oil. The idea of starting this work was enabling industrialization of this biomass in a more effective way that can change its concept from so called industrial waste to a valuable commodity.

The specific objectives for the accomplishment of our main objective are given below:

(i) Investigation of suitable methods for the removal of terpenes from orange peel.

(ii) Evaluation of extraction methods for hesperidin isolation from CPWO.

(iii) Application of classical way of acid hydrolysis to CPWO.

(iv) Determining suitable parameters for enzyme hydrolysis on bench scale.

(v) Evaluation of commercially available enzymes for obtaining fermentable sugars from CPWO.

(vi) Exploring enzymes produced by *Xanthomonas axonopodis* pv. *citri (Xac* 306*)*, a citrus pathogen, for hydrolysis of CPWO.

(vii) Comparison of acidic and enzymatic hydrolyses and their sugar yields.

(viii) Setting suitable parameters for fermentation on bench scale.

(ix) Application of different microorganism strains isolated from CPWO in mono- and co-culture fermentations of the hydrolyzates.

(x) Comparison of ethanol yields after fermentations and optimization of fermentation conditions.

*

CHAPTER III: MATERIALS AND METHODS

* Image taken in Chemical Biology Laboratory (LQB, IQ-UNICAMP) showing Bioethanol obtained from Orange Bagasse.

3. Materials and Methods

3.1. Citrus Processing Waste from Oranges (CPWO)

CPWO (peel, seeds and segment membranes) was collected at a local restaurant in Campinas (Sao Paulo, Brazil). It was ground in a food processor (Philco multiprocessor, 800W, China) to obtain 2-3 mm particle size in diameter (Fig. III-1). This CPWO was kept in plastic bags and stored frozen below 4°C. Besides this, CPWO was also obtained from orange juice factory (Citrosuco Matao, Sao Paulo, Brazil), for some comparative compositional analyses.

(a) (b) (c)

Figure III-1. (a) CPWO; (b) Food processor; (c) Homogenized CPWO.

3.2. Primary Characterization

Compositional analyses of starting material are important to be done, in order to know the exact nature of substrate involved in future biomass processing. Therefore primary characterization was performed following standard protocols. Pectin extraction procedure described by Sudhakar and Maini (2000) was used to extract and quantify pectin from CPWO, while total solids were calculated using standard procedure of NREL, Laboratory Analytical Procedure (2012). Ash contents were calculated applying standard AOAC method: 940.26 [AOAC, 2012]. Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) and Acid Detergent Lignins (ADL) were determined to estimate quantity of cellulose, hemicellulose and lignin proportion present in CPWO [ANKOM Technology, USA]. The difference between NDF and ADF corresponded to hemicellulose while difference between ADF and ADL gave amount of cellulose present in CPWO [Oberoi *et al*., 2010]. Total Protein contents in CPWO were determined by Bradford method (1976).

3.2.1. Determination of Total Solids

In order to determine total solids in CPWO, standard procedure described by NREL [National Renewable Energy Laboratory, 2012] for "Determination of Total Solids in Biomass" was used.

For triplicate analysis, 3 Petri dishes were pre-dried by placing them in a 105°C drying oven for a minimum of 30 minutes. After that, dishes

were cooled at room temperature in a desiccator and were weighed (M_1) on an analytical balance (Mettler AE 200). 17 g of homogenized CPWO was spreaded as evenly as possible on each petri dish and new weight was noted (M_2). Samples were placed in a convection oven at 105ºC for drying. After 4 hours, samples were removed from the oven and cooled down to room temperature in a desiccator. Weight of the dish containing dried CPWO was taken and the sample was placed back into the oven at 105ºC and dried to constant weight (M_3).

The total solids were calculated using equation III-1:

$$\% \text{ Total Solids} = \left[\frac{(M_3 - M_1)}{(M_2 - M_1)}\right] \times 100 \quad \text{(eq. III-1)}$$

Where:

M_1 = mass of empty Petri dish in g,

M_2 = mass of Petri dish + mass of CPWO sample in g,

M_3 = mass of Petri dish + mass of completely dried CPWO in g.

3.2.2. Determination of Moisture Contents

After drying 17 g of CPWO homogenized sample to constant weight using above mentioned procedure (3.2.1), moisture contents were calculated via equations III-2 or III-3:

$$\% \text{ Moisture} = \left[1 - \left(\frac{M_3 - M_1}{M_2 - M_1}\right)\right] \times 100$$

(eq. III-2)

Where:

M_1 = mass of empty Petri dish in g,

M_2 = mass of Petri dish + mass of CPWO sample in g,

M_3 = mass of Petri dish + mass of completely dried CPWO in g.

or

$$\% \text{ Moisture} = 100 - \% \text{ total solids}$$

(eq. III-3)

3.2.3. Determination of Ash Contents

The ash contents in CPWO samples were calculated according to the standard AOAC official method: 940.26. Three empty porcelain crucibles were weighed on an analytical balance and their mass were denoted as (M_1). In the next step, 17 g of CPWO samples were placed in each crucible and dried in oven to constant weight (M_2). Muffle furnace was pre-heated at 525^{o}C and samples were kept for 4 hours. After that, samples were removed, allowed to cool down in a desiccator and their mass was determined (M_3). Crucibles were placed back into the muffle furnace at 525^{o}C and ashed until constant weight.

Ash contents of CPWO were calculated using equation III-4:

$$\% \text{ Ash } = \left[\frac{(M_3 - M_1)}{(M_2 - M_1)}\right] \times 100$$

(eq. III-4)

Where:

M_1= mass of empty crucible in g,

M_2= mass of crucible + mass of dried CPWO in g,

M_3= mass of crucible with ash in g.

3.2.4. Determination of Pectin

Pectin determination was done in triplicate, in accordance with the protocol standardized by Sudhakar and Maini (2000). 17 g CPWO was boiled in extracting medium composed of 0.05M HCl (Merck) with peel and extractant ratio of 1:2 (m/V) for one hour at 100^{o}C. The extract was strained through a strainer, cooled and filtrate was set aside inside a beaker. From the residue left on the stainer, again second extraction was done using same procedure and stained again. The filtrates obtained in both extractions were mixed together and cooled down. Pectin was precipitated by adding two volumes of 95% ethanol (Quemis), acidified with 0.05M HCl. The resulting precipitates were filtered off on a pre-weighed filter paper (M_1) and the precipitated pectin was then washed at least twice with 65% acidified ethanol (acidified with 0.05M HCl). Finally, 95% ethanol was used to wash extracted pectin that was dried at 40^{o}C in an oven until constant weight (M_2).

Amount of pectin present in CPWO was finally calculated by equation III-5:

$$\%\ \text{pectin in CPWO} = \left(\frac{M_2 - M_1}{M_3}\right) \times 100$$

(eq. III-5)

Where:

M_1 = mass of filter paper in g,

M_2 = mass of filter paper + pectin in g,

M_3 = mass of CPWO in g.

3.2.5 Fiber Analyses

Fiber analyses were performed for determining cellulose, hemicellulose and lignin present in the biomass. For this purpose, three protocols for evaluating fiber types: "NDF" ("Neutral Detergent Fiber"), "ADF" ("Acid Detergent Fiber") and "ADL" ("Acid Detergent Lignins") were used.

3.2.5.1 Neutral Detergent Fiber (NDF)

NDF was determined applying Filter Bag Technique [ANKOM Technology Methods, 2012]. In this method, CPWO is treated with a detergent solution and the residue left is composed from fibers that are predominantly cellulose, hemicellulose and lignin.

Three filter bags (F57, ANKOM Technology) were weighed on an analytical balance (Mettler AE 200) as (M_1) and zero balance. 0.45-0.55 g of CPWO (M_2) was placed directly in filter bag. Using a heat sealer, the upper edge of the filter bag within 4 mm of the top was completely sealed. Two blank bags were weighed and included in run to determine blank bag correction (C_1). To a 1000 mL beaker, 500 mL of ND solution (Table III-1), 5 g of sodium sulfite (Synth) and 1 mL of alpha-amylase (Sigma) was added and all bags were suspended inside this solution. The beaker was covered and left for agitation at 100°C for about 75 minutes. After that the filter bags were removed from the solution and rinsed thrice with hot distilled water (70-90°C). Excess water from bags was gently pressed out and bags were placed in a 250 mL beaker and then soaked in acetone (Nuclear) for 3-5 min. After that they were first air-dried and then placed in oven at 102°C for drying (2-4 hours). All filter bags were cooled to ambient temperature in a desiccator and weighed (M_3). % NDF was calculated using equation III-6:

$$\%\ \text{NDF (in CPWO sample)} = \left[\frac{M_3 - (M_1 \times C_1)}{M_2}\right] \times 100$$

(eq. III-6)

Where:

M_1 = mass of filter bag in g,

M_2 = mass of CPWO in g,

M_3 = mass of dry filter bag with fiber after extraction process in g

C_1 = Blank bag correction

(final oven-dried weight /original blank bag weight)

Table III-1. Neutral detergent solution for determining NDF

Neutral Detergent Solution (ND solution) **500 mL**	
Sodium dodecyl sulfate (Acros) Ethylenediaminetetraacetic disodium salt, dehydrate (Bio Agency) Sodium borate (Synth) Anhydrous sodium phosphate dibasic (Synth) Triethylene glycol (Sigma)	15.0 g 9.31 g 3.40 g 2.28 g 5.0 mL
All reagents were diluted to 500 mL using distilled H_2O. The resulting solution was slightly heated and agitated for 5 minutes. pH range = 6.9 to 7.1	

3.2.5.2. Acid Detergent Fiber (ADF)

Acid Detergent Fiber was determined applying Filter Bag Technique [ANKOM Technology Methods, 2012]. This method determines ADF, which is the residue remaining after digestion of biomass with H_2SO_4 and CTAB. The residual fibers are predominantly cellulose and lignin.

Three filter bags (F57, ANKOM Technology) were weighed (M_1) and zero balance. 0.45-0.55 g of ground CPWO (M_2) was weighed directly in filter bag and sealed the upper edge of the filter bag (within 4 mm of the top) by heat sealer. Two blank bags were weighed and included in run to determine blank bag correction factor (C_1). To a 1000mL beaker, 500ml Acid detergent (AD) solution (Table III-2) was added and all bags were suspended inside this solution. The beaker was covered and left for agitation at 100°C for about 60 minutes. After that the filter bags were removed from the solution and rinsed with distilled water. During rinsing, agitation was turned on and bags were left in hot distilled water (70-90°C) for about 5 minutes. Rinsing process was done thrice and after that excess water from bags was gently pressed out and bags were placed in a 250 mL beaker and were soaked with acetone for 3-5 min. After that, they were first air-dried and then placed in an electric oven at 102°C for drying (2-4 hours). All filter bags were cooled to ambient temperature in a desiccator and weighed (M_3). % ADF in CPWO sample was calculated by equation III-7:

$$\%\ \text{ADF (in CPWO sample)} = \left[\frac{M_3 - (M_1 \times C_1)}{M_2}\right] \times 100 \qquad \text{(eq. III-7)}$$

Where:

M_1 = mass of filter bag in g,

M_2 = mass of CPWO in g,

M_3 = mass of dry filter bag with fiber after extraction process in g,

C_1 = Blank bag correction

(final oven-dried weight /original blank bag weight).

Table III-2. Acid detergent solution for determining ADL

AD solution, 500mL	
Cetyl trimethylammonium bromide (CTAB, Synth)	10 g
1.00N H_2SO_4 (Synth)	0.5 L
Both of these were agitated well to get a homogeneous solution	

3.2.5.3. Acid Detergent Lignin (ADL)

ADL was determined in order to estimate lignin contents [ANKOM Technology Methods, 2012], in starting biomass (CPWO).

For ADL analysis, three filter bags (F57, ANKOM Technology) were weighed (M_1) and zero balance. 0.45-0.55 g of air-dried CPWO (M_2) was weighed directly in filter bag and sealed the upper edge of the filter bag (within 4 mm of the top) by heat sealer. Two blank bags were weighed and included in run to determine blank bag correction factor (C_1). First, ADF determinations were performed and later all dried bags were placed into 250 mL beaker and sufficient quantity of 72% H_2SO_4 (Synth) was added to cover bags. A small 100 mL beaker was placed inside 250 mL beaker to keep all bags submerged and at 30 minutes interval, they were agitated by gently pushing and lifting 100mL beaker up and down about

10 times. After 3 hours, H_2SO_4 was poured off and bags were rinsed with tap water to remove all acid. Rinsing was repeated until pH was neutral. At neutral pH, bags were soaked in acetone (Nuclear) for 3 minutes to remove water. After that they were first air-dried and then placed in an electric oven at 105°C for drying (2-4 hours). All filter bags were cooled to ambient temperature in a desiccator and weighed (M_3). % ADL was calculated by using equation III-8:

$$\%\ \text{ADL (in CPWO sample)} = \left[\frac{M_3 - (M_1 \times C_1)}{M_2}\right] \times 100 \qquad \text{(eq. III-8)}$$

Where*:*

M_1 = mass of filter bag in g,

M_2 = mass of CPWO in g,

M_3 = mass of dry filter bag with fiber after extraction process in g,

C_1 = Blank bag correction

(final oven-dried weight/original blank bag weight).

3.2.6. Determination of Protein contents by Bradford

Protein determination in CPWO sample was done by Bradford protein assay. For standard curve, different concentrations of Bovine serum albumin (BSA-Sigma-Aldrich) were prepared. In the small test tubes, 100 µL of BSA solution and 3.0 mL of Bradford Reagent (Table III-3) were

added, mixed well by pipettor by drawing the mixture into the pipettor tip repeatedly and left for 5 minutes. Absorbance was noted at a wavelength of 595 nm on spectrophotometer (*Femto CIRRUS 80MB*) by using disposable *plastic cuvettes.* Water was used as blank and absorbance was plotted as a function of mass of BSA.

For the sample, same procedure was repeated and protein mass was measured by standard curve. All analyses were done in triplicate.

Table III-3. Reagents for preparing Bradford

Reagentes	**Quantities**
Coomassie blue (Sigma) Absolute methanol (Nuclear) Conc. phosphoric acid (Lufan) Distilled water	10 mg 5 mL 10 mL 100 mL
All reagents were added in this order and solution was filtered. The resulting solution has brown color that turned blue after adding protein solution.	

3.3. Hesperidin

Hesperidin was extracted by methodology of Ikan (1991). For extracting hesperidin from CPWO, approximately 200 g of biomass was oven dried at 70°C for about 8 hours to remove all moisture contents and powdered in a food processor.

3.3.1. Extraction

20 g of powdered CPWO was filled in the thimble made from thick filter paper and placed inside extraction tube of a Soxhlet extractor. The Soxhlet extractor was placed onto a 500 mL flask containing 250 mL petroleum ether (Nuclear) as the extraction solvent and magnetic stir bar. The Soxhlet was then equipped with a condenser.

The reaction mixture was stirred and heated for 4 hours under strong reflux. The petroleum ether extract was then removed. The content of the extraction sleeve was spread out in a crystallization dish in order to remove the adherent petroleum ether. Afterwards, the substrate was again filled in the thimble, placed inside extraction sleeve and this time extraction was done with 250 mL methanol (Nuclear). In the beginning the solvent leaving the extraction sleeve was of brown color. It took about 3 hours when the solvent leaving the extraction sleeve was colorless.

3.3.2. Work up

The orange brown methanol extract was evaporated in the rotary evaporator until syrup consistency was achieved. This syrup was mixed with 50 mL of 6% acetic acid (Nuclear) and left for precipitation. The precipitated Yellow color solid was the crude hesperidin. It was centrifuged at 5000 rpm for 5 minutes. The supernatant was drained off and hesperidin was washed with 6% acetic acid. Crude hesperidin was dried at 60°C until constant weight was achieved.

3.3.3. Recrystallization

For recrystallization of hesperidin, a 5% solution of the crude product in dimethyl sulfoxide (Merck) was stirred at 60-80°C. Heating was stopped and same amount of distilled water was added drop-wise under constant stirring to the hesperidin mixture with dimethyl sulfoxide. The resulting solution was left for cooling at room temperature and white crystals of hesperidin were precipitated. This solution was centrifuged at 5000 rpm for 5 minutes, supernatant was drained off and hesperidin was washed with a small portion of warm distilled water and then with iso-propanol (Acros) and dried in the desiccator until constant weight was achieved. The extracted hesperidin crystals were analyzed with infra-red spectroscopy (IR) and nuclear magnetic resonance spectroscopy (NMR).

3.4. Pretreatment for extracting essential oil

CPWO contains considerable quantity of essential oil, rich in D-limonene (more than 90%) that is a yeast growth inhibitor. Therefore, it must be removed before hydrolysis. Thus, 17 g of homogenized CPWO for every single hydrolysis was subjected to pretreatment for the removal of essential oils applying two methods.

In the first method, essential oil was extracted by steam distillation technique (Figure III-2). The steam produced in distillation flask was allowed to pass for about 20 minutes into the flask holding CPWO. The high temperature steam causes small sacs containing essential oil to burst. The oil vapors were carried by the steam out of the flask and after passing

through condenser, condense to liquid distillate. The oil was extracted from the distillate by using a separating funnel. Extraction was done with diethyl ether (Nuclear). After vigorous shaking, mixture was left for 5 minutes to allow separation of aqueous from organic phase. Aqueous layer was drained off, while organic layer was collected in a small collection flask. This extraction was repeated twice and collected ether fractions were dried using anhydrous sodium sulfate (Synth). In a rotary evaporator, ether was evaporated and the resulting oily fraction was weighed for the mass determination. The extracted oil was analyzed using gas chromatography coupled with mass spectrometry (GC-MS).

In the second case, in 250 mL Erlenmeyer flasks 17 g CPWO was subjected to auto-hydrolysis in an autoclave, at a temperature of 120˚C under high pressure of $9.4x10^4$ Pa for about 15 minutes.

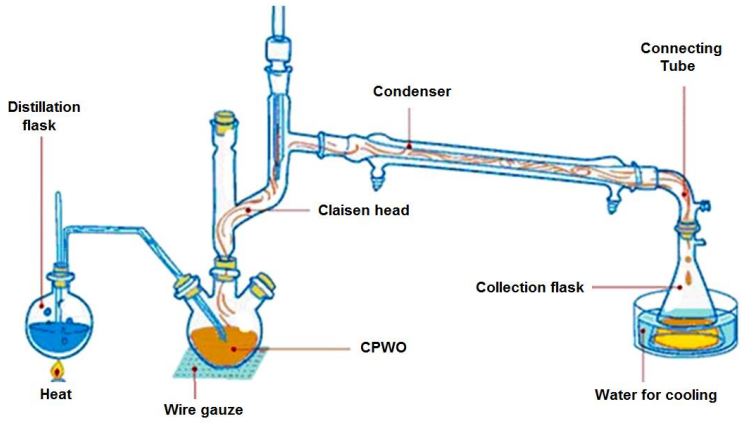


Figure III-2. Steam distillation technique for the extraction of essential oil (adapted from *NovaEscola website*, http://revistaescola.abril.com.br/ensino-medio/cheiros-milenares-500064.shtml. Web page accessed on January 5th, 2013).

3.5. Acid Hydrolyses

All acid pretreatments were done in triplicate. For each pretreatment, 17 g of CPWO (thawed) was mixed with 100 mL of distilled water in a 250 mL Erlenmeyer flask. The concentrated sulfuric acid (98%) was added in such a way that the resulting acid concentration was equal to: 0.5; 1; 1.5% (v v^{-1}) or 0% (control experiment without any acid). After that, Erlenmeyer flasks were autoclaved at 120°C and 9.4 $x10^4$ Pa for 15 or 30 min. The hydrolyzates were collected and analyzed with HPLC. Acid insoluble and acid soluble lignin contents were determined using standard protocol of NREL, determination of Structural Carbohydrates and Lignin in Biomass (2012).

3.5.1 Determination of acid insoluble lignin

For determining acid insoluble lignin, the hydrolyzates were filtered after hydrolysis using pre-weighed filter paper (M_1). The filtrate was collected in a 250 mL Erlenmeyer and was used for fermentations. The residues on the filter paper were rinsed 5 times with hot distilled water (80 mL) to remove all acid, soluble lignin and sugars. The filter paper along with acid insoluble residue was dried at 105°C in an electric oven for four hours and later, kept in a desiccator for cooling at room temperature. Samples were weighed and again kept in oven until a constant weight was achieved (M_2). This insoluble residue also contains ash since it is insoluble. So, the ash contents determined according to the methodology

described earlier (**3.2.3**) were subtracted for calculating acid insoluble lignin portion.

The percent of acid insoluble residue and acid insoluble lignin were calculated according to equations III-9 and III-10, respectively:

$$\%\ \text{acid insoluble residue} = \left(\frac{M_2 - M_1}{M_3}\right) \times 100 \quad \text{(eq. III-9)}$$

$$\%\ \text{acid insoluble lignin} = \%\ \text{acid insoluble residue} - \%\ \text{ash contents} \quad \text{(eq. III-10)}$$

Where:

M_1 = mass of filter paper in g,

M_2 = mass of filter paper + acid insoluble residue in g,

M_3 = dry mass of CPWO (17 g moisture contents).

3.5.2. Determination of acid soluble lignin

Aliquots of the filtered hydrolyzates were analyzed for acid soluble lignin using UV-Vis spectroscopy, within six hours of acid hydrolysis. A blank of deionized water was run on a UV-Vis spectrophotometer (Hitachi U-200), at 240 nm, in a 1 cm path length quartz cuvette. The samples were diluted with deionized water to have an absorbance in the range of 0.7-1.0.

Each sample was analyzed in triplicate. The percent of acid soluble lignin was calculated using equation III-11:

$$\%\ \text{acid soluble lignin} = \left(\frac{UV_{abs} \times V_{filtrate} \times \text{Dilution factor}}{\varepsilon \times M \times \text{Pathlength}}\right) \times 100$$

(eq. III-11)

Where:

UV_{abs} = UV-Vis absorbance at 240 nm wavelength,

$V_{filtrate}$ = volume of filtrate in L,

$\varepsilon = 25\ L.g^{-1}.cm^{-1}$ (absorptivity of biomass at 240 nm wavelength),

M = dry mass of CPWO in grams,

Pathlength = pathlength of UV-Vis cell in cm,

Dilution factor was calculated using equation III-12:

$$\text{Dilution factor} = \frac{Volume_{sample} + Volume_{solvent}}{Volume_{sample}}$$

(eq. III-12)

The total amount of lignin on an extractives free basis was calculated with equation III-13:

$$\%\ \text{total lignin} = \%\ \text{acid soluble lignin} + \%\ \text{acid insoluble lignin}$$

(eq. III-13)

3.6. Enzymes used for enzymatic hydrolyses of CPWO

The enzymatic hydrolysis was carried out using the following commercial enzymes: pectinase (EC 3.2.1.15, obtained from *Aspergillus niger*, Sigma-Aldrich), Celluclast 1.5 L (EC 3.2.1.4, obtained from *Trichoderma reesei* ATCC 26921, Sigma-Aldrich) and β-glucosidase (EC 3.2.1.21, obtained from *Aspergillus oryzae* , Sigma). Besides these three commercial enzymes, first time the enzymes present in protein lysates from bacterium *Xanthomonas axonopodis* pv. *citri* 306 (*Xac* 306; IBSBF 1594) were also explored.

