TEZ ŞABLONU ONAY FORMU
THESIS TEMPLATE CONFIRMATION FORM

1. Şablonda verilen yerleşim ve boşluklar değiştirilmemelidir.
2. **Jüri tarihi** Başlık Sayfası, İmza Sayfası, Abstract ve Öz'de ilgili yerlere yazılmalıdır.
3. İmza sayfasında jüri üyelerinin unvanları doğru olarak yazılmalıdır. Tüm imzalar **mavi pilot kalemle** atılmalıdır.
4. **Disiplinlerarası** programlarda görevlendirilen öğretim üyeleri için jüri üyeleri kısmında tam zamanlı olarak çalıştıkları anabilim dalı başkanlığının ismi yazılmalıdır. Örneğin: bir öğretim üyesi Biyoteknoloji programında görev yapıyor ve biyoloji bölümünde tam zamanlı çalışıyorsa, İmza sayfasına biyoloji bölümü yazılmalıdır. İstisnai olarak, disiplinler arası program başkanı ve tez danışmanı için disiplinlerarası program adı yazılmalıdır.
5. Tezin **son sayfasının sayfa** numarası Abstract ve Öz'de ilgili yerlere yazılmalıdır.
6. Bütün chapterlar, referanslar, ekler ve CV sağ sayfada başlamalıdır. Bunun için **kesmeler** kullanılmıştır. **Kesmelerin kayması** fazladan boş sayfaların oluşmasına sebep olabilir. Bu gibi durumlarda paragraf (¶) işaretine tıklayarak kesmeleri görünür hale getirin ve yerlerini **kontrol edin**.
7. Figürler ve tablolar kenar boşluklarına taşmamalıdır.
8. Şablonda yorum olarak eklenen uyarılar dikkatle okunmalı ve uygulanmalıdır.
9. Tez yazdırılmadan önce PDF olarak kaydedilmelidir. Şablonda yorum olarak eklenen uyarılar PDF dokümanında yer almamalıdır.
10. **Bu form aracılığıyla oluşturulan PDF dosyası arkalı-önlü baskı alınarak tek bir spiralli cilt haline getirilmelidir.**
11. Spiralli hale getirilen tez taslağınızdaki ilgili alanları imzalandıktan sonra, Tez Juri Atama Formu ile birlikte bölüm sekreterliğine teslim edilmelidir.
12. Tez taslaklarının kontrol işlemleri tamamlandığında, bu durum öğrencilere METU uzantılı öğrenci e-posta adresleri aracılığıyla duyurulacaktır.
13. Tez yazım süreci ile ilgili herhangi bir sıkıntı yaşarsanız, **Sıkça Sorulan Sorular (SSS)** sayfamızı ziyaret ederek yaşadığınız sıkıntıyla ilgili bir çözüm bulabilirsiniz.

1. Do not change the spacing and placement in the template.
2. Write the **defense date** to the related places given on Title page, Approval page, Abstract and Öz.
3. Write the titles of the examining committee members correctly on Approval Page. **Blue ink** must be used for all signatures.
4. For faculty members working in **interdisciplinary programs**, the name of the department that they work full-time should be written on the Approval page. For example, if a faculty member staffs in the biotechnology program and works full-time in the biology department, the department of biology should be written on the approval page. Exceptionally, for the interdisciplinary program chair and your thesis supervisor, the interdisciplinary program name should be written.
5. Write **the page number of the last page** in the related places given on Abstract and Öz pages.
6. All chapters, references, appendices and CV must be started on the right page. **Section Breaks** were used for this. **Change in the placement** of section breaks can result in extra blank pages. In such cases, make the section breaks visible by clicking paragraph (¶) mark and **check their position.**
7. All figures and tables must be given inside the page. Nothing must appear in the margins.
8. All the warnings given on the comments section through the thesis template must be read and applied.
9. Save your thesis as pdf and Disable all the comments before taking the printout.
10. **Print two-sided the PDF file that you have created through this form and make a single spiral bound.**
11. Once you have signed the relevant fields in your thesis draft that you spiraled, submit it to the department secretary together with your Thesis Jury Assignment Form.
12. This will be announced to the students via their METU students e-mail addresses when the control of the thesis drafts has been completed.
13. If you have any problems with the thesis writing process, you may visit our **Frequently Asked Questions (FAQ)** page and find a solution to your problem.

☒ **Yukarıda bulunan tüm maddeleri okudum, anladım ve kabul ediyorum. / I have read, understand and accept all of the items above.**

Name : **Zeynep Başak**
Surname : **Çetin**
E-Mail : **e194384@metu.edu.tr**
Date : **30.06.2022**
Signature : _____ _____

PRODUCTION OF LACTIC ACID BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF HORSE CHESTNUT SHELL BY USING *Lactobacillus casei*

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ZEYNEP BAŞAK ÇETİN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
FOOD ENGINEERING

AUGUST 2022

Approval of the thesis:

PRODUCTION OF LACTIC ACID BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF HORSE CHESTNUT SHELL BY USING *Lactobacillus casei*

submitted by **ZEYNEP BAŞAK ÇETİN** in partial fulfillment of the requirements for the degree of **Master of Science in Food Engineering, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Serpil Şahin
Head of the Department, **Food Engineering** _____

Prof. Dr. Deniz Çekmecelioğlu
Supervisor, **Food Engineering, METU** _____

Prof. Dr. Haluk Hamamcı
Co-Supervisor, **Gastronomy and Culinary Arts, Alanya HEP University** _____

Examining Committee Members:

Assoc. Prof. Dr. Yeşim Soyer
Food Engineering, METU _____

Prof. Dr. Deniz Çekmecelioğlu
Food Engineering, METU _____

Prof. Dr. Haluk Hamamcı
Gastronomy and Culinary Arts, Alanya HEP University _____

Prof. Dr. Şebnem Harsa
Food Engineering, İzmir Institute of Technology University _____

Prof. Dr. Özlem Osmanağaoğlu
Biology, Ankara University _____

Date: 01.08.2022

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name Last name : Zeynep Başak Çetin

Signature :

ABSTRACT

PRODUCTION OF LACTIC ACID BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF HORSE CHESTNUT SHELL BY USING *Lactobacillus casei*

Çetin, Zeynep Başak
Master of Science, Food Engineering
Supervisor : Prof. Dr. Deniz Çekmecelioğlu
Co-Supervisor: Prof. Dr. Haluk Hamamcı

August 2022, 124 pages

Lactic acid is a multifunctional organic acid used as an acidifier, flavoring, or preservative in textile, food, pharmaceutical, and cosmetic industries. Bacterial fermentation is the most preferred method for lactic acid production, but the often-used refined sugar increases the cost of production. For this reason, a new environmentally friendly, sustainable, and low-cost carbon source is being researched.

The seeds of horse chestnut are mostly used in the pharmaceutical industry for antioxidant production, while the shells are inert. In the present study, lactic acid production processes and efficiency were investigated from pre-treated horse chestnut shells as a promising raw material. In addition to separate hydrolysis and fermentation, simultaneous saccharification and fermentation technologies, a new method has been established by combining the advantages of these two processes. In the developed process, two bioreactors, one for enzymatic hydrolysis and the other for fermentation, were connected by hoses and the medium in the reactors was circulated through a pump. Thus, the optimum pH and temperature conditions

required for the enzyme in the reactor used for enzymatic hydrolysis and for the microorganism in the reactor used for fermentation were provided. In addition to the effective operation of the enzyme and microorganism, the medium circulation between the reactors prevented the decrease in enzyme activity due to irreversible product inhibition. In the experiment, lactic acid titers and yields were measured by changing the temperature and pH parameters. After enzymatic hydrolysis, the total sugar in the hydrolysate was converted to 18.25 g L^{-1} lactic acid equivalent to a yield of 0.18 g g^{-1} dry horse chestnut shell by *L. casei* in separate hydrolysis and fermentation (SHF). In the simultaneous saccharification and fermentation process, using the same parameters, 6.45 g L^{-1} lactic acid concentration was produced, while the yield was 0.065 g g^{-1} dry horse chestnut shell. The highest lactic acid titer (42.1 g L^{-1}) and yield (0.42 g g^{-1} dry horse chestnut shell) were obtained from the simultaneous saccharification and fermentation with a dual bioreactor process (SSF2). Based on the results obtained from this study, the simultaneous saccharification and fermentation with dual bioreactor method for microorganisms and enzymes, whose optimal conditions are not close to each other, gave promising results in obtaining valuable end products from lignocellulosic raw materials such as horse chestnut shells.

Keywords: Horse Chestnut Shell, Enzymatic Hydrolysis, Lactic Acid, Simultaneous Saccharification and Fermentation

ÖZ

AT KESTANESİ KABUĞUNDAN EŞ ZAMANLI SAKKARİFİKASYON VE FERMANTASYON YAPARAK *Lactobacillus casei* İLE LAKTİK ASİT ÜRETİMİ

Çetin, Zeynep Başak
Yüksek Lisans, Gıda Mühendisliği
Tez Yöneticisi: Prof. Dr. Deniz Çekmecelioğlu
Ortak Tez Yöneticisi: Prof. Dr. Haluk Hamamcı

Ağustos, 124 sayfa

Laktik asit, tekstil, gıda, ilaç ve kozmetik endüstrilerinde asitlendirici, aroma verici veya koruyucu olarak kullanılan çok işlevli bir organik asittir. Laktik asit üretimi için çoğunlukla tercih edilen yöntem bakteriyel fermantasyondur ancak sıklıkla kullanılan rafine şeker üretim maliyetini arttırmaktadır. Bu nedenle çevre dostu, sürdürülebilir ve düşük maliyetli yeni bir karbon kaynağı araştırılmaktadır.

At kestanesinin tohumları çoğunlukla ilaç ve antioksidan üretiminde kullanılır, kabukları ise atıldır. Mevcut çalışmada umut vaat edici hammadde olarak ön işleme tabi tutulmuş at kestanesi kabuğundan laktik asit üretim prosesleri ve verimliliği araştırılmıştır. Ayrı hidroliz ve fermantasyon ve eş zamanlı sakkarifikasyon ve fermantasyon teknolojilerinin yanı sıra, bu iki prosesin avantajlarını bir araya getiren yeni bir yöntem geliştirilmiştir. Geliştirilen yöntemde iki biyoreaktör, biri enzimatik hidroliz diğeri fermantasyon için, hortumlar aracılığıyla birbirlerine bağlanmış ve reaktörlerdeki besiyerleri pompa aracılığıyla sirküle edilmiştir. Böylece, enzimatik hidroliz için kullanılan reaktörde enzim için, fermantasyon için kullanılan reaktörde de mikroorganizma için gerekli optimal pH ve sıcaklık koşulları sağlanmıştır. Enzim

ve mikroorganizmanın efektif çalışmasına ek olarak, reaktörler arasındaki ortam sirkülasyonu enzim aktivitesinde geri dönüşü olmayan ürün inhibisyonu nedeniyle düşüşü önlemiştir. Deneyde sıcaklık ve pH parametreleri değiştirilirek elde edilen laktik asit derişimi ve verimi kıyaslanmıştır. Enzimatik hidrolizden sonra, hidrolizattaki toplam şeker, ayrı hidroliz ve fermantasyonda *L. casei* aracılığıyla 18.25 g L^{-1} laktik aside dönüştürüldü ve verim 0.0.18 g g^{-1} (kuru at kestanesi kabuğu) olarak hesaplandı. Aynı parametrelerin kullanıldığı eş zamanlı sakkarifikasyon ve fermantasyon prosesinde 6.45 g L^{-1} laktik asit elde edilirken verim 0.065 g g^{-1} olarak saptandı, en yüksek laktik asit titresi (42.1 g L^{-1}) ve verimi (0.42 g g^{-1}) ise iki biyoreaktörlü eş zamanlı sakkarifikasyon ve fermantasyon prosesinde oldu. Bu çalışmadan elde edilen sonuçlara göre, at kestanesi kabuğu gibi lignoselülozik hammaddelerden değerli son ürün elde edilmesinde, optimal koşulları birbirine yakın olmayan mikroorganizma ve enzim için iki biyoreaktörlü eş zamanlı sakkarifikasyon ve fermantasyon yöntemi umut vaat etmektedir.

Anahtar Kelimeler: At Kestanesi Kabuğu, Enzimatik Hidroliz, Laktik Asit, Eşzamanlı Sakkarifikasyon ve Fermantasyon

Dedicated to my family;

ACKNOWLEDGMENTS

I would like to express deepest gratitude and sincere regards to my supervisor, Prof. Dr. Haluk Hamamcı for giving me his valuable support. His knowledge and experience have been my guide not only in my thesis but also in every aspect of my life. I feel lucky for being his student.

I would like to give special thanks to my co-advisor Prof. Dr. Deniz Çekmecelioğlu for his support, interest, and valuable time.

I would like to thank my friends Aylin Özle, Alara Batıgün, Berfin Özışık, Burak Selçuk, Deniz Günalan, Emre Köse, Gizem Özcan, Imran Fakhar, Melis Erdir, Nilufer Kılınç and Utku Uysal for making all the hardships I have been through bearable and for being a good friendship, help, and source of motivation.

I would also like to express my gratitude to my dear parents Canan-İbrahim Oral, my sister Cemre Zeren Oral, my grandmother Safiye Oral and my grandfather Erdoğan Oral. They brought me to this day with their love, support, and patience. They are the best family anyone could imagine.

Last but not least, I would like to express my endless gratitude to Süleyman Çetin, who brought pure happiness to my life with his unlimited love and support.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS

Ara	Arabinose
ASA-BG	ASA-Biogazyme 2x
C	Carbon
°C	Degree Celcius
Ca^{2+}	Calcium (II) ion
D	D-Lactic acid
DL	DL-Lactic acid
DP	Degree of polymerization
Eqn	Equation
FDA	U.S. Food and Drug Administration
FPU	Filter paper unit
g	Gram
Gal	Galactose
Glu	Glucose
GRAS	Generally recognized as safe
h	Hour
HCS	Horse chestnut shell
kg	Kilogram
kt	Kiloton
L	Liter
LA	Lactic acid
LAB	Lactic acid bacteria
m	Meter
M	Molar
Man	Mannose
µL	Microliter

min	Minute
mg	Milligram
mL	Milliliter
mm	Millimeter
Mn^{2+}	Manganese (II) ion
Mn^{3+}	Manganese (III) ion
MRS	De Man Rogosa and Shape
nm	Nanometer
OD	Optical density
p	p-value
PLA	Polylactic acid
Ref	References
RH	Relative humidity
Rha	Rhamnose
rpm	Rounds per minute
s	Second
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
SSF2	Simultaneous saccharification and fermentation with dual bioreactor system
U	Unit of enzyme's catalytic activity
US$	United State dollar
v	Volume
w	Weight
Xyl	Xylose

CHAPTER 1

INTRODUCTION

Lactic acid, which was first noticed in sour milk by the Swedish chemist Karl Wilhelm Scheele in 1780 and added to the literature, took its place in the industry after Louis Pasteur declared that it could be produced by lactic acid bacteria (Alsaheb et al., 2015). Today, lactic acid (2-hydroxy propionic acid) is one of the most demanded organic acids with its GRAS status (the term indicating that it is safe to use in food), especially in the food industry, as well as in cosmetics, pharmaceutical, and chemical industries (Gobbetti & Minervini, 2014). Examples of lactic acid usage are as preservative and acidifier in beverages, softener, and emulsifier in pastries, or for elasticity in the leather industry. Apart from the examples given, lactic acid is also used in different fields (Cengiz, 2002; John et al., 2009). The production of polylactic acid (PLA), which contributes to the protection of the environment by biodegrading in addition to being used as an auxiliary in the specified industries, is increasing day by day to replace petroleum-based plastics (Abdel-Rahman et al., 2011).

Lactic acid can be produced by chemical methods or by microbial fermentation (Sreenath et al., 2001). The most widely used method in the chemical synthesis of lactic acid is the hydrolysis of lactonitrile with the help of strong acid (John et al., 2009). However, since lactonitrile is a petroleum-derived substance and is produced from a non-renewable resource, its chemical synthesis cannot meet the global energy demand. Furthermore, considering the damage it causes to the environment, it has led to the production of lactic acid through microbial fermentation (Marques et al., 2008). In comparison to the chemical method, the microbial fermentation method

allows the use of cheap raw materials, less energy consumption, and the opportunity to obtain optically pure D- or L-lactic acid (Abdel-Rahman et al., 2011).

In recent years, the importance given to the recycling of biological wastes has been increasing to obtain cheap raw materials. Among the reusable resources, especially lignocellulosic biomass is promising. Lignocellulosic biomass, generally composed of cellulose, hemicellulose, and lignin, is the most common renewable carbon source in the world, with an annual production of 181.5 billion tons (Paul & Dutta, 2018). In addition to being so widespread, easily accessible, and inexpensive, its use as a raw material contributes to reducing environmental contamination and pollution. Although it is the most common biomass in the world, its approximate consumption for feed or energy is limited to 7 billion tons (Dahmen et al., 2019). The main sources of lignocellulosic biomass can be listed as agricultural wastes, urban wastes, industrial wastes, energy crops, and forest residues (Y. Sun & Cheng, 2002).

Horse chestnut (*Aesculus hippocastanum L.*) is found in many countries in Europe, Asia, and America, thanks to its extraordinary ability to withstand harsh environmental conditions (Rafiq et al., 2016; Šedivá et al., 2013). In addition to its durability, its attractive appearance makes it preferred ornamental tree in parks, plots, and roadsides (Gullón et al., 2020). Horse chestnut seeds contain toxic substances called aesculin, which prevent blood from clotting (Underland et al., 2012). Therefore, consuming horse chestnut seeds creates a toxic effect on humans. After proper treatment to remove toxic substances, the seeds are still unfitted for human consumption. However, the substance aescin obtained from processed horse chestnut seeds can be used in pharmaceutical industry for various therapeutic properties, namely treatment of chronic venous insufficiency associated with inflammatory diseases such as arthritis, rheumatism, tendinitis, sports injuries, and skin inflammation, treatment of bladder and gastrointestinal diseases, and antimicrobial capabilities (Felipe et al., 2013; Kapusta et al., 2007; Küçükkurt et al., 2010). In addition to these beneficial health effects, horse chestnut seeds contain functional active compounds such as saponins, flavonoids, proanthocyanidins, coumarins, and essential oils (Morales et al., 2018). After the horse chestnut seeds are used in the

pharmaceutical industry, the remaining shells can be considered biodegradable lignocellulosic waste.

Although lignocellulosic biomass is a widely available or inexpensive raw material, it is unfortunately not suitable for use directly in microbial fermentation. Since it contains lignin in addition to cellulose and hemicellulose, it is necessary to subject the lignin structure to physical, chemical, and microbiological pretreatments for complete transformation. In this way, the surface area is increased, the degree of crystallinity decreases, and a porous structure is formed, which increases the population of cellulase and hemicellulase enzymes, enabling the conversion of lignocellulosic materials into sugar that can be used in fermentation (Bajpai, 2019). However, by-products formed during the enzymatic degradation of lignocellulosic materials may adversely affect the activity of enzymes. As a solution, increasing the amount of enzyme, removal of by-products from the environment by filtration techniques, increasing the concentration of β-glucosidases to provide cellobiose transformation or simultaneous saccharification and fermentation (SSF) are known ways (Ricardo Soccol et al., 2011). SSF prevents product inhibition by rapidly consuming the hydrolyzed sugar by microorganisms during fermentation (Abdel-Rahman et al., 2011). For this reason, its yield is higher than the traditional method of separated hydrolysis and fermentation (SHF). In addition, the SSF method is also economically advantageous as the process takes place in a single bioreactor (Kádár et al., 2004).

The objective of this study is to evaluate the production processes and efficiency of lactic acid with *Lactobacillus casei* from pre-treated horse chestnut shells. Besides, separate hydrolysis and fermentation, and simultaneous saccharification and fermentation technologies, a new method has been established by combining the advantages of these two processes. Optimum conditions for the highest lactic acid yield were investigated by conducting experiments with different temperatures and pH values in these three processes.

CHAPTER 2

LITERATURE REVIEW

2.1 Lignocellulosic Biomass

Biomass is generally defined as an organic matter from living or recently living organisms produced from atmospheric carbon dioxide and water using sunlight through photosynthesis. (Clarke & Preto, 2011). The vast majority of biomass consists of plant or plant-based substances. Since they are composed of cellulose, hemicellulose, and lignin, they can be specifically referred to as lignocellulosic biomass (O. V. Singh & Harvey, 2010).

Lignocellulosic materials, which were used for energy in the past, could not meet the increasing energy demand and left their place to fossil fuels, but due to environmental concerns caused by the accumulation of carbon dioxide in the atmosphere, increasing prices in the extraction of these resources and fossil fuels being a non-renewable energy source, as a result of the researches, it has taken its place on the stage of history again as one of the promising candidates that can be used instead of non-renewable energy sources (Eiteman & Ramalingam, 2015; Gandla et al., 2018).

It has been observed that the annual production of many lignocellulosic materials, such as trees, grasses, and residues after agricultural harvest, reaches 181.5 billion tons worldwide (Paul & Dutta, 2018). Of these substances, only 7 billion tons are used for energy needs such as heating or cooking, as animal feed, or in the paper industry to produce fiber (Szambelan et al., 2018). Lignocellulosic materials are one of the materials in the world with a high recyclability potential(Qian, 2013). Thanks

to this feature, it provides economic convenience in the production of materials such as biofuel, organic acid, or biodegradable and thermoplastic polymers.

2.1.1 Components of the Lignocellulosic Biomass

Lignocellulosic materials consist of cellulose, hemicellulose, and lignin, which are the three main biopolymers, together with a small amount of protein, pectin, inorganic compounds, extractives, and ash (Okolie et al., 2021). The amount of cellulose, hemicellulose and lignin contained in the lignocellulosic material may vary according to the type of plant, as well as depending on the plant's tissue or growing conditions (N. Sun et al., 2011). In general, when the amounts are considered, it varies between cellulose (40-50%), hemicellulose (20-40%), and lignin (18-35%) (Y. Sun & Cheng, 2002).

The percentage distribution of common lignocellulosic substances in terms of cellulose, hemicellulose, and lignin can be seen in the following Table 2.1. As can be seen, some lignocellulosic materials contain a high amount of cellulose, just like flax, while others are rich in lignin, like a walnut shell.

Table 2.1 Components of some lignocellulosic biomasses

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Albizzia wood	58.3	8.1	33.2	(Okolie et al., 2021)
Almond shell	50.7	28.9	20.4	(Demirbaş, 2002)
Aspen wood	60.7	19.1	14.8	(Shen et al., 2009)
Barley straw	32.5	25.7	23.0	(Naik et al., 2010)
Bamboo	26.0-43.0	30.0	21.0-31.0	(J. Yang et al., 2019)
Birch Branches	33.3	23.4	20.8	(Y. Lee et al., 2013)
Canola straw	42.4	16.4	14.2	(Adapa et al., 2009)
Chestnut shell	38.1	16.7	23.2	(Morales et al., 2018)
Coconut shell	20.0	48.8	30.0	(Okolie et al., 2021)
Coffee pulp	35.0	46.3	18.8	(Sánchez, 2009)
Corn cob	45.0	35.0	15.0	(Prasad et al., 2007)
Cornstalk	42.7	23.6	17.5	(J. Yang et al., 2019)
Esparto grass	33.0-38.0	27.0-32.0	17.0-19.0	(Sánchez, 2009)
Eucalyptus	48.0	14.0	29.0	(Qian, 2013)
Flax	71.0	18.6-20.6	2.2	(J. Yang et al., 2019)
Flax straw	28.7	26.8	22.5	(Naik et al., 2010)
Grasses	25.0-40.0	35.0-50.0	10.0-30.0	(Bajpai, 2019)

Table 2.1 (continued)

Hazelnut shell	26.8	30.4	42.9	(Demirbaş, 2002)
Hardwood	40.0-55.0	24.0-40.0	18.0-25.0	(Bajpai, 2019)
Hemp	68.0	15.0	10.0	(J. Yang et al., 2019)
Hemp hurds	44.5	32.8	21.0	(Stevulova et al., 2014)
Jute	41.0-48.0	21.0-24.0	18.0-22.0	(J. Yang et al., 2019)
Miscanthus straw	47.5	20.9	9.4	(Butler et al., 2013)
Oak	53.9	29.0	12.9	(Shen et al., 2009)
Oak straw	37.6	23.3	12.9	(Aqsha et al., 2017)
Oil palm	65.0	-	29.0	(J. Yang et al., 2019)
Olive husk	24.0	23.6	20.4	(Okolie et al., 2021)
Pine branches	32.0	32.0	21.5	(J. Yang et al., 2019)
Pinecone	32.7	37.6	24.9	(Okolie et al., 2021)
Pinewood	38.8	23.6	20.4	(Okolie et al., 2021)
Poplar	43.8	14.8	29.1	(Kumar et al., 2009)
Rice rusk	35-45	19-25	20.0	(J. Yang et al., 2019)
Rice straw	32.1	24.0	18.0	(Howard et al., 2003)
Salix straw	43.8	14.6	22.5	(Butler et al., 2013)

Table 2.1 (continued)

Sisal	65.0	12.0	9.9	(J. Yang et al., 2019)
Softwood	45.0-50.0	25.0-35.0	25.0-35.0	(Bajpai, 2019)
Spruce straw	49.4	4.7	27.7	(Butler et al., 2013)
Spruce branches	29.0	30.0	22.8	(J. Yang et al., 2019)
Sugarcane bagasse	42.4	35.3	20.8	(Esquivel-Hernández et al., 2022)
Sunflower shell	48.4	34.6	17.0	(Demirbaş, 2002)
Sweet sorghum	35.0	17.0	23.0	(Qian, 2013)
Switchgrass	30.0-50.0	10.0-40.0	5.0-20.0	(McKendry, 2002)
Timothy grass	34.2	30.1	18.1	(Okolie et al., 2021)
Walnut shell	25.6	22.1	52.3	(Demirbaş, 2002)
Wheat straw	39.1	24.1	16.3	(Okolie et al., 2021)

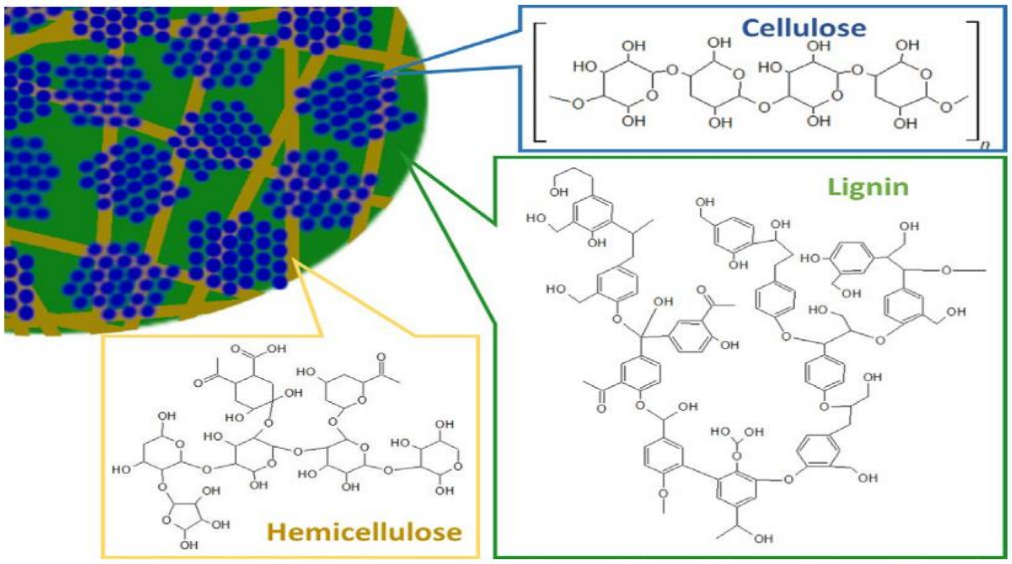


Figure 2.1 Representation of cellulose hemicellulose and lignin, the three main components of the microfibril in the cell wall of lignocellulosic materials (Dahmen et al., 2019)

2.1.1.1 Cellulose

Cellulose is considered one of the most important renewable resources in the world so its annual production is determined as 7.5×10^{10} tons (Okolie et al., 2021). It is an organic compound found as a structural element in plants, many algae, oomycetes, and some bacteria (Yousuf et al., 2020).

Cellulose is a polymer of glucose with the formula $(C_6H_{10}O_5)n$ where n expresses the degree of polymerization (DP) (Lisa Axelsson et al., 2012). DP is the number of glucose units in the molecule and has an important role in determining the properties of the polymer. Repeating units of β-D-glucopyranose are connected linearly via β-(1-4) glycosidic bonds and the β configuration forms the stretched chain structure. Then, through hydrogen bonds, a flat sheet structure is formed. In addition, van der Waals forces are present in the bond structure of the compound (J. Yang et al., 2019).

Units of glucose monomer in cellulose can vary between 7000 to 15000 so it is a long-chain polysaccharide (Moon et al., 2011).

Cellulose turns into fibril structures by hydrogen bonding of the monomer unit in the cellulose chain with the monomer unit in the neighboring chain, and with further aggregation, fibrils are ordered to a large unit of microfibrils and then into cellulose fibers (Habibi et al., 2010; Kumar et al., 2009). As a result, a stable configuration is formed. During the formation of this structure, the degree of crystallinity is determined by the degree of hydrogen bonding between the chains (Mäki-Arvela et al., 2012). Crystalline regions are less susceptible to hydrolysis than amorphous (Moon et al., 2011). Moreover, the surface structure and degree of polymerization affect hydrolysis of the cellulose (Kumar et al., 2009).

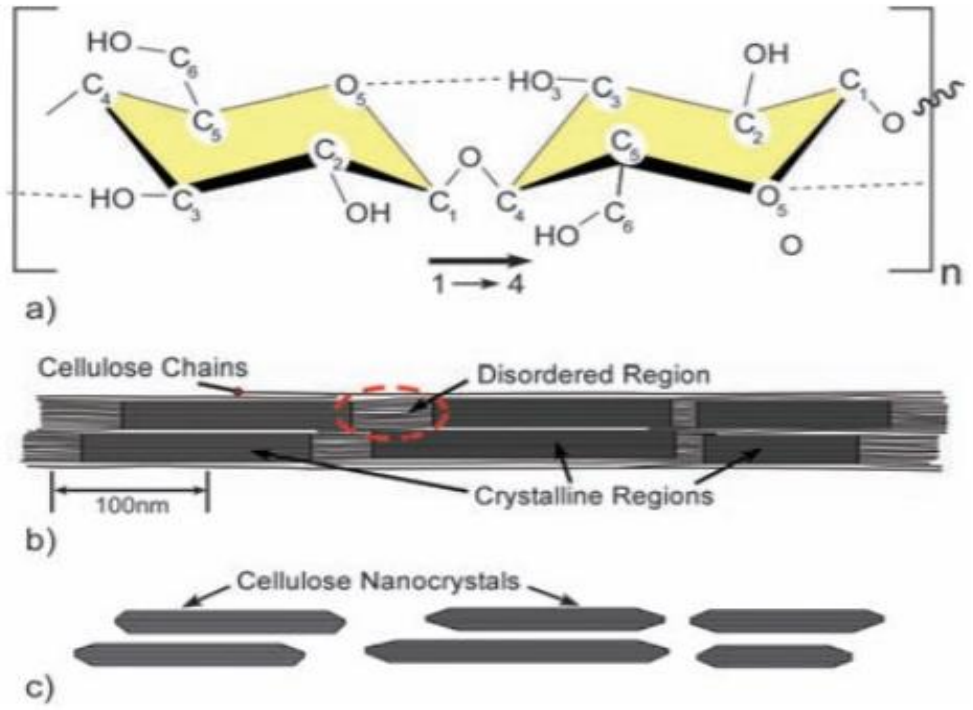


Figure 2.2 Schematic of (a) cellulose unit link β-1,4-glycosidic bonds linearly, (b) crystalline and amorphous regions of a cellulose microfibril, and (c) cellulose nanocrystals after hydrolysis (Moon et al., 2011)

Cellulose is insoluble in water or many solvents, unlike glucose, which is its building block, thanks to its crystalline structure, crystallinity, and DP. While this feature is important in dealing with microbial spoilage, it also makes diffusion of other decomposition products difficult (Brandt et al., 2013; Mudgil, 2017).

2.1.1.2 Hemicellulose

Hemicellulose is another biopolymer found in lignocellulosic materials, consisting of mixtures of sugar monosaccharides with five to six carbons, with short lateral branches either homopolymer or heteropolymer in its structure. The pentose sugars in hemicellulose are xylose, rhamnose, and arabinose, while the hexose sugars are glucose, mannose, and galactose. It also contains 4-o-methylglucuronic, D-glucuronic, and D-galacturonic acids (Balat, 2011; Okolie et al., 2021).

