MICROBIAL PRODUCTION OF ALKALINE PECTINASE FROM HAZELNUT SHELL

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

BY

SİBEL UZUNER

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
FOOD ENGINEERING

DECEMBER 2014

Approval of the thesis:

MICROBIAL PRODUCTION OF ALKALINE PECTINASE FROM HAZELNUT SHELL

submitted by **SİBEL UZUNER** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Food Engineering Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Alev Bayındırlı _____
Head of Department, **Food Engineering**

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

Examining Committee Members:

Prof. Dr. Faruk Bozoğlu _____
Food Engineering Dept., METU

Assoc. Prof. Dr. Deniz Çekmecelioğlu _____
Food Engineering Dept., METU

Prof. Dr. Alev Bayındırlı _____
Food Engineering Dept., METU

Assoc. Prof. Dr.Gülsün Evrendilek _____
Food Engineering Dept., Abant Izzet Baysal University

Assoc. Prof. Dr. Irfan Turhan _____
Food Engineering Dept., Akdeniz University

Date: 29.12.2014

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: Sibel Uzuner

Signature:

ABSTRACT

MICROBIAL PRODUCTION OF ALKALINE PECTINASE FROM HAZELNUT SHELL

Uzuner, Sibel

Ph. D., Department of Food Engineering

Supervisor : Assoc. Prof. Dr. Deniz Cekmecelioglu

December 2014, 217 pages

Utilization of cheap and abundant materials for enzyme production is one of the strategies that can reduce the product costs. Besides, use of renewable agro-food industrial wastes as a raw material provides not only low cost and sustainable value-added products but also is a solution to waste disposal problem.

In this study, fermentation medium composition and conditions for maximal production of pectinase enzyme from *Bacillus subtilis* in submerged fermentation were investigated. The potential use of crude enzyme for clarification of carrot juice was also evaluated. In order to enhance utilization of the hazelnut shells as carbon source in pectinase production, various pretreatment methods including dilute acid, alkaline, and ozone pretreatments were tested prior to enzymatic hydrolysis step.

After conversion of hazelnut shells to fermentable sugars, the "Plackett-Burman" (PB) design was used for screening of the eight factors; pH, fermentation time, temperature, inoculum volume (%v/v) and of pectin, yeast extract (YE), magnesium sulphate [$MgSO_4$], and dipotassium hydrogen phosphate [K_2HPO_4]. Five variables (pH, time, temperature, yeast extract concentration and K_2HPO_4), which were determined to be significant by PB design, were further optimized using Box-Behnken response surface method to maximize the PG activity.

The produced crude enzyme was tested in clarification of carrot juice, highly nutritious and worldwide consumed food material, afterwards. The carrot juice was treated with different concentration of crude pectinase (0.1-0.5%), pH (4-7), and time (2-6 h) for clarification.

Among the pretreatment methods tested, the dilute acid pretreatment (3.42 (w/w)% acid, 31.7 min, 130 ºC) was chosen the best with higher sugar conversion (62.8% saccharification yield) than sodium hydroxide and ozone pretreatment methods.

The pectinase optimization results indicated that a maximal PG activity of 5.60 U/mL was achieved at pH 7.0, 72 h, and 30 ºC using 0.5% (w/v) of yeast extract and 0.02% (w/v) of K_2HPO_4.

The results of clarification revealed that 100% clarity was achieved at 0.5% (w/v) enzyme load, 7.0 pH, and 6 h of clarification yield (%) with commercial enzyme reached only 78.18±3.14 %.

This study also proved that crude enzyme was equally effective as the purified commercial enzyme.

Keywords: Alkaline pectinase enzyme, bioconversion, carrot juice, depectinization, optimization

ÖZ

FINDIK KABUĞUNDAN MİKROBİYAL ALKALİ PEKTİNAZ ÜRETİMİ

Uzuner, Sibel
Doktora, Gıda Mühendisliği Bölümü
Tez Yöneticisi: Doç. Dr. Deniz Çekmecelioğlu

Aralık 2014, 217 sayfa

Enzim üretiminde ucuz ve bol bulunan maddelerin kullanımı ürün maliyetini düşürebilecek stratejilerden biridir. Üstelik ham madde olarak yenilenebilir gıda-tarım atıklarının kullanımı yalnızca düşük maliyetli ve sürdürülebilir katma değerli ürünler sağlamakla kalmaz aynı zamanda atık tasfiyesi için de çözüm olabilmektedir.

Bu çalışmada, *Bacillus subtili*s kullanarak derin kültür fermentasyon yönteminde pektinaz enzimi üretimini arttırmak amacıyla fermentasyon ortam kompozisyonu ve koşulları araştırılmıştır. Ham enzimin havuç suyunu berraklaştırma potansiyeli de değerlendirilmiştir. Pektinaz üretiminde fındık kabuğunun karbon kaynağı olarak kullanımını iyileştirmek amacıyla enzimatik hidroliz öncesi seyreltik asit, alkali ve ozon gibi farklı ön işlemler denenmiştir.

Fındık kabuklarının fermente edilebilir şekerlere dönüştürülmesinden sonra, pH, fermentasyon süresi, sıcaklık, inokulüm hacmi (%v/v), pektin, maya özütü, magnezyum sülfat [$MgSO_4$] ve dipotasyum hidrojen fosfat [K_2HPO_4] derişimi gibi 8 faktörün etkilerini araştırmada "Plackett-Burman" (PB)' tasarımı kullanılmıştır. PB tasarımıyla önemli bulunan beş faktör (pH, zaman, sıcaklık, maya özütü derişimi ve K_2HPO_4 derişimi), PG aktivitesini arttırmak amacıyla Box-Behnken yüzey yanıt yöntemiyle optimize edilmiştir.

Üretilen ham enzim daha sonar oldukça besleyici ve en çok tüketilen gıdalardan biri olan havuç suyunu berraklaştırmada denenmiştir. Havuç suyu, farklı ham enzim derişimi (0.1-0.5%), 4-7 pH ve 2-6 saat süre ile muamele edilmiştir.

Denenen ön işlemler arasında sodyum hidroksit ve ozondan daha yüksek şeker dönüşümü değerlerine (% 62.8 sakarifikasyon verimi) sahip olan seyreltik asit ön işlemi (%3.42 asit, 31.7 dakika, 130 ^{o}C) en iyi yöntem olarak seçilmiştir.

Pektinaz optimizasyon sonuçları, pH 7.0, 30 ^{o}C, 50.5 maya özütü, % 0.02 K_2HPO_4 ve 72 saat sonunda en yüksek PG aktivitesinin 5.60 U/mL olduğunu göstermiştir.

En yüksek berraklaştırma verimi %0.5 enzim ile pH 7.0 ve 6 saat süre sonunda % 100 olarak elde edilirken, ticari enzim ile berraklaştırma verimi % 78.18±3.14'e ulaşmıştır.

Ayrıca, bu çalışma ham enzimin saflaştırılmış ticari enzimle aynı derecede etkili olduğunu da kanıtlamıştır.

Anahtar Kelimeler: Alkali pektinaz enzimi, biyodönüşüm, havuç suyu, enzimatik durultma, optimizasyon

To my beloved parents…

ACKNOWLEDGEMENTS

First and foremost, my deepest gratitude is to my advisor, Assoc. Prof. Dr. Deniz Çekmecelioğlu for his endless support, understanding and encouragement throughout this study. I have been privileged to have an advisor who gave me the freedom to explore on my own.

I would like to express sincere thanks to Assoc. Prof. Dr. Ratna Sharma Shivappa for her great support, hospitality, kindness, and providing a great laboratory environment during my reseaarch at North Carolina University Agricultural and Biological Engineering Department.

I would like to express my appreciation to my Ph.D. Examining Committee members, Prof. Dr. Alev Bayındırlı and Assoc. Prof. Dr. Gülsün Akdemir Evrendilek for their enlightening comments and directions.

I would like to thank METU Council of Scientific Research, (BAP-03-14-2011-002) for funding this study, and Department of Food Engineering, METU for experimental support and to The Scientific and Technological Council of Turkey for granting the Science Fellowships and Grant Program (TÜBİTAK-BIDEB) during my research study.

I owe a debt of gratitude to my lab friend and office mate Ali Übeyitoğulları who actually suffered with me especially during writing my thesis. I am thankful to the valuable members of our research group, Abduvali Valiev, Oya Nihan Uncu, Gözde Ören Yardımcı and Önay Burak Doğan for their support and invaluable assistance.

I would also like to thank to all my colleagues and friends; Elif Yolaçaner, Alev Emine İnce, Özlem Yüce, Armağan Cabadağ, Hande Baltacıoğlu, Sezen Sevdin, Ece Bulut, and Sinem Acar, for their friendship and the invaluable stimuli that made me focus on my work again. Life would be boring without them.

I would like to express my deepest and biggest appreciation to my parents, Sema-Erol Uzuner and also to my sister, Selin Uzuner. I would like to thank them for always believing in me.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

AIL	Acid Insoluble Lignin
ASL	Acid Soluble Lignin
AFEX	Ammonia Fiber Explosion
ANN	Artificial Neural Network
ANOVA	Analysis Of Variance
BBD	Box Behnken Design
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
DNS	3,5-dinitrosalicylic Acid
FPU	Filter Paper Unit
GRAS	Generally Regarded As Safe
HMF	Hydroxymethylfurfural
HPLC	High Performance Liquid Chromatography
kDa	Kilo Dalton
LHW	Liquid Hot Water
MAE	Mean Absolute Error
MLP	Multi-layer Perceptron
MSE	Mean Squared Error
NaOH	Sodium Hydroxide
NRRL	Northern Regional Research Laboratory
OPEFB	Oil Palm Empty Fruit Bunch Fiber
PAL	Pectic Acid Lyases
PATE	Pectic Acid Trans Eliminase
PBD	Plackett Burman Design
PE	Pectin Esterases

PG	Polygalacturonase
PGL	Polygalacturonate Lyases
PMG	Polymethylgalacturonase
PMGL	Polymethylgalacturonate Lyases
RMSE	Root Mean Square Error
RPD	Root Percent Deviation
RSM	Response Surface Methodology
Rpm	Revolutions per Minute
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SLR	Solid-liquid Ratio
SmF	Submerged Fermentation
SSF	Solid-state Fermentation
U	Unit

CHAPTER 1

INTRODUCTION

Enzymes are key components of textile, paper, pulp, pharmaceutical and food industries. The total value of the food and beverage enzyme market is estimated as $320 million in 2013. Among the food enzymes, pectinases (or pectinolytic enzymes) account for 25% of food enzymes which are produced from microbial sources. Pectinases are also used in diverse applications such as extraction and clarification of fruit juices (Lee et al., 2006; Sandri et al., 2011), bleaching of paper pulp (Ahlawat et al., 2008), degumming of fibers (Sharma & Satyanarayana, 2012), oil extraction (Najafian et al., 2009), coffee and tea fermentation (Kashyap et al., 2001; Murthy &Naidu, 2011). One of the drawbacks of pectinase application in food, pharmaceutical and chemical industry is their high cost. Therefore it is of great importance to reduce the cost of enzyme production and optimize enzyme production conditions in order to meet the increasing demand. Production of enzymes with low production cost is still a new challenge area. A significant cost reduction may be achieved by using high yielding strains, optimal fermentation conditions and cheap raw materials as a carbon source for growing microorganisms.

Various agro-industrial by-products can be successfully utilized for microbial pectinolytic enzyme production since these by-products are relatively inexpensive, renewable, and widely available in nature. The agro-industrial byproducts are composed of complex polysaccharides, which are used for microbial growth to enhance the production of industrially important enzymes. The agro-industrial byproducts consist of many and varied wastes from agriculture and food industry, which in total account for over 25 million tonnes of waste per year in Turkey. These by-products still have a limited industrial use and cause potential environmental threat. Identification and bioconversion of new locally available agro-wastes is

advantageous to enhance both economic and environmental benefits. From the biotechnological point of view, a wide variety of hazelnut shells, which can be potential source of sugars such as xylose and glucose are available as potential candidate for production of value added bioproducts like bio-ethanol/bio-fuels, enzyme, organic acids, etc. The classification, mechanism and application of pectinolytic enzymes are well described and the definition of pectinases produced from fungal and bacterial sources are reviewed in Chapter 2.

To best of our knowledge no work has been reported on pectinase production from hazelnut shells as carbon source using *Bacillus subtilis*, which adds extra novelty to our work. Therefore, this thesis will be one of the initial studies working in this field.

The hypothesis is if a more fermentable sugar is recovered from hazelnut shells, then *B.subtilis* will grow faster and produce more PG activity. The goal of this research was to study the potential and performance of biomass product for pectinase production and to increase pectinase activity through increasing fermentable sugar recovery with high-efficiency pretreatment methods such as acid, alkali and ozone. Dilute acid, alkaline and ozone as pretreatment methods were used to increase sugar recovery from hazelnut shells. The effect of pretreatment on enzymatic hydrolysis, final sugar yield, and pectinase fermentation were studied. All of the pretreatment methods used in this study were optimized with respect to total reducing sugar yield (Chapter 3).

Fermentation medium and conditions were screened to select important factors which affect pectinase production. The key factors of fermentation medium and conditions for submerged fermentation are mentioned in Chapter 3. To investigate the clarification step, an experimental design was also set up and its results were analyzed with the statistical tools in Chapter 3.

Enzyme production conditions were optimized according to selective factors such as pH, time, temperature, yeast extract concentration and K_2HPO_4. Additionally, the constructed model was numerically optimized and validated by selecting various factors about pectinase production. The screening, optimization and

validation results of pectinase production are presented in Chapter 4. Furthermore characterization of the enzyme with respect to its optimum pH and temperature and the effect of these on the stability were also investigated and expressed in Chapter 4.

Raw juice is clarified to avoid turbidity, haze, and sediments in the final products before commercialization. There are several studies on optimization of enzymatic clarification of fruit juices using commercial pectinases of fungal origin. To the best of our knowledge, there is not any report on optimization of enzymatic clarification of fruit juices using crude pectinase produced from hazelnut shells. To determine the effectiveness of crude pectinase, the parameters such as enzyme concentration, temperature and time were optimized during clarification of carrot juice (Chapter 4).

CHAPTER 2

LITERATURE REVIEW

2.1 Pectin

Pectin is a complex polysaccharide made of linear chains of α-(1–4)-linked D-galacturonic acid D-xylose (xylogalacturonan) or D-apiose (apiogalacturonan), branching from the D-galacturonic acid backbone. They are important components of cell wall and middle lamella, and can be found in fruits and vegetables (Saad et al., 2007). The pectic substances account for about 0.5–4% of the weight of fresh material (Table 2.1). The raw juice is rich in insoluble particles mainly made up of pectic substances (Jayani et al., 2005).

Table 2.1 Composition of pectin in different fruits and vegetables (Jayani et al. 2005)

Fruit/Vegetable	Tissue	Pectic substance (%)
Apple	Fresh	0.5-1.6
Orange pulp	Dry matter	12.4-28.0
Strawberries	Fresh	0.6-0.7
Banana	Fresh	0.7-1.2
Peaches	Fresh	0.1-0.9
Tomatoes	Dry matter	2.4-4.6
Carrot	Dry matter	6.9-18.6

2.2 Pectinolytic Enzymes (Pectinases)

Enzymes cleaving pectic substances are called pectinolytic enzymes or pectinases which are of great industrial importance (Fogarty &Kelly, 1983; Saad et al., 2007). Pectin provides strength and structure to plant cells (Kaur et al., 2004).

Pectinases can be classified as pectinesterases, depolymerizing enzymes and protopectinases on the basis of their role in the degradation of pectin (Jayani et al., 2005). Different pectic enzymes and their mode of action are illustrated in Figure 2.1. Pectinesterases or pectin methyl hydrolases (PE) catalyze hydrolytic removal of the methyl ester group of pectin, forming pectic acid. Depolymerizing enzymes are the enzymes hydrolyzing or cleaving glycosidic linkages (Kashyap et al., 2001).

Hydrolysis of glycosidic linkages requires polymethylgalacturonase (PMG) and polygalacturonase (PG). PMG catalyzes the hydrolytic cleavage of α-1,4-glycosidic bonds and is classified as endo-PMG that causes random cleavage of α-1,4-glycosidic linkages of pectin, preferentially highly esterified pectin, and exo-PMG that causes sequential cleavage of α-1,4-glycosidic linkage of pectin from the non-reducing end of the pectin chain (Kashyap et al., 2001). PG which catalyzes hydrolysis of α-1,4-glycosidic linkages in pectic acid (polygalacturonic acid), are the most abundant pectinolytic enzymes (Jayani et al., 2005). This enzyme is also classified as endo-PG, known as poly (1,4-α-D-galacturonide) glycanohydrolase, which catalyzes random hydrolysis of α-1,4-glycosidic linkages in pectic acid and exo-PG known as poly (1,4-α-D-galacturonide) galacturonohydrolase, catalyzes hydrolysis in a sequential fashion of α-1,4-glycosidic linkages on pectic acid (Kashyap et al., 2001).

Cleavage of α-1,4-glycosidic linkages by trans-elimination, which results in galacturonide with an unsaturated bond between C4 and C5 at the non-reducing end of the galacturonic acid is formed by polymethylgalacturonate lyases (PMGL) and polygalacturonate lyases (PGL). PMGL catalyzing the breakdown of pectin by trans-eliminative cleavage are classified as endo-PMGL, known as poly

(methoxygalacturonide) lyase, which catalyzes random cleavage of α-1,4-glycosidic linkages in pectin, and exo-PMGL, which catalyzes stepwise breakdown of pectin by trans-eliminative cleavage. On the other hand PGL catalyzing the cleavage of α-1,4 glycosidic linkage in pectic acid by trans- elimination are also classified as endo-PGL, known as poly (1,4-α-D-galacturonide) endolyase, which catalyzes random cleavage of α-1,4-glycosidic linkages in pectic acid, and exo-PGL, known as poly (1,4-α-D galacturonide) exolyase, which catalyzes sequential cleavage of α-1,4-glycosidic linkages in pectic acid. Protopectinases solubilize protopectin forming highly polymerized soluble pectin (Kashyap et al., 2001).

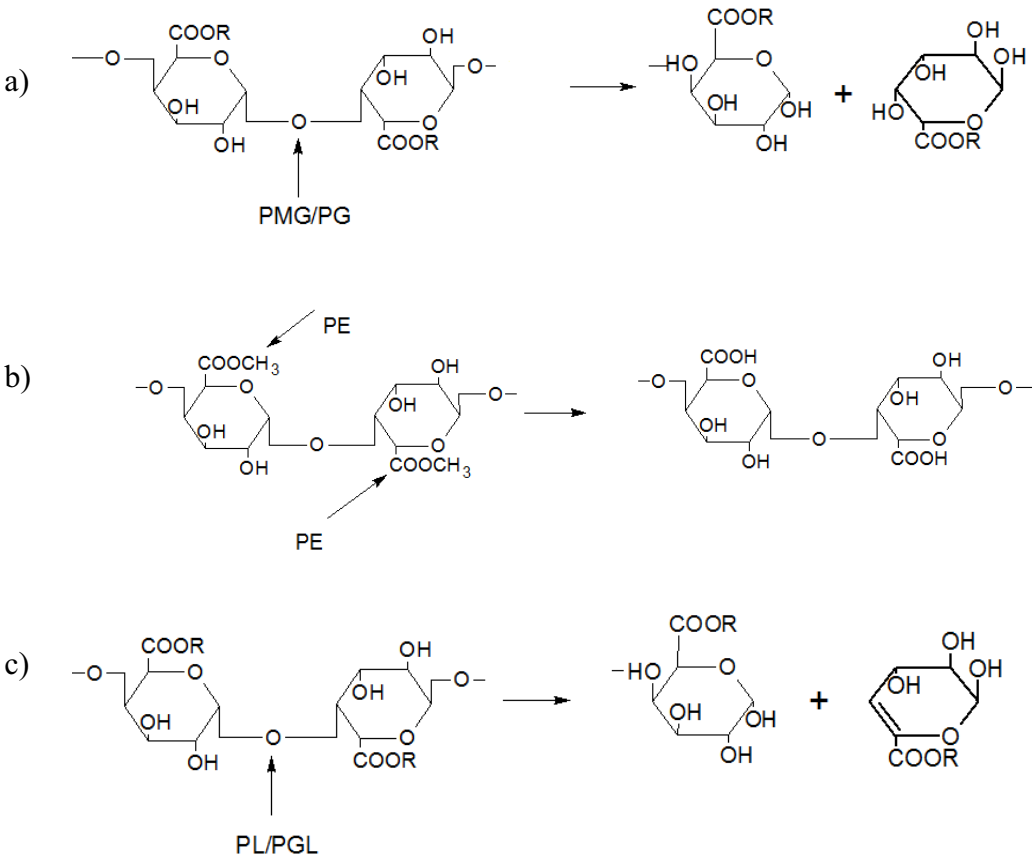


Figure 2.1 Mode of action of pectinases: (a) R = H for PG and CH_3 for PMG; (b) PE; and (c) R = H for PGL and CH_3 for PL. The arrow indicates the place where the pectinase reacts with the pectic substances. PMG, polymethylgalacturonases ; PG, polygalacturonases ; PE, pectinesterase ; PL, pectin lyase (Jayani et al., 2005).

2.2.1 Application of Pectinases

Pectinases have potential applications in fruit, paper and textile industries. Pectinases are mainly used for increasing filtration efficiency and clarification of fruit juices (Joslyn et al., 1952; Brawman, 1981) and used in maceration, liquefaction and extraction of vegetable tissues (Charley, 1969; Bohdziewiez & Bodzek, 1994). Pectinases play a crucial role in fruit juice industries in order to increase fruit juice yield and clarity (Alkorta et al., 1988). Pectinases are used in extraction and clarification of fruit juices (Lee et al., 2006; Sin et al., 2006; Sandri et al., 2011), bleaching of paper pulp (Ahlawat et al., 2008), degumming of fibers (Sharma & Satyanarayana, 2012), oil extraction (Najafian et al., 2009), coffee and tea fermentation (Kashyap et al., 2001; Murthy & Naidu, 2011). Novel use of pectinases in DNA extraction from plants and production of pectic oligosaccharides as functional/prebiotic food components also emerges (Combo et al., 2012; Sabajanes et al., 2012).

2.3 Pectinase Production

Pectinolytic enzymes are produced from plants and microorganisms such as bacteria, yeasts and moulds (Khairnar et al., 2009). The major sources of plant pectinases are tomatoes and oranges (Torres et al., 2005). In contrast to plant and animal sources, pectinases derived from microorganisms have advantages such as cheap production, easier gene manipulations, faster product recovery, free of harmful substances over plant and animal derived pectinases (Chaudhri & Suneetha, 2012). The important producers of pectinases as reported in the literature are given in Table 2.2. Almost all the commercial pectinolytic enzymes are produced by the fungi, namely, *Aspergillus* sp., *Aspergillus japonicus, Rhizopus stolonifer, Alternaria mali, Fusarium oxysporum, Neurospora crassa, Penicillium italicum* ACIM F-152, and many others (Jayani et al., 2005). Some of the bacterial

species producing pectinases are *Agrobacterium tumefaciens, Bacteroides thetaiotamicron, Ralstonia solanacearum, and Bacillus* sp. (Jayani et al., 2005).

Table 2.2 Pectinases produced by microorganisms (Kashyap et al., 2001)

Microorganism	**Type of pectinase**	**Optimum pH**	**Optimum temperature (^oC)**
Fungal pectinase			
Aspergillus niger CH4	Endo-pectinase Exo-pectinase	4.5-6.0	< 50
Penicillium frequentans	Endo-PG	4.5-4.7	50
Sclerotium rolfsii	Endo-PG	3-5	55
Rhizoctonia solani	Endo-PG	4.8	50
Mucor pusilus	PG	5	40
Bacterial pectinase			
Bacillus sp. RKG	PGL	10.0	-
Bacillus sp. NT-33	PG	10.5	75
Bacillus polymxa	PG	8.4-9.4	45
Bacillus pumilis	PATE	8.0-8.5	60
Bacillus sp. DT7	PMGL	8.0	60
Bacillus subtilis	PAL	9.5	60-65

PG: Polygalacturonase, PGL: Polygalacturonate lyase, PATE: Pectic acid transeliminase, PMGL: Polymethyl galacturonate lyase, PAL: Pectic acid lyases

A few commercial pectinases have been launched successfully worldwide as shown in Table 2.3.

Table 2.3 Commercial pectinases and their suppliers (Pedrolli et al., 2009)

Product Trade Name	**Supplier**	**Source Microorganism**	**Recommended pH/Temp.**	**Action Pattern**
Grindamyl 3PA	Danisco, Denmark	*A.niger*	4.0/55 ºC	PL
Pectinase CCM	Biocon, India	*A.niger*	4.0/50 ºC	PG
			6.0/40 ºC	PL
Pectinex 3XL	Novozyme Denmark	*A.niger*	4.7/50 ºC	PG
			5.0-6.5/35 ºC	PL
Rapidase C80	Gist Brocades Holland	*A.niger*	4.0/55 ºC	PG
			6.0/40-45 ºC	PL

The production of pectinase from microorganisms involves the following steps: isolation and screening of the microorganism, growth of microorganism on a culture medium, fermentation, purification steps. Pectinolytic enzymes are produced in two different methods: submerged fermentation (SmF) and solid-state fermentation (SSF). Bacterial pectinases are generally alkaline in nature and are

carried out by SmF, since SSF is generally suitable for fungi which require a low water activity (0.6) compared to bacteria (0.95). SSF provides higher enzyme yields than SmF method (Pedrolli et al., 2009). However, the industrial application of SSF suffers from complicated product purification resulting from heterogeneous fermentation medium, difficulty of scale up, and losses of enzyme in the solid residues (Gupta et al., 2008; Pedrolli et al., 2009). Besides, SSF requires long fermentation periods (e.g. 5-6 days) (Ustok et al., 2007). SmF is easier to control at a large scale and has been already successfully used for production of various metabolites since 1940s. In SmF method, pectinase production can be remarkably enhanced compared to SSF, although several studies have shown that SSF gives higher enzyme yields. Some of the alkaline pectinases from microbial sources documented in the literature are listed in Table 2.4. As can be seen from Table 2.4, agro-food wastes were employed for production of alkaline pectinase using many bacteria but especially *Bacillus spp*.

There are several studies comparing SmF to SSF and reporting promising enzyme activity with SmF method (Rangarajan et al., 2010). Rangarajan et al. (2010) compared pectinase production using *Aspergillus niger* in SmF and SSF methods both in shaker flask and reactor levels using orange peel as carbon source and varying amounts of organic and inorganic nitrogen sources. A maximal exo-pectinase activity of 5128 U/g and endo-pectinase activity of 793 U/g were reported with 4% soybean meal in SSF method, whereas maximal exo-pectinase activity of 5834 U/g and endo-pectinase activity of 951 U/g were achieved with SmF method using 4% peptone and 3% soybean meal, respectively. A similar trend was reported at reactor levels. *Aspergillus niger* has been used as pectinase producer in SmF system by others (Mojsov, 2010; Zeni et al., 2011). Use of *Bacillus* species in pectinase production by SmF resulted in promising results as reported by Sharma and Satyanarayana (2006), Ahlawat et al. (2009), and Joshi et al. (2013).

Table 2.4 Microbial sources of alkaline pectinases and their applications

Microorganism/ bacteria	**pH range**	**Substrate**	**Industrial applications**	**Reference**
B.polymyxa	8.4-9.4	Minerale medium	-	(Nagel & Vaughn, 1961)
B.pumilis	8-8.5	-	-	(Dave & Vaughn, 1971)
B.subtilis	8.5	Carrot	Vegetable meceration	(Chesson & Codner, 1978)
Bacillus sp.strains	6.0-7.0	Wheat bran	Fruit/vegetable juice extraction	(Soares et al., 2001)
*Bacillus sp.*DT-7	8	Wheat bran, rice bran and apple puree	-	(Kashyap et al., 2003)
Bacillus gibsonii	7-12	Sugar beet pulp	-	(Li et al., 2005)
B.pumilus dcsr1	10.5	Minerale medium	-	(Sharma & Satyanarayana, 2006)
Bacillus firmus-I-4071	6.0	Potato peel	-	(Bayoumi et al., 2008)
Bacillus sphaericus	6.8	-	-	(Jayani et al., 2010)
Bacillus subtilis CM5	7.0	-	Carrot juice extraction	(Swain & Ray, 2010)

However, several researchers have reported enhanced enzyme production by bacterial strains under SSF. Improved production of alkaline and thermotolerant pectinase has been reported by *Bacillus* sp. DT7 under SSF using wheat bran (Kashyap et al., 2003). Nadaroglu et al. (2010) investigated production of pectin lyase by *Bacillus pumilus* (P9) using solid state fermentation. They also determined the action of pectin lyase in fruit juice production. It was determined that yields of fruits juices significantly improved compared with control.

Jayani et al. (2010) reported that *Bacillus sphaericus*, a bacterium isolated from soil, produced a good amount of polygalacturonase activity (6.2 ±1.3 U/mL) after 72 h of incubation in production medium at 30°C and pH 6.8. Maximum enzyme production was with citrus pectin as carbon source and with casein hydrolysate and yeast extract together as nitrogen source (6.4±0.8 U/mL). Jayani et al. (2010) suggested that this enzyme with good activity at neutral pH would be potentially useful to increase the yield of banana, grape, or apple juice.

In the light of above, some of the researchers prefer to use some basal (synthetic) medium to produce pectinase production. However, basal medium or supplementary solutions may increase the cost of pectinase production. Therefore, current studies have been focused on agro-food wastes as the low-cost materials.

2.3.1 Production of Pectinases Using Agro-Industrial Wastes

In the industrial market, pectinases occupy almost 25% of the global enzyme sales (Jayani et al., 2005). Therefore in order to meet this high demand, it is highly important to produce pectinase enzyme in a cost effective and productive way (Gogus, 2006). Utilization of cheap and abundant materials for enzyme production to reduce product costs is one of the critical issues that the researchers have recently considered. Currently, degradation of the agricultural and food wastes by microorganisms is popular for the production of valuable compounds such as

proteins, polysaccharides, oligosaccharides, vitamins, hormones, enzymes and other raw materials for medicinal and industrial uses (El-Sheekh et al., 2009). Decreasing the capital investment by using low cost agricultural and fruit processing industrial waste as raw materials not only helpful to reduce the cost of production but also aids in solving disposal problems (Patill & Dayanand, 2006).

Also for SmF to be commercially viable, pectinases have been produced on low cost carbon sources such as citrus limetta peel (Joshi et al., 2013), orange peel extract (Rangarajan et al., 2010), mix of apple pulp and corn flour (Mojsov, 2010), wheat bran (Ahlawat et al., 2009), pumpkin oil cake (Pericin et al., 2007) and other agricultural wastes. Selection of appropriate source of carbon, nitrogen and other nutrients is also a critical stage in the development of an efficient and economic enzyme production process (Sharma & Satyanarayana, 2012). Besides, 30-40% of the enzyme production cost belongs to cost of the medium (Ustok et al., 2007). Thus, the solid agricultural substances are effective source of carbon, nitrogen, and minerals for enzyme production (Sharma & Satyanarayana, 2012).

2.4 Usability of Hazelnut By-Product As An Agro-Industrial Wastes

Lignocellulosic biomass is composed of three main components; cellulose, hemicellulose and lignin. Cellulose is the most abundant, and comprises 35-50% of the plant cell wall, whereas hemicellulose comprises 20-35%, and lignin comprises 12-37% (Yat et al., 2008). Hemicelluloses are heterogeneous and the composition of hemicelluloses within a substance depends on that source. Hemicelluloses vary significantly among hardwoods and softwoods according to the type and content in the wood cell walls. Generally, hardwoods contain a high proportion of xylose units and more acetyl groups than softwoods. By contrast, softwoods have a high proportion of mannose units and more galactose units (Sixta, 2006). Hemicellulose can be broken down into sugars, mainly xylose, as well as a few minor sugars, through a pretreatment process. Cellulose and hemicellulose are sugar rich fractions of interest for use in fermentation processes, since microorganisms may use the

sugars for growth and production of value added compounds such as ethanol, food additives, enzymes, organic acids, and others. The average values of the main components in some lignocellulosic wastes are shown in Table 2.5.

Table 2.5 Composition of some agricultural lignocellulosic materials

	Composition (%, dry basis)			
Lignocellulose waste	**Cellulose**	**Hemicellulose**	**Lignin**	**References**
Corn fiber	15	35	8	(Saha, 2003)
Hazelnut shell	25-30	25-30	30-40	(Arslan, 2011)
Corn stover	40	25	17	(Saha, 2003)
Rice straw	35	25	12	(Saha, 2003)
Sugarcane bagasse	40	24	25	(Saha, 2003)

Hazelnut shells are a potential feedstock for fermentation due to their high carbohydrate content (50-60%). The average structured analysis of hazelnut shell is as follows: hemicelluloses 30.4%, celluloses 26.8%, lignin 42.9% and extractive matter 3.3% (Demirbaş, 2006).

Hazelnut is Turkey's most important agricultural crop since Turkey is one of the main producer and exporter of hazelnuts in the world. Turkish hazelnuts account for 65-76% of the world hazelnut market followed by Italy, Spain, the USA, China, Iran, Greece and Russia (Koksal et al., 2006; Fischbach & Brasseur, 2012). The

shells account for about 44.5% of whole hazelnut and produced annually at 250,000-600,000 tonne in the Black Sea region of Turkey alone (Arslan & Saraçoglu, 2010; Demirbaş, 2002). Hazelnut shells still have a limited industrial use and mostly used as fuels in the Black Sea region.

2.5 Conversion of Lignocellulosic Biomass to Fermentable Sugars

Pretreatment, enzymatic hydrolysis, and fermentation are the three areas receiving the most attention through research. Of these, pretreatment is of significant importance because as a process step, it is upstream of both enzymatic hydrolysis and fermentation (Mosier et al., 2005).

The presence of lignin and hemicellulose makes the access of cellulose enzymes difficult, thus the efficiency of the hydrolysis is reduced. The cellulose is embedded in a matrix of lignin and hemicelluloses and this arrangement presents a major accessibility problem to cellulose enzymes. Therefore, a pretreatment (Figure 2.2) is necessary in order to improve the digestibility of the biomass for a subsequent enzymatic hydrolysis step (Mosier et al., 2005).

Hydrolysis can be improved by removal of lignin and hemicellulose, reduction of cellulose crystallinity, and increase of porosity by pretreatment processes. Of these various pretreatment technologies, some are focused on hemicellulose, some are focused on disrupting the highly ordered cellulose, and others are focused on disrupting the lignin-carbohydrate complex (Sun, 2002).

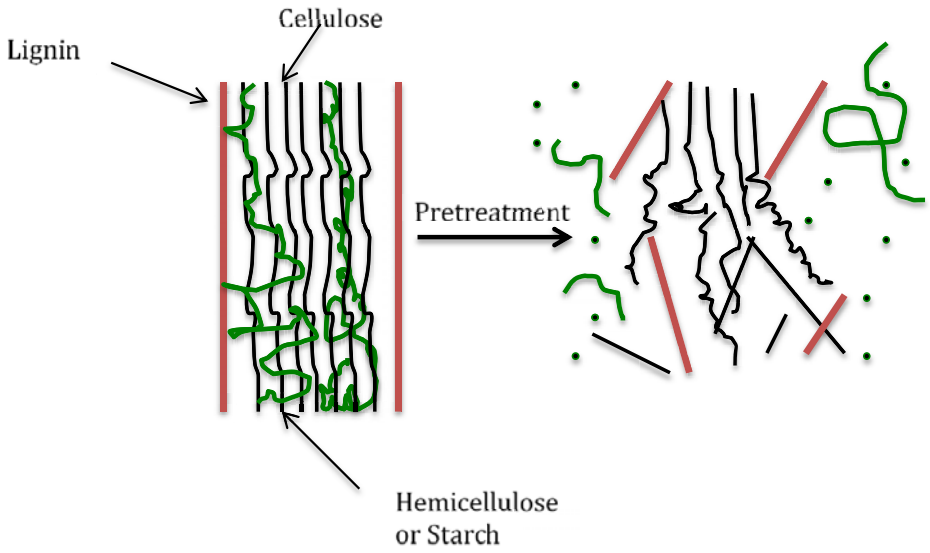


Figure 2.2 Action of pretreatment on lignocellulosic biomass- adapted from Mosier et al., 2005.

A good pretreatment will disrupt the biomass enough to allow for the maximum hydrolysis of both the hemicellulose and cellulose components into monomeric sugars with minimal generation of enzymatic hydrolysis and fermentation inhibitors (Hu et al., 2008). Table 2.6 highlights the advantages and disadvantages of the pretreatment technologies. As can be seen from Table 2.6, alkali and ozone pretreatment methods disrupted lignin structure of lignocellulosic biomass. However, using green solvents are not cost-effective pretreatment methods for lignin removal from lignocellulosic biomass. Searching novel technologies or improving these pretreatment methods can be solved some problems such as economical and environmental concerns and formation of toxic compounds described in the following section 2.5.

Table 2.6 Advantages and disadvantages of different pretreatment methods of lignocellulosic biomass (Brodeur et al., 2011)

Pretreatment method	**Advantages**	**Disadvantages**
Alkali	Efficient removal of lignin Low inhibitor formation	High cost of alkaline catalyst Alteration of lignin structure
Acid	High glucose yield Solubilizes hemicellulose	High costs of acids and need for recovery High costs of corrosive resistant equipment Formation of inhibitors
Green solvents	Lignin and hemicellulose hydrolysis Mild processing conditions (low temperatures) Ability to dissolve high loadings of different biomass type	High solvent costs Need for solvent recovery and recycle
Ozone	Reduces lignin content No toxic compounds generation	Large amount of ozone required
Steam	Cost effective Lignin transformation and hemicellulose solubilization High yield of glucose and hemicellulose	Partial hemicellulose degradation Toxic compound generation Acid catalyst needed to make process efficient with high lignin content material

2.6 Pretreatment Methods for Lignocellulosic Biomass

The most often studied pretreatment technologies are summarized in four categories which include physical pretreatments, chemical pretreatments, physio-chemical (combination) pretreatments, and biological pretreatments (Figure 2.3) (Sun & Cheng, 2002).

According to Lynd (1996), pretreatment is directly associated with the efficiency of hydrolysis. When the lignocellulosic biomass is instantly used in hydrolysis glucose yield is less than 20% of theoretical yield, but when pretreatment is applied before hydrolysis step, glucose yield even surpasses 90% of theoretical yield. Although the pretreatment is necessary for a higher glucose yield, the cost of the process should be considered in a detailed way.

I. Physical Methods

- Mechanical comminution (grinding&chipping)
- Pyrolysis

II. Physical-Chemical Methods

- Steam explosion
- Liquid hot water
- Ammonia fiber explosion
- Carbon dioxide explosion

III. Chemical Methods

- Ozonolysis
- Acid Hydrolysis (dilute or concentrated)
- Alkaline Hydrolysis
- Oxidative Delignification
- Wet Oxidation
- Organosolv Process

IV. Biological Methods

Figure 2.3 Classification of lignocellulose-pretreatment methods

2.6.1 Physical Methods

Physical methods such as milling (ball milling, hammer milling, colloid milling) and irradiation (gamma rays, ultrasound, microwaves or electron beam) increase surface area by reducing the particle size (Palmowski & Muller, 2000; Taherzadeh & Karimi, 2008). Final particle size of the biomass varies according to applied these physical methods. The size of materials is usually 10-30 mm after chipping and 0.2-2 mm after milling or grinding (Sun and Cheng, 2002).

They also cause shearing and reduce the degree of polymerization of cellulose, which can increase hydrolysis yield by 5-25% while reducing digestion time by 23-59% (Delgenes et al., 2002). Although no product inhibitors are produced during physical pretreatment, this method has a very high energy requirement, which makes it economically unsuitable (Hendriks & Zeeman, 2009).

2.6.2 Physical-Chemical Methods

Physical-chemical pretreatments that combine both chemical and physical processes consist of steam explosion, liquid hot water (LHW), ammonia fiber explosion (AFEX) and carbon dioxide explosion (McMillan, 1994). Steam explosion, also known as autohydrolysis, is performed with a saturated steam, at a temperature range of 160-290 °C and pressure range of 0.69-4.85 MPa during several seconds or minutes. During this treatment, hemicellulose is solubilized into oligomeric or monomeric sugars and released into the liquid phase which in turn increase the accessibility of cellulose found in the solid phase to enzyme activity (Galbe & Zacchi, 2007). A disadvantage of this method is the formation of inhibitory compounds from degradation of xylan (Mueller, 2009). These inhibitors affect the microbial growth, enzymatic hydrolysis and fermentation.

The other commonly used physical-chemical pretreatment methods are liquid hot water (LHW, thermohydrolysis) and ammonia fiber explosion (AFEX). For LHW, pressurized hot water is used at a process pressure of more than 5 MPa, and the temperatures are between 170-230°C. LHW pretreatment provides high pentose recovery rates without production of inhibitors as in steam explosion (da Costa Sousa et al., 2009). The major advantage of LHW pretreatment is that it does not require addition or use of chemicals (Suryawati et al., 2009).