3.7. Protein lysates of "*Xac* 306"

Xac 306 cells were cultured in modified, salt-free Luria-Bertani medium composed of 0.5% m v^{-1} Yeast extract (Neogen) and 1% m v^{-1} peptone (Neogen) in the presence of ampicillin. A stock solution of ampicillin with a concentration of 50 mg mL^{-1} was prepared from ampicillin sodium salt (Sigma-Aldrich) in sterile deionized water or a mixture of sterile deionized water and absolute ethanol (1:1; v v^{-1}). It was filtered and stored at -4°C. This solution was added in the culture medium in such a way that the final concentration of antibiotic in the medium was 50 µg mL^{-1}. In these conditions, *Xac* 306 cultures were allowed to grow at 32°C for 16 to 24 h in an incubator shaker until the A_{600} (600 nm) reached a value of approximately 0.8.

The cell culture was centrifuged at 4°C with 15,000 rpm agitation (Beckman Coulter Alegra X-22R) for 30 minutes, and the cell mass was suspended in a lysis buffer (50 mM Tris-HCl, 50 mM NaCl and 10 mM

EDTA solution) at pH 8 that was sterilized before use. The proportion of cell mass to lysis buffer was 0.8 % (v/v). This mixture was stored in a refrigerator at -80 ° C at least for 1 h and was defreezed at 4°C. After that cells were ruptured by sonication (Sonicator with probe Sonic VCX-750), with 30% pulse amplitude applied during 2 minutes (pulsing for 10 sec and resting for 40 sec) in an ice bath.

The lysate that was obtained was centrifuged at 4°C under agitation of 15,000 rpm for 15 minutes and the supernatant was dialyzed with water for a period of 48 hours, using cellulose membrane (with a porosity of 3.5 kDa, Dialysis Tubing – Fisher Scientific). Subsequently, this mixture was lyophilized in a lyophilizer (TERRONI LB 300TT) and stored at -20°C until used.

3.8. Enzymes activities

3.8.1.Pectinase and Polygalacturonase (PG) activities

This enzyme assay is based on the hydrolysis of citrus pectin and polygalacturonic acid, followed with the resulting galacturonic acid determination spectrophotometrically at 575 nm. This assay was applied by using pectin (Sigma-Aldrich) and polygalacturonic acid (Sigma) as substrates respectively [Phutela *et al*., 2005].

Citrate buffer was prepared in concentration of 0.05 M and pH 4.4. 1% substrate solution was prepared in this buffer and enzyme dilution was also made with a suitable concentration. Equal amounts (1.5-3 mL) of both these solutions were taken and mixed in a small test tube. This reaction

mixture was left in a water bath at 50ºC for about 30 minutes. At the end of indicated period, assay was stopped by addition of DNS solution and kept for 10 minutes in a boiling water bath. The assay mixture was cooled down and absorbance was read at 575 nm using UV-visible spectrophotometer (*Femto* CIRRUS 80MB). The quantity of released reducing sugar was calculated by plotting standard curve for galactouronic acid (Sigma). Pectinase and polygalacturonase enzyme's activity was calculated using equations III-14 and III-15:

$$\text{Volume activity}\left(\frac{\text{Units}}{\text{mL}}\right) = \frac{\mu\text{moles}_{\text{Gal.acid}} \times \text{V}_{\text{total}} \times \text{df}}{\text{V}_{\text{enz.}} \times \text{time}_{\text{assay}}} \quad \text{(eq. III-14)}$$

Or

$$\text{Weight activity}\left(\frac{\text{Units}}{\text{mg}}\right) = \left(\frac{\text{U}}{\text{mL}}\right) \times \left(\frac{1}{\text{C}}\right) \quad \text{(eq. III-15)}$$

Where:

df = dilution factor,

$V_{Enz.}$ = mL of enzyme solution used in the reaction,

V_{final} = final reaction volume,

$time_{assay}$ = time for assay in minutes,

$\mu moles_{Gal.acid}$ = micromoles of galacturonic acid released,

C = concentration of enzyme solution (mg mL^{-1}).

3.8.2. Beta-glucosidase Assay

Beta-glucosidase activity was measured with *p*-nitrophenyl-β-D-glucopiranoside (PNPG) substrate [Carrão-Panizzi and Bordingnon, 2000].

The assay is based on the release of *p*-nitrophenol (PNP) that is measured spectrophotometrically at 420 nm. Beta-glucosidase activity is measured in Units. One unit (U) is defined as the amount of enzyme that will produce 1 µmol of *p*-nitrophenol (PNP) per min at 40°C under the conditions described below.

1 mM of PNPG (Sigma-Aldrich) solution was made in sodium phosphate buffer (0.1 M, pH 6.7). Equal amounts of PNPG solution and suitably diluted enzyme solution were incubated in a test tube. The assay was left at 40ºC for 25 minutes.

Reaction was stopped by adding sodium carbonate solution (0.25 M, pH 9.0). The amount of released PNP was spectrophotometrically determined at 420 nm (*Femto* CIRRUS 80MB, Brazil) based on the yellow color appearance.

The blank was made using same amount of PNPG solution (used to make sample), adding it to Na_2CO_3 and then to enzyme solution.

Activity was calculated using the equation III-16:

$$\text{volume activity}\left(\frac{\text{Units}}{\text{mL}}\right) = \frac{\text{A} \times \text{V}_{\text{total}} \times \text{df}}{18.1 \times \text{V}_{\text{Enz}} \times \text{time}} \qquad \text{(eq. III-16)}$$

where:

df = dilution factor,

V_{Enz} = mL of enzyme solution used in the assay,

V_{final} = final reaction volume,

time = time for assay in minutes,

18.1 = millimolar extinction coefficient of PNP under the assay conditions (cm^2 μmol^{-1}).

3.8.3. Cellulase assay

Cellulase activity in terms of "filter paper units" (FPU) was measured applying standard procedure of National Renewable Energy laboratory based on the methodology of Ghose (1987) and Miller (1959).

The substrate used were filter paper strips; Whatman No. 1. 50 mg of substrate was added to test tubes and soaked with 1.0 mL of 0.05 M sodium citrate buffer (pH 4.8).

Tubes were maintained at 50°C and then 0.5 mL of suitable enzyme dilutions were added to them. Tubes were incubated with blank (citrate buffer) and controls (enzyme and substrate) at 50°C for exactly 60 min. At the end, 3.0 mL of DNS reagent (Table III-4) was added and tubes were placed in a boiling water bath. After 5 minutes, they were transferred to a cold ice-water bath. Samples were diluted according to absorbance at 540 nm that should be in the range of 0.1 to 1.0.

The amount of glucose released for each sample tube was obtained using glucose standard curve. Using the same curve, concentration of enzyme which would have released exactly 2.0 mg of glucose was estimated.

Table III-4. Preparation of DNS reagent

DNS Reagent	
Reagents	Quantity
Distilled water	1000 mL
3,5-dinitrosalicylic acid (Sigma)	10.6 g
Sodium hydroxide (Nuclear)	19.8 g
Phenol (melted at 50°C; Sigma-Aldrich)	7.6 mL
Sodium metabisulfite (Synth)	8.3 g
All reagents were mixed and kept in amber flask for protection from light, labeled as DNS stock solution.	
Rochelle salt was added just at the time of use.	
Reagents	Quantity
DNS stock solution	100 mL
Rochelle salts (sodium potassium tartrate; Nuclear)	30.6 g
After mixing both these reagents, distilled water was added to a final volume of 146 mL.	

Filter paper activity was calculated applying equation III-17:

$$\text{Filter paper activity}\left(\frac{\text{Units}}{\text{mL}}\right) = \frac{0.37}{\text{enzyme releasing 2 mg of glucose}} \quad \text{(eq. III-17)}$$

3.9. Enzyme Hydrolysis

For enzyme hydrolysis, the biomass was first treated to extract essential oil. In erlenmeyer flask, CPWO was diluted to 17% (w v^{-1}) with sterile water or sodium citrate buffer (50 mM, pH 4.8). The pH range was around 4.5-5.0. Chloramphenicol (30 µg mL^{-1}; Fluka) was added as an antibiotic in order to prevent contamination during enzyme hydrolysis. Enzymes were added in varying concentrations and erlenmeyer flasks were stirred at 45°C (35-40 rpm) for 48 h. To analyze the sugar contents, samples were collected at intervals: 0, 0.5, 1, 3, 6, 12, 24, 36 and 48 hours. The collected samples were kept in an electric oven at 110°C for 15 min in order to deactivate the enzymes. Later, samples were frozen at -20^{0}C until analyzed by HPLC. After 48 h when the hydrolysis was completed, the resulting biomass slurry was filtered and the hydrolyzates were fermented with microorganisms. All experiments were repeated thrice.

3.10. Yeast strains and growth medium

Twenty different types of microorganisms had been isolated from the CPWO by our research group. These microorganisms were screened for their fermentation ability in a medium enriched with carbohydrates. Among twenty, two microorganisms gave best results and were chosen for the CPWO acidic and enzymatic hydrolyzate fermentations. The two strains were identified as *Candida parapsilosis* IFM 48375; AB363782 and *Candida parapsilosis* NRRL Y-12969, U45754. Apart from these two strains, commercial yeast strain, *Saccharomyces cerevisiae* (Itaiquara

yeast), was also employed in our mono- and co-culture fermentation studies.

For growing any of the yeast strains, sterile Yeast Peptone Dextrose (YPD) medium was prepared in an erlenmeyer flask (Table III-5). Cells were aseptically inoculated in the laminar flow hood, chloramphenicol (30 µg mL^{-1}; Fluka) was added and erlenmeyer flasks were incubated at 32°C, for 14-18 h in an incubator shaker (Marconi, Brazil) with 120 rpm. When the absorbance, A_{600nm} of the growth medium reached the range of 0.8 to 1.5, growth medium was analyzed for the cell count. Cell count was performed using a Newbauer plate, and the cells were concentrated by centrifugation or diluted with sterilized YPD medium (2%) in order to achieve cell count of ~ 1×10^9 cells mL^{-1}. A fraction of 10% (v v^{-1}) inoculum was added to the hydrolyzates, yielding an inoculum concentration of ~ 1×10^8 cells mL^{-1} at the start of fermentation.

Table III-5. Preparation of growth medium for yeast cells

Yeast peptone dextrose (YPD) Media	
Yeast extract (Neogen)	10.8 g L^{-1}
Peptone (Neogen)	15 g L^{-1}
Dextrose (Synth)	20 g L^{-1}
Deionized water was used as a solvent and resulting medium was sterilized in autoclave at 120ºC for 15 min.	

3.11. Mono- and co-culture fermentations

For mono- and co-culture fermentation studies, pH of hydrolyzates was checked in each case and adjusted to pH 5 with solid $CaCO_3$. The microorganisms were inoculated with Chloramphenicol (30 µg mL^{-1}; Fluka) and hydrolyzates were left for fermentation in an incubator shaker (Marconi) at 35°C during 48 h, at a constant stirring rate of 120 rpm. All fermentation experiments were performed in triplicate, and samples were collected over intervals: 0, 0.5, 1, 3, 6, 9, 12, 24; 36 and 48 h for performing qualitative and quantitative analyses. After 48 hours, fermented hydrolyzates were distilled twice to extract 2-G ethanol and analyzed with ^{1}H and ^{13}C NMR.

3.12. HPLC Analysis

Bioethanol, sugars (glucose, fructose, arabinose, galactose), galacturonic acid, polygalacturonic acid, 5-hydroximethylfurfural (5-HMF), furfural and acetic acid were quantified using HPLC (Alliance e2695series, Waters Co., USA), a pre-column SH-G (6 x 50 mm) Shodex 1011 and column (300 mm x 8 mm) (Showa Denko Co., Japan). Sugars, ethanol, galacturonic acid, 5-HMF, furfural and acetic acid were detected using refractive index detector (Model-2414, Waters Co., USA). The oven temperature was maintained at 50ºC and 0.005 $molL^{-1}$ sulfuric acid (degassed) was used as an eluent. The eluent had a flow rate of 0.6 $mLmin^{-1}$. All hydrolysed or fermented samples, collected for HPLC analyses were first centrifuged (Eppendorf Centrifuge 5424, Hamburg, Germany), then

filtered (Maxcrom OEM; PTFE syringe filters 13 mm/0.45 μm) and kept into HPLC vials for analyses. Standard calibration curves were plotted employing working standards, which were purchased from Synth or Sigma Aldrich.

3.13. Gas chromatography–mass spectrometry (GC-MS)

Essential oil from CPWO was analyzed with gas chromatography coupled to a mass spectrometer (GC-MS).

Specifications of GC-MS operating system are as follows:

►GC:

Equipment: GCT – Premier Waters

Column: HP5 – MS

Conditions:

Injection Temperature: 270°C

Type of injection: Split (50:1)

Injected Volume: 1μL

Solvent: CH_2Cl_2

Carrier gas flow: 1mL min^{-1}

Temperature programming: maintaining 50°C for 3 min

Temperature elavation ratio: 15°C min^{-1} maitained upto 280°C.

►MS:

Equipment: GCT – Premier Waters

Conditions:

Inlet temperature: 250°C

Ion source Temperature: 200°C

Function: Full Scan

Full-Scan range: (m/z) 50-600

Interval between scans: 0.01sec.

The sample was prepared with a concentration of 10 mg mL^{-1} (oil solution in CH_2Cl_2) and injected in GCT – Premier Waters equipment with the above mentioned specifications. The obtained gas chromatogram and mass spectrum were compared with the available literature data to elucidate the oil components.

3.14. Nuclear magnetic resonance (NMR)

^{1}H NMR, ^{13}C NMR, COSY NMR and HSQC NMR were recorded in a Bruker Advance 600 MHz equipment at 25˚C. NMR experiments were done with equipment operating at 600 MHz frequency for ^{1}H and 150 MHz for ^{13}C. All experiments were done on a TBI (Triple Resonance Broadband Inverse) probe. Standard pulse sequences and parameters were used for the NMR experiments and all chemical shifts were reported in parts per million (ppm, δ). For data processing, ACD/NMR Processor Academic Edition, Version: 12.01 Software was used. Samples were prepared in DMSO-d6 (Cambridge Isotope Laboratories, Inc.) and the solvent peak at δ 2.5 was used as reference

Hesperidin NMR samples were prepared by dissolving 10-12 mg of a sample in 600 μL of DMSO-d6. For ^{1}H NMR, 32 scans were acquired while for ^{13}C NMR, a good resolution spectrum was obtained after 5120 scans. In case of COSY NMR, 16 scans were done while HSQC spectrum

has 32 scans. The pulse program used for the acquisition of COSY NMR was cosyqf while in case of HSQC it was hsqcedetgp.

3.15. Infrared spectroscopy (IR)

Infrared analysis of hesperidin sample was executed applying fourier transform infrared spectroscopy (FTIR) technique. The KBr disc was comprised of 1% g g^{-1} sample in KBr and placed to Bomem (MB100, Canada). Data was acquired in the transmittance mode. Spectrum was measured with 4 cm^{-1} resolution from 400 to 4000 cm^{-1}.

3.16. Statistical Analysis

Analysis of variance (ANOVA) was done by using GraphPad Prism5 Software for all experiments. Acid hydrolysis was performed considering two independent process variables: hydrolysis time and acid concentration. The enzymatic sacchacarification was done by taking into account three independent process variables: limonene pretreatment (steam distillation and autohydrolysis), Buffer solution or sterile water (to dilute 17 g CPWO) and effect of different enzyme loadings (individual commercial enzymes, their cocktails and *Xac* 306). Released sugars (glucose, cellobiose, fructose and arabinose) and sugar acids (polygalacturonic acid and D-galacturonic acid) were the dependent variables. In case of fermentation experiments, yeast species were the independent variables while ethanol yield was the dependent variable. The *P* value (level of significance) for all procedures were <0.05.

*

CHAPTER IV: RESULTS AND DISCUSSION

* Figure taken from Real Science website (http://www.realscience.us/2009/07/13/bridging-the-science-and-society-gap/. Web page accessed on January 15th, 2013).

4. RESULTS AND DISCUSSION

4.1. Compositional Analyses of CPWO

The CPWO contains significant amounts of polysaccharides, which can be easily hydrolyzed in acidic or enzymatic saccharification processes. Total solids in CPWO were determined in triplicate and account for about 19% of CPWO. Moisture content was equal to around 81%. These results are summarized in Table IV-1.

Table IV-1. Chemical composition of CPWO (local restaurant, Campinas)

pH of CPWO	**Moisture** **%**	**Total Solids** **%**	**Ash** **%**	**Pectin** **%**	**Proteins** **%**
4.1-4.3	80.93±0.53	19.07±0.41	1.95±0.35	4.65±0.52	6.40±0.63

The amount of cellulose, hemicellulose and lignin were obtained through NDF (Neutral Detergent Fiber), ADL (Acid Detergent lignin) and ADF (Acid Detergent Fiber) and are shown in Table IV-2. The difference between NDF (cellulose, hemicellulose and lignin) and ADF (cellulose and lignin) corresponded to hemicellulose, while the difference between ADF (cellulose and lignin) and ADL (estimated lignin contents) gave amount of cellulose present in CPWO (equation IV-1 and IV-2).

$$NDF - ADF = hemicellulose \quad \text{(eq. IV-1)}$$

$$ADF - ADL = cellulose \quad \text{(eq. IV-2)}$$

Table IV-2. Fiber analyses of CPWO

NDF **%**	**ADF** **%**	**ADL** **%**	**Cellulose** **(ADF-ADL)** **%**	**Hemicellulose** **(NDF-ADF)** **%**	**Lignin** **%**
13.00 ± 0.85	9.38 ± 0.57	4.40 ± 0.19	4.98±0.47	3.62±1.03	4.40 ± 0.19

Compared to other lignocellulosic biomass [Bampidis and Robinson, 2006], the CPWO has smaller proportion of lignin content. Low lignin level makes this substrate ideal for fermentation as less harsh pre-treatments are required to hydrolyze and it generates few by-products that could inhibit yeasts in fermentations. Pectins combined with proteins and other polysaccharides form strong skeletal plant tissues [Liu *et al.,* 2004]. In our case, CPWO collected from Sao Paulo gave pectin yield of about 5% as a whole and ~ 25% on dry matter (DM) basis. This percentage is about half of what was previously reported by Rivas *et al*. (2008) that was about 42.50%.

Many factors can affect nutrient levels of orange fruit. The most important among them are the source and method of processing the fruit [Ammerman and Henry, 1991]. As Brazil is a tropical country, the fruits require less time to grow and get mature. This shorter maturity time may result in a decreased level of pectin in fruits.

Comparative analyses were also performed with the industrial samples provided by Citrosuco (Matao, SP). Citrosuco Industry is been dedicated to orange production since 1932. They are one of the pioneers in the construction of an integrated system for bulk transportation of concentrated orange juice for storage, export and product distribution in the worldwide markets. In this orange processing factory, after juice extraction, CPWO left overs are crushed to a particle size ranging from 0.4 to 1 cm. These crushed biomass residues are further compressed by machines under high pressure and all soluble liquid fractions are removed inside a floater. The remaining biomass is converted into citrus pulp pallets. Two samples were analyzed for their composition: (i) one was industrial CPWO before compressing, (ii) while the other one was the solid residue of the floater. The results are shown in the Table IV-3.

Table IV-3. Chemical composition of industrial CPWO samples

Substrate	Moisture %	Total Solids %	Ash %	Pectin %
CPWO from Citrosuco before compressing	77.34±2.85	22.66±2.85	2.26±1.13	4.50±0.99
Solids left from the floater	89.70 ± 0.28	10.3 ± 0.28	0.81±0.59	0.53 ± 0.84

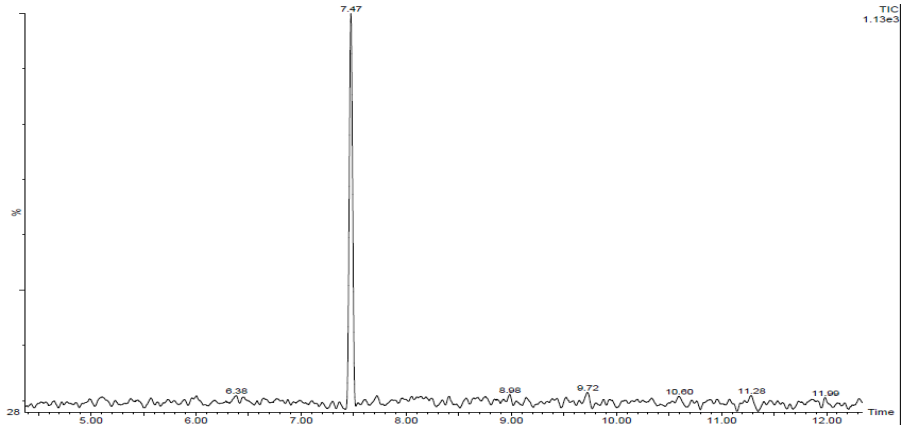
Obtained results show that the industrial CPWO (before compressing) gave results quite similar to the CPWO collected from a local restaurant. Industrial bagasse and CPWO biomass from local restaurant has similar

characteristics. However, solids lefts after compressing CPWO gave very different results with high moisture contents and low solid residues.

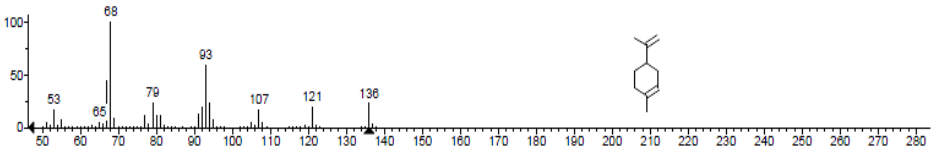
4.2. Extraction and characterization of essential oil from CPWO

The yield of essential oil obtained in various steam distillations performed individually or before hydrolysis was in the range of 0.8 to 1.5% (g g^{-1}). It was transparent in color with a very strong aroma. GC-MS analysis showed presence of about 99% of D-limonene. Figure IV-1 (a) shows chromatogram of the oily extract from CPWO with one main peak at retention time 7.47 min. The characteristic ion peaks of limonene observed in mass spectrum were, m/z: 68, 93 and 136.

D-limonene is a hydrophobic component that inhibits the growth of bacteria and yeasts [Pourbafrani *et al.,* 2007]. Its presence in the medium affects yeast more than bacteria [Subba *et al.,* 1967]. Biotransformation of D-limonene is known in several yeast species. However, these biotransformation reactions seem to be catalyzed by monooxygenases, that need oxygen [Duetz *et al*., 2003]. Therefore, oxidation of D-limonene under anaerobic conditions is not likely to occur and it is important to remove D-limonene prior to hydrolysis to prevent any inhibition of yeast cell growth during fermentation [Hillyer, 2012; Stewart *et al.*, 2013]. Moreover, by removing essential oil through autohydrolysis or steam distillation pretreatment, breakdown of lignocellulosic framework helps enzymes to penetrate the disintegrated tissues easily, hence increasing rate of enzyme hydrolysis [Grohmann *et al.,* 1995].



(a)



(b)

Figure IV-1. (a) Gas chromatogram of the extracted essential oil and(b) mass spectrum of the main peak in the oil extract (D-limonene).

4.3. Extraction and characterization of hesperidin

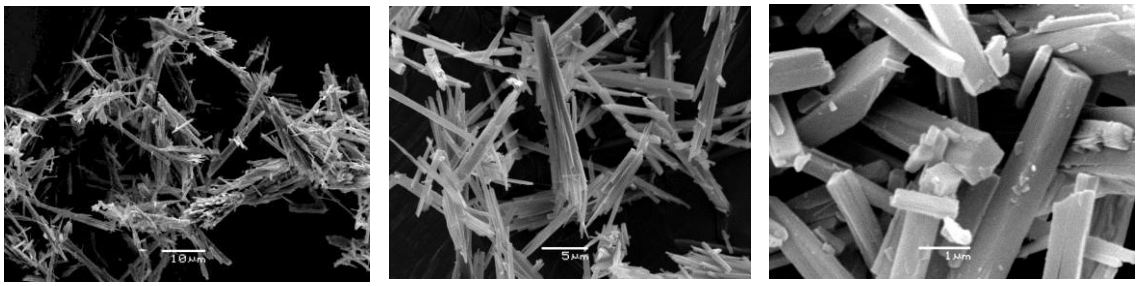
Hesperidin extraction from dried orange peel was done successfully. It was obtained as a yellow powder (Figure IV-2a) in a crude yield of 1.5% g g^{-1} from dry CPWO. Hesperidin was purified by recrystallization (Figure IV-2b).