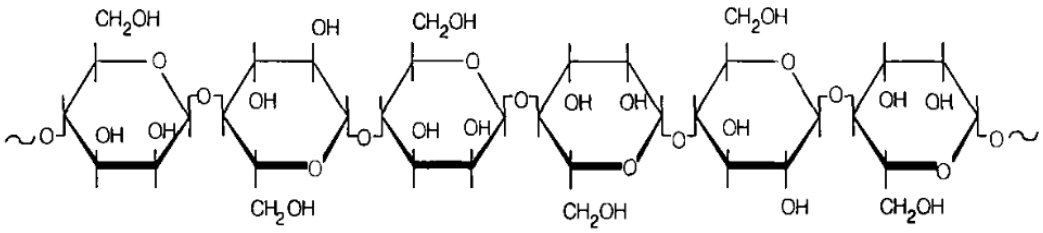


Figure 2.3 The chemical structure of glucomannan in hardwood (Puls, 1997)

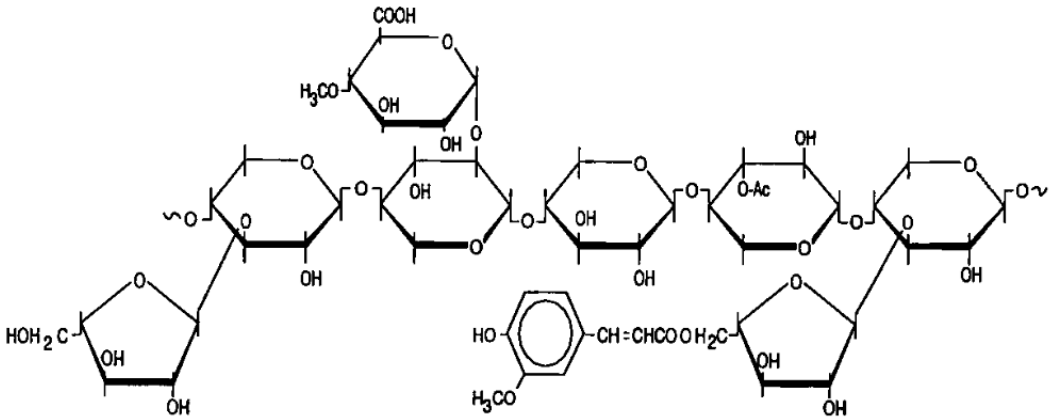


Figure 2.4 The chemical structure of arabinoxylan in grasses (Puls, 1997)

Hemicellulose is composed of approximately 500-3000 monomers, linked by β-(1,4)-glycosidic bonds or sometimes β-(1,3)-glycosidic bonds (J. Yang et al., 2019). Unlike cellulose chains, the polymer structure of hemicellulose does not agglomerate even if they form a crystalline structure (Kumar et al., 2009). In addition, its degree of polymerization range has changed between 50-200 so, it is amorphous in structure and can be easily hydrolyzed (Gibson, 2012; Kumar et al., 2009). However, having acetylated side chains in its structure causes it to have a lower molecular weight (Brandt et al., 2013; Kumar et al., 2009)

The content of hemicellulose varies according to the structure and type of biomass. A plant may contain more than one type of hemicellulose, and even the amount and type of hemicellulose may vary in different parts. Hemicellulose, rich in xylan, is more common in hardwoods and cereal, while hemicellulose, rich in glucomannan, is observed more in softwoods (R. Sun, 2010). Table 2.2 shows the monomers of hemicellulose found in some plants.

Table 2.2 Amount of hemicellulose monosaccharide hemicellulose in some plants

Plant Source	Xyl (w/w)	Man (w/w)	Ara (w/w)	Glu (w/w)	Gal (w/w)	Rha (w/w)	Ref.
Aspen	2.0	55.0	1.0	41.0	1.0	-	(Telemann et al., 2003)
Barley	66.1	0.5	10.6	7.6	3.7	0.4	(R. Sun, 2010)
Birch	1.0	68.0	2.0	28.0	1.0	-	(Telemann et al., 2003)
Corn stover	89.0	-	5.8	4.2	0.3	-	(Naran et al., 2009)
Date palm leaf	75.0	-	6.3	0.3	1.0	-	(Bendahou et al., 2007)
Larch	-	1.0	16.0	1.0	79.0	-	(Willför et al., 2002)
Maize	62.7	0.8	15.2	6.3	4.7	0.5	(R. Sun, 2010)
Passion fruit rind	29.0	9.0	1.0	42.0	15.0	-	(Placket t, 2011)
Pine	-	7.0	14.0	3.0	69.0	-	(Willför et al., 2002)
Oat	68.3	0.3	11.2	6.1	3.6	0.4	(R. Sun, 2010)

Table 2.2 (continued)

Rice	56.3	-	14.9	22.3	4.8	0.6	(R. Sun, 2010)
Rye	65.3	0.3	11.2	6.1	3.3	0.5	(R. Sun, 2010)
Spruce	-	30.0	11.0	8.0	35.0	-	(Willför et al., 2002)
Sugarcane bagasse	55.0	1.5	13.0	28.0	2.6	-	(Placket, 2011)
Wheat	60.5	0.4	13.8	9.8	4.5	0.8	(R. Sun, 2010)
Wheat straw	23.8	0.1	0.2	1.1	0.2	-	(Puls, 1997)

2.1.1.3 Lignin

Lignin is the third major component found in lignocellulosic materials. It has a complex structure due to the random and non-linear bonding of phenolic monomers to each other via ester bonds (Reinoso et al., 2018). It consists of three main monomers, which can be seen schematically in Figure 2.6, varying according to plant species, these are coniferyl alcohol (guaiacyl propanol), p-coumaryl alcohol (p-hydroxyphenyl propanol), and sinapyl alcohol (syringyl alcohol). These phenolic substances are linked with alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds. Also, lignin forms covalent bonds with hemicellulose, and stable ether bonds with arabinose and galactose found in xylan and mannan form a lignin carbohydrate complex (Kristensen et al., 2009; Kumar et al., 2009).

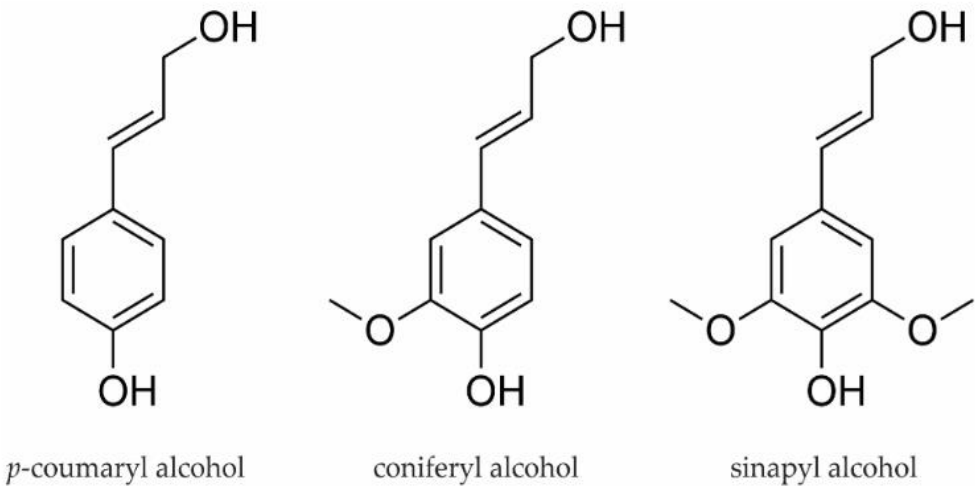


Figure 2.5 The chemical structure of the main building blocks of lignin (Grzybek et al., 2021)

Herbaceous plants such as grasses generally have the lowest lignin content (17-24%), while softwoods (25-35%) have the highest content. The content of lignin varies according to the plant species, for example, in softwoods, up to 90% of the lignin structure consists of coniferyl alcohol, and in the structure of hardwood lignin, sinapyl and coniferyl alcohol are formed at different rates (Harmsen et al., 2010). This difference in the composition of the lignin structure is highly effective in degradation.

Thanks to the cross-linking phenolic polymers in its structure, it has a rigid and hydrophobic structure. As a result of this feature, it is found in the primary cell wall by providing structural support, impermeability, and resistance against microbial attack (Kumar et al., 2009; Mäki-Arvela et al., 2012).

2.1.2 Types of Lignocellulosic Biomass

Lignocellulosic biomass raw materials can be catagorized as agricultural wastes, forest residues, food wastes and wastes left after industrial use. These materials are the most promising biomass due to their relatively low cost and easy availability. Table 2.3 shows examples of types of lignocellulosic materials.

Table 2.3 Types of lignocellulosic biomass (Cai et al., 2017)

Sector	Type	Examples
Agriculture	Energy crops	Switchgrass, reed, rapeseed, sugarcane, corn
	Crop waste	Leaves, stover, straws
Forest	Forest biomass	Cedar, poplar, willow, eucalyptus
	Forest waste	Barks, wood chips, sawdust, wood blocks
Industry	Agro-food waste	Rice husk, sugarcane bagasse, corn cob
	Wood-industry waste	Sawdust from a sawmill, recycled newspaper
Other	Lignocellulosic waste	Residues from parks, gardens

2.1.2.1 Horse Chestnut

Horse chestnut (*Aesculus hippocastanum L.*) is grown in many regions of the world. Thanks to its ability to withstand harsh environmental conditions, is a popular species from Southeast Europe to North America, from Asia, especially in Nepal, India, Pakistan, and Japan to Canada, and from New Zealand to Great Britain (Gullón et al., 2020; Rafiq et al., 2016; Šedivá et al., 2013). It is mostly seen as an ornamental tree in parks, squares, roadsides, and gardens, because of its durable structure, rapid

growth feature, regularly shaped crown leaves, and attractive appearance of thorny fruits emerging from white cluster flowers (Weryszko-Chmielewska & Chwil, 2017).

Horse chestnut seeds have been used for centuries in both modern medicine and alternative treatment methods. As a folk medicine in Turkey, horse chestnut was prepared as a tea to reduce kidney stones (Küçükkurt et al., 2010). While it was used as a fever reducer in the 18th century, its anti-hemorrhoidal property was discovered and started to be used in the 19th century (Kapusta et al., 2007). Although it is mostly used for chronic venous insufficiency today, it is widely used in the treatment of rheumatoid arthritis, sports injuries, and other inflammatory disorders such as inflammation of the skin (Küçükkurt et al., 2010; Margină et al., 2015). Moreover, it has been discovered that it helps in the treatment of bladder and gastrointestinal problems and has antiaging and antimicrobial properties (Felipe et al., 2013). It has such therapeutic properties because it is rich in various functional active compounds such as saponins, flavonoids, coumarins, and essential oils. Triterpene saponin glycosides such as aescin contained in horse chestnuts have anti-inflammatory, anti-edematous, and vasoprotective properties and flavonoids such as 7-O-methylkaempferol have antifungal properties (Chen et al., 2007; Čukanović et al., 2020; Kurkin et al., 2020). Apart from horse chestnut seeds, which are traditionally used in the pharmaceutical industry, horse chestnut shells, which are wasted every year, offer a great opportunity given the growing interest worldwide to find new and inexpensive raw materials to produce value-added compounds.

2.2 Pretreatment Methods

As mentioned earlier, lignocellulosic biomass is the most abundant and promising source of carbohydrates used in fermentation. However, due to its complex structure, it cannot be used directly by microorganisms. Deterioration of the crystal structure of cellulose and physical bonds between lignin and cell wall by physical, chemical, or biochemical pretreatment methods increases the surface area, porous structure,

and homogeneity, thus increasing the enzyme-substrate interaction and providing effective enzymatic hydrolysis. Pretreatment methods not only increase the effectiveness, but also contribute to the deterioration of the structure of toxic substances that may be harmful during the fermentation process, reduce the processing cost due to the use of less enzyme amount, and obtain a purer high-value product (Qian, 2013). Figure 2.7 shows the effect of pretreatment on lignocellulosic biomass.

Pre-treatment methods can be used separately or together, but it should be noted that a good pre-treatment method should not increase the cost, can be used in much lignocellulosic biomass, should not create by-products that will affect the next process, and should not cause loss by causing deterioration in carbohydrates (Bajpai, 2019). Pretreatment methods are shown in Figure 2.6, and their effects on lignocellulosic biomass are shown in Figure 2.7.

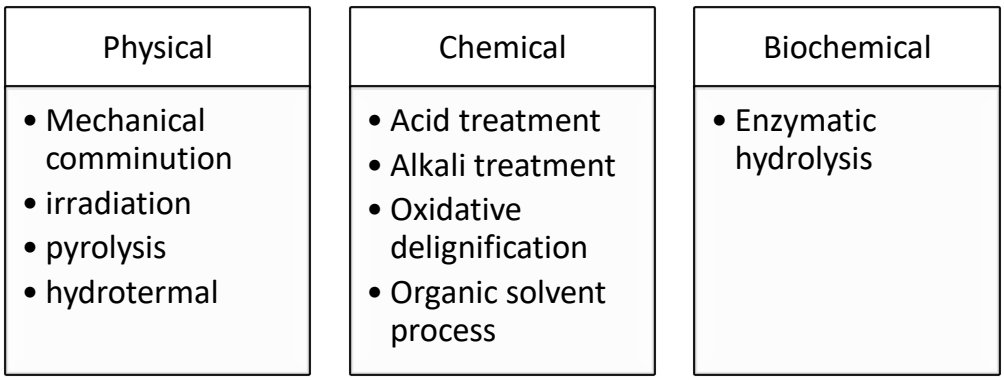


Figure 2.6 List of pretreatment methods of lignocellulosic biomass

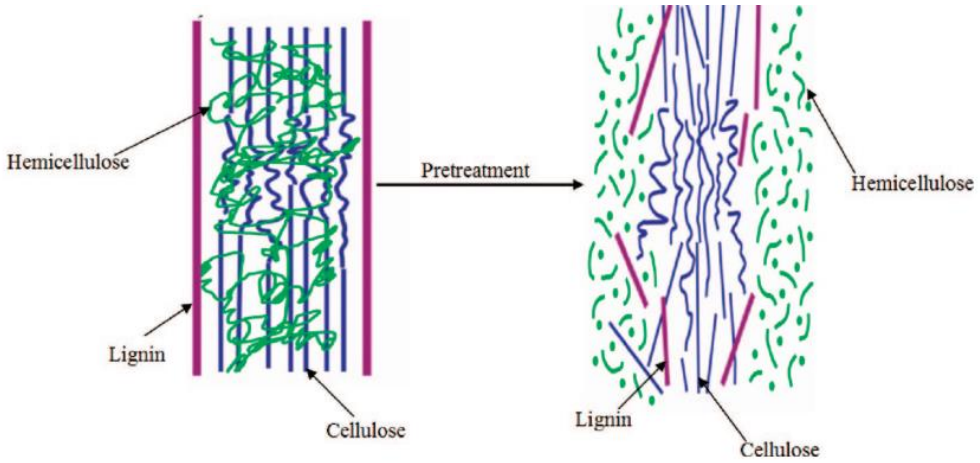


Figure 2.7 Effect of pretreatment on the lignocellulosic biomass (Kumar et al., 2009)

2.2.1 Physical Pretreatment Methods

Physical pretreatment methods provide structural deterioration in lignocellulosic materials, without making little or any chemical changes (Waldron, 2010). Physical pretreatment methods are mechanical comminution, irradiation (Yousuf et al., 2020) pyrolysis (Bajpai, 2019), and hydrothermal (Waldron, 2010).

In mechanical comminution processes, since the particle size is reduced by dry, wet, or vibratory ball milling, chipping, and shredding, the degree of polymerization and crystal structure of cellulose is reduced and the surface area accessible by the enzyme is increased (Bajpai, 2019; Waldron, 2010; Yousuf et al., 2020). Depending on the method and the structure of the lignocellulosic biomass, the particle size changes, and its effectiveness differs. For example, according to the research of Alvo and Belkacemi (1997), it was observed that the hydrolysis efficiency of alfalfa pretreated in the roller mill was increased by almost 25% when compared to the unpretreated one, while the same treatment changed the yield of timothy herb only 5%. Moreover, it is an important advantage that no inhibitor is formed during these processes.

Pyrolysis is another physical pretreatment method (Kumar et al., 2009). Depending on the type of lignocellulosic material, it is increased above 200-400°C, and decomposition is achieved. During processing, hemicellulose decomposes to xylan at 250-350°C, while cellulose decomposes to glucose, usually at 320-400 °C. The amount of heat required for lignin, which has the most stable structure, varies between 350-500 °C (Yogalakshmi et al., 2022). The remaining biochar after heat treatment can be used in fermentation, fertilizer, or electrochemical energy storage since it contains 65-90% carbon content (Akom, et al., 2020; Y. Lee et al., 2013).

Gamma-ray, ultrasound, microwave, electron beam, and ultraviolet light are other methods that provide physical pretreatment by giving lignocellulosic biomass irradiation. It allows most of the cellulose and hemicellulose to depolymerize, while lignin is partially depolymerized due to the destruction of the structure of the cell wall (Bajpai, 2019; Yousuf et al., 2020).

In the hydrothermal method, either steam (0.7 - 4.8 MPa and 160–240 °C) (Agbor et al., 2011) or liquid water (180-230 °C) (Mosier et al., 2005; Wyman et al., 2005) at high temperatures is applied to the lignocellulosic biomass, making the cellulose more accessible and hydrolysis of hemicellulose by removing the lignin structure.

Physical pretreatment methods are selected according to the nature of the lignocellulosic material. Meantime the hot liquid water method or mechanical grinding methods are more acceptable in cost, the irradiation method is more difficult to implement due to its high cost.

2.2.2 Chemical Pretreatment Methods

Chemical pretreatment is another method used in the depolymerization of lignocellulosic biomass. Acid treatment, alkali treatment, oxidative delignification, and organic solvent process are the main chemical pretreatment methods.

Concentrated or diluted hydrochloric acid, nitric acid, phosphoric acid, and sulfuric acid are preferred in the acid treatment method (S. B. Kim et al., 2000; Mosier et al., 2005; Torget et al., 1990). Although this method causes depolymerization of lignin structure and hemicellulose to pentoses, mainly xylose, which will facilitate the hydrolysis of cellulose, it has disadvantages such as being a high-cost process, corrosive effects, and formation of compounds that may be toxic during fermentation (Kumar et al., 2009).

The alkaline pretreatment method is also one of the frequently used chemical pretreatment methods. Hydrogen peroxide, sodium hydroxide, potassium hydroxide, calcium hydroxide (lime), aqueous ammonia, ammonia hydroxide, and sodium hydroxide are the principal preferred bases. In this method, amorphous regions (lignin and hemicellulose) are removed, thereby causing changes in the degree of polymerization, surface area, crystallinity and porosity of each component in the lignocellulosic biomass. Although it does not require extreme environmental conditions during its application, it has disadvantages such as the formation of salts that cannot be recovered as a result of the reactions that may occur, or the incorporation of these salts into the structure of the biomass. In addition, the processing time is long and the amount of monosaccharide degradation is low (Bajpai, 2019; Taherzadeh & Karimi, 2008; Waldron, 2010).

Oxidative delignification provides pretreatment of lignocellulosic biomass using oxidizing agents such as hydrogen peroxide, ozone, or oxygen (Bajpai, 2019; Bensah & Mensah, 2013; Kobayashi et al., 2004). While this pretreatment method ensures that lignin is converted into acid, the fact that this acid is an inhibitor in the fermentation medium makes the method ineffective. In addition, since almost all the hemicellulose is degraded, it reduces the yield of the final product (Lucas et al., 2012).

The organic solvent method is to pre-treat the biomass by mixing liquid organic solvent under certain temperatures and pressures (Alriols et al., 2010; Y. Sun & Cheng, 2002). It is possible to add catalysts such as acid-base or salt to the medium

during this process. The most preferred solvents are ethanol, methanol, and acetone. (Ichwan & Won Son, 2011). At the end of the process, the hemicellulosic syrup is obtained by breaking the glycosidic bonds in hemicellulose, pure lignin is obtained by breaking the lignin's bonds with the cell walls as well as hemicellulose, and cellulose becomes easily accessible (Conde-Mejía et al., 2012). After removing the lignin, the remaining solid material can be washed with water and used in appropriate processes. However, the disadvantage is the high cost of chemical materials and the formation of inhibitory by-products such as furfural and 5-hydroxymethyl, which are formed in the presence of an acid catalyst.

2.2.3 Biochemical Pretreatment

Biochemical pretreatment is another method that can prepare lignocellulosic materials for enzymatic hydrolysis by microorganisms or enzymes. Among these microorganisms, *Phanerochaete chrysosporium* (Hatakka, 1994) and *Ceriporia lacerate* (Y. Lee et al., 2013), which are the most preferred white-rot fungi, directly cause degradation in the lignin structure, while brown rot fungi such as *Serpula lacrymans* (Nurika et al., 2020) and *Coniophora puteana* (Ray et al., 2010) and soft rot fungi like *Paecilomyces sp.*(Zerva et al., 2014) and *Cadophora sp.*(Akhtar et al., 2016), they mainly cause degradation in the cellulose structure, causing minimal degradation in the lignin structure. It has been observed that *Bacillus circulans* (Zerva et al., 2014) and *Sphingomonas paucimobilis* (Taherzadeh & Karimi, 2008) are effective in the degradation of lignin, partial degradation of hemicellulose, and reducing the degree of polymerization of cellulose. In addition, laccase and peroxidase enzymes show supportive properties in the degradation of the lignin structure (Y. Sun & Cheng, 2002).

Although biochemical pretreatment is very advantageous in terms of low energy use, little or no chemical requirement, and less harm to the environment, its low efficiency is an obstacle to its preference (Østby et al., 2020).

2.3 Enzymatic Hydrolysis

Lignocellulosic biomass is a raw material with many advantages that can be used in the production of high value-added industrial products such as lactic acid, ethanol, or biodegradable plastic. In addition to its advantages, it also has disadvantages for instance requiring pretreatment and then hydrolysis of cellulose and hemicellulose for the partially delignified and depolymerized biomass to be used in fermentation by microorganisms (Galbe, 2002).

Lignocellulosic materials can be hydrolyzed by acid, alkaline, or enzyme. The conditions such as high pressure and/or high temperature required during chemical hydrolysis of cellulose, the difficulties during the recovery of chemicals, corrosion problems, or the possibility of decreased efficiency during the degradation of glucose to hydroxymethylfurfural have made enzymatic hydrolysis more preferable (Nieves et al., 1998).

Saccharification using enzymes is a more advantageous method compared to chemical processes, as the efficiency is higher, the by-product formation is much less, the energy requirement is not so much, and there is no corrosive effect (Bon & Ferrara, 2013). On the other hand, the adsorption of the enzyme on the substrate, inhibition of cellulase and β-glucosidase due to the rise in the amount of monomer, enzyme activity, enzyme load, pH, and temperature are the factors to be considered during enzymatic hydrolysis. Moreover, long processing time and enzyme costs must be taken into account. To obtain the carbon source from lignocellulosic biomass efficiently under optimum condition, it is important to understand both the structure of the biomass and the working mechanisms of cellulolytic enzymes (B. Yang et al., 2011).

2.3.1 Cellulase

Cellulases are enzymes that break down β-1,4 bonds in cellulose chains to obtain monomers. It can be obtained from microorganisms such as bacteria, fungi, or actinomycetes. Table 2.4 more detailed representation is available. Cellulase enzymes, which are mostly used in industry, are produced by *Trichoderma reesei* with its cellulase containing different cellobiohydrolases and endoglucanases for the hydrolysis of cellulose (Miettinen-Oinonen & Suominen, 2002).

Table 2.4 Microorganisms that can be used in cellulase production

Major group	Genus	References
Fungi	*Aspergillus sp.*	(Ong et al., 2004)
	Bulgaria sp.	(Lübeck, 2018)
	Cladosporium sp.	(Abrha & Gashe, 1992)
	Fusarium sp.	(Sukumaran et al., 2005)
	Humicola sp.	(Schülein, 1997)
	Myrothecium sp.	(Lübeck, 2018)
	Penicillium sp.	(Jørgensen et al., 2003)
	Schizophyllum sp.	(Lübeck, 2018)
	Trichoderma sp.	(Sukumaran et al., 2005)
Bacteria	*Acetobacter sp,*	(Lübeck, 2018)
	Acidothermus cellulolyticus	(Sukumaran et al., 2005)
	Bacillus sp.	(Mawadza et al., 2000)
	Bacteriodes sp.	(Lübeck, 2018)

Table 2.4 (continued)

	Clostridium sp.	(López-Contreras et al., 2004)
	Enterobacter sp.	(Lokapirnasari et al., 2015)
	Escherichia coli	(Amraini et al., 2017)
	Geobacillus sp.	(Potprommanee et al., 2017)
	Gluconacetobacter sp.	(Lübeck, 2018)
	Pseudomonas cellulosa	(Sukumaran et al., 2005)
	Rhodothermus marinus	(Bjornsdottir et al., 2006)
Actinomycetes	*Cellulomonas sp.*	(Rajoka & Malik, 1997)
	Streptomyces sp.	(Okeke & Paterson, 1992)
	Thermononospora sp.	(Sukumaran et al., 2005)

For complete hydrolysis of cellulose, endoglucanases ((EC 3.2.1.4), (EC 3.2.1.2)) which provide random breaking of amorphous regions in polysaccharide chains, exoglucanases including cellobiohydrolases ((EC 3.2.1.91), (EC 3.2.1.176)) and cellodextrinases (EC 3.2.1.74) that operably cleave cellooligosaccharides from chain ends and β-glucosidases (EC 3.2.1.21) to prevent irreversible product inhibition from cellobiose accumulation by hydrolyzing soluble cellobiose and cellobiose to glucose should be used (Bhat, 2000; Buckle et al., 1995; Dimarogona et al., 2012).

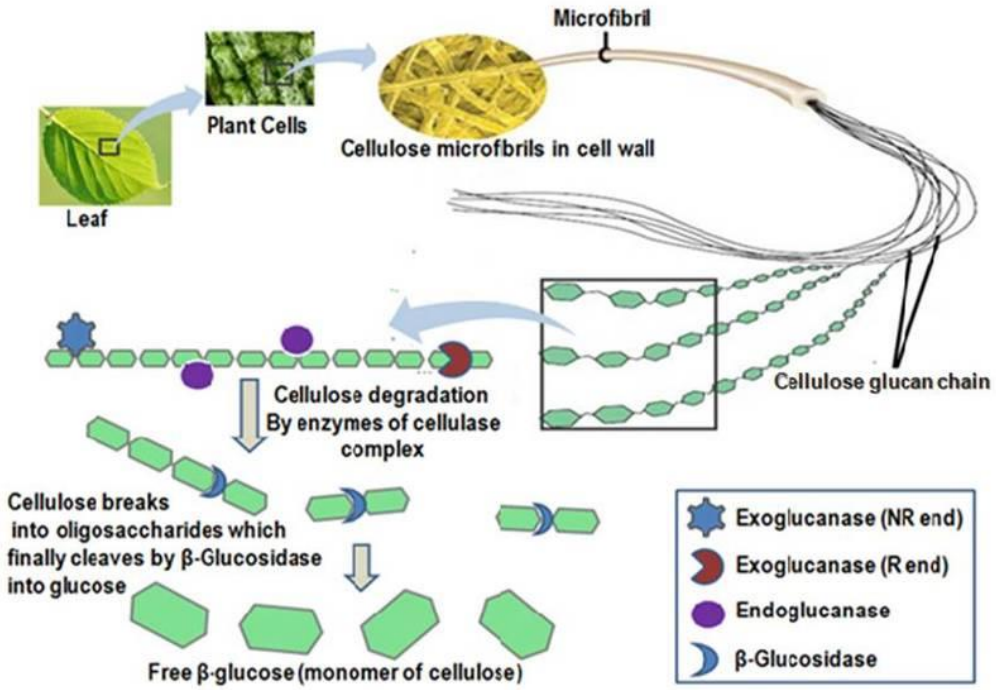


Figure 2.8 Hydrolysis mechanism of cellulases (G. Singh et al., 2016)

Teugjas and Väljamäe (2002) investigated the end-product inhibition by hydrolyzing bacterial celluloses of cellulases obtained from fungi in their studies. In their findings, they observed that cellobiose and glucose were affected primarily by cellobiohydrolases, and then by endoglucanases. Decreased enzyme activity due to product inhibition also causes a reduction in glucose yield. Besides the increase in cellobiose and glucose, Chia-wen et al. (2014) mentioned that other emerging monosaccharides such as mannose and galactose also affect the enzyme activity and cause a drop in yield. In addition, they noticed in their studies that exoglucanases and endoglucanases were most influenced by high monosaccharide concentration, and β-glucosidases were less affected by other monosaccharides concentrations than glucose.

Several methods have been developed to reduce the deactivation of cellulaFse. These can be listed as the addition of surfactants (polyoxyethylene glycol, sophorolipid, polysorbate 20 or 80) to the hydrolysis medium (Duff et al., 1995; Kristensen et al., 2009; Y. Sun & Cheng, 2002), the simultaneous saccharification and fermentation (SSF) method (Andrić et al., 2010), the creation of different enzyme mixtures (E. Kim et al., 1998), the use of membrane reactors (Andrić et al., 2010).

2.3.2 Hemicellulase

Hemicellulase is another type of enzyme that facilitates the access of cellulose, which is responsible for the hydrolysis of hemicelluloses such as xylan, xyloglucans, arabinoxylans, and glucomannans surrounding the cellulose, thus contributing to the rise of its productivity. The most common hemicellulose type is xylan. Therefore, hemicellulase can also be called xylanases. Just like cellulases, hemicellulase contains different types of enzymes. The most important of these are endoxylanases (EC 3.2.1.8), which randomly hydrolyze β-1,4 glycosidic bonds to form xylooligosaccharides and reduce the degree of polymerization, and β-xylosidases (EC 3.2.1.3), which hydrolyze the non-reducing ends of xylooligosaccharides to xylose (Buschle-Diller et al., 1999; Meena et al., 2017; Saha, 2003). In addition to these enzymes, it may contain a few auxiliary enzymes such as a-L-arabinofuranosidase, which removes arabinose from the xylan backbone, a-glucuronidase, which removes 4-O-methyl glucuronic acid, and esterase, which hydrolyzes acetyl ester bonds, feruloyl ester bonds and q-coumaryl ester bonds (Saha, 2003). Another main component of hemicelluloses is mannans. The enzymes used during the hydrolysis of mannans are called as endo-1,4-β-mannanase, β-mannosidases, β-glucosidases, α-galactosidases and acetyl mannan esterase (Kunamneni et al., 2014; Shallom & Shoham, 2003).

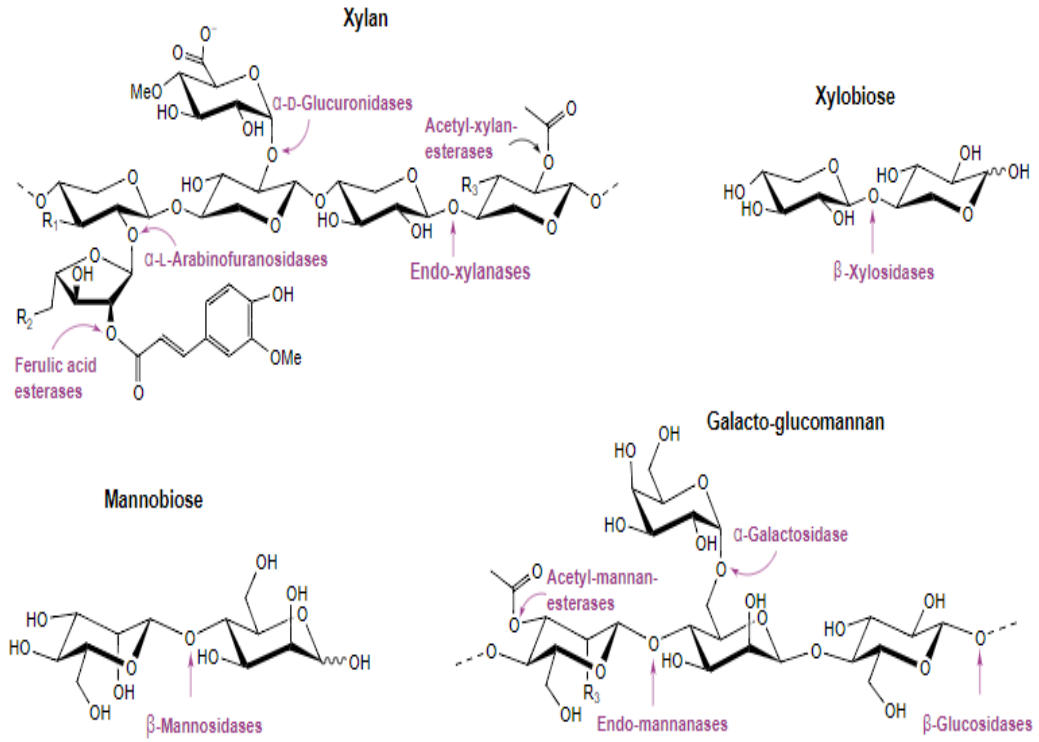


Figure 2.9 Hemicellulases are responsible for the hydrolysis of monosaccharides in hemicellulose (Shallom & Shoham, 2003)

While hemicellulases can be obtained from different microorganisms, saprophytic microbes generally contain this enzyme in nature. However, regarding industrial use, the most important sources are fungi and thermophilic bacteria in terms of adapting to different pH and temperatures. These microorganisms are shown in the Table 2.5 in more detail.