Ammonia-fiber explosion (AFEX) is an alkaline physico-chemical pretreatment process, in which lignocellulosic materials are exposed to liquid ammonia at high temperature (60-160 ^{o}C) and pressure from 5 to 30 min (Wyman et al., 2005;

Teymouri et al., 2005) and then the pressure is swiftly reduced. This process does not produce any inhibitory compounds. Vlasenko et al. (1997) studied for comparing acid-catalyzed steam explosion, dilute acid hydrolysis, and AFEX pretreatments for enzymatic hydrolysis of rice straw. The AFEX pretreatment does not significantly solubilize hemicellulose compared to acid pretreatment (Vlasenko et al., 1997). Thus, AFEX treated biomass requires enzymes that can hydrolyze the hemicelluloses and celluloses to produce fermentable sugars (Jorgensen et al., 2007).

2.6.3 Chemical Methods

Chemical pretreatment methods include use of acid, alkali, organosolvents, peroxides and ozone (Keshwani & Cheng, 2009).

Acid pretreatment is applied in two forms, concentrated or dilute. In both, the acid used is generally sulfuric acid or hydrochloric acid. Since concentrated acid pretreatment is found to be a harmful and expensive application because of the toxicity and corrosivity of the chemical (Sivers & Zacchi, 1995), instead of concentrated acid pretreatment, dilute acid is well-developed and is an effective method. The dilute acid process is conducted under high temperature and pressure, and has a reaction time in the range of seconds or minutes (Demirbaş, 2012). Dilute acid catalyzes the breakdown of long hemicellulose chains to shorter chain oligomers and then to sugar monomers that the acid can degrade. Rahman et al. (2007) studied the effect of acid concentration, temperature and time on release of xylose and glucose and formation of byproducts (acetic acid and furfural) during hydrolysis of oil palm empty fruit bunch fiber (OPEFB) biomass. The optimum reaction temperature, time and acid concentration were reported as 119 °C, 60 min and 2%, respectively. Under these conditions xylose yield was reported as 91.27%. Roberto et al. (2003) reported optimal conditions of 1% sulfuric acid, 121 ºC, and 27 min for rice straw resulting in 77% xylose yield.

Alkali, such as calcium hydroxide (lime), sodium hydroxide and ammonia, has been employed as a pretreatment method for many years. Alkaline pretreatment causes a swelling effect on the biomass, which is an intra crystalline swelling action penetrating both amorphous and crystalline structure of cellulose and resulting in irreversible change in the structure of cellulose (Kang, 2011). Alkaline pretreatment results in the breakage of bonds between lignin and carbohydrates leading to disruption of the lignin structure while retaining cellulose and a significant portion of hemicellulose in the recovered solids (Galbe & Zacchi, 2007; Hendriks & Zeeman 2009; Jorgensen et al., 2007). Sodium hydroxide pretreatment of coastal bermuda grass reduced lignin content by 86% and increased glucan and xylan conversions to 90.4 and 65.1%, respectively (Wang et al., 2010) while lime pretreatment of switchgrass reduced lignin content by 35.5% (Xu et al., 2010).

Other chemical pretreatments are oxidative delignification and wet oxidation, for which peroxidase with 2% hydrogen peroxide are used as oxidizing agents; and water, sodium carbonate or sulfuric acid are added with the presence of oxygen pressure respectively. Rather than bases or acids, solvent-addition is also tried and that procedure is named as organosolv process. Whereas almost all the hemicellulose is hydrolyzed and lignin is solubilized, organosolv pretreatment can not be opponent to other methods (Lynd et al., 1999) because of the economical disadvantage of solvent prices.

Ozonolysis is a pretreatment technique, which is reducing the lignin content of lignocellulosic wastes. Ozone treatment increases the digestibility of the treated material, and it does not produce toxic residues (Kumar et al., 2009). Ozone is a very strong oxidizing agent that can be produced by passing oxygen through an electrical discharge, where the oxygen molecules dissociate to form ozone (Eckert & Singh, 1975). Ozone has been used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw (Ben-Ghedalia & Miron, 1981), bagasse, green hay, peanut, pine (Neely, 1984), cotton straw (Ben-Ghedalia & Shefet, 1983) and poplar sawdust and rye straw (Garcia-Cubero et al., 2010). A net reduction of 66.8% of acid insoluble lignin concentration was observed when sugarcane bagasse was treated with an ozone concentration of 3.44% (v/v) with

40% moisture content for 120 min (Travaini et al., 2013). Garcia – Cubero et al. (2009) observed that ozonolysis increased enzymatic hydrolysis yield from 29 and 16% to 88.6 and 57% in wheat straw and rye straw, respectively with no furfural and HMF being detected. Kaur et al. (2012) achieved a reduction of over 42% in cotton stalk lignin content using ozone pretreatment.

2.6.4 Biological Methods

The fourth group of pretreatment type is biological pretreatment. Biological pretreatments are performed by employing lignin degrading microorganisms like white-, brown- and soft- rot fungi. These organisms secrete extracellular enzymes such as lignin peroxidases and laccases that remove lignin from biomass (Christian et al., 2005). This method is favorable in terms of the needs for low temperature and no chemicals (Taherzadeh & Karimi, 2008). Nevertheless, the rate of biological hydrolysis is usually very low, so this pretreatment requires long residence times. For instance in the study of Hatakka (1983), *Pleurotus ostreatus* was able to convert 35% of wheat straw into reducing sugars in 5 weeks. As Sun & Cheng (2002) mentioned while white-rot fungi are able to break down cellulose and lignin, brown-rot fungi usually destruct only cellulose.

2.7 Formation of Fermentation Inhibitors

Pretreatment of lignocellulosic biomass may produce degradation products with an inhibitory effect on downstream processes like enzymatic hydrolysis and fermentation. These inhibitors have toxic effects on the fermenting organisms, thus reducing the pectinase activity and productivity.

2.7.1 Sugar Degradation Products

During pretreatment at high severe conditions, pentose polymers are hydrolyzed into monomers, which can be further degraded into furfural. After prolonged period of pretreatment time, furfural can be degraded into levulinic acid. The same process occurs with hexoses as well, generating hydroxymethylfurfural (HMF) and formic acid respective of exposure to pretreatment conditions. Equations 1 and 2 below outline the pathway of sugar degradation (Redding, 2009).

Hemicellulose ➔ Pentose ➔ Furfural ➔ Levulinic Acid (1)

Cellulose and Hemicellulose ➔ Hexose ➔ HMF ➔ Formic Acid (2)

HMF is considered less toxic than furfural and its concentration in (hemi)cellulose hydrolysates is usually low. It is clear that extensive degradation of (hemi)cellulose is responsible for the formation of the latter inhibitor compounds. Kinetic studies have shown that the production of furfural strongly increases with temperature and reaction time (McKillip & Collin, 2002).

Generally, furfural and HMF have been reported as to cause a lag phase in yeast growth before sugar consumption begins because the yeast takes up the two degradation compounds first (furfural faster than HMF) before moving on to converting sugars to ethanol. Levulinic and formic acids are generally shown as helpful at levels up to 100 mmol and inhibitory after levels of 100-200 mmol. This has been confirmed by experiments done by Larsson et al. (1998), which showed furfural and HMF as not inhibitory to ethanol yield, while weak acids (formic, levulinic, and acetic) at a combined concentration greater than 100 mmol did inhibit the ethanol yield.

Lavarack et al. (2002) studied dilute acid hydrolysis of baggase hemicelluloses to produce xylose, arabinose, glucose, acid-soluble lignin, and furfural. They found

that H_2SO_4 is more efficient as a catalyst than hydrochloric acid for the degradation of xylose. Saha & Bothast (1999) used dilute acid and enzymatic saccharification method for conversion of corn fiber to fermentable sugars. They found that corn fiber pretreated with 0.5% H_2SO_4 at 121°C for 1 h facilitated commercial enzymes to highly hydrolyze remaining starch and hemicellulose components without generation of inhibitors such as furfural and hydroxymethyl furfural (HMF), which are generally considered inhibitors for fermentative microorganisms.

2.7.2 Lignin Degradation Products

In general, lignin degrades into phenolic compounds with a variety of molecular weights (Klinke et al., 2004). Details regarding the phenolic inhibition are limited due to a lack of accurate investigation, but it is suspected that low molecular weight phenolic compounds are more inhibitory to fermentation and that there are interaction effects with furfural and HMF, which increase the overall inhibitory effects of both compounds (Palmqvist & Hahn-Hagerdahl, 2000). Table 2.7 illustrates the maximum allowable concentration of various inhibitors for ethanol fermentation (Klinke et al., 2004).

2.8 Elimination of Inhibitors from Fermentation Medium

Sugar degradation not only reduces the sugar yield, but the degradation products such as furfural and other by-products can also inhibit the fermentation process. Various methods for detoxification of the hydrolyzates have been developed (Corredor, 2008). To decrease the toxicity caused by the inhibitory compounds, considerable efforts have been focused on detoxification methods prior to fermentation, including neutralization, over-liming, evaporation, ion exchange resins and charcoal adsorption (McMillan, 1994).

Table 2.7 Origin of various inhibitors and their maximum allowable concentrations for ethanol fermentation (Klinke et al., 2004)

Component	Inhibitor	Maximum concentration (g/l)	Remarks
Hemicellulose	Acetic acid	3	Inhibition of acetic acid depends on pH during fermentation
Xylose	Furfural	<0.25	
Mannose, galactose, glucose	HMF	0.25	
Cellulose	HMF	0.25	HMF may further degrade to formic acid and levulinic acid
Glucose			
Lignin	Aromatics	<0.1	Vanillic acid, caproic acid, caprylic acid, pelargonic acid, palmitic acid
	Poly-aromatics		
	Aldehydes		
	Phenolic compounds		

2.9 Enzymatic Hydrolysis

Enzymatic hydrolysis is the process by which several enzymes, such as cellulases and hemicellulases are used to hydrolyze polysaccharides (cellulose and hemicellulose) into their component monosaccharides (Taherzadeh & Karimi, 2007). Without any pretreatment, the conversion of cellulose into glucose is a slow and complex process since cellulose is embedded in a matrix of lignin and hemicellulose in macrofibrils (Figure 2.2) (Kang, 2011).

The products of hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45 to 50 ^{o}C) and does not cause a corrosion problem (Sun, 2002). Enzymatic hydrolysis has some major factors such as substrate utility, substrate concentration, enzyme activity, applied pretreatment method and hydrolysis conditions (enzyme load, pH and temperature) (Taherzadeh & Karimi, 2007). Palmarola-Adrados et al. (2005) pretreated starch free wheat bran with dilute acid and subsequently hydrolyzed using a mixture of Celluclast and Ultraflo (1:1) and the overall sugar yield of this combined hydrolysis method reached 80%. Aswathy et al. (2010) reported improved efficiency of 71% from combined dilute acid pretreatment and enzymatic hydrolysis of hyacinth biomass compared to dilute acid alone (57%). Yan et al. (2012) reported a high enzymatic digestibility of 93.12% from food waste hydrolysis prior to fermentation within 4 h. The optimum batch enzymatic conditions were found to be saccharification pH of 4.5, temperature of 55 ^{o}C , glucoamylase concentration of 120 U/g, α –amylase concentration of 10 U/g, solid-liquid ratio of 1: 0.75 (w/w) (Yan et al., 2012). Therefore, enzyme mixture or cocktails act both synergistically to saccharify polysaccharides from lignocellulosic biomass to fermentable sugars and more economical.

2.10 Optimization of Pectinase Production

It is essential to improve the efficiency of the fermentation process by increasing the yield while decreasing the cost of the enzyme production. Cost of enzyme production is highly affected by raw material, medium composition and cultivation conditions. Numerous studies have been conducted on the microbial production of pectinases using synthetic media, whereas few have been published about cost-effective production of the enzymes. A cost-effective substrate must be renewable, abundant and containing the majority of the nutrients essential to the microorganism, if not, it would be necessary to supplement them externally at an additional cost. It is also known that 30-40% of the enzyme production cost belongs to cost of the medium (Ustok et al., 2007). The selection of an appropriate source of carbon, nitrogen and other nutrients is one of the most critical stages in the development of an efficient and economic enzyme production process. Thus, reduction in the production costs can be achieved by usage of inexpensive lignocellulosic materials as a substrate, such as wheat bran, sugarcane bagasse, corncob, rice bran, wheat straw, rice straw, saw dust, orange peel, lemon peel etc (Smith & Aidoo, 1988; Pilar et al., 1999).

Optimization by traditional one-factor at a time method generally used in biotechnology involves varying a single factor while keeping the others constant. Thus, it is time consuming and expensive, when a various variables are to be evaluated. To overcome this difficulty and to evaluate the interactions between parameters, response surface methodology (RSM) has been widely used to decrease time and cost by providing fewer numbers of experiments (Haaland, 1989). RSM was widely used in the optimization of fermentation process (Kaushik et al., 2006).

The growth and enzyme production are strongly influenced by medium components thus optimization of media components and culture conditions is necessary to improve a biological process (Djekrif-Dakhmouche et al., 2006). Based on the literature, there are few studies about statistical methods that have been applied for

optimization of pectinase by using *Bacillus spp*. (Sharma & Satyanarayana, 2006, Swain & Ray, 2010, Mukesh Kumar et al., 2012, Andrade et al., 2012).

Sharma & Satyanarayana (2006) aimed to enhance the production of an alkaline and thermostable pectinase from *Bacillus pumilus* dcsr1 in submerged fermentation. As compared to pectinase production by *B. pumilus* dcsr1 in unoptimized medium, a 34-fold increase was recorded in RSM optimized medium in shake flasks. The pectinase production of 25.330 U/L in the laboratory fermenter was higher than the enzyme production attained in shake flasks (21.000 U/L). This could be attributed to uniform distribution of nutrients and improved aeration.

Swain & Ray (2010) investigated the exo-PG production by *Bacillus subtilis* CM5 isolated from cow ruminant microflora, which was comparable to marketed pectinase (Pectinex®, Novozyme, Denmark). The optimum temperature, pH, and incubation period for optimum exo-PG production (82.0–83.2 units) were found 50 °C, 7.0, and 36 h, respectively. *B. subtilis* strain CM5 isolated from culturable cow ruminant microflora produced moderately thermostable exo-PG, which is more efficient in extracting juice (13.3% more yield) from carrot than standard marketed pectinase enzyme (Pectinex, Novozyme, Denmark).

Mukesh Kumar et al. (2012) investigated pectinase production and optimization by bacterial strain MFW7 isolated from fruit market wastes. The bacterium *Bacillus sp*. MFW7 produced significant amount of pectinase (1.8 U/mL) after 96 h of incubation in fermentation medium at 35 °C and pH 6.5. The maximum enzyme production was obtained with lactose as carbon source, peptone as nitrogen source and cassava waste as substrate (2.5 U/mL). This study illustrated the usage of cassava wastes as a substrate for pectinase production. To conclude, this enzyme may be further scaled up for juice production after careful investigations.

Andrade et al. (2012) reported maximum polygalacturonase production by the thermophilic *Bacillus sp*. SMIA-2 cultivated in liquid cultures containing 0.5% (w/v) apple pectin supplemented with 0.3% (w/v) corn steep liquor, after 36 h with activity of 39 U/mL.

There were many studies in the literature using synthetic media for production of pectinase by using *Bacillus sp*. mutant strain. However, no study has been found to investigate neither usage of hazelnut shells as a substrate for pectinase production nor optimization of the fermentation medium and conditions to enhance the pectinase production by using wild-type *Bacillus subtilis*.

2.11 Objectives of The Study

The aim of this study was to produce alkaline pectinase enzyme using hazelnut shells as a substrate and to determine its potential in crude form for clarification of carrot juice.

Numerous studies have been conducted on the production of pectinases from various microorganisms. However, a few works have been published about cost-effective production of enzymes. The difficulties to obtain the appropriate substrate might be the biggest problem to develop such studies. A suitable substrate should be cost-effective and composed of all necessary nutrients to the microorganism, if not, it would be necessary to supplement them externally. Lignocellulosic biomass contains sugar rich fractions such as cellulose and hemicellulose. Various pretreatment methods and enzymatic hydrolysis are used to produce simple sugars such as glucose and xylose. Then, microorganisms may use these sugars for growth and production of pectinase. For this purpose, hazelnut shell as a low-cost substrate was selected and various pretreatment methods such as dilute acid, alkali and ozone may be improved and may result in better enzymatic saccharification first and then pectinase production.

Furthermore, industrial fermentation is moving forwards to a more knowledge-based and better-controlled process. Hence, for the development of an economically feasible bioprocess by rational design and optimization, a comprehensive understanding of the effect of process parameters on the final product has become inevitable. Different fermentation factors (temperature, time, medium pH,

inoculum level) are important. By this study, it was aimed to optimize the fermentation medium and conditions to enhance the alkaline pectinase production by using response surface methodology (RSM).

In this context, the potential of produced enzyme in crude form was also examined in the clarification of carrot juice and compared with Pectinex 3XL which is known as a commercial pectinase.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Lignocellulosic Biomass

Hazelnut shells of Tombul variety were kindly provided by a local plant in Ordu, a province of Turkey and dried at 70 °C in an oven for 24 h on arrival. The hazelnut shells were then ground by a laboratory type grinding mill (Thomas-Wiley Laboratory Mill, Model 4, Arthur H.Thomas Company, Philadelphia, PA, USA) to pass through a 1 mm sieve for easy reaction with acid. The ground hazelnut shells were stored inside plastic bags and kept at room temperature until use.

3.1.2 Microoganisms, Growth and Fermentation Media

Bacillus pumilus NRRL NRS-272 and *Bacillus subtilis* NRRL B-4219 were kindly provided by the ARS culture collection, Northern Regional Research Laboratory (NRRL), Peoria, Illinois, USA. Stock cultures of *B. pumilus* and *B.subtilis* were grown on nutrient agar at 30 ºC and maintained at 4 ºC.

B. subtilis and *B. pumilus* were maintained on modified media containing, yeast extract (1 g/L), glucose (10 g/L), K_2HPO_4 (0.4 g/L), KH_2PO_4 (0.2 g/L), $MgSO_4.7H_2O$ (0.4 g/L), and citrus pectin (2g /L) and incubated at 30 ºC for 20 h with agitation of 130 rpm (Kapoor and Kuhad, 2002). Stock cultures of *B. pumilus*

and *B.subtilis* were prepared with 20% of glycerol-water and stored at -80 ^{o}C for long term storage.

The submerged fermentation media were prepared in 500 mL Erlenmeyer flasks with the 100 mL medium consisting of hazelnut shell hydrolyzate, yeast extract (5g/L), pectin (2 g/L), $MgSO_4 7H_2O$ (0.2 g/L), and K_2HPO_4 (0.2 g/L). Each flask was inoculated with $3x10^8$ CFU/ 100 mL of inoculum and incubated under shaking (130 rpm) at 30, 35 and 40 ^{o}C for 24, 48 and 72 h.

3.1.3 Chemicals and Enzymes

All chemicals and enzymes used in this study are given in Table A.1.

3.1.4 Buffers and Solutions

The preparation of buffers and solutions used throughout the study is given in Table B.1.

3.2 Methods

3.2.1 Analytical Methods

3.2.1.1 Chemical Analysis of Biomass

Moisture, ash, fat, extractives, crude fiber, cellulose, hemicellulose, acid insoluble lignin (AIL) and acid soluble lignin (ASL) contents of hazelnut shells were determined as described in the following sections.

3.2.1.1.1 Moisture Content

Moisture content was determined using the Standard Official Methods of Analysis of the AOAC (1984). Approximately 1g of ground sample was placed in a pre-weighed aluminum foil dish and dried to a constant weight in a 105 ^{o}C oven. Analysis was done in triplicate. The moisture content of the sample was calculated using the following formula:

$$Moisture\ (\%)\ (w.b) = \left[\frac{\left(W_i - W_f\right)}{W_i}\right] \times 100 \qquad (3)$$

where; W_i= Weight of wet sample (g), and

W_f= Weight of dry sample (g)

3.2.1.1.2 Ash Content

Total ash content of ground samples was determined by incineration, as described by AOAC (1984). An oven dried sample of 1 g was placed into crucibles and combusted at 575 ^{o}C for 24 h until constant weight was reached. The amount of ash was then calculated using the formula:

$$Ash\ (\%)\ (d.b) = \left[\left(\frac{W_2 - W_1}{W_i \times \%TS}\right)\right] \times 100 \qquad (4)$$

where; W_1= Weight (g) of crucible

W_2=Final weight (g) after burning

W_i= Initial weight of sample (g)

TS= Total solids (%)

3.2.1.1.3 Amount of Extractives

Duplicate samples of 5 g oven dry material were extracted with 75 mL of ethanol and 225 mL of hexane for extracting ethanol and fat soluble solutes, respectively. The mixtures were allowed to stand 4 h in a reflux condenser and then cooled to room temperature and weighed.

3.2.1.1.4 Fat Content

The total fat in ground hazelnut shells was determined according to the Soxhlet method (Hara & Radin, 1978). Approximately five grams of oven dried sample was weighed into thimbles and placed in a Soxhlet apparatus filled with 300 mL of hexane and heated for 6 h. The hexane and oil mixture was placed into 105 ºC oven to dry. The fat percent of samples was calculated according to Equation (5),

$$Fat\ (\%) = \left(\frac{W_2 - W_1}{w} \right) \times 100 \qquad (5)$$

where; w= weight of sample, (g)

W_1=weight of balloon, (g)

W_2=Weight of fat and balloon, (g)

3.2.1.1.5 Crude Fiber

For crude fiber determination, samples were dissolved in boiling sulphuric acid

solution (0.25 N) for 30 min and subsequently filtered into a crucible and washed to remove the acid from the retentant inside the crucible. For calcination, samples were boiled in potassium hydroxide solution (0.31 N) for 30 min and then filtered and washed. The obtained residue was dried at 105 °C for 24 h and burned at 550 °C until constant weight. This procedure was also repeated for blank. The difference between the dry organic/inorganic residue weight and the ashed residue weight were used as fibrous material, and the fiber content was calculated as percentage by weight, C_{fibre}, by the formula:

$$C_{fibre} = \frac{[(b-c)\times 100]}{a} \tag{6}$$

where a is the mass (g) of the sample, b is the loss of mass after ashing, and c is the loss of mass after ashing for the blank.

3.2.1.1.6 Cellulose and Hemicellulose Content

The holocellulose content of the extractive-free samples was determined by the sodium chlorite method (Browning, 1967). Duplicate samples of 2.5 g were mixed with 80 mL of distilled water, 0.5 mL of glacial acetic acid and 1 g of 80% sodium chlorite and the mixture was kept at 70 °C for 6h. Further additions of acid and chlorite solutions were made hourly. After six hour, the samples were cooled in an ice bath. The obtained holocellulose was filtered and washed with 200 mL of ethanol and 25 mL of acetone. Then samples were oven dried overnight in an oven at 105 °C. The samples were re-weight and the holocellulose content calculated was on a dry basis.

To determine the cellulose content, duplicate samples of 1.5 g oven dry holocellulose were weighed and 100 mL of 17.5% sodium hydroxide solution were added. The extraction was carried out at 20 °C for 2 h with 120 rpm and subsequently, the samples were filtered. The cellulose retained in the crucible was further washed with 10 mL of 10% acetic acid and 200 ml distilled water and then

oven dried overnight at 105 °C. The samples were re-weighed and cellulose was calculated as a percentage of the oven dry sample.

The filtrate containing the hemicellulose was neutralized to pH 5.0 with acetic acid and 2 L of ethanol were added to precipitate the hemicellulose. The hemicelluloses were filtered and washed with water and were dried in an oven at 105 °C overnight and re-weighed. The hemicellulose were calculated as a percentage of the oven dry sample.

3.2.1.1.7 Acid Insoluble (Klason) and Acid Soluble Lignin Content

Acid insoluble lignin was determined by the TAPPI standard method (T2220S-74) (TAPPI, 1989). Dried samples of 1 g were mixed with 15 mL of 72% (w/w) sulfuric acid and stirred with a glass rod every 15 min for 2 h at 20 ºC. At the end of the initial hydrolysis, samples were transferred from beaker to the flask and diluted with water to 3% concentration of sulfuric acid completing to a total volume of 575 mL. The resulting hydrolysate was autoclaved at 121°C for 2 h and then cooled to room temperature, filtered through pre-weighed dry filters. The obtained residue was dried for 24 h at 105 °C to reach constant weight for Klason lignin calculation as follows:

$$Klason\ lignin\ content\ (\%) = \frac{W_2 - W_3}{W_1 \times \frac{T_f}{100}\%} \times 100\% \qquad (7)$$

where; W_1=initial sample weight

W_2=Weight of filter paper, acid-insoluble lignin and acid-insoluble ash

W_3=Weight of filter paper and acid-insoluble ash

T_f= % total solids content of the prepared sample used in the analysis, on a 105 ºC

dry weight basis.

The acid soluble lignin was measured by the TAPPI standard method (TAPPI, 1985). The soluble lignin was determined in the hydrolysate by spectroscopy at 205 nm wavelength. The 4% (w/w) sulfuric acid was used as the reference blank. The hydrolyzate was diluted appropriately to attain an absorbance between 0.2-0.7. The amount of acid soluble lignin (ASL) was then calculated as

$$ASL\ (\%) = \frac{UV_{abs} \times Volume_{filtrate} \times Dilution}{\varepsilon \times Pathlength \times W} \times 100 \quad (8)$$

where; UV abs= Absorbance for the sample at 205 nm

Volume $f_{iltrate}$= volume of filtrate

W=weight of sample (mg)

ε =Absorptivity coefficient of biomass at specific wavelength (110g/L cm)

The percentage of lignin reduction was calculated with the following equation:

$$The\ percentage\ of\ lignin\ reduction = \left(\frac{L_u - L_p}{L_u} \right) * 100 \quad (9)$$

where; L_p= weight of Klason lignin in the pretreated biomass

L_u= weight of Klason lignin in the untreated biomass

3.2.2 Total Reducing Sugar

Total reducing sugars were determined colorimetrically using dinitrosalicycilic acid reagent (Miller, 1959). The DNS reagent was prepared as described in Appendix B.

Reducing sugars were determined by using glucose as standard (Table C.1). Six different concentrations (0.15, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L) of glucose were prepared in distilled water. Three ml of diluted samples and 3ml of DNS reagent were mixed in a test tube. The mixture was heated for 15 min at 90 °C in a water bath. After the color had developed 1.0 mL of 40% Rochelle salt was added when the contents of the tubes were still hot and then the tubes were cooled. A blank was also prepared (3.0 mL each of distilled water, DNS and Rochelle salt solution). Absorbance was measured with spectrophotometer at 575 nm. Measurements were carried out in duplicate.

3.2.3 Inhibitory Compounds

Furfural, HMF and acetic acid were determined by a HPLC system (Prostar, Varian, CA, USA) equipped with a RI detector for HMF and furfural and a PDA (Diode array) detector for acetic acid. The column was Metacarb 87H column (300 mm X 7.8 mm, varian, S/N: 05517112, Varian) and the operation conditions were 35 °C with 0.08 N H_2SO_4 as the eluent flowing at 0.5 mL/min (Xie et al., 2011).

The Folin-Ciocalteu assay was used for determination of total phenol content in the hydrolyzate samples colorimetrically (Spanos & Wrolstad, 1990) at 760 nm. Phenolics were expressed as gallic acid equivalents prepared with gallic acid standards at 6 different concentrations ranging from 10 to 60 mg/L (Figure D.1).

3.2.4 Pectinase Assay

Polygalacturonase (PG) activity was assayed by measuring the release of reducing

groups from polygalacturonic acid using the 3,5-dinitrosalicylic acid (DNS) reagent assay (Miller, 1959). The number of reducing groups was expressed as galacturonic acid.

Galacturonic acid monohydrate was used as standard (Figure E.1). Polygalacturonase (PG) activity was evaluated by mixing 0.5 mL of enzymatic extract and 0.5 mL of polygalacturonic acid solution (1 % w/v polygalacturonic acid in 0.05 M acetate buffer at pH 7.0) and incubating at 50 °C for 30 min (Kapoor et al., 2000). The absorbance was measured at 575 nm. The blank was prepared in the same way except that the crude enzyme. One unit of enzymatic activity (U) was defined as the amount of enzyme which releases one µmol of galacturonic acid per minute under assay conditions.

3.2.5 Determination of Cell Density

Cell density was measured turbidometrically at 600 nm wavelength. Growth curves of *B.subtilis* and *B.pumilus* were shown in Appendix F.

3.2.6 Determination of Protein Content

The total protein content of the samples was determined by Lowry et al. (Lowry et al., 1951); the protein standard used was bovine serum albumin (BSA). The lowry solution was prepared as described in Appendix G. Sample of 0.5 mL was added to 0.7 mL Lowry Solution, mixed thoroughly and incubated for 20 min at room temperature in dark, and 0.1 mL Folin's reagent was later added. After mixing, it was left for 30 min at room temperature in dark and then absorbance of each sample was determined at 750 nm against blank.

3.2.7 Clarity Test (%)

Carrot juice samples of 200 mL were placed into a flask. After addition of the produced pectinase, samples were mixed under shaking (120 rpm) at 50 ºC. At the end of enzymatic treatment, the enzyme was inactivated by heating the suspension at 90 ºC for 5 min and centrifuged at 2000x *g* for 10 min.

The clarity of the juice was determined by measuring the absorbance at 660 nm. The degree of clarification was expressed by percentage of clarity calculated using Eqn (10)

$$Clarity = \frac{\left\lfloor Abs_{untreatedsample} - \left(Abs_{control} - Abs_{sample}\right)\right\rfloor}{Abs_{untreatedsample}} * 100 \qquad (10)$$

Abs $_{untreated}$: Sample with no enzyme

Abs $_{control}$: Sample with no enzyme heated 50 ºC

Abs $_{sample}$: Sample with enzyme heated 50 ºC

3.2.8 Pectin Degradation (Alcohol test) Test

An alcohol test was conducted to check for residual pectin after enzymatic treatment (clarification process). Ethanol-HCl of 100 mL prepared by adding 5 mL of 5% hydrochloric acid to 95 mL of 96% ethanol were mixed with 5 mL of carrot juice. For each sample, if breakdown of pectin was observed in 1-minute, depectinization would be considered incomplete (Acar & Gokmen, 2005).

3.3 Pretreatment Methods

3.3.1 Dilute-Acid Pretreatment

The schematic diagram of the set-up of the dilute acid pretreatment conducted in autoclave was shown in Figure 3.1. Ground hazelnut shells were pretreated with dilute sulfuric acid at concentrations of 1, 3, and 5 % (w/w) and a biomass solid loading of 1/20 (g dry weight/mL). The pretreatments were performed at 110 °C, 120 °C and 130 °C in an autoclave for 15, 37.5 and 60 min. After acid hydrolysis, the solid residue was separated by centrifugation (MPW-15 mini centrifuge, MPW Med. Instruments Co., Warsaw, Poland) (2000x *g*, 30 min) and pH (Hanna portable pH-meter, HANNA Instruments Inc, USA) of the supernatant was adjusted to 5.0 using 10 M NaOH.

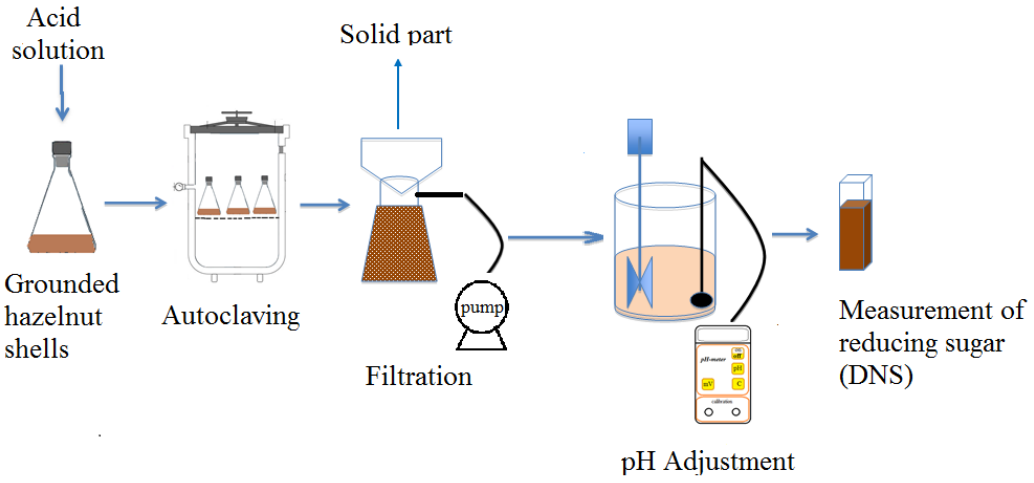


Figure 3.1 Schematic of dilute acid pretreatment set up

The flow chart of dilute acid hydrolysis is shown in Fig. 3.2 for better understanding of mass input and outputs.

Reducing sugar yield (%, w/w) for each pretreatment was calculated as follows:

$$Yield = \frac{a}{b} * 100 \quad (11)$$

a= Amount of reducing sugar in hydrolyzate obtained from pretreatment of dry biomass (g/g)

b=Amount of cellulose and hemicellulose in untreated dry biomass (g/g)

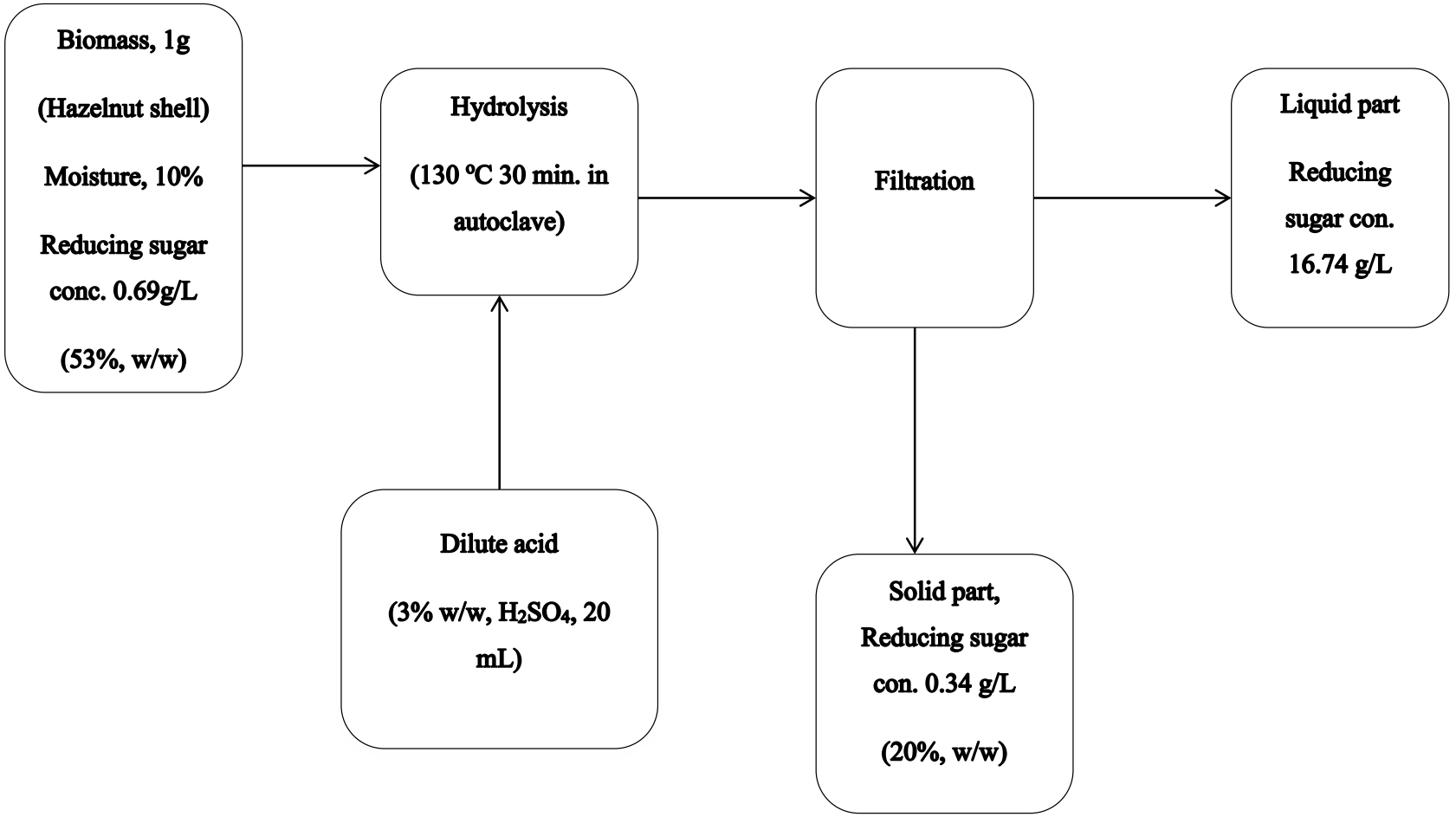


Figure 3.2 Flow chart of dilute acid pretreatment method.

3.3.1.1 Solid/liquid Ratio

Solid/liquid ratio was defined as the ratio of dry weight of hazelnut shells to volume of sulfuric acid. Three different solid/liquid ratios of 1/20, 1/10 and 1/7 (w/v) were investigated in acid pretreatment for comparing reducing sugar yields.

3.3.2 Alkaline Pretreatment

Ground hazelnut shells were pretreated with sodium hydroxide (NaOH) using two different methods.

First procedure is so called Method 1, in which ground hazelnut shells were pretreated by solutions of 1, 3, 5 and 7 % (w/v) NaOH at 30, 45 and 60 ^{o}C for 24 h at a solid/liquid ratio of 5% (g shells/mL NaOH solution).

Ground hazelnut shells were pretreated with 100 mL of NaOH solution at concentrations of 3, 5, and 7% (w/v) and biomass solid-liquid ratios (SLR) of 5, 10 and 15% (w/v, dry weight basis). The pretreatments were performed in crimp sealed, glass serum bottles at 121 $^{\circ}$C, 15 psi in an autoclave for treatment times of 30, 60 and 90 min. This procedure is so called Method 2.

The pretreated biomass was recovered by filtration and washed with 400 ml of deionized water in a Buchner funnel-vacuum pump assembly to remove excess alkali and dissolved by-products that might inhibit enzymes during subsequent hydrolysis. The residual biomass was analyzed for solid recovery and acid soluble and insoluble lignin contents using standard methods. After the pretreatment, samples were centrifuged at 2000x *g* for 30 min to separate the supernatant. Finally, total sugar content of the supernatant was measured using DNS method (Miller, 1959). The pH of the pretreated samples was adjusted to 6.5 using 5% (w/w) H_2SO_4.

3.3.3 Ozone Pretreatment

Figure 3.3 shows the ozonolysis reactor set-up used in this study at North Carolina State University at Biological and Agricultural Engineering Department. Ozonolysis was performed in a glass column reactor tube (Aceglassware, NJ, USA) with 5 cm diameter and 30 cm length. One end of the reactor was plugged with glass wool to support the biomass. Ozone, produced on-site by an ozone generator (Model: OL80 A Ozone lab instrument, Canada) supplied with industrial grade oxygen (Airgas National Welders, Raleigh, NC) at a flow rate of 0.25 L/min maintained using a mass flow controller (Model no:FMA5516, Omega, CT, USA) was introduced into the reactor from the top. The bottom of the reactor tube was connected to an ozone destructor through an exit line. Five gram (dry basis) of ground hazelnut shell was prepared for ozonolysis by adding different amounts of moisture and allowed to equilibrate for an hour. Three ozone concentration 30, 40, and 50 mg/L were tested for hazelnut shell. After each experiment, ozonated hazelnut shell was washed with 200 mL of distilled water. Pretreated samples were stored in ziplock bags at room temperature until further use for compositional analysis including total solids, acid insoluble lignin (AIL), acid soluble lignin (ASL), ash, reducing sugar content and enzymatic hydrolysis.