(a) **(b)**

Figure IV-2. Extracted samples of (**a**) crude and (**b**) recrystallized hesperidin.

The recrystallized sample was in the form of white powder (yield ~1% g g^{-1} dry CPWO). It has been characterized applying SEM, IR, ^{1}H NMR and ^{13}C NMR, including 2D-NMR experiments (COSY and HSQC).

The Scanning electron micrograph revealed hesperidin as needle like structures (Figure IV-3).



(a) (b) (c)

Figure IV-3. Scanning electron micrographs of hesperidin with (a) 10 µm (b) 5 µm and (iii) 1 µm scales.

The IR spectrum (Figure IV-4) showed a strong band of OH stretching (str) at 3544 and 3470 cm^{-1}, CH (aliphatic) at 2976, 2916 and 2848 cm^{-1}, C=C (aromatic) at 1606, 1519, 1467 and 1443 cm^{-1} and of C=O (str) at 1648 cm^{-1}, C-O (str) at 1298, 1276, 1240, 1203, 1182, 1154, 1131, 1094, 1050, 1033 and 1009 cm^{-1}. This data was in accordance with Aghel *et al.,* 2008.

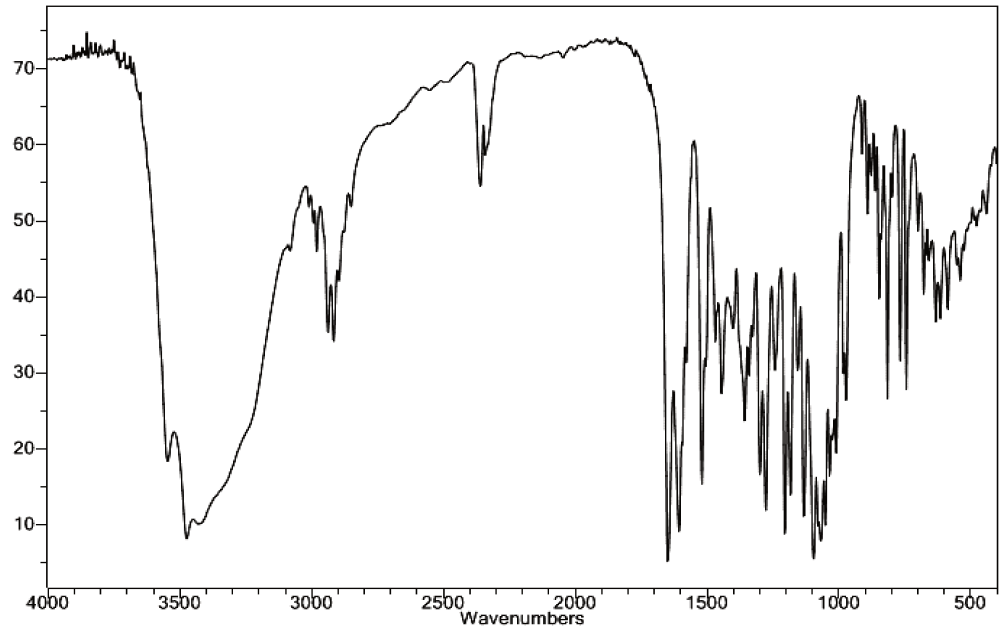


Figure IV-4. FTIR spectrum of hesperidin.

The ^{1}H NMR spectrum of hesperidin (Figure IV-5, Table IV-4) confirmed the structure of isolated compound. All hydrogen assignments are in excellent agreement with the literature data [Nizamutdinova *et al*., 2008; Kokotkiewicz *et al*., 2012]. Hesperidin has shown typical flavanone skeleton signals at δ 5.51 (1H, dd, J=12.1, 3.3 Hz, H-2), three protons of 1,3,4-trisubstituted ring at δ 6.91 (3H, m), two anomeric protons of glucose and rhamnose at δ 4.98 (1H, d, J=7.3 Hz) and 4.53 (1H, m), one methoxy signal at δ 3.8 (3H, s), one methyl signal at δ 1.09 (4H, d, J=6.2 Hz), and two aromatic hydroxyl signals at δ 12.02(1H, s, 5-OH) and 9.09 (1H, s).

The ^{13}C NMR also showed characteristic chemical shifts for hesperidin in agreement with the literature data [Aghel *et al.,* 2008] (Figure IV-6, Table IV-5).

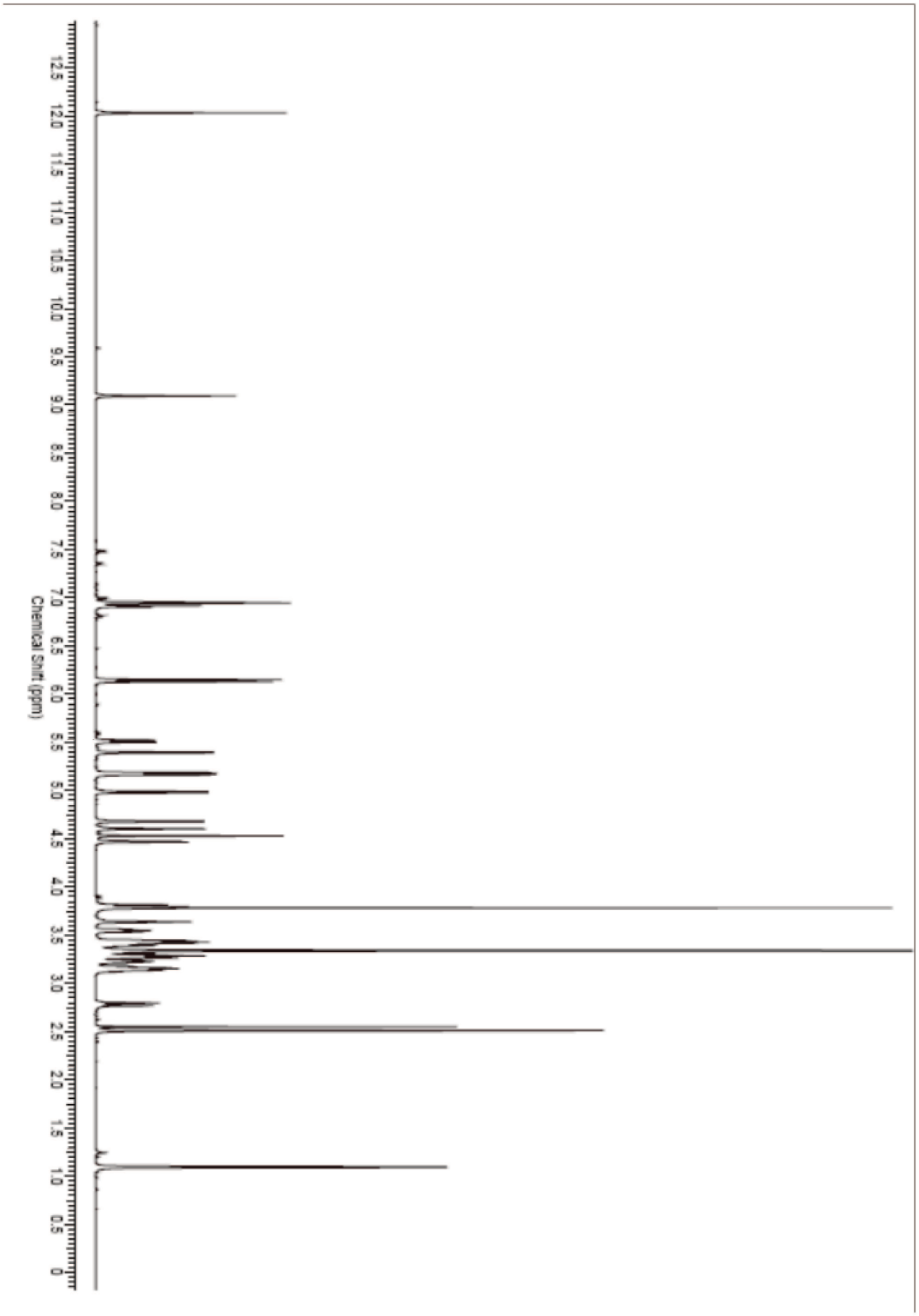
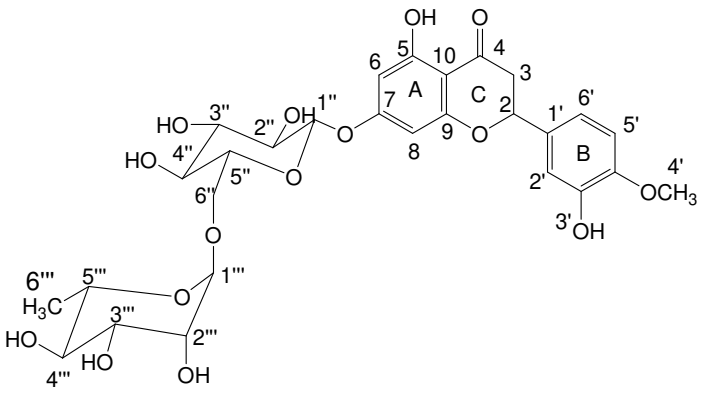


Figure IV-5. ^{1}H NMR spectrum of hesperidin.

Table IV-4. Hesperidin ^{1}H NMR chemical shifts and assignments

Assignment	Chemical shifts (ppm)	No. of Hydrogens	Assignment	Chemical shifts (ppm)	No. of Hydrogens
OH-5	*12.0 (s)*	*1*	H-1‴	*4.5 (d)*	*1*
OH-3′	*9.1 (s)*	*1*	4′-OCH_3	*3.8 (s)*	*3*
H-2′, 6′, 5′	*6.9 (m)*	*3*	H-2‴	*3.6 (m)*	*1*
H-3″	*3.3(m)*	*1*	H-5″	*3.5(m)*	*1*
H-3	*3.2(d)*	*1*	H-5‴	*3.4 (m)*	*1*
H-8	*6.1 (d)*	*1*	H-6″, H-3‴	*3.4 (m)*	*3*
H-6	*6.1 (d)*	*1*	H-2″	*3.2 (m)*	*1*
H-2	*5.5 (dd)*	*1*	H-4″, H-4‴	*3.2 (m)*	*2*
H-1″	*5.4 (d)*	*1*	H-6‴	*1.1 (d)*	*3*



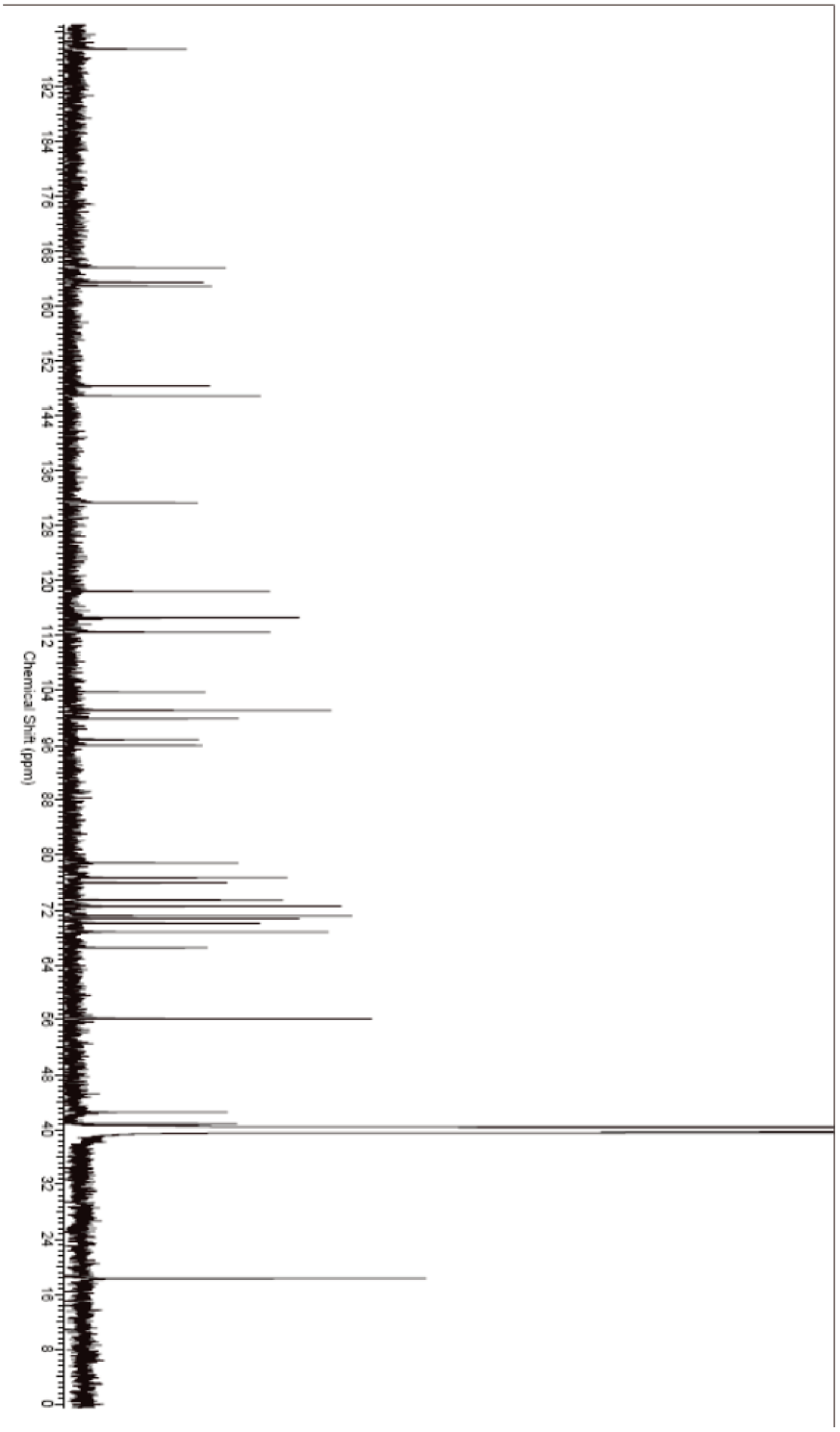
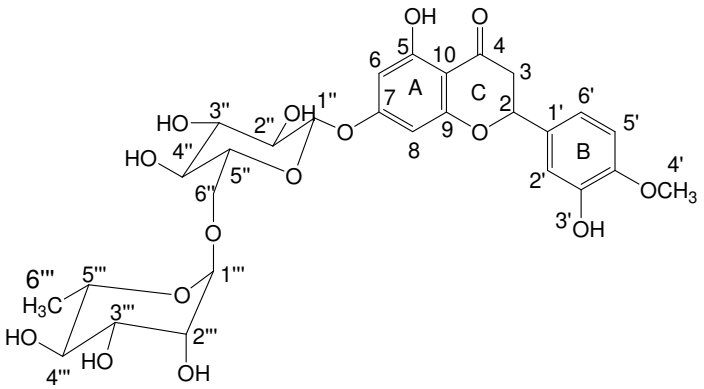


Figure IV-6. ^{13}C NMR spectrum of hesperidin.

Table IV-5. Hesperidin ^{13}C NMR chemical shifts and assignments

Chemical shift (ppm)	***Assignment***	***Chemical shift (ppm)***	***Assignment***
197.5	*C4*	***96.0***	*C8*
165.6	*C7*	***78.9***	*C2*
162.9	*C5*	***76.7***	*C3``*
162.4	*C9*	***76.0***	*C5``*
148.4	*C4`*	***73.4***	*C2``*
146.9	*C3`*	***72.5***	*C4```*
131.4	*C1`*	***71.2***	*C4``*
118.4	*C6`*	***70.8***	*C3```*
114.6	*C2`*	***66.5***	*C2```*
112.5	*C5`*	***68.8***	*C5```*
103.8	*C10*	***66.8***	*C6``*
101.1	*C1```*	***56.2***	*OCH3*
99.9	*C1``*	***42.5***	*C3*
96.9	*C6*	***18.3***	*C6```*
Solvent chemical shifts: 40.1-38			



COSY (COrrelation SpectroscopY) was performed for finding the correlation within coupled hydrogens. Coupling hydrogens were determined by cross peaks (correlation peaks) in the COSY spectrum that further confirmed the structure of pure hesperidin (Figure IV-7).

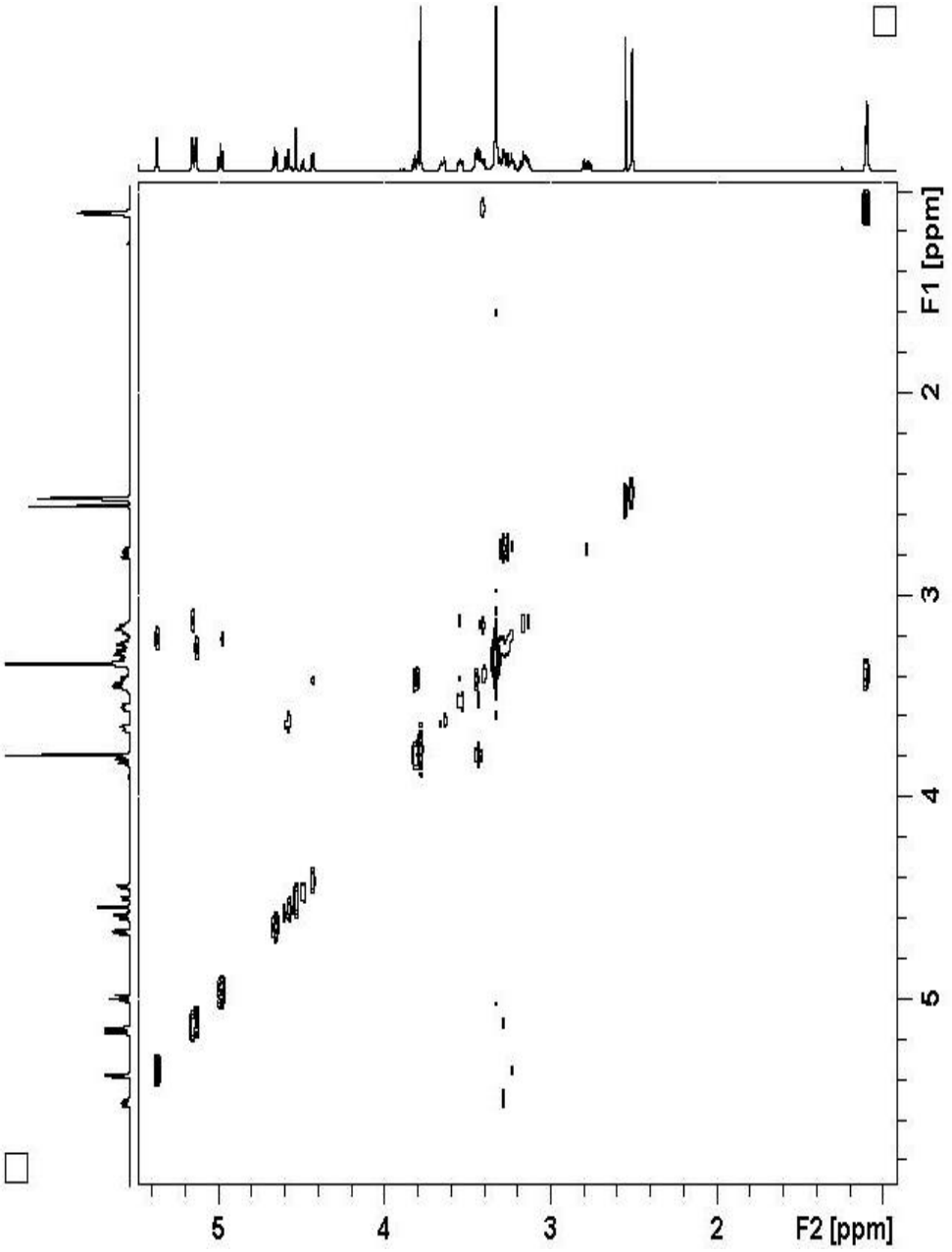


Figure IV-7. COSY NMR spectrum of hesperidin.

The Heteronuclear Single Quantum Coherence (HSQC) experiment is frequently used in the identification of organic molecules. This experiment was done in order to check correlation between the carbon and its attached protons (Figure IV-8). The data acquired after experiment showed clear interactions between carbon and hydrogens present in hesperidin, confirming its overall structure.

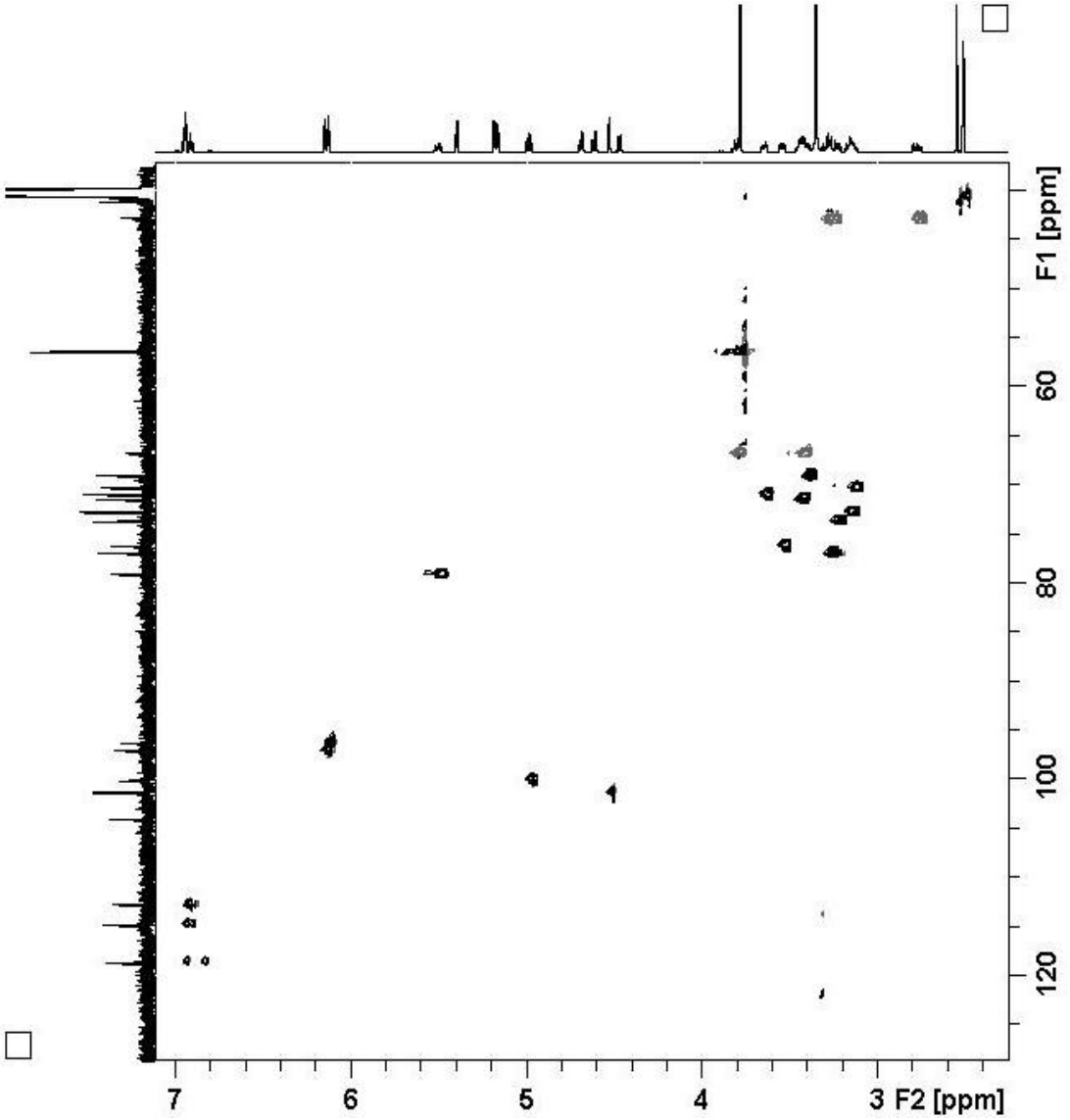


Figure IV-8. HSQC NMR spectrum of hesperidin.