Table 2.5 Microorganisms that can be used in hemicellulase production

Major group	Genus	References
Fungi	*Aspergillus niger*	(Kang et al., 2004)
	Humicola insolens	(Xia et al., 2015)
	Fusarium graminearum	(Conejo-Saucedo et al., 2011)
	Meripilus giganteus	(Conejo-Saucedo et al., 2011)
	Penicilium funiculosum	(Karboune et al., 2009)
	Trichoderma reesei	(Dekker, 1983)
Bacteria	*Agrobacterium tumefaciens*	(Shallom & Shoham, 2003)
	Bifidobacterium breve	(Shin et al., 2003)
	Clostridium thermocellum	(Kohring et al., 1990)
	Caulobacter crescentus	(Shallom & Shoham, 2003)
	Escherichia coli	(Conejo-Saucedo et al., 2011)
	Thermoanaerobacter ethanolicus	(Mai et al., 2000)
Actinomycetes	*Thermomonospora fusca*	(Irwin et al., 1994)

2.3.3 Lignin Modifying Enzymes and Lignin Degrading Auxiliary Enzymes

Enzymes used in the hydrolysis of lignin can be examined under two different headings. The first is enzymes that help lignin hydrolyzation, and the second is enzymes that modify the lignin structure (Dhagat & Jujjavarapu, 2021). Although auxiliary enzymes cannot degrade the lignin structure on their own, they contribute to hydrolysis by incorporating proteins through the production of oxidative hydrogen peroxide. Enzymes included in this category can be obtained from the secretomes of the white-rot fungus. Enzymes such as aryl alcohol oxidase, glucose oxidase, or pyranose 2-oxidase are cited as examples. Moreover, an aerobic environment is required for these enzymes to assist in hydrolysis (Levasseur et al., 2008). Lignin-modifying enzymes are also referred to as ligninases or lignases in the literature. Lignin-modifying enzymes, which can be found in bacteria and fungi and examined under 4 main headings: laccase, manganese peroxide, lignin peroxidase, and versatile peroxidase, provide partial degradation of lignin (Dhagat & Jujjavarapu, 2021).

Laccase (EC 1.10.3.2. diphenol: oxygen oxidoreductase) is an oxidase that uses molecular oxygen as an oxidizing agent, as well as enables the oxidation of phenolic rings to phenoxy radicals, thanks to the four copper atoms it contains in the catalytic site. It is involved in the oxidation of many important aromatic compounds such as phenolic moieties, aromatic amines, and hydroxylindoles (Baldrian, 2006).

Lignin peroxidases (EC 1.11.1.14) play an important role in many reactions such as breaking the β-0-4 ether bonds and Cα-Cβ bonds in the structure of lignins, as well as in the oxidation of benzyl alcohols, phenolic, and non-phenolic compounds. In addition, it has many different functions such as the hydroxyl group inclusion, forming the quinone structure, and breaking down aromatic rings. Thanks to its high redox potential, it can easily oxidize both phenolic, especially non-phenolic, and methoxy-substituted lignin (Wong, 2009).

Manganese peroxidase (EC 1.11.1.13) oxidizes Mn^{2+} to Mn^{3+}, and it can degrade the phenolic parts of lignin as an oxidant thanks to Mn^{3+} chelate and form phenoxy radicals. Manganese peroxidase is a type of heme enzyme and contains two a-helices, two Ca^{2+} ions, and five disulfide bridges in its structure.

Versatile peroxidases (EC 1.11.1.16) have the characteristics of catalytic activities of lignin peroxidases and manganese peroxidases. Just like lignin peroxidases, it can oxidize non-phenolic compounds thanks to its high redox activity, and Mn^{2+} like manganese peroxidase. Its versatile catalytic activity allows reactions to take place on aromatic substrates, regardless of low or high redox potential (Goszczynski et al., 1994; Martínez et al., 2005).

2.4 Lactic Acid

Karl Wilhelm Scheele, a Swedish chemist, noticed lactic acid (LA), an organic hydroxy acid type, in sour milk in 1780 and defined it as a component of milk. In 1808, Jöns Jakob Berzelius discovered that the liquid obtained from meat also contained LA, paving the way for Justus von Liebig to find it in dead muscle tissues. In the light of this information, the effects of LA on muscle contraction were investigated and it was observed that the amount increased in muscle tissues in an oxygen-free environment. In 1843, Johann Joseph Scherer proved that there is LA in the blood after death in pathological conditions, and Carl Folwarczny declared that it was also seen in people who lived in 1858(Kompanje et al., 2007). It was first started to be produced on an industrial scale in 1895 by the Boehringer Ingelheim company after Louis Pasteur stated that lactic acid could be produced by lactic acid bacteria (LAB) in the 1860s (Alsaheb et al., 2015).

Lactic acid (2-Hydroxypropanoic acid) is an alpha-hydroxy acid type. It has a wide range of applications and markets from food to pharmaceutical, cosmetics, and chemical industries (Martínez et al., 2005). Its industrial production was first started with its use for acidification and preservation in foods, and it received GRAS status

from the FDA (Miller et al., 2019). The usage areas of LA can be exemplified in leather tanning for removing hair from animal skin and providing elasticity to the skin, pharmaceutical and cosmetic applications for pH regulation, and metal separation. Apart from the examples given, lactic acid also has usage areas in different niches (Cengiz, 2002).

Along with the developed technologies and changing environmental factors, the trend toward a greener, biodegradable products started, and lactic acid was the source of research as a promising organic substance. Ethyl lactate ($C_5H_{10}O_3$), an ester of lactic acid, is used in many industries as an important green product with its non-toxic, biodegradable, and good solvent properties, in the production of nitrocellulose, as a food additive, and to produce chemicals required for flavoring (Dangpradab & Rattanaphanee, 2015). Another product that can be obtained by lactic acid synthesis, on which studies are intensive, is polylactic acid (PLA), an aliphatic polyester type. PLA is a green product with high potential, which has a wide range of uses from surgery and medicine to packaging or from film making to fiber formation. In addition, is considered safe for health by the FDA is an important plus (García Ibarra et al., 2016).

2.4.1 Lactic Acid in Global Market

Lactic acid is a valuable organic substance used in many industries and its demand in the global market is increasing day by day. Miller et al. (2018) reported that 75% of the lactic acid produced worldwide is produced in the fermentation facilities of Galactic, PURAC Corporation, Cargill Incorporated, and Archer Daniels Midland Company. While the price list varies according to the usage area of the product (food, medical, etc.) and the raw material from which it is produced the approximate sales price is 4.0 US$ kg^{-1} - 5.0 US$ kg^{-1}. Lactic acid consumption was determined as approximately 1220 tons in 2016, Alves de Oliveira et al. (2018) stated that the demand for lactic acid, which increased its annual global growth by 16.2 %, exceeded 1960.1 tons in 2025 and reached the global market for US$ 9.8 billion. In

addition to lactic acid, polylactic acid (PLA) has an important place in the global market. Leading PLA manufacturers can be listed as NatureWorks LLC, Corbion and Total, Galactic, and Cargill companies, respectively. Ratshoshi et al. (2021) stated that the approximate selling price of PLA is 5.14 US$ kg^{-1} and expressed that its production could be 328 kt $year^{-1}$ in 2024.

2.4.2 Physical and Chemical Properties of Lactic Acid

Lactic acid, with the molecular formula $CH_3CH(OH)COOH$, is a yellow to white color range, syrupy liquid to solid, odorless, and a member of hydroxycarboxylic acid. Its designation as a weak acid species is due to its partial dissociation in water as well as its associated dissociation constant (Komesu et al., 2017; *Regulations*, 2012).

Table 2.6 Some properties of lactic acid (Ameen & Caruso, 2020; Komesu et al., 2017)

Parameters	Description
Molecular Weight	90.08 mol g^{-1}
Density	1.249 g L^{-1} at 20°C
Boiling Point	122.0°C (DL) 103.0°C (D) at 15 mmHg
Dissociation Constant	3.83(D) 3.79 (L) pKa at 25°C
Heat Capacity	190 (DL) J/mol.°C at 20°C
Heat of Fusion	16.86 (L) 11.33(DL) (kJ/mol)

In its molecular structure, it has a chiral atom to which a hydroxyl group is attached, and two terminal carbon atoms, one belonging to the carboxylic group and the other to the methyl group. In this way, L(+) lactic acid (dextrorotatory) and D(-) lactic acid (levorotatory) exist in optically isomeric form. Although both forms have the same

molecular formula, different physical properties are observed. Chemically occurring lactic acid is in racemic form, while biologically occurring lactic acid can usually approach enantiomeric purity (Ameen & Caruso, 2020; Miller et al., 2019; Teixeira et al., 2021).

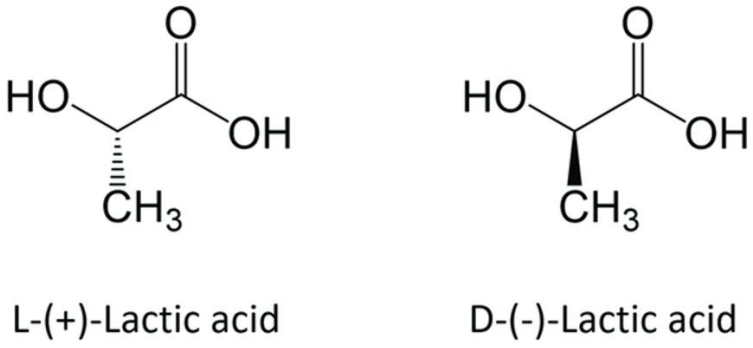


Figure 2.10 Enantiomers of lactic acid (Casalini et al., 2019)

2.4.3 Synthesis of Lactic Acid

Lactic acid can be produced by chemical methods or by microbial fermentation (Sreenath et al., 2001).

The most widely used method in the chemical synthesis of lactic acid is the hydrolysis of lactonitrile(2-hydroxypropanenitrile, $CH_3CHOHCN$) with the aid of strong acid (John et al., 2009). Firstly, hydrogen cyanide must be added nucleophilic to the liquid phase of acetaldehyde under high pressure and in an alkaline medium to obtain lactonitrile (Eqn. 2.1). After recovery of lactonitrile, it is hydrolyzed with strong and concentrated acid (hydrochloric acid or sulfuric acid) to obtain ammonium sulfate salt and crude lactic acid (Eqn. 2.2). In the purification of crude lactic acid, methyl lactate ester is obtained by using methanol (Eqn. 2.3). In the next step, after the methyl lactate ester distillation and purification process are completed, it is hydrolyzed in the presence of an acidic aqueous solution to obtain a racemic mixture of lactic acid (Eqn. 2.4) (Ameen & Caruso, 2020; Narayanan et al., 2004).

$$CH_3CHO + HCN \xrightarrow{catalyst} CH_3CHOHCN \quad \text{(Eqn. 2.1)}$$

$$CH_3CHOHCN + H_2O + \frac{1}{2}(H_2SO_4) \rightarrow CH_3CHOHCOOH + \frac{1}{2}((NH_4)_2SO_4) \quad \text{(Eqn. 2.2)}$$

$$CH_3CHOHCOOH + CH_3OH \rightarrow CH_3CHOHCOOCH_3 + H_2O \quad \text{(Eqn. 2.3)}$$

$$CH_3CHOHCOOCH_3 + H_2O \rightarrow CH_3CHOHCOOCH + CH_3OH \quad \text{(Eqn. 2.4)}$$

Another option used in the chemical synthesis of lactic acid is the oxidation of propane in the presence of oxygen and nitric acid to obtain α-nitropropionic acid (Eqn. 2.5), and then crude lactic acid as a result of further hydrolysis (Eqn. 2.6) (Vaidya et al., 2005).

However, considering that lactonitrile and propene are substances produced from petroleum-derived and non-renewable resources, it becomes clear that it is not possible to use chemical synthesis to meet global energy demand. In addition, considering the damage they cause to the environment, it has highlighted the production of lactic acid through microbial fermentation (Marques et al., 2008).

$$CH_3CH_2CH_3 + HNO_3 + O_2 \rightarrow CH_3CH(NO_2)COOH \quad \text{(Eqn. 2.5)}$$

$$CH_3CH(NO_2)COOH + H_2O \rightarrow CH_3CHOHCOOH \quad \text{(Eqn. 2.6)}$$

2.4.3.1 Microbial of Lactic Acid Production and Microorganisms

Fermentation is defined as the biodegradation of carbohydrates by microorganisms, resulting in the formation of metabolites such as ethanol, citric acid, and lactic acid. Industrial production of lactic acid by fermentation gained momentum after the discovery of *Lactobacillus sp.* and became the subject of research. Today, 90% of the worldwide production of LA is manufactured through microbial fermentation (Hofvendahl & Hahn-Hägerdal, 2000). Additionally, the advantages of the microbial fermentation method are listed as the possibility of using cheap raw materials, less energy consumption, the possibility of obtaining optically pure D- or L-lactic acid, and less damage to the environment (Abdel-Rahman et al., 2013).

In lactic acid production, different carbon sources can be used together with various species of bacterium (*Lactobacillus sp., E. coli*), fungi (*Rhizopus sp.*) cyanobacterium (*Corynebacterium glutamicum*), and yeast (*Kluyveromyces lactis*), depending on the desired yield or purity (Abdel-Rahman et al., 2013; Abedi & Hashemi, 2020; Miller et al., 2019).

Many bacterial species can produce LA as a product of primary or secondary fermentation. Amen and Caruso (2017) identified Lactic acid bacteria (LAB) generally used in lactic acid production as *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Carnobacterium*, *Enterococcus*, *Tetragenococcus*, *Aerococcus*, *Vagococcus*, *Leuconostoc*, *Oenococcus*, and *Weissella sp*. The fact that LAB has GRAS status makes them frequently preferred in the food industry (Abdel-Rahman et al., 2013). Lactic acid bacteria, which can be isolated from different sources in nature, have the characteristics of gram-positive, non-sporing, generally immobile, cocci, coccobacilli, or rods (L Axelsson & Narvhus, 2003).

LAB is generally examined under two main headings, homofermentative (homolactic) species such as *Lactococcus*, *Pediococcus*, and some *Lactobacillus* that produce only lactic acid during fermentation, and heterofermentative (heterolactic) species of *Leuconostoc*, *Weissella*, and *Carnobacterium sp.* (Komesu et al., 2017). In heterolactic species, they are divided into two as obligatory and facultative. During homolactic LAB metabolizes carbon source, it prefers the Embden-Meyerhof-Parnas pathway (glycolysis) shown in Figure 2.11, while obligate heterolactics use the 6-phosphogluconate/phosphoketolase pathway (Figure 2.12) (Ameen & Caruso, 2020). The facultative heterolactic LAB can use both paths. *L. fermentum*, *L. parabuchneri*, and *L. reuteri* are an example of obligate heterofermentative LAB. Facultative heterofermentative LAB is *L. alimentarius*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactococcus lactis*, *Lactobacillus pentosus* and *Lactobacillus xylosus* (Castillo Martinez et al., 2013; Komesu et al., 2017).

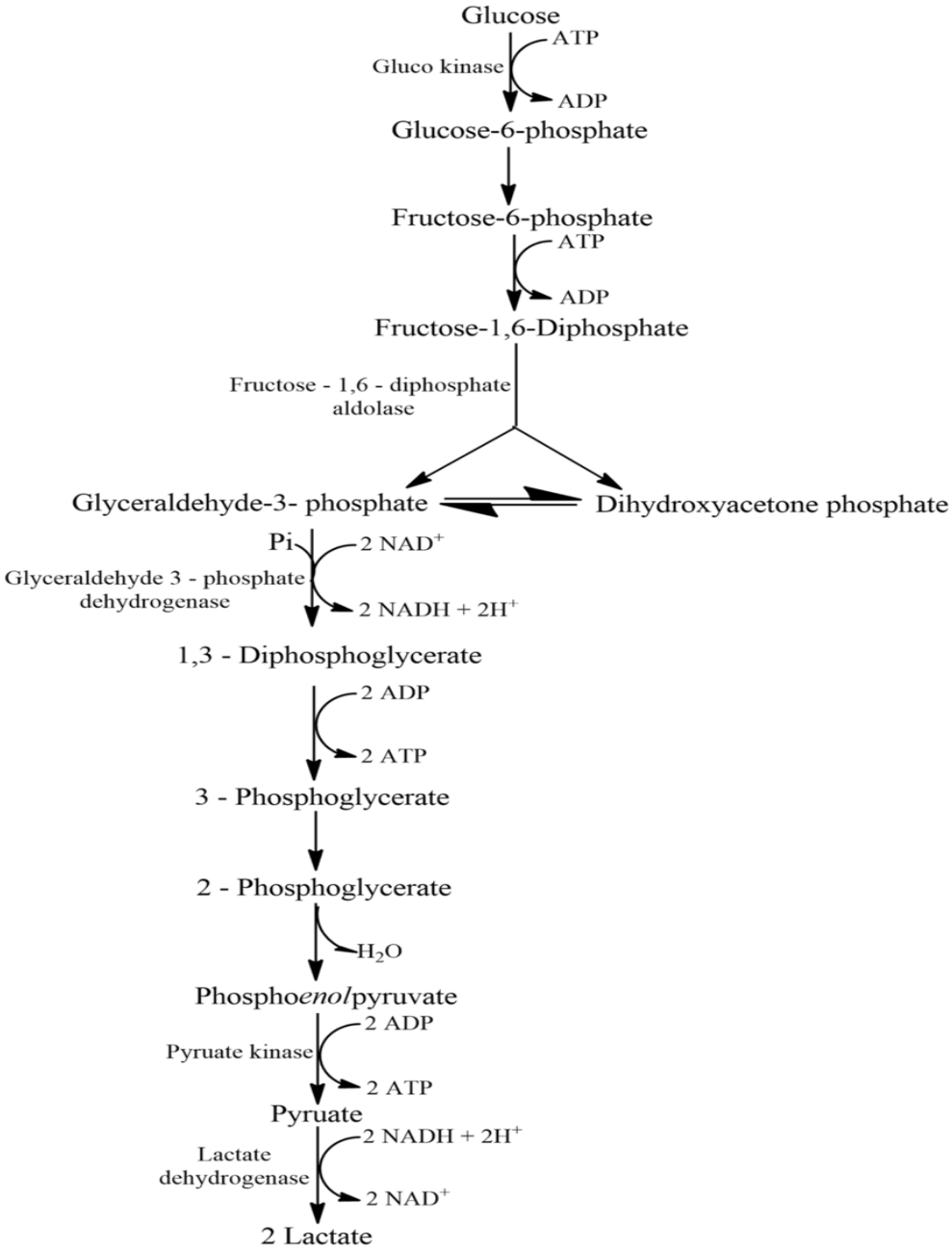


Figure 2.11 Illustration of homolactic lactic acid bacteria metabolizing glucose by the Embden–Meyerhof–Parnas pathway (glycolysis) (Vivek et al., 2019)

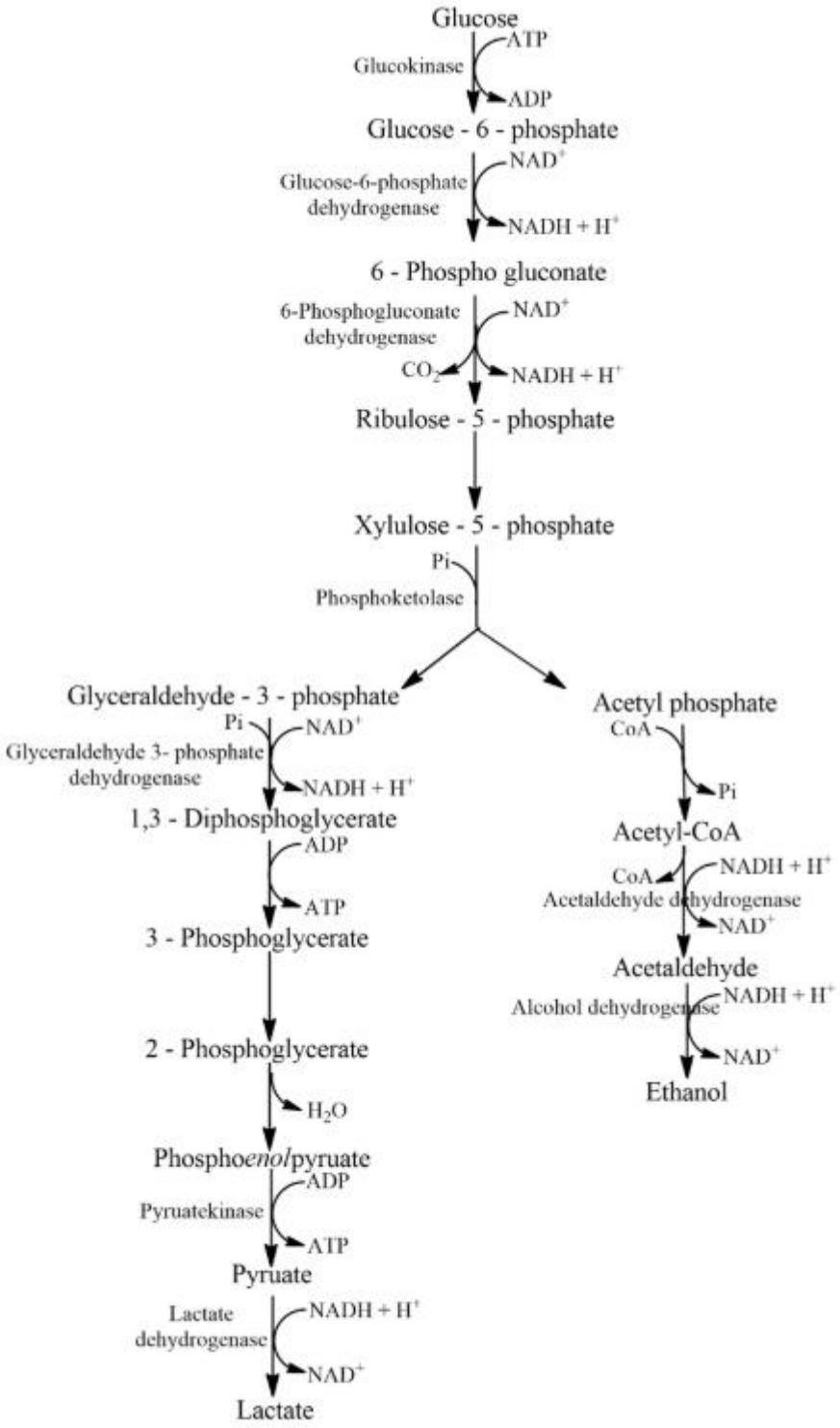


Figure 2.12 Illustration of heterolactic lactic acid bacteria metabolizing glucose by the 6-phospho-gluconate/phosphoketolase pathway (Vivek et al., 2019)

2.5 Simultaneous Saccharification and Fermentation

The use of lignocellulosic raw materials with high carbon content in fermentation technology has brought along some problems, and different methods have been developed to find solutions to these problems. Simultaneous saccharification and fermentation (SSF) is one of the most promising methods. Lignocellulosic biomass is converted to sugar that can be used in fermentation by cellulase and hemicellulase enzymes, but these enzymes are very sensitive to feedback inhibition. SSF prevents the irreversible inhibition of enzymes by allowing the hydrolyzed sugar to be used by microorganisms immediately during fermentation (Abdel-Rahman et al., 2011). Compared to the separate hydrolysis and fermentation (SHF) method, which is the traditional production method of fermentative products, it also has an economic advantage due to the fact that the process takes place in a single bioreactor and the production time is short (Kádár et al., 2004). Furthermore, since the sugar concentration is kept at low levels, it leads to a decrease in the osmotic pressure of the cells, leading to the manufacturing of products with high added value (Miller et al., 2019). In addition to the advantages of SSF, it also has the disadvantage of finding an equilibrium point, since the temperature and pH required for hydrolysis and fermentation differ (Abdel-Rahman et al., 2011). Table 2.7 shows the alteration in the amount of organic acid obtained as a result of the production of some lignocellulosic materials by SHF and SSF methods. As can be seen from Table 2.7, the SSF process generally gives more effective results in the production of valuable end products from lignocellulosic biomass than the SHF process.

Table 2.7 Organic acid production from some lignocellulosic biomass using SHF and SSF methods

Materials	Products	SHF	SSF	References
Aspen sawdust	Succinic acid	41.0 (g L^{-1})	46.0 (g L^{-1})	(Maslova et al., 2019)
Barley straw	Ethanol	0.71 (g g^{-1})	0.83 (g g^{-1})	(Won et al., 2012)
Birch sawdust	Lactic acid	27.2 (g L^{-1})	37.3 (g L^{-1})	(Maslova et al., 2019)
Cassava pulp	Ethanol	21.16 (g L^{-1})	29.27 (g L^{-1})	(Zhu et al., 2012)
Cellulose	Ethanol	0.35 (g g^{-1})	0.41 (g g^{-1})	(Drissen et al., 2009)
Corn grain	Ethanol	0.640 (g g^{-1})	0.710 (g g^{-1})	(Szambelan et al., 2018)
Corn stover	Lactic acid	0.64 (g g^{-1})	0.78 (g g^{-1})	(Öhgren et al., 2007)
Jerusalem artichoke tubers	Fumaric acid	42.5 (g L^{-1})	50.2 (g L^{-1})	(Maslova et al., 2019)
Rice straw	Butanol	3.05 (g L^{-1})	5.24 (g L^{-1})	(Valles et al., 2020)
Seaweed	Ethanol	8.6 (g L^{-1})	7.6 (g L^{-1})	(Cho et al., 2013)
Sorghum	Ethanol	21.6 (g L^{-1})	26.8 (g L^{-1})	(Ghaffar et al., 2014)
Wheat straw	Lactic acid	28.0 (g L^{-1})	34.7 (g L^{-1})	(Maslova et al., 2019)

2.6 Aim of the Study

The thesis study aims to obtain fermentable sugar from horse chestnut shells with cellulolytic enzymes via three different processes by *Lactobacillus casei*. Besides, separate hydrolysis and fermentation, and simultaneous saccharification and fermentation technologies, a new method has been established by combining the advantages of these two processes. In the developed method, two bioreactors were connected by hoses, and the medium was passed through each other via a peristaltic pump. In this way, optimum conditions are provided for both the enzyme and the microorganism while performing simultaneous saccharification and fermentation. Different substrate load, temperature, and pH were investigated to determine the appropriate hydrolysis and fermentation conditions to metabolize horse chestnut shell, which is a lignocellulosic substance that has not been used for such a purpose before, to lactic acid by green methods. All three methods were compared in terms of the efficiency of production LA.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Horse chestnuts (Aesculus hippocastanum) were collected from Middle East Technical University campus (Turkey) in September 2020.

ASA Biogazyme 2x cellulolytic enzyme was supplied by ASA Spezialenzyme GmbH in Wolfenbüttel, Germany.

For this study, *Lactobacillus casei* NRRL B-441 (Northern Regional Research Lab, Illinoi, USA) was obtained from H2biotek Limited Company.

3.1.1 Chemicals

The chemicals used during the study were all analytical grades. The list of chemicals is listed in Appendix A.

3.1.2 Enzymes

ASA Biogazyme 2x (From ASA Spezialenzyme GmbH in Wolfenbüttel, Germany) enzyme used in industry, *Trichoderma sp.* origin. Its composition mixture of cellulase and hemicellulase enzymes are exo-cellulase (EC 3.2.1.91), endo-glucanase (EC 3.2.1.4), β-glucosidase (EC 3.2.1.21), xylanase (EC 3.2.1.8).

Table 3.1 Activity of ASA Biogazyme 2x (*Herstellung Organischer Säuren Für Die Polyestersynthese*, 2017)

Enzyme	Activity (U/g)
Exo-cellulase	1050
Endo-glucanase	117000
β-glucosidase	290
Xylanase	150000

3.2 Methods

3.2.1 Pretreatment of Horse Chestnut Shell

Fresh horse chestnuts were first autoclaved at 121°C for 15 minutes to facilitate separation from their shells and to get rid of aescin, which has an anti-microbial effect. Before being hydrolyzed by enzyme, physical processes were applied during saccharification to increase the yield. In this way, the surface area was increased, and the crystal structure of cellulose was disrupted (Harmsen et al., 2010). After shell and seed separation, the moisture content of the shells was reduced to 4% with 1 m s^{-1} air velocity at 80 °C in a tray dryer (EKSİS Industrial Drying Systems, Isparta, Turkey). The dried shells (FRITSCH Industries. 8 55743 Idar-Oberstein, Germany) were ground to a particle size of 1 mm and sieved to obtain homogenized particles. Finally, the shell powders are packaged and preserved at 4 °C for further use.

3.2.2 Bacterial Strain and Medium Preparations

For this study, *Lactobacillus casei* NRRL B-441 (Northern Regional Research Lab, Illinoi, USA) was obtained from H2biotek Limited Company. Bacteria were maintained as stock cultures in a 1:1 ratio of 50% glycerol and liquid growth medium

at -80°C. To revive the bacterial culture, the inoculum was made from stock culture into pre-sterilized for 15 min at 121°C, 5 ml de Man, Rogosa, and Sharpe (MRS, Merck Germany) broths, and incubated for 16 hours at 40 °C and 160 rpm.

Bacterial cultures grown in MRS broth were used as inoculum in growth media containing synthetic or horse chestnut shells. For rapid use, the cells were grown on MRS agar at 37 °C and preserved at 4 °C, and regularly renewed every month. The growth medium of Hujanen et al. was modified (Hujanen et al., 2001). The content of the growth medium is the following: 20 g L^{-1} glucose, 12 g L^{-1} yeast extract, 1 g L^{-1} Tween 80, 0.2 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 0.05 g L^{-1} $MnSO_4 \cdot 4\ H_2O$, 0.5 g L^{-1} $C_2H_3NaO_2$, 1.5 g L^{-1} KH_2PO_4, 1.5 g L^{-1} K_2HPO_4, 10 g L^{-1} casein peptone, 30 g L^{-1} $CaCO_3$. For fermentation medium, glucose was replaced with dried horse chestnut shell dust.

To obtain growth curve, *Lactobacillus casei* strain was grown at 40°C in 250 mL Erlenmeyer flasks containing 100 mL of sterilized MRS broth. Its optical density was recorded every two hours with a spectrophotometer (UV 1202, Shimadzu, Japan) at 620 nm.

3.2.3 Separate Hydrolysis and Fermentation

3.2.3.1 Enzymatic Hydrolysis of Horse Chestnut Shell

To obtain sugars for use in fermentation, pre-treated dried horse chestnut shells (HCS) were first hydrolyzed by an enzyme. Enzymatic hydrolysis was carried out in shaker incubators (Infors HT, Switzerland) with a working volume of 100 ml in a 250 ml Erlenmeyer flask. The experiments were done in duplicates to get the best approach. Hydrolysis was carried out with 10% (w/v) solid pretreated HCS load at 150 rpm at different temperatures and pH. Samples containing HCS pretreated with sodium citrate buffer were autoclaved at 121°C for 15 min and then allowed to cool and 65 FPU g^{-1} ASA-BG (contains exo-cellulase, endo-glucanase, β-glucosidase,

and xylanase) enzyme was added. A sample was taken every 8 hours to monitor the amount of sugar. A constant amount of monosaccharide was observed after approximately 24 h.

3.2.3.1.1 Effect of the pH and Temperature on the Enzymatic Hydrolysis

To observe the effect of pH on enzymatic hydrolysis, 0.05 M sodium citrate buffer was prepared using 5 M NaOH as 4.5 ± 0.1, 4.8 ± 0.1, 5.0 ± 0.1, 5.5 ± 0.1. Each pH was tested sequentially at 50, 55, and 60 °C.

3.2.3.2 Batch Lactic Acid Fermentation

Fermentation was done in 250 mL Erlenmeyer flasks with a working volume of 100 mL. The fermentation medium contains the same components as the growth medium except for glucose. HCS was used as a fermentable sugar source instead of glucose. The chemicals used in the fermentation medium were added to the hydrolysate, the pH was adjusted to 6.0 ± 0.2 with 5 M NaOH, and in addition, 3% (w/v) calcium carbonate was added to the fermentation medium to eliminate the negative effect of acidity on fermentation by keeping the pH constant. The prepared medium was sterilized by autoclaving at 121°C for 15 min before adding 5% (v/v) inoculum to ensure aseptic conditions. The experiment was carried out in a shaking incubator (Infors HT, Switzerland) at 200 rpm at 40 °C for 48 h.

3.2.4 Simultaneous Saccharification and Fermentation

3.2.4.1 Single Bioreactor System

In this part of the experimental set, serial batch experiments were performed under sterile conditions using 1.5 L of the 3 L volume of the jacketed benchtop bioreactor for enzymatic hydrolysis and fermentation. 10% (w/v) pretreated horse chestnut shell

powder was determined as a solid load. Components in the fermentation medium, horse chestnut shells, and bioreactor were autoclaved separately at 121 °C for 15 min. After cooling, the experiment was started by adding 5% (v/v) inoculum and 65 FPU g^{-1} ASA-BG. It was tested at different temperatures and pHs. The shaking speed was kept at 200 rpm.

3.2.4.1.1 Effect of pH and Temperature on Simultaneous Saccharification and Fermentation

An experimental set was set up to determine how pH and temperature changed the lactic acid concentration during SSF. The pH was tested at 4.8 ± 0.1, 5.5 ± 0.1, and the temperature was set at 50, 55, and 60°C.

3.2.4.2 Dual Bioreactor System

In this part of the experiment, serial batch experiments were carried out using 2 different bioreactors for enzymatic hydrolysis and fermentation. It was developed based on the working mechanism of the SSF process. Two 3 L bioreactors with a working volume of 1.5 L were connected to each other via hoses, the transition was made with a peristaltic pump with almost 0.17 L h^{-1} flow rate, thus creating a single-stage process. In this way, optimum conditions for both hydrolysis and fermentation were established. Figure 3.1 shows process diagram. Pretreated horse chestnut shells with 0.05 M sodium citrate buffer at pH 4.8 and fermentation medium were autoclaved at 121 °C for 15 minutes. In addition, both bioreactors were autoclaved by adding deionized water to prevent clogging in the lines and to protect the pH probes and were evacuated with a peristaltic pump to keep them sterile. After reducing the temperature, 65 FPU g^{-1} ASA-BG was added externally to the bioreactor for hydrolysis, and saccharification was started at 200 rpm for different temperatures and different solid loading. Simultaneously, the fermentation process was started by adding 5% (v/v) inoculum. The sugar requirement for fermentation

was provided by HCS. The pH of the bioreactor used for hydrolysis was controlled with the help of sodium citrate buffer and monitored automatically. The bioreactor employed for fermentation was also kept constant at 6.0 ± 0.2 using 5 M KOH, and the stirring speed and temperature were kept constant at 200 rpm and 40°C.