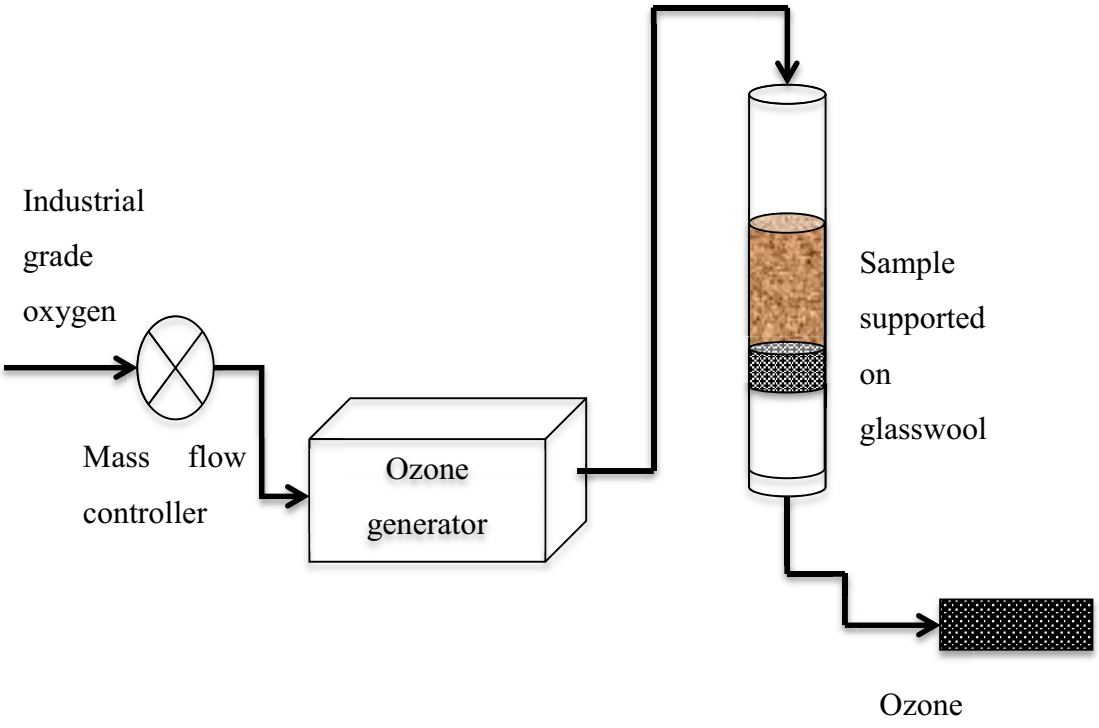


Figure 3.3 Schematic of ozonolysis reactor set up –adapted from Panneerselvam et al. (2013)

3.4 Enzymatic Hydrolysis

The untreated ground samples and solid residues recovered after sulfuric acid pretreatment were separately hydrolyzed using Viscozyme L containing cellulase and xylanase enzymes at 50 °C and 120 rpm for 24 h in 250 mL flasks with enzyme loadings of 66.6, 100 and 200 U/g dry substrate and solid loadings of 1/20, 1/10 and 1/7 (w/v) (g shells/g liquid). Cellulase and xylanase activity were assayed as carboxymethly cellulose units (U) (Ghose, 1987) and xylan units from beechwood (Bailey et al., 1992), respectively. The activities of the enzymes were measured as

2250 U/mL and 1400 U/mL at 50 ºC and pH 5.0 after 30 min-incubation for cellulase and xylanase, respectively. The sodium acetate buffer (Merck, Darmstadt, Germany) (50 mM) was used to maintain the pH at 5.0. The initial pH was adjusted to 5.0 because Viscozyme L works well with pH 3.0–5.0. Samples were taken out every 3h for reducing sugar analysis. Hazelnut shell without any pretreatment was also subjected to saccharification as the control. After the enzymatic hydrolysis, samples were heated to 100 °C for 15 min to inactivate the enzymes (Ghose, 1987).

Enzymatic hydrolysis was also performed on alkali and ozone pretreated hazelnut shells at 5% solid loading (dry basis) in 10 mL volume made up by 50 mM sodium citrate buffer (pH 4.8), 40 µg/mL tetracycline hydrochloride (an antibiotic added to avoid microbial contamination), Cellic® Ctec2 cellulase enzyme complex (110 FPU/mL) (density 1.1457 g/mL) at a dosage of 0.5 g enzyme/g biomass and HTec2 xylanase enzyme complex (density 1.1548 g/mL) at a dosage of 0.2 g enzyme/g biomass. Samples were hydrolyzed in 50 mL centrifuge tubes in a shaking water bath (Model:89032-226, VWR International, PA, USA) at 50 °C and 150 rpm for 72 h. Untreated biomass with equivalent enzyme loading was also hydrolyzed as the control. After hydrolysis, the samples were centrifuged at 10,000 rpm for 10 min and the recovered supernatant was used for fermentable sugar yield determination by measuring reducing sugars. Enzymatic conversion (η) (%) was then calculated (eqn 12) as;

$$Enzymatic\ \ conversion\ \ (\eta)\ (\%) = \frac{Conc.\ of\ reducing\ \ sugar\ \ in\ \ hydrolysate,\ g/mL}{Conc.\ of\ reducing\ \ sugar\ \ in\ pretreated\ \ solids,\ g/mL} \times 100 \qquad (12)$$

3.5 Pectinase Production By Submerged Fermentation (SmF)

The schematic diagram of the set-up of the submerged fermentation (SmF) process conducted in the shake-flask bioreactors was presented in Figure 3.4. A 500 mL shake-flask bioreactors containing 100 mL of fermentation medium similar to

growth medium but only amended with 5% hazelnut shell hydrolysate instead of glucose was used for fermentation. Before inoculation, the flasks and medium were sterilized by autoclaving at 121 ^{o}C for 15 min. After sterilization step, fermentation medium was inoculated with 1 mL of overnight grown bacterial culture containing 10^{6} CFU/mL and incubated under agitation of 130 rpm at 30 ^{o}C (Fig. 3.4). After fermentation, the biomass was separated by centrifugation at 2000x*g* for 20 minutes and the supernatant was used as a crude enzyme in the pectinase assay described in section 3.2.4.

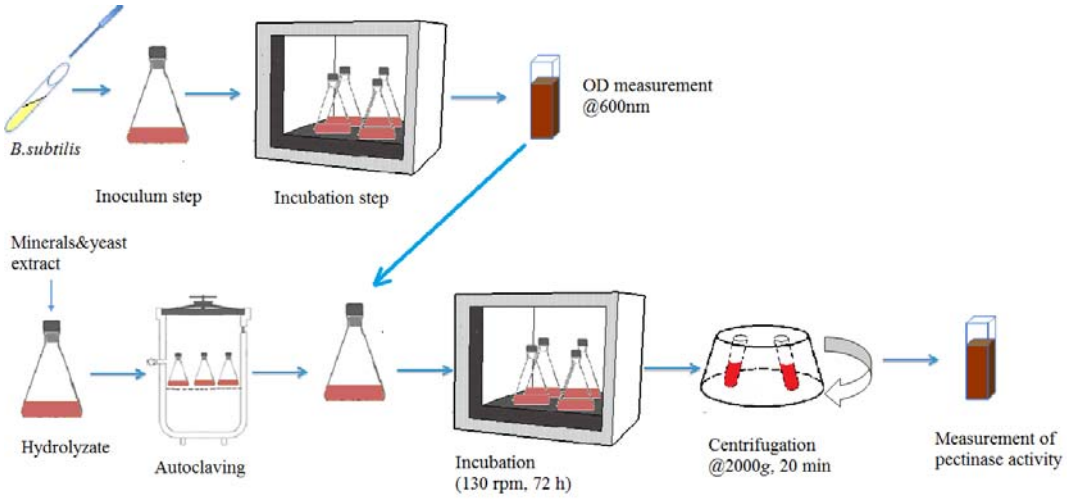


Figure 3.4 Schematic of pectinase production steps

3.5.1 Effect of Substrate Type

Effect of polymeric substrate (pectin) and organic wastes (hazelnut shells and its hydrolysate) as a carbon source were carried out for selecting substrate.

3.5.2 Effect of pH on Pectinase Activity and Stability

To determine the effect of pH on pectinase activity, the enzyme assays were performed at various pH range of 5.0-9.0 using 50 mM of the following buffers: sodium acetate (pH 5.0), sodium phosphate (pH 7.0) and carbonate-bicarbonate (pH 9.0).

The pH stability of *Bacillus subtilis* pectinase was investigated in the pH range of 5.0-9.0 during 7 hours. After incubations, activity was determined by the standard activity method and was reported as the ratio of enzyme activity after pH treatment to the initial maximum activity at pH 7.0.

3.5.3 Effect of Temperature on Pectinase Activity and Stability

Effect of temperature on pectinase activity was determined in the range of 40-60^{o}C. In order to determine the effect of temperature on pectinase stability, enzyme samples were incubated at 40-60 ^{o}C for 7 h. After incubation, the activity was determined by the standard activity method and was reported as the ratio of enzyme activity after temperature treatment to the initial maximum activity at 50 ^{o}C. From a semilogarithmic plot of residual activity versus time, the inactivation rate constants (k_d) were calculated (from slopes), and apparent half lives were estimated using equation 13. The half-life ($t_{1/2}$) is known as the time where the residual activity reaches 50%.

$$t_{1/2} = \frac{\ln 2}{k_d} \tag{13}$$

3.6 Extraction of Carrot Juice

The carrots used in this study were purchased from a local market in Ankara, Turkey. The carrot juice extraction illustrated in Figure 3.5 was carried out in the

pilot plant of Ankara University. The extract of crude enzyme and Pectinex 3XL were used in processing of carrot juice. The extract of crude enzyme was obtained from fermentation step as described in section 3.5.

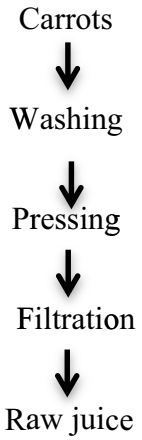


Figure 3.5 Flow chart of carrot juice extraction

3.7 Experimental Design

3.7.1 Experimental Design for Dilute Acid Pretreatment

The optimization of dilute acid pretreatment was carried out in several steps. The first step was the selection of hydrolysis conditions (acid concentration, temperature, and time) and their levels by using one-factor-at- a-time approach. The second step was estimation of the optimum values by Box-Behnken design (BBD) of Response Surface Methodology (RSM) to obtain the maximum reducing sugar concentration. The third step was the use of Artificial Neural Network **(**ANN) to predict reducing sugar concentration in comparison to RSM model.

3.7.1.1 The Conventional One Factor At A Time Approach

One factor at a time approach involves varying one factor while keeping the other factors at a constant level. The effects of acid concentration, time, and temperature on hydrolysis conditions of hazelnut shells were examined.

3.7.1.2 Response Surface Methodology

One of the best models to describe chemical processes by analytical methods is quadratic model in terms of linear and cubic equation. A set of 15 experiments was carried out in two randomized replicates giving total experimental runs of 30 with 6 center runs. The individual and combined effects of temperature, acid concentration and treatment time on reducing sugar production were studied using Box–Behnken response surface method (Box & Behnken, 1960) (BBD) using MINITAB 16.0 (Minitab Inc. State College, PA, USA) (Table 3.1). All reducing sugar analyses were conducted in triplicate.

Table 3.1 Coded and uncoded variables of dilute acid pretreatment by Box-Behnken surface design

	Factors					
Exp.No	**Temperature (°C)**		**Acid conc. (%)**		**Time (min)**	
	coded	**uncoded**	**coded**	**uncoded**	**coded**	**uncoded**
1	0	120	+1	5	+1	60
2	0	120	+1	5	-1	15
3	+1	130	-1	1	0	37.5
4	+1	130	+1	5	0	37.5
5	-1	110	-1	1	0	37.5
6	0	120	-1	1	+1	60
7	-1	110	0	3	-1	15
8	0	120	-1	1	-1	15
9	-1	110	+1	5	0	37.5
10	-1	110	0	3	+1	60
11	+1	130	0	3	+1	60
12	+1	130	0	3	-1	15
13	0	120	0	3	0	37.5
14	0	120	0	3	0	37.5
15	0	120	0	3	0	37.5

A quadratic mathematical model was developed (Equation 14) to fit the experimental data using the response optimizer function of Minitab:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

(14)

where Y is the reducing sugar concentration, b's are regression coefficients, and X_1,X_2,X_3 are temperature, acid concentration and treatment time, respectively. Analysis of variance (ANOVA) and regression were performed at 95% confidence interval to define the coefficients of the predictive model and significant terms. The optimum conditions for maximizing the reducing sugar concentration was determined using Response Optimizer tool in MINITAB 16.0.

3.7.1.2.1 Model Validation

The constructed model was verified by conducting additional fermentation experiments, which were not present in the design matrix and comparing the results of experiments to the predicted values. The coefficient of determination (R^2) was calculated to check for the variability between the predicted vs. observed reducing sugar concentration values. A statistical difference measure test was also carried out to evaluate the performance of the model by calculating root mean square error (RMSE) and mean absolute error (MAE) values which indicate the goodness of the predictions as follows:

$$RMSE = \left(\frac{1}{N} \sum_{i=1}^{N} \left(X_{pred,i} - X_{\exp,i} \right)^2 \right)^{0.5} \quad (15)$$

$$MAE = \frac{1}{N} \sum_{i=1}^{N} \left| X_{pred,i} - X_{\exp,i} \right| \quad (16)$$

where X_{exp} is the experimental value and X_{pred} is the predicted value of total reducing sugar concentration, N is the number of data.

The reproducibility of the PG production was calculated using coefficient of variation (CV) values as follows

$$CV = \frac{\sigma}{\bar{X}} * 100 \quad (17)$$

where σ is sample standard deviation, and $\bar{X}$ is sample mean. Lower values of CV indicate higher reproducibility.

3.7.1.3 Artificial Neural Network (ANN) Modeling

ANN is a mathematical means of simulating the biological neurons in a brain learn relationship between input and output data after training with example input and output data sets (Kashaninejad et al., 2009). Especially in the past two decades, it has been greatly used in numerous fields of science and engineering. ANNs consist of many simple computational elements, called nodes or neurons, organized in layers and operating in parallel (Silva et al., 2008). Neurons are connected by weights that are modified during learning phase (Kashaninejad et al., 2009). All neural networks have three main layers, which are called input, hidden and output layers (Mehdizadeh & Movaghernejad, 2011). A number of classes of neural networks exist in the literature such as, feed forward back propagation, recurrent neural networks, cascade correlation neural networks and radial basis function neural networks. All types of these network architectures have the same elements: neurons, layers and weights. The most common type of ANN in engineering application is multi-layer perceptron (MLP), which is a back propagation feed forward network (Singh et al., 2008). In this work, a multi-layer feed forward neural network has been used. MLP network consist of input and output layers, with several but usually only one-hidden layer (Rurnelhart et al., 1986). In this particular ANN, information moves only in one direction, forward from the input layer, through the hidden layer and to the output layer (Mehdizadeh & Movaghernejad, 2011). The number of neurons in the input layer depends on the number of

independent variables and the number of neurons in the output layer corresponds to dependent variables. Several ANNs with differing numbers of hidden layer neurons was developed for describing dilute acid pretreatment. Input data were randomized into three sets: learning, validation and testing. Usually 30% of data are used for testing and remaining 70% for training and validation (Mehdizadeh & Movaghernejad, 2011). The experimental data, 43 data points, was divided such that 70% was used to train the model, 15% was used to validate the model and 15% was used to test the generalization ability of the model. The activation function of hidden layer was "logsig" and the one in output layer was "purelin".

$$\text{logsig}(x) = \frac{1}{1+e^{-x}} \tag{18}$$

$$\text{purelin}(x) = x \tag{19}$$

Training of the network was performed with the function of "trainlm", that updates weight and bias values according to Levenberg-Marquardt optimization. In learning of network "learngd" as adaption learning function was used. The maximum training epochs were 1000, and mean square error was 0.0001. The other parameters of neural network were taken as defaults of neural network toolbox, MATLAB R2011a (The MathWorks, USA).

It is necessary to train an artificial neural network before using it for a particular application (Mehdizadeh & Movaghernejad, 2011). Feed forward network training starts by applying the input vector to the input layer having network processing element (Singh et al., 2008). During the training, the network learns to create new outputs through a repetitive method (Mehdizadeh & Movaghernejad, 2011). The generated outputs by network are compared with the target. The network is adjusted, based on a comparison of the output and the target, until the network output matches the target. Basically the purpose of training patterns is to reduce the global error (Kashaninejad et al., 2009).

The performance of the ANN was statistically measured by mean squared error (MSE), root percent deviation (RPD) and the coefficient of determination (R^2). The

R^2 represents how well the approximated function predicts the response versus just using the response mean. Values closest to 1 are the best. The MSE is a representation of the difference between the predicted and actual values and gives a sense of how close the predicted values are to the observed values in the units of those values. Lower values of MSE are good and was calculated using the formula in equation 20. The RPD represents the percent that the error was of the value being estimated. Lower values are good and was calculated using the formula in equation 21.

$$MSE = \frac{\sum_{i=1}^{n}\left(X_{pre,i} - X_{\exp,i}\right)^2}{n} \tag{20}$$

$$RPD = \frac{100}{n}\sum_{i=1}^{n}\frac{\left(X_{pre,i} - X_{\exp,i}\right)}{X_{\exp,i}} \tag{21}$$

Where $X_{pre,i}$ is the predicted output from observation i, $X_{exp,i}$ is the experimental (target) output from observation i, $\overline{X}$ is the average value of experimental output, and n is the total number of data.

3.7.2 Optimization of Alkaline Pretreatment

Individual and combined effects of NaOH concentration, solid-liquid ratio (biomass loading) and treatment time on solid recovery, lignin reduction (based on AIL) and total reducing sugar (after enzyme hydrolysis), were studied by Box–Behnken Design (BBD) using MINITAB 16.0. Optimal alkaline pretreatment conditions were identified using the response optimizer function under DOE-RSM. The coded and uncoded forms of independent factors are given in Table 3.2. A set of 30 experiments were carried out. The measurement of each response variable was reported as the average of three replicates. Quadratic mathematical equation (Eqn.

22) as expressed below were also obtained by Response Surface Methodology (RSM) analysis for predictive modeling:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (22)$$

where Y is the response (solid recovery, lignin reduction and total reducing sugar yield after hydrolysis), b's are regression coefficients, and X_1,X_2, and X_3 are NaOH concentration, solid-liquid ratio (SLR) and treatment time, respectively. Analysis of variance (ANOVA) and regression were performed at 95% confidence interval to define the coefficients of the predictive model and significant terms.

From regression analysis of Box-Behnken design, optimal conditions for alkaline production were defined. To validate the model, additional trials at the defined pretreatment conditions were carried out in triplicate. All experiments in this study were conducted in triplicate with a 95 % confidence level.

Table 3.2 Coded and uncoded variables of alkaline pretreatment by using response surface design.

	Factors					
Exp.No	**NaOH conc. (%)**		**SLR (%)**		**Time (min)**	
	coded	**uncoded**	**coded**	**uncoded**	**coded**	**uncoded**
1	0	5	0	10	0	60
2	0	5	0	10	0	60
3	0	5	0	10	0	60

Table 3.2 (continued)

4	+1	7	+1	15	0	60
5	+1	7	-1	5	0	60
6	-1	3	+1	15	0	60
7	-1	3	-1	5	0	60
8	-1	3	0	10	+1	90
9	+1	7	0	10	+1	90
10	0	5	+1	15	+1	90
11	0	5	-1	5	+1	90
12	-1	3	0	10	-1	30
13	+1	7	0	10	-1	30
14	0	5	-1	5	-1	30
15	0	5	+1	15	-1	30

3.7.3 Experimental Design and Optimization of Ozone Pretreatment

The optimization of ozone concentration, moisture and treatment time for solid recovery, lignin reduction and total reducing sugar yield were carried out in two steps. The first step was the selection of physical parameters such as ozone concentration, moisture concentration and treatment time. The second step was the estimation of the optimum values by using full factorial design to obtain maximal reducing sugar yield.

Initial screening of factors was performed with BBD technique to identify the crucial parameters affecting lignin reduction, solid recovery and total reducing sugar yield and subsequently full factorial design was used to determine the optimal total reducing sugar production using the selected process variables.

3.7.3.1 BBD Design

The individual and combined effects of ozone concentration, moisture and hydrolysis time on total reducing sugar yield, solid recovery and lignin reduction were studied using Box–Behnken response surface method (Table 3.3). A quadratic predictive model was fit to experimental data to simulate reducing sugar yield, solid recovery and lignin reduction as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (23)$$

where Y is the response (solid recovery, lignin reduction and total reducing sugar yield), b's are regression coefficients, and X_1,X_2,X_3 are ozone concentration, moisture concentration and hydrolysis time.

Table 3.3 Box-Behnken design matrix for identifying key process variables

	Factors					
Exp.No	**Ozone conc.(%)**		**Moisture (mg/L)**		**Time (min)**	
	coded	**uncoded**	**coded**	**uncoded**	**coded**	**uncoded**
1	-1	25	0	40	+1	120
2	0	30	-1	30	+1	120
3	0	30	+1	50	+1	120
4	+1	35	0	40	+1	120
5	-1	25	-1	30	0	90
6	-1	25	+1	50	0	90
7	0	30	0	40	0	90
8	0	30	0	40	0	90
9	0	30	0	40	0	90
10	+1	35	-1	30	0	90
11	+1	35	+1	50	0	90
12	-1	25	0	40	-1	60
13	0	30	-1	30	-1	60
14	0	30	+1	50	-1	60
15	+1	35	0	40	-1	60

3.7.3.2 Full-Factorial Design

Based on BBD results, the full factorial design was constructed for 2 factors (ozone concentration and time) each having 3 levels (-1, 0 and 1) with 15 experimental designs (12 points of the factorial design and 3 center points to establish the experimental errors) as shown in Table 3.4. All experiments were carried out in triplicate and the results are reported in terms of mean values.

Table 3.4 Experimental levels of the variables studied in the full factorial design

Factor	Low (-1)	Center (0)	Upper (+1)
Ozone concentration (mg/L)	30	40	50
Time (min)	60	90	120

3.7.4 Optimization of Pectinase Production

The optimization of medium components and culture conditions for pectinase production by *B.subtilis* and *B.pumilus* was carried out in two steps. The first step was the selection of physical parameters such as pH, fermentation time, temperature, inoculum volume (%, v/v) and medium components such as pectin, yeast extract, magnesium sulphate [$MgSO_4$], and dipotassium hydrogen phosphate [K_2HPO_4].

The second step was the estimation of the optimum values using Box-Behnken design (BBD) under RSM to obtain the maximum pectinase activity.

Initial screening of factors (step 1) was performed with Plackett-Burman Design (PBD) to identify the primary parameters affecting pectinase production and subsequently BBD technique was used to determine the optimal pectinase production using the selected factors (step 2).

3.7.4.1 Plackett Burman Design (PBD)

A two level PBD experimental matrix was set up to identify the significant factors of pectinase production. The PB design provides a linear model, where only main effects are taken into account. Selection of appropriate carbon, nitrogen and other nutrients is one of the most critical stages in the development of an efficient and economic process. In this study, 8 independent variables were selected, namely pH, fermentation time, temperature, inoculum volume (%, v/v) and media components of pectin, yeast extract, magnesium sulphate [$MgSO_4$], and dipotassium hydrogen phosphate [K_2HPO_4]. The concentration range of each nutrient was based on various studies in the literature. Table 3.5 illustrates the design matrix of various components with coded and uncoded values. Table 3.6 shows PB experimental design in coded variables form. Pareto chart was then plotted to highlight the most significant factors responsible for pectinase production (Figure J.1).

Table 3.5 Coded and uncoded variables of independent factors in PBD.

Variable	**Low (-1)**	**High (+1)**
pH	5	9
Fermentation time (h)	24	72
Temperature(°C)	30	40
Inoculum volume (%, v/v)	1	5
Pectin (%, w/v)	0.2	0.5
Yeast extract (%, w/v)	0.1	0.5
$MgSO_4$ $7H_2O$ (%, w/v)	0.02	0.08
K_2HPO_4 (%, w/v)	0.02	0.04

Table 3.6 Plackett Burman Design for screening major factors of pectinase production.

Run No.	pH	Time (h)	Temperature (°C)	Inoculum Volume (%v/v)	Pectin (%w/v)	Yeast extract (%w/v)	$MgSO_4$ (%w/v)	K_2HPO_4 (%w/v)
1	+1	-1	+1	-1	-1	-1	+1	+1
2	+1	+1	-1	+1	-1	-1	-1	+1
3	-1	+1	+1	-1	+1	-1	-1	-1
4	-1	-1	+1	+1	+1	-1	+1	+1
5	-1	-1	-1	+1	+1	+1	-1	+1
6	-1	+1	-1	-1	-1	+1	+1	+1
7	+1	+1	-1	+1	+1	-1	+1	-1
8	-1	+1	+1	+1	-1	+1	+1	-1
9	-1	-1	-1	-1	-1	-1	-1	-1
10	+1	-1	-1	-1	+1	+1	+1	-1
11	+1	+1	+1	-1	+1	+1	-1	+1
12	+1	-1	+1	+1	-1	+1	-1	-1

3.7.4.2 BBD Design and Optimization

BBD technique is a statistical tool used to develop a quadratic model including major factors and their interactions to estimate pectinase production (Box & Behnken, 1960). Based on Pareto chart results (Figure J.1), BBD matrix was constructed for the five significant factors (pH, fermentation time, fermentation temperature, yeast extract and dipotassium hydrogen phosphate [K_2HPO_4]). The ranges for each factor studied were 5.0-9.0 for pH, 24-48 h for fermentation time, 30-40 ^oC for temperature, 0.1-0.5 (%, w/v) for yeast extract and 0.02-0.04 (%,w/v) for K_2HPO_4.

A set of 46 experiments were conducted and the results given in Table 3.7 were analyzed by MINITAB 16.0. Each response was reported as the average of two replicates.

A second order predictive model was fitted to represent linear, interaction and quadratic effects of variables on enzyme production (Eqn 24):

$$Y= b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{55}X_5^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{15}X_1X_5 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{25}X_2X_5 + b_{34}X_3X_4 + b_{35}X_3X_5 + b_{45}X_4X_5 \quad (24)$$

where Y is the response (pectinase activity), b's are regression coefficients, and X_1,X_2,X_3, X_4, X_5 are pH, fermentation time, fermentation temperature, yeast extract and dipotassium hydrogen phosphate [K_2HPO_4], respectively. The analysis of variance (ANOVA) and regression analysis were performed to define the coefficients of the predictive model and significant terms using MINITAB 16.0.

From regression analysis of Box-Behnken design, optimal conditions for pectinase production were defined. To validate the model, additional trials at the defined fermentation conditions were carried out in triplicate.

Table 3.7 Experimental design for optimization of pectinase production using Box-Behnken response surface method (RSM).

Run Order	pH (A)	Temperature (B)	Time (h) (C)	Yeast extract (%w/v) (D)	K_2HPO_4 (%w/v) (E)
1	0	0	+1	-1	0
2	0	+1	0	0	+1
3	0	+1	0	-1	0
4	0	0	+1	0	-1
5	0	-1	0	0	-1
6	+1	0	+1	0	0
7	0	0	0	0	0
8	+1	0	0	+1	0
9	0	+1	+1	0	0
10	+1	+1	0	0	0
11	+1	0	0	0	+1
12	0	-1	0	+1	0
13	0	0	0	0	0
14	+1	0	-1	0	0
15	0	0	-1	0	+1
16	-1	0	+1	0	0
17	-1	0	-1	0	0
18	0	+1	0	+1	0
19	-1	0	0	0	-1
20	0	0	+1	0	+1
21	0	0	0	0	0
22	0	+1	0	0	-1

Table 3.7 (continued)

23	0	-1	0	0	+1
24	0	-1	+1	0	0
25	0	0	0	0	0
26	0	0	0	0	0
27	0	+1	-1	0	0
28	-1	0	0	+1	0
29	0	0	0	-1	-1
30	0	0	0	+1	+1
31	+1	-1	0	0	0
32	0	0	-1	0	-1
33	0	-1	0	-1	0
34	0	0	+1	+1	0
35	+1	0	0	-1	0
36	0	0	0	-1	+1
37	0	0	-1	+1	0
38	-1	+1	0	0	0
39	-1	0	0	-1	0
40	0	0	0	+1	-1
41	+1	0	0	0	-1
42	-1	0	0	0	+1
43	-1	-1	0	0	0
44	0	0	-1	-1	0
45	0	-1	-1	0	0
46	0	0	0	0	0

3.7.5 Optimization of Clarification Process

A BBD matrix was constructed for the three process variables (enzyme concentration (%), pH and time (h). The ranges for each factor were 0.1-0.5 % for enzyme concentration, 4.0-7.0 for pH, and 2-6 h for time (Table 3.8). The optimal clarification conditions were defined using a quadratic mathematic equation expressed as;

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (25)$$

where Y is the response clarity, b's are regression coefficients, and X_1,X_2,X_3 are enzyme concentration, pH and time. From regression analysis of Box-Behnken design, optimal conditions for clarity were defined. To validate the model, additional trials at the defined clarification conditions were carried out in triplicate.

Table 3.8 Coded and uncoded variables of clarification by using response surface design

	Factors					
Exp.No	**Enzyme Conc. (%)**		**pH**		**Time (h)**	
	coded	**uncoded**	**coded**	**uncoded**	**coded**	**uncoded**
1	0	0.3	-1	4.0	+1	6
2	0	0.3	+1	7.0	+1	6

Table 3.8 (continued)

3	0	0.3	0	5.5	0	4
4	+1	0.5	-1	4.0	0	4
5	+1	0.5	+1	7.0	0	4
6	+1	0.5	0	5.5	-1	2
7	0	0.3	-1	4.0	-1	2
8	0	0.3	+1	5.5	0	4
9	-1	0.1	+1	5.5	-1	2
10	-1	0.1	-1	4.0	0	4
11	-1	0.1	0	5.5	+1	6
12	0	0.3	+1	7.0	-1	2
13	+1	0.5	0	5.5	+1	6
14	-1	0.1	+1	7.0	0	4
15	0	0.3	0	5.5	0	4

3.8 Statistical Analysis

Statistical analyses were accomplished using MINITAB 16.0 to test the significance of different acidic, alkaline, ozone, enzymatic hydrolysis, pectinase activity and clarification treatments. The pairwise comparisons were made by Tukey's test with a significance level of 0.05.

3.9 Cost Analysis

Cost of dilute acid pretreatment providing the highest reducing sugar conversion was also estimated for better assessment. The total cost of dilute acid pretreatment was calculated using the following Eqn (26) according to study of Vavouraki et al. (2014):

$$TC\,(in\ TL) = M_G * P_G - M_{ch} * P_{ch} - El * P_{El} \tag{26}$$

where TC represents the total cost (in Turkish Liras, TL) for the dilute acid pretreatment, M_G denotes the mass (in g) of glucose obtained at the end of pretreatment, P_G is market prices of glucose (in TL/g) considered as 0.21 TL/g, M_{ch} denotes the amount of chemical used for pretreatment (H_2SO_4) and P_{ch} its cost, considered as 20 TL/L H_2SO_4. El represents the estimated electrical energy consumed (in KWh) for dilute acid pretreatment, while P_{El} is the cost of electricity (0.18 TL/KWh) (Vavouraki et al., 2014).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Composition of Raw Material

The hazelnut shells contained 24.20±0.99% (w/w) cellulose, 28.20±0.14 % (w/w) hemicellulose and 34.64±0.43 % (w/w) lignin on a wet basis (Table 4.1), which approved their potential as the source of sugars such as xylose and glucose. These results agreed well with study of Demirbaş (2006). The chemical composition of hazelnut shells varies with geographic location, season, harvesting practice, as well as analysis procedures (Agblevor et al., 2003).

Table 4.1 Constituents of the hazelnut shell used in the experiments, expressed as percent of wet basis.

Constituent	**Content[a] (%, w/w, wb)**
Dry solid	90.30±0.24
Extractive	6.09 ±0.16
Fat	6.06±0.48
Crude fiber	68.22±1.86

Table 4.1 (continued)

Cellulose	24.20±0.99
Hemicellulose	28.20±0.14
Total lignin	34.64±0.43
Ash content	1.13±0.04

[a] Results belong to two replicates

4.2 Effect of Pretreatment Methods on Fermentable Sugar Production

4.2.1 Effect of Dilute Acid Pretreatment

4.2.1.1 Effect of Particle Size on Reducing Sugar Concentration

Particle size in pretreatments and enzymatic hydrolysis is important for yield of fermentable sugars. Table 4.2 shows the effects of the particle size on concentration of reducing sugars. When particle size decreases, surface area increases. Thus, reaction sites, where molecules can collide and interact increase. For particle size larger than 1mm, the penetration of acid was slower with result of declining the reducing sugar concentration. It was clear that the reducing sugar concentration decreased with increasing particle size, and at the particle size 1 mm, the reducing sugar concentration was the highest (16.74 g/L). Thus, a particle size of 1mm was chosen as the best for reducing sugars.

Table 4.2 Reducing sugar concentration with respect to particle size (3.42%, w/w, acid, 130 ^{o}C, 31.7 min).

Particle size (mm)	Reducing sugar concentration (g/L)*
0.18	15.81±0.14[b]
0.71	15.35±0.06[c]
1	16.74±0.13[a]
2	15.11±0.10[c]

* Results belong to two replicates

4.2.1.2 Effect of Solid/Liquid Ratio On Reducing Sugar Yield

Three different solid/liquid ratios of 1/20, 1/10 and 1/7 (w/v) were investigated in dilute acid pretreatment for reducing sugar yield (Figure 4.1). It was observed that the reducing sugar yield decreased with increasing solid/liquid ratio and the highest yield was achieved at the solid/ liquid ratio of 1/20, giving 63.2±0.5 % (w/w) of reducing sugar yield. Similar observations were reported by Ferrer et al. (2013) and Amenaghawon et al. (2014) who reported that maximum reducing sugar concentration were obtained using low liquid to solid ratio. At the ratio of 1/7, the reducing sugar yield decreased to 57.7 % (w/w). High solid/liquid ratio can result in mixing problems, which are reflected by slow solid liquefaction. Therefore, a ratio of 1/20 was chosen as the best ratio for production of reducing sugars. Mansilla et al. (1998) studied the effect of acid hydrolysis on rice hull to produce furfural. They reported that the optimal furfural production obtained with a solid liquid ratio of 1/25.

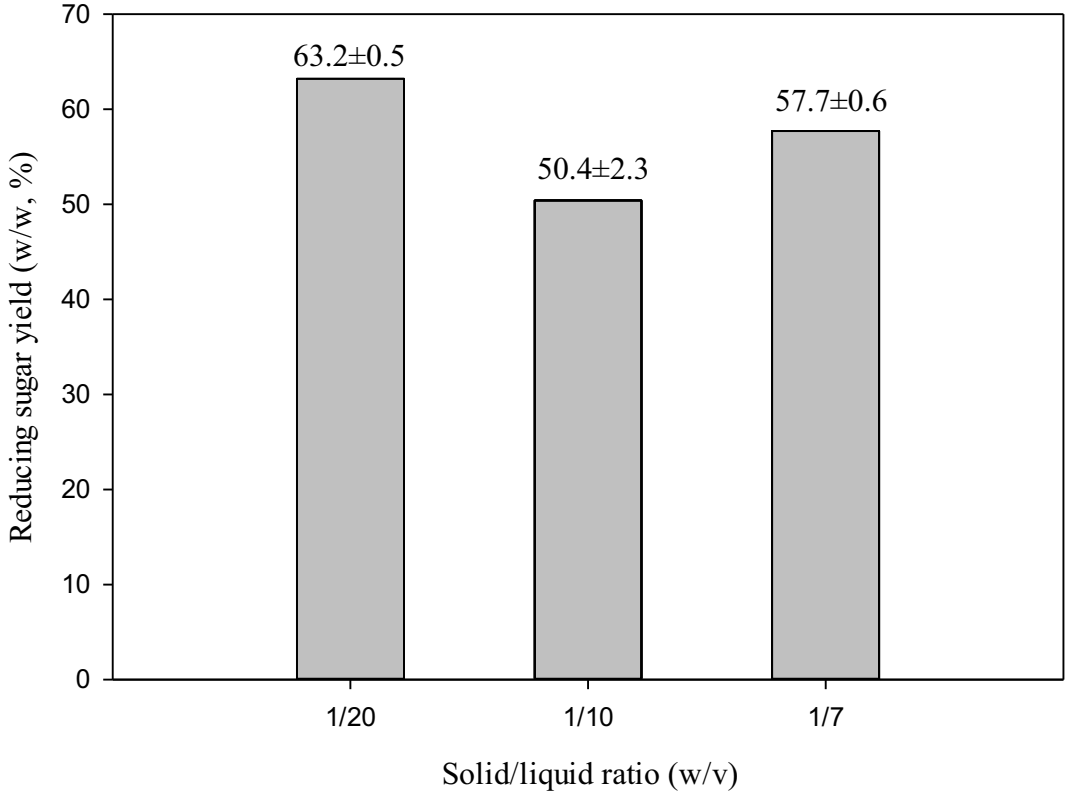


Figure 4.1 Reducing sugar yield with respect to solid/liquid ratio (3%, w/w, acid, 130 ºC, 30 min)

4.2.1.3 Screening The Factors Affecting Dilute Acid Pretreatment

The effects of acid concentration, time, and temperature on hydrolysis conditions of hazelnut shells were examined using one factor at a time approach. To determine the temperature effect, three different temperatures (110, 120 and 130 °C) were applied at 3% (w/w) acid concentration for 37.5 min. As temperature increased, reducing sugar concentration increased (Fig. 4.2). The amount of reducing sugar was increased at high temperature since reducing sugars released from

hemicellulose (Taherzadeh & Karimi, 2007; Sirikarn et al., 2012). For temperatures of 110–120 °C, the amounts of reducing sugar were not significantly different (P>0.05). The reducing sugar concentration at 130 °C (16.74 g/L) was significantly higher than the concentration at 110 °C (9.67 g/L) (P<0.05).

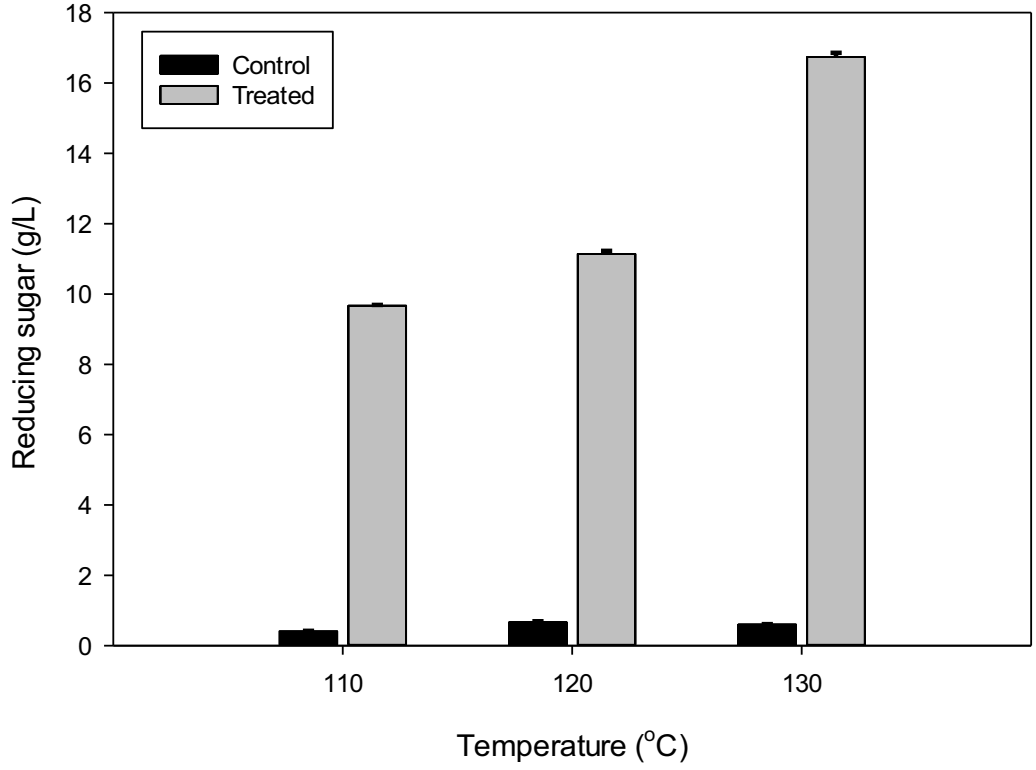


Figure 4.2 Hydrolysis of hazelnut shell at various temperatures (3%, w/w, 37.5 min)

Different acid (H_2SO_4) concentrations (1%, 3% and 5%, w/w) were then used at 130 ^oC for 37.5 min to assess the impact of acid concentration. The average results are shown in Fig. 4.3. According to ANOVA results acid concentration had significant effect on reducing sugar concentration ($P<0.05$). Increasing the acid concentration from 1% to 3% (w/w) increased the reducing sugar concentration from 12.56 g/L to 16.74 g/L whereas increasing the acid concentration from 3% to 5% (w/w) decreased the reducing sugar concentration from 16.74 g/L to 15.18 g/L. This may be explained by formation of organic acids (formic acid and acetic acid) and 5-HMF and furfural which, are byproducts of hexose and pentose degredation. Thus, the 3% acid concentration was chosen as the best level.

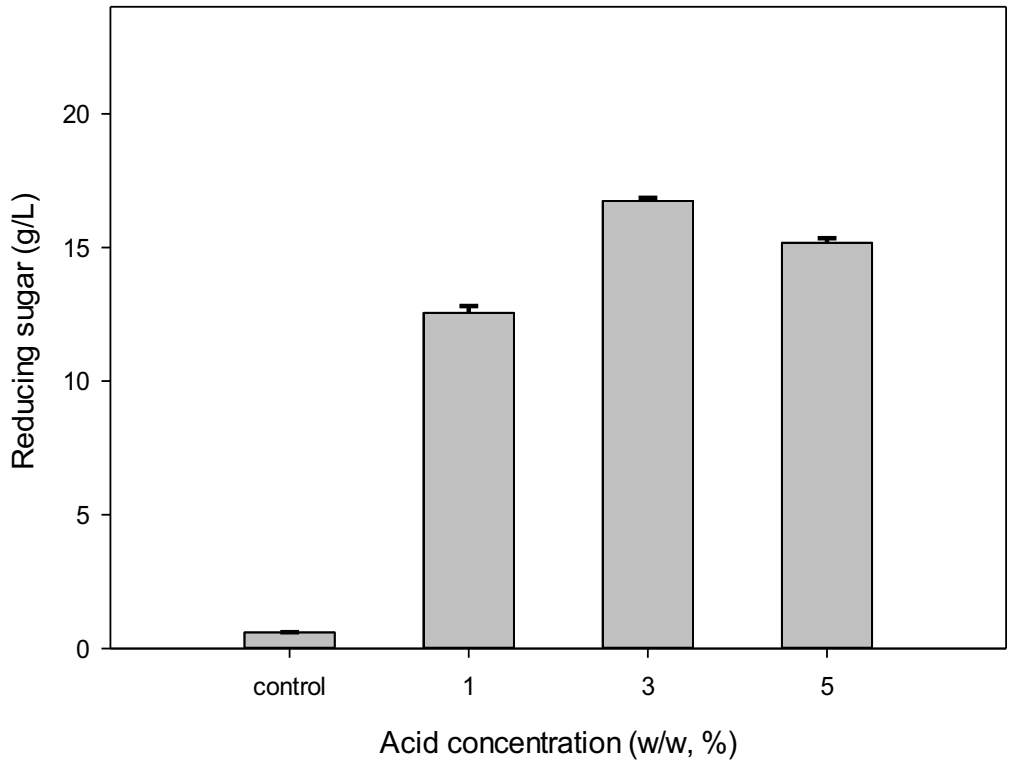


Figure 4.3 Hydrolysis of hazelnut shell at various acid concentrations (130 ^oC, 37.5 min)

Increasing the acid concentration leads to increase in the concentration of hydrogen ions, which increase in turn increase the rate of the hydrolysis reaction and consequently the glycosidic bonds breakage resulting in a high conversion of hemicellulose fraction into fermentable sugars (Kumar et al., 2009; Mosier et al., 2002). Hu et al. (2010) investigated the acid hydrolysis of sugar maple wood extract at atmospheric pressure using dilute sulfuric acid. They showed that increasing the acid concentration resulted in an increase in the concentration of fermentable sugars. Maximum xylose concentration was found as 161.58 g/L with 6.2% H_2SO_4 at 95 ^oC for 50 min (Hu et al., 2010).