Comparative analyses of industrial samples provided by Citrosuco (Matao, SP) and CPWO from local restaurant (Campinas, SP) have shown almost equal hesperidin yields. Hesperidin has novel pharmaceutical properties and is one of the promising phytotherapeutic agents. Its extraction in Brazil has not started. Therefore, extraction and characterization done in this project are very important in terms of analyzing the important procedures and data for the industrial extraction of hesperidin from the CPWO. Various foreign suppliers of laboratory chemicals such as Sigma-Aldrich have very high prices for this valuable compound. Thus, an industrial process for extraction of a new commodity in Citrus processing factories in Brazil can be a very profitable to the Brazilian economy.

4.4. Hydrolyses of CPWO

Hydrolyses of CPWO is a powerful mean of saccharification of biomass into soluble sugar fractions that can be further fermented into bioethanol. During hydrolyses, the fractions were collected at different time intervals to monitor released sugars. Samples collected at the start of the reaction were usually a clear liquid, later they had solid particles. When these samples were stored in eppendorf, solids used to settle down at the bottom of the Eppendorfs. So, before HPLC analyses, all samples were filtered with syringe filters (13 mm and 0.45 μm) in order to avoid any solid particles interference in the soluble fraction analyses. The chromatogram shown in Figure IV-9 exhibits retention times (RT) of different sugars, sugar acids, furfurals and ethanol in fermentation samples.

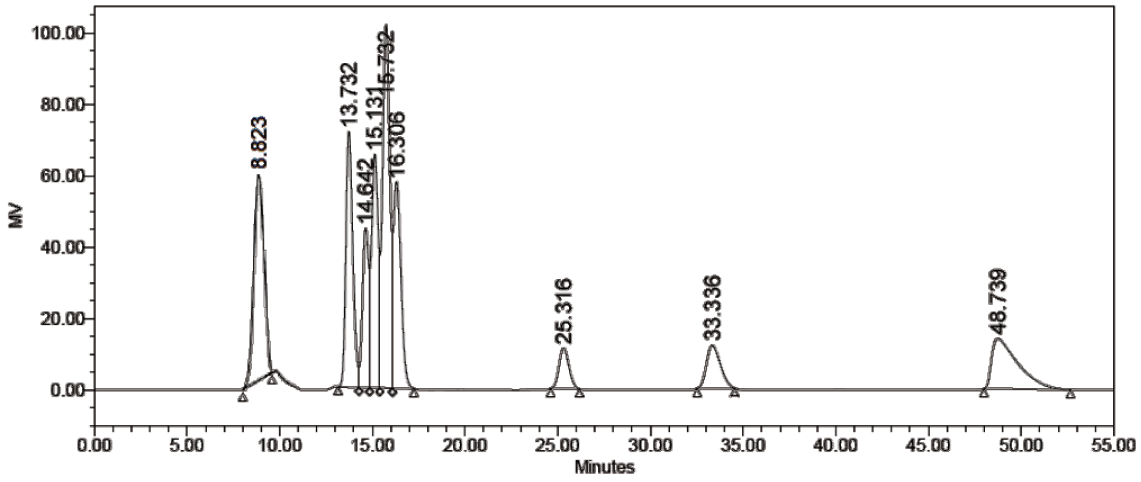
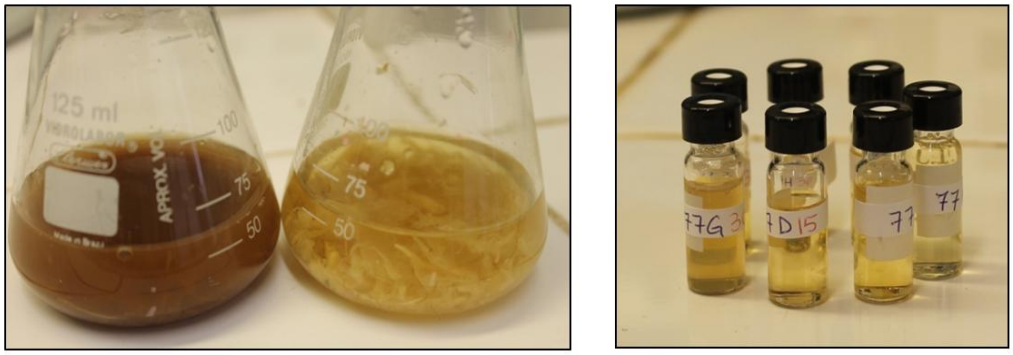


Figure IV-9. HPLC (High Performance Liquid Chromatography) Chromatogram showing profile of glucose (RT=15.131), fructose (RT=15.732), arabinose (RT=16.306), cellobiose (RT=13.732), galacturonic acid (RT=14.642), polygalacturonic acid (RT=8.823), 5-hmf (RT=33.336), furfural (RT=48.739) and ethanol (RT=25.316).

4.4.1. Acid Hydrolysis

After removal of the essential oil, the resulting suspension was subjected to acid hydrolysis process at a temperature of 120^{o}C and pressure of 9.4 x 10^{4} Pa. Acid hydrolysis was performed considering two independent variables: hydrolysis time (15 or 30 minutes) and acid concentration (0.5 to 1.5% v v^{-1}). When compared control sample (untreated CPWO) and acid treated, it was observed that the untreated CPWO appeared unchanged while the acid treated had acquired a reddish brown color. After filtration and drying, the acid treated CPWO showed different texture from that of the untreated (control) sample (Figure IV-10a). All hydrolyzates were filtered in HPLC vials and analyzed using HPLC.



(a) (b)

Figure IV-10. (a) Acid treated (brown color) and untreated (pale color) CPWO hydrolyzates (b) HPLC vials with hydrolyzates for analyses.

The principle soluble sugars observed in HPLC analyses were glucose, fructose, arabinose and cellobiose. The concentrations of these carbohydrates are shown in Table IV-6, represented as mean values (triplicates).

It was observed that no drastic variation in concentrations of soluble carbohydrates occurred with respect to increased acid (H_2SO_4) concentration. The highest amount of fermentable sugars (13.3 g L^{-1} or 40% g g^{-1} dry weight of CPWO) have been released after acid hydrolysis with 1% acid (H_2SO_4) performed at a temperature 120^0C, pressure of $9.4x10^4$ Pa, after 30 minutes.

Table IV-6. Sugar concentrations obtained after acid hydrolyses of CPWO

Conditions	Sugars (% g g^{-1})			
	Glucose	**Fructose**	**Cellobiose**	**Arabinose**
0.5 % H_2SO_4; 15 min	13.4 ± 1.6	16.7 ± 1.8	2.2 ± 0.2	3.1 ± 0.6
0.5 % H_2SO_4; 30 min	15.6 ± 0.5	18.1 ± 0.1	2.1 ± 0.1	4.2 ± 1.2
1.0 % H_2SO_4; 15 min	17.1 ± 1.1	17.8 ± 0.9	3.0 ± 0.6	6.8 ± 0.8
1.0 % H_2SO_4; 30 min	20.5 ± 0.5	20.4 ± 0.4	2.9 ± 0.3	4.4 ± 1.2
1.5 % H_2SO_4; 15 min	13.3 ± 0.9	13.1 ± 1.2	1.7 ± 0.6	5.2 ± 1.1
1.5 % H_2SO_4; 30 min	19.2 ± 0.1	15.3 ± 0.7	2.5 ± 0.1	6.1 ± 0.3
Control	14.2 ± 0.3	13.9 ± 0.5	4.9 ± 0.2	-

Although acid hydrolysis generates high quantities of fermentable sugars, it also forms inhibitory compounds as a result of sugar decomposition (Figure IV-11). These inhibitors are furfurals and products of secondary reactions, for example, products of the acid catalyzed dehydration and rehydration reactions of hexoses [Kuster, 1990]. The formation of these side products not only reduces yields of the desired sugar monomers [Grohmann *et al.*, 1995], but also produces toxins that inhibit cell growth during the fermentation of sugars [Taherzadeh and Karimi, 2007].

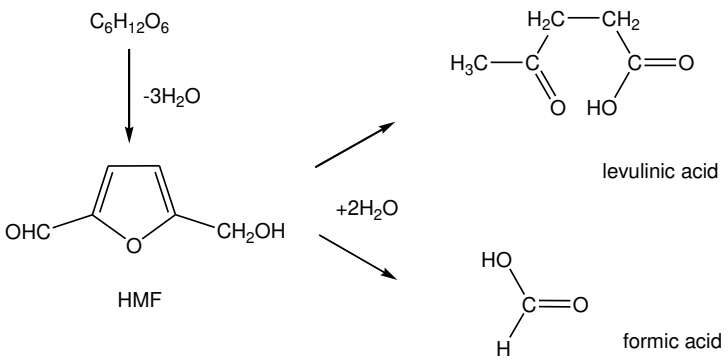


Figure IV-11. Dehydration products of hexoses and acid-catalyzed secondary reactions [Kuster, 1990].

In our studies, during acid hydrolysis, none furfural unlike previous reports was formed [Scriban, 1985; Taherzadeh *et al*. 2000; Talebnia *et al*. 2008]. However, 5-hydroxymethylfurfural (5-HMF), another potent yeast inhibitor, was identified during acid hydrolysis. The quantity of 5-HMF ranged from 0.56 to 1.56 g L^{-1} depending upon the acid concentration used (Figure IV-12).

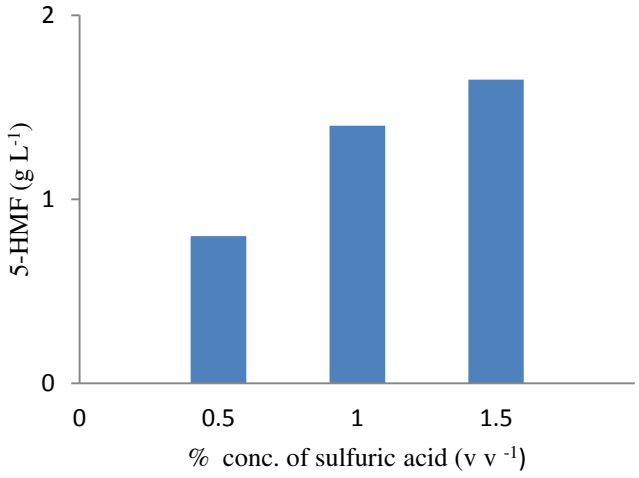


Figure IV-12. Graphical representation of released 5-HMF concentration (g L^{-1}) as a function of H_2SO_4 concentration (v v^{-1}).

This inhibitor is reported in many articles [Scriban, 1985; Taherzadeh *et al*. 2000; Talebnia *et al*. 2008]. The higher the acid concentration was, higher amount of 5-HMF was detected in the hydrolyzates (Figure IV-12).

Acid hydrolysis was also applied to industrial CPWO and obtained results are shown in the Table IV-7. Hydrolysis was performed considering two independent variables: hydrolysis time (15 and 30 minutes) and acid concentration (0.5 to 3 % v v^{-1}).

Results show that with increasing acid concentration, concentration of cellobiose starts to decrease and concentration of glucose increases. This shows that acid hydrolysis is effective not only to disrupt the main cellulosic wall for releasing monomeric sugars but it also breaks the bonds in cellobiose molecule (a dimer of glucose), hence increasing glucose concentration.

It should be noted that there was a correlation between acid concentration and glucose released during hydrolysis while concentration of fructose was maximum at 1% acid concentration (after 15 or 30 minutes). Arabinose that is a 5-carbon sugar and an important component of biopolymers, such as hemicellulose and pectin, is also released because of acid pretreatment. The control sample (standard sample) did not show presence of arabinose in the absence of acid. Maximum amount of fermentable sugars (glucose and fructose) were released when acid concentration was 1 or 1.5%. These results are similar to those shown for CPWO in Table IV-6 (collected from the local area canteen). Although maximum quantity of fermentable sugars (~30% g g^{-1}; condition 4, Table

IV-7) released from industrial CPWO were lower than the one reported in Table IV-6 (~40% g g^{-1} after 30 minutes of hydrolysis).

Table IV-7. Sugar concentrations obtained after acid hydrolyses of industrial CPWO

H_2SO_4 Conc. (%)	Time of hydrolysis	Cellobiose % g g^{-1}	Glucose % g g^{-1}	Fructose % g g^{-1}	Arabinose % g g^{-1}
control	**15 min**	3.8	9.9	9.3	0
0.5		1.2	8.3	10.1	4.0
1		0	14.4	13.4	5.8
1.5		0	16.7	13.0	4.5
3		0	17.0	9.8	4.9
control	**30 min**	4.0	11.2	8.4	0
0.5		2.3	11.1	12.8	4.9
1		0	14.4	13.1	4.8
1.5		0	15.4	12.0	5.2
3		0.4	16.7	9.8	5.9

4.4.2. Enzyme hydrolysis involving commercial enzymes

Activities of the enzymes used for hydrolysis of CPWO were determined and celluclast 1.5 L exhibited cellulase activity of 68 FPU mL^{-1}, pectinase exhibited pectinase activity of 0.141 U mg^{-1} and beta-glucosidase activity was 1.6 I U mg^{-1}.

The enzymatic hydrolysis was carried out following the procedure described in section 3.9. The influences of enzyme loadings on the saccharification of CPWO were tested first with commercial enzymes

applied individually, one by one. The temperature (45°C) and pH (4.8) were chosen within the range of optimum enzymes activities.

Hydrolyses were carried out for 48 h and the resulting sugar yields were expressed in % g g^{-1} of dry CPWO. The main carbohydrates released during saccharification were glucose, fructose, arabinose and cellobiose along with galacturonic and polygalacturonic acid. Table IV-8 shows the obtained yields expressed as means with standard deviation.

Pectinase, cellulase and beta-gucosidase hydrolyses results (triplicates) were compared with the control sample data. Pectinase enzyme loading influenced all yields (p<0.05) except fructose in comparison to control experiment, while cellulase and beta-glucosidase loadings have significant effects (p<0.05) on the yields of all sugars and sugar acids. Galacturonic acid (GA) and arabinose were not observed when used cellulase, while in case of beta-glucosidase enzyme treatment, no Galacturonic acid (GA) was observed that was observed with pectinase enzyme treatment.

Comparing effects of different enzyme loadings (Table IV-8), it was noted that pectinase loadings significantly affected (p<0.05) glucose yields; and cellobiose, fructose, arabinose, polygalacturonic acid and galacturonic acid did not suffer significant increase with increase of pectinase loadings. Highest glucose yield was obtained when 25 mg of pectinase was applied. Cellulase loadings had significant effect (p<0.05) on glucose, fructose and cellobiose yields. Finally, beta-glucosidase loadings had significant effects (p<0.05) on polygalacturonic acid and cellobiose yields. Among three enzymes, cellulase loadings gave higher yields of fermentable sugars

(glucose and fructose) in comparison to pectinase and beta glucosidase. Maximum C-6 fermentable sugars (glucose and fructose) were released when cellulase was used and were ~39% g g^{-1} dry mass of CPWO.

Table IV-8. Sugar Yields (% g g^{-1} dry CPWO) obtained in hydrolyses with pectinase, cellulase and beta-glucosidase after 48 h of enzyme hydrolyses

Enzyme loadings[a, c]	Poly-galacturonic acid[b] %	Cellobiose[b] %	Galacturonic acid[b] %	Glucose[b] %	Fructose[b] %	Arabinose[b] %
Control	28.5±1.3	22.7±0.5	-	11.1±0.5	10.8±0.7	-
Pectinase [a]						
10	5.3±0.4	14.4±0.3	16.4±0.5	10.8±0.2	11.1±0.2	3.8±0.5
15	5.4±0.3	14.7±0.7	17.1±2.8	11.4±0.3	11.1±0.4	3.9±0.6
20	5.1±0.9	14.0±0.1	16.3±0.6	11.7±0.1	11.4±0.3	4.2±1.2
25	5.2±0.1	14.2±0.9	16.1±1.4	12.4±0.2	11.4±0.3	4.1±0.8
Cellulase [a]						
20	2.8±0.8	22.1±0.1	-	17.7±0.3	14.3±0.4	-
40	3.4±1.4	21.5±0.1	-	18.7±0.6	17.4±0.9	-
60	3.8±1.2	21.1±0.2	-	19.1±0.2	19.6±0.1	-
Beta-glucosidase [a]						
2	18.8±0.3	31.5±0.5	-	16.3±0.1	15.5±0.1	2.1±0.1
4.15	20.4±0.2	29.6±0.9	-	16.6±0.3	16.2±0.4	2.8±0.1
6.3	21.0±0.5	25.7±1.6	-	16.2±0.3	16.2±0.5	3.1±0.1

[a] mg protein/g dry CPWO,
[b] yield expressed in % g g^{-1} , reported as mean along with their standard deviation,
[c] enzyme hydrolysis at 45°C, steam distilled sample of CPWO, with buffer at pH 4.8.

4.4.3. Enzyme hydrolysis involving enzyme cocktails

Enzyme hydrolysis was also performed using different enzyme combinations, *i.e.* enzyme cocktails and these treatments have significant effects ($p<0.05$) on sugar yields as shown in Table IV-9. Treatment with a combination of three enzymes cellulase, pectinase and beta-glucosidase, loading 2 with buffer, gave high C-6 sugar yields (~40% g g^{-1} dry CPWO). This shows that for the complete hydrolysis of the starting biomass, pectinase is important to break the pectin polymer and to release long chains of cellulose polymer. Later, cellulase and beta-glucosidase enzymes catalyze the depolymerization of long chains of cellulose converting them in to sugar monomers. The high quantity of sugars obtained after 48 h of enzyme hydrolysis shows synergy that exists between enzymes present in cocktail.

Enzyme cocktail 1 (Table IV-9) composed from pectinase and beta-glucosidase gave better sugar yield of around 37% g g^{-1} (dry CPWO) than enzyme cocktail 3 (beta-glucosidase in combination with cellulase). Hydrolysis with enzyme cocktail 4 was carried out in sterile water, without any buffer added, and limonene was removed with autohydrolysis. Comparing results obtained from cocktail 4 and 2 (Table IV-9), except arabinose, all other sugars and sugar acids exhibited significant difference ($p<0.05$).

High level of galacturonic acid was produced in the absence of buffer. In all previous cases of enzymes hydrolysis where buffer was used, galacturonic acid was formed only in case where pectinase enzyme was

present. In the case of enzyme cocktail 4, the highest galacturonic acid (pKa = 3.51) yield was obtained from pectin, after 48 h of hydrolysis and pH of the hydrolyzate was decreased to 3.7.

Table IV-9. Sugar Yields (% g g^{-1} dry CPWO) after 48 h of enzyme hydrolyses with enzyme cocktails

Enzyme coctails	Poly-galacturonic acid % g g^{-1}	Cellobiose % g g^{-1}	Galacturonic acid % g g^{-1}	Glucose % g g^{-1}	Fructose % g g^{-1}	Arabinose % g g^{-1}
1	4.3±0.1	14.9±2.9	5.4±0.5	17.7±0.6	19.6±0.5	4.2±0.1
2	4.8±0.6	17.2±0.2	4.8±0.2	20.1±0.1	20.0±0.3	2.9±1.5
3	28.9±0.2	21.7±0.7	-	15±0.4	13.8±0.2	-
4	2.4±0.7	2.1±0.9	25.7±0.3	19.7±0.6	17.9±0.7	2.8±0.5

1. enzyme preparation consisting of 2 mg g^{-1} b-glucosidase and 2 mg g^{-1} pectinase; hydrolysis at 45ºC, steam distilled sample of CPWO, with buffer at pH 4.8;
2. enzyme preparation consisting of 2 mg g^{-1} b-glucosidase, 2 mg g^{-1} of pectinase and 8 mg g^{-1} cellulase; hydrolysis at 45ºC, steam distilled sample of CPWO, with buffer at pH 4.8;
3. enzyme preparation consisting of 2 mg g^{-1} b-glucosidase and 15 mg g^{-1} cellulase; hydrolysis at 45ºC, steam distilled sample of CPWO, with buffer at pH 4.8;
4. enzyme preparation consisting of 1 mg g^{-1} pectinase, 2 mg g^{-1} b-glucosidase and 15 mg g^{-1} celulase; enzyme hydrolysis at 55ºC, autohydrolysed, without buffer; initial pH= 4.12, final pH=3.7.

4.4.4. Enzyme hydrolysis involving *Xac* 306 enzymes

The lyophilized *Xac* 306 protein extract (Figure IV-13) was tested for its different enzyme activities. It gave pectinase activity equal to 58 U g^{-1}, polygalacturonase activity equal to 78 U g^{-1} and cellulase activity equal to 8

FPU mL^{-1}. Enzyme hydrolyses utilizing *Xac* 306 gave good yields of sugars as shown in Table IV-10.

Figure IV-13. Lyophilized *Xac* 306 protein extract.

Three different conditions were used for exploring *Xac* hydrolyzing abilities. Comparing the control sample (Table IV-10) with the *Xac 306* hydrolysis yields ($p<0.05$), it is obvious that *Xac* 306 hydrolysis was successful in releasing fermentable sugars for ethanol fermentation. However, increase in *Xac* 306 enzymes loadings had no significant effect on yields of glucose and fructose for hydrolysis at 45°C with buffer. Comparing conditions a, b and c (Table IV-10) polygalacturonic acid, cellobiose and fructose yields had shown significant change ($p<0.05$), with changing environment.

Xac 306 enzymes treatment gave good yields of C-6 sugars (~34% g g^{-1} dry CPWO). CPWO hydrolysed at 45°C without buffer at *Xac* 306 loading 5.6 mg g^{-1} dry CPWO (condition b, Table IV-10), showed excellent fermentable sugar yields. Initial pH of this sample was 4.1. It shows that *Xac* 306 enzymes can tolerate low pH and could work within broad range of pH.