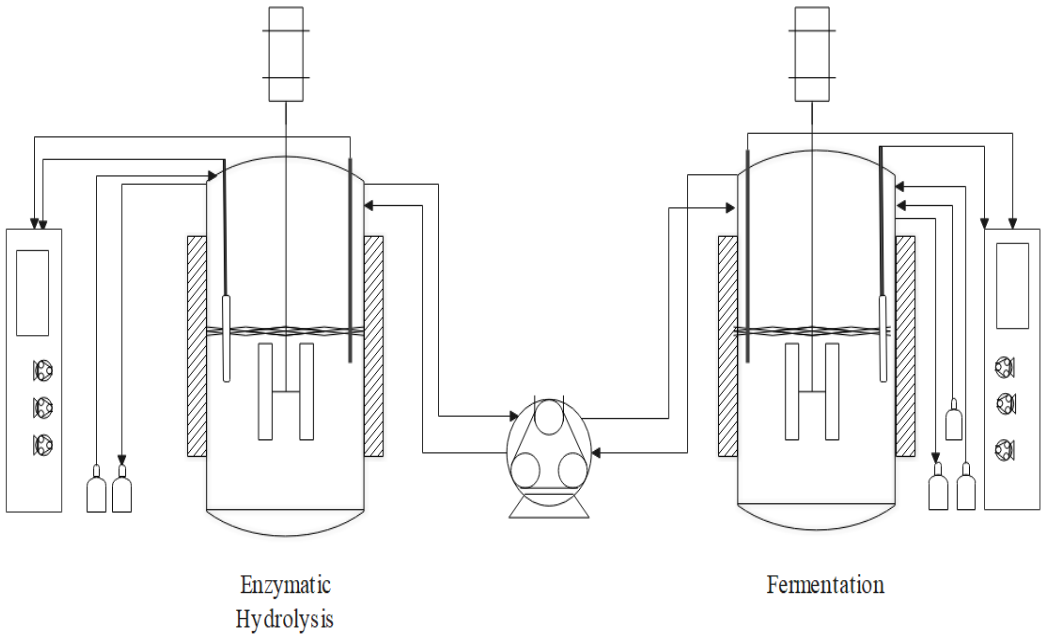


Figure 3.1 Schematic diagram of the two-bioreactor system with simultaneous saccharification and fermentation

3.2.4.2.1 Effect of Temperature on Enzymatic Hydrolysis and Fermentation Yield

Solid load and enzyme load were determined as 10% (w/v), 65 FPU/g respectively. For enzymatic hydrolysis, 0.05 M sodium citrate buffer (at pH 4.8) was used to control pH. To observe the effect of temperature change on the hydrolysis rate, experiments were carried out at 50 and 55 °C, and its effects on fermentation yield were investigated. Fermentation was carried out at 40°C, pH 6.0. 5 M KOH was used for pH control. Bacterial load was determined as 5% (v/v). The experiment period was continued until to obtain a constant monosaccharide and lactic acid concentration.

3.2.4.2.2 Effect of Solid Load on Enzymatic Hydrolysis and Fermentation Yield

In order to monitor the effect of substrate amount on enzymatic hydrolysis and lactic acid production, pretreated horse chestnut shell was added as 8%, 10%, 12%, and 14 % (w/v). Enzyme load was selected as 65 FPU/g. The temperature of the bioreactor used for hydrolysis was determined as 55 °C. pH was controlled by 0.05 M sodium citrate buffer (at 4.8 pH). Fermentation was carried out at 40°C, pH 6.0. 5 M KOH was used for pH control. Bacterial load was determined as 5% (v/v). The experiment period was continued until to obtain a constant monosaccharide and lactic acid concentration.

3.2.5 Analytical Methods

The pH of samples was measured by benchtop pH meter (PL-700 PC, Gondo Electronic Co., Taiwan). Samples taken from the experimental sets at certain time intervals were first centrifuged at 14000 rpm for 5 minutes with a laboratory-type centrifuge (Mikro 220 R, Hettich Lab Technology, 34 Germany). The supernatant

was diluted 100 times and passed through 0.22 μm nylon filters in order not to deform the column and not cause contamination in the channels. The carbohydrate and organic acid concentrations of the prepared samples were analyzed by high-performance liquid chromatography (Agilent Technologies, USA) equipped with a refractive index detector at 35 °C with a 10 μL sample injection. A Rezex™ ROA-Organic Acid H+ (8%) column (300 × 7.8 mm) was used at 55 °C, and 50 mM H_2SO_4 mobile phase flows at 0.6 mL min^{-1}. To improve the precision of HPLC analysis, 0.5 g L^{-1} succinic acid internal standard (known concentration of a succinic acid was added in a sample to measure components of the sample) was used.

3.2.6 Data Analysis

The results obtained in the experiments were shown as the mean values of the replicates. Analysis of variance (ANOVA) was used for statistical interpretation via Minitab 19 (Minitab Inc., UK), and the results were evaluated with the Tukey test with a 95% confidence level. When a p-value was lower or equal to 0.05, statistical differences between the results were considered significant.

CHAPTER 4

RESULTS AND DISCUSSION

This chapter contains the experimental data on the enzymatic hydrolysis of horse chestnut shells through cellulolytic enzyme and the fermentation of lactic acid via SHF, SSF, and SSF2 by *Lactobacillus casei* using hydrolyzate as a carbon source. The influence of parameters and methods in experiments were discussed extensively in the following subsections. In addition;

- All experiments were performed in duplicate and the results in graphs and tables were prepared by taking the mean values, in addition, all results were shown with standard error data.
- Yield and productivity calculations are given in Appendix B
- Calibrations of HPLC analyses for the components observed during the experiment were made and are given in Appendix C.
- Growth curve of *Lactobacillus casei* NRRL B-441 is given in Appendix D.
- Enzymatic hydrolysis, fermentation and glucose consumption rates are given in Appendix E.
- HPLC Chromogram samples are given in Appendix F.
- ANOVA results of the experiments are given in Appendix G.

4.1 Separate Hydrolysis and Fermentation

Cellulolytic enzyme from ASA Spezialenzyme GmbH was used in all experiments. The working pH range for ASA-BG specified in the ASA(2012) product page varies between 4.5 and 6.0, while the temperature range is specified as 50 – 60°C.

For the separate hydrolysis and fermentation part, all experiments were carried out in shaker bottles, and the hydrolyzate contents formed by applying different pH and temperatures were compared.

4.1.1 Effect of pH and Temperature on Enzymatic Hydrolysis

The amount of substrate, temperature, pH, the structure of the salts in the medium, and ionic forces are among the factors that determine enzyme activity. Cheng (1998) investigated the effects of buffers K_2HPO_4/KH_2PO_4, acetic acid/sodium acetate, and citric acid/sodium citrate on the activity of cellulase enzyme. Since the citric acid/sodium citrate buffer provides the highest enzyme activity, it was chosen as the buffer medium in this study.

In all experiments, 10% (w/v) solid load of horse chestnut shell by dry weight and working volumes of 100 mL were prepared in 250 mL bottles and 65 FPU g^{-1} ASA-BG enzyme was added. For the experiment, four different pH values determined as 4.5, 4.8, 5.0, and 5.5 were used at 50 °C, and then the same procedures were followed at 55 °C and 60 °C. The pH of the hydrolysis medium was kept constant by 0.05 M sodium citrate buffer and the temperature was controlled through a shaker incubator. Figures 4.1, 4.2, and 4.3 show the effect of pH and temperature on enzymatic hydrolysis. While determining the hydrolysis time, samples were taken at 8-hour intervals and the constant amount of monosaccharide was reached at the 24th hour.

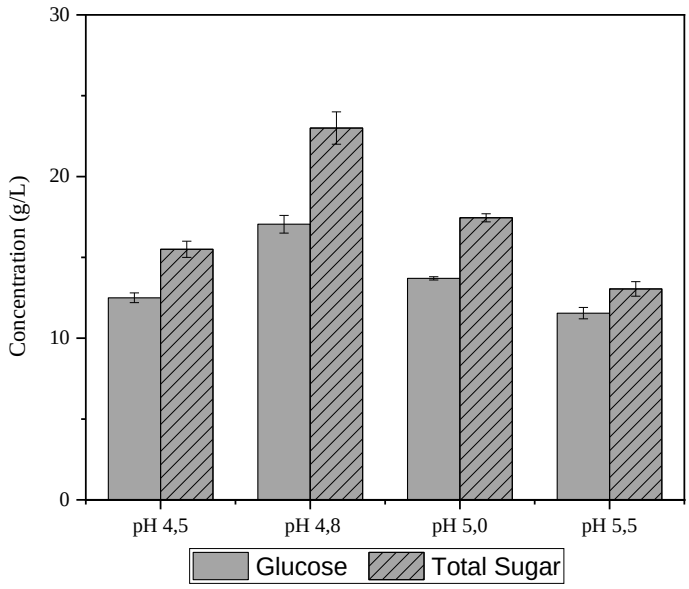


Figure 4.1 Glucose and total sugar conversion in g/L at 50 °C for 4.5, 4.8, 5.0, and 5.5 pH ($p \leq 0.05$, two-way ANOVA were applied independently for glucose and total conversion data, and the results were given in the graph with their standard errors.)

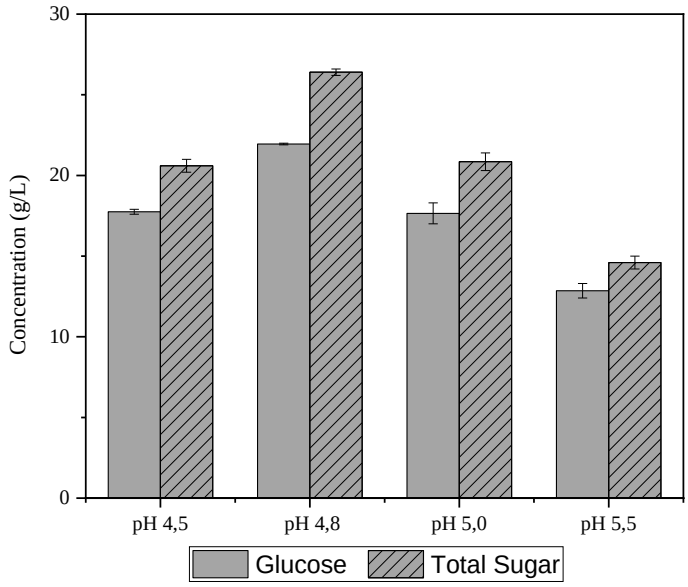


Figure 4.2 Glucose and total sugar conversion in g/L at 55°C for 4.5, 4.8, 5.0, and 5.5 pH ($p \leq 0.05$, two-way ANOVA were independently for glucose and total conversion data, and the results were given in the graph with their standard errors.)

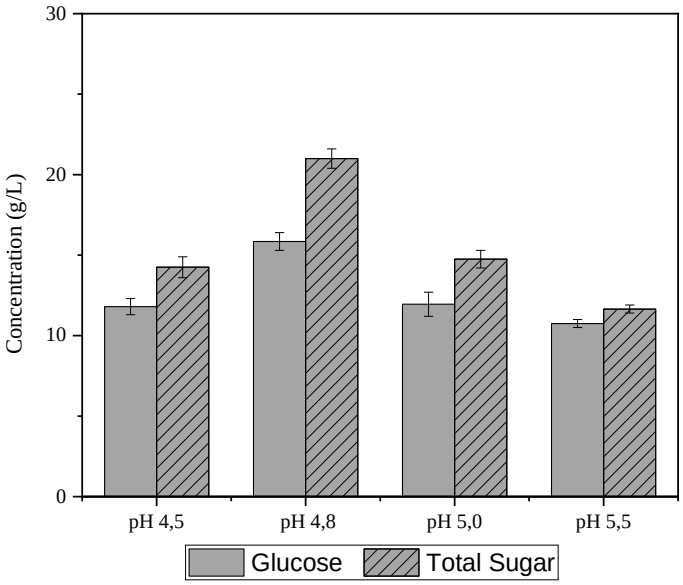


Figure 4.3 Glucose and total sugar conversion in g/L at 60 °C for 4.5, 4.8, 5.0, and 5.5 pH ($p \leq 0.05$, two-way ANOVA were applied independently for glucose and total conversion data, and the results were given in the graph with their standard errors.)

Cellobiose, xylose, fructose, and arabinose were also observed during hydrolysis, but since the concentration was very low compared to glucose, it was included in the total sugar concentration. Based on the results, it is aimed to find optimum pH and temperature values for the ASA-BG enzyme. At the end of the 24-hour hydrolysis, for the same temperature, pH 4.8 was the optimum value, almost the same sugar concentration was observed for pH values of 4.5 and 5.0, and a decrease in sugar concentration was detected when pH increased to 5.5. Besides, for all pH values, the optimum temperature was found to be 55°C. When compared to 50°C and 60°C, the sugar concentration was slightly higher at 50 °C. This difference can be explained by the denaturing of the enzyme protein with the increment in temperature (Scopes, 2002). At 4.8 pH and 55 °C, the glucose concentration, total sugar concentration, and yield were found as respectively 21.95 g L^{-1}, 26.40 g L^{-1}, 0.26 g g^{-1}. Hydrolysis results are shown in Table 4.1.

Table 4.1 Converted glucose and total sugar concentrations, and yields according to pH and temperatures change in enzymatic hydrolysis

Temperature (°C)	pH	Glucose Concentration (g L^{-1})	Total Sugar Concentration ** (g L^{-1})	Yield * (g g^{-1})
50	4.5	12.50 ±0.30	15.50±0.50	0.16±0.01
	4.8	17.05 ±0.55	23.00±1.00	0.23±0.01
	5.0	13.70±0.10	17.45±0.25	0.18±0.00
	5.5	11.55±0.35	13.05±0.45	0.13±0.01
55	4.5	17.75±0.15	20.60±0.40	0.21±0.00
	4.8	21.95±0.05	26.40±0.20	0.26±0.00
	5.0	17.65±0.65	20.85±0.55	0.21±0.01
	5.5	12.85±0.45	14.60±0.40	0.15±0.00
60	4.5	11.80±0.50	14.25±0.65	0.13±0.00
	4.8	15.85±0.55	21.00±0.60	0.20±0.01
	5.0	11.95±0.75	14.75±0.55	0.15±0.01
	5.5	10.75±0.25	11.65±0.25	0.12±0.00

** Grams of total sugar produced/grams of horse chestnut shell added.*

*** Total sugar: xylose, arabinose, fructose, and cellobiose*

To observe the effect of temperature and pH on enzymatic hydrolysis, two-way ANOVA at a 95% confidence level was applied and noticed that pH (p=0.00) and temperature (p=0.00) had the same effect for both glucose and total sugar concentrations. In addition, it was determined that the interaction of pH and temperature could not be evaluated independently of each other for glucose concentration (p=0.00) and total sugar concentrations (p=0.02). Furthermore, the coefficient of determination (R^2) was found 98.73% for glucose and 98.70% for total sugar.

4.1.2 Batch Fermentation of Lactic Acid

Initially, the necessary components for the fermentation medium were added to the hydrolyzate obtained by enzymatic hydrolysis, and the temperature was risen to 80°C, for 1 h to stop the enzyme activity. After decreasing the temperature to 50°C, 55°C and 60°C, the inoculum 5% (v/v) was added under sterile conditions, the fermentation process was started. Initial glucose amounts vary in all experiments, working volumes were prepared to be 100 mL in 250 mL bottles. Initial pH was adjusted to 6.0 ± 0.2 with 5 M NaOH and 30 g L^{-1} calcium carbonate was added as a buffer. The results of the experiments are illustrated in Figures 4.4, 4.5, and 4.6.

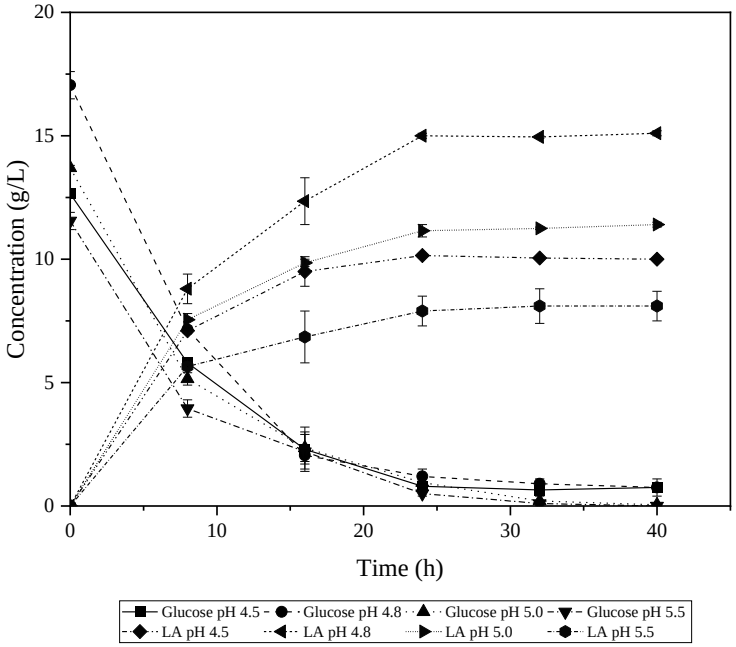


Figure 4.4 Result of lactic acid production by shake flask fermentation of horse chestnut shell hydrolyzed at 50 °C

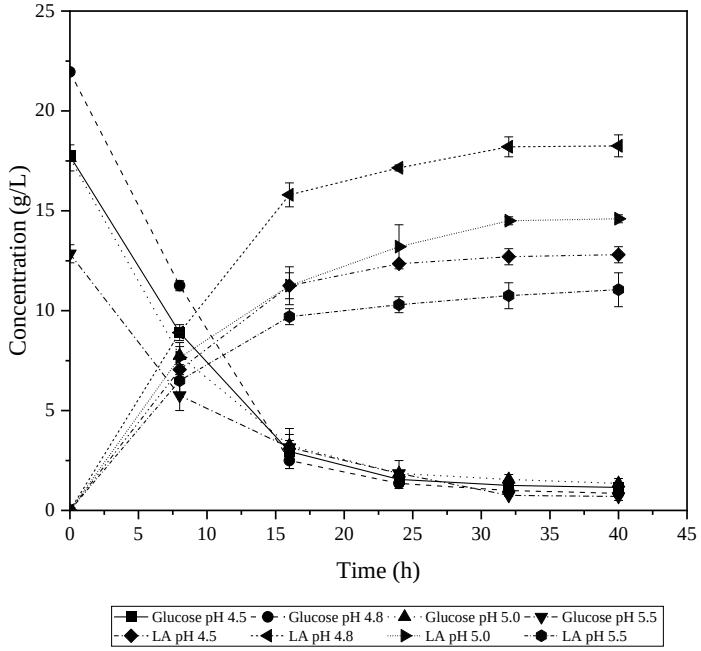


Figure 4.5 Result of lactic acid production by shake flask fermentation of horse chestnut shell hydrolyzed at 55 °C

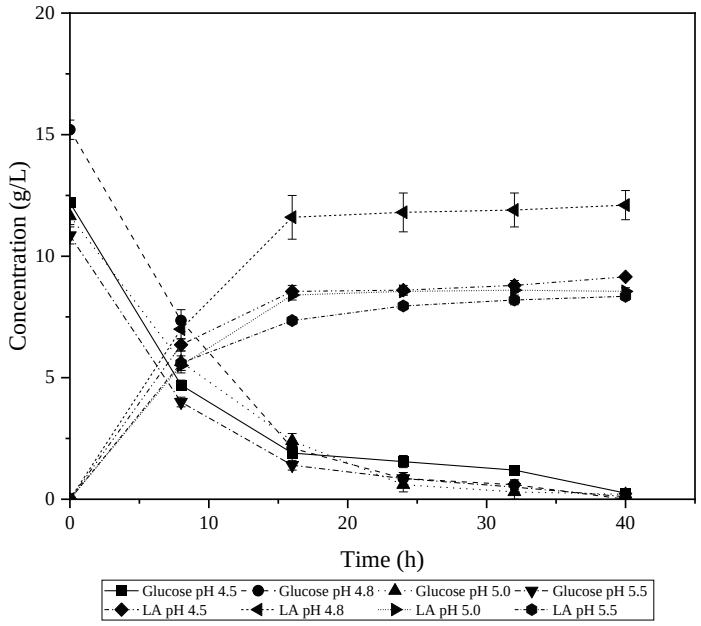


Figure 4.6 Result of lactic acid production by shake flask fermentation of horse chestnut shell hydrolyzed at 60 °C

As a result of the experiment, another organic acid production was not observed except lactic acid. The fermentation process almost ended at the end of 24 hours and stopped completely at the end of the 40th h. Table 4.2 shows the result of lactic acid fermentation. Since the experiment time was the same for all shake flasks, the highest lactic acid production and productivity were achieved as 18.25 g L^{-1}, 0.46 g L^{-1} h^{-1} as a result of fermentation from hydrolyzate (at 55 °C, 4.8 pH) which has a high sugar concentration.

In the experiment where all shake flasks had the same fermentation medium and inoculum size, hydrolysates with different sugar ratios were used. The highest lactic acid yield was 0.18 g g^{-1} with the hydrolyzate obtained from 5.5 pH at 55 °C. Medium supplementation, pH-temperature control, and microbial culture are the main factors affecting lactic acid yield (Ghaffar et al., 2014; Mussatto et al., 2008). Several studies have indicated that using different concentrations of carbon source results in similar lactic acid yields when all conditions same (Dey et al., 2012; Yun et al., 2003). Although temperature control was provided in the experiments and calcium carbonate was used as a buffer for pH control, the pH of the environment decreased from 6.0 to 4.3-5.1 due to the accumulation of lactic acid in the environment over time. Even though all conditions are the same, the inability to get similar lactic acid yields from different sugar concentrations can be explained by the lack of pH control.

Table 4.2. Batch fermentation (at 40°C, 6.0 pH) results using different hydrolyzed in fermentation media

Temperature of Hydrolysate (°C)	pH of Hydrolysate	Total Sugar Concentration (g L^{-1})	Lactic Acid Concentration (g L^{-1})	Productivity (g L^{-1} h^{-1})	Yield* (g g^{-1})	Yield** (g g^{-1})
50	4.5	15.65 ±0.35	10.00±0.00	0.25±0.00	0.64±0.01	0.10±0.00
	4.8	23.00 ±1.00	15.10±0.10	0.38±0.00	0.66±0.02	0.15±0.00
	5.0	17.45±0.25	11.40±0.10	0.29±0.00	0.65±0.00	0.11±0.00
	5.5	13.05±0.45	8.10±0.60	0.20±0.02	0.62±0.03	0.08±0.01

Table 4.2 (continued)

55	4.5	20.60±0.40	12.80±0.40	0.32±0.01	0.62±0.01	0.13±0.00
	4.8	26.40±0.20	18.25±0.55	0.46±0.01	0.69±0.03	0.18±0.01
	5.0	20.85±0.55	14.60±0.20	0.37±0.01	0.70±0.01	0.15±0.00
	5.5	14.60±0.40	11.05±0.85	0.28±0.02	0.76±0.08	0.11±0.01
60	4.5	14.65±0.35	9.15±0.05	0.23±0.00	0.63±0.01	0.09±0.00
	4.8	20.35±0.45	12.10±0.60	0.30±0.02	0.59±0.02	0.12±0.01
	5.0	14.45±0.55	8.55±0.15	0.21±0.00	0.59±0.03	0.09±0.00
	5.5	11.80±0.30	8.35±0.15	0.21±0.00	0.71±0.01	0.08±0.00

**Gram of lactic acid produced per gram of glucose hydrolyzed.*

***Gram of lactic acid produced per gram of dry horse chestnut shell added*

4.2 Simultaneous Saccharification and Fermentation

The pH and temperatures used in simultaneous saccharification and fermentation experiments with a single bioreactor were selected according to the operating temperature and pH ranges of ASA-BG, and the systems were set up accordingly.

4.2.1 Effect of Temperature and pH

In order to determine the effect of temperature on the lactic acid yield gained by simultaneous saccharification and fermentation method by *L. casei*, 4.8 pH 50, 55, 60°C, and 5.5 pH 50, 55, 60°C experiments were carried out. To keep the pH constant in the system, 5.0 M KOH was used, and it was automatically controlled with the temperature. Dried horse chestnut shell was added as the solid load at 10% of the working volume.

After the start of the fermentation process, samples were taken at 8 and 16 h intervals, and glucose and lactic acid concentrations were monitored (Figure 4.7). At the 40th hour of enzymatic hydrolysis and fermentation, the concentration change almost stopped, and stable results were obtained at 72nd h. In addition, cellobiose was detected in the medium, but it was not specified due to its low concentration. At the end of 72 h, glucose and lactic acid were determined at different concentrations of

50, 55, and 60 °C at 4.8 pH. The concentration of lactic acid at 50, 55, and 60 °C were 16.35 g L^{-1}, 6.45 g L^{-1}, and 2.55 g L^{-1} respectively. It was noticed that the yield and productivity of lactic acid concentration achieved decreased significantly with the increase in temperature. Unused glucose amount, which was found to be unexpectedly high, was determined as 17.55 g L^{-1}, 23.00 g L^{-1}, 15.50 g L^{-1} at 50, 55, and 60 °C, respectively. Increase in temperature did not reduce the residual glucose concentration, the maximum amount was obtained at 55 °C. A similar trend was observed for lactic acid and non-consumed glucose concentrations at 50, 55, and 60°C at 5.5 pH, just like at 4.8 pH. The highest lactic acid concentration was found as 18.25 g L^{-1} at 50 °C, while the highest residual glucose concentration was determined as 16.45 g L^{-1} at 55 °C. The results are given in Table 4.3.

Table 4.3. Effect of pH and temperature on the lactic acid production by SSF with single bioreactor

pH	Temperature (°C)	Residual Glucose Concentration (g L^{-1})	Lactic Acid Concentration (g L^{-1})	Productivity (g L^{-1} h^{-1})	Yield* (g/g)
4.8	50	17.55 ± 0.45	16.35 ± 0.55	0.23 ± 0.01	0.16 ± 0.01
	55	23.00 ± 0.00	6.45 ± 0.15	0.09 ± 0.00	0.07 ± 0.00
	60	15.50 ± 0.20	2.55 ± 0.05	0.04 ± 0.00	0.03 ± 0.00
5.5	50	14.25 ± 0.25	18.25 ± 0.25	0.25 ± 0.00	0.18 ± 0.00
	55	16.45 ± 0.55	8.10 ± 0.10	0.11 ± 0.00	0.08 ± 0.00
	60	13.40 ± 0.10	3.05 ± 0.15	0.04 ± 0.00	0.03 ± 0.00

**Gram of lactic acid produced per gram of dry horse chestnut shell added.*

It has been stated in many studies that there is a drop in LA yield due to the departure of *L. casei* from the optimum temperature value (Hao et al., 2021). Although *Lactobacillus sp*. can maintain its viability up to 65°C, serious reductions in yield have been detected above 50°C (Haddaji et al., 2015; Qin et al., 2012). To examine the thermal inactivation, research was carried out on *Lactobacillus plantarum* and *Lactobacillus paracasei*; no decrease in the cell number was noticed in *L. paracasei* kept at 50°C, but a linear decrease in the cell number was detected after 15 minutes

when the temperature rose to 60°C. For *L. plantarum*, thermal inactivation started 2 minutes after 57.5 °C (Capra et al., 2006). For this reason, it is typical for the lactic acid concentration to decrease with increasing temperature. Since the cells were exposed to high temperatures for a long time, they started to lose their viability after a period, and the amount of lactic acid produced lessened with the increase in temperature.

On the other hand, residual glucose amounts were similar to glucose concentrations in the hydrolysis part of SHF experiments (Figure 4.7). The fact that the lactic acid production rate of *L. casei* was lower than the rate of enzymatic hydrolysis caused a reduction in enzyme activity due to product inhibition of cellulase, just like in SHF (Appendix E). Cellulase contains more than one enzyme. Among them, β-glucosidase, which converts cellobiose to glucose, has an important role. As the amount of glucose accumulated in the medium rises, the β-glucosidase activity declines, in this case, the amount of cellobiose increases in the medium and leads to secondary inhibition of cellulase (Kristensen et al., 2009). In the hydrolysis of softwood with cellulase, 10 g/L glucose concentration has been reported to decrease the enzyme activity by 80% (Xiao et al., 2004). In another study, Solka Floc SW 40 was hydrolyzed with the cellulase for 96 hours, as a result, it was noticed that the hydrolysis rate decreased from 9.1 to 1.3 g L^{-1} h^{-1} within the first 4 h due to product inhibition. It was stated that the subsequent decrease in rate remained below 0.5 g L^{-1} h^{-1} (Fan & Lee, 1983). In this case, it can be said that glucose, which cannot be used in the production of lactic acid, reduces the enzyme activity, and almost stops hydrolysis. This prevented a further increment in glucose concentration.

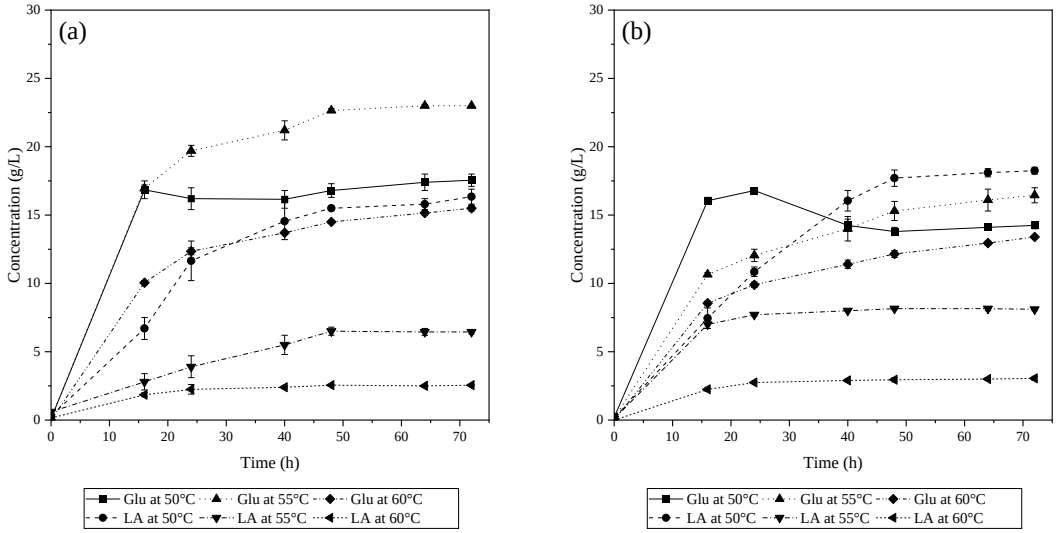


Figure 4.7 Effect on temperature on SSF with single bioreactor a) 50, 55 60°C at 4.8 b) 50, 55, 60°C at 5.5 pH

At 4.8 pH, less lactic acid concentration was obtained than at 5.5 pH at all temperatures, as expected since 5.5 pH closer to the optimal pH of *L. casei*. However, the difference in concentration was not very large. As can be seen from the test results in Table 4.3, the maximum amount of lactic acid was 18.3 g L^{-1} at 50 °C, 5.5 pH, and 16.4 g L^{-1} at 50 °C, 4.8 pH. In addition to the fact that *L. casei* is known to withstand a wide pH range, in a study conducted for four different pH values in the 4.5 - 6.5 range, it was stated while the LA concentration did not change, only the glucose utilization time varied (Büyükkileci & Harsa, 2004; Hossein Nezhad et al., 2010).

It has been noticed that ASA-BG is more sensitive to pH change than *L. casei*. The maximum unused glucose concentration was 23.0 g L^{-1} at 55 °C, 4.8 pH, and 16.5 g L^{-1} at 55 °C, 5.5 pH. Similar results were obtained in the literature. Increasing the pH from 5.0 to 5.5 during hydrolysis of pure cellulose with cellulase from T*richoderma reesei* (optimal pH 5, operating range 4-6) resulted in a reduction in sugar yield from 31.7 g kg^{-1} to 24.7 g kg^{-1}. In another study, it was expressed that when sawdust was hydrolyzed with cellulase obtained from *Aspergillus niger* (pH

range 4-6), the enzyme activity at 4.5 pH was 0.0925 IU mL^{-1}, while at 5.0 pH the enzyme activity reduced to 0.0444 IU mL^{-1} (Acharya et al., 2008).

As a result of a three-way ANOVA performed at a 95% confidence interval, the variation of lactic concentration with time, pH, and the temperature was investigated. Statistically significant results were obtained when all three variables were individual, and it was determined that these three variables affected LA concentration. Moreover, all the binary and triple interactions of these three variables gave a significant result. Furthermore, time, pH, temperature, and all interactions were significant in the residual glucose concentration ($p \leq 0.05$).

4.3 Simultaneous Saccharification and Fermentation with Dual Bioreactor System

The SSF method was developed to prevent product inhibition of the cellulase enzyme. However, although it prevents product inhibition, the achieved yield decreases because the optimum pH and temperature conditions required for enzymatic hydrolysis and lactic acid fermentation are different. For this reason, a different system was developed with 2 bioreactors, and it was aimed to achieve higher lactic acid production.