Finally, the time effect was examined using the aforementioned ideal temperature and acid concentration levels for periods of 15, 37.5 and 60 min. The results are shown in Fig. 4.4.

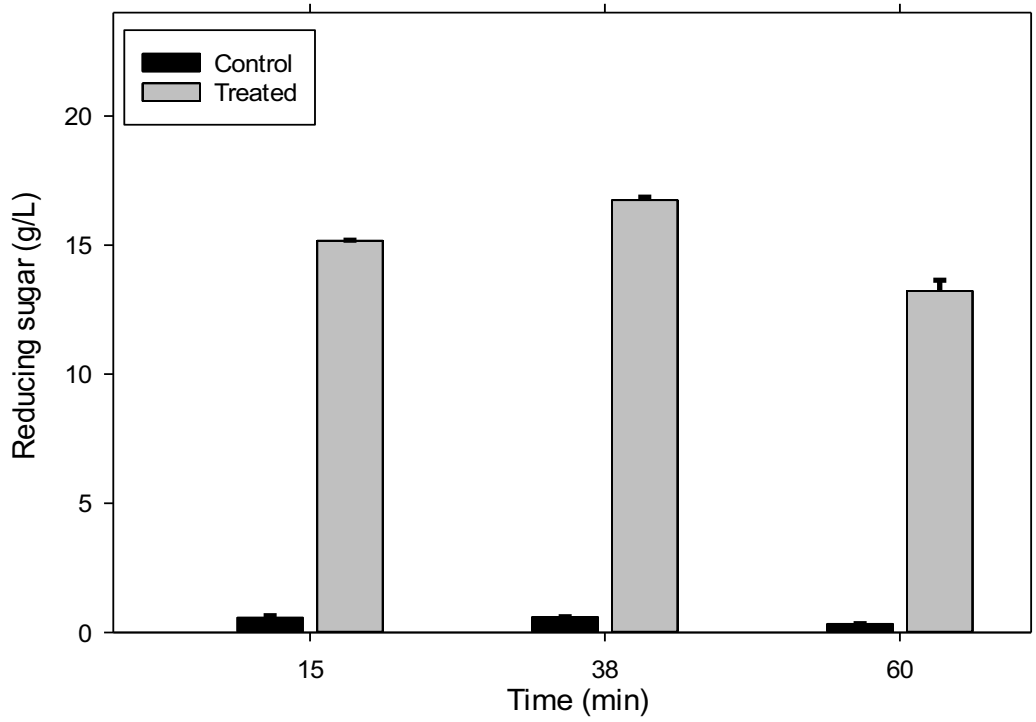


Figure 4.4 Hydrolysis of hazelnut shells at various time (130 ^oC, 3%, w/w, acid)

It was clear that, the time had a limited impact, yielding a decrease in reducing sugar concentration after a 60 min hydrolysis. A period of 37.5 min showed the highest reducing sugar concentration (16.74 g/L). Therefore, it was chosen as the best time among the tested levels for production of high fermentable sugars.

These results indicated that a temperature of 130 ^{o}C, a period of 37.5 min and 3% (w/w) acid concentration revealed the highest reducing sugar concentration (16.74 g/L) which was also equal to 63.2% yield. Arslan and Saraçoglu (2010) studied the hydrolysis of hazelnut shells using 3-5% H_2SO_4 at 100-120 ^{o}C with the 1/7 solid to liquid ratio and reported that the highest reducing sugar concentration was obtained at 120 ^{o}C, 5% H_2SO_4 and 30 min. When the overall reducing sugar yield of this study was compared with the previous studies conducted with hazelnut shell (57.7%) (Arslan & Saraçoglu, 2010) and cassava bagasse (62.4%) (Woiciechowski et al., 2002) it was found that the overall sugar yield of this study was higher (62.8%) (Table 4.3). The difference among results is due to the type of the raw material and difference in hydrolysis conditions.

Table 4.3 Saccharification yields for various agro-residues at different dilute acid pretreatment conditions.

Temperature (^{o}C)	Acid conc. (%)	Time (min)	Raw material	Sacc. Yield	References
140	6	15	Cotton stalk	47.9	(Akpinar et al., 2011)
120	5	30	Hazelnut shell	57.7	(Arslan & Saraçoglu, 2010)
121	1	27	Rice straw	77	(Roberto et al., 2003)

Table 4.3 (continued)

120	1	10	Cassava bagasse	62.4	(Woiciechowski et al., 2002)
130	3.42	31.7	Hazelnut shell	62.8	This study

As a result, acid concentration, time and temperature are important parameters which affect the sugar yield for dilute acid hydrolysis. For comprehensive understanding of interactions between each factor and for complete optimization, further study was carried out to maximize the sugar yields using response surface method.

4.2.1.4 Response Surface Optimization

The experimental levels of temperature (X_1), acid concentration (X_2), and time (X_3) tested for optimization of reducing sugar production are shown in Table 3.3. Overall, 2.84 to 15.24 g/L reducing sugar were obtained when hazelnut shells were treated with dilute acid, depending on pretreatment conditions shown in Table 4.4. The highest reducing sugar of about 15.24 g/L was obtained at 130 ºC, 5% (w/w) acid solution, and 37.5 min of time, whereas the poorest conditions were 110 ºC, 1% (w/w), and 37.5 min, giving about 2.84 g/L of reducing sugars.

Table 4.4 Experimental design for optimization of dilute acid pretreatment using response surface method (RSM)

	Independent Variables			Response (Reducing sugar, g/L)	
Exp. No	**Temperature (^oC)**	**Acid conc. (%)**	**Time (min)**	**Experimental[a]**	**Predicted**
1	120	5	60	10.47±0.12 G	11.18
2	120	5	15	10.99±0.15 FG	11.02
3	130	1	37.5	12.57±0.18 DE	12.69
4	130	5	37.5	15.24±0.06 A	14.65
5	110	1	37.5	2.84±0.15 J	3.43
6	120	1	60	7.23±0.19 H	7.20
7	110	3	15	6.15±0.13 H	6.25
8	120	1	15	4.94±0.11 I	4.24
9	110	5	37.5	12.36±0.17 DE	12.23
10	110	3	60	11.70±0.16 EF	11.13
11	130	3	60	13.75±0.23 BC	13.65
12	130	3	15	14.84±0.32 AB	15.41
13	120	3	37.5	12.89±0.06 CD	13.07
14	120	3	37.5	12.89±0.30 CD	13.07
15	120	3	37.5	13.42±0.25 CD	13.07

[a] Results belong to two replicates

To fit the experimental data to Eqn. (26), regression analysis was performed and the resulting model was evaluated by ANOVA (Table 4.5).

Table 4.5 Significance of term coefficients for BBD using coded values; X_1: Temperature (°C); X_2: Acid concentration (w/w); X_3: Time (min)

Term	Coefficient	Standard Error Coefficient	T Value	P Value
Constant	13.07	0.2320	56.313	0.000
X_1	2.92	0.1421	20.550	0.000
X_2	2.69	0.1421	18.927	0.000
X_3	0.78	0.1421	5.467	0.000
X_1* X_2	-1.71	0.2009	-8.535	0.000
X_1* X_3	-1.66	0.2009	-8.261	0.002
X_2* X_3	-0.70	0.2009	-3.502	0.000
X_1^2	0.44	0.2092	2.109	0.048
X_2^2	-2.76	0.2092	-13.197	0.000
X_3^2	-1.90	0.2092	-9.085	0.000

*Result is significant when $p<0.05$.

According to ANOVA results, the insignificant terms were excluded and the final form of the equation was given as:

$$Y = 13.07 + 2.92X_1 + 2.69X_2 + 0.78X_3 + 0.44X_1^2 - 2.76X_2^2 - 1.90X_3^2 - 1.71X_1X_2 - 1.66X_1X_3 - 0.70X_2X_3 \quad (27)$$

where Y is the predicted reducing sugar concentration, X_1 , X_2 and X_3 are coded values for temperature, concentration and time, respectively. The insignificant lack of fit (P = 0.226 >0.05) and high values of R^2=0.9846 proved that the model fitted well to the experimental data (Table 4.6). All factors showed significant effects (P <0.05) on reducing sugar concentration and the interactions between temperature-temperature, concentration–concentration, time interactions and temperature–concentration showed significant effects (Table 4.5). The complete experimental design, results and predicted values are given in Table 4.4.

Table 4.6 ANOVA results for dilute acid pretreatment using coded values

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F Ratio	P Value
Model					
Linear Effects	3	261.803	87.268	270.14	0.000
Interaction Effects	3	49.537	16.512	51.11	0.000
Quadratic Effects	3	81.897	27.299	84.51	0.000
Residual	19	6.138	0.323		
Lack of Fit	15	5.493	0.366	2.27	**0.226**
Pure Error	4	0.645	0.161		
Total	29	399.457			

R-Sq = 0.9846 % R-Sq(adj) = 0.9765 % R-Sq(pred) = 0.9582 %

Estimation of reducing sugar at varying process variables (temperature, acid concentration, and time) are pictured in response surfaces plots shown in Figures 4.5-7. Figure 4.5 shows the effect of acid concentration and time on reducing sugar at the mid-point temperature of 120 °C. The reducing sugar content of the hydrolyzates were strongly influenced by both variables, as indicated by sharp increasing line and decreasing curvature around higher ends. Increasing the acid concentration during hydrolysis leads to a corresponding increase in the concentration of hydrogen ions, which in turn increases the rate of the hydrolysis reaction. Thus, the glycosidic bonds are broken to result in a high conversion of hemicellulose fraction into fermentable sugars (Mosier et al., 2002). An increase in acid concentration from 1 to 3% (w/w) gave the highest reducing sugar of 12.5 g/L at about 37.5 min, but above 3% (w/w) a decrease was observed. Three percent dilute acid could be considered as a high acid concentration for this pretreatment due to conversion of reducing sugars into 5-HMF or furfural, which caused a decrease in reducing sugar yield.

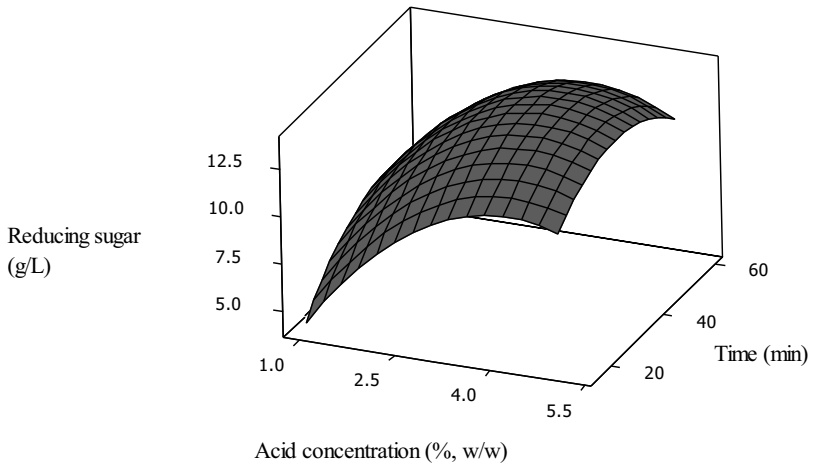


Figure 4.5 Response surface plot for the effects of acid concentration, and time on reducing sugar (temperature is constant at 120 °C)

On the other hand, the effect of temperature and time on reducing sugar is shown in Figure 4.6, where acid concentration was set at 3% (w/w) as the center point. A remarkable increase in reducing sugar was observed as temperature and time increased and this increase was limited beyond 50 min (Figure 4.6). The sugar yield increased with increase of temperature and time, due to hemicellulose removal, which is transformed to hexose and pentose (Idrees et al., 2014). The maximum reducing sugar concentration of 14.84 g/L was obtained at 130 °C and 15 min. This result agrees with Clausen & Gaddy (1993), who also observed that the hydrolysis of cellulose to D-glucose was highly dependent upon temperature (50-100 ˚C).

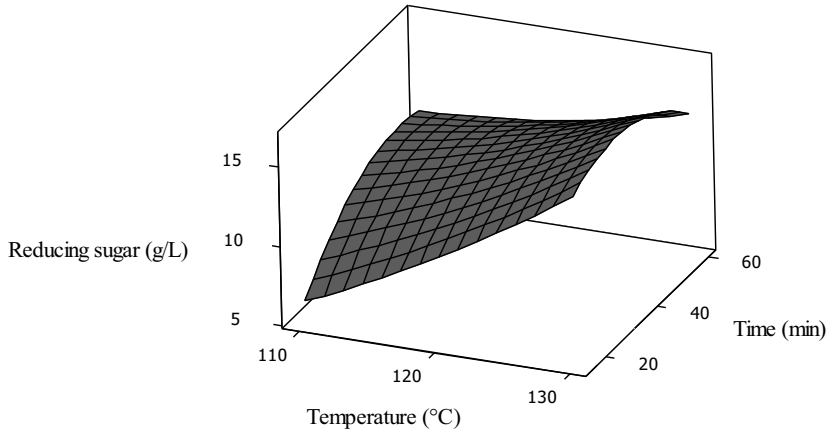


Figure 4.6 Response surface plot for the effects of temperature and time on reducing sugars (acid concentration is constant at 3%, w/w).

A similar positive effect of temperature was observed in Figure 4.7, showing the effect of temperature and acid concentration on reducing sugar, where 3% (w/w) acid concentration was the limit for this increase (Figure 4.7). Under elevated temperatures and acidic medium, monosaccharide degradation occurs, thus the production of reducing sugars is adversely affected.

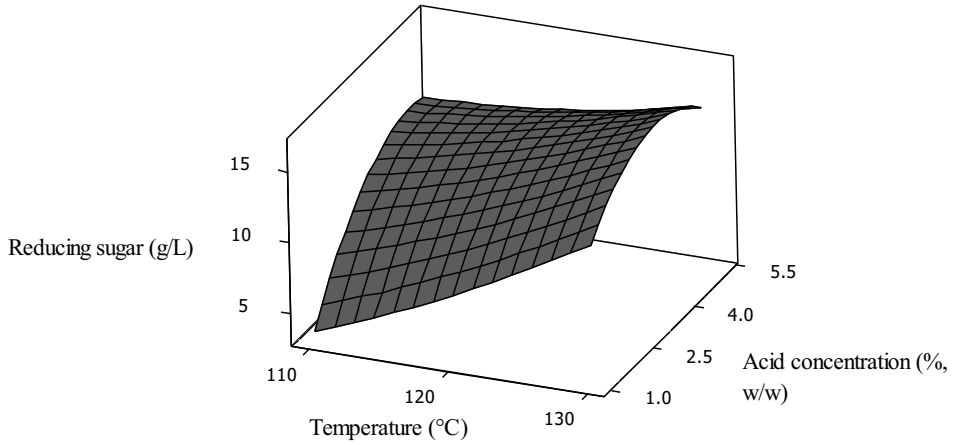


Figure 4.7 Response surface plot for the effects of temperature and acid concentration on reducing sugars (time is constant at 37.5 min).

The interactive effects of significant variables were represented in contour plots as shown in Fig. 4.8. The maximum response is referred to a surface confined in the smallest ellipse in a contour plot. The perfect interaction between the independent variables can be shown when elliptical contours are obtained (Zahangir et al., 2009). An increase in acid concentration from 1% to 3% (w/w) increased the reducing sugar to 12.5 g/L (Fig. 4.8.a). It can be seen that for the 30 min of time, when the temperature was increased from 120 to 130 °C, the reducing sugar concentration increased from >12 g/L to 16 g/L (Fig. 4.8.b).

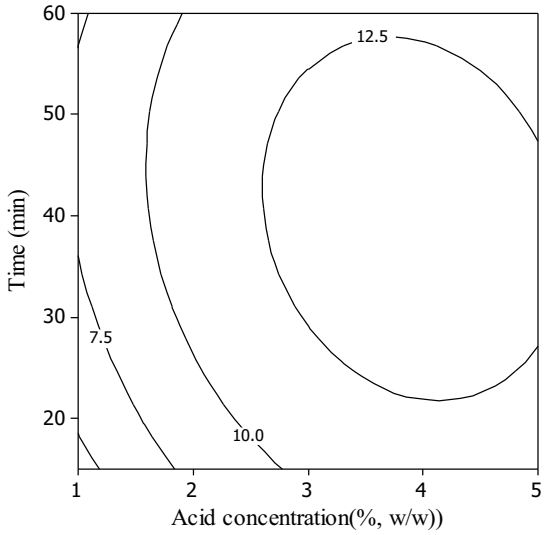


Figure 4.8.a Contour plot showing interactive effect of time and acid concentration (%, w/w) on reducing sugar concentration.

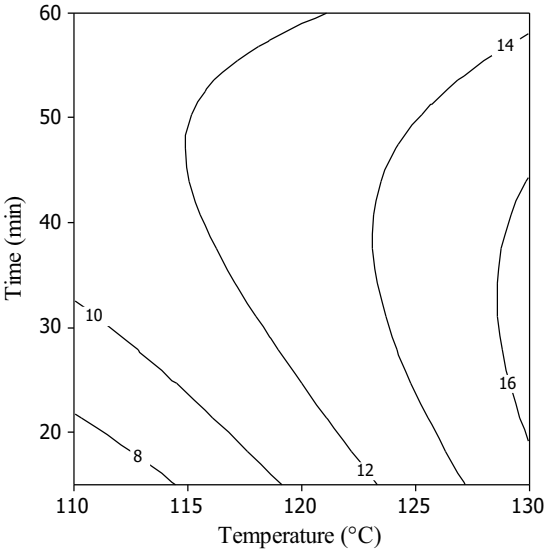


Figure 4.8.b Contour plot showing interactive effect of time and temperature on reducing sugar concentration

Fig. 4.8.c shows that the effect of acid concentration and temperature on reducing sugar concentration. The interactive effect of acid concentration with temperature had positive significant effect. When the acid concentration was increased from 2% (w/w) to 3 % (w/w), the reducing sugar concentration increased from 7.5 g/L to 10 g/L (Fig. 4.8.c).

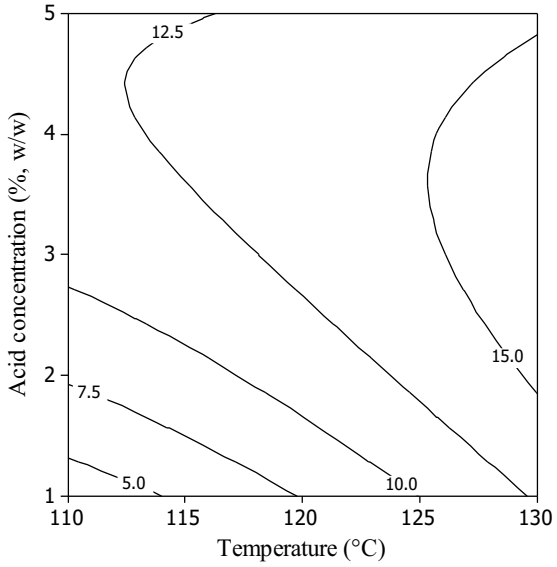


Figure 4.8.c Contour plot showing interactive effect of acid concentration and temperature on reducing sugar concentration

To confirm the validity of RSM model, eleven optimum check points were selected by intensive grid search performed over the entire experimental domain (Table 4.7). The resultant experimental data were compared with that of the predicted values in

Figure 4.9, which gave high correlation with R^2=0.9578 and slope about 1.00. The constructed model was also assessed using error analysis. The RMSE and MAE values were calculated as 0.61 and 0.15, respectively. Low values of RMSE and MAE also indicate that the model was successful in predicting reducing sugar concentration. Furthermore, it was found that error was systematic (RMSEs=0.02<RMSEu=0.61), of which experimental values were slightly higher than the predicted values.

Table 4.7 Verification experiments of dilute acid pretreatments

	Independent Variables			**Response (Reducing sugar,g/L)**	
Exp. No	**Temperature (^{o}C)**	**Acid conc. (%)**	**Time (min)**	**Experimental**	**Predicted**
1	120	5	37.5	13.77	12.49
2	130	3	30	16.74	17.54
3	125	4	35	14.87	14.48
4	130	1	60	10.88	10.04
5	125	1.5	35	12.16	11.22
6	115	2	35	9.36	8.64
7	115	4	35	12.13	12.23
8	110	3	30	9.67	9.29
9	110	1	60	6.50	4.30
10	120	5	30	13.80	12.33
11	120	3	15	9.50	10.13

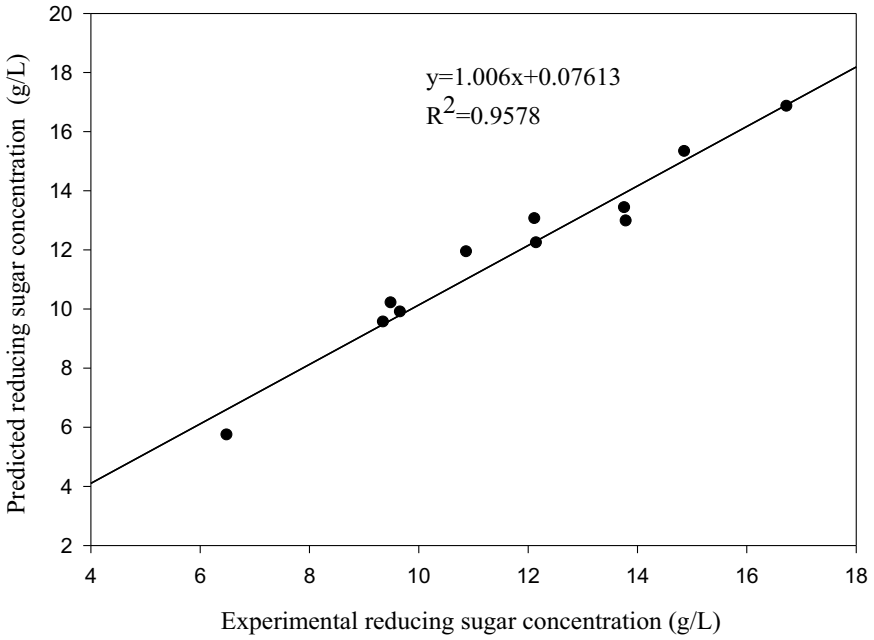


Figure 4.9 Predicted vs. experimental reducing sugar concentration for validation of the response surface model.

Therefore, to determine the optimal pretreatment conditions, the response optimizer tool in MINITAB® 16.0 was used. The optimum conditions for dilute acid pretreatment were found as 130 °C of temperature, 3.42% (w/w) of acid concentration and 31.7 min of time giving 62.8% (w/w) yield (or 16.65 g/L) at minimum cost (-0.25 TL). It was concluded that the total cost of dilute acid pretreatment at optimum conditions is negligible. The profit can be improved furthermore when xylose is also targeted at production in addition to glucose.

4.2.1.5 Artificial Neural Network (ANN) Modeling

4.2.1.5.1 Training Data Set

Various ANN models were tabulated to show the effect of the number of neurons in the hidden layer on the ability of the model to predict the response variables (Table 4.8). The coefficient of determination (R^2), mean squared error (MSE), and the root percent deviation (RPD) statistics from the training data are also shown in Table 4.8.

Generally, as the number of hidden layer neurons in the ANN increases, the R^2 values increase slightly and the MSE values decrease slightly. The best network model was the network with minimal MSE and RPD and maximum R^2 of predicted and experimental data (Table 4.8)

Table 4.8 Artificial neural network (ANN) models and their MSE, R^2 and RPD values from training data

Model name	Statistical criteria	Reducing sugar concentration (g/L)
ANN 3 Neuron	MSE	0.6299
	R^2	0.9796
	RPD (%)	4.7627
ANN 4 Neuron	MSE	0.1389
	R^2	0.9949
	RPD (%)	1.7474
ANN 5 Neuron	MSE	0.0433
	R^2	0.9983
	RPD (%)	0.4647
ANN 6 Neuron	MSE	0.0263
	R^2	0.9991
	RPD (%)	0.5192
ANN 7 Neuron	MSE	0.0265
	R^2	0.9990
	RPD (%)	0.5423
ANN 8 Neuron	MSE	0.0431
	R^2	0.9985
	RPD (%)	1.0657
ANN 10 Neuron	MSE	0.0229
	R^2	0.9989
	RPD (%)	0.2700
ANN 12 Neuron	MSE	0.1125
	R^2	0.9954
	RPD (%)	0.8245

From the training data, the ANN model with 6 hidden layer neurons appears to be the best predictor based on the simplicity of this ANN versus 12 neurons as well as the low MSE and RPD values (Table 4.8). Therefore, a 3-6-1 network that could accurately model the dilute acid pretreatment among the training samples was built for modeling. Figure 4.10 shows an example of the architecture for feed forward backpropagation ANN, which includes an input layer with three inputs, a hidden layer that contains six neurons, and an output layer.

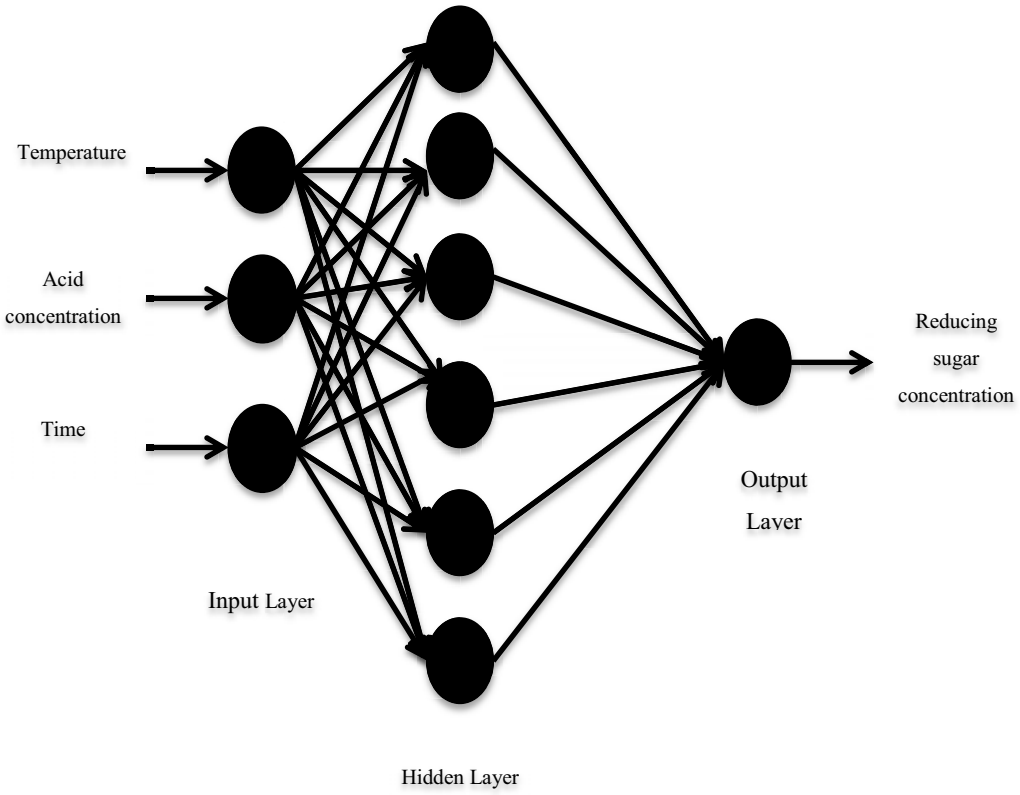


Figure 4.10 Schematic representation of ANN to simulate the dilute acid pretreatment of hazelnut shells.

4.2.1.5.2 Testing of Data Set

After the training stage had been completed, the processed networks were tested and the performances were evaluated by determining mean square errors (MSE) and coefficient of determination of both training and testing processes (Table 4.9). For the best network model, minimum MSE and maximum regression coefficient values using predicted and experimental data were selected as optimal for further applications.

Table 4.9 Performance of Neural Network model

Response	Statistical Parameter	Training	Testing	Validation	Overall
Reducing sugar concentration	R^2	0.9991	0.9939	0.9939	0.9974
	MSE	0.0263	0.2151	0.1798	0.0033

4.2.1.5.3 Comparison of RSM and ANN

After the model was built, a comparative study was performed between RSM and ANN models for predicting reducing sugar concentration. After training and testing, Table 4.10 shows the predicted values by RSM and ANN models. The ANN model generated a better prediction than the RSM model in terms of relative error. Thus, the accuracy of ANN model was higher. The experimental error produced by RSM exceeded that of ANN with 1% acid concentration at 110 ºC for 37.5 min (27.51% error).

Table 4.10 BBD matrix of three factors and experimental data, RSM and ANN for determined values of maximum reducing sugar concentration

Factors			Reducing sugar concentration (g/L)				
				RSM		ANN	
Temp. (°C)	Acid conc. (%)	Time (min)	Experimental Value	Model value	Relative Error (%)	Model value	Relative Error (%)
120	5	60	10.59	11.18	5.57	10.65	0.55
120	5	60	10.35	11.18	8.02	10.41	0.57
120	5	15	11.14	11.02	1.08	11.20	0.53
120	5	15	10.85	11.02	1.57	10.91	0.54
130	1	37.5	12.39	12.69	2.42	12.45	0.47
130	1	37.5	12.74	12.69	0.39	12.80	0.46
130	5	37.5	15.18	14.65	4.25	15.36	0.38
130	5	37.5	15.30	14.65	3.49	15.24	0.38
110	1	37.5	2.99	3.43	14.72	3.05	1.93
110	1	37.5	2.69	3.43	27.51	2.75	2.15
120	1	60	7.03	7.20	2.42	7.09	0.83
120	1	60	7.42	7.20	2.96	7.48	0.79
110	3	15	6.02	6.25	3.82	6.08	0.48
110	3	15	6.28	6.25	0.48	6.34	0.42
120	1	15	5.04	4.24	15.87	5.09	0.77
120	1	15	4.83	4.24	12.22	4.89	0.84
110	5	37.5	12.19	12.23	0.33	12.25	0.27
110	5	37.5	12.53	12.23	2.39	12.59	0.29
110	3	60	11.54	11.13	3.55	11.60	0.23
110	3	60	11.85	11.13	6.08	11.91	0.25
130	3	60	13.97	13.65	2.29	14.03	0.36
130	3	60	13.52	13.65	0.96	13.58	0.34
130	3	15	15.16	15.41	6.13	15.22	0.39
130	3	15	14.52	15.41	1.65	14.58	0.41
120	3	37.5	12.95	13.07	0.93	13.01	0.31
120	3	37.5	12.83	13.07	1.87	12.89	0.31
120	3	37.5	13.19	13.07	0.91	13.25	0.33
120	3	37.5	12.59	13.07	3.81	12.65	0.29
120	3	37.5	13.17	13.07	0.76	13.23	0.32
120	3	37.5	13.67	13.07	4.39	13.73	0.35

Though both models performed well and offered stable response in prediction; the ANN model was better in predict on than the RSM model. Table 4.10 shows lower RPD values or relative errors obtained by neural network compared to that of RSM model. Thus the accuracy of ANN model was higher and better fitted the data than the RSM method.

Figure 4.11 shows the plot of predicted reducing sugar concentration by ANN and RSM against the experimental values. The results showed that ANN predictions gave higher correlation with r=1.0 and slope about 1.00 than those of RSM (r=0.98). ANN is a superior and more accurate modeling technique compared to RSM, as it represents the non-linearity in a much better way (Bas and Boyacı, 2007, Bas et al., 2007 and Mingzhi et al., 2009).

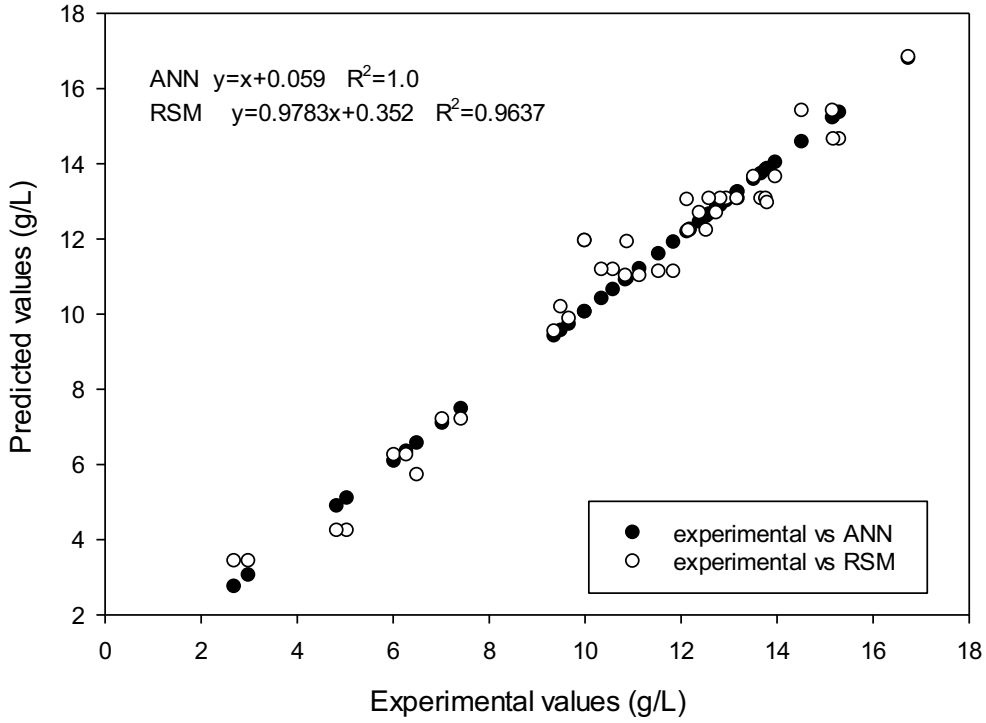


Figure 4.11 Combined training, validation and testing predicted versus actual experimental values for an ANN with 6 neurons in the hidden layer and RSM

4.2.1.6 Combined Dilute Acid Pretreatment and Enzymatic Hydrolysis

The dilute acid pretreatment method was followed by enzymatic hydrolysis. To determine the necessary process time for enzymatic hydrolysis, aliquot samples were taken hourly and analyzed for reducing sugar concentration. Glucose production was monitored until the glucose concentration reached a constant value. The concentration of glucose increased gradually with time and reached a constant value within 20 h for all solid-liquid ratios (Figure 4.12). Thus, the enzymatic hydrolysis was carried out for 20 h in all subsequent experiments. Moshe (1967) found the process time as 8-10 h for hydrolysis of cellulose.

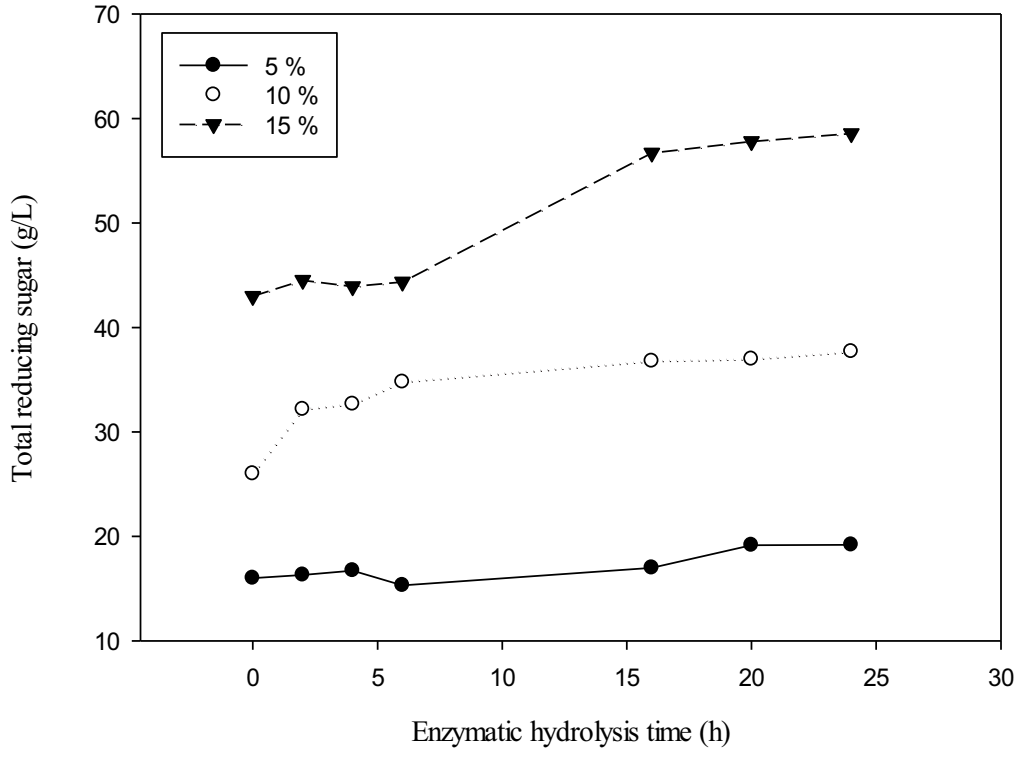


Figure 4.12 Effect of solid/liquid ratio on the enzymatic hydrolysis time of hazelnut shells.

However in another study by Aiduan et al. (2007) the optimum hydrolysis time was stated as 3h for bioconversion of municipal solid waste to glucose. The difference among results can be due to the type of the raw material and difference in enzymatic hydrolysis conditions.

However, total reducing sugar concentration was much higher at solid/liquid ratio of 15% than that of 5% (Figure 4.12). The total reducing sugar yield was obtained as 72.4 and 71.5 g/g at the solid-liquid ratio of 5 and 15%, respectively. Reducing sugar yield from the enzymatic hydrolysis of cellulose was investigated as a function of cellulose enzyme loading (66.6-200 U/g dry substrate) at solid/liquid ratios of 1/20, 1/10 and 1/7 (w/v)) for 20 h using the dilute sulfuric acid hydrolysate (3.42 % (w/w) $H_2 SO_4$, 31.7 min, 130 ^oC). Reducing sugar yield of pretreated samples after 20 h of enzymatic hydrolysis is shown in Figure 4.13. Sugar yield after 20 h ranged from 63.2 g/g at the low solid-liquid ratio (1/20) and low enzyme loading (66.6 U/g dry substrate) to 72.4 g/g at the high solid-liquid ratio (1/7) and high enzyme loading (200 U/g dry substrate). The highest total reducing sugar yield was obtained as 76 g/g at 1/10 solid-liquid ratio and 100U/g dry substrate. However, these solid-liquid ratio may still be too high to make the process economic. Moreover, these values were not found statistically different from each other ($p>0.05$). The best results of reducing sugar yield was observed at the solid loading of 1/20 (w/v) as shown in Figure 4.13.