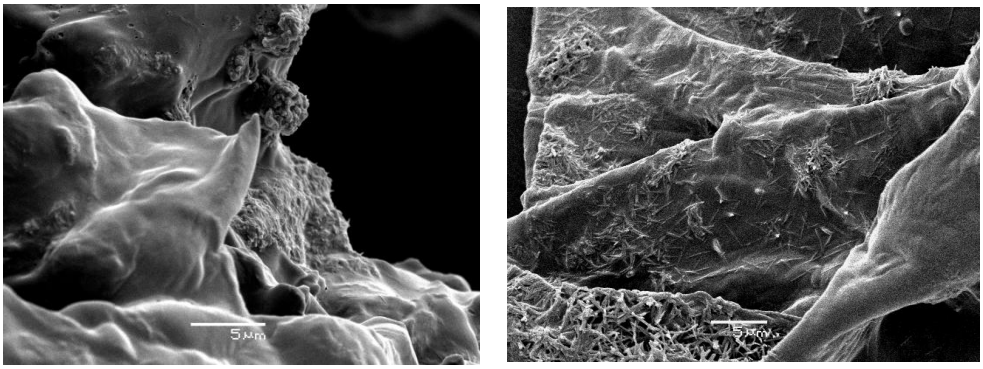
Table IV-10. Sugar yield (% g g^{-1} dry CPWO) after 48 h of enzyme hydrolyses with *Xac 306* enzymes

***Xac* 306 loadings**		**Conditions**	**Poly-galacturonic acid % g g^{-1}**	**Cellobiose % g g^{-1}**	**Glucose % g g^{-1}**	**Fructose % g g^{-1}**
mg added to 17 g CPWO	**mg g^{-1} dry CPWO**					
18	5.6	**A**	17.4 ± 0.8	17.9 ± 0.8	17.0 ± 0.5	12.4 ± 0.3
36	11.2		17.8 ± 0.3	22.9 ± 0.7	18.2 ± 1.1	12.3 ± 1.2
54	16.8		18.9 ± 0.4	22.1 ± 1.4	17.6 ± 0.5	13.2 ± 0.2
18	5.6	**B**	15.5 ± 0.8	3.3 ± 0.3	16.9 ± 0.5	16.9 ± 1.0
18	5.6	**C**	2.9 ± 0.9	3.9 ± 0.4	17.2 ± 1.8	13.9 ± 0.6

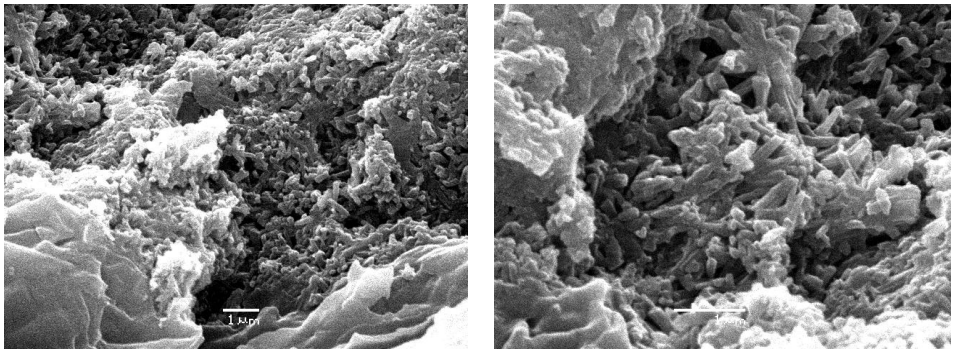
a = steam distilled sample of CPWO employed for enzyme hydrolysis at 45°C, with buffer at pH 4.8, 48 h of incubation,
b = CPWO autohydrolysed for 15 min before adding *Xac 306*; enzyme hydrolysis at 45°C *w*ithout antibiotic; no buffer; Initial pH = 4.1; 48 h of incubation,
c = CPWO autohydrolysed for 15 min before adding *Xac 306*; enzyme hydrolysis at 55°C without antibiotic; no buffer; Initial pH = 4.1; 72 h of incubation.

After hydrolysis of CPWO with *Xac* 306 enzymes, the residue was filtered, washed with water and dried in oven at 60°C along with non-treated CPWO. It can be seen (Figure IV-14), that hydrolyzed CPWO is brown in color and completely different in texture when compared to unhydrolyzed CPWO. It shows how efficiently *Xac* 306 hydrolytic enzymes disrupt the fruit cell wall.

Figure IV-14. Photos of (a) unhydrolyzed and (b) hydrolyzed CPWO with *Xac* 306 enzymes.



(a)



(b)

Figure IV-15. SEM images of: (a) unhydrolyzed CPWO and (b) hydrolyzed CPWO using *Xac* 306 enzymes.

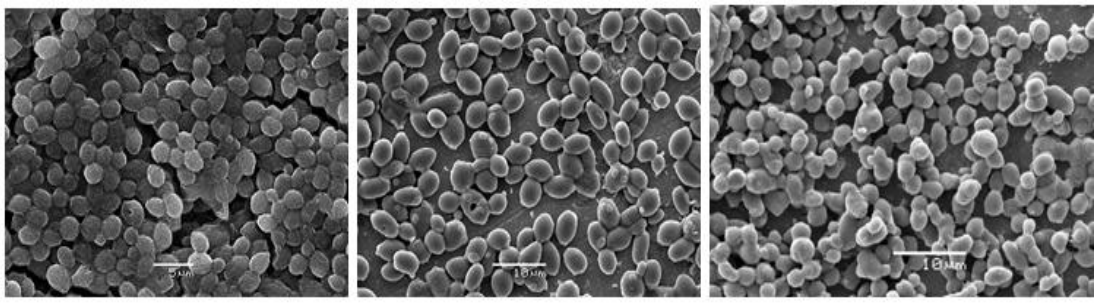
Both, hydrolyzed and unhydrolyzed samples were analyzed using scanning electron microscopy (SEM). Figure IV-15a shows SEM images of unhydrolyzed CPWO where lignocellulosic sheet like structures are present. However, image b (Figure IV-15) shows disrupted structure in contrast to the first one. The lignocellulosic sheets are no more present and *Xac* 306 have shown successful degradation of biomass.

4.5. Submerged Fermentations of Hydrolyzates

The presence of fermentable sugars in appropriate quantities and low levels of lignin make CPWO a useful substrate for ethanol production. The proteins present in CPWO may act as organic nutrients and can allow growth of yeast microorganisms, thus favoring the fermentation process [Oberoi *et al*., 2010]. Although conventional yeast strains can operate over a wide range of temperatures, they show better performance in the range 30-35°C [Ingram *et al.*, 1997].

In this study, all submerged fermentations (SmF) were performed at a temperature of 37^{0}C, during 48 to 96 hours and pH adjusted to 5-6, to favor maximum ethanol production. The SmF were performed with two types of CPWO hydrolyzates, one hydrolyzed with acid and the other with enzymes. Fermentations were performed using mono and co-culture yeast samples. Three yeasts strains were utilized for this purpose. One was the commercial yeast *Saccharomyces cerevisiae* while the other two were isolated from CPWO: *Candida parapsilosis* IFM 48375 and *Candida parapsilosis* NRRL Y-12969 (Figure IV-16). *Saccharomyces cerevisiae* was chosen for fermentation because it is commonly used all over the world from centuries.

The reason for isolation and utilization of other two yeast strains of *Candida* from orange bagasse was to investigate if these strains can work better than *Saccharomyces cerevisiae.* As isolated from CPWO, these strains would have the ability to work better in the presence of essential oils. It was also assumed that these microorganisms could show more resistance to environmental factors and can help in better ethanol production in terms of quantity as well as quality.



(a) (b) (c)

Figure IV-16. SEM micrographs of (a) *Candida parapsilosis* IFM 48375*; (b) Saccharomyces cerevisiae; (c) Candida parapsilosis* NRRL Y-12969.

Acid hydrolyzates were only fermented with yeast mono-cultures while enzyme hydrolyzates were fermented with both mono and co-culture strains. The main objectives of mono- and co-culture fermentations were:

(i) to screen the best strain for ethanolic conversion of biomass;

(ii) to analyze the differences between activity of isolated yeast strains and compare it to *Saccharomyces cerevisiae*, most widely used all over the world.

The fermented samples were analyzed with HPLC for monitoring the ethanol concentration. The percentage of ethanol theoretical yield was calculated on dry matter basis, considering 3.24 g of CPWO (dry weight corresponding to 17 g CPWO) as sugar input for yeast fermentation to proceed. According to the Reaction IV-1, the theoretical maximum yield is 0.51 g ethanol per g of C-6 sugar:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

(Reaction IV-1)

Hence, for 3.24 g CPWO, the ethanol yield (%) was calculated using the following expression:

$$\text{percentage of ethanol theoretical yield } = \left(\frac{\text{EtOH}}{0.511 \times 3.24}\right) \times 100$$

Where:

EtOH = ethanol concentration, g g^{-1},

0.511 = conversion factor from glucose to ethanol,

and 3.24 = dry weight corresponding to 17 g of CPWO.

4.5.1. Results of Submerged fermentations of acid hydrolyzates

Yeast samples (*Saccharomyces cerevisiae* or *Candida parapsilosis* sp.) were inoculated (10% v v^{-1}) in the form of mono-cultures. In order to investigate the best strains for ethanol production from acid hydrolyzates,

each yeast strain was applied to 15 min hydrolyzed CPWO samples with acid concentration 0.5, 1 and 1.5% that counted for 9 fermentations. In the next round, individual strains were applied to 30 minutes hydrolyzed CPWO samples with acid concentration 0.5, 1 and 1.5% that again gave 9 fermentations. Thus, in total 18 fermentations were executed with each set in triplicate. It is important to note that after acid hydrolysis, the pH of hydrolyzates was adjusted in the range of 5-6 to facilitate yeast growth.

The results obtained from mono-fermentations of the acid hydrolyzates showed that ethanol was produced in the range of 18-25 g g^{-1}, depending on the type of yeast used and the concentration of soluble sugars obtained during the hydrolysis step.

Fermentations involving *Saccharomyces cerevisiae,* gave maximum ethanol yield with CPWO sample, which was hydrolyzed with 0.5% sulfuric acid for 15 minutes. The ethanol was produced in a yield of 22% g g^{-1} (Dry CPWO) accounting for 43% of the theoretical yield after 72 hours of fermentation (Figure IV-17). The concentration of starting fermentable sugar contents in this case at 0 h was 11g L^{-1}.

Figure IV-17 shows a typical pattern of yeast growth during the fermentation. In the first 36 h, *lag* phase was observed. During the *lag* phase, no significant cell growth occurs as newly pitched yeast cells mature and get adapted to the environment. This phase was followed by the *log* phase (or exponential growth phase) that occurred from 36 to 72 h, where cells started to grow rapidly and divide producing more quantity of ethanol. The exponential phase occurred because yeast rapidly started the sugar consumption. Nutrients were in sufficient quantity relative to cell number at this stage. As cell number increased, cell growth began to slow down after

72 h and, eventually, cells entered into death phase and no increase in ethanol concentration was observed.

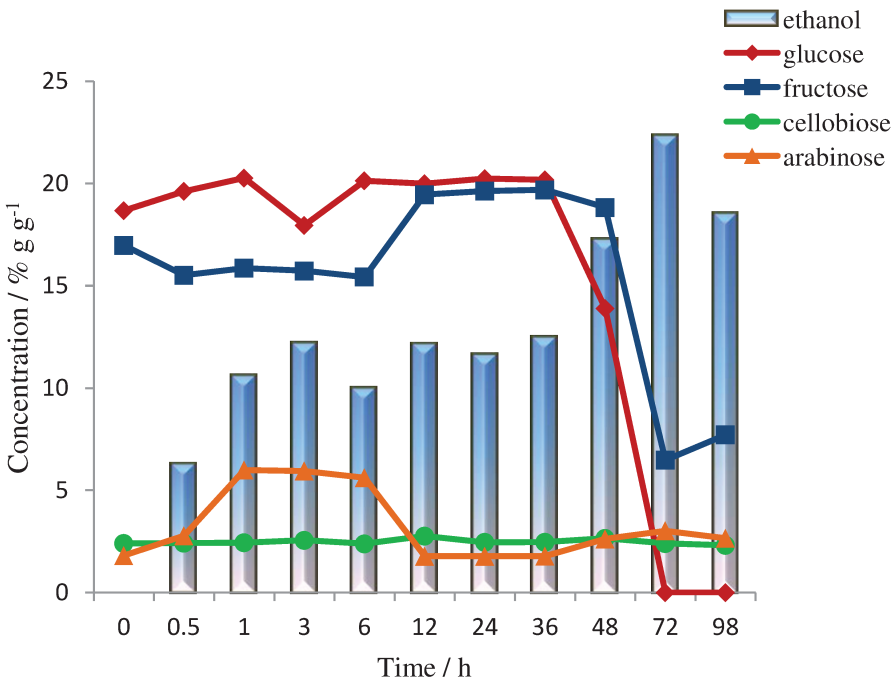


Figure IV-17. A plot of sugar and 2G-ethanol concentrations in relation to time in a *Saccharomyces cerevisiae* fermentation of 0.5% v v^{-1} (15 min) acid hydrolyzed sample.

Compared with the control analysis, acid hydrolysis (0.5% v v^{-1}) effectively released hexoses and pentoses from CPWO as shown in section 4.4.1, and higher acid concentrations gave better sugar yields. However, it was observed that fermentation of hydrolyzates with highest acid concentration resulted in a slower rate of ethanol production. The ethanol yield was also lower when compared to what was observed with 0.5% acid hydrolyzates. These results show that the higher concentration of side products (5-HMF) that increased with increasing acid concentration, inhibited growth of *Saccharomyces cerevisiae*.

For yeast *Candida parapsilosis* IFM 48375, the maximum ethanol yield was obtained from CPWO hydrolyzed with 0.5% sulfuric acid for 15 minutes (Figure IV.18).

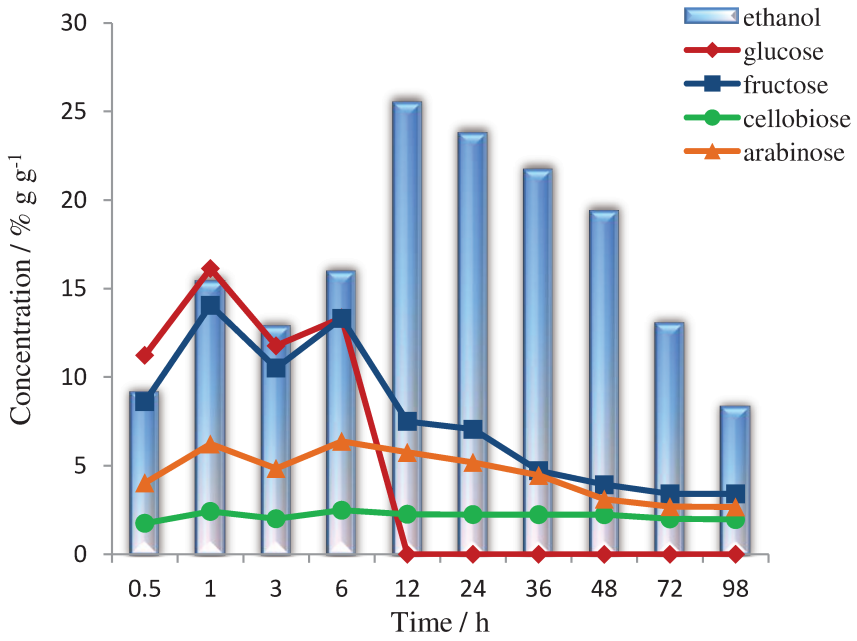


Figure IV-18. A plot of sugar and 2G-ethanol concentrations in relation to time in *Candida parapsilosis* IFM 48375 fermentation of 0.5% v v^{-1} (15 min) acid hydrolyzed sample.

The ethanol produced was around 26% g g^{-1} (Dry CPWO) and 51% of the theoretical yield was obtained after 12 h of fermentation (Figure IV-18). Fermentation was started with the initial C-6 sugar concentration (glucose and fructose) of 6.5 g L^{-1}.

Figure IV-18 shows that in the first 6 h, *lag* phase was observed where no significant cell growth occurred. After that the *log* phase or exponential growth phase started that lasted up to 12 h, where cells started rapidly to grow and divide, producing more quantity of ethanol. Yeast rapidly started to consume glucose followed by fructose. Cell growth began to slow down

after 12 h. A gradual decrease in quantity of ethanol was observed from 12 to 98 h that probably have occurred because of evaporation due to fermentation temperature (35-37ºC).

Fermentations involving *Candida parapsilosis* NRRL Y-12969, also gave maximum ethanol yield with CPWO that was hydrolyzed with 0.5% sulfuric acid for 15 minutes (Figure IV-19).

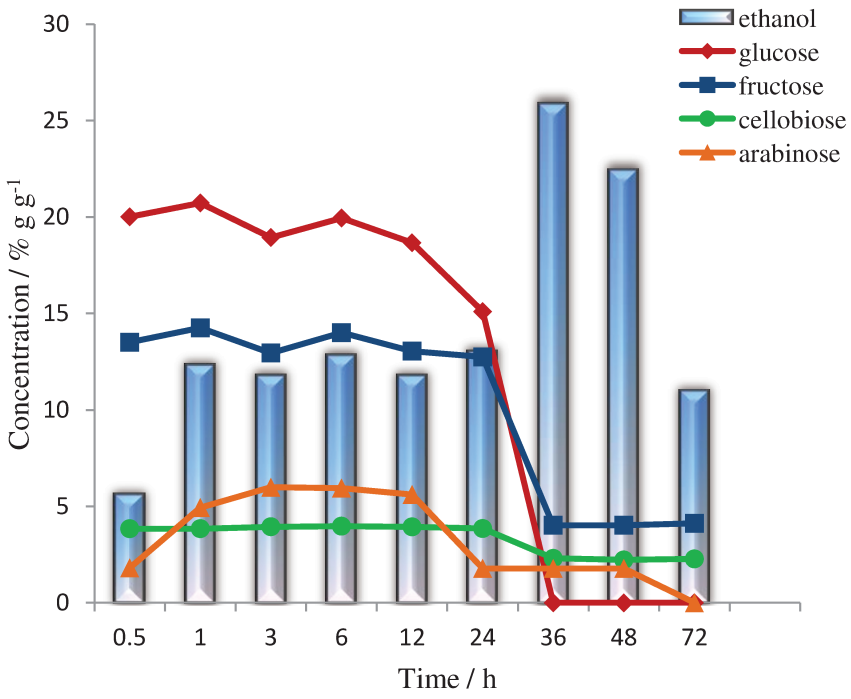


Figure IV-19. A plot of sugar and 2G-ethanol concentration in relation to time in *Candida parapsilosis* NRRL Y-12969 fermentation of 0.5% v v^{-1} (15 min) acid hydrolyzed sample.

The ethanol yield was around 27% g g^{-1} (Dry CPWO) and 52% of the theoretical yield, with initial C-6 sugar concentration of ~11 g L^{-1}. The time at which ethanol yield was the highest was after 36 h (Figure IV-19).

Comparing the three best results obtained after mono-culture fermentations of acid hydrolyzates, it is clear that the isolated *Candida* strains showed better performance in comparison to the commercial yeast. A rapid consumption of glucose within 6-12 h of incubation was observed with *Candida parapsilosis* IFM 48375 or within 24-36 h with other *Candida* strain. On the other hand, commercial yeast showed high glucose consumption in 48-72 h of incubation, which shows that the *Candida* strains have shorter *lag* phase and thus adapt faster with the fermentation media as compared to *Saccharomyces cerevisiae.* Also, it is clear that in comparison to *Saccharomyces cerevisiae*, both *Candida* strains gave significantly high yields ($p<0.05$) of ethanol.

In all fermentations, the first consumed sugar was glucose followed by fructose. Arabinose was present in small quantities and its consumption was not significant. The same behavior was observed in experimental work carried out by Oberoi *et al.* (2010). In the presence of arabinose in the medium, no significant increase in ethanol concentration is observed when used commercial yeast.

Hahn-Hagerdal *et al.* (2007) reported that the pentose-fermenting yeasts were slower in their activities as compared with hexoses-fermenting yeast, and even genetically modified organisms (GMOs) were not able to produce a substantial amount of ethanol from the pentoses. Also, it has been observed that the strains of pentose fermenting yeasts are very sensitive to concentrations of inhibitor compounds, besides being dependent on the presence of oxygen [Huang *et al.* 2009].

The results in this section show that the fermenting capacities of *Candida* species were better and faster as compared to *Saccharomyces cerevisiae*. Moreover, the *Candida parapsilosis* IFM 48375 showed higher resistance against the inhibitor, 5-HMF. Therefore, it can be concluded that the fermentation depends on the successful growth of the yeast cells. Delegenes *et al.* (1996) reported that the growth of *Pichia stipitis* (xylose fermenting yeast) decreased approximately 43% when the concentration of 5-HMF was 0.5 g L^{-1}. The highest concentration of 5-HMF (more than 1 g L^{-1}) observed in acid hydrolyzed samples had drastically effected the growth of *Saccharomyces cerevisiae* and its ability to ferment. Another reason for lower efficiency of *Saccharomyces cerevisiae* can be CO_2 accumulation during fermentation, which might have affected the yeast fermentation ability by lowering pH of the medium [Chen and Gutmanis, 1976].

4.5.2. Results of Submerged fermentations of enzyme hydrolyzates

The enzyme hydrolyzates were subjected to mono and co-culture fermentations, which are presented and discussed in separate sub-sections.

4.5.2.1. Mono-culture fermentations

After enzyme hydrolysis, yeast samples (*Saccharomyces cerevisiae* or *Candida parapsilosis* sp) were inoculated (10% v v^{-1}) first in the form of mono-cultures. The enzyme hydrolyzates that were employed in this section were the one that gave excellent fermentable sugar yields in enzymatic

saccharification. Thus, hydrolyzates with pectinase loading of 25 mg g^{-1} dry CPWO, cellulase loading of 60 mg g^{-1} dry CPWO, beta-glucosidase loading of 4.15 mg g^{-1} dry CPWO, and the mixture of these three enzymes (enzyme cocktail 2, Table IV-9) were used. A total of 12 sets of fermentations were performed. The quantity of C-6 sugars (glucose and fructose) in the enzyme hydrolyzates and the ethanol concentrations measured using HPLC analyses are shown in the Table IV-11.

Table IV-11. Comparative analysis of mono-culture fermentation results obtained from enzymatic hydrolyzates

Enzyme hydrolysates	**Pectinase**	**Cellulase**	**Beta-glucosidase**	**Condition 2 from Table IV-9**
C-6 Sugars liberated during hydrolyses (% g g^{-1})	24.0	38.5	32.8	40.2
****Ef*** ***(Saccharomyces cerevisiae)***	18.3	20.6	20.2	26.2
****Ef*** ***(Candida parapsilosis* IFM48375)**	20.4	23.0	23.6	26.8
****Ef*** ***(Candida parapsilosis* NRRL12969)**	18.6	19.3	20.9	23.2

**Ef* = final ethanol yield, % g g^{-1}.

It is clear (Table IV-11) that *Candida parapsilosis* IFM48375 showed the best fermentation results. *Candida parapsilosis* NRRL-12969 showed similar ethanol yields as that obtained from *Saccharomyces cerevisiae,* but adaptation of this strain to the media was faster.

The best mono-fermentation result was obtained *Candida parapsilosis* IFM 48375 during fermentation of CPWO sample that was hydrolyzed with enzymes combination (2 mg g^{-1} beta-glucosidase + 2 mg g^{-1} pectinase + 8 mg g^{-1} cellulase). The sugar and ethanol profiles throughout fermentation process are shown in Figure IV-20 and 52% theoretical ethanol yield was obtained after 12 hours of hydrolysis.

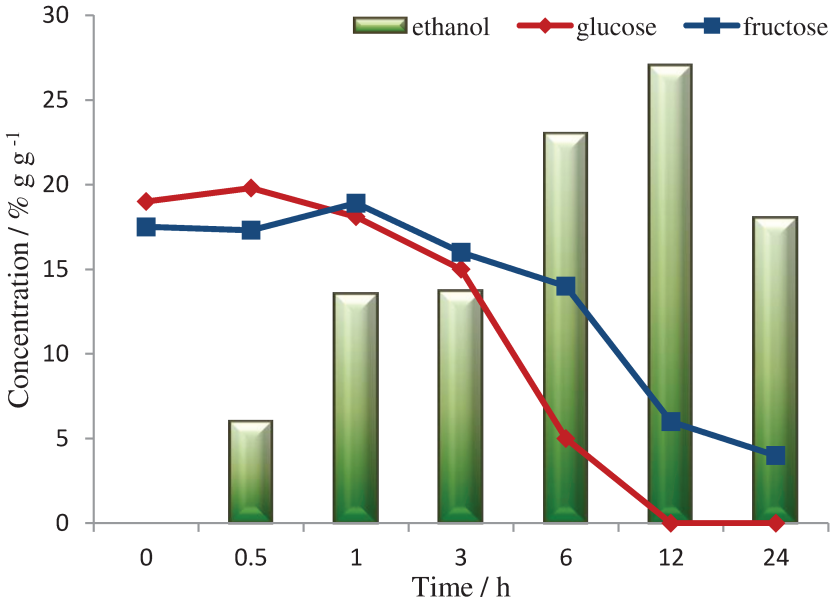


Figure IV-20. Fermentation of CPWO enzymatically treated and fermented with *Candida parapsilosis* IFM 48375, theoretical ethanol yield was 52% in 12 h.