4.3.1 Effect of Temperature

The system was prepared for the enzymatic hydrolysis reactor at 50, 55°C, 4.8 pH, 10% (w/v) solids load, and the optimum conditions (40°C, 6.0 pH) were kept constant in the bioreactor employed for fermentation (Figure 4.8). In this way, it is aimed to observe the effects of temperature change on enzymatic hydrolysis and lactic acid concentration.

Due to the solution transition between the two reactors, the medium of some enzymes and microorganisms has changed. As in SHF experiments, the highest hydrolysis

efficiency for ASA-BG was determined at 55 °C, 4.8 pH. However, this determined temperature and pH are quite different from the optimum (40°C, 6.0 pH) conditions for *Lactobacillus casei* in lactic acid production.

To reduce the effect of the sudden temperature difference caused by the medium change of *L. casei* and to alleviate the environmental stress, the first hydrolysis temperature used in the experiment was reduced from 55 °C to 50 °C. Although this has a positive effect on lactic acid production, it has a more negative effect on enzymatic hydrolysis. According to the test results, lactic acid produced at 55 °C had the highest production and productivity (42.1 g L^{-1} 0.59 g L^{-1} h^{-1}) with a yield of 0.42 g g^{-1}. To examine the effect of temperature and time on lactic acid concentration, a two-way ANOVA with a 95 % confidence interval was applied, $p \leq 0.05$ and R^2 as 94.7 %. In the light of this finding, it was determined that enzyme activity was more sensitive to temperature change than *L. casei*. The results are given in Table 4.4.

Table 4.4. Effect of temperature on the lactic acid production by SSF with dual bioreactor system

Temperature (°C)	Concentration (g L^{-1})	Productivity (g L^{-1} h^{-1})	Yield* (g g^{-1})
50	32.55 ± 0.55	0.45 ± 0.01	0.33 ± 0.01
55	42.10 ± 0.20	0.59 ± 0.00	0.42 ± 0.00

**Gram of lactic acid produced per gram of dry horse chestnut shell added.*

It has been reported that sudden changes in pH and temperature create environmental stress on the microorganisms and resulted in a decrease in lactic acid concentration (Hao et al., 2021). In addition to environmental stress, moving away from the optimum temperature and pH leads to a reduction in LA yield. Qin and co-workers (2012) investigated the effect of temperature on the lactic acid production of *L. casei* and noticed the lactic acid yield reduced from 0.94 g g^{-1} at 41 °C to 0.35 g g^{-1} at 50 °C. Although the temperature increment decreased the yield, *L. casei* can

withstand heat shocks up to 75°C and cultivated up to 65°C, as well as to adapt to environmental stress by changing the synthesis rate of different specific proteins (Haddaji et al., 2015). Besides the temperature, pH changes generate alter in yield, but it has been stated that *L.casei* is less sensitive to pH swap and can grow up to 3.0 pH (Hossein Nezhad et al., 2010). Moreover, Büyükkileci and Harsa (2004) investigated the effect of different pH (5.0, 5.5, 6.0, and 6.5) on lactic acid fermentation by *L. casei*, and observed that similar lactic acid concentrations were obtained at different fermentation times. Fermentation took 23 hours at pH 5.0, lasted 12 hours at pH 5.5, 6.0 and 6.5. Thus, it can be said that the change in pH influences the fermentation time rather than the concentration of lactic acid.

Considering this information, it can be said that *L. casei* has a high chance of growing and surviving under environmental stress and can repair the damage caused by stress thanks to its modified proteins. Therefore, lactic acid production at 55 °C can be expected to exceed 50 °C. While the enzyme was working effectively at 55 °C, *L. casei*, which were transferred to a different medium, were able to repair the damage and continue fermentation when they returned to the fermentation medium.

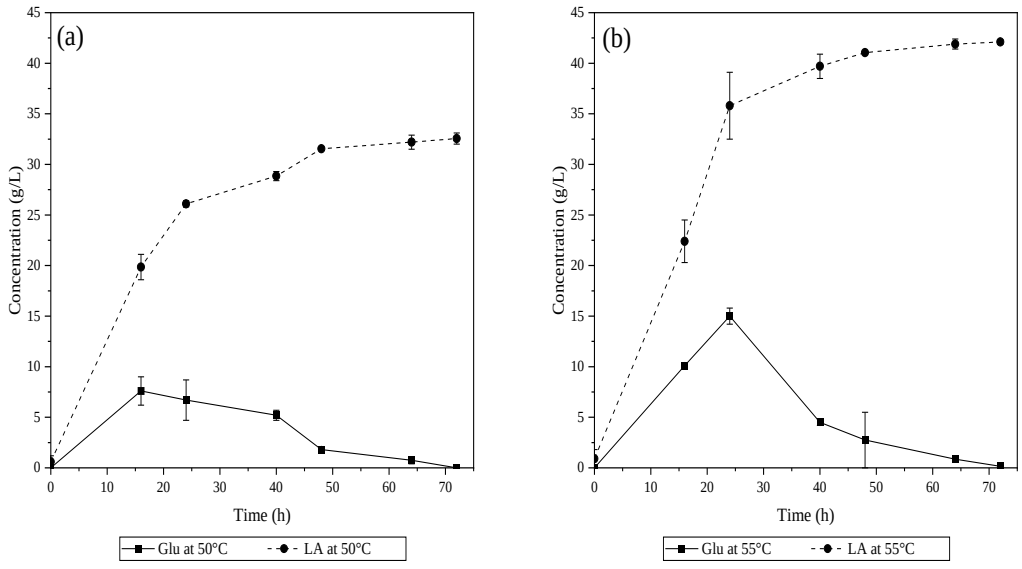


Figure 4.8 Illustrations of SSF with dual bioreactor system experiments with varied temperatures (40°C, 6.0 pH used in bioreactor for fermentation) a) 50°C b) 55°C

As shown in Figure 4.8, at the beginning of the SSF process (0-15 hours for 50 °C, 0-25 hours for 55 °C), glucose release from the pretreated HCS occurred rapidly, with concentrations of 7.6 g L^{-1} and 15 g L^{-1}, respectively. At this stage, the adaptation of *L. casei* cells to the environment led to the fermentation rate not reaching the enzymatic hydrolysis rate. In the following time, the glucose concentration decreased gradually with the decrease in the amount of hydrolysable substrate in HCS and the adaptation of the microorganisms to new environment, thus the rate of fermentation preceded the rate of hydrolysis.

4.3.2 Effect of Solid Load

In this part of the experimental set, it was aimed to obtain a glucose titer that would provide higher lactic acid concentration by changing the amount of horse chestnut shell.

The optimum conditions (at 55°C and 4.8 pH) determined for hydrolysis in the SHF method were applied for the bioreactor where the enzymatic hydrolysis took place, and the optimum conditions for the lactic acid fermentation of *Lactobacillus casei* (40°C and 6.0 pH) were applied in the other bioreactor. While preparing 0.05 M sodium citrate buffer for enzymatic hydrolysis, 30 g L^{-1} calcium carbonate was used for lactic acid fermentation and pH was controlled with 5 M KOH. Since the solution transition between the reactors was slow and the pH dropped due to lactic acid accumulation over time, the pH equalization of the two reactors was close to the termination of lactic acid production. Thus, the enzyme activity was almost unaffected.

As the solid load increases, more sugar is hydrolyzed, resulting in more glucose and carbon sources available for lactic acid formation. For this reason, it was assumed that the increase in lactic acid concentration was dependent on the amount of solid load. Therefore, SSF experiments were carried out with 8%, 10%, 12%, and 14% (w/v) HCS solids load at 72 hours. As seen in Figure 4.9, there is an increase in lactic acid concentration as the solid load increases. Statistical results are significant ($p \leq$

0.05) by obtaining similar results in repeated experiments. After the regression analysis, it was determined that there was a linear relationship between LA concentration and solid load (Figure 4.10). Due to the problems arising from the viscosity, it was not possible to carry out experiments above 12% (w/v) solids load. In the experiments prepared with different solid-liquid ratios, it was declared that lactic acid and hydrolysis efficiency decreased when it was exceeded 20% (w/v) (Alves de Oliveira et al., 2018). This is explained by the fact that mixing cannot be effective due to the increase in viscosity when the solid load is increased by 20% (w/v) or more (Kristensen et al., 2009). The concentration, yield, and productivity of fermentations carried out with different solid loads are shown in Table 4.5. Although the lactic acid yields were similar at the end of the experiment, a rise in the solid load escalated the concentration and fermentation rates (Appendix E) and thus the productivity.

Table 4.5. SSF with dual bioreactor system results using different solid loads.

Solid Load (%)	Lactic acid Concentration (g L^{-1})	Productivity (g L^{-1} h^{-1})	Yield* (g g^{-1})
8	34.65 ± 0.15	0.48 ± 0.00	0.43 ± 0.00
10	42.10 ± 0.20	0.59 ± 0.00	0.42 ± 0.00
12	50.85 ± 0.35	0.71 ± 0.01	0.43 ± 0.00

**Gram of lactic acid produced per gram of dry horse chestnut shell added.*

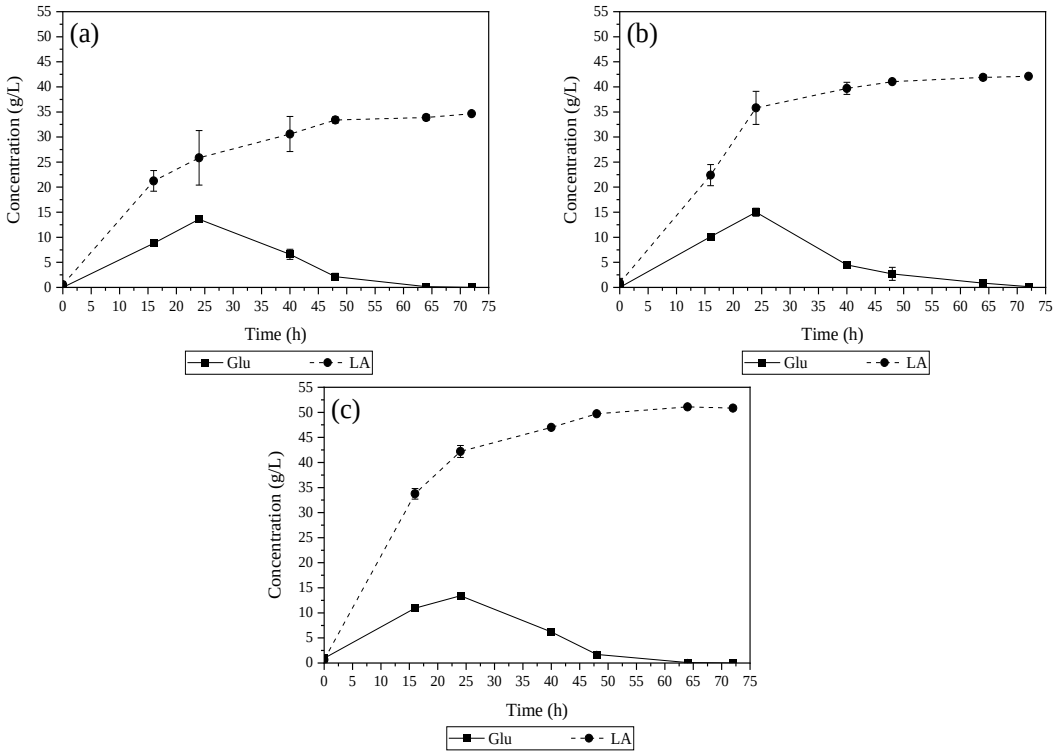


Figure 4.9 Effect of different solid loads on lactic acid accumulation by simultaneous saccharification and fermentation method with dual bioreactor system a) 8% b) 10 % c) 12% (($p \leq 0.05$) and two-way ANOVA was applied for lactic acid accumulation data.)

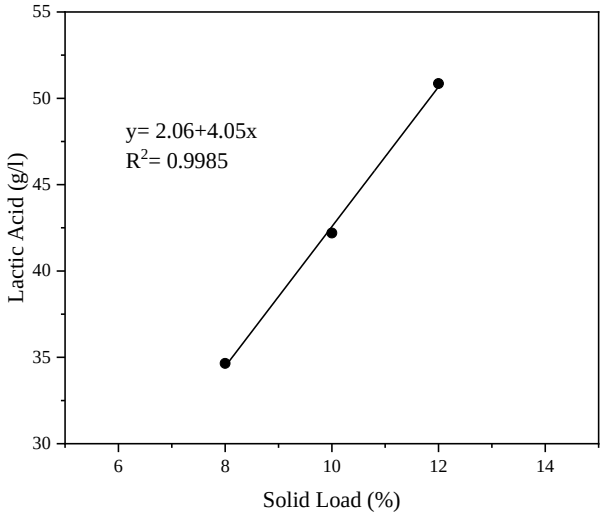


Figure 4.10 Relationship between lactic acid concentration and solid load.

4.4 Comparison in Terms of Lactic Acid Production of SHF, SSF, and SSF with Dual Bioreactor System

In experiments based on the operating temperature and pH of the ASA-BG enzyme (in the range of 50-60 C 4.5-6.0 pH), SHF, SSF, and SSF with dual bioreactor processes were used in lactic acid production, and compared in terms of concentration, yield, and productivity. The results attained from the study are summarized in Table 4.6. In the experiments performed at 55 and 50°C and 4.8 pH, the highest yields were achieved as 0.42 g g^{-1} and 0.33 g g^{-1} respectively in the SSF system with dual bioreactor. SHF followed it with 0.18 g g^{-1} (55°C), and 0.15 g g^{-1} (50°C), while the lowest yield was found in SSF with 0.07 g g^{-1} (55°C) and 0.16 g g^{-1} (50°C). In addition, it was observed that the fermentation rate (at 4.8 pH, 55ºC) of SSF2 was almost 1.2 times higher than the fermentation rate of SHF, and almost 4.1 times higher than that of SSF. On the other hand, when fermentation rates at 50 °C and 4.8 pH were compared, it was observed that SSF2 was approximately 1.1 times higher than SHF and approximately 1.5 times higher than SSF (Appendix E). Since the SSF process was developed to prevent product inhibition that occurs in SHF, higher yields are expected. However, in the literature, when SHF and SSF processes are compared, SSF has not always been a more efficient process for converting inert materials to valuable end products, in some studies, more efficient results have been procured with the SHF process (Table 4.7). In a study of ethanol production, where wheat straw is used as raw material, the SHF yield is 0.50 g g^{-1}, while the SSF yield is 0.39 g g^{-1} (Saha et al., 2011). In another study, in the production of butanol from the same raw material, 133 g kg^{-1} yield was determined in SHF and 143 g kg^{-1} yield in SSF(Qi et al., 2019). Since the microorganisms and enzyme brands used in these two studies were different, the processes with higher yields varied. In the study where high yield SHF is obtained, the optimum conditions between the enzyme and the microorganism are far from each other, on the other hand in the study where the high yield is gained from SSF, the optimum conditions are closer.

Table 4.6 Lactic acid concentration, productivity, and yield results of SHF, SSF, and SSF2 processes

Process	Temperature (°C)	Fermentation Time (h)	Total Time (h)	Lactic Acid Concentration ($g\ L^{-1}$)	Productivity Based on Fermentation Time ($g\ L^{-1}\ h^{-1}$)	Productivity Based on Total Time	Yield* (g/g)
SHF	50	40	64	15.10 ± 0.10	0.38 ± 0.00	0.24 ± 0.00	0.15 ± 0.00
	55	40	64	18.25 ± 0.55	0.46 ± 0.01	0.29 ± 0.01	0.18 ± 0.00
SSF	50	72	72	16.35 ± 0.55	0.23 ± 0.01	0.23 ± 0.01	0.16 ± 0.01
	55	72	72	6.45 ± 0.15	0.09 ± 0.00	0.09 ± 0.00	0.07 ± 0.00
SSF2	50	72	72	32.55 ± 0.55	0.45 ± 0.01	0.45 ± 0.01	0.33 ± 0.01
	55	72	72	42.10 ± 0.20	0.59 ± 0.03	0.59 ± 0.03	0.42 ± 0.00

** Gram of lactic acid produced per gram of dry horse chestnut shell added.*

The low SSF yield achieved in the experiment was due to the different temperature and pH requirements for ASA-BG and *Lactobacillus casei*. The carbon source in the medium was not used in the production of lactic acid by the microorganisms that were damaged by the high temperature. This situation also affected enzyme activity, just like in SHF, and prevented the increment of the glucose concentration. In order to obtain high efficiency in the SSF process, it is necessary to choose the optimum conditions of the microorganism and the enzyme close to each other, if the optimum conditions cannot be approached, it is necessary to encapsulate the microorganism to reduce damage, or to change the process mechanism used (Hetényi et al., 2011; Maslova et al., 2019).

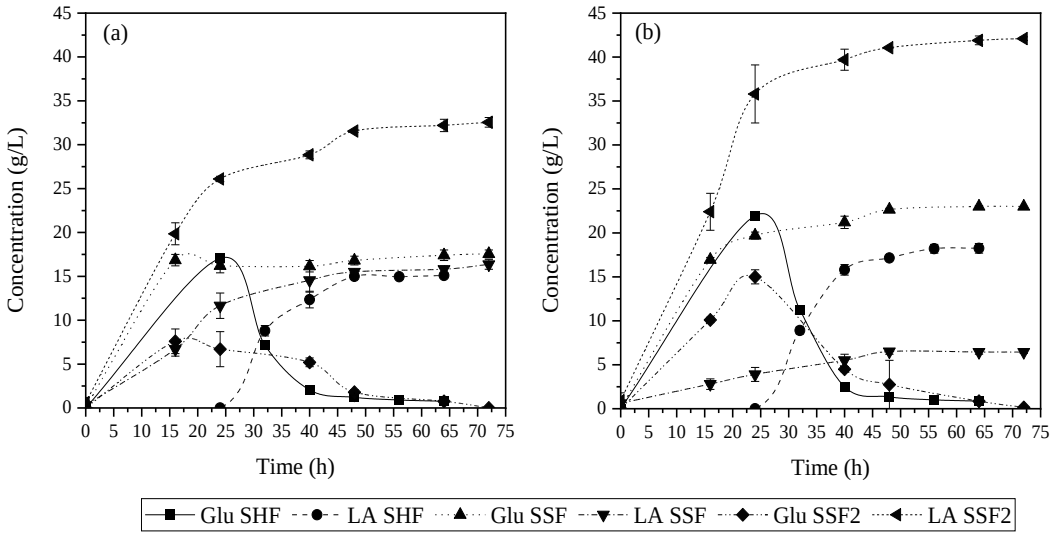


Figure 4.11 Variation of lactic acid and glucose concentrations of SHF (64 h), SSF (72 h), and SSF2 (72 h) processes over time (for SSF2 pH of bioreactor for hydrolysis at 4.8, and pH of bioreactor for fermentation at 6.0) a) at 50°C, 4.8 pH b) at 55°C, 4.8 pH

The SSF with dual bioreactor process has been developed to produce valuable end products from lignocellulosic biomass with high efficiency while using enzymes and microorganisms with such different optimum conditions. Although the extraction of copper (II) ion (Parus, 2018), and proanthocyanidins which have anti-obesity (Kimura et al., 2011) and retinal protective effects (Ishihara et al., 2018) from horse chestnut shell, has been studied in the literature, there are no articles in which it is used as a carbohydrate source in the production of organic acids, but there are a few studies in which chestnut shell is exposed to enzymatic hydrolysis. As a result of hydrolysis of different pretreated chestnut shells, an amount of glucose in the range of 27.6 - 57.8% was determined (He, Liu, Di, et al., 2016; He, Liu, Gong, et al., 2016; K. H. Lee et al., 2021; Maurelli et al., 2013). Similar glucose levels could be expected in HCS. In this case, it can be said that almost maximum efficiency was achieved in the experiment made by SSF2 since in the study performed by adding 10% solid load (w/v), the achieved lactic acid concentration was 42.1 g L^{-1} at 55 °C and 32.6 g L^{-1} at 50 °C. The difference between the two temperatures is due to the enzyme activity,

the hydrolysis rate at 55 °C was determined to be approximately 1.7 times higher than at 50 °C (Appendix E).

In addition to the yield gained, the processes have features that are superior to each other. Since SSF and SSF2 are single-stage processes, the risk of contamination is low, whereas the risk of SHF, which is a two-stage process, is higher. The redundancy of the equipment used, the area occupied by this equipment, and the energy consumed are similar in SHF and SSF with dual bioreactor, while SSF is more advantageous in this regard. The constant lactic acid concentration was observed as 64 hours for SHF and 72 hours for SSF2 and SSF in the experiment. However, the concentration changes after 48 hours in all three processes can be negligible. The lactic acid concentration in SSF2 was 41.1 g/L at the 48th hour and 42.1 g/L at the 72nd hour.

Table 4.7 Compilation of studies on the production of valuable end products from lignocellulosic biomass by using SHF and SSF processes

Substrate	Process	Enzyme	Microorganism	Product	Concentration ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)	Yield ($g\ g^{-1}$)	Ref.
Wheat Straw	SSF	Celluclast 1.5L and β-glucosidase	*R. oryzae* F-814	Lactic acid	33.4	0.84 ± 0.03	0.29 ± 0.01	(Maslova et al., 2019)
Wheat Straw	SHF	Celluclast 1.5L and β-glucosidase	*R. oryzae* F-814	Lactic Acid	29.2	0.73 ±0.03	0.33 ± 0.01	(Maslova et al., 2019)
Wheat Straw	SSF	Celluclast 1.5L and Novozym188	*E. coli* FBR5	Ethanol	17.4	0.18	0.20	(Saha et al., 2011)
Wheat Straw	SHF	Celluclast 1.5L and Novozym188	*E. coli* FBR5	Ethanol	21.9 ± 0.3	0.24	0.25	(Saha et al., 2011)
Wheat Straw	SSF	Cellulase and xylanase from Imperial Jade Bio-Technology	*C. acetobutylicum* ATCC 824	Acetone Butanol Ethanol	19.18	0.15	0.14	(Qi et al., 2019)
Wheat Straw	SHF	Cellulase and xylanase from Imperial Jade Bio-Technology	*C. acetobutylicum* ATCC 824	Acetone Butanol Ethanol	17.75	0.11	0.13	(Qi et al., 2019)

Table 4.7 (continued)

Corncob	SSF	Cellulase from Meiji Pharmaceutic	*A. thermophilus* TCC 24622 *Rhizopus sp.* MK-96-1196	Lactic Acid	24	0.33	0.24	(Miura et al., 2004)
Corncob	SHF	Cellulase from Meiji Pharmaceutic	*Rhizopus sp.* MK-96-1196	Lactic Acid	28	0.38	0.28	(Miura et al., 2004)
Corncob	SSF	Cellic® CTec2	*R. oryzae* NLX-M-1	Lactic Acid	60.3	1.00	0.60	(Zhang et al., 2015)
Corncob	SHF	Cellic® CTec2	*R. oryzae* NLX-M-1	Lactic Acid	34.0	0.71	0.34	(Zhang et al., 2015)
Wood Chips	SSF	glutase-AN and Cellic® CTec 2	*C. acetobutylicum* NBRC13948	Acetone Butanol Ethanol	13.8	0.10	-	(Sasaki et al., 2014)
Wood Chips	SHF	glutase-AN and Cellic CTec 2	*C. acetobutylicum* NBRC13948	Acetone Butanol Ethanol	12.1	0.16	-	(Sasaki et al., 2014)
Horse Chestnut Shell	SSF	ASA-BG	*L. casei* NRRL B-441	Lactic Acid	6.45 ± 0.2	0.09 ± 0.00	0.07 ± 0.00	This Study
Horse Chestnut Shell	SHF	ASA-BG	*L. casei* NRRL B-441	Lactic Acid	18.3 ± 0.6	0.29 ± 0.01	0.18 ± 0.01	This Study
Horse Chestnut Shell	SSF*	ASA-BG	*L. casei* NRRL B-441	Lactic Acid	42.1 ± 0.2	0.59 ± 0.00	0.42 ± 0.00	This Study

**Simultaneous saccharification and fermentation with dual bioreactor*

The findings of studies using lignocellulosic biomass in the production of valuable end products by SSF and SHF methods are listed in Table 4.7 together with the results obtained from this study. In some studies, the results obtained from the SSF process were found to be higher, while in others, the SHF process gave higher results. Depending on the optimal condition difference of the microorganism and enzyme, the results obtained from the SSF process were found to be higher in some studies, while the SHF process gave higher results in others.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

In this study, fermentative lactic acid production was investigated through separate hydrolysis and fermentation, simultaneous saccharification and fermentation, and simultaneous saccharification and fermentation with dual bioreactor processes using dried horse chestnut shell as the main carbon source. *Lactobacillus casei* was used as the lactic acid-producing microorganism.

During the enzymatic hydrolysis experiments in the SHF process, the effects of temperature and pH on the saccharification of dried horse chestnut shells were investigated, and hydrolysis was carried out at 50, 55, 60 °C at pH 4.5, 4.8, 5.0, and 5.5. In the experiment performed with a working volume of 100 ml in a 250 ml flask, the highest total sugar and glucose content was found as 21.95 g L^{-1} and 26.40 g L^{-1} at 55 °C and 4.8 pH, respectively. Thus, optimum conditions for the commercial cellulase enzyme (ASA-BG) employed were determined and considered for other processes in the study. Then, batch fermentation was started and as a result, the highest lactic acid concentration was found as 18.25 g L^{-1} from the hydrolyzate obtained at 55 °C, 4.8 pH. The yield calculated with dried horse chestnut shell was 0.18 g g^{-1}. In addition, according to the two-way ANOVA results, it was determined that both glucose and total sugar conversion were significantly affected by pH and temperature ($p < 0.05$). The hydrolysis time was determined as 24 hours and the total process time as 64 hours.

In SSF experiments where hydrolysis and fermentation were carried out simultaneously, experiments were performed at 50, 55, and 60°C, 4.8 and 5.5 pH to measure how AS-BG and *Lactobacillus casei* reacted to temperature and pH. A decrease in lactic acid production was noticed with increasing temperature. In

addition, while the lactic acid concentration obtained at pH 4.8 for each temperature was low, it was higher at pH 5.5. It has also been observed that the microorganism reacts more to temperature change rather than pH. The highest residual glucose concentration was found at 23.0 g L^{-1} at 55°C, 4.8 pH. However, since microorganisms were negatively affected by high temperature, lactic acid production was limited (6.45 g L^{-1}). The best yield was 0.18 g g^{-1} at 50°C, 5.5 pH. In this condition, the lactic acid concentration was found to be 18.25 g L^{-1}, and the residual glucose concentration was 14.25 g L^{-1}. In the three-way ANOVA, the effects of temperature, pH, and time on lactic acid and residual glucose concentration were individually significant ($p \leq 0.05$). Moreover, double, and triple intersections gave significant results ($p \leq 0.05$).

The effects of temperature and solid load on lactic acid concentration were investigated in experiments carried out in SSF2. First of all, the temperature of the bioreactor employed in hydrolysis was set to 50, 55°C, and the temperature of the bioreactor used for fermentation was kept constant at 40°C. The lactic acid concentration and yield achieved were 42.1 g L^{-1}, 0.42 g g^{-1} at 55°C, 32.55 g L^{-1}, 0.33 g g^{-1} at 50°C. As in the SSF experiments, higher lactic acid concentration was not obtained at 50°C because the continuous transition between the bioreactors allowed the bacteria to maintain their viability even though they were damaged by the high temperature. Thus, there was no decrease in lactic acid production rate (Appendix E), and hydrolysis and fermentation could be carried out under optimum conditions.

Secondly, SSF2 experiments were carried out with 8%, 10%, 12%, and 14%(w/v) solids load to investigate the effect of substrate amount on lactic acid production. However, due to the difficulties in handling the HCS, the amount of solid load could not be increased above 12% (w/v). A linear increase in lactic acid concentration was observed as the solid load amount increased. Two-way ANOVA was applied to solid loads-time and temperatures-time. The results showed both of them were significant ($p \leq 0.05$)

The three processes used in the study were compared with each other at 55°C and 50°C, at 4.8 pH, with 10% (w/v) solids load. At 55°C, 4.8 pH, the highest lactic concentration and yield were achieved in SSF2 (42.10 g L^{-1}, 0.42 g g^{-1}), followed by SHF (18.25 g L^{-1}, 0.18 g g^{-1}), and SSF (6.45 g L^{-1}, 0.07 g g^{-1}) was the lowest among them. At 50 °C, maximum results were also obtained at SSF2 (32.55 g/L, 0.33 g/g), while SSF (16.35 g L^{-1}, 0.16 g g^{-1}) and SHF (15.10 g L^{-1}, 0.15 g g^{-1}) had little difference between. At both temperatures, the results in SSF2 were way ahead of other processes by a large margin. It was noticed that the fermentation rate of SSF2 (at 4.8 pH, 55ºC) was approximately 1.2 times higher than that of SHF and approximately 4.1 times higher than SSF. Fermentation rate of SSF2 at 50 °C and 4.8 pH were found to be approximately 1.1 times higher than SHF and 1.5 times higher than SSF (Appendix E). In this case, the newly developed SSF2 method has proven to be an ideal system to produce valuable end products from lignocellulosic raw material for enzymes and microorganisms whose optimum conditions do not have approximate values. In addition, it has been determined that the horse chestnut shell is promising lignocellulosic biomass in the production of valuable end products by fermentation. However, further studies should be done before using the SSF2 method and horse chestnut shells in large-scale production. The effect of applying different pre-treatment processes on horse chestnut shells, strain change, and enzyme load on yield should be examined. Moreover, the rate of transition between bioreactors and supplementation of nitrogen/vitamin/mineral sources should be optimized. After investigating the effects of these parameters on the cost, an economical lactic acid production bioprocess can be developed.

REFERENCES

Abdel-Rahman, M. A., Tashiro, Y., & Sonomoto, K. (2011). Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: Overview and limits. *Journal of Biotechnology*, *156*(4), 286–301. https://doi.org/10.1016/j.jbiotec.2011.06.017

Abdel-Rahman, M. A., Tashiro, Y., & Sonomoto, K. (2013). Recent advances in lactic acid production by microbial fermentation processes. *Biotechnology Advances*, *31*(6), 877–902. https://doi.org/10.1016/j.biotechadv.2013.04.002

Abedi, E., & Hashemi, S. M. B. (2020). Lactic acid production – producing microorganisms and substrates sources-state of art. *Heliyon*, *6*(10), e04974. https://doi.org/10.1016/j.heliyon.2020.e04974

Abrha, B., & Gashe, B. A. (1992). Cellulase production and activity in a species of *Cladosporium*. *World Journal of Microbiology & Biotechnology*, *8*(2), 164–166. https://doi.org/10.1007/BF01195839

Acharya, P. B., Acharya, D. K., & Modi, H. A. (2008). Optimization for cellulase production by *Aspergillus niger* using sawdust as substrate. *African Journal of Biotechnology*, *7*(22), 4147–4152.

Adapa, P., Tabil, L., & Schoenau, G. (2009). Compaction characteristics of barley, canola, oat and wheat straw. *Biosystems Engineering*, *104*(3), 335–344. https://doi.org/10.1016/j.biosystemseng.2009.06.022

Agbor, V. B., Cicek, N., Sparling, R., Berlin, A., & Levin, D. B. (2011). Biomass pretreatment: Fundamentals toward application. *Biotechnology Advances*, *29*(6), 675–685. https://doi.org/10.1016/j.biotechadv.2011.05.005

Akhtar, N., Gupta, K., Goyal, D., & Goyal, A. (2016). *Recent Advances in Pretreatment Technologies for Efficient Hydrolysis of Lignocellulosic Biomass*. *35*(2), 489–511. https://doi.org/10.1002/ep

Akom, A., Fanyin - Martin, A., Boateng, C. O.-, Otoo, E., & Dawoe, E. (2020). Yield and Cost of Biochar Produced by a Locally Fabricated Reactor. *Journal of Agriculture and Environmental Sciences*, *9*(2), 1–5. https://doi.org/10.15640/jaes.v9n2a1

Alriols, M. G., García, A., Llano-ponte, R., & Labidi, J. (2010). Combined organosolv and ultrafiltration lignocellulosic biorefinery process. *Chemical Engineering Journal*, *157*(1), 113–120. https://doi.org/10.1016/j.cej.2009.10.058

Alsaheb, R. A. A., Aladdin, A., Othman, N. Z., Malek, R. A., Leng, O. M., Aziz, R., Enshasy, H. A. El, & El, H. A. (2015). Lactic acid applications in pharmaceutical and cosmeceutical industries. *Journal of Chemical and Pharmaceutical Research*, *7*(10), 729–735.