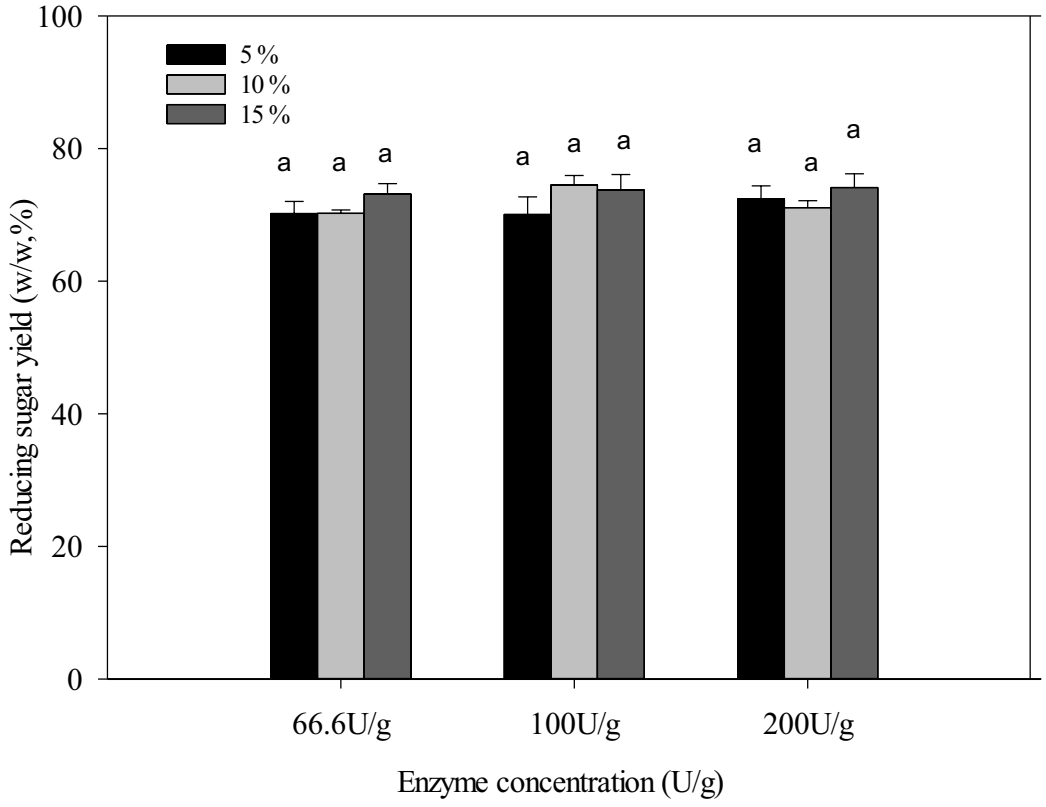


Figure 4.13 Enzymatic hydrolysis at various solid concentration (5, 10 and 15%) of hazelnut shell and enzyme loadings (50 ºC, pH 5.0, 20 h at 130 rpm)

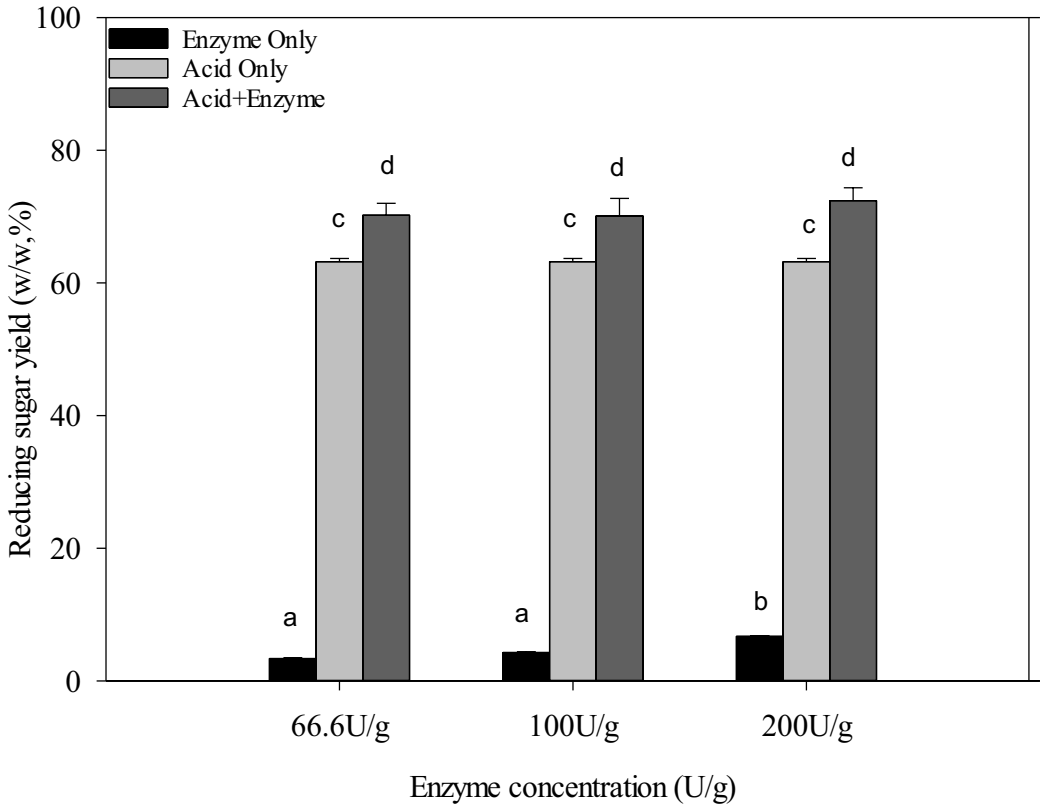


Figure 4.14 Acid hydrolysis and combination of acid and enzymatic hydrolysis at 5% solid concentration and various enzyme loadings (50 ºC, pH 5.0, 20 h at 130 rpm)

Figure 4.14 indicates that combined acid and enzyme treatment gave significantly higher reducing sugar yield than only acid pretreatment or enzymatic hydrolysis for all enzyme loads (P<0.05). The yield values also increased as enzyme loading increased. The highest reducing sugar concentration and yield were obtained as 19.2 g/L and 72.4 g/g, respectively with 200 U/g of enzyme load.

The analysis of results also revealed that pretreatment alone resulted in 62.8% reducing sugar yield, but this value increased to 72.4% when combined with enzymatic hydrolysis. Palmarola-Adrados et al. (2005) pretreated starch free wheat bran with dilute acid and subsequently hydrolyzed using a mixture of Celluclast and Ultraflo (1:1) (2g enzyme mixture/100 g slurry) and the overall sugar yield of this combined hydrolysis method reached 80%. Aswathy et al. (2010) reported the highest value of 639.42 mg/g reducing sugar attained with an enzyme loading of 12 FPUs and 2400 U of cellulose and β-glucosidase, respectively. An efficiency of 57% was obtained by dilute acid pretreatment of water hyacinth biomass and subsequently enhanced from 57% to 71% with combined acid and enzymatic hydrolysis in the study of Aswathy et al. (2010). The differences in yield values compared to results of our study were clearly due to raw material characteristics, enzyme type used and processing conditions. As a result, the saccharification yield of our study (72.4%) seems to be comparable, as hazelnut shell is a high lignin containing raw material.

4.2.1.7 Effect of Dilute Acid Pretreatment on Amount of Inhibitory Compounds

Furfural, HMF and acetic acid are known as fermentation inhibitors, which are formed as a result of hemicellulose, and cellulose degradation during pretreatment of biomass (Karunanithy & Muthukumarappan, 2011). Figures H.1 and H.2 represent the chromatograms of the HMF, furfural and acetic acid standard and the pretreated samples with dilute acid after pretreatment as an example. At optimal temperature, time and acid concentration of 130 ^{o}C, 31.7 min, and 3.42 (w/w) % respectively, the acetic acid level was measured as 2.59 ±0.09 g/L, HMF level was measured as 0.0145±0.0007 g/L, phenolic compounds were measured as 0.15±0.00 g/L and no furfural were detected in the hydrolyzate (Table 4.11).

Mouta et al. (2011) studied the hydrolysis of sugarcane leaves straw using 2.9% H_2SO_4 (w/v) at 130 ^oC for 30 min with the 1/4 solid to liquid ratio. They reported that acetic acid, furfural and HMF were measured as 3.19, 0.56 and 0.15, respectively. When the amount of inhibitory compounds of this study was compared with the previous studies conducted with rice straw (Baek & Kwon, 2007) and sugarcane leaves straw (Mouta et al., 2011) it was found that the amount of furfural and HMF of this study was lower (Table 4.11). The difference among results is due to the type of the raw material and difference in hydrolysis conditions.

Table 4.11 Dilute acid pretreatment of various agro-residues for the production of hemicellulosic-derived products.

Agro-residue	Conditions for acid hydrolysis	Inhibitors (g/L)	References
Rice straw	1.5% H_2SO_4, 130 ^oC, 30 min, S:L=1:10	Acetate, 1.43, HMF, 0.15; Furfural, 0.25	Baek and Kwon (2007)
Sorghum straw	2% H_2SO_4, 122 ^oC, 71 min	Furfural, 0.2; acetic acid, 0.00	Sepülveda-Huerta et al. (2006)
Corn stover	2.13 % H_2SO_4, 121 ^oC, 180 min, S:L=1:10	Acetic acid, 1.48; Furans, 0.56; Phenolics, 0.08	Cao et al. (2009)
Sugarcane leaves straw	2.9%H_2SO_4 (w/v), 130 ^oC, 30 min, S:L=1:4	Acetic acid, 3.19; furfural, 0.56; HMF, 0.15.	Mouta et al. (2011)
Hazelnut shell	3.42%H_2SO_4 (w/w), 130 ^oC, 31.7 min, S:L=1:5	Acetic acid, 2.59; furfural, nd; HMF, 0.0145; phenolics, 0.15.	This study

nd=not detected

Temperature and acid concentration are also responsible for degradation of pentose and hexose sugars to furfural and hydroxymethyl furfural compounds, which strongly affect the microbial metabolism at >0.25 g/L (Klinke et al., 2004). Thus, to minimize those inhibitory compounds it is advised to run the hydrolysis process at less severe conditions. The toxic compounds present in the hazelnut shell hydrolysate can be efficiently removed by a simple detoxification strategy based on pH alteration. This indicated that the hydrolysate would impose no risk to fermentation microorganisms.

4.2.2 Effect of Alkaline Pretreatment

The reducing sugar yields obtained under different alkaline conditions are compared in Table 4.12 and Table 4.13 which illustrate the alkaline pretreatment conditions and corresponding reducing sugar yields (%, w/w) for Method 1 and Method 2, respectively. The highest yield of reducing sugar (2.9%) was observed when the hazelnut shells were subjected to 3% (w/v) NaOH at 60 ^{o}C for 24 h (Table 4.12). The reducing sugar yield was calculated by dividing the concentration of the reducing sugar obtained with reducing sugar in hazelnut shells multiplied by 100.

In Method 1, the reducing sugar yield increased as NaOH concentration was increased from 1% to 3% (w/v) but decreased thereafter at 7% (w/v) (Table 4.12). Increase in NaOH concentration with decreasing reducing sugar yield was observed due to high solid loss (Wang et al., 2008). The results also indicated a positive effect of temperature on reducing sugar yields at 5% (w/v) NaOH. The highest reducing sugar yield of 2.9% (w/w) was obtained at 3% (w/v) NaOH, 60 ^{o}C, and 24 h for Method 1. However, the highest total reducing sugar yield of 61.2% (w/w) was obtained at 15% SLR treated with 3% (w/v) NaOH for 60 min by Method 2. Lower temperatures for NaOH pretreatment was not favorable to enhance sugar yield because the crosslinkages between lignin and carbohydrates were not

disrupted sufficiently to reach a high sugar production (Wang, 2009). Reducing sugar yield with NaOH pretreatment at high temperature (121 ^{o}C) was much higher than at lower temperatures (<121 ^{o}C). Based on reducing sugar yield, Method 2 was used for comprehensive understanding of interactions between each factor. For completion of optimization, further study was carried out to maximize the sugar yields.

Table 4.13 illustrates the reducing sugar concentration in the pretreated hazelnut shell solids at various alkaline conditions. Total reducing sugar in untreated hazelnut shells was 290.9±6.9 mg/g dry pretreated material (Table 4.13). Sugars in the recovered solids varied depending on the pretreatment combination and impact of NaOH concentration (X_1), SLR (X_2) and treatment time (X_3) on reducing sugar concentration was statistically evaluated. Sodium hydroxide concentration, SLR and treatment time showed significant effects on total reducing sugars recovered in pretreated solids. While interactions between NaOH concentration x SLR and SLR x time had no significant effects ($P>0.05$), interactions between SLR x time showed significant effects ($P<0.05$).

The highest total reducing sugar recovered after pretreatment (estimated equivalent from 320.6 mg/g dry biomass) was obtained at 15% SLR treated with 3% (w/v) NaOH for 60 min (Table 4.13). This value was significantly higher than that reported by Arslan and Saraçoglu (2010), who pretreated hazelnut shell using 1-3% NaOH at room temperature with a solid to liquid ratio of 1:5 and reported reducing sugar concentration between 130-140 mg/g sugar (estimated equivalent from 26.40-28.86 g/L).

Table 4.12 Total reducing sugar of hazelnut shell pretreated with different NaOH concentration at different temperatures for 24 h

Treatments*	Total reducing sugar (mg/g dry raw material)	Reducing sugar yield (%,w/w)
Alkaline pretreatment at 30 °C		
Untreated	3.34±0.09	0.6
1% NaOH	7.93±0.60	1.5
3% NaOH	8.88±1.14	1.7
5% NaOH	6.73±0.38	1.3
7% NaOH	6.56±0.29	1.2
Alkaline pretreatment at 45 °C		
Untreated	5.00±0.94	1.0
1% NaOH	10.65±0.17	2.0
3% NaOH	8.19±0.17	1.5
5% NaOH	9.19±0.76	1.7
7% NaOH	7.72±0.18	1.5
Alkaline pretreatment at 60 °C		
Untreated	7.59±0.60	1.5
1% NaOH	7.39±0.11	1.7
3% NaOH	15.03±0.68	2.9
5% NaOH	13.33±0.81	2.5
7% NaOH	12.30±0.30	2.3

* Results belong to two replicates and solid/liquid ratio=1:20 (5%).

Table 4.13 Total reducing sugar of hazelnut shell pretreated with different NaOH concentration, time and solid-liquid ratio at 121 ºC, 15 psi (autoclave).

Treatments	Total reducing sugar (mg/g pretreated biomass)	Reducing sugar yield (%,w/w)
Untreated	290.9±6.9	33.6±0.6
3% NaOH		
5% SLR 60 min	347.1±4.3	53.0±1.2
10% SLR 30 min	352.3±22.4	50.3±1.4
10% SLR 90 min	308.1±21.3	51.4±1.2
15% SLR 60 min	363.5±3.7	61.2±1.3
5% NaOH		
5% SLR 30 min	321.0±18.4	39.1±2.2
5% SLR 90 min	337.0±4.1	48.4±0.9
10% SLR 60 min	397.5±22.1	58.1±2.5
15% SLR 30 min	342.2±14.4	49.5±1.6
15% SLR 90 min	303.0±30.7	46.1±3.4
7% NaOH		
5% SLR 60 min	353.8±20.9	47.7±1.3
10% SLR 30 min	309.9±21.1	42.1±2.7
10% SLR 90 min	301.3±17.5	45.2±1.3
15% SLR 60 min	374.4±24.3	55.8±0.1

* Results belong to triplicate.

4.2.2.1 Alkaline Pretreatment of Hazelnut Shells and Response Surface Modeling for Optimization

Optimization of alkaline pretreatment conditions focused on three aspects: solid recovery, lignin removal, and total reducing sugar production after enzymatic hydrolysis.

4.2.2.1.1 Effect of Alkali Pretreatment Conditions on Solid Recovery

Solid recovery is an important parameter for evaluating pretreatment performance as it determines the total amount of biomass that can be eventually converted to sugars via enzymatic hydrolysis (Zhang, 2012).

RSM is a frequently used technique for modeling and determining optimal process conditions. Response surface analysis of experimental results for solid recovery in hazelnut shells, treated with NaOH at various concentrations, SLR and treatment time per BBD (Table 4.14), were further elucidated to identify the best treatment conditions in the ranges tested. Overall, 63.9 to 85.2% solids were recovered when hazelnut shells were treated with NaOH, depending on pretreatment intensity (Table 4.14).

Table 4.14 BBD matrix of conditions for alkali pretreatment of hazelnut shells and corresponding solid recovery (%) and lignin reduction (%)

Run No.	NaOH conc. (%) (X_1)	SLR (%) (X_2)	Time (min) (X_3)	Solid recovery[a] (%) (Y_1)	Lignin reduction[a] (%) (Y_2)
1	5	10	60	73.7±3.5	19.3±0.3
2	5	10	60	72.2±3.2	19.7±1.4
3	5	10	60	75.4±1.1	19.0±1.0
4	7	15	60	75.9±2.2	16.7±0.9
5	7	5	60	71.8±4.8	13.7±1.8
6	3	15	60	84.4±6.4	14.2±1.4
7	3	5	60	80.1±2.6	13.9±1.3
8	3	10	90	85.2±1.7	15.5±1.3
9	7	10	90	81.8±1.6	14.8±0.5
10	5	15	90	79.7±1.5	10.2±0.9
11	5	5	90	75.9±1.0	9.6±0.7
12	3	10	30	75.0±4.9	6.9±0.3
13	7	10	30	71.3±0.5	7.6±0.7
14	5	5	30	63.9±0.1	7.3±0.3
15	5	15	30	75.9±4.0	8.3±0.1

[a] Results belong to triplicate

Table 4.15 shows the effect of process variables and interactions on solid recovery. NaOH concentration, SLR and treatment time showed significant effects on solid recovery which was also significantly affected ($P < 0.05$) by interaction between SLR and treatment time.

Table 4.15 ANOVA results and estimated regression coefficients for the coded solid recovery model

Term	Coefficient	P
Regression		0.000
Linear		0.000
Square		0.000
Interaction		0.009
Lack-of-fit		0.518
Constant	74.31	0.000*
NaOH conc. (X_1)	-2.995	0.000*
SLR (X_2)	3.109	0.000*
Time (X_3)	4.635	0.000*
X_1*X_1	4.241	0.000*
X_2*X_2	0.818	0.281
X_3*X_3	-1.304	0.092
X_1*X_2	-1.373	0.085
X_1*X_3	-0.949	0.195
X_2*X_3	-2.036	0.005*

*result is significant when $P<0.05$

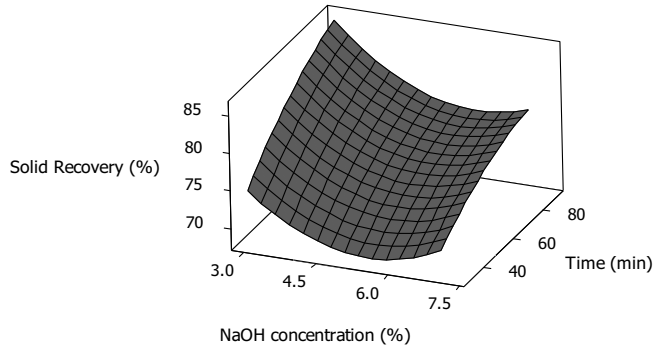
Another run with excluded insignificant terms according to Table I.1 expressed by Eqn 28;

$$Y_1 = 74.01 - 3,08X_1 + 3.12X_2 + 4.56X_3 + 4.14X_1^2 - 2.04X_2X_3 \quad (28)$$

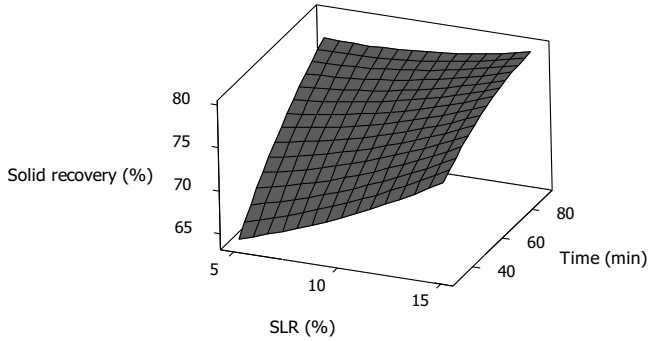
where Y_1 is predicted solid recovery, X_1 is NaOH concentration (%), X_2 is solid-liquid ratio (%), and X_3 is time (min). Equation 28 was found fairly adequate to represent the data with R^2 of 0.85. The insignificant lack of fit for solid recovery was ($P = 0.45 > 0.05$) also proved that the model fit the experimental data well.

Figure 4.15 shows the response-surface plots for the effects of various NaOH pretreatment conditions on solid recovery. It was observed that solid recovery decreased significantly ($P<0.05$) with increase in NaOH concentration, but increased with increase in time (Fig. 4.15.a). Ester bonds between lignin and carbohydrates in the biomass were disrupted during NaOH pretreatment. During this process, lignin is solubilized and high solid losses occur (Keshwani, 2009). Solid loss decreased with increase in SLR and treatment time (Fig. 4.15.b) but there was more loss of solids when NaOH concentration was higher even in the presence of higher solids (Fig. 4.15.c). Xu et al. (2010b) showed the same pattern of solid loss which is decreased with high temperature and NaOH concentration during NaOH pretreatment. Xu et al. (2010b) reported that 19.5-53.9% of biomass was lost during NaOH pretreatment. According to Mcintosh and Vancov (2011), wheat straw was lost at a range of 25% (1% NaoH, 60 min, 60 ^{o}C) to 57% (2% NaOH, 121 ^{o}C).

a



b



c

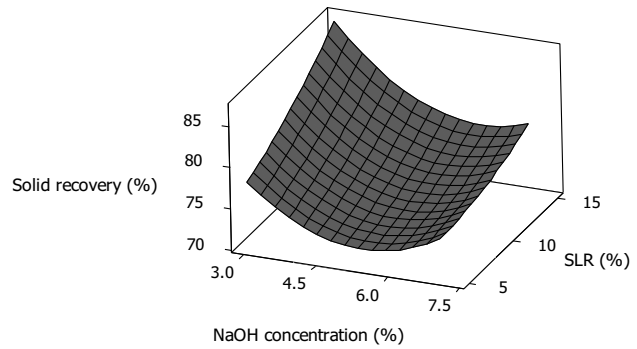


Figure 4.15 Response surface plots for the effects of alkaline pretreatment conditions on solid recovery

The correlation between the experimental and predicted values of solid recovery showed that predicted values were within the model's designed ranges (R^2=0.94) (Fig.4.16).

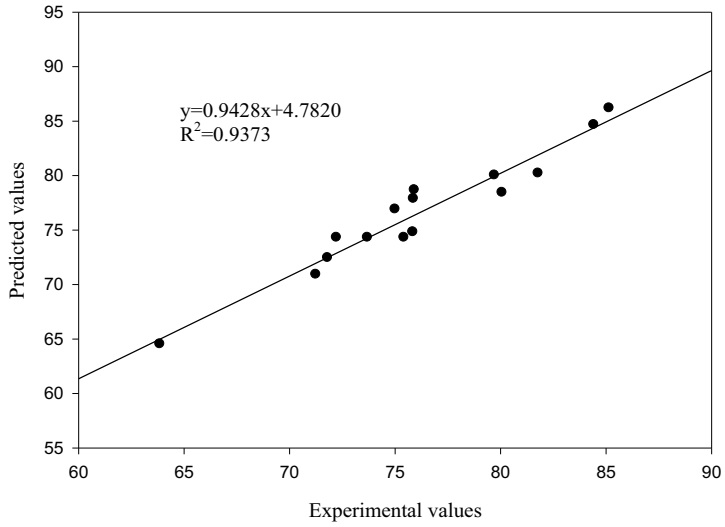


Figure 4.16 Experimental versus RSM predicted values for solid recovery (%)

The conditions for obtaining maximum solid recovery of 89.1%, as predicted by the model, were found as 3% NaOH concentration, 15% SLR for a 90 min treatment time. These conditions were experimentally tested in triplicate to validate the model's predictive ability and a solid recovery of 88.4±0.5%, which indicated no significant difference between the experimental and model predicted values (P>0.05).

4.2.2.1.2 Effect of Alkali Pretreatment Conditions on Delignification

The major effect of alkaline pretreatment on lignocellulosic materials is to reduce the lignin content of the biomass (Chang and Holtzapple, 2000). Lignin analysis of the solids recovered after pretreatment indicated that 6.9 to 19.7% of the initial lignin in the untreated hazelnut shell was removed after NaOH treatment (Table 4.14).

The highest lignin reduction (19.7%) was obtained upon treatment of hazelnut shells at 10% SLR with 5% NaOH for 60 min at 121 °C. This condition also corresponded to low biomass recovery. Second-order polynomial equations (quadratic model) were established to identify the relationship between lignin reduction and the three pretreatment variables: NaOH concentration (X_1), SLR (X_2) and time (X_3). The ANOVA results and the estimated regression coefficients for the coded lignin reduction model are shown in Table 4.16. Among the variables studied, only time showed significant effect (P <0.05) on lignin reduction (Table 4.16). The interaction between the variables did not have a significant effect (P>0.05) on lignin reduction.

Table 4.16 ANOVA results* and estimated regression coefficients for the coded lignin reduction model

Term	Coefficient	P
Regression		0.000
Linear		0.000
Square		0.000
Interaction		0.837
Lack-of-fit		0.069
Constant	19.36	0.000
NaOH conc. (X_1)	-0.024	0.941
SLR (X_2)	0.181	0.558
Time (X_3)	2.502	0.000*
X_1^2	-2.047	0.000*
X_2^2	-3.315	0.000*
X_3^2	-6.850	0.000*
X_1*X_2	0.214	0.629
X_1*X_3	0.355	0.451
X_2*X_3	-0.066	0.876

*result is significant when $P<0.05$

Another run with excluded insignificant terms according to Table I.2 and the equation (Eqn 29) was reduced to:

$$Y_2 = 19.3611+2.5019X_3-2.0466X_1^2 -3.3152X_2^2 -6.8501X_3^2 \quad (29)$$

where Y_2 is predicted lignin reduction, X_1 is NaOH concentration, X_2 is SLR and

X_3 is time. Equation 28 was found fairly adequate to represent the data with R^2 of 0.94. The insignificant lack of fit for lignin reduction was (P = 0.086 >0.05), which also proved that the model fit the experimental data well.

The surface plots showing the effect of the pretreatment conditions on lignin reduction are presented in Fig. 4.17. Lignin reduction increased with increase in NaOH concentration from 3% to 5% (w/v) and time from 30 min to 60 min (Fig. 4.17.a) but decreased when treatment time or NaOH concentration was increased further.

a

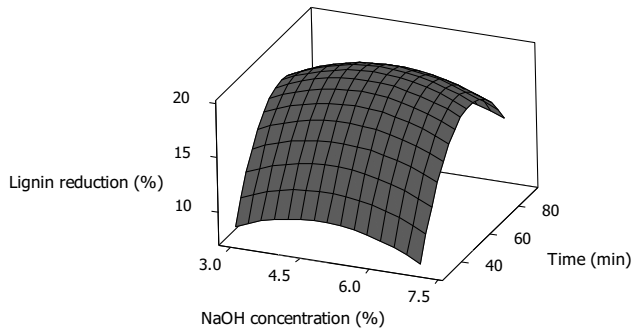
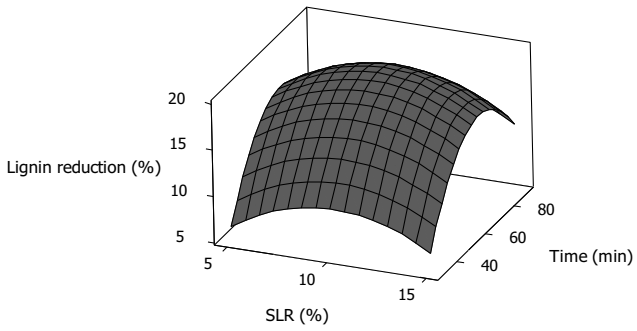


Figure 4.17 Response surface plots for the effects of alkaline pretreatment conditions on lignin reduction

b



c

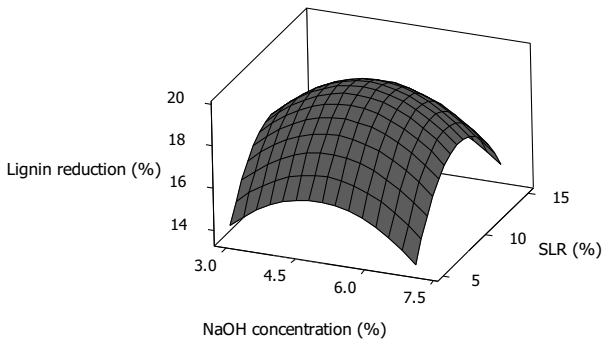


Figure 4.17 (Continued)

Kaur et al. (2012) reported that increased alkali concentration and pretreatment time led to decreased lignin content and increased relative cellulose content in cotton stalk solid residue. However, the increased alkali concentration and time negatively affected the biomass available for hydrolysis due to a significant reduction in available biomass caused by higher solubilization (Kaur et al., 2012). The effect of pretreatment time on lignin removal was reduced when a higher solid-liquid ratio was used (Fig.4.17.b). Fig. 4.17.c showed that lignin reduction increased with alkali concentration and SLR up to 5% (w/v) and 10%, respectively. Then, lignin reduction decreased with high alkali concentration and SLR. Correlation between the experimental and predicted values of lignin reduction showed that predicted values were within the model's designed ranges (R^2=0.94) (Fig.4.18).

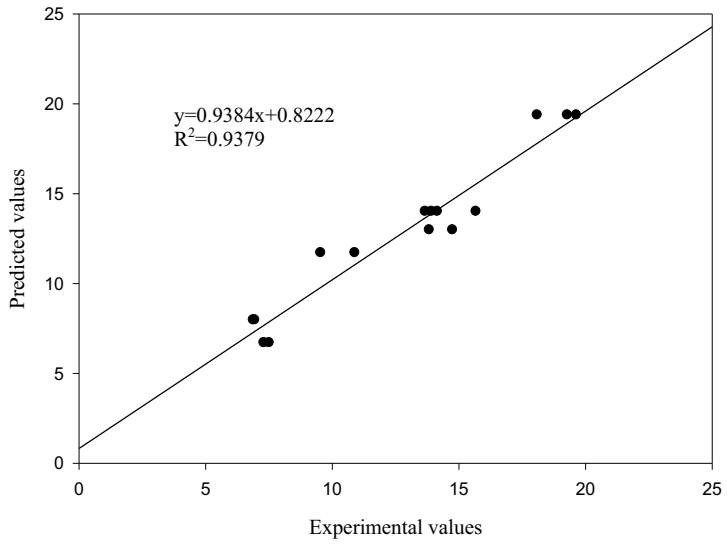


Figure 4.18 Experimental versus RSM predicted values for lignin reduction (%)

The model predicted conditions for optimum lignin reduction of 19.6% were found to be 5% of NaOH concentration and 10% SLR with 65 min pretreatment time. When these conditions were verified, lignin reduction was 18.1±0.03%, which was also close to the corresponding predicted value of 19.6%. These results indicate that the RSM models could be used for determining the conditions best suited for reducing lignin while recovering an optimal level of solids when hazelnut shells are pretreated with NaOH, at least within the ranges of this study.

4.2.2.1.3 Effect of Alkali Pretreatment Factors on Reducing Sugar Yield Combined with Enzymatic Hydrolysis

Enzymatic hydrolysis of the pretreated hazelnut shell was performed to generate fermentable sugars (Table 4.17). Total reducing sugars in the hydrolyzate were measured and used to evaluate overall pretreatment effectiveness. Hydrolysis of

untreated biomass resulted in 120±10 mg reducing sugar/g dry pretreated biomass and the total reducing sugar yield from various pretreated samples ranged at 172.2-318.3 mg/g dry pretreated biomass.

The highest sugar yield (318.6 mg/g dry pretreated biomass) was obtained from biomass at 5% SLR treated with 3% (w/v) NaOH for 60 min and the corresponding enzymatic conversion efficiency was 91.7%. However, enzymatic conversion was found as 97.2% at 10% SLR treated with 3% (w/v) NaOH for 90 min. No significant differences in enzymatic conversion were observed using either Run 7 an 8 (P>0.05) (Table 4.17). Considering economical concerns, the highest sugar yield (318.6 mg/g dry pretreated biomass) with at 5% SLR treated with 3% (w/v) NaOH for 60 min was selected. When alkali is used at high concentration, degradation and alkaline hydrolysis of the polysaccharides takes place which ultimately leads to the destruction of hemicellulose thus releasing sugars (Fengel and Wegener, 1984). However, this yield was not significantly different (P>0.05) from Run 12, which resulted in 316.3 mg/g reducing sugars with shorter treatment time and higher SLR. It was noted that although overall enzymatic conversion efficiency increased slightly with treatment time and SLR, it decreased with increasing NaOH concentration (Table 4.17, Run 4 and 6). No significant (P>0.05) changes in enzymatic efficiency were observed between 5% and 15% SLR or 30 and 90 min. Pretreatments with 3% (w/v) NaOH concentration led to significantly (P<0.05) higher carbohydrates conversion efficiency than those with 5% and 7% (w/v) NaOH concentration.

The sodium hydroxide concentration, SLR and treatment time showed significant effects (P<0.05), on reducing sugars in the hydrolysate. Interactions between NaOH concentration- SLR and SLR-time also had significant effects (P<0.05).

Table 4.17 Comparison of reducing sugars recovered in pretreated hazelnut shell solids and reducing sugar yield after enzymatic h121ydrolysis(E.H)

Run No.	NaOH conc. (%)	SLR (%)	Time (min)	Reducing sugar before E.H (mg/g dry pretreated biomass)	Reducing sugar after E.H (mg/g dry pretreated biomass) (Y_3)	Enzymatic conversion efficiency (%)
1	5	10	60	415.6±13.7	289.6±26.8	65.2±1.5 F
2	5	10	60	387.7±31.5	283.9±17.5	73.3±2.4 DEF
3	5	10	60	389.2±5.4	272.8±13.1	70.1±3.5 EF
4	7	15	60	376.4±24.3	303.6±10.2	80.9±5.9 BCDE
5	7	5	60	353.8±20.9	217.2±13.2	62.4±5.9 F
6	3	15	60	363.5±3.7	308.2±5.7	84.8±1.0 ABCD
7	3	5	60	347.1±4.3	318.3±14.2	91.7±4.9 AB
8	3	10	90	308.1±21.3	298.6±13.8	97.2±6.8 A
9	7	10	90	301.3±17.5	207.4±5.2	69.1±5.6 EF
10	5	15	90	303.0±30.7	184.7±9.9	76.4±6.2 CDEF
11	5	5	90	337.0±4.1	232.6±3.2	70.3±0.0 DEF
12	3	10	30	352.3±22.4	316.3±7.8	89.9±3.6 ABC
13	7	10	30	309.9±21.1	235.1±25.3	75.3±2.9 DEF
14	5	5	30	321.0±18.4	172.2±8.6	42.3±2.6 G
15	5	15	30	342.2±14.4	220.7±6.6	64.6±3.1 F

[a] Results belong to triplicate

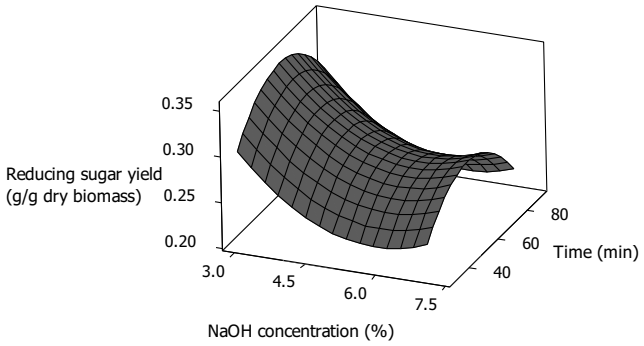
As with lignin reduction, a second-order polynomial equation (Eqn 30) was developed to establish correlations between reducing sugar yield and the pretreatment variables. The model equation adequately represented the data with an R^2=0.92 and insignificant lack of fit (P = 0.555 >0.05). Detailed ANOVA calculations is given in the Table I.3. The final equation without insignificant (p>0.05) terms was:

$$Y_3 = 0.2769 - 0.0368X_1 + 0.0071X_2 + 0.0383X_1^2 - 0.0245X_2^2 - 0.0509X_3^2 + 0.0281X_1X_2 - 0.0252\,X_2X_3 \quad (30)$$

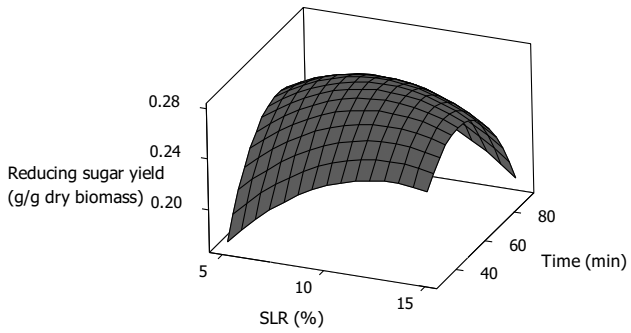
where Y_3 is predicted reducing sugar yield, X_1 is NaOH concentration, X_2 indicates the SLR (%) and X_3 represents time (min).

Fig. 4.19.a shows that reducing sugar yield decreased significantly (P<0.05) with increase in NaOH concentration, but increased with increase in treatment time up to 60 min. the yield was also significantly (P<0.05) improved with the increase of solid liquid ratio, but decreased (P<0.05) when time was attained for more than 60 min (Fig. 4.19.b). Fig. 4.19.c shows that reducing sugar yield decreased significantly (P<0.05) with increase in NaOH concentration, but increased with increase of solid liquid ratio. Increased NaOH concentration helps the increase of surface area and formation of pores to permit easier enzyme access and attack on carbohydrates for reducing sugar production (Goh et al., 2010). When alkali is used at high concentration, cellulose and hemicellulose components were irreversibly lost through dissolution and degredation of hemicellulose (Chen et al., 2013).

a



b



c

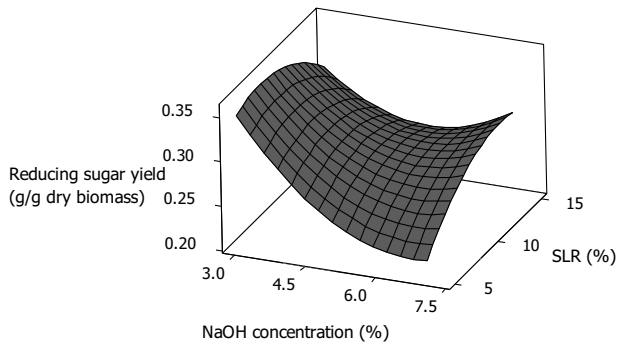


Figure 4.19 Response surface plots for the effects of alkaline pretreatment conditions on reducing sugar yield (g/g dry biomass)

The correlation between the experimental and predicted values of reducing sugar yield showed that predicted values were within the model's designed ranges (R^2=0.93) (Fig. 4.20).

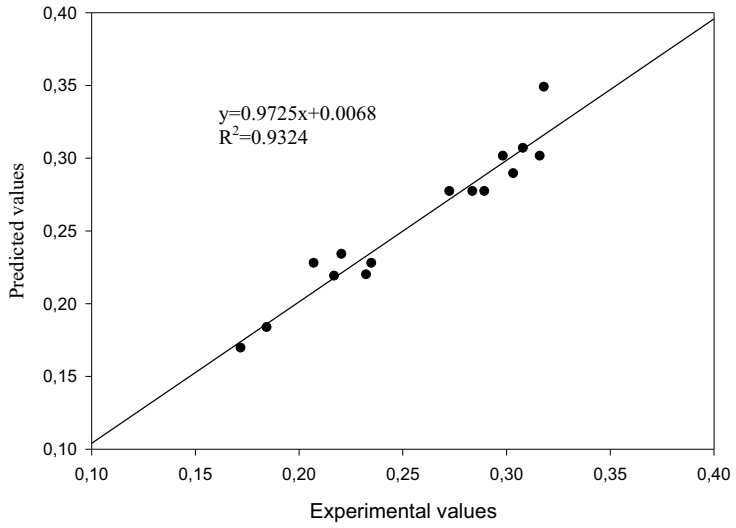


Figure 4.20 Experimental versus RSM predicted values for total reducing sugar yield (g/g dry biomass)

The optimum condition for maximal reducing sugar yield, as predicted by the model was 357.2 mg/g dry pretreated biomass, and obtained with 3% (w/v) NaOH concentration, 7.5% SLR and 63 min treatment time.

To validate the models predictive ability and optimum condition, a verification run was performed in triplicate and the reducing sugar yield was obtained as 243.3±10.2 mg/g dry pretreated biomass. A significant difference was observed between the model predicted and experimental verification values potentially due to non-

homogeneous untreated material characteristics. It could also be inferred that the pretreatment parameter range was not sufficient to completely evaluate the effect of fermentable sugar production.

Although optimizations were performed to determine the most suitable conditions for individually enhancing delignification and reducing sugar yield, an overall evaluation of the results of this study indicated that subsequent research on hazelnut shell-to-sugar conversion processs should target a process that can improve the cost-effectiveness while maintaining the efficiency high.

4.2.3 Effect of Ozone Pretreatment

4.2.3.1 Effect of Ozonolysis on Biomass Recovery in Hazelnut Shell

Ozonolysis of hazelnut shells resulted in 4.54-10.87% loss of the biomass during pretreatment depending on the pretreatment conditions (Table 4.18). Ozonolysis with 25 % moisture content showed a higher biomass recovery than that with 35 % moisture content. Solid recovery significantly ($P<0.05$) decreased with the increase in pretreatment time and moisture content. The highest biomass recovery (95.5%) was obtained at an ozone concentration of 30 mg/L with 25% moisture content for a treatment time of 90 min. Among the factors, moisture content and time showed significant effects ($P <0.05$) on biomass recovery.

4.2.3.2 Effect of Ozonolysis on Lignin Content in Hazelnut Shell

It was observed that ozonolysis reduced AIL concentration in hazelnut shell for all pretreated samples. Lignin removal after ozone pretreatment ranged from 9.79% to 20.52% of the initial lignin in the untreated hazelnut shell (Table 4.18). AIL content

significantly (P<0.05) decreased with the increase of time and ozone concentration. Garcia-Cubero et al. (2010) reported that ozonloysis of wheat, rye, barley and oats in a fixed bed reactor resulted in a 38, 50, 41 and 40% reduction in lignin, respectively. Travaini et al. (2013) observed a higher net reduction of 66.8% AIL in sugarcane bagasse pretreated at an ozone concentration of 3.44% (v/v) with 40% moisture content for 120 min.