At the start of fermentation, there was low cell growth and this refers to the *lag* phase of microbial growth in a newly inoculated media. The

length of the *lag* phase depends upon the microbial strain history. Among the three strains, in the majority of fermentations *Candida parapsilosis* IFM 48375 showed short *lag* phase as compared to other two. The *lag* phase is actually the adaptation phase to the new environment.

4.5.2.2. Co-culture fermentations

After mono-culture fermentations, co-cultures of microorganisms were used in order to evaluate compatibility of different yeast species with one another. Also, one of the reasons for testing these co-cultures was to see their effects on the ethanol yield.

In the co-culture fermentations, yeast samples (*Saccharomyces cerevisiae* or *Candida parapsilosis sp*) were inoculated in the form of co-cultures (5% v v^{-1} of each yeast strain). In order to find out the best co-culture strains for ethanol production, 6 sets of fermentations were performed. Three CPWO samples hydrolyzed with commercial enzymes and three CPWO samples hydrolysed by *Xac 306* protein enzymes were fermented applying co-culture strategy (Table IV-12).

All fermentations were executed in triplicate. Maximum ethanol yield obtained was 61% in fermentation carried out with co-culture of *Saccharomyces cerevisiae* and *Candida parapsilosis NRRL Y12969*. It was also observed that co-culture fermentation of CPWO sample hydrolyzed with commercial enzymes and hydrolyzed with *Xac* 306 proteins had almost the same results. For this reason Table IV-12, just represents *Xac* 306 co-culture fermented samples compared to the mono-culture fermentations.

Table IV-12 shows final ethanol concentration in % g g^{-1} and theoretical yield of ethanol on dry matter basis obtained with different single and mixed yeast cultures. The fermentation time was 48 h for all strains and ethanol produced by individual strains ranged approximately from 45 to 52%.

Table IV-12. Comparative analysis of fermentation results obtained in mono and co-culture treatments of enzymatically hydrolyzed CPWO

Parameters	***S.C[a]***	***C.P IFM 48375[b]***	***C.P. NRRL Y12969[c]***	***S.C + C.P IFM 48375***	***S.C + C.P. NRRL Y12969***	***C.P IFM 48375 + C.P. NRRL Y12969***
****Ef***	26	27	23	29	31	19
*****E.Y***	51	52	45	57	61	38
$TIME_{max}$	24	12	24	6	6	12

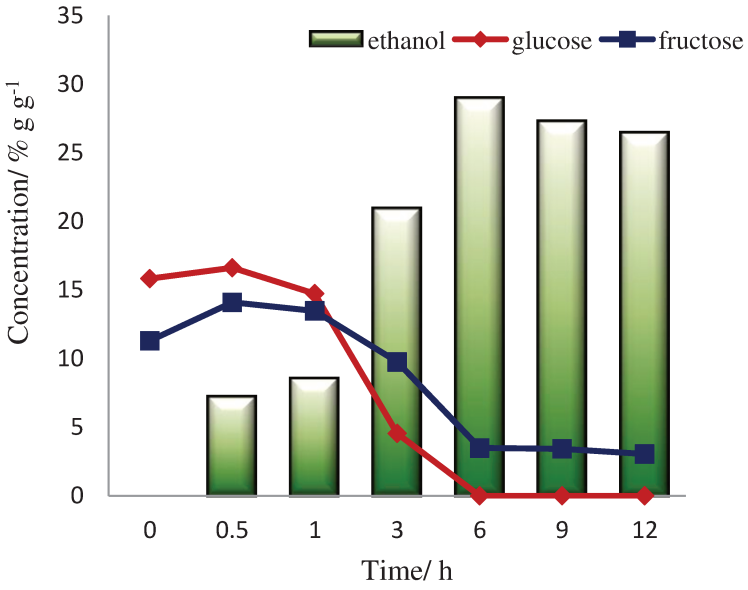
**Ef= final ethanol yield, % g g^{-1},*
***E.Y= percentage of theoretical yield of ethanol on dry matter basis,*
TIMEmax= time at which maximum ethanol yield was obtained,
a= Saccharomyces cerevisiae,
b= Candida parapsilosis IFM48375,
c= Candida parapsilosis NRRL-12969.

In comparison to single strain fermentation with *Saccharomyces cerevisiae*, mixed yeast cultures gave significantly high yields ($p<0.05$) of ethanol (from 57 to 61% of theoretical yield). Fermentation with *Candida parapsilosis* IFM48375 resulted in 2G-ethanol yield similar to fermentation executed with *Saccharomyces cerevisiae*, but fermentation time was reduced to 12 hours. In all these fermentations, glucose was consumed first, followed by fructose.

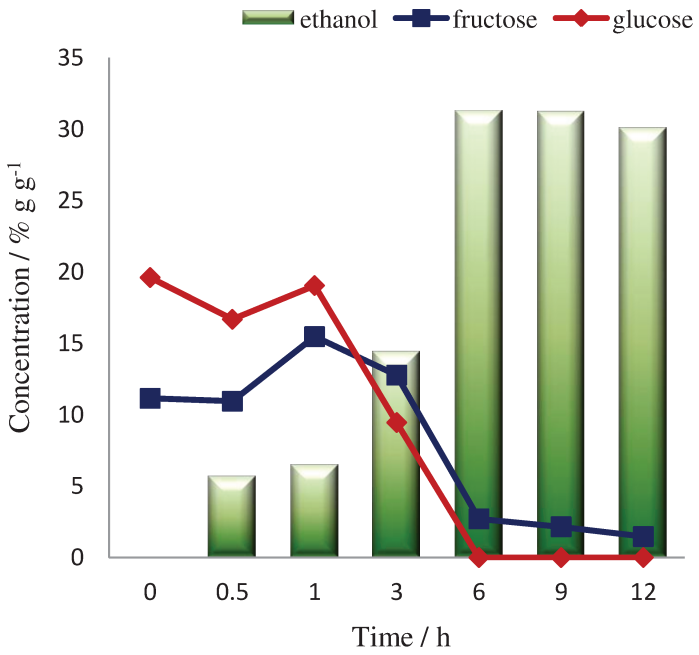
In co-culture fermentations, concentration of 2G-ethanol produced with respect to C-6 sugars (glucose and fructose) is represented in Figure IV-21 and IV-22, as microorganisms did not consume other monosaccharides.

When *Saccharomyces cerevisiae* is incubated with *Candida parapsilosis* IFM48375 strain (Figure IV-21a), rapid ethanol production is observed after 1 h of incubation that goes upto 3 h but then ethanol productivity slows down. At 6 h maximum theoretical ethanol yield 57% was obtained and after that ethanol production was stopped due to the growth inhibition of microorganisms. In this case, ethanol yield of 29% g g^{-1} (57% of theoretical) in 6 h is significant (p<0.05) in comparison to yield obtained by mono-culture of *Saccharomyces cerevisiae* and it can be the cause of growth inhibition of yeast cells. The high ethanol yield obtained with co-culture shows that the two yeast cultures behave synergistically.

Similar behavior was observed in the next case of co-culture fermentation with *Saccharomyces cerevisiae* with *Candida parapsilosis* NRRL-12969 (Figure IV-21b), but in that case rapid ethanol productivity is more significant between 3 to 6 h of incubation producing 61% ethanol theoretical yield at 6 h, after which it became somewhat constant. The trend observed can be explained as the yeast cells first take some time to adapt with the medium in which they are growing so ethanol productivity in the beginning is slower. The ethanol yield after a certain level becomes constant towards the end of the plot because inhibition has set in and yeast cells are no longer producing fresh ethanol. Plot a and b (Figure IV-21) shows a very minute decrease in ethanol yield at 12 h that might have occurred because of evaporation of ethanol at 35ºC after 6 h of inhibition period.



(a)



(b)

Figure IV-21. Ethanol yield with respect to C-6 sugars (glucose and fructose) produced by co-culture fermentation of *Xac* 306 enzyme hydrolyzates with (a) yeast *Saccharomyces cerevisiae* and *Candida parapsilosis* IFM48375, and (b) *Saccharomyces cerevisiae* and *Candida parapsilosis* NRRL-12969.

Fermentation results shown by co-culture of *Candida parapsilosis* IFM48375 and *Candida parapsilosis* NRRL-12969 did not show high compatibility that *Saccharomyces cerevisiae* had shown with both of these isolated *Candida* strains. Theoretical yield obtained was 38% that was produced after 12 h of incubation. Glucose was consumed first and later was followed by fructose. Trend in ethanol production can be seen in Figure IV-22 that shows initial slower production of ethanol followed by rapid growth of yeast cells that increase ethanol concentration to a considerable extent upto 6 h of incubation. After that, there is a gradual decrease in ethanol productivity that shows product inhibition and after 12 h ethanol yield remained constant.

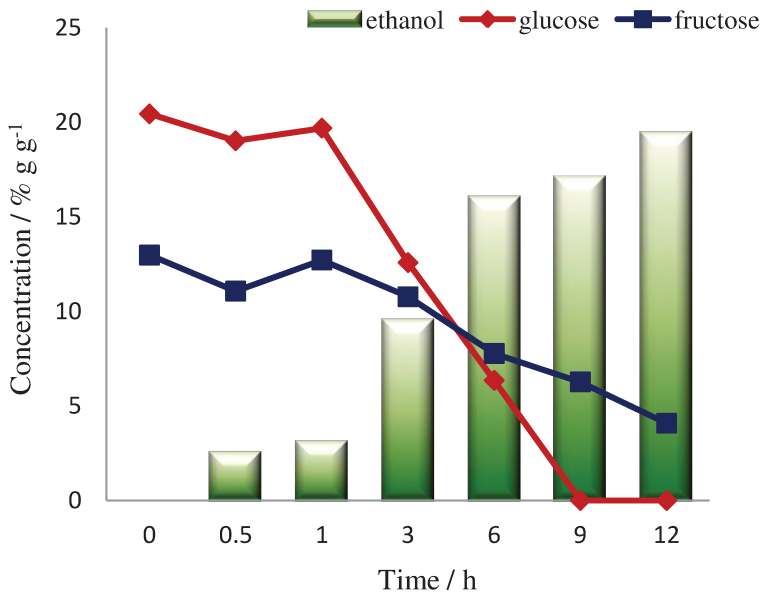


Figure IV-22. Ethanol yields with respect to C-6 sugars (glucose and fructose) produced using co-culture fermentation of *Xac* 306 enzyme hydrolyzates with *Candida parapsilosis* IFM48375 and NRRL-12969.

Figure IV-23 shows a time line study of employed co-culture fermentations. *Saccharomyces cerevisiae* with *Candida* strains in co-culture

fermentations showed greater yields in less time than all other fermentations. It might be because of the fact that *Saccharomyces cerevisiae* takes a little bit more time to get adapted with environmental perturbations, such as changes in temperature, osmotic pressure, pH, and nutrients of the hydrolyzate medium to grow. For rapid cell growth, adaptation to environmental changes is conceived by mechanisms that are adjusted by cells in their intracellular physiology corresponding to the external environment [Dinh *et al*., 2008]. *Candida* species might would accelerate *Saccharomyces cerevisiae* to get adapted faster for ethanol productivity and both of them attribute together for the increase in product yield. On the other hand, where both *Candida* strains are together, the decreased amount of ethanol yield and more time consumption can be attributed to some by-products produced by one of the strain that can poison growth of the other, thus limiting ethanol production.

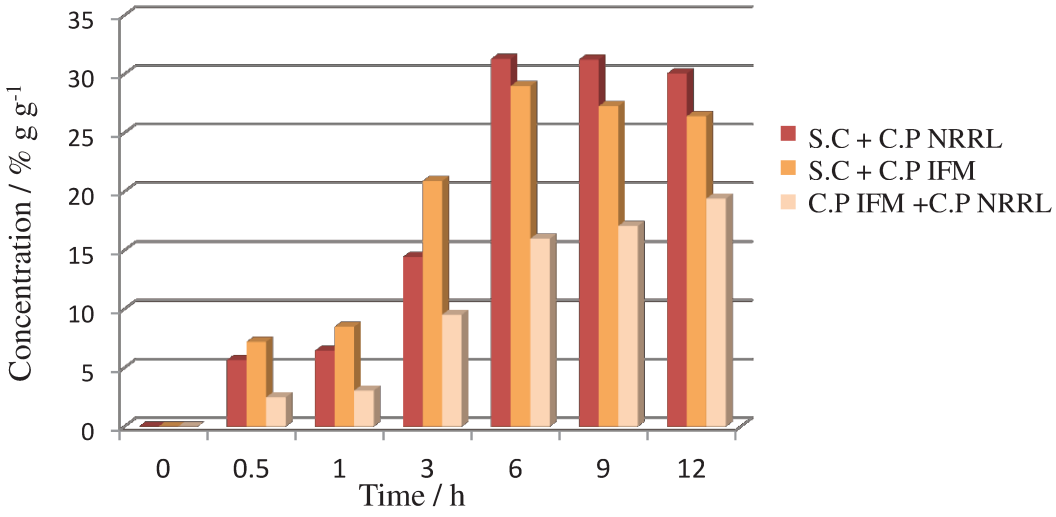


Figure IV-23. Comparison of co-culture fermentations on *Xac* 306 hydrolyzates (where S.C = *Saccharomyces cerevisiae*; C.P IFM = *Candida parapsilosis* IFM48375; C.P NRRL = *Candida parapsilosis* NRRL-12969).

4.6. 2G-Ethanol quality

After completion of fermentation, the fermented samples were distilled and then analyzed applying nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C NMR). The spectra of the distillate obtained showed pure ethanol, without presence of any impurity and residual water signal (Figure IV-24).

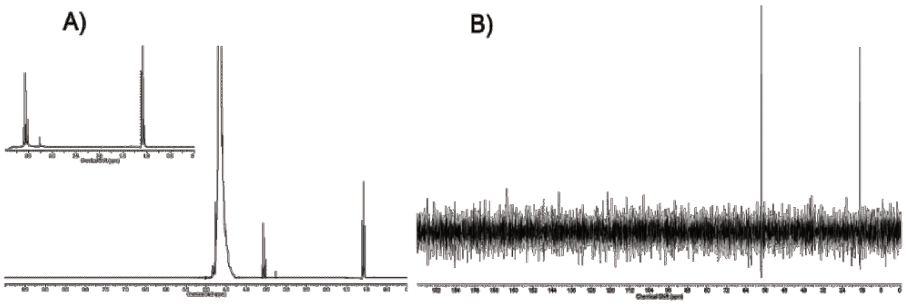


Figure IV-24. (A) ^{1}H NMR and (B) ^{13}C NMR spectra of 2G-ethanol obtained from fermentations of CPWO hydrolyzates.

*

CHAPTER V: FINAL REMARKS

* Figure taken from Youngster website (http://www.youngester.com/2010/07/green-technology-green-planet.html. Web page accessed on January 15th, 2013).

5. Final Remarks

Herein, we have proved that CPWO is a suitable and low cost source for biorefinery. Results obtained points great potential of this biomass to yield several valuable products using different procedures. The most important is the second-generation ethanol, obtained as very pure and in excellent yield.

Overall, we can conclude as:

- CPWO saccharifications (acidic or enzymatic) were optimized and provided good yields of glucose, fructose, cellobiose, arabinose, polygalacturonic and galacturonic acids.
- Furfurals formation during acid hydrolysis was observed and low quantities of 5-HMF were detected.
- For the first time, the lysates of *Xanthomonas axonopodis* pv. *citri* (*Xac* 306) were explored for the saccharification of CPWO at laboratory scale. Comparative studies showed that *Xac* 306 proteins were capable of hydrolyzing CPWO as good as enzyme cocktails.
- Submerged mono-culture fermentations showed best results in terms of ethanol production with *Candida* strains isolated from CPWO.
- Submerged co-culture fermentations involving commercial yeast *Saccharomyces cerevisiae* with any of the two isolated yeast strains from CPWO, *Candida parapsilosis* IFM 48375 or *Candida parapsilosis* NRRL

Y-12969, gave much better ethanol yields (61%) when compared to results obtained using mono-strains.

- By employing co-culture fermentation strategy, apart from getting better bioethanol yields, fermentation time was also reduced, to 6 h for maximum ethanol production, that makes this process feasible and cost-effective.
- Our results showed successful extraction of 1 % g g^{-1} pure hesperidin from dried CPWO. Various preliminary studies reveal novel pharmaceutical properties for this flavanoid. Its recovery from citrus industry by-products is important for two reasons: its bioactive pharmaceutical properties and the reduction of the amount of residues.

*

CHAPTER VI: REFERENCES

*Figure taken from EUCLock Information System website, http://www.bioinfo.mpg.de/euclis/ (Webpage accessed on January 15th, 2013).

6. References

Aghel, N.; Ramezani, Z.; Beiranvand, S. *Pak. J. Biol. Sci*. **2008**, *11,* 2451-2453.

Alegria, M. C.; Souza, D. P.; Andrade, M. O.; Docena, C.; Khater, L.; Ramos, C. H. I.; da Silva, A. C. R.; Farah, C. S. *J. Bacteriol.* **2005**, *187,* 2315–2325.

Ali, J.; Abid, H.; Hussain, A. *J. Chem. Soc. Pak.* **2010**, *32*, 83-86.

Ammerman, C. B.; Henry, P. R. Citrus and vegetable products for ruminant animals. In: Proceedings of the Alternative Feeds for Dairy and Beef Cattle Symposium, St. Louis, MO, USA, 1991. pp. 103-110.

Andorra, I.; Berradre, M.; Rozes, N.; Mas, A.; Guillamon, J. M.; Esteve-Zarzoso, B. *Eur. Food Res. Technol.* **2010,** *231*, 215-224.

Andric, P.; Meyer, A. S.; Jensen, P. A.; Dam-Johansen, K. *Biotechnol. Adv.* **2010**, *28*, 308-324.

ANKOM Technology Methods` Procedure for "NDF" ("Neutral Detergent Fiber") http://www.ankom.com/media/documents/Method_6_NDF_4013011_A200,A200I.pdf (Accessed on May 15th, 2012).

ANKOM Technology Methods` Procedure for "ADF" ("Acid Detergent Fiber") http://www.ankom.com/media/documents/Method_5_ADF_4-13-11_A200,A200I.pdf (Accessed on May 15th, 2012).

ANKOM Technology Methods` Procedure for "ADL" ("Acid Detergent Lignins"). http://www.ankom.com/media/documents/Method_8_Lignin_in_beakers_4-13-11.pdf (Accessed on May 15th, 2012).

AOAC International. www.aoac.org (Accessed on December 20th, 2012).

Arroyo-López, F. N.; Orlíc, S.; Querol, A.; Barrio, E. *Int. J. Food Microbiol.* **2009**, *131*, 120-127.

Asikin, Y.; Taira, I.; Inafuku, S.; Sumi, H.; Sawamura, M.; Takara, K.; Wada, K. *J. Food Sci.* **2012,** *77*, 469-475.

Asztalos, A.; Daniels, M.; Sethi1, A.; Shen, T.; Langan, P.; Redondo, A.; Gnanakaran, S. *Biotechnol. Biofuels.* **2012**, *5,* 1-5.

Bampidis, V.A.; Robinson, P. H. *Anim. feed Sci. Tech.* **2006**,*128*, 175-217.

Bansal, N.; Tewari, R.; Gupta, J. K.; Soni, R.; Soni, S. K. *BioResources.* **2011**, *6*, 552-569.

Baurhoo, B.; Ruiz-Feria, C. A.; Zhao, X. *Anim. Feed Sci. Technol*. **2008**, *144*, 175-184.

Bermejo, A.; Llosa, M. J.; Cano, A. *Food Sci. Tech. Int*. [online]. **2011**, *17*, 55-62. http://fst.sagepub.com/content/17/1/55 (Accessed on May 15th, 2012).

Bradford, M.M. *Anal. Biochem*. **1976**, *72*, 248-254.

Breuil, C.; Chan, M.; Gilbert, M.; Saddler, J. N. *Bioresour. Technol.* **1992**, *39*, 139-142.

Brunings, A. M.; Gabriel, D. W. *Mol. Plant Pathol.* **2003**, *4,* 141-157.

Carvalho, R. N. L. Dilute acid and enzymatic hydrolysis of sugarcane bagasse for biogas production. Master's dissertation, *Instituto superior technico*, Technical University of Lisboa, 2009.

Cano, A.; Medina, A.; Bermejo, A. *J. Food Compos. Anal*. **2008**, *21*, 377-381.

Carpita, N.; Tierney, M.; Campbell, M. *Plant Mol. Biol.* **2001**, *47*, 1-5.

Carrao-Panizzi, M. C.; Bordingnon, J. R. *Pesq. Agropec. Bras*. **2000**, *35,* 873-878.

Chandra, R. P.; Bura, R.; Mabee1, W. E.; Berlin, A.; Pan, X.; Saddler, J. N. *Adv. Biochem. Engin/Biotechnol.* **2007,** *108*, 67-93.

Chandra, R.; Takeuchi, H.; Hasegawa, T. *Renew. Sust. Energ. Rev*. **2012**, *16,* 1462-1476.

Chang, V. S.; Holtzapple, M. T. *Appl. Biochem. Biotechnol*. **2000**, *84-86*, 5-37.

Charoenchai, C.; Fleet, G.; Henschke, P. A. *Am. J. Enol. Vitic.* **1998**, *49*, 283-288.

Chaudhary, N.; Qazi, J. I. *Afr. J. Biotechnol.* **2011**, *10,* 1270-1274.

Chen, S. L.; Gutmanis, F. *Biotechnol. Bioeng*. **1976**, *18,* 1455-1462.

Citrus production report Wikipedia. http://en.wikipedia.org/wiki/Citrus_production (Accessed on January 12th, 2013).

Clark, L. *J. Wildl. Manage.* **1997**, *61*, 1352-1358.

Converse, A.O. *Bioconversion of forest and agricultural plant residues;* Saddler, J. N., Ed.; Walllingford: CAB International, 1993; pp 93-106.

Cordeiro de Melo, T. C.; Machado, G. B.; Belchior, C. R. P.; Colaço, M. J.; Barros, J. E. M.; de Oliveira, E. J.; de Oliveira, D. G. *Fuel*, **2012**, *97*, 796-804.

CTBE- Brazilian Bioethanol Science and Technology Laboratory, http://www.bioetanol.org.br/english/index.php (Accessed on September 5th, 2012).

Cubero, J.; Graham, J. H.; Gottwald, T. R. *Appl. Environ. Microbiol*. **2001**, *67*, 2849-2852.