Alves de Oliveira, R., Komesu, A., Vaz Rossell, C. E., & Maciel Filho, R. (2018). Challenges and opportunities in lactic acid bioprocess design—From economic to production aspects. *Biochemical Engineering Journal*, *133*, 219–239. https://doi.org/10.1016/j.bej.2018.03.003

Ameen, S., & Caruso, G. (2020). *Lactic Acid in the Food Industry* (Issue January 2017). https://doi.org/10.1007/978-3-319-58146-0

Amraini, S. Z., Ariyani, L. P., Hermansyah, H., Setyahadi, S., Rahman, S. F., Park, D. H., & Gozan, M. (2017). Production and characterization of cellulase from *E. coli* EgRK2 recombinant based oil palm empty fruit bunch. *Biotechnology and Bioprocess Engineering*, *22*(3), 287–295. https://doi.org/10.1007/s12257-017-0034-2

Andrić, P., Meyer, A. S., Jensen, P. A., & Dam-Johansen, K. (2010). Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis. II. Quantification of inhibition and suitability of membrane reactors. *Biotechnology Advances*, *28*(3), 407–425. https://doi.org/10.1016/j.biotechadv.2010.02.005

Aqsha, A., Tijani, M. M., Moghtaderi, B., & Mahinpey, N. (2017). Catalytic pyrolysis of straw biomasses (wheat, flax, oat and barley) and the comparison of their product yields. *Journal of Analytical and Applied Pyrolysis*, *125*(March), 201–208. https://doi.org/10.1016/j.jaap.2017.03.022

Axelsson, L, & Narvhus, J. . (2003). *ENCYVLOPEDIA OF FOOD SCIENCES AND NUTRITION (Second Edition)* (B. Caballero (Ed.)). Academic Press. https://doi.org/https://doi.org/10.1016/B0-12-227055-X/00673-8.

Axelsson, Lisa, Franzén, M., Ostwald, M., Berndes, G., Lakshmi, G., & Ravindranath, N. H. (2012). Perspective: Jatropha cultivation in southern India: Assessing farmers' experiences. *Biofuels, Bioproducts and Biorefining*, *6*(3), 246–256. https://doi.org/10.1002/bbb

Bajpai, P. (2019). *Pretreatment of Lignocellulosic Biomass for Biofuel Production* (K. S. Sharma (Ed.)). https://doi.org/10.1007/978-981-10-0687-6

Balat, M. (2011). Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. *Energy Conversion and Management*, *52*(2), 858–875. https://doi.org/10.1016/j.enconman.2010.08.013

Baldrian, P. (2006). Fungal laccases-occurrence and properties. *FEMS Microbiology Reviews*, *30*(2), 215–242. https://doi.org/10.1111/j.1574-4976.2005.00010.x

Bendahou, A., Dufresne, A., Kaddami, H., & Habibi, Y. (2007). Isolation and structural characterization of hemicelluloses from palm of *Phoenix dactylifera L. Carbohydrate Polymers*, *68*(3), 601–608. https://doi.org/10.1016/j.carbpol.2006.10.016

Bensah, E. C., & Mensah, M. (2013). Chemical pretreatment methods for the production of cellulosic ethanol: Technologies and innovations. *International Journal of Chemical Engineering*, *2013*. https://doi.org/10.1155/2013/719607

Bhat, M. K. (2000). Cellulases and related enzymes in biotechnology.

Biotechnology Advances, *18*(5), 355–383. https://doi.org/10.1016/S0734-9750(00)00041-0

Bjornsdottir, S. H., Blondal, T., Hreggvidsson, G. O., Eggertsson, G., Petursdottir, S., Hjorleifsdottir, S., Thorbjarnardottir, S. H., & Kristjansson, J. K. (2006). *Rhodothermus marinus*: Physiology and molecular biology. *Extremophiles*, *10*(1), 1–16. https://doi.org/10.1007/s00792-005-0466-z

Bon, E. P. S., & Ferrara, M. A. (2013). *Bioethanol Production via Enzymatic Hydrolysis of Cellulosic Biomass*. *October 2007*, 1–11.

Brandt, A., Gräsvik, J., Hallett, J. P., & Welton, T. (2013). Deconstruction of lignocellulosic biomass with ionic liquids. *Green Chemistry*, *15*(3), 550–583. https://doi.org/10.1039/c2gc36364j

Buckle, K. E. N., Camire, M. E., Heymann, H., Hutkins, R., Lund, D. B., Weaver, C., Kinsella, J. E., & Taylor, S. L. (1995). Advisory Boards Series Editors. In *Advances in Food and Nutrition Research* (Vol. 57). http://dx.doi.org/10.1016/S1043-4526(09)57007-6

Buschle-Diller, G., Fanter, C., & Loth, F. (1999). Structural Changes in Hemp Fibers as a Result of Enzymatic Hydrolysis with Mixed Enzyme Systems. *Textile Research Journal*, *69*(4), 244–251. https://doi.org/10.1177/004051759906900403

Butler, E., Devlin, G., Meier, D., & McDonnell, K. (2013). Characterisation of spruce, salix, miscanthus and wheat straw for pyrolysis applications. *Bioresource Technology*, *131*, 202–209. https://doi.org/10.1016/j.biortech.2012.12.013

Büyükkileci, A. O., & Harsa, S. (2004). Batch production of L(+) lactic acid from whey by *Lactobacillus casei* (NRRL B-441). *Journal of Chemical Technology and Biotechnology*, *79*(9), 1036–1040. https://doi.org/10.1002/jctb.1094

Cai, J., He, Y., Yu, X., Banks, S. W., Yang, Y., Zhang, X., Yu, Y., Liu, R., &

Bridgwater, A. V. (2017). Review of physicochemical properties and analytical characterization of lignocellulosic biomass. *Renewable and Sustainable Energy Reviews*, *76*(January), 309–322. https://doi.org/10.1016/j.rser.2017.03.072

Capra, M. L., Quiberoni, A., & Reinheimer, J. (2006). Phages of *Lactobacillus casei/paracasei*: Response to environmental factors and interaction with collection and commercial strains. *Journal of Applied Microbiology*, *100*(2), 334–342. https://doi.org/10.1111/j.1365-2672.2005.02767.x

Casalini, T., Rossi, F., Castrovinci, A., & Perale, G. (2019). A Perspective on Polylactic Acid-Based Polymers Use for Nanoparticles Synthesis and Applications. *Frontiers in Bioengineering and Biotechnology*, *7*(October), 1–16. https://doi.org/10.3389/fbioe.2019.00259

Castillo Martinez, F. A., Balciunas, E. M., Salgado, J. M., Domínguez González, J. M., Converti, A., & Oliveira, R. P. de S. (2013). Lactic acid properties, applications and production: A review. *Trends in Food Science and Technology*, *30*(1), 70–83. https://doi.org/10.1016/j.tifs.2012.11.007

Cengiz, N. (2002). DERi SANAYiiNDE KULLANILAN KiMYASALLARIN İŞÇİ VE ÇEVRE SAGLIGI DZERiNE ETKiLERi. *DERi SANAYiiNDE KULLANILAN KiMYASALLARIN İŞÇİ VE ÇEVRE SAGLIGI üZERiNE ETKiLERi*, *3*(1). https://doi.org/10.18229/ktd.87369

Chen, J., Li, W., Yang, B., Guo, X., Lee, F. S. C., & Wang, X. (2007). Determination of four major saponins in the seeds of *Aesculus chinensis Bunge* using accelerated solvent extraction followed by high-performance liquid chromatography and electrospray-time of flight mass spectrometry. *Analytica Chimica Acta*, *596*(2), 273–280. https://doi.org/10.1016/j.aca.2007.06.011

Cheng, C. (1998). Cellulase Activity in Different Buffering Media during Waste Paper Hydrolysis by HPLC. *Journal of the Chinese Chemical Society*, *45*(5),

679–688. https://doi.org/10.1002/jccs.199800103

Cho, Y., Kim, M.-J., & Kim, S.-K. (2013). Ethanol Production from Seaweed, Enteromorpha intestinalis, by Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) with *Saccharomyces cerevisiae*. *KSBB Journal*, *28*(6), 366–371. https://doi.org/10.7841/ksbbj.2013.28.6.366

Clarke, S., & Preto, F. (2011). Biomass Burn Characteristics. *Ministry of Agriculture, Food and Rural Affairs - Ontario*, *11*, 6. http://www.range-road.ca/Documents/Biomass Burn Characteristics.pdf

Conde-Mejía, C., Jiménez-Gutiérrez, A., & El-Halwagi, M. (2012). A comparison of pretreatment methods for bioethanol production from lignocellulosic materials. *Process Safety and Environmental Protection*, *90*(3), 189–202. https://doi.org/10.1016/j.psep.2011.08.004

Conejo-Saucedo, U., Cano-Camacho, H., & Lara, A. (2011). Hemicellulases of fungi : A vision of their function in the coordinated degradation of polysaccharides of plant cell walls. *Current Trends in Microbiology*, *7*(February 2016).

Čukanović, J., Tešević, V., Jadranin, M., Ljubojević, M., Mladenović, E., & Kostić, S. (2020). Horse chestnut (*Aesculus hippocastanum L.*) seed fatty acids, flavonoids and heavy metals plasticity to different urban environments. *Biochemical Systematics and Ecology*, *89*(October 2019), 103980. https://doi.org/10.1016/j.bse.2019.103980

Dahmen, N., Lewandowski, I., Zibek, S., & Weidtmann, A. (2019). Integrated lignocellulosic value chains in a growing bioeconomy: Status quo and perspectives. *GCB Bioenergy*, *11*(1), 107–117. https://doi.org/10.1111/gcbb.12586

Dangpradab, B., & Rattanaphanee, P. (2015). Efficiency Comparison of Different Design Schemes of Reactive Distillation Process for Ethyl Lactate Production

from Fermentation-Derived Magnesium Lactate. In *Computer Aided Chemical Engineering* (Vol. 37, Issue June). Elsevier. https://doi.org/10.1016/B978-0-444-63577-8.50025-5

Dekker, R. F. H. (1983). Bioconversion of hemicellulose: Aspects of hemicellulase production by *Trichoderma reesei* QM 9414 and enzymic saccharification of hemicellulose. *Biotechnology and Bioengineering*, *25*(4), 1127–1146. https://doi.org/10.1002/bit.260250419

Demirbaş, A. (2002). Fuel characteristics of olive husk and walnut, hazelnut, sunflower, and almond shells. *Energy Sources*, *24*(3), 215–221. https://doi.org/10.1080/009083102317243601

Dey, P., Sikder, J., Roy, S., & Pal, P. (2012). Fermentative lactic acid production from a renewable carbon source under response surface optimized conditions without alkali addition: A membrane-based green approach. *Clean Technologies and Environmental Policy*, *14*(5), 827–835. https://doi.org/10.1007/s10098-011-0448-z

Dhagat, S., & Jujjavarapu, S. E. (2021). Utility of lignin-modifying enzymes: a green technology for organic compound mycodegradation. *Journal of Chemical Technology and Biotechnology*, *March*. https://doi.org/10.1002/jctb.6807

Dimarogona, M., Topakas, E., & Christakopoulos, P. (2012). Cellulose degradation by oxidative enzymes. *Computational and Structural Biotechnology Journal*, *2*(3), e201209015. https://doi.org/10.5936/csbj.201209015

Drissen, R. E. T., Maas, R. H. W., Tramper, J., & Beeftink, H. H. (2009). Modelling ethanol production from cellulose: Separate hydrolysis and fermentation versus simultaneous saccharification and fermentation. *Biocatalysis and Biotransformation*, *27*(1), 27–35. https://doi.org/10.1080/10242420802564358

Duff, S. J. B., Moritz, J. W., & Casavant, T. E. (1995). Effect of surfactant and

particle size reduction on hydrolysis of deinking sludge and nonrecyclable newsprint. *Biotechnology and Bioengineering*, *45*(3), 239–244. https://doi.org/10.1002/bit.260450308

Eiteman, M. A., & Ramalingam, S. (2015). Microbial production of lactic acid. *Biotechnology Letters*, *37*(5), 955–972. https://doi.org/10.1007/s10529-015-1769-5

Esquivel-Hernández, D. A., García-Pérez, J. S., López-Pacheco, I. Y., Iqbal, H. M. N., & Parra-Saldívar, R. (2022). Resource recovery of lignocellulosic biomass waste into lactic acid - Trends to sustain cleaner production. *Journal of Environmental Management*, *301*(January 2021). https://doi.org/10.1016/j.jenvman.2021.113925

Fan, L. T., & Lee, Y. -h. (1983). Kinetic studies of enzymatic hydrolysis of insoluble cellulose: Derivation of a mechanistic kinetic model. *Biotechnology and Bioengineering*, *25*(11), 2707–2733. https://doi.org/10.1002/bit.260251115

Felipe, M. B. M. C., de Carvalho, F. M., Félix-Silva, J., Fernandes-Pedrosa, M. F., Scortecci, K. C., Agnez-Lima, L. F., & Batistuzzo de Medeiros, S. R. (2013). Evaluation of genotoxic and antioxidant activity of an *Aesculus hippocastanum L.* (*Sapindaceae*) phytotherapeutic agent. *Biomedicine and Preventive Nutrition*, *3*(3), 261–266. https://doi.org/10.1016/j.bionut.2012.10.014

Galbe, M. M. (2002). *A review of the production of ethanol from softwood*. 618–628. https://doi.org/10.1007/s00253-002-1058-9

Gandla, M. L., Martín, C., & Jönsson, L. J. (2018). Analytical Enzymatic Saccharification of Lignocellulosic Biomass for Conversion to Biofuels and Bio-Based Chemicals. *Energies*, *11*(11). https://doi.org/10.3390/en11112936

García Ibarra, V., Sendón, R., & Rodríguez-Bernaldo De Quirós, A. (2016). Antimicrobial Food Packaging Based on Biodegradable Materials. In

Antimicrobial Food Packaging (Issue Figure 1). Elsevier Inc. https://doi.org/10.1016/B978-0-12-800723-5.00029-2

Ghaffar, T., Irshad, M., Anwar, Z., Aqil, T., Zulifqar, Z., Tariq, A., Kamran, M., Ehsan, N., & Mehmood, S. (2014). Recent trends in lactic acid biotechnology: A brief review on production to purification. *Journal of Radiation Research and Applied Sciences*, *7*(2), 222–229. https://doi.org/10.1016/j.jrras.2014.03.002

Gibson, L. J. (2012). The hierarchical structure and mechanics of plant materials. *Journal of the Royal Society Interface*, *9*(76), 2749–2766. https://doi.org/10.1098/rsif.2012.0341

Gobbetti, M., & Minervini, F. (2014). *Lactobacillus: Lactobacillus casei*. In *Encyclopedia of Food Microbiology: Second Edition* (pp. 432–438). Elsevier Inc. https://doi.org/10.1016/B978-0-12-384730-0.00180-4

Goszczynski, S., Paszczynski, A., Pasti-Grigsby, M. B., Crawford, R. L., & Crawford, D. L. (1994). New pathway for degradation of sulfonated azo dyes by microbial peroxidases of *Phanerochaete chrysosporium* and *Streptomyces chromofuscus*. *Journal of Bacteriology*, *176*(5), 1339–1347. https://doi.org/10.1128/jb.176.5.1339-1347.1994

Grzybek, J., Sepperer, T., Petutschnigg, A., & Schnabel, T. (2021). *Organosolv Lignin from European Tree Bark : Influence of Bark Pretreatment is usually*.

Gullón, P., Gullón, B., Muñiz-Mouro, A., Lú-Chau, T. A., & Eibes, G. (2020). Valorization of horse chestnut burs to produce simultaneously valuable compounds under a green integrated biorefinery approach. *Science of the Total Environment*, *730*. https://doi.org/10.1016/j.scitotenv.2020.139143

Habibi, Y., Lucia, L. A., & Rojas, O. J. (2010). Cellulose nanocrystals: Chemistry, self-assembly, and applications. *Chemical Reviews*, *110*(6), 3479–3500. https://doi.org/10.1021/cr900339w

Haddaji, N., Krifi, B., Lagha, R., Khouadja, S., & Bakhrouf, A. (2015). Effect of high temperature on viability of *Lactobacillus casei* and analysis of secreted and GroEL proteins profiles. *African Journal of Bacteriology Research*, *7*(3), 29–34. https://doi.org/10.5897/JBR2015.0155

Hao, F., Fu, N., Ndiaye, H., Woo, M. W., Jeantet, R., & Chen, X. D. (2021). Thermotolerance, Survival, and Stability of Lactic Acid Bacteria After Spray Drying as Affected by the Increase of Growth Temperature. *Food and Bioprocess Technology*, *14*(1), 120–132. https://doi.org/10.1007/s11947-020-02571-1

Harmsen, P., Bermudez, L., & Bakker, R. (2010). Literature Review of Physical and Chemical Pretreatment Processes for Lignocellulosic Biomass. *Biomass*, *January*, 1–49.

He, Y. C., Liu, F., Di, J. H., Ding, Y., Gao, D. Z., Zhang, D. P., Tao, Z. C., Chong, G. G., Huang, M. Z., & Ma, C. L. (2016). Effective pretreatment of dilute NaOH-soaked chestnut shell with glycerol-HClO4-water media: Structural characterization, enzymatic saccharification, and ethanol fermentation. *Bioprocess and Biosystems Engineering*, *39*(4), 533–543. https://doi.org/10.1007/s00449-015-1535-z

He, Y. C., Liu, F., Gong, L., Di, J. H., Ding, Y., Ma, C. L., Zhang, D. P., Tao, Z. C., Wang, C., & Yang, B. (2016). Enzymatic in situ saccharification of chestnut shell with high ionic liquid-tolerant cellulases from *Galactomyces sp*. CCZU11-1 in a biocompatible ionic liquid-cellulase media. *Bioresource Technology*, *201*, 133–139. https://doi.org/10.1016/j.biortech.2015.11.034

Herstellung organischer Säuren für die Polyestersynthese. (2017). https://literatur.thuenen.de/digbib_extern/dn059306.pdf

Hetényi, K., Németh, Á., & Sevella, B. (2011). Investigation and modeling of lactic acid fermentation on wheat starch via SSF, CHF and SHF technology. *Periodica Polytechnica Chemical Engineering*, *55*(1), 11–16.

https://doi.org/10.3311/pp.ch.2011-1.02

Hofvendahl, K., & Hahn-Hägerdal, B. (2000). Factors affecting the fermentative lactic acid production from renewable resources. *Enzyme and Microbial Technology*, *26*(2–4), 87–107. https://doi.org/10.1016/S0141-0229(99)00155-6

Hossein Nezhad, M., Stenzel, D. J., & Britz, M. L. (2010). Effect of growth at low pH on the cell surface properties of a typical strain of *Lactobacillus casei* group. *Iranian Journal of Microbiology*, *2*(3), 144–151.

Howard, R. L., Abotsi, E., Van Rensburg, E. L. J., & Howard, S. (2003). Lignocellulose biotechnology: Issues of bioconversion and enzyme production. *African Journal of Biotechnology*, *2*(12), 702–733. https://doi.org/10.5897/ajb2003.000-1115

Hujanen, M., Linko, S., Linko, Y. Y., & Leisola, M. (2001). Optimisation of media and cultivation conditions for L(+)(S)-lactic acid production by *Lactobacillus casei* NRRL B-441. *Applied Microbiology and Biotechnology*, *56*(1–2), 126–130. https://doi.org/10.1007/s002530000501

Ichwan, M., & Won Son, T. (2011). Study on organosolv pulping methods of oil palm biomass. *International Seminar on Chemistry*, *2011*(November), 24–25.

Irwin, D., Jung, E. D., & Wilson, D. B. (1994). Characterization and sequence of a *Thermomonospora fusca* xylanase. *Applied and Environmental Microbiology*, *60*(3), 763–770. https://doi.org/10.1128/aem.60.3.763-770.1994

Ishihara, T., Kaidzu, S., Kimura, H., Koyama, Y., Matsuoka, Y., & Ohira, A. (2018). Protective effect of highly polymeric A-type proanthocyanidins from seed shells of Japanese horse chestnut (*Aesculus turbinata BLUME*) against light-induced oxidative damage in rat retina. *Nutrients*, *10*(5), 1–14. https://doi.org/10.3390/nu10050593

John, R. P., G.S., A., Nampoothiri, K. M., & Pandey, A. (2009). Direct lactic acid

fermentation: Focus on simultaneous saccharification and lactic acid production. *Biotechnology Advances*, *27*(2), 145–152. https://doi.org/10.1016/j.biotechadv.2008.10.004

Jørgensen, H., Eriksson, T., Börjesson, J., Tjerneld, F., & Olsson, L. (2003). Purification and characterization of five cellulases and one xylanase from *Penicillium brasilianum* IBT 20888. *Enzyme and Microbial Technology*, *32*(7), 851–861. https://doi.org/10.1016/S0141-0229(03)00056-5

Kádár, Z., Szengyel, Z., & Réczey, K. (2004). Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. *Industrial Crops and Products*, *20*(1), 103–110. https://doi.org/10.1016/j.indcrop.2003.12.015

Kang, S. W., Park, Y. S., Lee, J. S., Hong, S. I., & Kim, S. W. (2004). Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. *Bioresource Technology*, *91*(2), 153–156. https://doi.org/10.1016/S0960-8524(03)00172-X

Kapusta, I., Janda, B., Szajwaj, B., Stochmal, A., Piacente, S., Pizza, C., Franceschi, F., Franz, C., & Oleszek, W. (2007). Flavonoids in horse chestnut (*Aesculus hippocastanum*) seeds and powdered waste water byproducts. *Journal of Agricultural and Food Chemistry*, *55*(21), 8485–8490. https://doi.org/10.1021/jf071709t

Karboune, S., L'Hocine, L., Anthoni, J., Geraert, P. A., & Kermasha, S. (2009). Properties of selected hemicellulases of a multi-enzymatic system from *Penicillium funiculosum*. *Bioscience, Biotechnology and Biochemistry*, *73*(6), 1286–1292. https://doi.org/10.1271/bbb.80808

Kim, E., Irwin, D. C., Walker, L. P., & Wilson, D. B. (1998). Factorial optimization of a six-cellulase mixture. *Biotechnology and Bioengineering*, *58*(5), 494–501. https://doi.org/10.1002/(SICI)1097-0290(19980605)58:5<494::AID-BIT5>3.0.CO;2-8

Kim, S. B., Yum, D. M., & Park, S. C. (2000). Step-change variation of acid concentration in a percolation reactor for hydrolysis of hardwood hemicellulose. *Bioresource Technology*, *72*(3), 289–294. https://doi.org/10.1016/S0960-8524(99)00081-4

Kimura, H., Ogawa, S., Sugiyama, A., Jisaka, M., Takeuchi, T., & Yokota, K. (2011). Anti-obesity effects of highly polymeric proanthocyanidins from seed shells of Japanese horse chestnut (*Aesculus turbinata Blume*). *Food Research International*, *44*(1), 121–126. https://doi.org/10.1016/j.foodres.2010.10.052

Kobayashi, F., Take, H., Asada, C., & Nakamura, Y. (2004). Methane production from steam-exploded bamboo. *Journal of Bioscience and Bioengineering*, *97*(6), 426–428. https://doi.org/10.1016/S1389-1723(04)70231-5

Kohring, S., Wiegel, J., & Mayer, F. (1990). Subunit composition and glycosidic activities of the cellulase complex from *Clostridium thermocellum* JW20. *Applied and Environmental Microbiology*, *56*(12), 3798–3804. https://doi.org/10.1128/aem.56.12.3798-3804.1990

Komesu, A., Oliveira, J. A. R. de, Martins, L. H. da S., Wolf Maciel, M. R., & Maciel Filho, R. (2017). Lactic Acid Production to Purification: A Review. *BioResources*, *12*(2), 4364–4383. https://doi.org/10.15376/biores.12.2.komesu

Kompanje, E. J. O., Jansen, T. C., Van Der Hoven, B., & Bakker, J. (2007). The first demonstration of lactic acid in human blood in shock by Johann Joseph Scherer (1814-1869) in January 1843. *Intensive Care Medicine*, *33*(11), 1967–1971. https://doi.org/10.1007/s00134-007-0788-7

Kristensen, J. B., Felby, C., & Jørgensen, H. (2009). Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnology for Biofuels*, *2*, 1–10. https://doi.org/10.1186/1754-6834-2-11

Küçükkurt, I., Ince, S., Keleş, H., Küpeli Akkol, E., Avci, G., Yeşilada, E., & Bacak, E. (2010). Beneficial effects of *Aesculus hippocastanum L*. seed extract on the body's own antioxidant defense system on subacute

administration. *Journal of Ethnopharmacology*, *129*(1), 18–22. https://doi.org/10.1016/j.jep.2010.02.017

Kumar, P., Barrett, D. M., Delwiche, M. J., & Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial and Engineering Chemistry Research*, *48*(8), 3713–3729. https://doi.org/10.1021/ie801542g

Kunamneni, A., Plou, F. J., Alcalde, M., & Ballesteros, A. (2014). *Trichoderma* Enzymes for Food Industries. In *Biotechnology and Biology of Trichoderma*. Elsevier. https://doi.org/10.1016/B978-0-444-59576-8.00024-2

Kurkin, V. A., Belov, P. V., Ryzhov, V. M., & Braslavskii, V. B. (2020). HPLC Determination of Rhamnocitrin in Common Horse Chestnut (*Aesculus hippocastanum L*.) Buds. *Pharmaceutical Chemistry Journal*, *53*(12), 1128–1132. https://doi.org/10.1007/s11094-020-02135-9

Lee, K. H., Lee, S. K., Lee, J., Kim, S., Park, C., Kim, S. W., & Yoo, H. Y. (2021). Improvement of enzymatic glucose conversion from chestnut shells through optimization of KOH pretreatment. *International Journal of Environmental Research and Public Health*, *18*(7). https://doi.org/10.3390/ijerph18073772

Lee, Y., Park, J., Ryu, C., Gang, K. S., Yang, W., Park, Y. K., Jung, J., & Hyun, S. (2013). Comparison of biochar properties from biomass residues produced by slow pyrolysis at 500°C. *Bioresource Technology*, *148*, 196–201. https://doi.org/10.1016/j.biortech.2013.08.135

Levasseur, A., Piumi, F., Coutinho, P. M., Rancurel, C., Asther, M., Delattre, M., Henrissat, B., Pontarotti, P., Asther, M., & Record, E. (2008). FOLy: An integrated database for the classification and functional annotation of fungal oxidoreductases potentially involved in the degradation of lignin and related aromatic compounds. *Fungal Genetics and Biology*, *45*(5), 638–645. https://doi.org/10.1016/j.fgb.2008.01.004

Lokapirnasari, W. P., Nazar, D. S., Nurhajati, T., Supranianondo, K., & Yulianto,

A. B. (2015). Production and assay of cellulolytic enzyme activity of *Enterobacter cloacae* WPL 214 isolated from bovine rumen fluid waste of Surabaya abbatoir, Indonesia. *Veterinary World*, *8*(3), 367–371. https://doi.org/10.14202/vetworld.2015.367-371

López-Contreras, A. M., Gabor, K., Martens, A. A., Renckens, B. A. M., Claassen, P. A. M., Van Der Oost, J., & De Vos, W. M. (2004). Substrate-induced production and secretion of cellulases by *Clostridium acetobutylicum*. *Applied and Environmental Microbiology*, *70*(9), 5238–5243. https://doi.org/10.1128/AEM.70.9.5238-5243.2004

Lübeck, M. (2018). *Cellulases: Methods and Protocols* (M. Lübeck (Ed.)).

Lucas, M., Hanson, S. K., Wagner, G. L., Kimball, D. B., & Rector, K. D. (2012). Evidence for room temperature delignification of wood using hydrogen peroxide and manganese acetate as a catalyst. *Bioresource Technology*, *119*, 174–180. https://doi.org/10.1016/j.biortech.2012.05.086

Mai, V., Wiegel, J., & Lorenz, W. W. (2000). Cloning, sequencing, and characterization of the bifunctional xylosidase-arabinosidase from the anaerobic thermophile *Thermoanaerobacter ethanolicus*. *Gene*, *247*(1–2), 137–143. https://doi.org/10.1016/S0378-1119(00)00106-2

Mäki-Arvela, P., Salminen, E., Riittonen, T., Virtanen, P., Kumar, N., & Mikkola, J. P. (2012). The challenge of efficient synthesis of biofuels from lignocellulose for future renewable transportation fuels. *International Journal of Chemical Engineering*, *2012*. https://doi.org/10.1155/2012/674761

Margină, D., Olaru, O. T., Ilie, M., Grădinaru, D., Guțu, C., Voicu, S., Dinischiotu, A., Spandidos, D. A., & Tsatsakis, A. M. (2015). Assessment of the potential health benefits of certain total extracts from *Vitis vinifera*, *Aesculus hyppocastanum* and *Curcuma longa*. *Experimental and Therapeutic Medicine*, *10*(5), 1681–1688. https://doi.org/10.3892/etm.2015.2724

Marques, S., Santos, J. A. L., Gírio, F. M., & Roseiro, J. C. (2008). Lactic acid

production from recycled paper sludge by simultaneous saccharification and fermentation. *Biochemical Engineering Journal*, *41*(3), 210–216. https://doi.org/10.1016/j.bej.2008.04.018

Martínez, Á. T., Speranza, M., Ruiz-Dueñas, F. J., Ferreira, P., Camarero, S., Guillén, F., Martínez, M. J., Gutiérrez, A., & Del Río, J. C. (2005). Biodegradation of lignocellulosics: Microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *International Microbiology*, *8*(3), 195–204. https://doi.org/10.13039/501100003339

Maslova, O., Stepanov, N., Senko, O., & Efremenko, E. (2019). Production of various organic acids from different renewable sources by immobilized cells in the regimes of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SFF). *Bioresource Technology*, *272*(August 2018), 1–9. https://doi.org/10.1016/j.biortech.2018.09.143

Maurelli, L., Ionata, E., La Cara, F., & Morana, A. (2013). Chestnut shell as unexploited source of fermentable sugars: Effect of different pretreatment methods on enzymatic saccharification. *Applied Biochemistry and Biotechnology*, *170*(5), 1104–1118. https://doi.org/10.1007/s12010-013-0264-5

Mawadza, C., Hatti-Kaul, R., Zvauya, R., & Mattiasson, B. (2000). Purification and characterization of cellulases produced by two *Bacillus* strains. *Journal of Biotechnology*, *83*(3), 177–187. https://doi.org/10.1016/S0168-1656(00)00305-9

McKendry, P. (2002). Energy production from biomass (part 1): Overview of biomass. *Bioresource Technology*, *83*(1), 37–46. https://doi.org/10.1016/S0960-8524(01)00118-3

Meena, M., Zehra, A., Dubey, M. K., Aamir, M., & Upadhyay, R. S. (2017). Penicillium enzymes for the food industries. In *New and Future Developments*

in Microbial Biotechnology and Bioengineering: Penicillium System Properties and Applications. Elsevier B.V. https://doi.org/10.1016/B978-0-444-63501-3.00009-0

Miettinen-Oinonen, A., & Suominen, P. (2002). Enhanced production of Trichoderma reesei endoglucanases and use of the new cellulase preparations in producing the stonewashed effect on denim fabric. *Applied and Environmental Microbiology*, *68*(8), 3956–3964. https://doi.org/10.1128/AEM.68.8.3956-3964.2002

Miller, C., Fosmer, A., Rush, B., McMullin, T., Beacom, D., & Suominen, P. (2019). Comprehensive Biotechnology:Industrial Production of Lactic Acid. In Murray Moo-Young (Ed.), *Comprehensive Biotechnology*. https://doi.org/10.1016/B978-0-444-64046-8.00248-2

Miura, S., Arimura, T., Itoda, N., Dwiarti, L., Feng, J. B., Bin, C. H., & Okabe, M. (2004). Production of L-lactic acid from corncob. *Journal of Bioscience and Bioengineering*, *97*(3), 153–157. https://doi.org/10.1263/jbb.97.153

Moon, R. J., Martini, A., Nairn, J., Simonsen, J., & Youngblood, J. (2011). Cellulose nanomaterials review: Structure, properties and nanocomposites. In *Chemical Society Reviews* (Vol. 40, Issue 7). https://doi.org/10.1039/c0cs00108b

Morales, A., Gullón, B., Dávila, I., Eibes, G., Labidi, J., & Gullón, P. (2018). Optimization of alkaline pretreatment for the co-production of biopolymer lignin and bioethanol from chestnut shells following a biorefinery approach. *Industrial Crops and Products*, *124*(July), 582–592. https://doi.org/10.1016/j.indcrop.2018.08.032

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*, *96*(6), 673–686. https://doi.org/10.1016/j.biortech.2004.06.025

Mudgil, D. (2017). The Interaction Between Insoluble and Soluble Fiber. In *Dietary Fiber for the Prevention of Cardiovascular Disease: Fiber's Interaction between Gut Micoflora, Sugar Metabolism, Weight Control and Cardiovascular Health*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-805130-6.00003-3

Mussatto, S. I., Fernandes, M., Mancilha, I. M., & Roberto, I. C. (2008). Effects of medium supplementation and pH control on lactic acid production from brewer's spent grain. *Biochemical Engineering Journal*, *40*(3), 437–444. https://doi.org/10.1016/j.bej.2008.01.013

Naik, S., Goud, V. V., Rout, P. K., Jacobson, K., & Dalai, A. K. (2010). Characterization of Canadian biomass for alternative renewable biofuel. *Renewable Energy*, *35*(8), 1624–1631. https://doi.org/10.1016/j.renene.2009.08.033

Naran, R., Black, S., Decker, S. R., & Azadi, P. (2009). Extraction and characterization of native heteroxylans from delignified corn stover and aspen. *Cellulose*, *16*(4), 661–675. https://doi.org/10.1007/s10570-009-9324-y

Narayanan, N., Roychoudhury, P. K., & Srivastava, A. (2004). L (+) lactic acid fermentation and its product polymerization. *Electronic Journal of Biotechnology*, *7*(2). https://doi.org/10.2225/vol7-issue2-fulltext-7

Nieves, R. A., Ehrman, C. I., Adney, W. S., Elander, R. T., & Himmel, M. E. (1998). *Technical Communication : Survey and analysis of commercial cellulase preparations suitable for biomass conversion to ethanol*. *14*, 301–304.