Panneerselvam et al. (2013) pretreated energy grasses with ozone and observed the net reduction in AIL ranged between 16.9-27.8% for washed samples. It was noted that solid recovery ranged between 61.2–89.9% and 58.1–85.6% for AIL and glucan with washed samples, respectively. The highest lignin reduction (20.52%) was obtained at ozone concentration of 50 mg/L with 30% moisture treated for 120 min, which also resulted in high biomass recovery (94.01%) (Table 4.18). It was indicated that different type of biomass responded differently to the ozonolysis process conditions. Delignification results were found to be comparable with conventional alkaline (NaOH) pretreatment. It was also clear that the use of NaOH ranging from 3% to 7% (w/v) decreased lignin reduction from 19.7% (5%, w/v, NaOH, 10% solid/liquid ratio, 60 min) to 6.9% (3%, w/v, NaOH, 10% solid/liquid ratio, 30 min). Among the factors, ozone concentration and time showed significant effects (P <0.05) on lignin reduction. It can be seen from Table 4.18 that the AIL content significantly (P<0.05) decreased with the increase of ozone concentration and time.

It was observed that when AIL decreased, acid soluble lignin (ASL) increased (Table 4.18). This was expected, since ozone's reaction with lignin converts an AIL fraction into ASL by inserting hydrophilic functional groups (Garcia-Cubero et al., 2009; Yu et al., 2011). Overall, ASL content of ozone pretreated hazelnut shells increased with increase in ozone concentration, reaching a maximum in Run 3, where it increased from 1.29% in the raw material to 1.91% after treatment (Table 4.18).

Table 4.18 Box-Behnken design matrix for identifying key process variables and solid recovery (%), acid insoluble lignin (%) and total reducing sugar (mg/g) in ozone treated samples.

Run No.	Moisture content (%)	Ozone conc. (mg/L)	Time (min)	Solid recovery (%)	AIL (%)	ASL (%)	After hydrolysis RD (mg/g)
1	25	40	120	91.02±0.76	40.18±1.84	1.54±0.05	256.2±8.0
2	30	30	120	92.54±0.55	40.95±0.64	1.45±0.06	278.2±10.8
3	30	50	120	94.02±1.40	37.51±0.93	1.91±0.09	196.3±1.0
4	35	40	120	89.13±2.32	41.60±1.41	1.41±0.05	243.9±6.0
5	25	30	90	95.46±2.06	42.19±1.13	1.34±0.10	284.3±15.0
6	25	50	90	93.23±1.51	41.44±0.93	1.29±0.04	293.7±7.3
7	30	40	90	92.97±1.64	40.20±0.22	1.49±0.06	270.9±14.0
8	30	40	90	91.57±0.94	40.29±0.05	1.57±0.03	292.4±13.9
9	30	40	90	93.32±0.99	41.46±2.36	1.53±0.03	275.6±11.2
10	35	30	90	92.77±0.47	43.07±1.02	1.24±0.09	274.9±11.1
11	35	50	90	92.37±1.37	39.31±1.51	1.50±0.03	296.2±13.3
12	25	40	60	94.81±1.40	40.51±0.25	1.79±0.06	228.8±12.9
13	30	30	60	93.63±0.50	42.98±1.24	1.27±0.11	304.0±6.3
14	30	50	60	92.84±1.82	40.88±1.26	1.44±0.11	263.1±6.9
15	35	40	60	93.00±0.45	41.21±0.28	1.69±0.01	261.6±9.2

4.2.3.3 Effect of Ozonolysis on Enzymatic Saccharification

Total reducing sugar yield also compared to analyze the influence of ozone pretreatment on enzymatic saccharification. Table 4.19 shows that the reducing sugar production after hydrolysis significantly (P<0.05) decreased with the increase of time and ozone concentration. However, as the moisture content was increased from 25 to 35%, there was no significant influence on total reducing sugar (P>0.05). Moisture content played a major role during ozonolysis of biomass such as bagasse (Neely, 1984), poplar sawdust (Vidal and Molinier, 1988) and wheat and rye straw (Garcia-Cubero et al., 2009). Optimal moisture content for ozone pretreatment varies with the biomass such as 25 to 35% for oak sawdust (Neely, 1984) and 30% for wheat and rye straws (Garcia-Cubero et al., 2009). Higher consumption of ozone was required using excessive moisture without any increase in pretreatment efficiency (Garcia-Cubero et al., 2009). Thus, the moisture content was set at 30% based on results of this study and Garcia-Cubero et al. (2009) who reported that the optimum moisture content for ozonolysis as 30% and an increase in moisture content above 30% evidenced no significant effects of ozonolysis pretreatment on reducing sugar production.

The highest reducing sugar production (304.0 mg/g dry pretreated biomass) was obtained at an ozone concentration of 30 mg/L, 30% moisture content and 60 min. This was significantly higher than sugar produced by untreated material (119.9±6.2 mg/g raw material) (Table 4.19). Ozonolysis resulted in an improvement in reducing sugar production. Enzymatic conversion increased by 100% at 30 mg/L ozone concentration and 60 min (Table 4.19). While there were variations in ozonolysis and hydrolysis conditions, ozonolysis used herein resulted in higher sugar yield than ozone pretreatments of wheat straw (about 40%, Garcia-Cubero et al., 2010), rye straw (between 40-57%, Garcia-Cubero et al., 2012), coastal Bermuda grass (63%, Lee et al., 2010), cotton stalk (60%, Kaur et al., 2012) and Japanese cedar sawdust (80%, Sugimoto et al., 2005). It was shown that ozonolysis of hazelnut shell was also able to enhance fermentable sugar production.

Table 4.19 Total reducing (RD) sugar from saccharification of hazelnut shell pretreated with ozonolysis.

Treatments	Before hydrolysis Total RD sugar (mg/g dry biomass)	After hydrolysis Total RD sugar (mg/g dry biomass)	Conversion (%)
Untreated	290.9±6.2	119.9±6.2	
Ozone treated with 25% MC			
30 mg/l for 90 min	319.1±18.2	284.3±15.0	89.2±2.9
40 mg/l for 60 min	262.9±8.8	228.8±12.9	83.2±4.9
40 mg/l for 120 min	325.6±13.5	256.2±8.0	81.9±1.9
50 mg/l for 90 min	322.4±8.2	293.7±7.3	91.2±3.5
Ozone treated with 30% MC			
30 mg/l for 60 min	302.1±4.5	304.0±6.3	100.6±2.1
30 mg/l for 120 min	325.0±7.6	278.2±10.8	85.5±2.4
40 mg/l for 90 min	323.3±4.2	270.9±14.0	82.2±1.7
40 mg/l for 90 min	318.2±12.3	292.4±13.9	93.8±2.4
40 mg/l for 90 min	319.7±6.5	275.6±11.2	86.2±1.9
50 mg/l for 60 min	317.7±5.6	263.1±6.9	84.1±1.8
50 mg/l for 120 min	238.2±6.3	196.3±1.0	83.4±2.5
Ozone treated with 35% MC			
30 mg/l for 90 min	309.1±6.1	274.9±11.1	88.9±3.0
40 mg/l for 60 min	263.2±9.7	261.6±9.2	99.4±1.5
40 mg/l for 120 min	323.1±8.8	243.9±6.0	75.6±3.8
50 mg/l for 90 min	322.6±5.1	296.2±13.3	91.3±7.1

[a] Results belong to triplicate

Regression analysis showed that ozone concentration and time, with P values lower than 0.05, had a substantial effect on reducing sugar production. It was observed that data analysis by BBD-RSM did not provide good correlations between the process variables (ozone concentration, moisture content and treatment time) and dependent variables (solid recovery, lignin reduction and total reducing sugar yield). Hence, to identify optimal process treatment conditions for ozonolysis of hazelnut shell, process variables that had a significant effect on reducing sugar production after enzymatic hydrolyses of ozonated samples were selected for further evaluation. A full factorial design was established by listing experimental conditions previously tested with RSM.

ANOVA (Table 4.20) suggested that there was a significant difference between the levels of pretreatment time, ozone concentration and their interactions.

Table 4.20 Analysis of variance results for reducing sugar production based on full factorial design conditions

Source	**Coef.**	**Seq SS**	**Adj SS**	**Adj MS**	**F value**	**P value**
Ozone concentration (mg/L)	-0.0307	0.0113	0.0113	0.0113	319.41	0.000
Time(min)	-0.0232	0.0065	0.0065	0.0065	183.56	0.000
Ozone conc.*Time (min)	-0.0102	0.0013	0.0013	0.0013	35.30	0.000

R-Sq = 0.9834% R-Sq(adj) = 0.9768% R-Sq(pred) = 0.9627%

The regression Eqn. (31) represented the best correlation between main 2 interaction effects of process variables on reducing sugar after the elimination of non-significant parameters (P> 0.05).

$$Y_1=0.2603-0.0306X_1-0.0232X_2-0.0102X_1X_2 \quad (31)$$

where Y_1 is predicted reducing sugar production (mg/g dry pretreated biomass), X_1 and X_2 are coded values for ozone concentration and time, respectively. The optimum conditions for reducing sugar were found as 30% moisture content, 30 mg/L of ozone concentration and 60 min of time. The reducing sugar results were found to be comparable with conventional alkaline (NaOH) pretreatment, which led to the highest reducing sugar yield of 318.3 mg/g dry biomass (and 91.7% enzyme conversion efficiency) from biomass pretreated with 3.0% NaOH at 5% solid liquid ratio for 60 min.

4.2.4 Comparison of All Pretreatment Methods

The effectiveness of dilute acid, alkaline and ozone pretreatments on conversion of hazelnut shell hydrolzates to reducing sugars with minimum sugar loss was also assessed.

The sulfuric acid pretreatment resulted in the highest sugar conversion (62.8% for 3.42 (w/w)% acid, 31.7 min, 130 ºC) whereas sugar conversion of 49.6% (%3 NaOH, 15% solid/liquid ratio, 60 min, 121 ºC/15psi) and 53.4% were obtained from NaOH and ozone pretreatment, respectively. Total reducing sugar conversion was enhanced from 62.8% to 72.4% with combined acid and enzymatic hydrolysis. After enzymatic hydrolysis, sugar conversion increased from 49.6 to 61.2% and 53.4 to 58.5% for NaOH and ozone pretreatment, respectively. Ozone pretreatment resulted in significantly lower (P<0.05) sugar conversion (58.5%) than sodium hydroxide and dilute acid pretreatment. Among all chemical pretreatments, sulfuric acid at 3.42% (w/w) gave the maximum sugar production. Dilute acid pretreatment (3.42 (w/w) % acid, 31.7 min, 130 ºC) was more effective at sugar conversion than

sodium hydroxide and ozone pretreatment. Eisenhuber et al. (2013) investigated that effect of different pretreatment methods such as steam explosion, acid and alkaline on sugar conversion. They reported that acid pretreatment led to the highest xylose concentration of 120.9 g/kg (at 100 ^{o}C, 30 min, 10% sulphuric acid), followed by a xylose concentration of 94.7 g/kg using steam exploded wheat straw (at 180 ^{o}C, 20 min). The lowest sugar yield was achieved by alkaline pretreatment (at 100 ^{o}C, 30 min, 5% NaOH). Tutt et al. (2012) reported that alkaline pretreatment method showed lower hydrolysis efficiency compared to the dilute acid pretreatment methods.

4.3 Selection of the Best Carbon Source of PG Production by *B.subtilis*

To determine the best inducing carbon source, different carbon sources such as pectin, hazelnut shell and hazelnut shell hydrolysate, are shown in Fig. 4.21. The highest PG production was obtained with hazelnut shell hydrolysate (4.8±0.03 EU/mL). It was clear from Fig. 4.21 that hydrolysis of hazelnut shell was effective compared to ground form based on PG production increased from 0.39 EU/mL to 4.8 EU/mL.

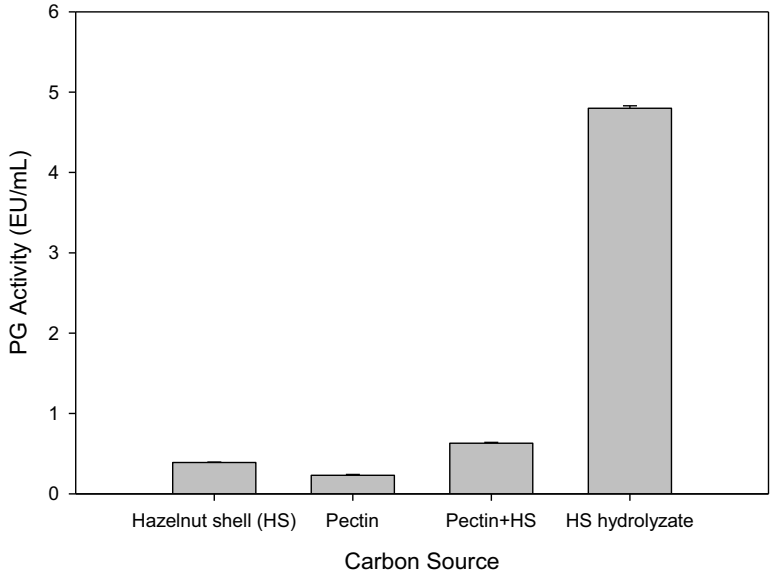


Figure 4.21 Effect of Carbon sources on PG activity at 30 ºC pH 7.0 and 130 rpm after 72 h fermentation

4.4 Evaluation of Key Variables Affecting Enzyme Production

4.4.1. Plackett Burman Design (PBD)

A two level PBD experimental matrix was set up to identify the factors and estimate their significance in pectinase production. PBD predicts linear model where only main effects are taken into consideration (Eqn. 32).

$$\text{Response} = a + \Sigma\, bi * Xi \qquad (32)$$

The response indicates the dependent variable in terms of overall pectinase production (EU/mL), a is the model intercept, and X_i represents the levels of independent variables. Selection of appropriate carbon, nitrogen and other nutrients is one of the most critical stages in the development of an efficient and economic

process. In order to obtain an industrially low cost medium, hazelnut shell with optimum blend of nutrients and culture conditions were screened for potential high pectinase activity. In this respect, 8 independent variables were selected; pH, fermentation time, temperature, inoculum volume (%v/v), pectin, yeast extract, magnesium sulphate [$MgSO_4$], and dipotassium hydrogen phosphate [K_2HPO_4]. The concentration of each nutrient was based on the literature. Table 4.21 represents their actual values while Table 4.22 illustrates the design matrix of various components with coded values as low (-1) and high (+1) levels.

Table 4.21 Independent variables with their coded and uncoded levels used in PBD

Serial Number	Variable	Low (-1)	Medium (0)	High (+1)
1	pH	5	7.0	9
2	Time (h)	24	48	72
3	Temperature (^oC)	30	35	40
4	Inoculum volume (%v/v)	1	3	5
5	Pectin (%w/v)	0.2	0.35	0.5
6	Yeast extract (%w/v)	0.1	0.3	0.5
7	$MgSO_4$ $7H_2O$ (%w/v)	0.02	0.05	0.08
8	K_2HPO_4 (%w/v)	0.02	0.03	0.04

A maximum pectinase activity of 3.96±0.06 EU/mL was found when *B.subtilis* was fermented with pectin 0.5 (%w/v), yeast extract 0.1 (%w/v), $MgSO_4 7H_2O$ 0.02 (%w/v), K_2HPO_4 0.02 (%w/v), pH 5.0, 40 ^oC, inoculum volume 1% (v/v) and 72h.

On the other hand, the maximum pectinase activity using *B.pumilus* was found as 3.93±0.02 EU/mL using pectin 0.5 (%w/v), yeast extract 0.5 (%w/v), $MgSO_4 7H_2O$ 0.08 (%w/v), K_2HPO_4 0.02 (%w/v), pH 9.0, 30 ^oC, inoculum volume 1% (v/v), and 24 h as fermentation components. No significant differences in maximum pectinase production were observed using either *B. pumilus* or *B. subtilis* (P>0.05).

Table 4.22 Plackett Burman Design for screening of factors for *B.subtilis* and *B.pumilus*

Run No.	pH	Time (h)	Temperature (°C)	Inoculum Volume (%v/v)	Pectin (%w/v)	Yeast extract (%w/v)	$MgSO_4$ (%w/v)	K_2HPO_4 (%w/v)	Pectinase Activity* (EU/mL)	
									B.subtilis	***B.pumilus***
1	+1	-1	+1	-1	-1	-1	+1	+1	3.26±0.07 ghi	3.54±0.01bcdefg
2	+1	+1	-1	+1	-1	-1	-1	+1	3.40±0.04 defgh	3.52±0.01bcdefg
3	**-1**	**+1**	**+1**	**-1**	**+1**	**-1**	**-1**	**-1**	**3.96±0.06 a**	3.62±0.02 bcde
4	-1	-1	+1	+1	+1	-1	+1	+1	3.40±0.13 defgh	3.69±0.01abcd
5	-1	-1	-1	+1	+1	+1	-1	+1	2.20±0.09 j	3.80±0.03ab
6	-1	+1	-1	-1	-1	+1	+1	+1	2.11±0.09 j	3.31±0.02fghi
7	+1	+1	-1	+1	+1	-1	+1	-1	3.57±0.09 bcdef	3.53±0.02bcdefg
8	-1	+1	+1	+1	-1	+1	+1	-1	3.52±0.09 bcdefg	3.33±0.02efghi
9	-1	-1	-1	-1	-1	-1	-1	-1	3.04±0.22 i	3.50±0.02cdefg
10	**+1**	**-1**	**-1**	**-1**	**+1**	**+1**	**+1**	**-1**	3.16±0.07 hi	**3.93±0.02 a**
11	+1	+1	+1	-1	+1	+1	-1	+1	3.31±0.08 fghi	3.71±0.02abc
12	+1	-1	+1	+1	-1	+1	-1	-1	3.46±0.07cdefgh	3.38±0.02efgh

[a] Results belong to replicate.

The pH of the juices generally falls within the optimum range of the commercial enzyme preparations. In any case, adjustment of the pH with alkali is not recommended for the production of fruit juices (Reed, 1966). pH 9.0 is not suitable for fruit juices and some vegetables (Table 4.23). Thus, *B.pumilus* was abandoned in further trials of pectinase production.

Table 4.23 pH value and pectin substances of various fruit/vegetables

Fruit/Vegetable	Pectin substance (%)	Approximate pH
Apple	0.5-1.6	3.30-4.00
Orange pulp	12.4-28.0	3.50-4.15
Strawberries	0.6-0.7	3.00-3.90
Banana	0.7-1.2	4.50-5.20
Peaches	0.1-0.9	3.30-4.05
Tomatoes	2.4-4.6	4.30-4.90
Carrot	6.9-18.6	5.88-6.40

The variability in the five factors (pH, fermentation time, temperature, yeast extract and K_2HPO_4) was found significant by PB analysis as illustrated in *Pareto* chart (Figure J.1).

These factors were further optimized by BBD response surface method. Sharma and Satyanarayana (2006) reported that C:N ratio, K_2HPO_4 and pH affected significantly pectinase production of *B. pumilus* dcsr1 when analyzed 11 variables

by PB method. Variability in the initial amount of yeast extract was not determined significant in their study, and time and temperature were not varied either. For *B. subtilis*, 8-factor and 2-level Plackett-Burman design was conducted. The factors such as pH, fermentation time, temperature, yeast extract and K_2HPO_4 with T values above threshold (2.145 in this case) and P values lower than 0.05 as represented by regression analysis (Table 4.24) had a substantial effect on enzyme activity and were considered for further evaluation by BBD, while the rest of the variables did not have a remarkable contribution to enzyme production.

Table 4.24 Regression analysis* for Plackett Burman design variables for *B.subtilis*

Term	Effect	Coefficient	T Value	P Value
Intercept		0.320	91.88	**0.000**
Block		-0.066	-1.88	0.081
pH	0.319	0.159	4.58	**0.000**
Fermentation time	0.225	0.113	3.24	**0.006**
Temperature	0.571	0.286	8.20	**0.000**
% Inoculum (v/v)	0.119	0.060	1.71	0.109
Pectin	0.134	0.067	1.93	0.074
Yeast extract	-0.481	-0.241	-6.91	**0.000**
Magnesium sulphate	-0.056	-0.028	-0.80	0.438
Di-potassium hydrogen phosphate	-0.504	-0.252	-7.24	**0.000**

*Result is significant when $P<0.05$.

The model considering the main effects (equation not shown) was found fairly accurate with a R^2 value of 0.93 and a R_{adj}^2 value of 0.89 (Table 4.25).

Table 4.25 Plackett Burman Design for screening of factors for *B.subtilis*

Serial	pH	Time (h)	Temperature (°C)	Inoculum Volume (%v/v)	Pectin (%w/v)	Yeast extract (%w/v)	$MgSO_4$ (%w/v)	K_2HPO_4 (%w/v)	Response variable Pectinase Activity (EU/mL)	
									Experimental*	Model predicted
1	+1	-1	+1	-1	-1	-1	+1	+1	3.26±0.07 BCD	3.36
2	+1	+1	-1	+1	-1	-1	-1	+1	3.40±0.04 BCD	3.19
3	-1	+1	+1	-1	+1	-1	-1	-1	3.96±0.06 A	3.97
4	-1	-1	+1	+1	+1	-1	+1	+1	3.40±0.13 BCD	3.30
5	-1	-1	-1	+1	+1	+1	-1	+1	2.20±0.10 E	2.30
6	-1	+1	-1	-1	-1	+1	+1	+1	2.11±0.09 E	2.22
7	+1	+1	-1	+1	+1	-1	+1	-1	3.57±0.09 AB	3.78
8	-1	+1	+1	+1	-1	+1	+1	-1	3.52±0.09 BC	3.41
9	-1	-1	-1	-1	-1	-1	-1	-1	3.04±0.22 D	3.03
10	+1	-1	-1	-1	+1	+1	+1	-1	3.16±0.07 CD	2.95
11	+1	+1	+1	-1	+1	+1	-1	+1	3.31±0.08 BCD	3.30
12	+1	-1	+1	+1	-1	+1	-1	-1	3.46±0.07 BC	3.56

[a] Results belong to replicate.

4.4.2 Effect of Fermentation Time

pH, fermentation time, temperature, inoculum volume (%v/v), pectin, yeast extract, magnesium sulphate [MgSO4], and dipotassium hydrogen phosphate [K_2HPO4] were kept at a constant, enzyme activities were measured at the end of 6, 12, 24, 36, 48, 60, 72, and 84 h to determine the effect of time on PG production (Figure 4.22). The results indicated that the highest enzyme production (4.8 EU/mL) was achieved after 72 h at 30 ^oC, pH 7.0 and with agitation of 130 rpm.

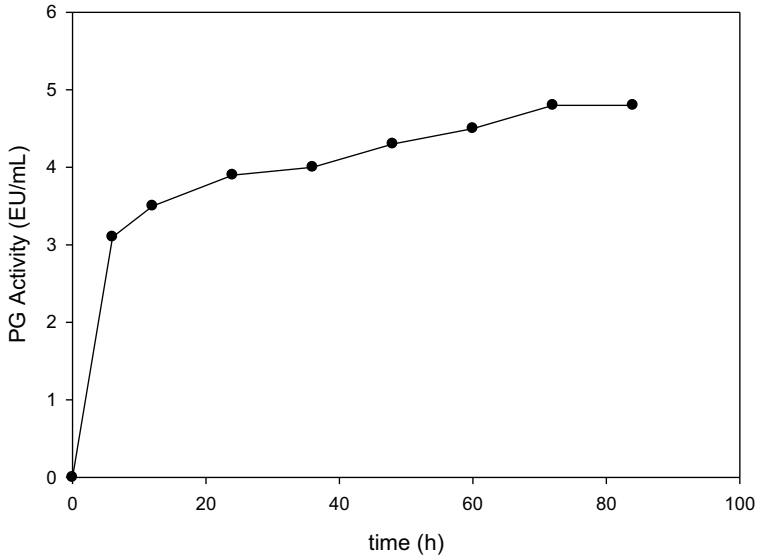


Figure 4.22 Time course of pectinase production by *B.subtilis* at pH 7.0, 30 ^oC and with shaking at 130 rpm.

4.4.3 Optimization of Fermentation Medium Components and Conditions for Pectinase Production by Response Surface Method (RSM)

In industrial perspective of enzyme production, the economy is another important point to consider and can be addressed by optimization of the bioprocess. The experimental plan and the results of pectinase production for various combination of pH (*A*), temperature (*B*), time (*C*), yeast extract (*D*) and dipotassium hydrogen phosphate [K_2HPO_4] (E) are shown in Table 4.26. The lowest PG activity (3.35±0.03 EU/mL) was obtained at pH 7.0, temperature 35 ºC with yeast extract 0.1 (w/w, %) and K_2HPO_4 0.03 (w/w, %) for 72 h. However, the highest PG activity (4.77±0.03 EU/mL) was observed at pH 7.0, temperature 35 ºC with yeast extract 0.5 (w/w, %) and K_2HPO_4 0.03 (w/w, %) for 72 h (Table 4.26).

A second order polynomial model fit to the experimental data for optimizing pectinase production by RSM predicts the response as a function of five variables and their interactions in terms of their coded values. The ANOVA results and estimated regression coefficients for the coded pectinase activity model are shown in Table 4.27. Another run with excluded insignificant terms according to Table K.1 expressed by Eqn. (33) and the final form of the equation in terms of coded values of factors was given as:

$$Y=4.271-0.075A^{a}0.233B+0.162C+0.137D+0.137E -0.221A^{a} -0.231B^2 – 0.166C^2- 0.258D^2 + 0.096AB -0.111AC -0.093AD-0.379AE–0.296BC -0.218BD +0.218BE + 0.478CD– 0.381DE \quad (33)$$

where Y is predicted pectinase activity, A,B,C,D,E are the coded values for pH, fermentation time, temperature, yeast extract and [K_2HPO_4] respectively.

Table 4.26 Experimental design for optimization of pectinase production using response surface method (RSM)

Run Order	pH (A)	Temperature (B)	Time (h) (C)	Yeast extract (%w/v) (D)	K_2HPO_4 (%w/v) (E)	Experimental Response Pectinase Activity (EU/mL)	Model predicted Response (EU/mL)
1	0	0	+1	-1	0	3.35±0.03	3.40
2	0	+1	0	0	+1	4.02±0.03	4.18
3	0	+1	0	-1	0	3.70±0.03	3.64
4	0	0	+1	0	-1	4.04±0.01	4.14
5	0	-1	0	0	-1	4.41±0.04	4.37
6	+1	0	+1	0	0	3.98±0.04	3.86
7	0	0	0	0	0	4.24±0.04	4.29
8	+1	0	0	+1	0	3.68±0.04	3.77
9	0	+1	+1	0	0	3.46±0.03	3.51
10	+1	+1	0	0	0	3.65±0.03	3.61
11	+1	0	0	0	+1	3.72±0.03	3.75
12	0	-1	0	+1	0	4.41±0.05	4.37
13	0	0	0	0	0	4.28±0.04	4.29
14	+1	0	-1	0	0	3.84±0.02	3.76
15	0	0	-1	0	+1	4.28±0.04	4.09
16	-1	0	+1	0	0	4.15±0.03	4.24
17	-1	0	-1	0	0	3.55±0.06	3.69
18	0	+1	0	+1	0	3.49±0.03	3.47
19	-1	0	0	0	-1	3.69±0.04	3.62
20	0	0	+1	0	+1	4.39±0.03	4.42
21	0	0	0	0	0	4.23±0.02	4.29

Table 4.26 (continued)

22	0	+1	0	0	-1	3.51±0.05	3.47
23	0	-1	0	0	+1	4.04±0.02	4.20
24	0	-1	+1	0	0	4.58±0.04	4.57
25	0	0	0	0	0	4.34±0.16	4.29
26	0	0	0	0	0	4.35±0.07	4.29
27	0	+1	-1	0	0	3.65±0.03	3.78
28	-1	0	0	+1	0	4.08±0.03	4.10
29	0	0	0	-1	-1	3.39±0.03	3.37
30	0	0	0	+1	+1	3.79±0.04	3.92
31	+1	-1	0	0	0	3.84±0.03	3.89
32	0	0	-1	0	-1	3.69±0.02	3.82
33	0	-1	0	-1	0	3.75±0.05	3.67
34	0	0	+1	+1	0	4.77±0.03	4.63
35	+1	0	0	-1	0	3.54±0.03	3.68
36	0	0	0	-1	+1	4.46±0.03	4.41
37	0	0	-1	+1	0	3.52±0.01	3.35
38	-1	+1	0	0	0	3.69±0.03	3.57
39	-1	0	0	-1	0	3.56±0.03	3.64
40	0	0	0	+1	-1	4.24±0.04	4.41
41	+1	0	0	0	-1	4.24±0.04	4.23
42	-1	0	0	0	+1	4.69±0.04	4.65
43	-1	-1	0	0	0	4.28±0.03	4.23
44	0	0	-1	-1	0	4.02±0.04	4.03
45	0	-1	-1	0	0	3.58±0.04	3.65
46	0	0	0	0	0	4.32±0.05	4.29

The derived polynomial equation was found adequate in representing the experimental data (R^2=0.9398). The coefficients of the equation shown in Table 4.27 indicated the contribution of individual and combinations of components in the pectinase production.

Table 4.27 ANOVA results of BBD in coded values

Term	Coefficient	P Value
Constant	4.291	0.000
pH (A)	-0.075	0.000*
Temp. (B)	-0.233	0.000*
Time (C)	0.162	0.000*
Yeast extract (D)	0.137	0.000*
K_2HPO_4 (E)	0.137	0.000*
A*B	-0.096	0.012*
A*C	-0.111	0.004*
A*D	-0.093	0.015*
A*E	-0.379	0.000*
B*C	-0.296	0.000*
B*D	-0.218	0.000*
B*E	0.218	0.000*
C*D	0.478	0.000*
C*E	-0.060	0.115
D*E	-0.381	0.000*
A^2	-0.229	0.000*
B^2	-0.229	0.000*
C^2	-0.174	0.000*
D^2	-0.266	0.000*
E^2	-0.028	0.274

*Result is significant when $P<0.05$

The most important factors determining the pectinase activity (EU/mL) were fermentation time with highest coefficient (0.233), which indicates that it is the most dominant factor influencing the overall pectinase production from hazelnut shells followed by temperature (0.162), [K_2HPO_4] (0.137), yeast extract (0.137) and pH (0.075) (Table 4.27).

The insignificant lack of fit ($P = 0.135 > 0.05$) also proved that the model fitted well to the experimental data. This signifies the model with 95% level of confidence ($\alpha=0.05$) and all effects namely linear, interaction and quadratic are exhibited. The quality of fit model was estimated by R_{adj}^2 and predicted R^2 (R_{pred}^2) values were found as 0.92 and 0.89 respectively, which are fairly high and accurate measures of precision (Ohtani, 2000). This indicates that only 8% variation in response cannot be suitably explained by the model. This indicated that model equation very well corresponded to BBD experimental data. It was also observed that the five-major factors and almost all interactions significantly affect the PG production with low P values (Table 4.27).

The three dimensional (3D) response surface curves were plotted to depict the relationship among the various selected factors and to determine their optimum values for attaining the maximum activity of pectinase. Figure 4.23 represents the response surface plots for pectinase production at varying concentration of a) [K_2HPO_4] vs yeast extract concentration, b) [K_2HPO_4] vs time, c) [K_2HPO_4] vs pH, d) [K_2HPO_4] vs temperature, e) yeast extract concentration vs time, f) yeast extract concentration vs temperature, g) yeast extract concentration vs pH, h) time vs temperature, i) time vs pH and j) temperature vs pH, respectively. The fifth factor, in all cases, was held constant at the center point (i.e. pH 7.0, time 48 h, temperature 35 ^oC, yeast extract concentration 0.3 (%w/v) and K_2HPO_4 0.03 (%w/v) respectively).

A rapid increase in pectinase production was observed when yeast extract was increased up to mid-values and stayed constant thereafter, whereas a slight increase was observed with K_2HPO_4 (Fig. 4.23.a). Joshi et al. (2013) also reported high pectinase production at 2.5% yeast extract. A similar trend was reported for

K_2HPO_4 (Sharma & Satyanarayana, 2006), where a maximum increase in pectinase was attained at 0.5% K_2HPO_4. Figure 4.23.b shows that pectinase activity increased with the increase of K_2HPO_4, and starts to decrease beyond 50 h.

Pectinase production is directly proportional to microbial growth and is high in the late exponential phase of growth and decreases thereafter (Joshi et al., 2013). Varying optimal fermentation time in the range of 24 h to 6 days are available for *Bacillus subtilis* (Ahlawat et al., 2009, Joshi et al., 2013, Swain & Ray, 2009).

A rapid increase in pectinase activity was observed as pH was increased to about 7.0, and a slight decrease occurs beyond pH 8 (Fig. 4.23.c,g,i,j). A similar positive effect of pH was reported by others (Sharma & Satyanarayana, 2006). High pH values accounts typically for PG and pectate lyase production (Joshi et al., 2013).

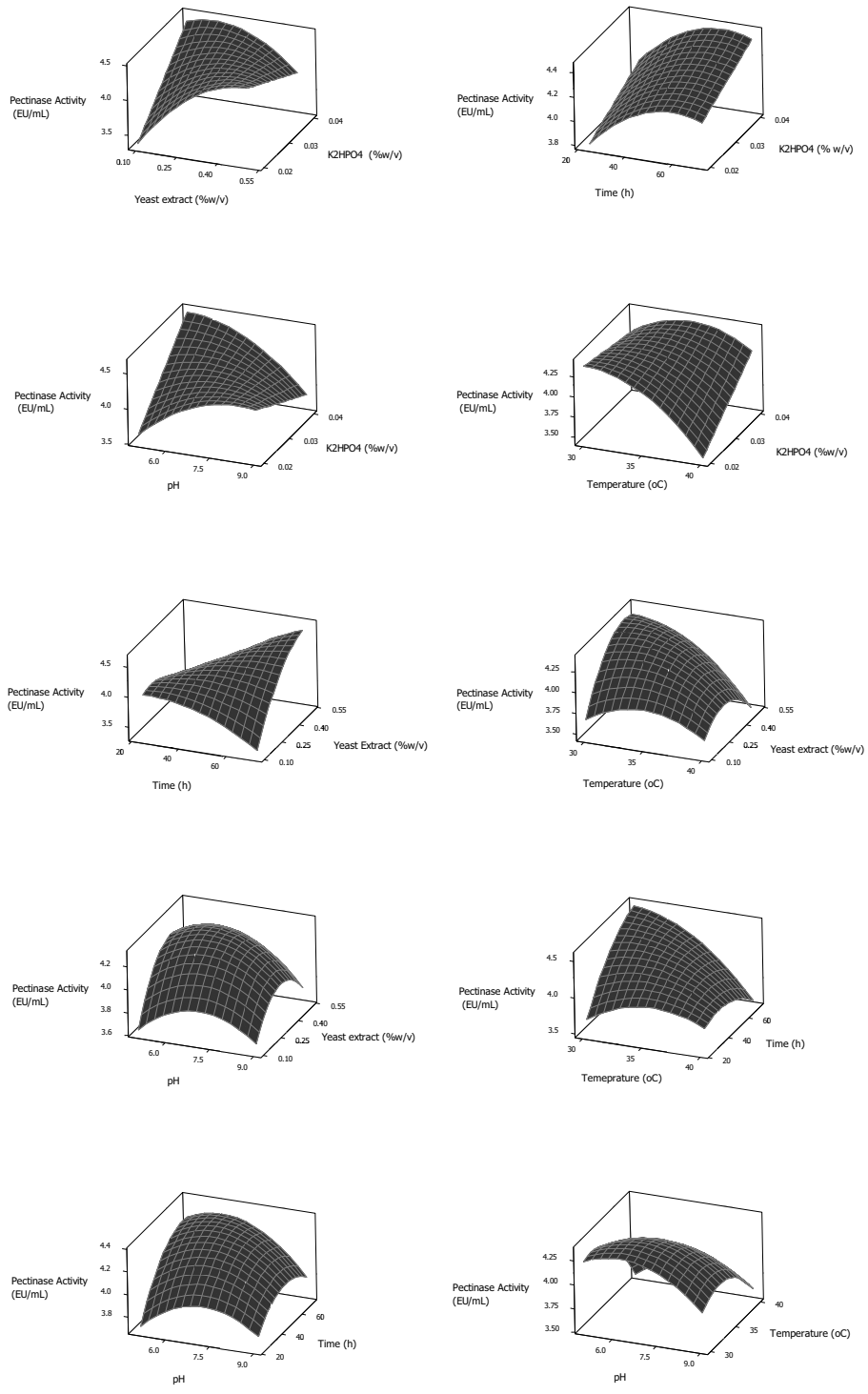


Figure 4.23 Surface plots showing the effect of a) [K_2HPO_4] and yeast extract concentration, b) [K_2HPO_4] and time, c) [K_2HPO_4] and pH, d) [K_2HPO_4] and temperature, e) yeast extract concentration and time, f) yeast extract concentration and temperature, g) yeast extract concentration and pH, h) time and temperature, i) time and pH and j) temperature and pH on pectinase production.

Temperature exerted a profound effect on pectinase production, giving high enzyme yields around 30-35 ^{o}C and decreasing at higher ends (Fig. 4.23.d,f,h). Similar mild temperatures were reported elsewhere (Ahlawat et al., 2009, Jayani et al., 2010, Joshi et al., 2013). Bayoumi et al. (2008) also obtained maximum pectinase activity in *Bacillus* at 37 °C at neutral pH. The influence of temperature is associated with the growth of the organism (Swain & Ray, 2009). It was also observed that pectinase activity increased when K_2HPO_4 increased and temperature decreased (Fig. 4.23.d).

An increase in yeast extract concentration led to the increasing PG activity, whereas this increase was limited by time (Fig. 4.23.e). Yeast extract contains vitamins, minerals and amino acids, which are necessary for bacterial growth and enzyme production, thus facilitating cell growth and enzyme production (Rehman et al., 2012). Fig. 4.23.f-j showed that as the levels of the variables increased, pectinase activity increased nonlinearly. Figure 4.23.f shows that pectinase activity initially increased and then decreased with the increase of yeast extract for a specified temperature. A similar nonlinear effect of yeast extract and pH on pectinase activity was observed in Fig. 4.23.g. Nonlinearity for different combinations of temperature, time and pH also existed in Fig 4.23.h-j. High temperature (35 ^{o}C) increases the solubility and diffusivity of proteins and no protein loss is indicated through thermal denaturation (Castilho et al., 2000). The enzyme activity decreased gradually as the temperature was raised to 40 ^{o}C. For most of the bacteria, pH optimum for growth and pectinase production is in the range of 7.0 to 10 (Ahlawat et al., 2009).

Three dimensional (3D) response contour plots were then plotted to observe the interaction effect of variables in pairs on pectinase production prior to determining the optimal conditions (Figure L.1). The remaining three factors were held constant at their center levels (i.e. pH 7.0, time 48 h, temperature 35 ^{o}C, yeast extract 0.3 (%w/v) and K_2HPO_4 0.03 (%w/v), respectively).

The shapes of the contour plots, which could be circular, elliptical, or saddle, provide another tool to indicate the significance of the interactions between the

variables. While a circular contour plot indicates negligible interaction, elliptical or saddle contour plot indicates a significant interaction between the corresponding variables (Murthy et al., 2000). In Figure I.1, the contour plot showed significant interaction between [K_2HPO_4]-yeast extract concentration, [K_2HPO_4]-pH, [K_2HPO_4]-temperature, yeast extract concentration- time, yeast extract concentration- temperature, yeast extract concentration-pH, time-temperature, time-pH and temperature-pH with the more elliptical or saddle shape than [K_2HPO_4]-time. In Figure I.1.b, the shape of the contour plots is rather circular, and [K_2HPO_4]-time interaction could be assumed negligible for pectinase production.

The optimum conditions giving maximal PG activity (5.60 EU/mL) were identified as 0.5 % (w/v) of yeast extract, pH 7.0, 72 h of fermentation time, 30 ^oC of temperature and 0.02 % (w/v) of K_2HPO_4. Thus, a 2.7 fold (or 166%) increase in pectinase production of *Bacillus subtilis* was achieved in shake flasks by PB and RSM optimization compared to unoptimized culture conditions. A 3.4 fold increase in PG production by *Bacillus subtilis* RCK was reported due to RSM optimization (Gupta et al., 2008). A 48% increase in enzyme activity of *A. sojae* ATCC 20235 was reported after optimizing culture conditions by RSM (Ustok et al., 2007). Similarly, a 1.5-fold increase in pectinase secretion of *Kluyveromyces wickerhamii* was reported in the study of Moyo et al. (2003) by RSM. These resulted proved that the PG production achieved in our study is comparable to those reported in the literature. Slight variation in the enzyme activity is inevitable due to differences in nature of the organism cultivated, varying culture conditions tested in RSM, and raw material used as carbon or nitrogen source.

To validate the model, additional runs were carried out for pectinase production at the optimal conditions predicted by RSM. The experimental PG activity of 4.84 EU/mL was slightly lower than the predicted value of 5.60 EU/mL. However, a low value of coefficient of variation, which was 10.6 %, indicated the adequacy of the model.