Daniel, G. *FEMS Microbiol. Rev*. **1994**, *13*, 199-233.

da Silva, A. C. R.; Ferro, J. A.; Reinach, F. C.; Farah, C. S.; Furlan, L. R.; Quaggio, R. B.; Monteiro-Vitorello, C. B.; Van Sluys, M. A.; Almeida, N. F.; Alves, L. M.; do Amaral, A. M.; Bertolini, M. C.; Camargo, L. E.; Camarotte, G.; Cannavan, F.; Cardozo, J.; Chambergo, F.; Ciapina, L. P.; Cicarelli, R. M.; Coutinho, L. L.; Cursino-Santos, J. R.; El-Dorry, H.; Faria, J. B.; Ferreira, A. J.; Ferreira, R. C.; Ferro, M. I.; Formighieri, E. F.; Franco, M. C.; Greggio, C. C.; Gruber, A.; Katsuyama, A. M.; Kishi, L. T.; Leite, R. P.; Lemos, E. G.; Lemos, M. V.; Locali, E. C.; Machado, M. A.; Madeira, A. M.; Martinez-Rossi, N. M.; Martins, E. C.; Meidanis, J.; Menck, C. F.; Miyaki, C. Y.; Moon, D. H.; Moreira, L. M.; Novo, M. T.; Okura, V. K.; Oliveira, M. C.; Oliveira, V. R.; Pereira, H. A.; Rossi, A.; Sena, J. A.; Silva, C.; de Souza, R. F.; Spinola, L. A.; Takita, M. A.; Tamura, R. E.; Teixeira, E. C.; Tezza, R. I.; Trindade dos Santos, M.; Truffi, D.; Tsai, S. M.; White, F. F.; Setubal, J. C.; Kitajima, J. P. *Nature.* **2002**, *417*, 459-463.

Deejing, S.; Ketkorn, W. *Chiang Mai J. Sci*. **2009**, *36*, 384-394.

Delgenes, J.; Moletta, R.; Navarro, J. M. *Enzyme Microb. Technol*. **1996**, *19,* 220-225.

Del Rio, J. A.; Fuster, M. D.; Sabater, F.; Porrass, I.; Garcia-lidon, A.; Ortuno, A. *J. Agric. Food. Chem.* **1995**, *43*, 2030-2034.

Del Rio, L. F.; Chandra, R. P.; Saddler, J. N. *Biotechnol. Bioeng*. **2011**, *108,* 1549-1558.

Dhillona, G. S.; Brara, S. K. *Food Chem.***2011**, *128*, 864-871.

Dinh, T. N.; Nagahisa, K.; Hirasawa, T.; Furusawa, C.; Shimizu, H. Adaptation of Saccharomyces cerevisiae Cells to High Ethanol Concentration and Changes in Fatty Acid Composition of Membrane and Cell Size. *PLoS ONE* [online] **2008,** *3,* e2623. (Accessed on December 15th, 2012). http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0002623.

Divne, C.; Ståhlberg, J.; Teeri, T. T.; Jones,T. A. *J. Mol. Biol*. **1998**, *275*, 309-325.

Doherty, W. O. S.; Mousavioun, P.; Fellows, C. M. *Ind. Crop.s Prod.* **2011**, *33*, 259-276.

Duetz, W. A.; Bouwmeester, H.; van Beilen, J. B.; Witholt, B. *Appl. Microbiol. Biotechnol.* **2003**, *61*, 269-77.

Duff, S.J.B.; Murray, W.D. *Bioresour. Technol*. **1996**, *55*, 1-33.

Edwards, M. C.; Doran-Peterson, J. *Appl. Microbiol. Biotechnol.* **2012,** *95*, 565-575.

Fan, L. T.; Gharpuray, M. M.; Lee, Y. H. *In Cellulose Hydrolysis Biotechnology Monographs*; Springer: Berlin, 1987; p 57.

Farrell, A. E.; Plevin, R. J.; Turner, B. T.; Jones, A. D.; O'Hare, M.; Kammen, D. M. *Science*, **2006**, *311*,506-508.

Fattori, J.; Prando, A.; Assis, L. H. P.; Aparicio, R.; Tasic, L. *Protein J.* **2011**, *30,* 324-333.

Fierobe, H. P.; Bayer, E. A.; Tardif, C.; Czjzek, M.; Mechaly, A.; Belaich, A.; Lamed, R.; Shoham, Y.; Belaich, J. P. *J. Biol. Chem*. **2002**, *277*, 49621-49630.

Fleet, G. H.; Heard, G. M. *Wine Microbiology and Biotechnology*; Fleet, G.H., Ed.; Harwood Academic Publishers, Chur Switzerland, 1993, pp. 42-43.

Galbe, M.; Zacchi, G. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 618-628.

Gan, Q.; Allen, S. J.; Taylor, G. *J. Chem. Technol.Biot.* **2005**, *80,* 688-698.

Gardner, K. H.; Blackwell, J. *Biopolymers*, **1974,** *13,* 1975-2001.

Garde-Cerdán, T.; Ancín-Azpilicueta, C. *Eur. Food Res.Technol*. **2006**, *222*, 15-25.

Ghose, T. K. *Pure Appl. Chem.* **1987,** *59,* 257-268.

Giraldo, M. A.; da Silva, T. M.; Salvato, F.; Terenzi, H. F.; Jorge, J. A.; Guimaraes, L. H. S. *World J. Microb. Biot.* **2012,** *28*, 463-472.

Greil, P. *J. Eur. Ceram. Soc.* **2001**, *21*, 105-118.

Grohmann, K.; Baldwin, E. A. *Biotecnol Lett*, **1992,** *14*, 1169-1174.

Grohmann, K.; Baldwin, E. A.; Buslig, B. S. *Appl. Biochem. Biotechnol*. **1994**, *45/46,* 315-327.

Grohmann, K.; Cameron, R.G.; Buslig, B.S. *Bioresour Technol*, **1995**, *54,* 129-141.

Gusakov, A.V.; Sinitsyn, A. P.; Berlin, A. G.; Markov, A. V.; Ankudimova, N. V. *Enzyme Microb. Tech*. **2000**, *27,* 664-671.

Hahn-Hagerdal, B.; Karhumma, K.; Fonseca, C; Spencer-Martins, I.; Marie, F. *Appl. Microbiol. Biotechnol*. **2007**, *74*, 937-953.

Han, Y.; Chen, H. *Bioresour. Technol*. **2011**, *102,* 4787-4792.

Harris, E. E.; Beglinger, E.; Hajny, G. J.; Sherrard, E. C. *Ind. Eng. Chem.* **1945**, *37,* 12-23.

Harris, J.; Baker, A.; Zerbe, J. *Energy Biomass Wastes*, **1984**, *8*, 1151-1170.

Hillyer, G. L. Ethanol production from citrus waste through limonene reduction. U.S. Patent 8,252,566 B2, August 28th, 2012.

Hsu, T. *Handbook of Bioethanol: Production and utilization*; Wyman, C.E., Ed.; Taylor and Francis: Washington, DC, 1996; pp 179-212.

Hu, J.; Arantes, V.; Saddler, J. N. *Biotechnol. Biofuels.* **2011***, 4,* 1-13.

Huang, C. F.; Lin, T. H.; Guo, G. L.; Hwang, W. S. *Bioresour. Technol*. **2009**, *100*, 3914-3920.

IBGE, Instituto Brasileiro de Geografia e Estatística. www.ibge.gov.br (Accessed on December 15th, 2012).

Ibrahim, M. A.; Kainulainen, P.; Aflatuni, A.; Tiilikkala, K.; Holopainen, J. K. *Agric. Food Sci. Finl*. **2001**, *10, 243*-259.

Ikan, R. *Natural products: a laboratory guide*; 2nd ed.; Academic press limited: London, 1991, pp 9-10.

Ingram, L. O.; Lai, X.; Moniruzzaman, M.; Wood, B. E.; York, S. W. Fuel ethanol production from lignocellulose using genetically engineered bacteria. In *Food and chemicals from biomass;* Saha, B. C.; Woodward, J., Ed.; ACS Symposium Series, American Chemical Society: Washington, DC, 1997; vol. 666, pp 57-73.

Jafari, V.; Labafzadeh, S. R.; Jeihanipour, A.; Karimi, K.; Taherzadeh, M. J. *Renew. Energ.* **2011**, *36*, 2771- 2775.

Jalak, J; Kurasin, M; Teugjas, H.; Väljamäe, P. *J. Biol. Chem.* **2012**, *287,* 28802-28815.

Jalan, N.; Aritua, V.; Kumar, D.; Yu, F.; Jones, J. B.; Graham, J. H.; Setubal, J. C.; Wang, N. *J. Bacteriol*. **2011,** *193,* 6342-6357.

Jayani, R. S.; Saxena, S.; Gupta, R. *Process Biochem*. **2005**, *40*, 2931–2944.

Jeoh, T.; Ishizawa, C. I.; Davis, M. F.; Himmel, M. E.; Adney, W. S.; Johnson, D. K. *Biotechnol. Bioeng*. **2007**, *98*, 112-122.

Jørgensen, H.; Kristensen, J.; Felby, C. *Biofuel. Bioprod. Bior*. **2007**, *1*, 119-134.

Joshi, B.; Bhatt, M. R.; Sharma, D.; Joshi, J.; Malla, R.; Sreerama, L. *Biotechnol. Mol. Biol. Rev*, **2011**, *6,* 172-182.

Kale, P. N.; Adsule, P. G. *Handbook of Fruit Science and Technology: Production, Composition, Storage, and Processing*; Salunkhe, D. K.; Kadam, S. S., Eds.; Marcel Dekker, Inc., New York, 1995, pp 39-65.

Keller, F.A. Integrated bioprocess development for bioethanol production. In *Handbook on bioethanol: production and utilization*; Wyman, C. E., Ed.; Taylor and Francis: Bristol, Pennsylvania, 1996, pp 351-379.

Khater, L.; Alegria, M. C.; Borin, P. F. L.; Santos, T. M.; Docena, C.; Tasic, L.; Farah, C. S.; Ramos, C. H. I. *Arch. Microbiol.* **2007**, *188*, 243-250.

Kirk, T. K. *Annu. Rev. Phytopathol*. **1971**, *9*, 185-210.

Koizumi, M. *Proc. Intern. Soc. Citric*. **1977,** *3,* 924-928.

Koizumi, M.; Kuhara, S. *Bull. Tree Fruit Res. Sta. D.* **1982**, *4*, 73-92.

Kokotkiewicz, A.; Luczkiewicz, M.; Sowinski, P.; Glod, D.; Gorynski, K.; Bucinski, A. *Food Chem.* **2012**, *133*, 1373-1382.

Kumar, R.; Singh, S.; Singh, O. V. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 377-391.

Kumar, A.; Mishra, S. *Indian J. Microbiol.* **2010,** *50*, 88-92.

Kuster, B. F. M. *Starch,* **1990**, *42*, 314-321.

Laivins, G. V.; Scallan, A. M. The mechanism of hornification of wood pulps. In: *Products of papermaking, transactions of the 10th fundamental research symposium*; Baker, C. F., Ed.; Pira International, Oxford, UK, 1993.

Lawford, H. G.; Rousseau, J. D. *Appl. Biochem. Biotechnol.* **1998**, *70–72*, 161-172.

Lawford, H. G.; Rousseau, J. D.; McMillan, J. D. *Appl. Biochem. Biotechnol*. **1997**, *63-65*, 269-286.

Lee, P. R.; Ong, Y. L.; Yu, B.; Curran, P.; Liu, S. Q. *Food Microbiol*. **2010**, *27*, 853-861.

Lee, P; Saputra, A.; Yu, B.; Curran, P.; Liu, S. *Food Sci. Technol.* **2012**, *46,* 84-90.

Lenihan, P.; Orozco, A.; O'Neill, E.; Ahmad, M. N. M.; Rooney, D. W.; Walker, G. M. *Chem. Eng. J*. **2010**, *156*, 395-403.

Li, J. Y.; Yei, X. M.; Ruan, S. M.; Cai, L. L.; Chang, Z. Z. *Enzymatic Hydrolysis of Rice Straw Pretreated with Ammonia ;* Proceedings of the Conference on Environmental Pollution and Public health, Scientific Research Publishing, USA, 2010; vol. 1-2, pp 482-485.

Li, J.; Sun, F. H.; Li, X. Z.; Yan, Z. Y.; Yuan, Y. X.; Liu, X. F. *J. Chem. Tech. Biotechnol.* **2012,** *87,* 1687-1693.

Lin, H. C.; Hsu, S. T.; Chang, H.; Tzeng, K. C. *J. Plant Pathol*. **2010**, *92,* 149-156.

Lin, Y.; Tanaka, S. *Appl. Microbiol. Biotechnol*. **2006,** *69,* 627-642.

Liu, L. P.; Deng, Z. N.; Qu, J. W.; Yan, J. W.; Catara, V.; Li, D. Z.; Long, G. Y.; Li, N. *Curr. Microbiol*. **2012,** *65*, 304-312.

Liu, L. S.; Won, Y. J.; Cooke, P. H.; Coffin, D. R.; Fishman, M. L.; Hicks, K. B.; Ma, P. X. *Biomaterials*, **2004**, *25*, 3201-3210.

Lynd, L.R. *Appl. Biochem. Biotechnol.* **1990,** *24-25,* 695-719.

Lynd, L. R.; Weimer, P. J.; Zyl, W. H.; Pretorius, I. S. *Microbiol. Mol. Biol. R.* **2002**, *66*, 506-577.

Maeda, R. N.; Serpa, V. I.; Rocha, V. A. L.; Mesquita, R. A. A.; Anna, L. M. M. S.; Castro, A. M.; Driemeier, C. E.; Pereira, N. J.; Polikarpov, I. *Process Biochem.* **2011,** *46,* 1196-1201.

Mansfield, S.D.; Mooney, C.; Saddler, J. N. *Biotechnol. Progr.* **1999**, *15,* 804-816.

Marín, F. R.; Soler-Rivas, C.; Benavente-García, O.; Castillo, J.; Pérez-Álvarez, J. A. *Food Chem*. **2007**, *100*, 734-741.

Maurer, S. A.; Bedbrook, C. N.; Radke, C. J. *Ind. Eng. Chem. Res*. **2012**, *51*, 11389-11400.

Mazzaferro, L. S.; Breccia, J. D. *Food Chem*. **2012**, *134*, 2338-2344.

Mcmillan, J. D. *Pretreatment of lignocellulosic biomass. In Enzymatic Conversion of Biomass for Fuels Production*; Himmel, M. E.; Baker, J. O.; Overend, R. P., Eds.; American Chemical Society: Washington, DC, 1994, pp. 292-324.

Mendgen, K.; Deising, H. *New Phytol*. **1993**, *124*, 193-213.

Merle, H.; Moro´n, M.; Bla´zquez, M. A.; Boira, H. *Biochem. Syst. Ecol.* **2004**, *32*, 491-497.

Miller, G. L. *Anal. Chem*. **1959,** *31*, 426-428.

Mooney, C. A.; Mansfield, S. D.; Touhy; M. G.; Saddler, J. N. *Bioresour. Technol*. **1998**, *64*, 113-119.

Moreira, L. M.; de Souza, R. F.; Almeida, N. F. Jr.; Setubal, J. C; Oliveira, J. C. F.; Furlan, L. R.; Ferro, J. A.; da Silva, A. C. R. *Annu. Rev. Phytopathol*. **2004**, *42,* 163–84.

Moreira, N.; Mendes, F.; Guedes de Pinho, P.; Hogg, T.; Vasconcelos, I. *J. Food Microbiol*. **2008**, *124*, 231-238.

Mussatto, S. I.; Teixeira, J. A., *Lignocellulose as raw material in fermentation processes , Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, Formatex Research Center, 2010, Vol. 2, pp 897-907.

Nam, K. H.; Sung, M. W.; Hwang, K. Y. *Biochem. Bioph. Res. Co.* **2010**, *391*, 1131-1135.

Nikolic, S.; Mojovic, L.; Pejin, D.; Rakin, M.; Vucurovic, V. *Food Technol. Biotechnol.* **2009,** *47*, 83-89.

Nizamutdinova, I. T.; Jeong, J. J.; Xu, G. H.; Lee, S.; Kang, S. S.; Kim, Y. S.; Chang, K. C.; Kim, H. J. *Int. Immunopharmacol,* **2008**, *8*, 670-678.

NREL, Laboratory Analytical Procedure (LAP) for 'Determination of Total solids in Biomass'. http://www.nrel.gov/biomass/pdfs/42621.pdf (Accessed on May 15th, 2012).

NREL, Determination of Structural Carbohydrates and Lignin in Biomass. http://www.nrel.gov/biomass/pdfs/42618.pdf (Accessed on May 15th, 2012).

Numan, M. T.; Bhosle, N. B. *J. Ind. Microbiol. Biotechnol*. **2006**, *33,* 247-260.

Oberoi, H. S.; Vadlani, P. V.; Madl, R. L.; Saida, L.; Abeykoon, J. P. *J. Agric. Food Chem.* **2010**, *58*, 3422-3429.

Ogeda, T. L.; Petri, D. F. S. *Quim. Nova,* **2010**, *33,* 1549-1558.

Ooshima, H.; Burns, D.; Converse, A. O. *Biotechnol. Bioeng*. **1990**, *36*, 446-452.

Ortega, N.; Busto, M.; Perez-Mateos, M. *Int. Biodeter. Biodegr*. **2001**, *47,* 7-14.

Ovissipour, M.; Abedian, A.; Motamedzadegan, A.; Rasco, B.; Safari, R.; Shahiri, H. *Food Chem.* **2009,** *115*, 238-242.

Panagiotopoulos, I. A.; Lignos, G. D.; Bakker, R. R.; Koukios, E. G. *J. Clean. Prod*. **2012**, *32*, 45-51.

Pandey, A.; Soccol, C. R.; Mitchell, D. *Process Biochem*. **2000**, *35,*1153-1169.

Phutela, U.; Dhuna, V.; Sandhu, S.; Chadha, B. S. *Braz. J. Microbiol.* **2005**, *36*, 63-69.

Plomion, C.; Leprovost, G.; Stokes, A. *Plant Physiol*. **2001**, *127*, 1513-1523.

Pourbafrani, M.; Talebnia, F.; Niklasson, C.; Taherzadeh, M. J. *Int. J. Mol. Sci.* **2007**, *8*, 777-787.

Qin, W. Master's Dissertation, The University of British Colombia, 2010.

Rakin, M.; Mojovic, L.; Nikolic, S.; Vukasinovic, M.; Nedovic, V. *Afr. J. Biotecnol*. **2009**, *8*, 467-471.

Reverbel-Leroy, C.; Pages, S.; Belaich, A.; Belaich, J-P.; Tardif, C. *J. Bacteriol.* **1997**, *179*, 46-52.

Rivas, B.; Torrado, A.; Torre, P.; Converti, A.; Dominguez, J. M. *J. Agr. Food Chem.* **2008**, *56*, 2380-2387.

Rogers, P. L.; Lee, K. J.; Tribe, D. E. *Biotechnol. Lett.* **1979**, *1*, 165-170.

Sadoudi, M.; Tourdot-Maréchal, R.; Rousseaux, S.; Steyer, D.; Gallardo-Chacón, J.; Ballester, J.; Vichi, S.; Guérin-Schneider, R.; Caixach, J.; Alexandre, H. *Food Microbiol.* **2012,** *32*, 243-253.

Saha, B.; Iten, L.; Cotta, M.; Wu, Y. *Biotechnol. Progr.* **2005,** *21*, 816-822.

Samayam, I. P.; Hanson, B. L.; Langan, P.; Schall, C. A. *Biomacromolecules,* **2011**, *12*, 3091-3098.

Santos, M. M.; Rosa, A. S.; Dal'boit, S.; Mitchell, D. A.; Krieger, N. *Bioresour. Technol*. **2004**, *93*, 261-268.

Sarita, C. R. PhD. Thesis, University of Campinas, Sao paulo, Brazil, 2010.

Sawada, T.; Nakamura, Y.; Kobayashi, F.; Kuwahara, M.; Watanabe, T.; *Biotechnol. Bioeng*. **1995**, *48*, 719-724.

Schaad, N. W.; Postnikova, E.; Lacy, G.; Sechler, A.; Agarkova, I.; Stromberg, P. E.; Stromberg, V. K.; Vidaver, A. K. *Syst. Appl. Microbiol.* **2006,** *29*, 690-695.

Science business report, http://www.sciencebusiness.net/Assets/2de40450-5768-4b63-8bf5-4006a37a66be.pdf . (Accessed on January 15th, 2013).

Scriban, R. Biotechnology, ed.; Manole Ltd: Sao paulo, Brazil, 1985, p 489.

Shrestha, P. Enhanced bioprocessing of lignocellulose: Woodrot fungal saccharification and fermentation of corn fiber to ethanol. Ph.D. thesis, Lowa State University; 2008.

Singhania, R. R.; Sukumaran, R. K.; Patel, A. K.; Larroche, C.; Pandey, A. *Enzyme Microb. Tech.* **2010**, *46*, 541-549.

Smook, G. A. *Handbook for Pulp and Paper Technologies,* 3rd ed.; Angus Wilde Publications Inc.: Vancouver, British Columbia, 2002.

Smyth, T. A.; Lambert, D. R. Biodegradable liquid degreaser and parts cleaner composition. U.S. Patent Application 09,108,588; Patent no. 5965512, December 10, 1998.

Soccol, C. R.; Vandenberghe, L. P.; Medeiros, A. B. P.; Karp, S. G.; Buckeridge, M.; Ramos, L. P.; Pitarelo, A. P.; Ferreira-Leitão, V.; Gottschalk, L. M. F.; Ferrara, M. A.; da Silva Bon, E. P.; de Moraes, L. M. P.; Araújo, J.; Torres, F. A. G. *Bioresource Technol*. **2010**, *101,* 4820-4825.

Sternberg, D. *Biotechnol. Bioeng. Symp*. **1976**, *6*, 35-53.

Stewart, D.; Raton, B.; Widmer, W. W.; Grohmann, K.; Wilkins, M. R. Ethanol Production from Citrus Processing Waste. US Patent 2006/0177916, August 10, 2006.

Stewart, D.; Raton, B.; Widmer, W. W.; Grohmann, K.; Wilkins, M. R. Ethanol Production from Solid Citrus Processing Waste. US Patent 2008/0213849, September 4th, 2008.

Stewart, D.; Widmer, W.; Grohmann, K.; Wilkins, M. Ethanol production from solid citrus processing waste. US Patent 8,372,614 B2, February 12th, 2013.

Subba, M. S.; Soumithri, T. C.; Rao, R. S. *J. Food Sci.* **1967**, *32*, 225-227.

Sudhakar, D. V.; Maini, S. B. *J. Food Process Pres.* **2000**, *24*, 209-227.

Sun, Y.; Cheng, J. *Bioresource Technol*. **2002**, *83*, 1-11.

Taherzadeh, M. J.; Eklund, R.; Gustafsson, L.; Niklasson, C.; Lidén, G. *Ind. Eng. Chem. Res.* **1997**, *36,* 4659-4665.

Taherzadeh, M.; Gustafsson, L.; Niklassonm C.; Liden, G. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 701-708.

Taherzadeh, M. J.; Karimi, K. *Bioresource.* **2007**, *2,* 472-499.

Talebnia, F.; Pourbafrani, M.; Lundin, M.; Taherzadeh, M. *Bioresources.* **2008,** *3*, 108-122.

Tanahashi, M. *Wood Res*. **1990**, *77*, 49-117.

Tanaka, M.; Ikesaka, M.; Matsuno, R. *Biotechnol. Bioeng*. **1988**, *32*, 698-706.

Tasic, L.; Borin, P. F. L.; Khater, L.; Ramos, C. H. I. *Protein Expres. Purif.* **2007,** *53*, 363-369.

Tonn, S. T.; Philippidis, G. P.; Ho, N. W. Y.; Chen, Z.; Brainard, A.; Lumpkin, R. E.; Riley, C. J. *Appl. Biochem. Biotechnol.* **1997**, *63-65*, 243-255.