Nurika, I., Suhartini, S., & Barker, G. C. (2020). Biotransformation of Tropical Lignocellulosic Feedstock Using the Brown rot Fungus *Serpula lacrymans*. *Waste and Biomass Valorization*, *11*(6), 2689–2700. https://doi.org/10.1007/s12649-019-00581-5

Öhgren, K., Bura, R., Lesnicki, G., Saddler, J., & Zacchi, G. (2007). A comparison

between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochemistry*, *42*(5), 834–839. https://doi.org/10.1016/j.procbio.2007.02.003

Okeke, B. C., & Paterson, A. (1992). Simultaneous production and induction of cellulolytic and xylanolytic enzymes in a *Streptomyces sp. World Journal of Microbiology & Biotechnology*, *8*(5), 483–487. https://doi.org/10.1007/BF01201945

Okolie, J. A., Nanda, S., Dalai, A. K., & Kozinski, J. A. (2021). Chemistry and Specialty Industrial Applications of Lignocellulosic Biomass. *Waste and Biomass Valorization*, *12*(5), 2145–2169. https://doi.org/10.1007/s12649-020-01123-0

Ong, L. G. A., Abd-Aziz, S., Noraini, S., Karim, M. I. A., & Hassan, M. A. (2004). Enzyme production and profile by *Aspergillus niger* during solid substrate fermentation using palm kernel cake as substrate. *Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology*, *118*(1–3), 73–79. https://doi.org/10.1385/abab:118:1-3:073

Østby, H., Hansen, L. D., Horn, S. J., Eijsink, V. G. H., & Várnai, A. (2020). Enzymatic processing of lignocellulosic biomass: principles, recent advances and perspectives. In *Journal of Industrial Microbiology and Biotechnology* (Vol. 47, Issues 9–10). Springer International Publishing. https://doi.org/10.1007/s10295-020-02301-8

Parus, A. (2018). Copper(II) ions' removal from aqueous solution using green horse-chestnut shell as a low-cost adsorbent. *Chemistry and Ecology*, *34*(1), 56–69. https://doi.org/10.1080/02757540.2017.1396452

Paul, S., & Dutta, A. (2018). Challenges and opportunities of lignocellulosic biomass for anaerobic digestion. *Resources, Conservation and Recycling*, *130*(March 2017), 164–174. https://doi.org/10.1016/j.resconrec.2017.12.005

Plackett, D. V. (2011). Biopolymers : new materials for sustainable films and

coatings LK - https://univdelosandes.on.worldcat.org/oclc/916392135. In *Ta - Tt* -.

Potprommanee, L., Wang, X. Q., Han, Y. J., Nyobe, D., Peng, Y. P., Huang, Q., Liu, J. Y., Liao, Y. L., & Chang, K. L. (2017). Characterization of a thermophilic cellulase from *Geobacillus sp*. HTA426, an efficient cellulase-producer on alkali pretreated of lignocellulosic biomass. *PLoS ONE*, *12*(4), 1–16. https://doi.org/10.1371/journal.pone.0175004

Prasad, S., Singh, A., & Joshi, H. C. (2007). Ethanol as an alternative fuel from agricultural, industrial and urban residues. *Resources, Conservation and Recycling*, *50*(1), 1–39. https://doi.org/10.1016/j.resconrec.2006.05.007

Puls, J. (1997). Relationship Between Hemicellulose Structljre. *Structure*, *196*, 183–196.

Qi, G., Huang, D., Wang, J., Shen, Y., & Gao, X. (2019). Enhanced butanol production from ammonium sulfite pretreated wheat straw by separate hydrolysis and fermentation and simultaneous saccharification and fermentation. *Sustainable Energy Technologies and Assessments*, *36*(19), 100549. https://doi.org/10.1016/j.seta.2019.100549

Qian, E. W. (2013). Pretreatment and Saccharification of Lignocellulosic Biomass. In *Research Approaches to Sustainable Biomass Systems*. Elsevier. https://doi.org/10.1016/B978-0-12-404609-2.00007-6

Qin, H., Gong, S. S., Ge, X. Y., & Zhang, W. G. (2012). The effect of temperature on L-lactic acid production and metabolite distribution of *Lactobacillus casei*. *Preparative Biochemistry and Biotechnology*, *42*(6), 564–573. https://doi.org/10.1080/10826068.2012.665114

Rafiq, S. I., Singh, S., & Saxena, D. C. (2016). Effect of heat-moisture and acid treatment on physicochemical, pasting, thermal and morphological properties of Horse Chestnut (*Aesculus indica*) starch. *Food Hydrocolloids*, *57*, 103–113. https://doi.org/10.1016/j.foodhyd.2016.01.009

Rajoka, M. I., & Malik, K. A. (1997). Cellulase production by *Cellulomonas biazotea* cultured in media containing different cellulosic substrates. *Bioresource Technology*, *59*(1), 21–27. https://doi.org/10.1016/S0960-8524(96)00136-8

Ray, M. J., Leak, D. J., Spanu, P. D., & Murphy, R. J. (2010). Brown rot fungal early stage decay mechanism as a biological pretreatment for softwood biomass in biofuel production. *Biomass and Bioenergy*, *34*(8), 1257–1262. https://doi.org/10.1016/j.biombioe.2010.03.015

Regulations (Vol. 8, Issue 2). (2012).

Reinoso, F. A. M., Rencoret, J., Gutiérrez, A., Milagres, A. M. F., Del Río, J. C., & Ferraz, A. (2018). Fate of p-hydroxycinnamates and structural characteristics of residual hemicelluloses and lignin during alkaline-sulfite chemithermomechanical pretreatment of sugarcane bagasse. *Biotechnology for Biofuels*, *11*(1), 1–12. https://doi.org/10.1186/s13068-018-1155-3

Reviews, F. M., Societies, E. M., & Chemistry, A. (1994). *Lignin-modifying enzymes from selected white-rot fungi : production and role in lignin degradation*. *13*, 125–135.

Ricardo Soccol, C., Faraco, V., Karp, S., Vandenberghe, L. P. S., Thomaz-Soccol, V., Woiciechowski, A., & Pandey, A. (2011). Lignocellulosic bioethanol: Current status and future perspectives. In *Biofuels* (1st ed.). Elsevier Inc. https://doi.org/10.1016/B978-0-12-385099-7.00005-X

Saha, B. C. (2003). Hemicellulose bioconversion. *Journal of Industrial Microbiology and Biotechnology*, *30*(5), 279–291. https://doi.org/10.1007/s10295-003-0049-x

Saha, B. C., Nichols, N. N., Qureshi, N., & Cotta, M. A. (2011). Comparison of separate hydrolysis and fermentation and simultaneous saccharification and fermentation processes for ethanol production from wheat straw by recombinant *Escherichia coli* strain FBR5. *Applied Microbiology and*

Biotechnology, *92*(4), 865–874. https://doi.org/10.1007/s00253-011-3600-0

Sánchez, C. (2009). Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnology Advances*, *27*(2), 185–194. https://doi.org/10.1016/j.biotechadv.2008.11.001

Sasaki, C., Kushiki, Y., Asada, C., & Nakamura, Y. (2014). Acetone-butanol-ethanol production by separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) methods using acorns and wood chips of *Quercus acutissima* as a carbon source. *Industrial Crops and Products*, *62*, 286–292. https://doi.org/10.1016/j.indcrop.2014.08.049

Schülein, M. (1997). Enzymatic properties of cellulases from *Humicola insolens*. *Journal of Biotechnology*, *57*(1–3), 71–81. https://doi.org/10.1016/S0168-1656(97)00090-4

Scopes, R. K. (2002). Enzyme Activity and Assays. *ELS*, 1–6. https://doi.org/10.1038/npg.els.0000712

Šedivá, J., Vlašínová, H., & Mertelík, J. (2013). Shoot regeneration from various explants of horse chestnut (*Aesculus hippocastanum L.*). *Scientia Horticulturae*, *161*, 223–227. https://doi.org/10.1016/j.scienta.2013.06.030

Shallom, D., & Shoham, Y. (2003). Microbial hemicellulases. *Current Opinion in Microbiology*, *6*(3), 219–228. https://doi.org/10.1016/S1369-5274(03)00056-0

Shen, D. K., Gu, S., Luo, K. H., Bridgwater, A. V., & Fang, M. X. (2009). Kinetic study on thermal decomposition of woods in oxidative environment. *Fuel*, *88*(6), 1024–1030. https://doi.org/10.1016/j.fuel.2008.10.034

Shin, H. Y., Park, S.-Y., Sung, jong H., & Kim, D.-H. (2003). Purification and characterization of ginsenoside Ra-hydrolyzing β-D-Xylosidase from *Bifidobacterium breve* K-110, a human intestinal anaerobic bacterium. *Biological and Pharmaceutical Bulletin*, *26*(8), 1170–1173. https://doi.org/10.1248/bpb.26.1170

Singh, G., Verma, A. K., & Kumar, V. (2016). Catalytic properties, functional attributes and industrial applications of β-glucosidases. *3 Biotech*, *6*(1), 1–14. https://doi.org/10.1007/s13205-015-0328-z

Singh, O. V., & Harvey, S. P. (2010). Sustainable biotechnology: Sources of renewable energy. *Sustainable Biotechnology: Sources of Renewable Energy*, *May*, 1–323. https://doi.org/10.1007/978-90-481-3295-9

Sreenath, H. K., Moldes, A. B., Koegel, R. G., & Straub, R. J. (2001). Lactic acid production by simultaneous saccharification and fermentation of alfalfa fiber. *Journal of Bioscience and Bioengineering*, *92*(6), 518–523. https://doi.org/10.1016/S1389-1723(01)80309-1

Stevulova, N., Cigasova, J., Estokova, A., Terpakova, E., Geffert, A., Kacik, F., Singovszka, E., & Holub, M. (2014). Properties characterization of chemically modified hemp hurds. *Materials*, *7*(12), 8131–8150. https://doi.org/10.3390/ma7128131

Sukumaran, R. K., Singhania, R. R., & Pandey, A. (2005). Microbial cellulases - Production, applications and challenges. *Journal of Scientific and Industrial Research*, *64*(11), 832–844.

Sun, N., Rodríguez, H., Rahman, M., & Rogers, R. D. (2011). Where are ionic liquid strategies most suited in the pursuit of chemicals and energy from lignocellulosic biomass? *Chemical Communications*, *47*(5), 1405–1421. https://doi.org/10.1039/c0cc03990j

Sun, R. (2010). *Cereal Straw as a Resource for Sustainable Biomaterials and Biofuels* (First edit).

Sun, Y., & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresource Technology*, *83*(1), 1–11. https://doi.org/10.1016/S0960-8524(01)00212-7

Szambelan, K., Nowak, J., Szwengiel, A., Jeleń, H., & Łukaszewski, G. (2018).

Separate hydrolysis and fermentation and simultaneous saccharification and fermentation methods in bioethanol production and formation of volatile by-products from selected corn cultivars. *Industrial Crops and Products*, *118*(March), 355–361. https://doi.org/10.1016/j.indcrop.2018.03.059

Taherzadeh, M. J., & Karimi, K. (2008). Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. In *International Journal of Molecular Sciences* (Vol. 9, Issue 9). https://doi.org/10.3390/ijms9091621

Teixeira, S., Eblagon, K. M., Miranda, F., Pereira, M. F. R., & Figueiredo, L. (2021). *Technical Applications*.

Teleman, A., Nordström, M., Tenkanen, M., Jacobs, A., & Dahlman, O. (2003). Isolation and characterization of O-acetylated glucomannans from aspen and birch wood. *Carbohydrate Research*, *338*(6), 525–534. https://doi.org/10.1016/S0008-6215(02)00491-3

Torget, R., Werdene, P., Himmel, M., & Grohmann, K. (1990). Dilute acid pretreatment of short rotation woody and herbaceous crops. *Applied Biochemistry and Biotechnology*, *24–25*(1), 115–126. https://doi.org/10.1007/BF02920238

Underland, V., Sæterdal, I., & Nilsen, E. S. (2012). Cochrane summary of findings: Horse chestnut seed extract for chronic venous insufficiency. *Global Advances In Health and Medicine*, *1*(1), 122–123. https://doi.org/10.7453/gahmj.2012.1.1.018

Vaidya, A. N., Pandey, R. A., Mudliar, S., Kumar, M. S., Chakrabarti, T., & Devotta, S. (2005). Production and recovery of lactic acid for polylactide - An overview. *Critical Reviews in Environmental Science and Technology*, *35*(5), 429–467. https://doi.org/10.1080/10643380590966181

Valles, A., Álvarez-Hornos, F. J., Martínez-Soria, V., Marzal, P., & Gabaldón, C. (2020). Comparison of simultaneous saccharification and fermentation and separate hydrolysis and fermentation processes for butanol production from

rice straw. *Fuel*, *282*(June), 118831. https://doi.org/10.1016/j.fuel.2020.118831

Vivek, N., Hazeena, S. H., Rajesh, R. O., Godan, T. K., Anjali, K. B., Nair, L. M., Mohan, B., Nair, S. C., Sindhu, R., Pandey, A., & Binod, P. (2019). Genomics of Lactic Acid Bacteria for Glycerol Dissimilation. *Molecular Biotechnology*, *61*(8), 562–578. https://doi.org/10.1007/s12033-019-00186-2

Waldron, K. (2010). Bioalcohol production: Biochemical conversion of lignocellulosic biomass. In *Bioalcohol Production: Biochemical Conversion of Lignocellulosic Biomass*. https://doi.org/10.1533/9781845699611

Weryszko-Chmielewska, E., & Chwil, M. (2017). Structure of floral nectaries in *Aesculus hippocastanum L. Acta Botanica Croatica*, *76*(1), 41–48. https://doi.org/10.1515/botcro-2016-0049

Willför, S., Sjöholm, R., Laine, C., & Holmbom, B. (2002). Structural features of water-soluble arabinogalactans from Norway spruce and Scots pine heartwood. *Wood Science and Technology*, *36*(2), 101–110. https://doi.org/10.1007/s00226-001-0137-x

Won, K. Y., Kim, Y. S., & Oh, K. K. (2012). Comparison of bioethanol production of simultaneous saccharification and fermentation and separation hydrolysis and fermentation from cellulose-rich barley straw. *Korean Journal of Chemical Engineering*, *29*(10), 1341–1346. https://doi.org/10.1007/s11814-012-0019-y

Wong, D. W. S. (2009). Structure and action mechanism of ligninolytic enzymes. In *Applied Biochemistry and Biotechnology* (Vol. 157, Issue 2). https://doi.org/10.1007/s12010-008-8279-z

Wyman, C. E., Dale, B. E., Elander, R. T., Holtzapple, M., Ladisch, M. R., & Lee, Y. Y. (2005). Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. *Bioresource Technology*, *96*(18 SPEC. ISS.), 2026–2032.

https://doi.org/10.1016/j.biortech.2005.01.018

Xia, W., Shi, P., Xu, X., Qian, L., Cui, Y., Xia, M., & Yao, B. (2015). High level expression of a novel family 3 neutral β-xylosidase from *Humicola insolens* Y1 with high tolerance to D-xylose. *PLoS ONE*, *10*(2), 1–14. https://doi.org/10.1371/journal.pone.0117578

Xiao, Z., Zhang, X., Gregg, D. J., & Saddler, J. N. (2004). Effects of sugar inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. *Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology*, *115*(1–3), 1115–1126. https://doi.org/10.1385/ABAB:115:1-3:1115

Yang, B., Dai, Z., Ding, S., & Wyman, C. E. (2011). *Enzymatic hydrolysis of cellulosic biomass*. *2*, 421–449.

Yang, J., Ching, Y. C., & Chuah, C. H. (2019). Applications of Lignocellulosic Fibers and Lignin in. *Polymers*, *11*, 1–26.

Yogalakshmi, K. N., Poornima, D. T., Sivashanmugam, P., Kavitha, S., Yukesh Kannah, R., Varjani, S., AdishKumar, S., Kumar, G., & J, R. B. (2022). Lignocellulosic biomass-based pyrolysis: A comprehensive review. *Chemosphere*, *286*(P2), 131824. https://doi.org/10.1016/j.chemosphere.2021.131824

Yousuf, A., Pirozzi, D., & Sannino, F. (2020). *Lignocellulosic Biomass to Liquid Biofuels* (R. Zanol (Ed.)). Katie Hammon.

Yun, J. S., Wee, Y. J., & Ryu, H. W. (2003). Production of optically pure L(+)-lactic acid from various carbohydrates by batch fermentation of *Enterococcus faecalis* RKY1. *Enzyme and Microbial Technology*, *33*(4), 416–423. https://doi.org/10.1016/S0141-0229(03)00139-X

Zerva, A., Savvides, A. L., Katsifas, E. A., Karagouni, A. D., & Hatzinikolaou, D. G. (2014). Bioresource Technology Evaluation of *Paecilomyces variotii*

potential in bioethanol production from lignocellulose through consolidated bioprocessing. *BIORESOURCE TECHNOLOGY*, *162*, 294–299. https://doi.org/10.1016/j.biortech.2014.03.137

Zhang, L., Li, X., Yong, Q., Yang, S. T., Ouyang, J., & Yu, S. (2015). Simultaneous saccharification and fermentation of xylo-oligosaccharides manufacturing waste residue for l-lactic acid production by *Rhizopus oryzae*. *Biochemical Engineering Journal*, *94*, 92–99. https://doi.org/10.1016/j.bej.2014.11.020

Zhu, M., Li, P., Gong, X., & Wang, J. (2012). A comparison of the production of ethanol between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using unpretreated cassava pulp and enzyme cocktail. *Bioscience, Biotechnology and Biochemistry*, *76*(4), 671–678. https://doi.org/10.1271/bbb.110750

APPENDICES

A. INFORMATION ON CHEMICALS AND PRODUCERS

Table A.1 The list of chemicals and producers

Chemicals	**Producers**
D-(+)-Glucose monohydrate	Sigma-Aldrich (St. Lois, MO, USA)
Citric acid monohydrate	Merck (Darmstadt, Germany)
Trisodium citrate dihydrate	Merck (Darmstadt, Germany)
Sodium hydroxide	Merck (Darmstadt, Germany)
L-(+)-Arabinose	Fluka Chemie GmbH (Germany)
Yeast Extract	Merck (Darmstadt, Germany)
D- (+)-Galactose	Fluka Chemie GmbH (Germany)
D- (+)-Xylose	Sigma-Aldrich (St. Lois, MO, USA)
D- (-)-Fructose	Merck (Darmstadt, Germany)
D- (+)-Cellobiose	AppliChem GmbH (Germany)
MRS Broth	Merck (Darmstadt, Germany)
Peptone from casein	Condalab (Madrid, Spain)
Tween 80	Merck (Darmstadt, Germany)
Glycerol	Merck (Darmstadt, Germany)
Sulfuric acid	Merck (Darmstadt, Germany)
D- (-)-Lactic acid	Sigma-Aldrich (St. Lois, MO, USA)
Succinic acid disodium salt	Sigma-Aldrich (St. Lois, MO, USA)
Potassium hydroxide	Emir Kimya (Turkey)
Dipotassium hydrogen phosphate	Merck (Darmstadt, Germany)
Potassium dihydrogen phosphate	Merck (Darmstadt, Germany)
Magnesium sulfate heptahydrate	Merck (Darmstadt, Germany)
Manganese (II) sulfate	Horosan Kimya
Calcium carbonate	Unknown
Glycerol	Merck (Darmstadt, Germany)
Dihydrogen potassium sulfate	Sigma-Aldrich (St. Lois, MO, USA)

B. PRODUCTIVITY AND YIELD CALCULATIONS OF ENZYMATIC HYDROLYSIS AND FERMENTATION

The yield and productivity calculations are defined in this section.

$$\text{Yield of enzymatic hydrolysis} = \frac{\text{total sugar produced (g/L)}}{\text{dried horse chestnut shell added (g/L)}}$$

$$\text{Yield of lactic acid} = \frac{\text{lactic acid produced (g/L)}}{\text{dried horse chestnut shell added (g/L)}}$$

$$\text{Productivity of lactic acid} = \frac{\text{lactic acid produced (g/L)}}{\text{total time (h)}}$$

C. STANDARD CURVES OF HPLC

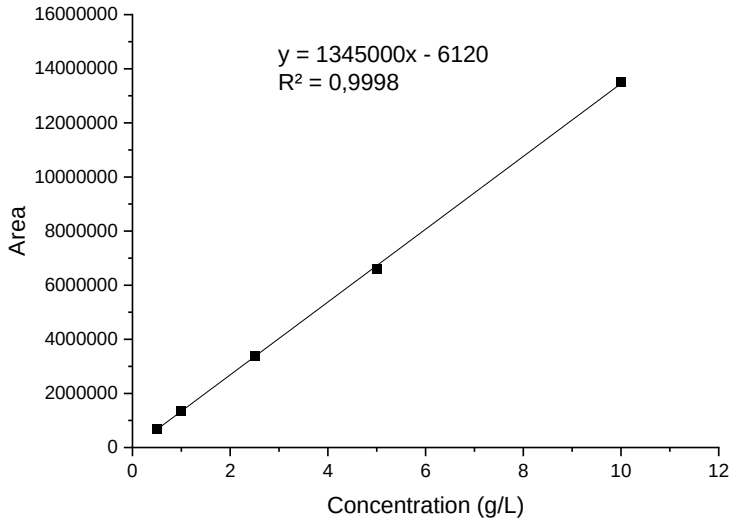


Figure C.1 Standard curve of glucose

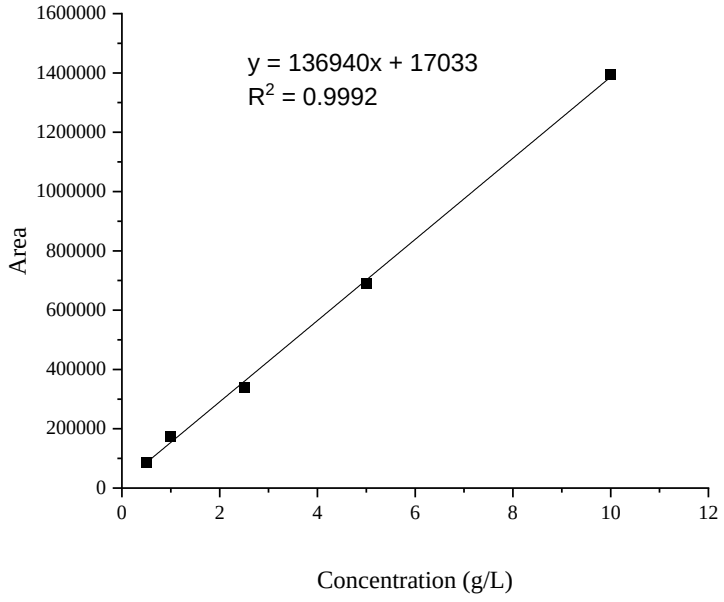


Figure C.2 Standard curve of xylose

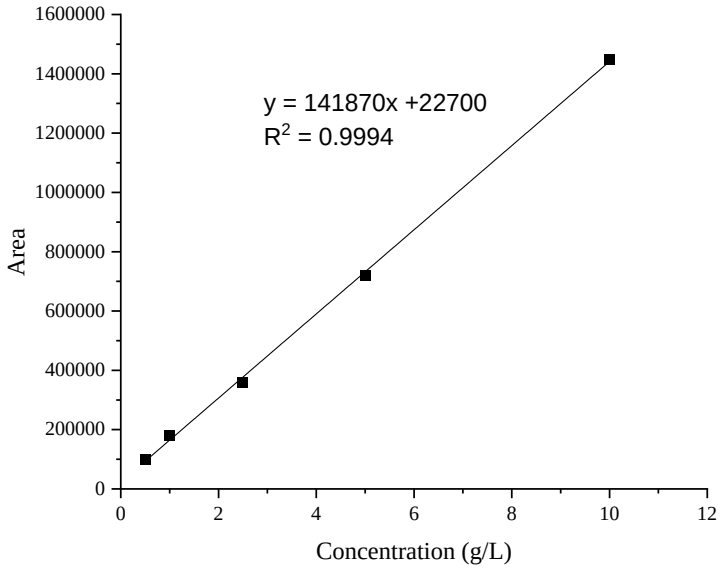


Figure C.3 Standard curve of cellobiose

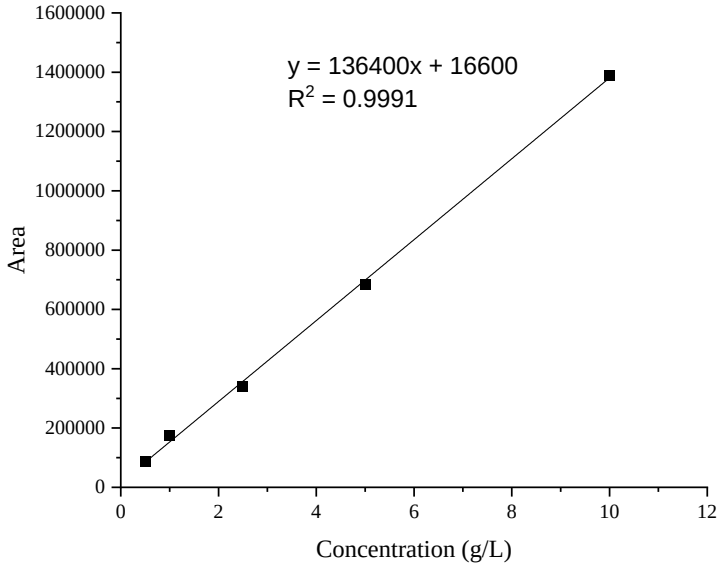


Figure C.4 Standard curve of fructose

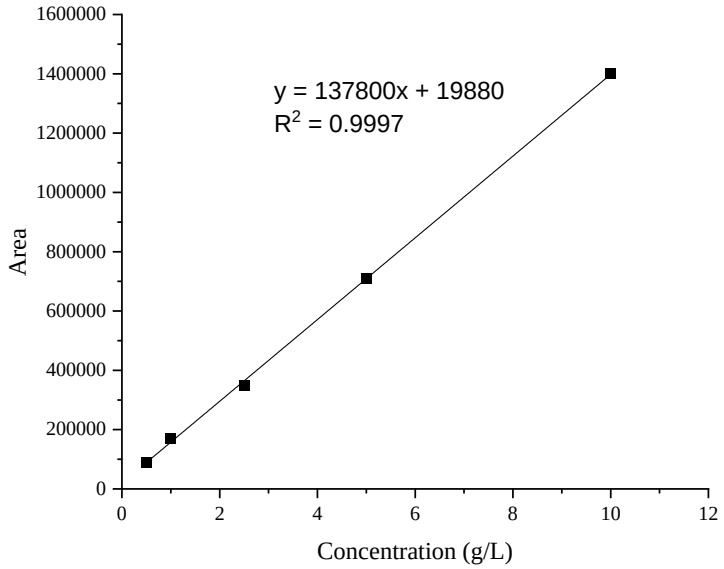


Figure C.5 Standard curve of arabinose

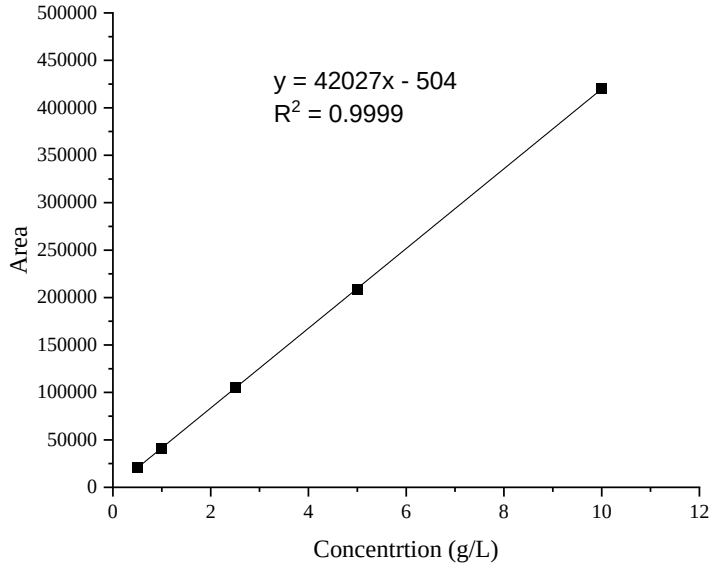


Figure C.6 Standard curve of succinic acid

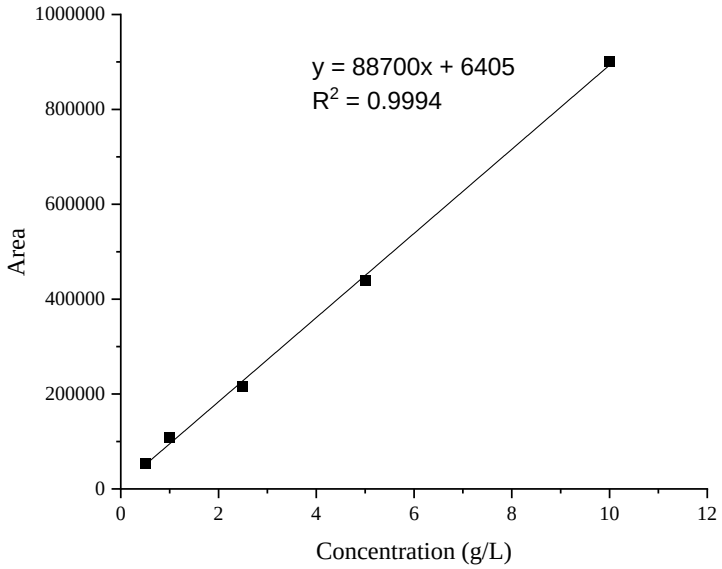


Figure C.7 Standard curve of lactic acid

D. OPTICAL DENSITY ANALYSIS

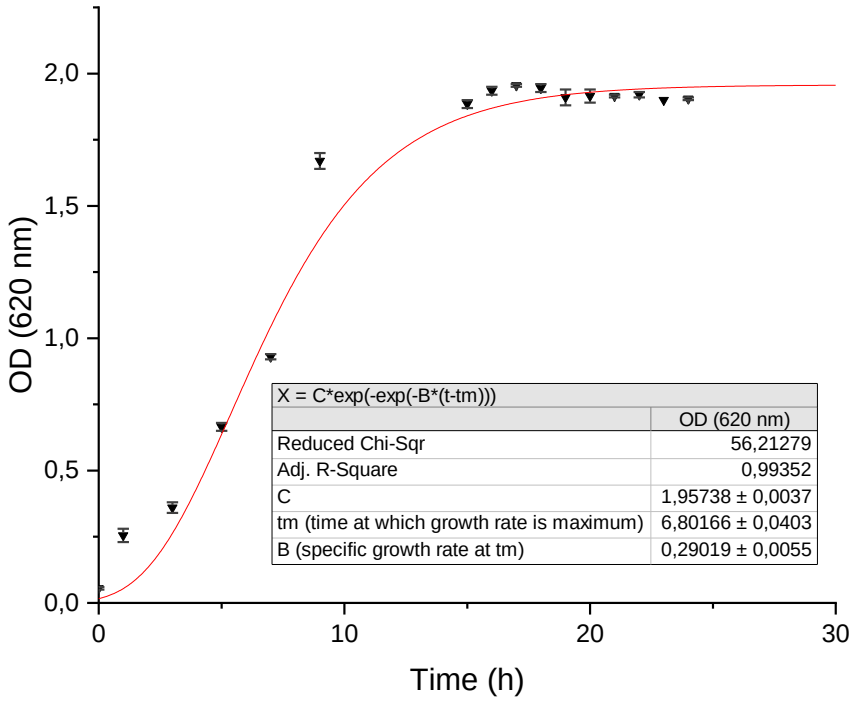
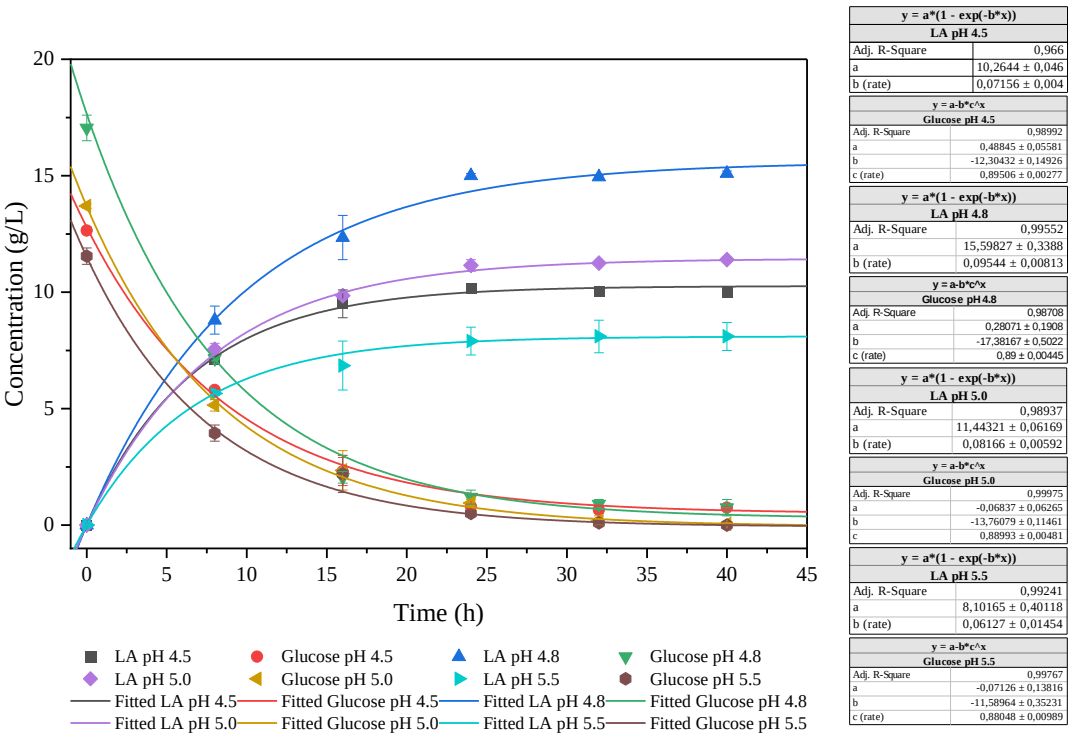


Figure D.1 Growth curve of *Lactobacillus casei* NRRL B-441 at 40ºC fitted with the Gompertz model.