Soares et al. (1999) selected six strains of *Bacillus sp*. as good PG producers. The activities varied from 0.3 to 4.0 U/mL. Galiotou-Panayotou and Kapantai (1993)

achieved 3.0 U/mL of PG by submerged fermentation of *A. niger* in medium composed by pectin of citrus. Kashyap et al. (2001) obtained 15.7 U/mL after 30 h of fermentation by strains of *Bacillus sp*. Martins et al. (2007) found a maximum activity of 5.0 U/mL using *Thermoascus aurantiacus*. According to another study conducted by Jayani et al. (2010), the polygalacturonase activity was observed between 4.3-6.2 U/mL by *Bacillus sphaericus* (MTCC 7542). In this study, 16-hour-old inoculum was used at 7.5% (v/v) in synthetic medium and incubated in shaking conditions (160 rpm) for 72 h with the optimal temperature and pH as 30 ◦C and 6.8, respectively, for bacterial growth and polygalacturonase production (Jayani et al., 2010). Zeni et al. (2011) obtained polygalacturonase activity as 4.2 U/mL after 48 h of fermentation by strains of *Aspergillus sp*.

Comparing the results of work presented here with those reported in the literature, higher pectinolytic activities were obtained in this study.

4.4.3 Biochemical Characterization of the Crude Pectinase

The purpose of the biochemical characterization was to understand the activity or stability behavior of the produced enzyme under different conditions in order to evaluate its potential industrial applications in the area of biotechnology or food engineering (Gummadi & Panda, 2003).

4.4.3.1 Effect of pH on The Activity and Stability of Pectinase

The effect of pH on pectinase activity and stability were determined in a pH range of 5.0-9.0 under standard assay conditions. The highest pectinase activity was observed at pH 7.0 as shown in Figure 4.24.

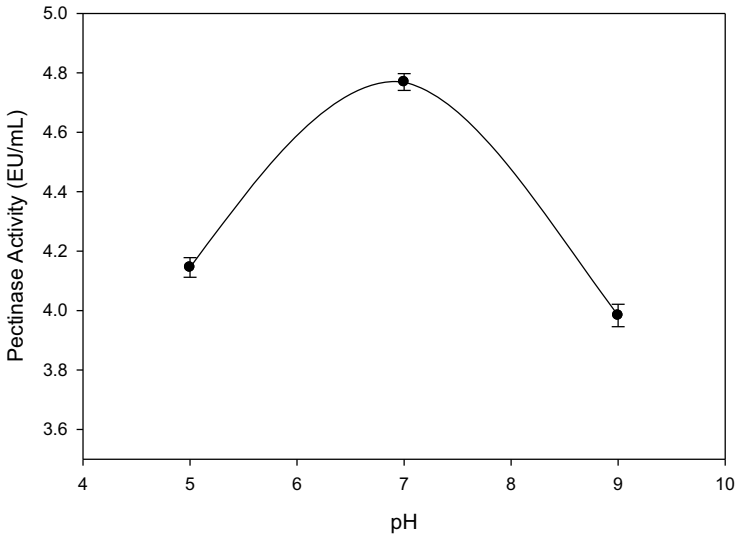


Figure 4.24 Effect of pH on the pectinase activity

The preference for a neutral pH for pectinase production was similar to that of *B.subtilis* MFW7 (Mukesh kumar et al., 2012), *B.subtilis* CM5 (Swain and Ray, 2010), *Bacillus sphaericus* (Jayani et al., 2010), *Bacillus spp*. (Kobayashi et al., 1999) and other *Bacillus strains* (Soares et al., 1999). Figure 4.25 illustrates the retained pectinase activity after 7 h incubation at different pH values. Pectinase was retained c.93% of its activity at pH 7.0 after 7h incubation.

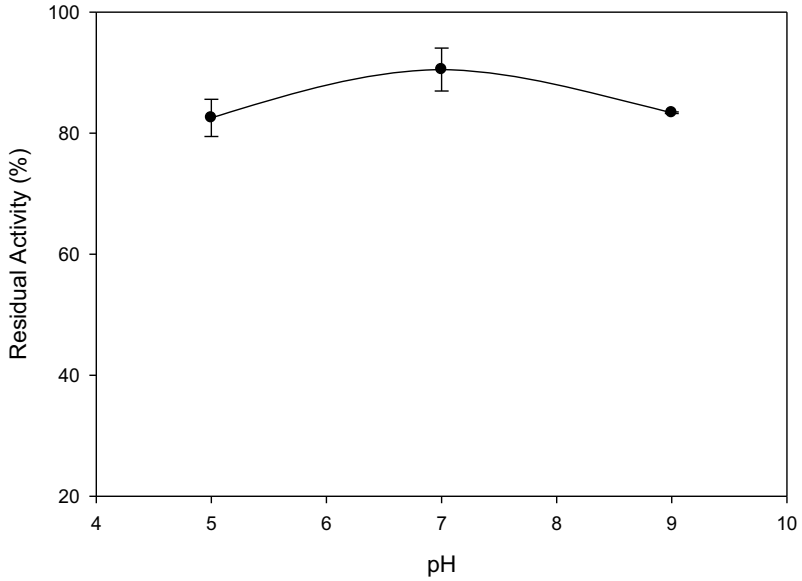


Figure 4.25 Effect of pH on the pectinase activity

4.4.3.2 Effect of Temperature on Pectinase Activity and Stability

The effect of temperature on pectinase activity and stability were determined at a temperature range of 30-40 ºC under standard assay conditions. The highest pectinase activity was observed at 30 ºC as shown in Figure 4.26. Several studies showed that the optimum temperature of pectinase activity obtained from *B.subtilis* was found as 30 ºC (Mukesh kumar et al., 2012; Kashyap et al., 2000). The optimum temperature of activity of *B.subtilis* is very suitable for fruit juice clarification applications which are generally held between 30-50 ºC.

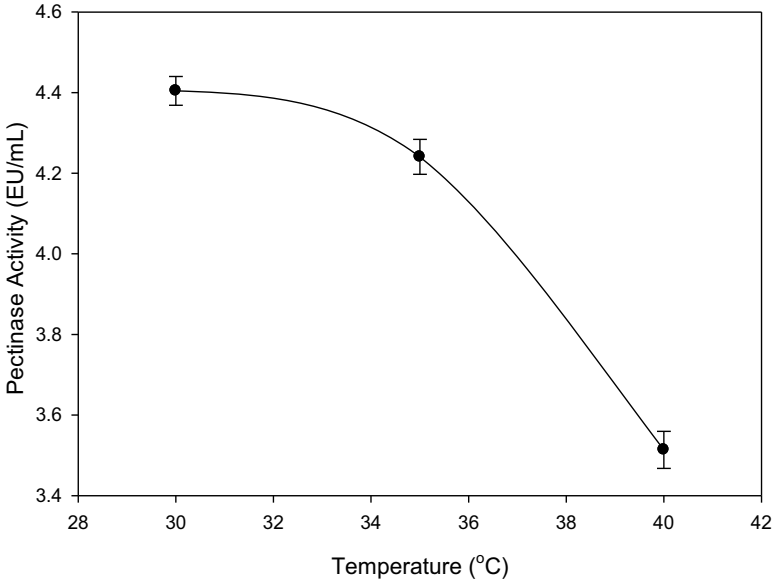


Figure 4.26 Temperature dependence of pectinase activity

The effect of temperature on pectinase stability was determined at a temperature range of 40-60 ºC under standard assay conditions Figure 4.27 illustrates the retained pectinase activity after 7 h incubation at different temperature values. Pectinase was retained c.93% of its activity at 50 ºC after 7h incubation. After this temperature, pectinase lost its stability sharply (79%).

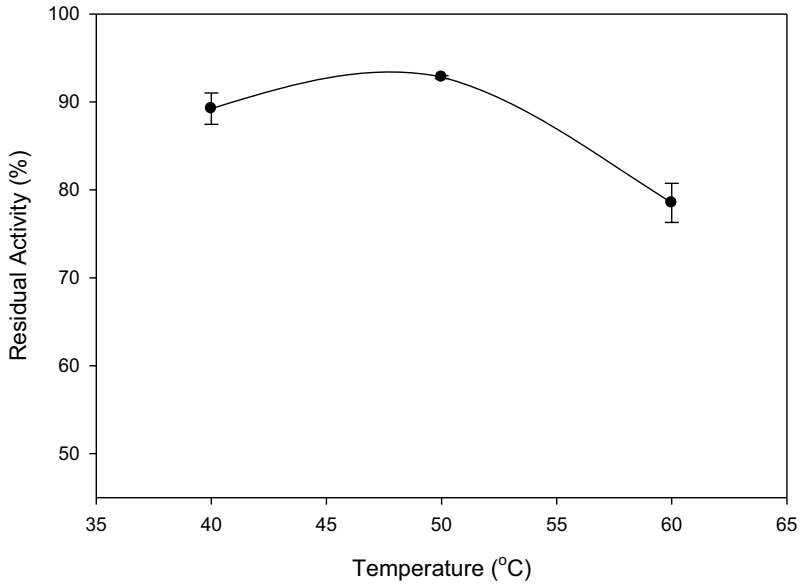


Figure 4.27 Temperature dependence of pectinase stability

This property indicated that the pectinase has sufficient thermostability at the enzymatic clarification of fruit juices held at 50 ºC. The half-life of pectinase at 50 ºC and 60 ºC were 82.5 and 21 h, respectively. Besides the half-life of pectinase at 50 ºC was higher than that of pectinase at 60 ºC showing that the enzyme was more stable at 50 ºC and easier to inactivate at 60 ºC.

4.4.3.3 Molecular Weight of Pectinase

Crude extract of pectinase exhibited 72.9 mg protein and a specific activity of 0.91 U/mg. After using 50 kDa ultrafiltration membranes, the enzyme purity increased 1.06 fold, with a specific activity of 0.96 U/mg (Table 4.28). According to the results, the molecular weight of pectinase was found around 50 kDa. There were

wide variations in molecular mass of exo-PG recorded from different microbial sources, that is, *Bacillus spp*. (20.3 kDa) (Kobayashi et al.,1999), *Aspergillus japonicum* (38 and 65 kDa) (Semenova et al., 2003), and *Thermotoga maritime* (151.2 kDa) (Kluskens et al., 2003).

Table 4. 28 Summary of the Pectinase Concentration from *B.subtilis*

Steps	Total Volume (mL)	Total activity (U)	Total Protein (mg)	[1*]Specific activity (U/mg)	[2*] Degree of Purification	[3*] Yield
Crude enzyme	15	4.86	72.90	0.91	1.00	100
<50 kDa	5	4.86	24.30	0.96	1.06	35.2
>50 kDa	5	6.35	31.75	0.77	0.84	36.7

[1*] Specific Activity (U/mg protein): PG activity (U/mL)/ Protein concentration (mg/mL)

[2*] Degree of Purification: Specific PG activity (U/mg)/Crude specific PG activity (U/mg)

[3*]Yield (%): [Total PG activity (U/mL)/Crude total PG activity (U/mL)]*100

4.5 Application of Crude Pectinase in Carrot Juice Clarification

The crude enzyme was applied for clarification of carrot juice and its effectiveness was studied after the optimization. The effect of varying enzyme concentration, pH and time on clarity (%) was evaluated.

4.5.1 Optimization of Clarification Variables Using Crude Pectinase Enzyme

The crude enzyme was applied to clarification of carrot juice and its effectiveness was evaluated by RSM optimization. The effect of varying enzyme concentration, pH and time was investigated in terms of clarity (%). Clarity is an important parameter of clarified juice (Sin et al., 2006). The experimental values for clarity under different conditions are presented in Table 4.29. Clarification of carrot juice in this study ranged between 78.77-97.73% treated with crude pectinase, irrespective of the enzyme concentration, pH and time. The maximum clarity of carrot juice (97.73%) was obtained at maximum conditions of enzyme concentration (0.3%), pH (7.0) and time (6h) (Table 4.29).

Table 4.29 BBD experimental design (in coded variables) employed for clarification of carrot juice

Serial number	Independent variables			Dependent variable
	Enzyme concentration (%)	pH	Time (h)	Clarity (%)
	X_1 (x_1)	X_2 (x_2)	X_3 (x_3)	Y
1	0.3 (0)	4.0 (-1)	6 (+1)	95.93±0.29 AB
2	0.3 (0)	7.0 (+1)	6 (+1)	97.73±0.47 A
3	0.3 (0)	5.5 (0)	4 (0)	85.68±0.37 F
4	0.5 (+1)	4.0 (-1)	4 (0)	82.71±0.34 G

Table 4.29 (continued)

5	0.5 (+1)	7.0 (+1)	4 (0)	94.00±0.79 BCD
6	0.5 (+1)	5.5 (0)	2 (-1)	86.15±0.29 F
7	0.3 (0)	4.0 (-1)	2 (-1)	78.77±0.48 H
8	0.3 (0)	5.5 (0)	4 (0)	86.24±0.42 F
9	0.1 (-1)	5.5 (0)	2 (-1)	84.58±0.96 FG
10	0.1 (-1)	4.0 (-1)	4 (0)	89.84±0.62 E
11	0.1 (-1)	5.5 (0)	6 (+1)	94.34±0.05 BC
12	0.3 (0)	7.0 (+1)	2 (0)	91.56±1.57 DE
13	0.5 (+1)	5.5 (0)	6 (+1)	95.82±0.46 ABC
14	0.1 (-1)	7.0 (+1)	4 (0)	93.22±0.62 CD
15	0.3 (0)	5.5 (0)	4 (0)	84.95±0.76 F

x represent the coded level of variables.
X represent the actual level of variables.
Figures in parentheses denote coded level of variables

The regression coefficients for the second order polynomial equation and results for the linear, quadratic and interaction terms are presented in Table 4.30. The statistical analysis indicated that the proposed model was adequate, possessing no significant lack of fit and with very satisfactory values of the R^2 for the response. The insignificant lack of fit for % clarity was ($P = 0.132 > 0.05$), which also proved that the model fit the experimental data well.

Table 4.30 ANOVA results* and estimated regression coefficients for the coded clarification of carrot juice model

Term	Coefficient	P Value
Regression		0.000
Linear		0.000
Square		0.000
Interaction		0.000
Lack-of-fit		0.119
Constant	85.62	0.000
Enzyme conc.(%)	-0.412	0.231
pH	3.657	0.000*
Time (h)	5.344	0.000*
Enzyme con. *Enzyme conc.	1.769	0.002*
pH*pH	2.547	0.000*
Time*Time	2.826	0.000*
Enzyme conc.*pH	1.978	0.000*
Enzyme conc.*Time	-0.021	0.964
pH*Time	-2.749	0.000*

R-Sq = 0.9631 % *R*-Sq(adj) = 0.9466 % *R*-Sq(pred) = 0.9128 %

*Result is significant when $P<0.05$

The derived polynomial equation was found adequate in representing the experimental data (R^2=0.9631). Another run with excluded insignificant terms according to Table M.1 expressed by Eqn. 34 and the final form of the equation in terms of coded values of factors was given as :

$$Y=85.62 +3.66X_2+5.34X_3+1.77X_1^2 +2.55X_2^2 +2.83X_3^2+ 1.98X_1X_2 -2.75X_2X_3 \quad (34)$$

where Y is predicted clarify (%), X_1, X_2, X_3 are coded values for enzyme concentration (%) , pH and time (h), respectively.

4.5.2 Effects of Enzyme Concentration, pH and Time

The effect of different enzyme treatment conditions on the clarity are reported (Table 4.30) by the regression coefficients. Linear (P=0.000), quadratic (P=0.000), and interaction effects (P=0.000) were found statistically significant. Among the variables studied, pH and treatment time showed significant effect (P<0.05) on clarity. The clarity depends on the enzyme concentration, where its linear (P>0.05) effect was not significantly affected and quadratic (P<0.05) effect was significantly affected by crude pectinase concentration. Interaction between enzyme concentration and pH and pH and time had significant effects (P<0.05) on clarity (Table 4.30).

The results showed significant interaction effects between pH and enzyme concentration at P<0.05 with a positive effect, therefore the pH was dependent on enzyme concentration. It showed a significant interaction effect between pH and time at P<0.05 with a positive effect (Table 4.30).

The most important factors determining the % clarity were time with highest coefficient (5.34), which indicates that it is the most dominant factor influencing the overall clarification of carrot juice using *B.subtilis* pectinase produced from hazelnut shell followed by pH (3.66) (Table 4.30).

To aid visualization, the response surface plots for clarity are shown in Figure 4.28-4.30. According to Kilara (1982), temperature may cooperate in the rate of enzymatic clarification process as the temperature is below denaturation temperature (40–60 ^{o}C). Therefore, moderate temperature could and should be used during clarification of carrot juice.

The positive linear and quadratic effects of time and pH explained the observed nature of the curve shown in Fig. 4.28. The clarity increased with increase of pH and time at constant enzyme concentration.

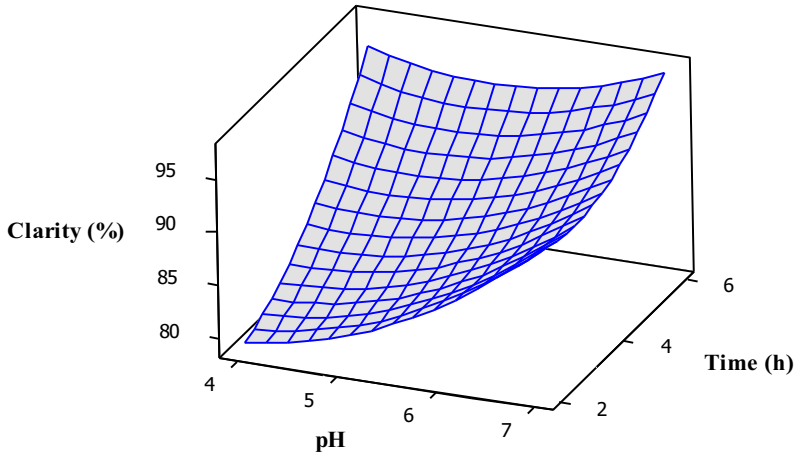


Figure 4.28 Surface plots showing the effect of pH and time on clarification of carrot juice

At constant pH, the clarity was found to decrease with enzyme concentration up to 0.40 (%) and increases slowly thereafter (Fig. 4.29). Increase in enzyme concentration may increase the rate of clarification due to the exposure of the positively charged protein beneath, thus reducing electrostatic repulsion between cloud particles, which cause these particles to aggregate to larger particles and eventually settle out (Sin et al., 2006). It may also be observed from the Fig. 4.29 that clarity increased with increasing time.

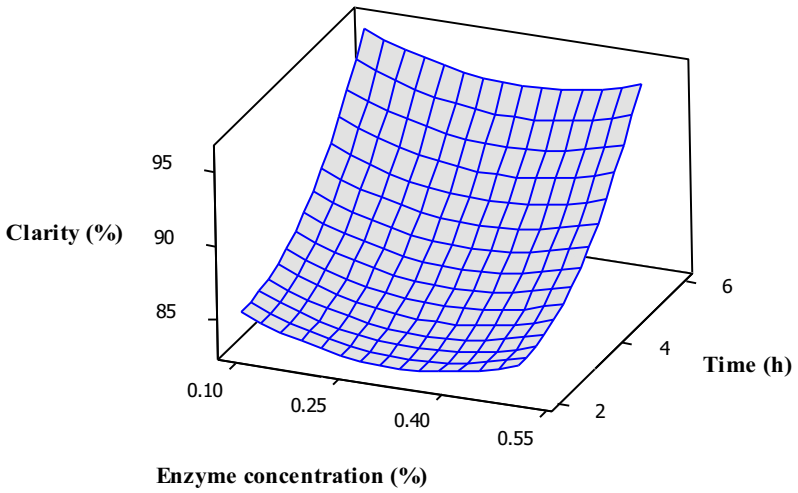


Figure 4.29 Surface plots showing the effect of enzyme concentration and time on clarification of carrot juice.

Sin et al. (2006) investigated the optimum conditions (0.1% enzyme concentration at 40 ^{o}C for 120 min) for clarification of sapodilla juice which was treated with pectinase enzyme at different incubation times (30–120 min), temperature (30–50 ^{o}C) and enzyme concentration (0.03–0.10%). A similar behaviour for the clarity was observed for the changes in incubation time with Sin et al. (2006). As time increased, fine particles in juice may also slowly settle down (Sin et al., 2006).

At the lowest level of enzyme concentration, the clarity of carrot juice was found to increase rapidly at the beginning with an increase in pH (Fig. 4.30). Degradation of pectin causes a decrease in water holding capacity, and consequently, free water is released to the system, which increases the clarity of juice (Demir et al., 2000).

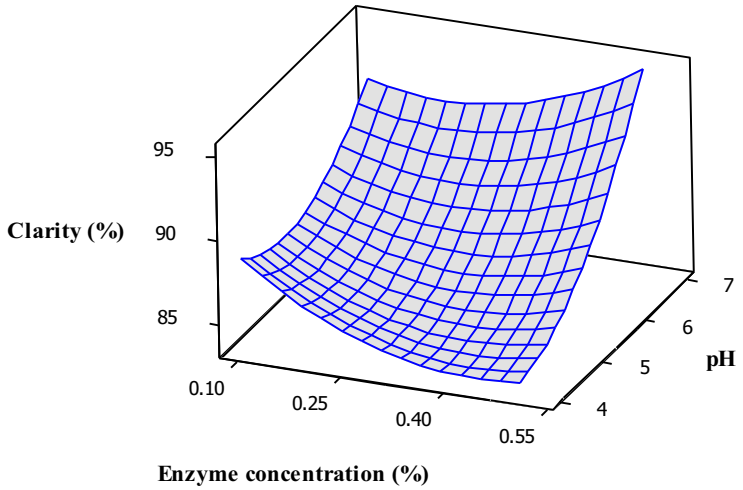


Figure 4.30 Surface plots showing the effect of enzyme concentration and pH on clarification of carrot juice.

The optimum conditions for clarification of carrot juice predicted by the model was found as 0.5 % enzyme concentration, 7.0 pH and 6 h. To confirm the validity of RSM model, six optimum check points were selected by intensive grid search performed over the entire experimental domain. Linear regression plots between the observed and predicted values of the response are drawn (Fig. 4.31). The constructed model was also assessed using error analysis. The root mean square error and mean absolute error values were calculated as 1.99 and 0.76, respectively. A high value of coefficient of determination (R^2=0.9631) showed that the model was successful in predicting clarity of the carrot juice (Fig. 4.31).

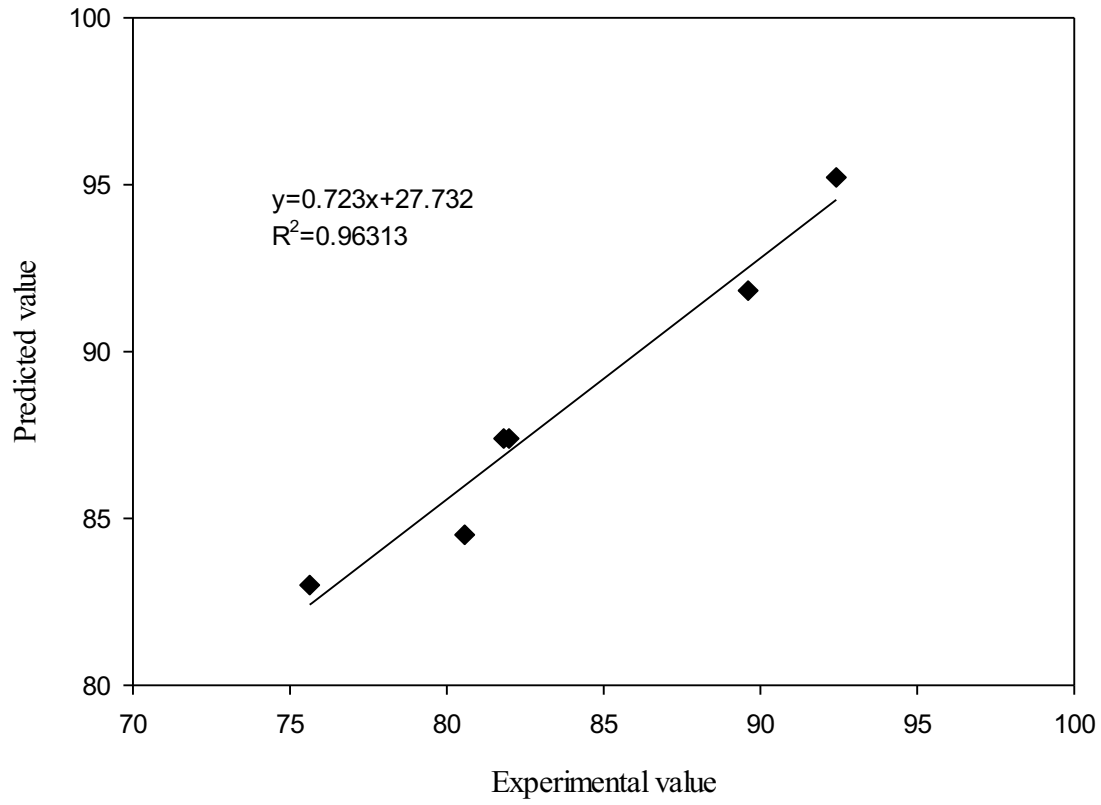


Figure 4.31 Verification of the model obtained by RSM

The clarification yield (%) using commercial enzyme was obtained as 78.18±3.14, whereas clarification yield (%) using crude enzyme was obtained as 94.47±0.01 at pH 7.0 with 0.5% enzyme concentration for 6 h. According to Swain and Ray (2010), yield of carrot juice was found as 70 and 95 % in Pectinex 3XL and *B.subtilis* polygalacturonase treated samples, respectively, over control (no enzyme treated sample) after 8 h of incubation. Incubation time of 2-16 h was found optimum for clarification of juice from apple, pears, and grapes (Kaur et al., 2004). This study also showed that crude pectinase provided good clarification of carrot juice as well. The crude pectinase was also as effective and competitive as commercial pectinase.

CHAPTER 5

CONCLUSIONS

This study investigated the potential use of hazelnut shell as carbon source in pectinase production from *Bacillus subtilis* by submerged fermentation. For this purpose, the pretreatment methods such as ozone, alkaline and dilute acid were employed to produce sugar monomers for enhancing the pectinase production.

Based on the produced reducing sugar yields, dilute acid pretreatment method was selected as the best chemical pretreatment method. An optimum reducing sugar concentration of 16.65 g/L (62.8 % saccharification yield) was achieved with low levels of acetic acid, HMF and no furfurals during acidic pretreatment. The overall sugar yield of combined acid and enzymatic hydrolysis reached 72.4% of the theoretical. The combined dilute acid and enzymatic hydrolysis was also found more effective than acid hydrolysis alone. The results indicated that hazelnut shell is an attractive source of fermentable sugars (more than 50 %) and dilute-acid hydrolysis is an effective process to produce sugars from hazelnut shells for production of various value added products.

Hazelnut shells, an inexpensive agro-residue, was shown to serve as an appropriate substrate for production of PG by *Bacillus subtilis* by optimizing submerged fermentation medium composition and conditions using PB and RSM approach.

It was concluded that PG production was influenced significantly by all factors and their interactions, except for time vs. K_2HPO_4 and squared K_2HPO_4. Significant squared terms also reflect the limiting increase in pectinase production by pH, temperature, time, and yeast extract levels, where upper and lower values resulted in low pectinase production, while K_2HPO_4 possessed linear effects in all plots.

A 2.7 fold enhancement in enzyme production was achieved compared to

unoptimized fermentation trials. The optimization results indicated that a maximal PG activity of 5.60 EU/mL was achieved at pH 7.0, 72 h, and 30 ^{o}C, 0.5 % (w/v) yeast extract and 0.02 % (w/v) of K_2HPO_4.

B. subtilis produced remarkable level of PG activity at neutral pH; hence, it can potentially be used to increase the extraction yield of banana, carrot, grape, or apple juice. The RSM results revealed that 100% clarity was attained under optimal clarification conditions of 0.5% (w/v) enzyme concentration, 7.0 pH, and 6 h of clarification period. The results also indicated that the produced crude pectinase was equally effective and competitive as the commercial enzyme.

CHAPTER 6

RECOMMENDATIONS

Kinetic studies of submerged pectinase production from *B.subtilis* using hazelnut shell hydrolyzate are required for better understanding and describing the behaviour of pectinase produced.

This study provided a preliminarily study of cost analysis for dilute acid pretreatment. Thus, further detailed analysis is necessary to determine the economic feasibility of dilute acid pretreatment and enzymatic hydrolysis.

The medium composition and culture conditions were optimized using shake-flask bioreactors. Thus, the optimum factors should be implemented at large scale bioreactors with strictly controlled extrinsic parameters such as aeration rate, pH etc. Also, purification and characterization of pectinase produced by *B. subtilis* may be carried out.

REFERENCES

Acar, J., & Gokmen, V. (2005). Meyve ve sebze işleme teknolojisi, Vol 1, Meyve ve sebze suları üretimi, Hacettepe Üniversitesi yayınları, Ankara, Türkiye.

Agblevor, F.A., Batz, S., & Trumbo, J. (2003). Composition and ethanol production potential of cotton gin residues. *Applied Biochemistry Biotechnology*, 105-108, 219-230.

Ahlawat, S., Dhiman, S.S., Battan, B., Mandhan, R.P., & Sharma, J. (2009). Pectinase production by *Bacillus subtilis* and its potential application in biopreparation of cotton and micropoly fabric. *Process Biochemistry*, 44, 521-526.

Ahlawat, S., Mandhan, R.P., Dhiman, S.S., Kumar, R., & Sharma, J. (2008). Potential application of alkaline pectinase from *Bacillus subtilis* SS in pulp and paper industry. *Applied Biochemistry and Biotechnology*, 149, 287-293.

Aiduan, L., Blanca, A.L., & Majeda, K. (2007). Bioconversion of municipal solid waste to glucose for bio-ethanol production. *Bioprocess and Biosystems Engineering*, 30, 189-196.

Akpinar, O., Levent, O., Bostanci, S., Bakir, U., & Yılmaz, L. (2011). The optimization of dilute acid hydrolysis of cotton stalk in xylose production. *Applied Biochemistry Biotechnology, 163, 313-325.*

Alkorta, I., Garbisu, C., Liama, M.J., & Serra, J.L.(1998). Industrial applications of pectin enzymes: a review. *Process Biochemistry*, 33(1), 21-28.

Andrade, M.V.V., Delatorre, A.B., Ladeira, S.A., & Martins, M.L.L. (2011). Production and partial characterization of alkaline polygalacturonase secreted by thermophilic *Bacillus sp*. SMIA-2 under submerged culture using pectin and corn steep liquor. Cienc. Technol. Aliment. Campinas 31(1):204-208.

Amenaghawon, A.N., Okieimen, C.O., & Ogbeide, S.E. (2014). Modeling and statistical optimization of dilute acid hydrolysis of eucalyptus wood chips using response surface methodology.*The Pacific Journal of Science and Technology,* 15, 1, 245-256.

AOAC (1984). Official methods of analysis. Association of Official Analytical Chemists. Washington, DC.

Arslan, Y., & Saraçoglu, N.E. (2010). Effects of pretreatment methods for hazelnut shell hydrolysate fermentation with *Pichia stipitis* to ethanol. *Bioresource Technology*, 101, 8664–8670.

Arslan, Y., Takac, S., & Saraçoglu, N.E. (2011). Kinetic study of hemicellulosic sugar production from hazelnut shells. *Chemical Engineering Journal*, 185-186, 23-28.

Aswathy, U.S., Sukumaran, R.K., Devi, G.L., Rajasree, K.P., Singhania, R.R., & Pandey, A. (2010). Bioethanol from water hyacinth biomass: An evaluation of enzymatic saccharification strategy. *Bioresource Technology*, 101, 925-930.

Baek, S.C., & Kwon, Y.J. (2007). Optimization of the pretreatment of rice straw hemicellulosic hydrolyzates for microbial production of xylitol. *Biotechnology and Bioprocess Engineering*, 12, 404–419.

Bailey, M.J., Biely, P., & Pountanen, K. (1992). Interlaboratory testing of methods for assay xylanase activity. *Biochimica et Biophisica Acta*, 117, 252-270.

Bas¸ D., & Boyaci, İ.H. (2007). Modeling and optimization II: Comparison of estimation capabilities of response surface methodology with artificial neural networks in a biochemical reaction. *Journal of Food Engineering*, 78, 846-854.

Bas, D., Dudak, F. C., & Boyaci, I. H. (2007). Modeling and optimization III: Reaction rate estimation using artificial neural network (ANN) without a kinetic mode. *Journal of Food Engineering*, 79, 622-628.

Bayoumi, R.A., Yassin, H.M., Swelim, M.A., & Abdel-All, E.Z. (2008). Production of bacterial pectinases from agro-industrial wastes under solid state fermentation conditions. *Journal of Applied Sciences Research*, 4(12), 1708-1721.

Ben-Ghedalia, D., & Miron, J. (1981). The effect of combined chemical and enzyme treatments on the saccharification and in vitro digestion rate of wheat straw. *Biotechnology and Bioengineering*, 23, 823–831.

Ben-Ghedalia, D., & Shefet, G. (1983). Chemical treatments for increasing the digestibility of cotton straw: 2. Effect of ozone and sodium hydroxide treatments on the digestibility of cell-wall monosaccharides. *Journal of Agricultural Science,* 100, 401-406.

Bohdziewiez, J., & Bodzek, M. (1994). Ultrafiltration preparation of pectinolytic enzymes from citric acid fermentation broth. *Process Biochemistry*, 29, 99-107.

Box, G., & Behnken, D. (1960). Some new three level designs for the study of quantitative variables. *Technometrics*, 2, 455-475.

Brawman, J.W. (1981). Application of enzymes in fruit juice technology. In: Birch GG, Blakcorough N, Barker JK, editors. Enzymes and food processing. London: Applied Science, 247-261.

Browning, B.L. (1967). In Methods of Wood Chemistry: Determination of Sugars, New York: Inter-Science, 589–590.

Brodeur, G., Yau, E., Badal, K., Collier, J., Ramachandran, K.B., & Ramakrishnan, S. (2011). Chemical and physicochemical pretreatment of lignocellulosic biomass: a review. *Enzyme Reseach*, doi:10.4061/2011/787532.

Cao, G., Ren, N., Wang, A., Lee, D.J., Guo, W., Liu, B., Feng, Y., & Zhao, Q. (2009). Acid hydrolysis of corn stover for biohydrogen production using *Thermoanaerobacterium thermosaccharolyticu* W16. *International Journal of Hydrogen Energy*, 34, 7182–7188.

Castilho, L.R., Medronho, R.A., & Alves, T.L.M. (2000). Production and extraction of pectinases obtained by solid state fermentation of agroindustrial residues with *Aspergillus niger*. Bioresource Technology, 71, 45-50

Chang, V.S., & Holtzapple, M.T. (2000). Fundamental factors affecting biomass enzymatic reactivity. *Applied Biochemistry and Biotechnology*. - Part A Enzyme Engineering and Biotechnology, 84-86, 5-37.

Charley, V.L.S. (1969). Advances in food processing using pectic and other enzymes. *Chemistry and Industry*, 20, 635-641.

Chaudhri, A., & Suneetha, V. (2012). Microbially derived pectinases: A review. *Journal of Pharmacy and Biological Sciences*, 2 (2), 1-5.

Chen, Y., Stevens, M.A., Zhu, Y., Holmes, J., & Xu, H. (2013). Understanding of alkaline pretreatment parameters for corn stover enzymatic saccharification. *Biotechnology for Biofuels*, 6, 8, 1-10.

Chesson, A., & Codner, R.C. (1978). Maceration of vegetable by a strain of *Bacillus subtilis*. *Journal of Applied Bacteriology*, 45, 219-230.

Christian, V., Shrivastava, R., Shukla, D., Modi, H.A., & Vyas, B.R. (2005). Degradation of xenobiotic compounds by lignin-degrading white-rot fungi: enzymology and mechanisms involved. *Indian Journal of Experimental Biology*, 43(4), 301-12.

Clausen, E.C., & Gaddy, J.L. (1993). Concentrated sulfuric acid process for lignocellulosic materials to sugars. *United States Patent, 5, 188-673.*

Combo, A.M.M., Aguedo, M., Goffin, D., Wathelet, B., & Paquot, M. (2012). Enzymatic production of pectic oligosachharides from polygalacturonic acid with commercial pectinase preparations. *Food and Bioproducts Processing*, 90, 588-596.

Corredor, D.Y. (2008). Pretreatment and enzymatic hydrolysis of lignocellulosic biomass. PhD thesis, Kansas State University, USA.

da Costa Sousa, L., Chundawat, S.P., Balan, V., & Dale, B.E. (2009). Cradle-to-grave assessment of existing lignocellulose pretreatment technologies. *Current Opinion in Biotechnology*, 20(3), 339-47.

Dave, B.A., & Vaughn, R.H. (1971). Purification and properties of a polygalacturonic acid *trans*-eliminase produced by *Bacillus pumilus*. *Journal of Bacteriology*, 108(1), 166-174.

Delgenes, J.P., Penaud, V., & Moletta, R. (2002). Pretreatments for the enhancement of anaerobic digestion of solid wastes. Biomethanization of the organic fraction of municipal solid wastes: IWA Publishing. p 201-228.

Demir, N., Acar, J., Sarioglu, K., & Mutlu, M. (2000). Use of commercial pectinase in fruit juice industry—part 3: immobilized pectinase for mash treatment. *Journal of Food Engineering*, 47, 4, 275–280.

Demir, H., Gögüş, N., Tarı, C., Heerd, D., & Lahore, M.F. (2012). Optimization of the process parameters for the utilization of orange peel to produce polygalacturonzase by solid-state fermentation from an *Aspergillus sojae* mutant strain. *Turkish Journal of Biology*, 36, 394-404.

Demirbaş¸ E., Kobya, M., Oncel, S., & Sencan, S. (2002). Removal of Ni(II) from aqueous solution by adsorption onto hazelnut shell activated carbon: equilibrium studies. *Bioresource Technology*, 84, 291–293.

Demirbaş, A. (2005). Bioethanol from cellulosic materials: A renewable motor fuel from biomass. *Energy Sources*, 27, 327-337.

Demirbaş, A. (2006). Furfural production from fruit shells by acid catalyzed hydrolysis. *Energy Source*, 28, 157–165.

Djekrif-Dakhmouche, S., Gheribi-Aoulmi, Z., Meraihi, Z., & Bennamoun, L. (2006). Application of a statistical design to the optimization of culture medium for

a amylase production by *Aspergillus niger* ATCC 16404 grown on orange waste powder. *Journal of Food Engineering*, 73, 190–197.

Eckert, R.C., & Singh, R.P. (1975). Ozone reactions in relation to the aromatic structure of lignin: a review of selected topics in ozone chemistry. In: Gratzl JS, Nakano J, Singh RP, editors. Chemsitry of delignification with oxygen, ozone and peroxides, Raleigh, NC. Uni publishers.

Eisenhuber, K., Krennhuber, K., Steinmüller, V., & Jager, A. (2013). Comparison of different pretreatment methods for separating hemicellulose from straw during lignocellulose bioethanol production. *Energy Procedia*, 40, 172-181.

El-Sheekh, M.M., Ismail, A.S., El-Abd, M.A., Hegazy, E.M., El-Diwany, A.I. (2009). Effective technological pectinases by Aspergillus carneus NRC1 utilizing the Egyptian orange juice industry scraps. *International Biodeterioration and Biodegradation*, 63(1), 12-18.

Fengel, D., & Wegener, G. (1984). Wood, Chemistry, Ultrastructure, Reactions. De Gruyter, Berlin.

Ferrer, A., Requejo, A., Rodriguez, A., & Jimenez, L. (2013). Influence of temperature, time, liquid/solid ratio and sulphric acid concentration on the hydrolysis of palm empty fruit bunches. *Bioresource Technology*, 129, 506-511.

Fischbach, J., & Brasseur, K. (2012). Processing American and hybrid hazelnuts: A guide for hazelnut growers in the Upper-West. *Upper-West Hazelnut Development Initiative*, 1-21.

Fogarty, W.M., & Kelly, C.T. (1983). Pectic enzymes. In: Microbial enzymes and biotechnology, W.M. Fogarty, (Ed.), pp. 131-182, Applied Science Publishers, ISBN: 0853341850, London, England.

Galbe, M., & Zacchi, G. (2007). Pretreatment of lignocellulosic materials for efficient bioethanol production. Olsson L, editor. Berlin: Springer.

Galiotou-Panayotou, M.P.R., & Kapantai, M. (1993) Enhanced polygalacturonase production by *Aspergillus niger* NRRL-364 grown on supplemented citrus pectin. *Letters in Applied Microbiology*, 17, 145–148.

Garcia-Cubero, M.A., Gonzalez-Benito, G., Indacoechea, I., Coca, M., & Bolado, S. (2009). Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. *Bioresource Technology*, 100(4), 1608-1613.

Garcia-Cubero, M.T., Coca, M., Bolado, S., & Gonzalez-Benito, G. (2010). Chemical oxidation with ozone as pre-treatment of lignocellulosic materials for bioethanol production. *Chemical Engineering Transactions*, 21.

Garcia-Cubero, M.T., palacin, L.G., Gonzalez-benito, G., Bolado, S., Lucaz, S., & Coca, M. (2012). An analysis of lignin removal in a fixed bed reactor by reaction of cereal straws with ozone. *Bioresource Technology*, 107, 229-234.

Ghose, T.K. (1987). Meausrement of cellulase activities. *Pure and Applied Chemistry*, 59, 257-268.

Goh, C.S., Lee, K.T., & Bhatia, S. (2010). Hot compressed water pretreatment of oil palm fronds to enhance glucose recovery for production of second generation bio-ethanol. *Bioresource Technology*,101, 7362-7367.

Gummadi, S.N., & Panda, T. (2003). Purification and biochemical properties of microbial pectinases- a review. *Process Biochemistry*, 38, 987-996.