US department of energy website, 2013. Biomass program; http://www1.eere.energy.gov/biomass/printable_versions/technology_background.html (Accessed on January 15th, 2013).

USDA report 2012, http://www.fas.usda.gov/psdonline/circulars/citrus.pdf (Accessed on December 5th, 2012).

Van Dyk, J. S.; Pletschke, B. I.; *Biotechnol. Adv.* **2012**, *30*, 1458-1480.

Vandame, E. Agro-Industrial Residue Utilization for Industrial Biotechnology Products. In *Biotechnology for Agro-Industrial Residues Utilisation*; Nigam, P.; Pandey, A., Ed.; Springer: Netherlands, 2009.

Vastrad, B. M.; Neelagund, S. E. *Int. J. Pharm. Tech.* **2011,** *3,* 3655-3667.

Vauterin, L.; Hoste, B.; Kersters, K.; Swings, J. *Int. J. Syst. Bacteriol*. **1995**, *45,*472-489.

Verendel, J. J.; Church, T. L.; Andersson, P. G.; *Synthesis,* **2011**, *11*, 1649-1677.

Vinzant, T. B.; Ponfick, L.; Nagle, N. J.; Ehrman, C. I.; Reynolds, J. B.; Himmel, M. E. *Appl. Biochem. Biotech.* **1994**, *45-46*, 611-626.

Vitali, J.; Schick, B.; Kester, H. C. M.; Visser, J.; Jurnak, F. *Plant Physiol.* **1998**, *116,* 69-80.

Vourc'h, G.; de Garine-Wichatitsky, M.; Labbé, A.; Rosolowski, D.; Martin, J-L.; Fritz, H. *J. Chem. Ecol*. **2002**, *28*, 2411-2427.

Walfridsson, M. PhD. Thesis, Department of Applied Microbiology, Lund University Sweden, 1996.

Wilkins, M. R.; Widmer, W. W.; Camero, R. G.; Grohmann, K. *Proc. Fla. State Hort. Soc.* **2005**, *118*, 419-422.

Wilkins, M. R.; Widmer, W. W.; Grohmann, K.; Cameron, R.G. *Bioresource Tecnol*. **2007a**, *98*, 1596-1601.

Wilkins, M. R.; Suryawati, L.; Maness, N. O.; Chrz, D. *World J. Microbiol. Biotechnol*. **2007b**, *23*, 1161-1168.

Wilkins, M. R. *Biomass Bioenerg.* **2009**, *33*, 538-541.

Willats, W. G. T.; Knox, J. P.; Mikkelsen, J. D. *Trends Food Sci. Technol.* **2006**, *17*, 97-104.

Wood, T. M.; *Biotechnol Bioeng Symp.* **1975,** *5*, 111-137.

Wyman, C. E. *Handbook on Bioethanol: Production and Utilization Applied Energy Tecnology Series,* Taylor & Francis Washington, D.C., 1996.

Wyman, C. E. *Annu. Rev. Energ. Env.* **1999**, *24*, 189-226.

Xiao, Z.; Zhang, X.; Gregg, D. J.; Saddler, J. N.; *Appl. Biochem. Biotech.* **2004**, *115,* 1115-1126.

Zeng, M. J.; Mosier, N. S.; Huang, C. P.; Sherman, D. M.; Ladisch, M. R.; *Biotechnol. Bioeng*. **2007**, *97,* 265-278.

Zhang, Y. H. P.; Lynd, L. R. *Biotechnol.Bioeng*. **2004,** *88,* 797-824.

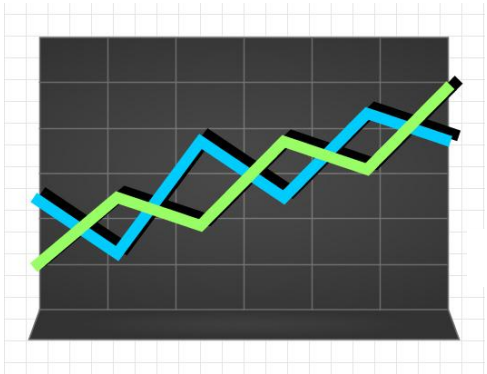
Zhao, X.; Zhang, L.; Liu, D. *Biofuels, Bioprod. Bioref***. 2012**, *6,* 465-482.

Zhong, R.; Ye, Z. H. Secondary Cell Walls. In *Encyclopedia of Life Sciences,* John Wiley and Sons, Ltd: Chichester, England, 2009.

Zhu, L. Fundamental study of structural features affecting enzymatic hydrolysis of Lignocellulosic biomass. Ph.D. Thesis, Texas A&M University, USA, 2005.

Zhu, J. Y.; Gleisner, R.; Scott, C. T.; Luo, X. L.; Tian, S. *Bioresour. Technol.* **2011**, *102,* 8921-8929.

Zvaigzne, G.; Karklina, D.; Seglina, D.; Krasnova, I. *Chem. Technol*. **2009**, *3,* 56-61.



*

APPENDIX

*http://www.smashingmagazine.com/2010/09/29/creating-graphs-with-adobe-illustrator/ (Web page accessed on January 15th, 2013).

Plots of 2G-ethanol from fermentations of acid hydrolyzates

Saccharomyces cerevisiae fermentations:

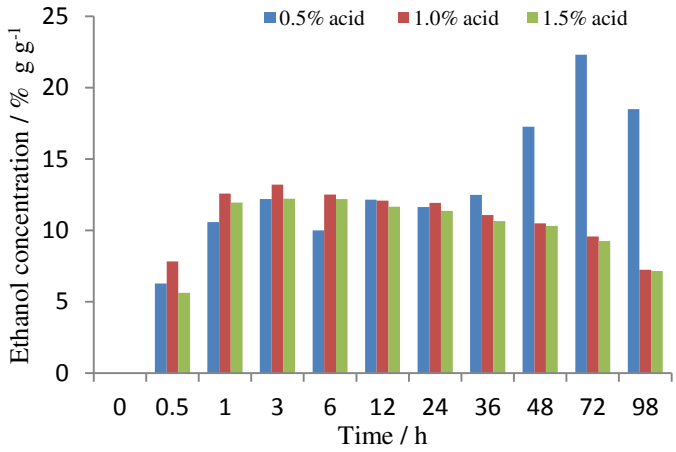


Figure A1. A plot of 2G-ethanol concentration in relation to time in *Saccharomyces cerevisiae* fermentations of 0.5, 1 and 1.5% v v^{-1} (15 min) acid hydrolyzed samples. Max. ethanol yield 22% g g^{-1} obtained after 72 hours of fermentation from 0.5% acid hydrolyzed sample with 11g L^{-1} initial fermentable sugar concentration.

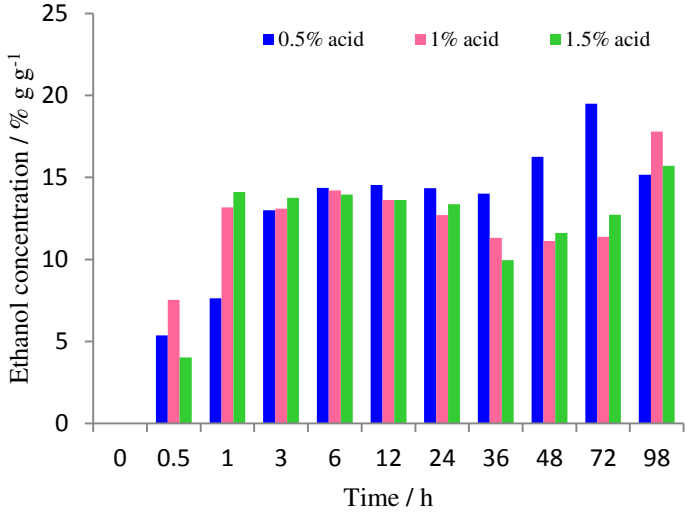


Figure A2. A plot of 2G-ethanol concentration in relation to time in *Saccharomyces cerevisiae* fermentations of 0.5, 1 and 1.5% v v^{-1} (30 min) acid hydrolyzed samples. Max. ethanol yield 19.5 % g g^{-1} obtained after 72 hours of fermentation from 0.5% acid hydrolyzed sample.with 14 g L^{-1} initial fermentable sugar concentration.

Candida parapsilosis NRRL Y-12969 fermentations:

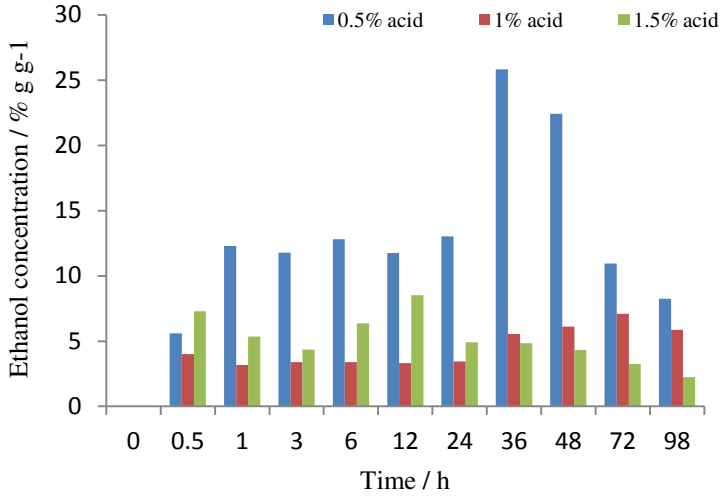


Figure A3. A plot of 2G-ethanol concentration in relation to time in *Candida parapsilosis* NRRL Y-12969 fermentations of 0.5, 1 and 1.5% v v^{-1} (15 min) acid hydrolyzed samples. Max. ethanol yield 27% g g^{-1} obtained after 36 hours of fermentation from 0.5% acid hydrolyzed sample with 11 g L^{-1} initial fermentable sugar concentration.

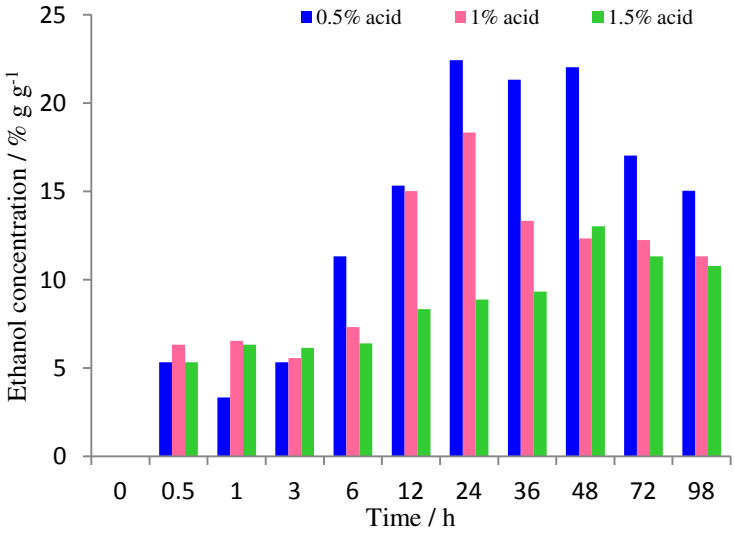


Figure A4. A plot of 2G-ethanol concentration in relation to time in *Candida parapsilosis* NRRL Y-12969 fermentations of 0.5, 1 and 1.5% v v^{-1} (30 min) acid hydrolyzed samples. Max. ethanol yield 22.4 % g g^{-1} obtained after 24 hours of fermentation from 0.5% acid hydrolyzed sample with 13 g L^{-1} initial fermentable sugar concentration.

Candida parapsilosis IFM 48375 fermentations:

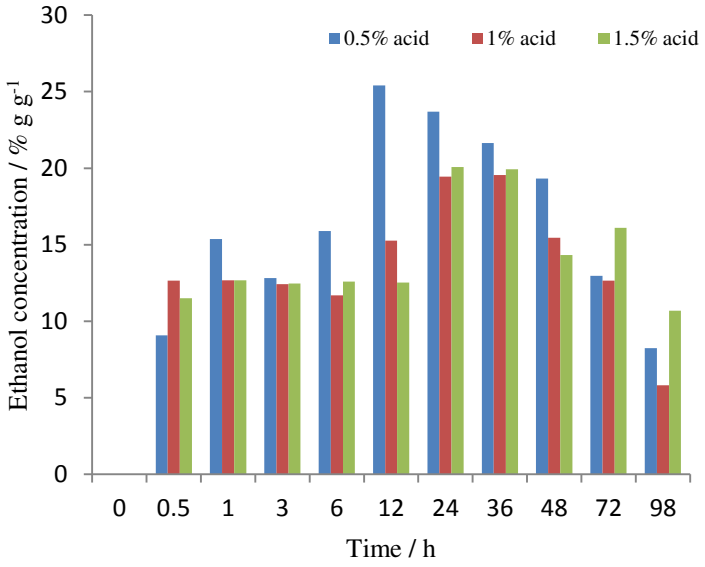


Figure A5. A plot of 2G-ethanol concentration in relation to time in *Candida parapsilosis* IFM 48375 fermentations of 0.5, 1 and 1.5% v v^{-1} (15 min) acid hydrolyzed samples. Max. ethanol yield 26% g g^{-1} obtained after 12 hours of fermentation from 0.5% acid hydrolyzed sample.with 13 g L^{-1} initial fermentable sugar concentration.

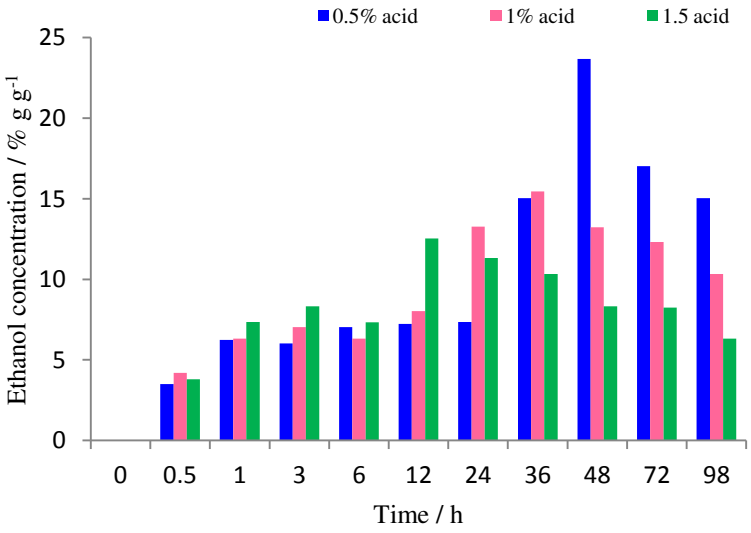


Figure A6. A plot of 2G-ethanol concentration in relation to time in *Candida parapsilosis* IFM 48375 fermentations of 0.5, 1 and 1.5% v v^{-1} (30 min) acid hydrolyzed samples. Max. ethanol yield 24% g g-1 obtained after 48 hours of fermentation from 0.5% acid hydrolyzed sample.with 10.8 g L^{-1} initial fermentable sugar concentration.

Plots of 2G-ethanol from fermentations of enzyme hydrolyzates

Fermentations of Enzyme cocktail 2 (Table IV-9) hydrolyzates

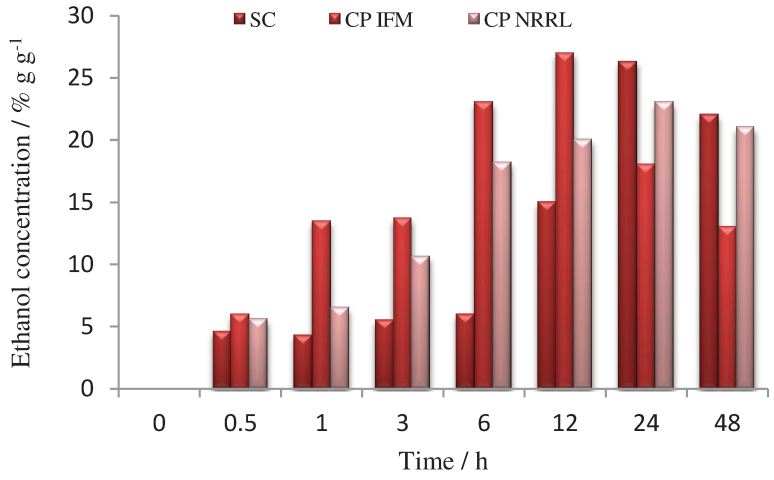


Figure A7. Comparison of mono-culture fermentations on Enzyme cocktail 2 hydrolyzates (where SC = *Saccharomyces cerevisiae*; CP IFM = *Candida parapsilosis* IFM48375; CP NRRL = *Candida parapsilosis* NRRL-12969).

Table A7. Comparative analysis of starting sugar concentration and ethanol obtained from mono-culture fermentations on Enzyme cocktail 2 hydrolyzates

Yeast strain	Starting C-6 sugars (% g g^{-1})	**Emax* (% g g^{-1})	***TIMEmax* (h)
SC	42.6	26.3	24
CP IFM	40.2	27	12
CP NRRL	39.0	23	24

*Emax= final ethanol yield, % g g^{-1},
****TIMEmax*= time at which maximum ethanol yield was obtained.

Fermentations of beta-glucosidase enzyme hydrolyzates

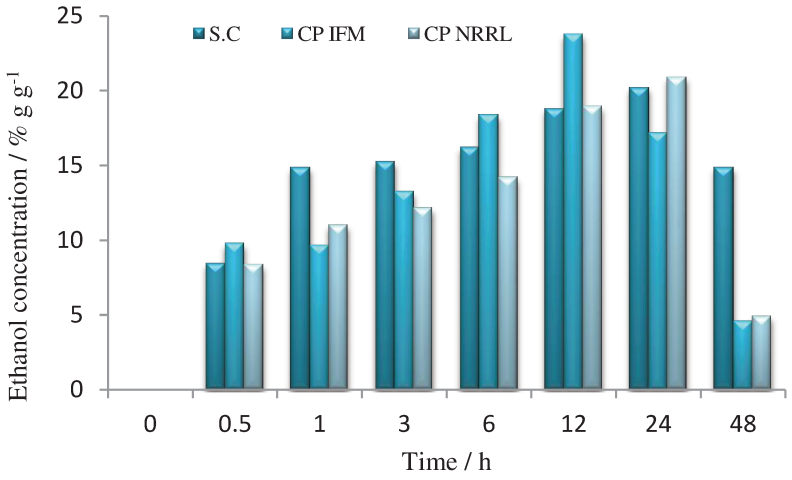


Figure A8. Comparison of mono-culture fermentations on beta-glucosidase enzyme (4.15 mg g^{-1} dry CPWO) hydrolyzates (where SC = *Saccharomyces cerevisiae*; CP IFM = *Candida parapsilosis* IFM48375; CP NRRL = *Candida parapsilosis* NRRL-12969).

Table A8. Comparative analysis of starting sugar concentration and ethanol obtained from mono-culture fermentations on beta-glucosidase enzyme hydrolyzates

Yeast strain	Starting C-6 sugars (% g g^{-1})	**Emax* (% g g^{-1})	***TIMEmax (h)*
SC	32.8	20.2	24
CP IFM	30.32	23.8	12
CP NRRL	34.2	20.9	24

**Emax*= final ethanol yield, % g $g^{-1,}$
***TIME max*= time at which maximum ethanol yield was obtained.

Fermentations of pectinase enzyme hydrolyzates

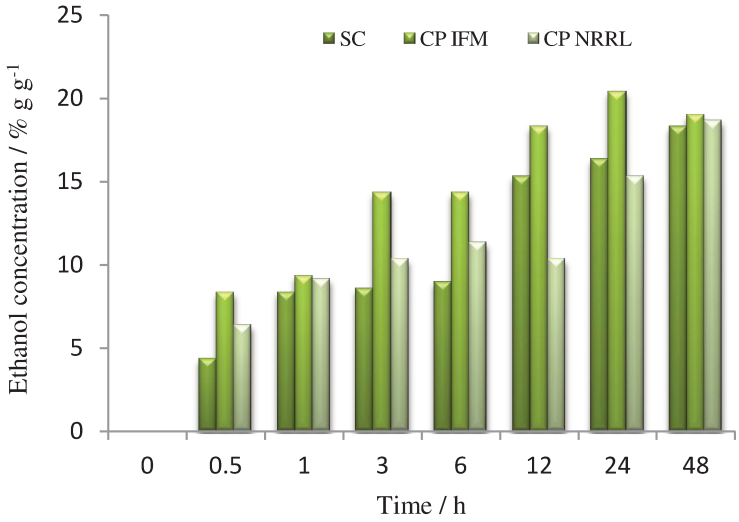


Figure A9. Comparison of mono-culture fermentations on pectinase enzyme (25 mg g^{-1} dry CPWO) hydrolyzates (where SC = *Saccharomyces cerevisiae*; CP IFM = *Candida parapsilosis* IFM48375; CP NRRL = *Candida parapsilosis* NRRL-12969).

Table A9. Comparative analysis of starting sugar concentration and ethanol obtained from mono-culture fermentations on pectinase enzyme hydrolyzates

Yeast strain	Starting C-6 sugars (% g g^{-1})	**Emax* (% g g^{-1})	***TIMEmax* (h)
SC	20	18.3	48
CP IFM	24.0	20.4	24
CP NRRL	19.3	18.65	48

**Emax*= final ethanol yield, % g g^{-1},
***TIME max*= time at which maximum ethanol yield was obtained.

Fermentations of cellulase enzyme hydrolyzates

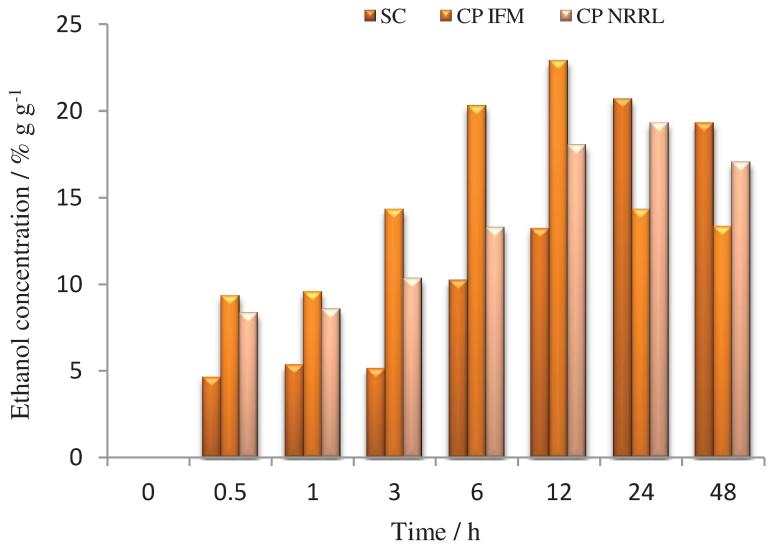


Figure A10. Comparison of mono-culture fermentations on cellulase enzyme (60 mg g^{-1} dry CPWO) hydrolyzates (where SC = *Saccharomyces cerevisiae*; CP IFM = *Candida parapsilosis* IFM48375; CP NRRL = *Candida parapsilosis* NRRL-12969).

Table A10. Comparative analysis of starting sugar concentration and ethanol obtained from mono-culture fermentations on cellulase enzyme hydrolyzates

Yeast strain	Starting C-6 sugars (% g g^{-1})	**Emax* (% g g^{-1})	***TIMEmax* (h)
SC	35.3	20.7	24
CP IFM	38.5	22.9	12
CP NRRL	37.2	19.32	24

**Emax*= final ethanol yield, % g g^{-1},
***TIME max*= time at which maximum ethanol yield was obtained.