E. ENZYMATIC HYDROLYSIS AND FERMENTATION RATES

The rates of enzymatic hydrolysis and fermentation were found by curve fitting approach.



y = a*(1 - exp(-b*x))	
LA pH 4.5	
Adj. R-Square	0,966
a	10,2644 ± 0,046
b (rate)	0,07156 ± 0,004

y = a-b*c^x	
Glucose pH 4.5	
Adj. R-Square	0,98992
a	0,48845 ± 0,05581
b	-12,30432 ± 0,14926
c (rate)	0,89506 ± 0,00277

y = a*(1 - exp(-b*x))	
LA pH 4.8	
Adj. R-Square	0,99552
a	15,59827 ± 0,3388
b (rate)	0,09544 ± 0,00813

y = a-b*c^x	
Glucose pH 4.8	
Adj. R-Square	0,98708
a	0,28071 ± 0,1908
b	-17,38167 ± 0,5022
c (rate)	0,89 ± 0,00445

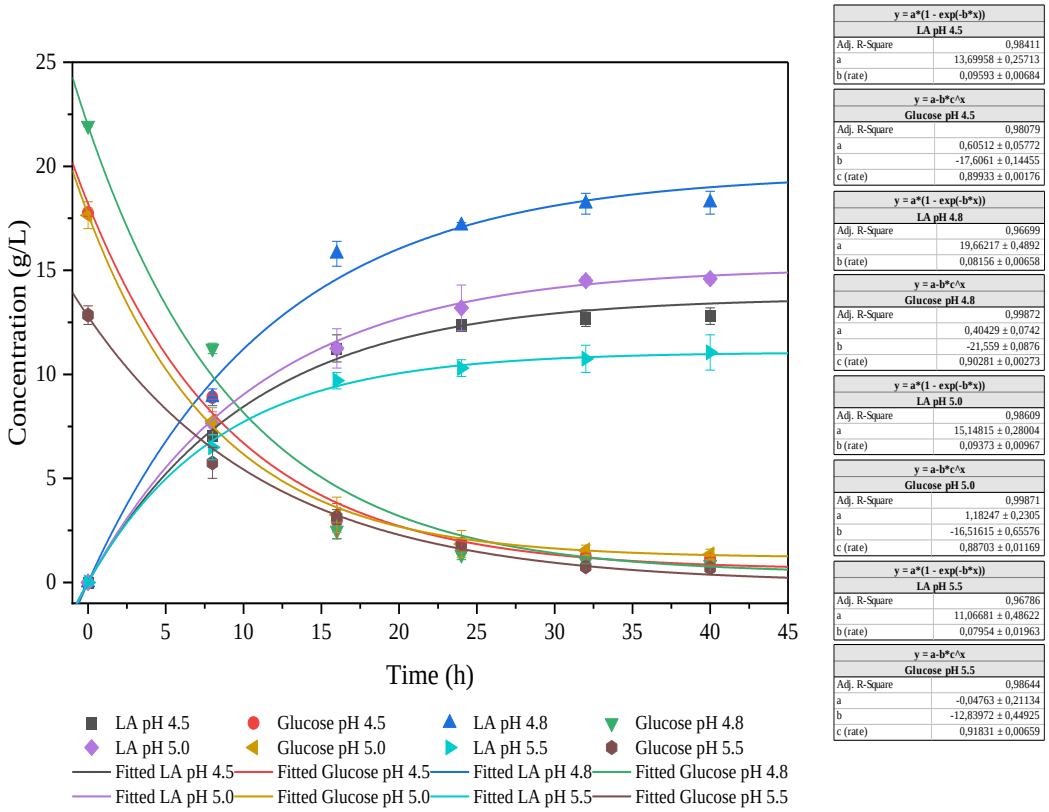
y = a*(1 - exp(-b*x))	
LA pH 5.0	
Adj. R-Square	0,98937
a	11,44321 ± 0,06169
b (rate)	0,08166 ± 0,00592

y = a-b*c^x	
Glucose pH 5.0	
Adj. R-Square	0,99975
a	-0,06837 ± 0,06265
b	-13,76079 ± 0,11461
c	0,88993 ± 0,00481

y = a*(1 - exp(-b*x))	
LA pH 5.5	
Adj. R-Square	0,99241
a	8,10165 ± 0,40118
b (rate)	0,06127 ± 0,01454

y = a-b*c^x	
Glucose pH 5.5	
Adj. R-Square	0,99767
a	-0,07126 ± 0,13816
b	-11,58964 ± 0,35231
c (rate)	0,88048 ± 0,00989

Figure E.1 Fermentation and glucose consumption rates in the separate hydrolysis and fermentation process (hydrolysate from solution at 50ºC, 4.5, 4.8, 5.0, and 5.5 pH, fermentation at 40ºC, 6.0 pH)



y = a*(1 - exp(-b*x))	
LA pH 4.5	
Adj. R-Square	0,98411
a	13,69958 ± 0,25713
b (rate)	0,09593 ± 0,00684

y = a-b*c^x	
Glucose pH 4.5	
Adj. R-Square	0,98079
a	0,60512 ± 0,05772
b	-17,6061 ± 0,14455
c (rate)	0,89933 ± 0,00176

y = a*(1 - exp(-b*x))	
LA pH 4.8	
Adj. R-Square	0,96699
a	19,66217 ± 0,4892
b (rate)	0,08156 ± 0,00658

y = a-b*c^x	
Glucose pH 4.8	
Adj. R-Square	0,99872
a	0,40429 ± 0,0742
b	-21,559 ± 0,0876
c (rate)	0,90281 ± 0,00273

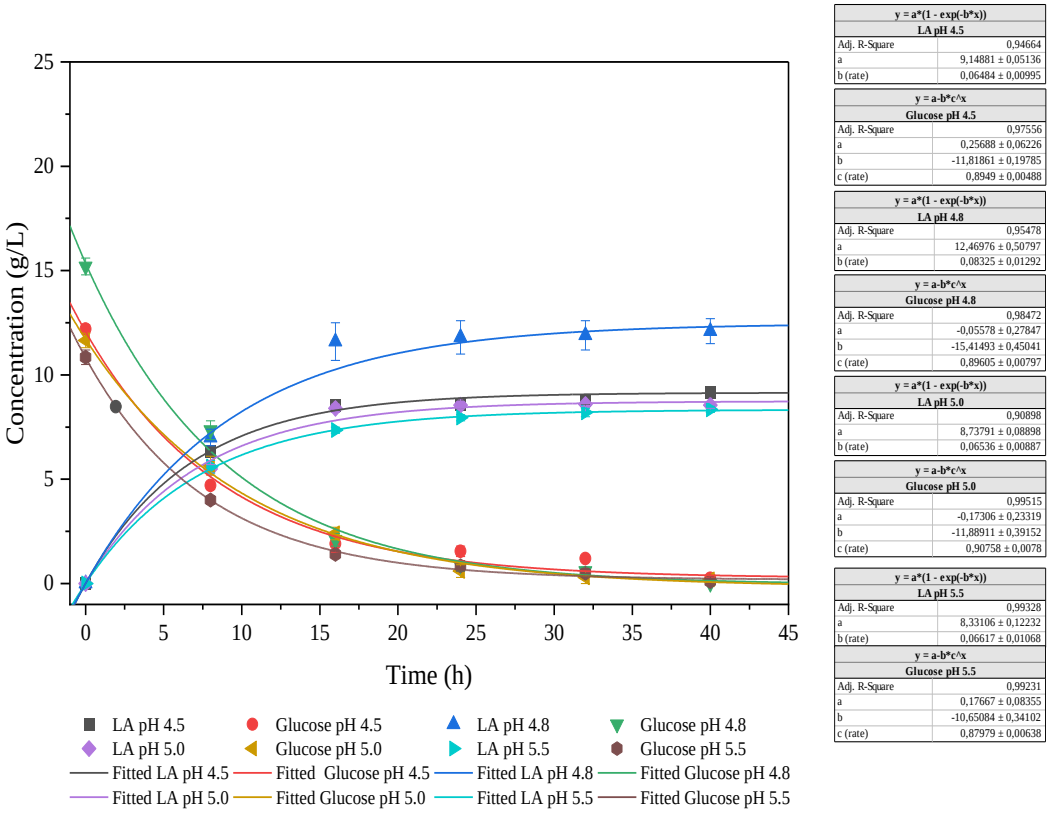
y = a*(1 - exp(-b*x))	
LA pH 5.0	
Adj. R-Square	0,98609
a	15,14815 ± 0,28004
b (rate)	0,09373 ± 0,00967

y = a-b*c^x	
Glucose pH 5.0	
Adj. R-Square	0,99871
a	1,18247 ± 0,2305
b	-16,51615 ± 0,65576
c (rate)	0,88703 ± 0,01169

y = a*(1 - exp(-b*x))	
LA pH 5.5	
Adj. R-Square	0,96786
a	11,06681 ± 0,48622
b (rate)	0,07954 ± 0,01963

y = a-b*c^x	
Glucose pH 5.5	
Adj. R-Square	0,98644
a	-0,04763 ± 0,21134
b	-12,83972 ± 0,44925
c (rate)	0,91831 ± 0,00659

Figure E.2 Fermentation and glucose consumption rates in the separate hydrolysis and fermentation process (hydrolysate from solution at 55ºC, 4.5, 4.8, 5.0, and 5.5 pH, fermentation at 40ºC, 6.0 pH)



y = a*(1 - exp(-b*x))	
LA pH 4.5	
Adj. R-Square	0,94664
a	9,14881 ± 0,05136
b (rate)	0,06484 ± 0,00995

y = a-b*c^x	
Glucose pH 4.5	
Adj. R-Square	0,97556
a	0,25688 ± 0,06226
b	-11,81861 ± 0,19785
c (rate)	0,8949 ± 0,00488

y = a*(1 - exp(-b*x))	
LA pH 4.8	
Adj. R-Square	0,95478
a	12,46976 ± 0,50797
b (rate)	0,08325 ± 0,01292

y = a-b*c^x	
Glucose pH 4.8	
Adj. R-Square	0,98472
a	-0,05578 ± 0,27847
b	-15,41493 ± 0,45041
c (rate)	0,89605 ± 0,00797

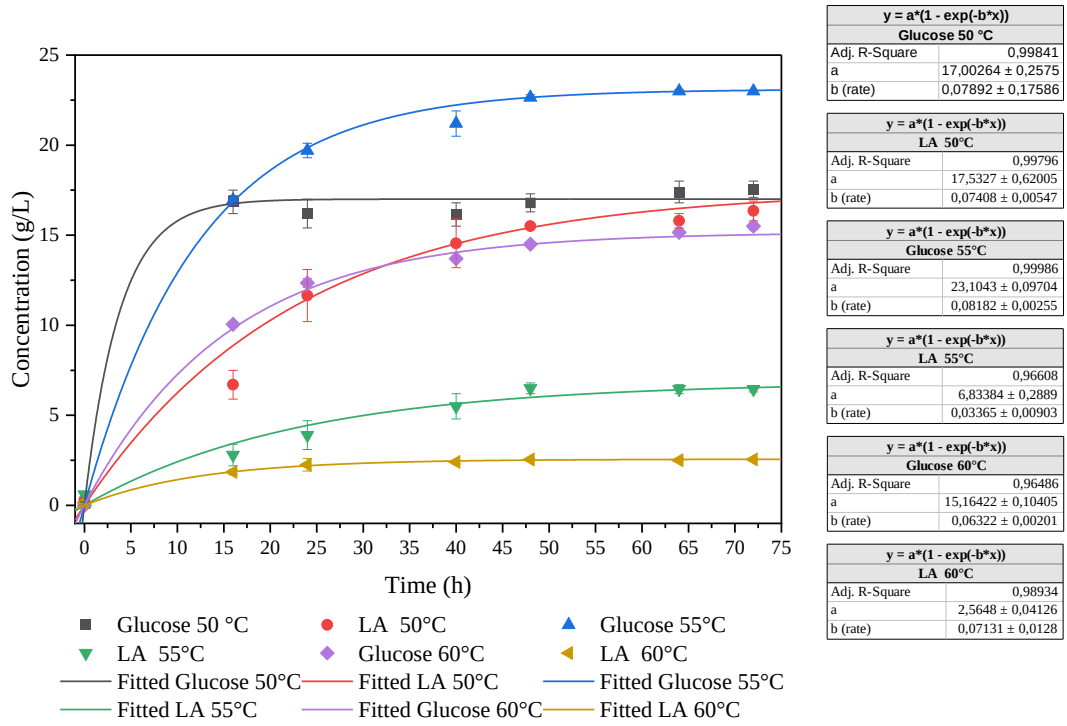
y = a*(1 - exp(-b*x))	
LA pH 5.0	
Adj. R-Square	0,90898
a	8,73791 ± 0,08898
b (rate)	0,06536 ± 0,00887

y = a-b*c^x	
Glucose pH 5.0	
Adj. R-Square	0,99515
a	-0,17306 ± 0,23319
b	-11,88911 ± 0,39152
c (rate)	0,90758 ± 0,0078

y = a*(1 - exp(-b*x))	
LA pH 5.5	
Adj. R-Square	0,99328
a	8,33106 ± 0,12232
b (rate)	0,06617 ± 0,01068

y = a-b*c^x	
Glucose pH 5.5	
Adj. R-Square	0,99231
a	0,17667 ± 0,08355
b	-10,65084 ± 0,34102
c (rate)	0,87979 ± 0,00638

Figure E.3 Fermentation and glucose consumption rates in the separate hydrolysis and fermentation process (hydrolysate from solution at 60ºC, 4.5, 4.8, 5.0, and 5.5 pH, fermentation at 40ºC, 6.0 pH)



y = a*(1 - exp(-b*x))	
Glucose 50 °C	
Adj. R-Square	0,99841
a	17,00264 ± 0,2575
b (rate)	0,07892 ± 0,17586

y = a*(1 - exp(-b*x))	
LA 50°C	
Adj. R-Square	0,99796
a	17,5327 ± 0,62005
b (rate)	0,07408 ± 0,00547

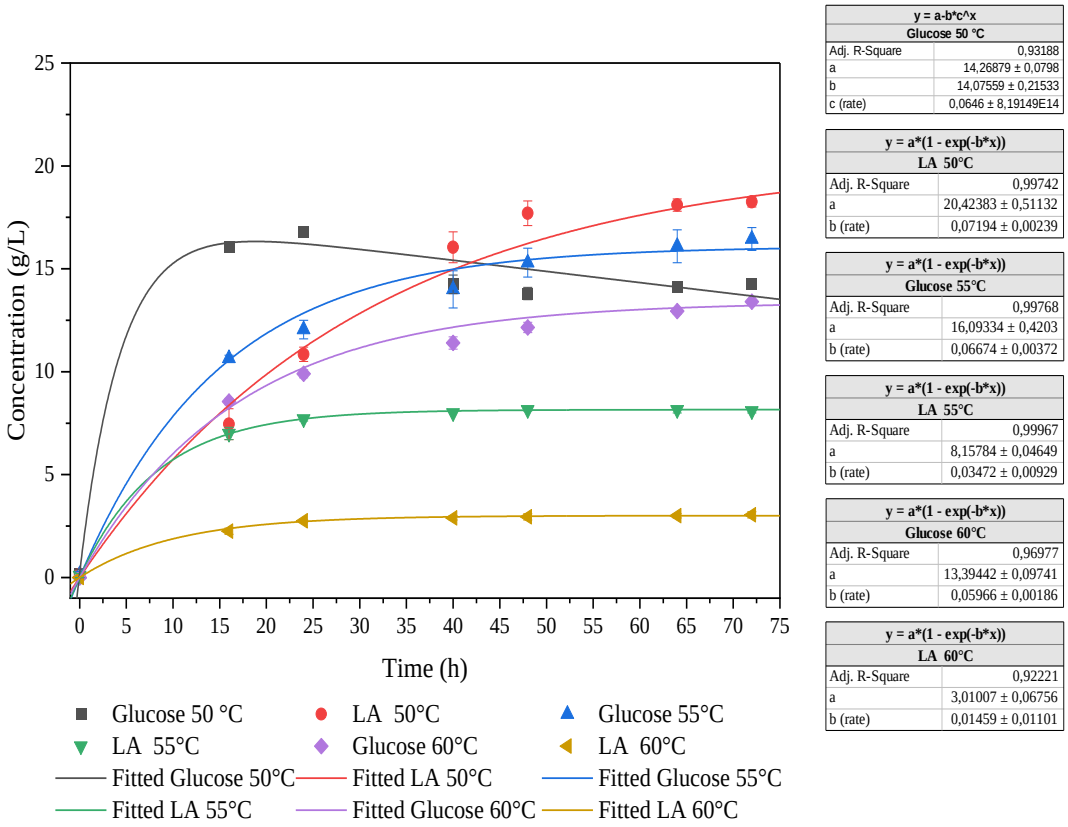
y = a*(1 - exp(-b*x))	
Glucose 55°C	
Adj. R-Square	0,99986
a	23,1043 ± 0,09704
b (rate)	0,08182 ± 0,00255

y = a*(1 - exp(-b*x))	
LA 55°C	
Adj. R-Square	0,96608
a	6,83384 ± 0,2889
b (rate)	0,03365 ± 0,00903

y = a*(1 - exp(-b*x))	
Glucose 60°C	
Adj. R-Square	0,96486
a	15,16422 ± 0,10405
b (rate)	0,06322 ± 0,00201

y = a*(1 - exp(-b*x))	
LA 60°C	
Adj. R-Square	0,98934
a	2,5648 ± 0,04126
b (rate)	0,07131 ± 0,0128

Figure E.4 Hydrolysis and fermentation rates in the simultaneous saccharification and fermentation process (at 4.8 pH, 50, 55, and 60 ºC)



y = a-b*c^x	
Glucose 50 °C	
Adj. R-Square	0,93188
a	14,26879 ± 0,0798
b	14,07559 ± 0,21533
c (rate)	0,0646 ± 8,19149E14

y = a*(1 - exp(-b*x))	
LA 50°C	
Adj. R-Square	0,99742
a	20,42383 ± 0,51132
b (rate)	0,07194 ± 0,00239

y = a*(1 - exp(-b*x))	
Glucose 55°C	
Adj. R-Square	0,99768
a	16,09334 ± 0,4203
b (rate)	0,06674 ± 0,00372

y = a*(1 - exp(-b*x))	
LA 55°C	
Adj. R-Square	0,99967
a	8,15784 ± 0,04649
b (rate)	0,03472 ± 0,00929

y = a*(1 - exp(-b*x))	
Glucose 60°C	
Adj. R-Square	0,96977
a	13,39442 ± 0,09741
b (rate)	0,05966 ± 0,00186

y = a*(1 - exp(-b*x))	
LA 60°C	
Adj. R-Square	0,92221
a	3,01007 ± 0,06756
b (rate)	0,01459 ± 0,01101

Figure E.5 Hydrolysis and fermentation rates in the simultaneous saccharification and fermentation process (at 5.5 pH, 50, 55, and 60 ºC)

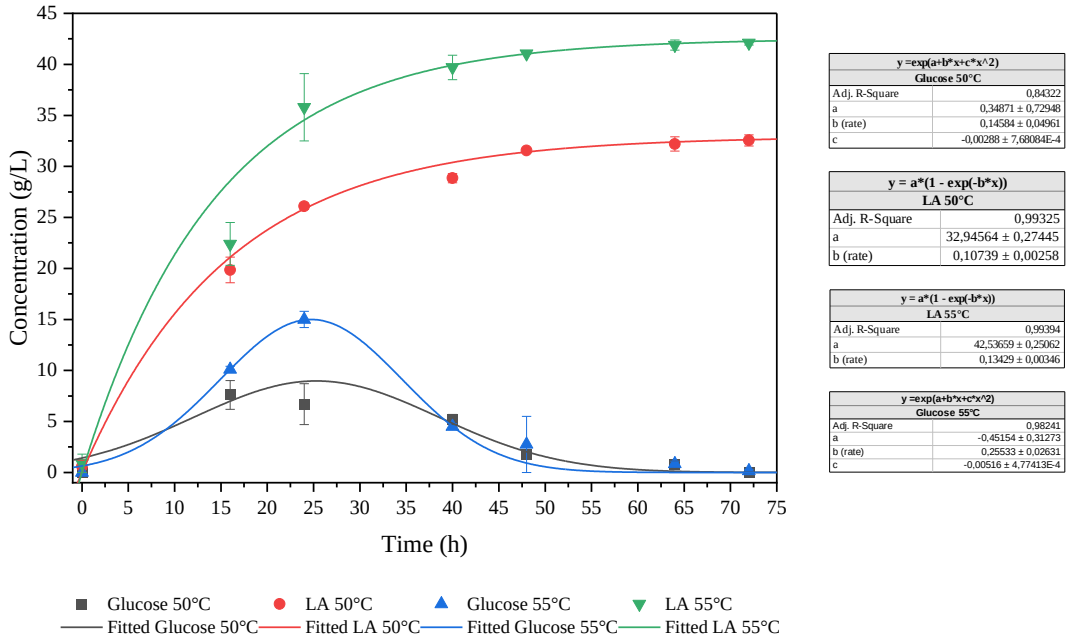
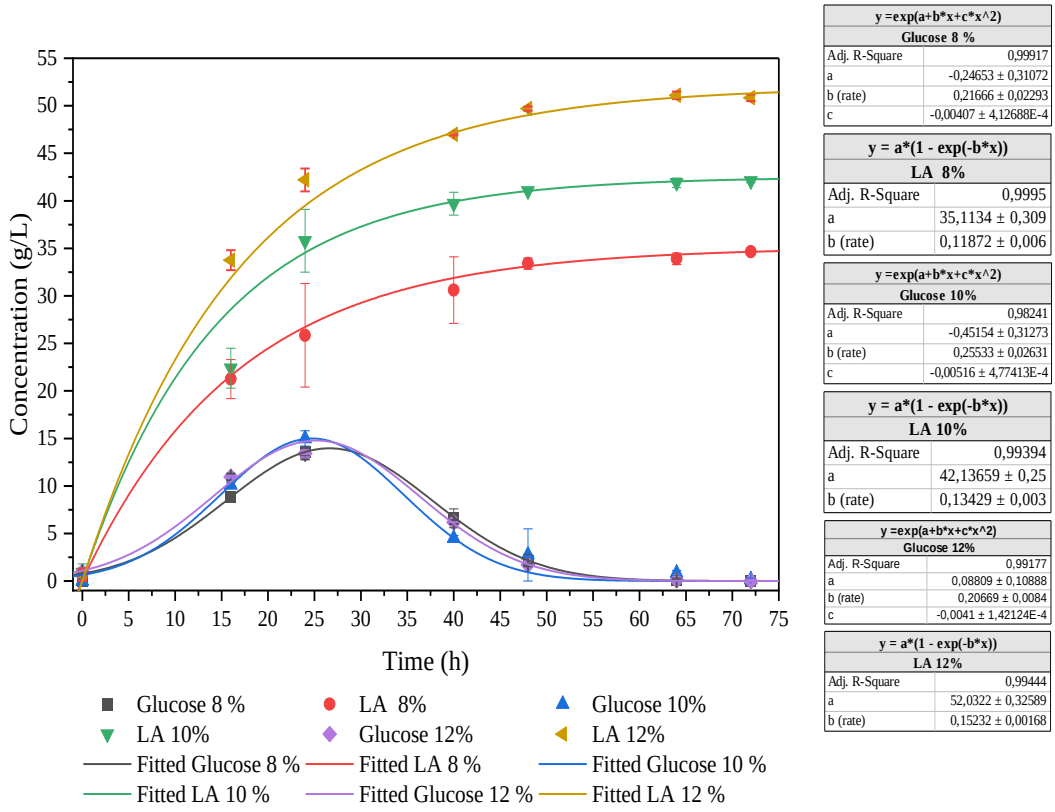


Figure E.6 Hydrolysis and fermentation rates in the simultaneous saccharification and fermentation with dual bioreactor process (hydrolysis bioreactor at 4.8 pH, 50 and 55 ºC, fermentation bioreactor at 6.0 pH, 40 ºC)



y =exp(a+b*x+c*x^2)	
Glucose 8 %	
Adj. R-Square	0,99917
a	-0,24653 ± 0,31072
b (rate)	0,21666 ± 0,02293
c	-0,00407 ± 4,12688E-4

y = a*(1 - exp(-b*x))	
LA 8%	
Adj. R-Square	0,9995
a	35,1134 ± 0,309
b (rate)	0,11872 ± 0,006

y =exp(a+b*x+c*x^2)	
Glucose 10%	
Adj. R-Square	0,98241
a	-0,45154 ± 0,31273
b (rate)	0,25533 ± 0,02631
c	-0,00516 ± 4,77413E-4

y = a*(1 - exp(-b*x))	
LA 10%	
Adj. R-Square	0,99394
a	42,13659 ± 0,25
b (rate)	0,13429 ± 0,003

y =exp(a+b*x+c*x^2)	
Glucose 12%	
Adj. R-Square	0,99177
a	0,08809 ± 0,10888
b (rate)	0,20669 ± 0,0084
c	-0,0041 ± 1,42124E-4

y = a*(1 - exp(-b*x))	
LA 12%	
Adj. R-Square	0,99444
a	52,0322 ± 0,32589
b (rate)	0,15232 ± 0,00168

Figure E.7 Hydrolysis and fermentation rates in the simultaneous saccharification and fermentation with dual bioreactor process with 8, 10, and 12 % (w/v) solid loads (hydrolysis bioreactor at 4.8 pH, 50 and 55 ºC, fermentation bioreactor at 6.0 pH, 40 ºC)

F. HPLC CHROMATOGRAM SAMPLES

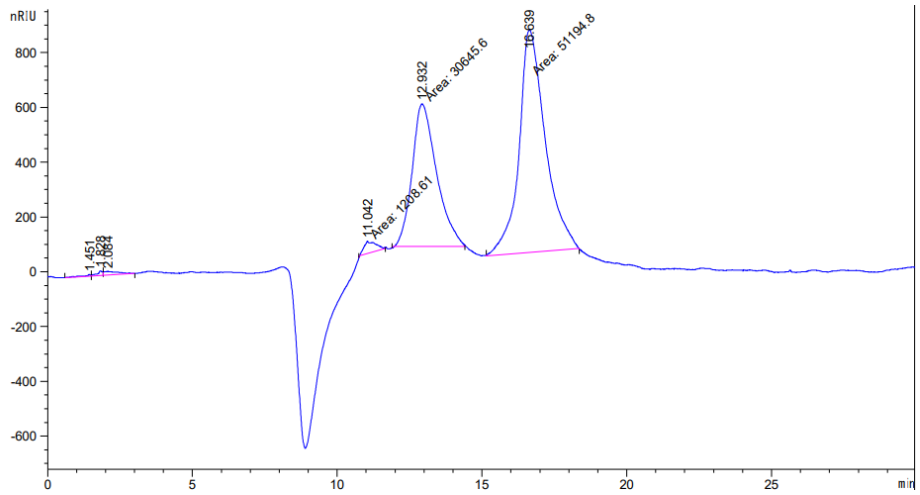


Figure F.1 Chromatogram of enzymatic hydrolysis in SHF (xylose at 11.04 min, glucose at 12.93 min, and succinic acid at 16.63 min)

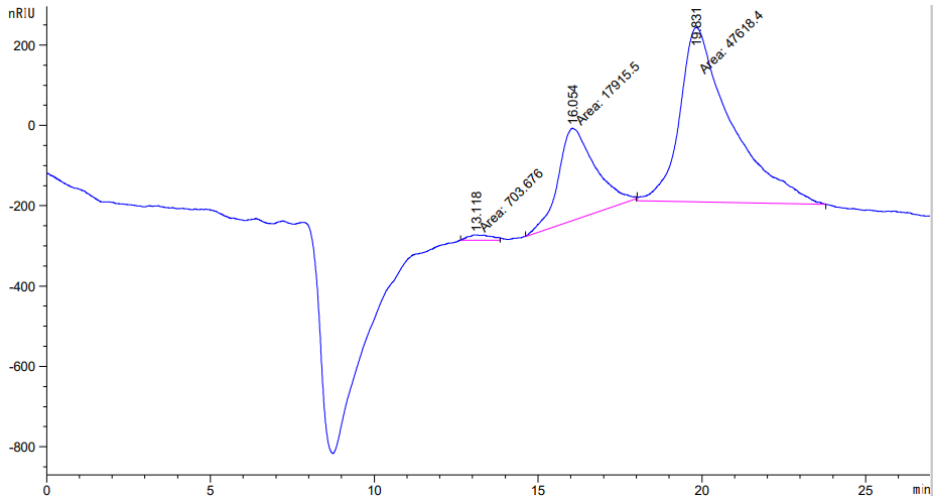


Figure F.2 Chromatogram of SSF2 (glucose at 13.12 min, succinic acid at 16.63 min, and lactic acid at 19.83 min)

G. STATISTICAL ANALYSIS OF DATA

Table G.1 Two-way ANOVA for pH and temperature effect on total sugar concentration in the hydrolysis of SHF

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	3	312.778	104.259	216.08	0.000
Temperature	2	115.067	57.534	119.24	0.000
pH*Temperature	6	12.029	2.005	4.16	0.017
Error	12	5.790	0.483		
Total	23	445.665			
S = 0.6946		R-Sq = 98.70 %			R-sq(adj) = 97.51 %

Table G.2 Two-way ANOVA for pH and temperature effect on glucose concentration in the hydrolysis of SHF

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	3	122.468	40.8228	151.20	0.000
Temperature	2	112.210	56.1050	207.80	0.000
pH*Temperature	6	16.747	2.7911	10.34	0.000
Error	12	3.240	0.2700		
Total	23	254.665			
S = 0.5196		R-Sq = 98.73 %			R-sq(adj) = 97.56 %

Table G.3 Three-way ANOVA for time, pH, and temperature effect on lactic acid concentration in the SSF process

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	6	773.82	128.969	303.20	0.000
pH	1	30.60	30.601	71.94	0.000
Temperature	2	1406.72	703.360	1653.58	0.000
Time*pH	6	8.22	1.371	3.22	0.011
Time*Temperature	12	393.72	32.810	77.13	0.000
pH*Temperature	2	11.02	5.508	12.95	0.000
Time*pH*Temperature	12	15.63	1.303	3.06	0.004
Error	42	17.87	0.425		
Total	83	2657.59			
S = 0.6522		R-Sq = 99.33 %			R-sq(adj) = 98.67 %

Table G.4 Three-way ANOVA for time, pH, and temperature effect on hydrolyzed glucose concentration in the SSF process

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	6	2486.66	414.444	1001.82	0.000
pH	1	216.00	216.001	522.13	0.000
Temperature	2	273.32	136.659	330.34	0.000
Time*pH	6	43.12	7.186	17.37	0.000
Time*Temperature	12	130.17	10.848	26.22	0.000
pH*Temperature	2	87.94	43.968	106.28	0.000
Time*pH*Temperature	12	26.17	2.181	5.27	0.000
Error	42	17.37	0.414		
Total	83	3280.76			
S = 0.6432		R-Sq = 99.47 %			R-sq(adj) = 98.95 %

Table G.5 Two-way ANOVA for time and temperature effect on lactic acid concentration in the hydrolysis and fermentation of SSF2

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	6	4300.82	716.803	240.39	0.000
Temperature	1	388.52	388.517	130.30	0.000
Time*Temperature	6	105.43	17.572	5.89	0.003
Error	14	41.75	2.982		
Total	27	4836.51			
S = 1.7268		R-Sq = 99.14 %			R-sq(adj) = 98.34 %

Table G.6 Two-way ANOVA for time and temperature effect on glucose concentration in the hydrolysis and fermentation of SSF2

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	6	463.10	77.184	36.29	0.000
Temperature	1	18.24	18.241	8.58	0.011
Time*Temperature	6	58.32	9.721	4.57	0.009
Error	14	29.78	2.127		
Total	27	569.45			
S = 1.4585		R-Sq = 94.77 %			R-sq(adj) = 89.91 %

Table G.7 Two-way ANOVA for time and solid load effect on lactic acid concentration in the hydrolysis and fermentation of SSF2

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	6	8277.8	1379.63	212.72	0.000
Solid load	2	1293.5	646.75	99.72	0.000
Time*Solid load	12	264.5	22.04	3.40	0.007
Error	21	136.2	6.49		
Total	41	9971.9			
S = 2.5467		R-Sq = 98.63 %			R-sq(adj) = 97.33 %

Table G.8 Two-way ANOVA for time and solid load effect on glucose concentration in the hydrolysis and fermentation of SSF2

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	6	1084.17	180.695	376.64	0.000
Solid load	2	0.65	0.326	0.68	0.518
Time*Solid load	12	14.83	1.236	2.58	0.028
Error	21	10.07	0.480		
Total	41	1109.73			
S = 0.6927		R-Sq = 99.09 %			R-sq(adj) = 98.23 %