Gupta, S., Kapoor, M., Sharma, K.K., Nair, L.M., & Kuhad, R.C. (2008). Production and recovery of an alkaline exo-polygalacturonase from *Bacillus subtilis* RCK under solid-state fermentation using statistical approach. *Bioresource Technology*, 99, 937-945.

Haaland, P.D. (1989). Statistical problem solving. In: Experimental design in biotechnology. New York: Marcel Dekker Inc. 1–18.

Hara, A. & Radin, N.S. (1978). Lipid extraction of tissues with a low toxicity solvent. Analytical Biochemistry, 90, 420-426.

Hatakka, A.I., (1983). Pretreatment of wheat straw by white-rot fungi for enzymic saccharification of cellulose. *European Journal of Applied Microbiology and Biotechnology,* 18 (6), 350-357.

Hendriks, A.T.W.M., & Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology*, 100, 10-18.

Hu, G., Heitmann, J., & Roja, O. (2008). Feedstock pretreatment strategies for producing ethanol from wood, bark, and forest residues. *BioResources*, 3(1), 270-294.

Hu, R., Lin, L., Liu, T., & Liu, S. (2010). Dilute sulfuric acid hydrolysis of sugar maple wood extract at atmospheric pressure. *Bioresource Technology*, 101(10), 3586-3594.

Idrees, M., Adnan, A., Bokhari, S.A., & Qureshi, F.A. (2014). Production of fermentable sugars by combined chemo-enzymatic hydrolysis of cellulosic material for bioethanol production. *Brazilian Journal of Chemical Engineering*, 31, 2, 355-363.

Jayani, R.S., Saxena, S., & Gupta, R. (2005). Microbial pectinolytic enzymes: A review. *Process Biochemistry,* 40, 2931-2944.

Jayani, R.S., Shukla, S.K., & Gupta, R. (2010). Screening of bacterial strains for polygalacturonase activity: Its production by *Bacillus sphaericus* (MTCC 7542). *Enzyme Research*, DOI: 10.4061/2010/306785.

Jorgensen, H., Kristensen, J.B., & Felby, C. (2007). Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels, Bioproducts and Biorefining*, 1, 119-134.

Joshi, M., Nerurkar, M., & Adivarekar, R. (2013). Use of citrus limetta peels for pectinase production by marine *Bacillus subtilis*. *Innovative Romanian Food Biotechnology*, 12, 75-83.

Joslyn, N.A., Mist, S., & Lambart, E. (1952). Clarification of apple juice by fungal PG preparations. *Food Technology*, 6, 133-139.

Kang, L. (2011). Bioconversion of pulp and paper mills sludge and prehydrolysate stream into ethanol and cellulose enzyme. PhD thesis, Auburn University, Alabama, USA.

Kapoor, M., Beg, Q.K., Bhushan, B., Dadhich, K.S., & Hoondal, G.S. (2000). Production and partial purification and characterization of a thermo-alkali stable polygalacturonase from *Bacillus* sp. MG-cp-2. *Process. Biochemistry*, 36, 467-473.

Kapoor M., & Kuhad R.C. (2002). Improved polygalacturonase production from *Bacillus* sp. MG-cp-2 under submerged (SmF) and solid state (SSF) fermentation. *Letters in Applied Microbiology*, 34, 317-322.

Karunanithy, C., & Muthukumarappan, K. (2011). Optimization of alkali soaking and extrusion pretreatment of prairie cord grass for maximum sugar recovery by enzymatic hydrolysis. Biochemical Engineering Journal, 54, 71-82.

Kashaninejad, M., Dehghani, A., & Kashiri, M. (2009). Modeling of wheat soaking using two artificial neural networks (MLP and RBF). *Journal of Food Engineering,* 91, 602-607.

Kashyap D.R., Chandra S., Kaul A., & Tewari R. (2000). Production, purification and characterization of pectinase from a Bacillus sp. DT7. *World Journal of Microbiology and Biotechnology*, 16, 277-282.

Kashyap, D.R., Vohra, P.K., Chopra, S., & Tewari, R. (2001). Applications of pectinases in the commercial sector: a review. Bioresource Technology, 77, 215-227.

Kashyap, D.R., Soni, S.K., & Tewari, R. (2003). Enhanced production of pectinase by *Bacillus* sp. DT7 using solid state fermentation. *Bioresource Technology*, 88, 251-254.

Kaur, G., Kumar, S., & Satyanarayana, T. (2004). Production, characterization and application of a thermostable polygalacturonase of a thermophilic mould *Sporotrichum thermophile* Apinis. *Bioresource Technology*, 94, 3, 239-243.

Kaur, U., Oberoi, H.S., Bhargav, V.K., Sharma-Shivappa, R., & Dhaliwal, S.S. (2012). Ethanol production from alkali- and ozone-treated cotton stalks using thermotolerant *Pichia kudriavzevii* HOP-1. *Industrial Crops and Products*, 37, 219–226.

Kaushik, R., Saran, S., Isar, J., & Saxena, R.K. (2006). Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipase production by *Aspergillus carneus*. *Journal of Molecular Catalysis B: Enzymatic*. 40, 121–126.

Keshwani, D.R. (2009). Microwave pretreatment of switchgrass fr bioethanol production. PhD thesis in Biological and Agricultural Engineering, North Carolina University, Raleigh, USA.

Keshwani, D.R., & Cheng, J.J. (2009). Switchgrass for bioethanol and other value-added applications: a review. *Bioresource Technology*, 100(4), 1515-23.

Khairnar Y., Krishna V., Boraste A., Gupta N., Trivedi S., Patil P., Gupta G., Gupta M., Jhadav A., Mujapara A., Joshi B., & Mishra D. (2009). Study of pectinase production in submerged fermentation using different strains of *Aspergillus niger*. *International Journal of Microbiology Research*, 1 (2), 13-17.

Kilara, A. (1982). Enzymes and their uses in the processed apple industry: A review. *Process Biochemistry*, 23, 35–41.

Klinke, H.B., Thomsen, A.B., & Ahring, B.K. (2004). Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pretreatment of biomass. *Applied Microbiology and Biotechnology*, 66, 10–26.

Kluskens, L.D., van Alebeek, G.J.W.M., Voragen, A.G.J., De Vos, W.M., Van Der Oost, J. (2003). Molecular and biochemical characterization of the thermo active family 1 pectate lysase from the hyperthermophilic bacterium *Thermotoga maritima*. *Biochemical Journal*, 370, 651–659.

Kobayashi, T., Koike, K., Yoshimatsu, T., Higaki, N., Suzumatsu, A., Ozawa, T., Hatada, Y., & Ito, S. (1999). Purification and properties of a low-molecular-weight, high-alkaline pectate lyase from an alkaliphilic strain of Bacillus. *Bioscience, Biotechnology and Biochemistry*, 63 (1), 65–72.

Koksal, A.I., Artik, N., Simsek, A., & Gunes, N. (2006). Nutrient composition of hazelnut (*Corylus avellana* L.) varieties cultivated in Turkey. *Food Chemistry*, 99, 509-515.

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*. 48:3713–3729

Kumar, R., & Wyman, C.E. (2009). Does change in accessibility with conversion depend on both the substrate and pretreatment technology? *Bioresource Technology*, 100(18), 4193-4202.

Larsson, S., Reimann, A., Nilverbrant, N., & Jonsson, L. (1999). Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce. *Applied Biochemistry and Biotechnology*, 77-79, 91-103.

Lavarack, B.P., Griffin, G.J., & Rodman, D. (2002). The acid hydrolysis of sugarcane bagasse hemicellulose to produce xylose, arabinose, glucose and other products. *Biomass Bioenergy,* 23, 367-380.

Lee, W.C., Yusof, S., Hamid, N.S.A., & Baharin, B.S. (2006). Optimizing conditions for enzymatic clarification of banana juice using response surface methodology (RSM). *Journal of Food Engineering*, 73, 55-63.

Lee, J. M., Jameel, H., & Venditti, R. A. (2010). Effect of ozone and autohydrolysis pretreatments on enzymatic digestibility of coastal Bermuda grass. *Bioresources*, 5 (2), 1084–1101.

Li, Z., Bai, Z., Zhang, B., Xie, H., Hu, O., Hao, C., Xue, W., & Zhang, H. (2005). Newly isolated *Bacillus gibsonii* S-2 capable of using sugar beet pulp for alkaline pectinase production. *World Journal of Microbiology Biotechnology*, 21, 1483-1486.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with the Folin Phenol Ragent. *Journal of Biological Chemistry*, 193, 265-275.

Lynd, L.R. (1996). Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment, policy. *Annual Review of energy and the Environment*, 21, 403-465.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., & Pretorious, I.S. (1999). Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews,* 66(3), 506-577.

Mansilla, H.D., Baeza, J., Urzúa, S., Maturana, G.,. Villaseñor, J., & Durán, N. (1998). Acid-catalysed hydrolysis of rice hull: Evaluation of furfural production. *Bioresource Technology*, 66(3), 189-193.

Martins, E.S., Silva, D., da Silva, R., Leite, R.S., & Gomes, E. (2007). Purification and characterization of polygalacturonase produced by thermophilic *Thermoascus aurantiacus* CBMAI-756 in submerged fermentation. Antonie Van Leeuwenhoek 91, 291–299.

Mcintosh, S., & Vancov, T. (2011). Optimization of dilute alkaline pretreatment for enzymatic saccharification of wheat straw. *Biomass and Bioenergy*, 35, 3094-3103.

McKillip, W.J., & Collin, G. (2002). Ullmann's Encyclopedia of Industrial Chemistry, Sixth edition, Weinheim, Germany, Wiley-VCH.

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

Mehdizadeh, B., & Movagharnejad, K. (2011). A comparison between neural network method and semi empirical equations to predict the solubility of different compounds in supercritical carbon dioxide. *Fluid Phase Equilibria*, 303, 40-44.

Miller, G.L. (1959). Use of dinitrosalysilic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31 (3), 426-428.

Mingzhi, H., Ma, Y., Jinquan, W., & Yan, W. (2009). Simulation of a paper mill wastewater treatment using a fuzzy neural network. *Journal Expert Systems with Applications*, 36 (3), 5064-5070.

Mojsov, K. (2010). Experimental investigations of submerged fermentation and synthesis of pectinolytic enzymes by *Aspergillus niger*: Effect of inoculum size and age of spores. *Applied Technologies and Innovations*, 2(2), 40-46.

Moshe, K., & Reese, E.T. (1967). Production of glucose by enzymatic hydrolysis of cellulose. *American Society of Microbiology*, 16, 419-420.

Mosier, N.S., Ladisch, C.M. & Ladich, M.R. (2002). Characterization of acid catalytic domains for cellulose hydrolysis and glucose degradation. *Biotechnology and Bioengineering*. 6(79), 610-618.

Mosier, N., Wyman, C., & Dale, B. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*, 96 (6), 673–686.

Mouta, R.O., Chandel, A.K., Rodrigues, R.C.L.B., Silva, M.B., Rocha, G.J.M., & Silva, S.S. (2011). Statistical optimization of sugarcane leaves hydrolysis into simple sugars by dilute sulfuric acid catalyzed process. *Sugar Tech*. doi:10.1007/s12355-011-0116-y

Moyo, S., Gashe, B.A., Collison, E.K., & Mpuchane, S. (2003). Optimizing growth conditions for the pectinolytic activity of *Kluyveromyces wickerhamii* by using response surface methodology. *International Journal of Food Microbiology*, 15, 87-100.

Mueller, M. (2009). Fermentation of xylose and xylans by *Kluveromyces marxianus* IMB strains. Stillwater: Oklahoma State University.

Murthy, M.S.R.C., Swaminathan, T., Rakshit, S.K., & Kosugi, Y. (2000). Statistical optimization of lipase catalyzed hydrolysis of methyloleate by response surface methodology. *Bioprocess Engineering*, 22, 35-39.

Murthy, P.S., & Naidu, M.M. (2011). Improvement of robusta coffee fermentation with microbial enzymes. *European Journal of Applied Sciences*, 3, 130-139.

Mukesh Kumar, D.J., Saranya, G.M., Suresh, K., Andal Priyadharshini, D., Rajakumar, R., & Kalaichelvan, P.T. (2012). Production and Optimization of Pectinase from *Bacillus sp*. MFW7 using Cassava Waste. *Asian Journal of Plant Science and Research*, 2 (3), 369-375.

Nadaroglu, H., Taşkın, E., Adıgüzel, A., Güllüce, M., & Demir, N. (2010). Production of a novel pectin lyase from *Bacillus pumilus* (P9), purification and characterization and fruit juice application. *Romanian Biotechnological Letters*, 15(2), 5167-5176.

Nagel, C.W., & Vaughn, R.H. (1961). The characteristic of a polygalacturonase produced by *Bacillus polymyxa*. *Archives of Biochemistry and Biophysics*, 93, 344-352.

Najafian, L., Ghodsvali, A., Khodaparast, M.H.H., & Diosady, L.L. (2009). Aqueous extraction of virgin olive oil using industrial enzymes. *Food Research International*, 42, 171-175.

Neely, W.C. (1984). Factors affecting the pretreatment of biomass with gaseous ozone. *Biotechnology and Bioengineering*, 26 (1), 59–65.

Ohtani, K. (2000). Bootstrapping R^2 and adjusted R^2 in regression analysis. *Economic Modelling*, 17, 473-483.

Palmarola-Adrados, B., Chotěborská, P., Galbe, M., & Zacchi, G. (2005). Ethanol production from non-starch carbohydrates. *Bioresource Technology*, 96, 843-850.

Palmowski, L., & Muller, J. (2000). Influence of the size reduction of organic waste on their anaerobic digestion. *Water Science and Technology*, 41(3):155-162.

Palmqvist, E. & Hahn-Hagerdal, B. (2000). Fermentation of lignocellulosic hydrolysates.I: inhibition and detoxification: review. *Bioresource Technology*, 74, 17–24.

Panneerselvam, A., Sharma-Shivappa, R.R., Kolar, P., Ranney, T., Peretti, S. (2013). Potential of ozonolysis as a pretreatment for energy grasses. *Bioresource Technology,* 148, 242–248.

Patil, S.R., & Dayanand, A. (2006). Optimization of process for the production of fungal pectinases from deseeded sunflower head in submerged and solid-state conditions. *Bioresource Technology*, 97, 2340-2344.

Pedrolli, D.B., Monteiro, A.C., Gomes, E., & Carmona, E.C. (2009). Pectin and pectinases: production, characterization and industrial application of microbial pectinolytic enzymes. *The Open Biotechnology Journal*, 3, 9-18.

Pericin, D.M., Madarev, S.Z., Radulovic, L.M., & Skrinjar, M. (2007). Production of exo-pectinase by *Penicillium roqueorti* using pumkin oil cake. *Proceedings of the National Academy of Sciences*, 113, 313-320.

Pilar, B., Carmen, S., & TomaËs, G. (1999). Villa Production of pectic enzymes in yeasts FEMS. *Microbiology Letters*, 175, 1-9.

Rahman, S.H.A., Choudhury, J.P., Ahmad, A.L., & Kamaruddin, A.H. (2007). Optimization studies on acid hydrolysis of oil palm empty fruit bunch fiber for production of xylose. *Bioresource Technology*, 98(3), 554–559.

Rangarajan, V., Rajasekharan, M., Ravichandran, R., Sriganesh, K., & Vaitheeswaran, V. (2010). Pectinase production from orange peel extract and dried orange peel solid as substrates using *Aspergillus niger*. *International Journal of Biotechnology & Biochemistry*, 6, 445-453.

Redding, A. (2009). An assessment of dilute acid pretreatment of coastal bermudagrass for bioethanol production. MSc thesis, North Carolina State University, Raleigh, USA.

Reed, G. (1966). Enzymes in Food Processing. New York, Academic Press. p. 311.

Rehman, H.U., Qader, S.A., & Aman, A. (2012). Polygalacturonase: production of pectin depolymerising enzyme from *Bacillus licheniformis* KIBGE IB-21. *Carbohydrate Polymers*, 90, 387-391.

Roberto, I.C., Mussatto, S.I., & Rodrigues, R.C.L.B. (2003). Dilute acid hydrolysis for optimization of xylose recovery from rice straw in a semi-pilot reactor. *Industrial Crops and Products*, 17, 171–176.

Rumelhart, D.E., Hintont, G.E., & Williams, R.J. (1986). Learning representations by back-propagating errors. *Nature*, 323, 533-536.

Saad, N., Briand, M., Gardarin, C., Briand, Y., & Michaud, P. (2007). Production, purification and characterization of an endopolygalacturonase from *Mucor rouxii* NRRL 1894. *Enzyme and Microbial Technology*, 41(6-7), 800-805.

Sabajanes, M.M., Yanez, R., Alonso, J.L., & Parajo, J.C. (2012). Pectic oligosaccharides production from orange peel waste by enzymatic hydrolysis. *International Journal of Food Science and Technology*, 47, 747-754.

Saha, B.C., & Bothast, R.J. (1999). Pretreatment and enzymatic saccharification of corn fiber. *Applied Biochemistry and Biotechnology,* 76, 65-77.

Saha, B.C. (2003). Hemicelluloses bioconversion. *Journal of Industrial Microbiology and Biotechnology*, 30, 279-291.

Sandri, I.G., Fontana, R.C., Barfknecht, D.M., & Da Silveira, M.M. (2011). Clarification of fruit juices by fungal pectinases. *LWT-Food Science and Technology*, 44, 2217-2222.

Semenova, M. V., Grishutin, S. G., Gusakov, A. V., Okunev, O. N., & Sinitsyn, A. P. (2003). Isolation and properties of pectinases from the fungus *Aspergillus japonicus*. Biochemistry, 68(5), 559-569.

Sepúlveda-Huerta, E., Tellez-Luis, S.J., Bocanegra-García, V., Ramírez, J.A., & Vázquez, M. (2006). Production of detoxified sorghum straw hydrolysates for fermentative purposes. *Journal of the Science of Food and Agricultural*, 86, 2579–2586.

Sharma, D.C., & Satyanarayana, T. (2006). A marked enhancement in the production of a highly alkaline and thermostable pectinase by *Bacillus pumilus* dcsr1 in submerged fermentation by using statistical methods. *Bioresource Technology*, 97, 727-733.

Sharma, D.C., & Satyanarayana, T. (2012). Biotechnological potential of agro residues for economical production of thermoalkali-stable pectinase by *Bacillus pumilus* dcsr1 by solid-state fermentation and its efficacy in the treatment of ramie fibres. *Enzyme Research*, DOI: 10.1155/2012/281384.

Silva, J. A., Costa Neto, E.H., Adriano, W.S., Ferreira, A.L.O., & Gonçalves, L.R.B. (2008). Use of neural networks in the mathematical modelling of the enzymic synthesis of amoxicillin catalysed by penicillin G acylase immobilized in chitosan. *World Journal of Microbiology and Biotechnology*, 24, 1761-1767.

Sin, H.N., Yusof, S., Hamid, N.S.A., & Rahman, R.A. (2006). Optimization of enzymatic clarification of sapodilla juice using response surface methodology. *Journal of Food Engineering*, 73, 313-319.

Singh, A., Tatewar, D., Shastri, P., & Pandharipande, S. (2008). Application of ANN for prediction of cellulase and xylanase production by *Trichoderma reesei* under SSF condition. *Indian Journal of Chemical Technology*, 15, 53-58.

Sirikarn S, Apanee L, & Sujitra W. (2012). Effect of temperature and time on dilute acid pretreatment of corn cobs. Int J Chem Biol Eng, 6, 333-337.

Sivers, M.V. & Zacchi, G. (1995). A techno-economical comparison of three processes for the production of ethanol from pine. *Bioresource Technology*, 51, 43-52.

Sixta, H. (2006). Handbook of pulp, Wiley-VCH, Weinheim.

Smith, J.E., & Aidoo, K.E. (1988). Growth of fungi on solid substrates. Physiology of Industrial Fungi. Blackwell. Oxford, England, 249-269.

Soares, M.M.C.N., da Silva, R., & Gomes, E. (1999). Screening of bacterial strains for pectinolytic activity: Characterization of the polygalactoronase produced by *Bacillus* sp. *Annual Review of Microbiology*, 30 (4), 299-303.

Soares, M.M.C.N., Da Silva, R., Carmona, E.C., & Gomes, E. (2001). Pectinolytic enzyme production by *Bacillus* species and their potential application on juice extraction. *World Journal of Microbiology & Biotechnology*, 17, 79-82.

Spanos, G.A., & Wrolstad, R.E. (1990). Influence of processing and storage on the phenolic composition of Thompson seedless grape juice. *Journal of Agricultural and Food Chemistry*, 38, 1565-1571.

Sugimoto, T., Magara, K., & Hosoya, S. (2005). Ozone pretreatment for ethanol production using lignocellulosic materials. 10th Biomass—Asia workshop. Retrieved from http://www.biomass-asia-workshop.jp/ biomassws/01workshop/material/No.3-FFPRI.pdf on June 20, 2013.

Sun, Y., & Cheng, J. (2002). Hydrolysis of lignocellulosic material for ethanol production: a review. *Bioresource Technology,* 83, 1-11.

Suryawati, L., Wilkins, M., Bellmer, D., Huhnke, R., Manness, N., & Banat, I. (2009). Effect of hydrothermolysis process conditions on pretreated switchgrass compostion and ethanol yield by SSF with *Kluveromyces marxianus* IMB4. *Process Biochemistry*, 44(5), 540-545.

Swain, M.R., Kar, S., & Ray, R.C. (2009). Exo-polygalacturonase production by *Bacillus subtilis* CM5 in solid state fermentation using cassava bagasse. *Brazilian Journal of Microbiology*, 40, 636-648.

Swain, M.R., Ray, R.C. (2010). Production, Characterization and application of a thermostable exo-polygalacturonase by *Bacillus subtilis* CM5. *Food Biotechnology*, 24, 37-50.

Taherzadeh, M.J., & Karimi, K. (2007). Acid-based hydrolysis processes for ethanol from lignocellulosic materials: a review. *Bioresource Technology*, 2, 472–499.

Taherzadeh, M.J., & Karimi, K. (2008). Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. *International Journal of Molecular Sciences*, 9, 1621–1651.

TAPPI (1985). TAPPI Useful Method UM 250: Acid-soluble lignin in wood and pulp, TAPPI Useful Methods, TAPPI, Atlanta, GA.

TAPPI Test Method T222 om-88, (1989). Acid-Insoluble Lignin in Wood and Pulp, In Tappi Test Methods. Atlanta, GA: Technical Association of the Pulp and Paper Industry.

Travaini, R., Otero, M.D., Coca, M., Da-Silva, R., & Bolado, S. (2013). Sugarcane bagasse ozonolysis pretreatment: effect on enzymatic digestibility and inhibitory compound formation. *Bioresource Technology*, 133, 332–339.

Teymouri, F.,Laureano-Perez, L., Alizadeh, H., & Dale,B.E. (2005). Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. *Bioresource Technology*, 96 (18), 2014-2018.

Torres, E.F., Aguilar, C., Esquivel, J.C.C & Gonzalez, G.V. (2005). Pectinases. In: Enzyme Technology, A. Pandey, C. Webb, C.R. Soccol, C. Larroche (Eds.), Enzyme Technology, Asiatech Publishers Inc., New Delhi, pp. 273–296.

Tutt, M., Kikas, T., & Olt, J. (2012). Comparison of different pretreatment methods on degredation of rye straw. 11th International Scientific Conference on Engineering for rural development, Jelgava.

Ustok, F.I., Tari, C., & Gogus, N. (2007). Solid-state production of polygalacturonase by *Aspergillus sojae* ATCC 20235. *Journal of Biotechnology*, 127(2),322-334.

Vavouraki, A.I., Volioti, V., & Kornaros, M.E. (2014). Optimization of thermo-chemcial pretreatment and enzymatic hydrolysis of kitchen wastes. *Waste Management*, 34, 167-173.

Vidal, P.F., & Molinier, J. (1988). Ozonolysis of lignin - Improvement of in vitro digestibility of poplar sawdust. *Biomass*, 16.

Vlasenko, E.Yu., Ding, H., Labavitch, J.M., & Shoemaker, S.P. (1997). Enzymatic hydrolysis of pretreated rice straw. *Bioresource Technology*, 59, 109-119.

Wang, Z., Keshwani, D., Redding, A., & Cheng, J. (2010). Sodium hydroxide pretreatment and enzymatic hydrolysis of coastal Bermuda grass. *Bioresource Technology*, 101(10), 3583-3585.

Woiciechowski, A.L., Nitsche, S., Pandey, A., & Ricardo, C. (2002). Acid and enzymatic hydrolysis to recover reducing sugar from cassava bagasse: an economic study. *Brazillian Archives of Biology and Technology*, 45 (3), 393-400.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., & Lee, Y.Y. (2005). Coordinated development of leading biomass pretreatment technologies. *Bioresource Technology*, 96(18),1959-1966.

Xie, R., Tu, M., Wu, Y., & Adhikari, S. (2011). Improvement in HLPC separation of acetic acid and levulinic acid in the profiling of biomass hydrolysate. *Bioresource Technology*, 102, 4938-4942.

Xu, J., Cheng, J.J., Sharma-Shivappa, R.R., & Burns, J.C. (2010a). Lime pretreatment of switchgrass at mild temperatures for ethanol production. *Bioresource Technology*, 101(8), 2900-2903.

Xu, J., Cheng, J.J., Sharma-Shivappa, R.R. & Burns, J.C. (2010b). Sodium hydroxide pretreatment of switchgrass for ethanol production. *Energy Fuels,* 24, 2113-2119.

Yan, S., Yao, J., Yao, I., Zhi, Z., Chen, X., & Wu, J. (2012). Fed batch enzymatic saccharification of food waste improves the sugar concentration in the hydrolysates and eventually the ethanol fermentation by *Saccharomyces cerevisiae* H058. *Brazillian Archives of Biology and Technology*, 55(2), 183-192.

Yat, S. C., Berger, A., & Shonnard, D. R. (2008). Kinetic characterization for dilute sulfuric acid hydrolysis of timber varieties and switchgrass. *Bioresource Technology,* 99, 3855-3863.

Yu, Z., Jameel, H., Chang, H., & Park, S. (2011). The effect of delignification of forest biomass on enzymatic hydrolysis. *Bioresource Technology*, 102 (19), 9083-9089.

Zahangir, A.M., Suleyman A. M. & Rosmaziah, W. (2009). Statistical optimization of process conditions for cellulase production by liquid state bioconversion of domestic wastewater sludge. *Bioresource Technology*, 99, 4709-4716.

Zeni, J., Cence, K., Grando, C.E., Tiggermann, L., Colet, R., Lerin, L.A., Cansian, R.L., Toniazzo, G., De Oliveira, D., & Valduga, E. (2011). Screening of pectinase-producing microorganisms with polygalacturonase activity. *Applied Biochemistry and Biotechnology*, 163, 383-392.

Zhang, X. (2012). Pretreatment of Corn Stover for Sugar Production by Using the Combination of Alkaline Reagents and Switchgrass-Derived Black Liquor. Dissertation, North Carolina State University.

APPENDIX A

CHEMICALS, ENZYMES AND SUPPLIER INFORMATION

Chemicals/Enzymes	Supplier
Acetic acid (glacial)	Merck
Acetone	Merck
Bovine serum albumine	Sigma-Aldrich
Cellic® Ctec2	Novozymes
Celluclast	Sigma-Aldrich
Citrus Pectin	Sigma-Aldrich
$CuSO_45H_2O$	Merck
D-glucose	Merck
dipotassium hydrogen phosphate (K_2HPO_4)	Merck
DNS (3,5-dinitrosalicylic acid)	Sigma-Aldrich
Ethanol	Merck
Folin-ciocalteu	Sigma-Aldrich
Furfural	Sigma-Aldrich
Galacturonic acid monohydrate	Sigma-Aldrich
Gallic acid	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Hexane	Merck
HMF	Sigma-Aldrich
HTec2	Novozymes
Magnesium sulphate ($MgSO_4.7H_2O$)	Merck
Nutrient Agar	Merck
Nutrient Broth	Merck
Oxygen (industrial grade)	Airgas
Pectinex 100L	Sigma-Aldrich

Phenol	Sigma-Aldrich
Potassium di-hydrogen phosphate (KH_2PO_4)	Merck
Potassium hydroxide	Merck
Rochelle salt	Merck
Sodium acetate	Merck
Sodium bicarbonate	Merck
Sodium carbonate	Merck
Sodium chlorite	Sigma-Aldrich
Sodium hydroxide	Merck
Sodium phosphate dibasic	Merck
Sodium phosphate monobasic	Merck
Sodium sulfite	Merck
Sulphuric acid	Merck
Viscozyme L	Sigma-Aldrich
Yeast extract	Merck

APPENDIX B

COMPOSITION OF BUFFERS AND SOLUTIONS

Buffers and Solutions

Composition of Acetate Buffer (pH 5)

Stock solutions:

A: 0.1 M solution of acetic acid

B: 0.1 M solution of sodium acetate

14.8 ml of solution A and 35.2 ml of solution B are mixed in 100 ml total volume to adjust the pH 5.0.

Composition of Phosphate Buffer (pH 7)

Stock solutions:

A: 0.1 M solution of sodium phosphate monobasic

B: 0.1 M solution of sodium phosphate dibasic

39 ml of solution A and 61 ml of solution B are mixed in 200 ml total volume to adjust the pH 7.0.

Composition of Carbonate-Bicarbonate Buffer (pH 9 &pH 11)

Stock solutions:

A: 0.1 M solution of sodium carbonate

B: 0.1 M solution of sodium bicarbonate

4 mL of solution A and 46 ml of solution B are mixed in 200 ml total volume to adjust the pH 9.0.

42.5 mL of solution A and 7.5 ml of solution B are mixed in 200 ml total volume to adjust the pH 11.0.

Composition of DNS Reagent:

Dinitrosalicylic Acid Reagent Solution, 1%

Dinitrosalicylic acid: 10 g

Phenol: 2 g

Sodium sulfite: 0.5 g

Sodium hydroxide: 10 g

Add water to: 1 liter

Potassium sodium tartrate solution, 40%

APPENDIX C

STANDARD CURVE FOR TOTAL REDUCING SUGAR

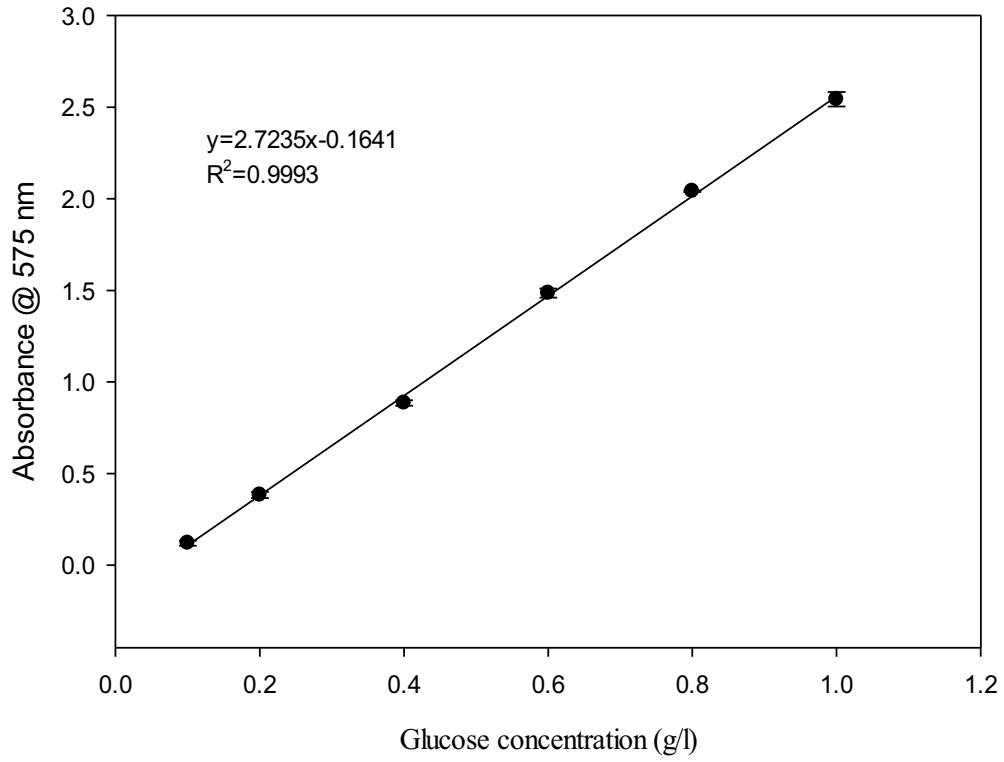


Figure C.1 The standard curve for DNS method

The total reducing sugar concentration was calculated as below:

The total reducing sugar concentration (g/L)= ((absorbance +0.1641)/2.7235)*dilution

APPENDIX D

STANDARD CURVE FOR TOTAL PHENOLIC COMPOUNDS

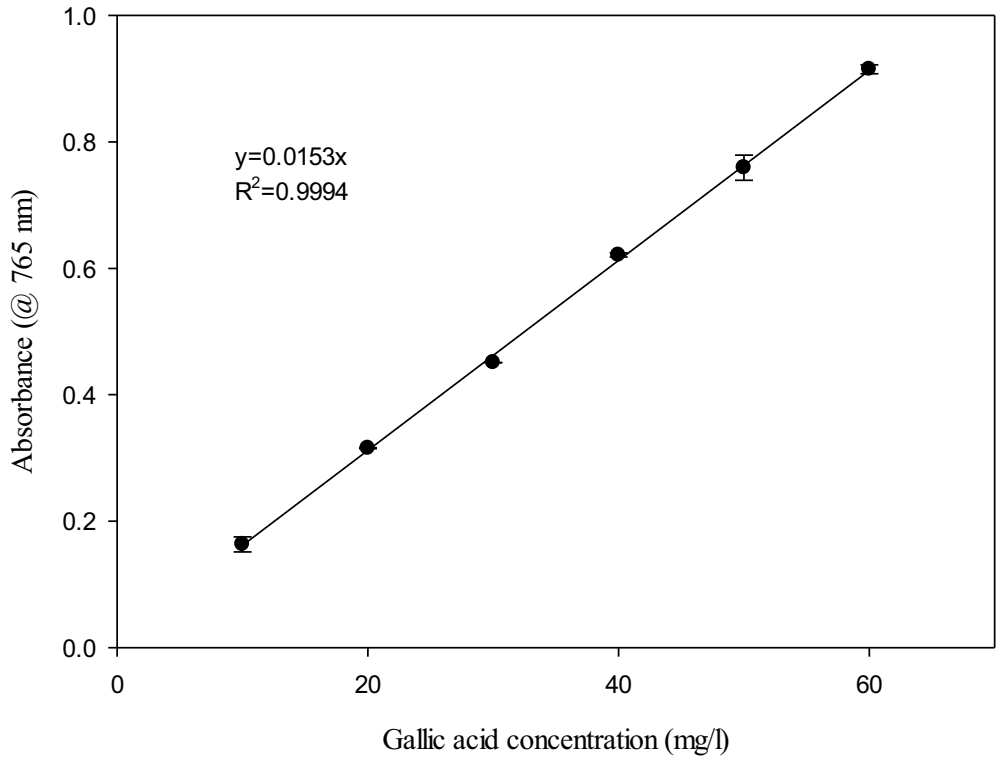


Figure D.1 Gallic acid standard curve for determination of total phenol content

APPENDIX E

STANDARD CURVE FOR ENZYME ACTIVITY

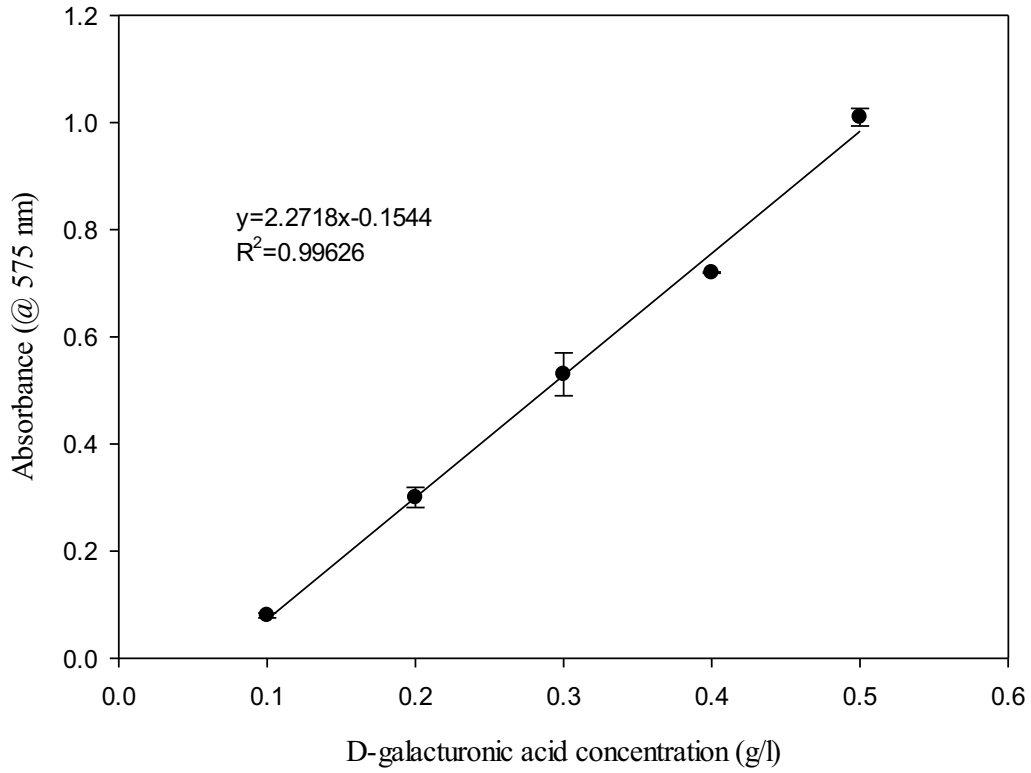


Figure E.1 D-Galacturonic acid standard curve for determination of enzyme activity

U/L=Absorbance*F*(1/incubation time)*DF*(1/212.12)*R_v

F= a factor to convert absorbance to g of galacturonic acid

Incubation time= time of incubation of the enzyme with substrate (30 min)

1/212.12= conversion from grams of galacturonic acid to moles of galacturonic acid

DF=Dilution factor

R_v =Amount of enzyme in the reaction mixture (ml)

APPENDIX F

GROWTH CURVE OF *Bacillus spp.*

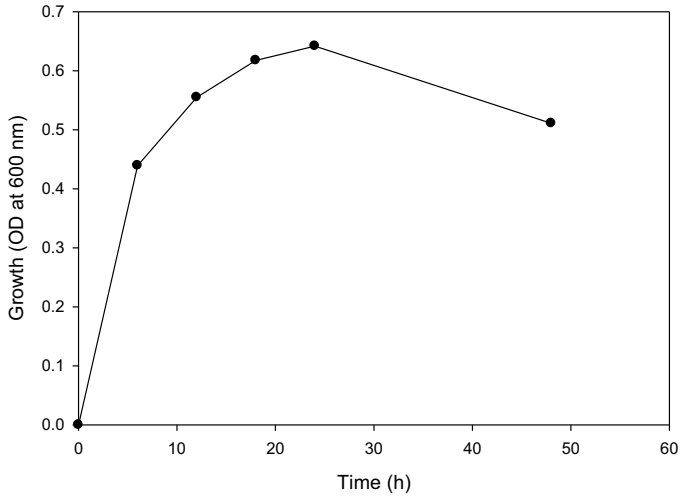


Figure F.1 Growth curve of *B.subtilis*

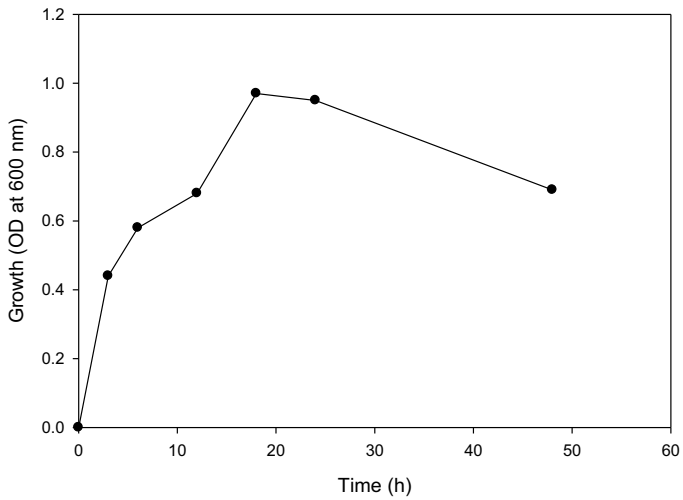


Figure F.2 Growth curve of *B.pumilus*

APPENDIX G

LOWRY PROTEIN ASSAY

SOLUTIONS

Solution A (alkaline solution, for 500 ml)

2.8598 g NaOH

14.3084 g Na_2CO_3

Solution B (for 100 ml)

1.4232 g $CuSO_45H_2O$

Solution C (for 100 ml)

2.85299 g Na_2Tartarate.2(H_2O)

Lowry Solution (fresh:0.7 lm/sample)

Solution A+Solution B+Solution C with a ratio (v/v) of (100:1:1)

Folin Reagent (instant fresh, 0.1 ml/sample)

5 ml of 2N Folin Ciocalteu Phenol Reagent + 6 ml distilled water. This solution light-sensitive. So it should be prepared at the last 5 min of the first sample incybation and kept in an amber container.

BSA Standard: 1mg BSA in 1 ml of water

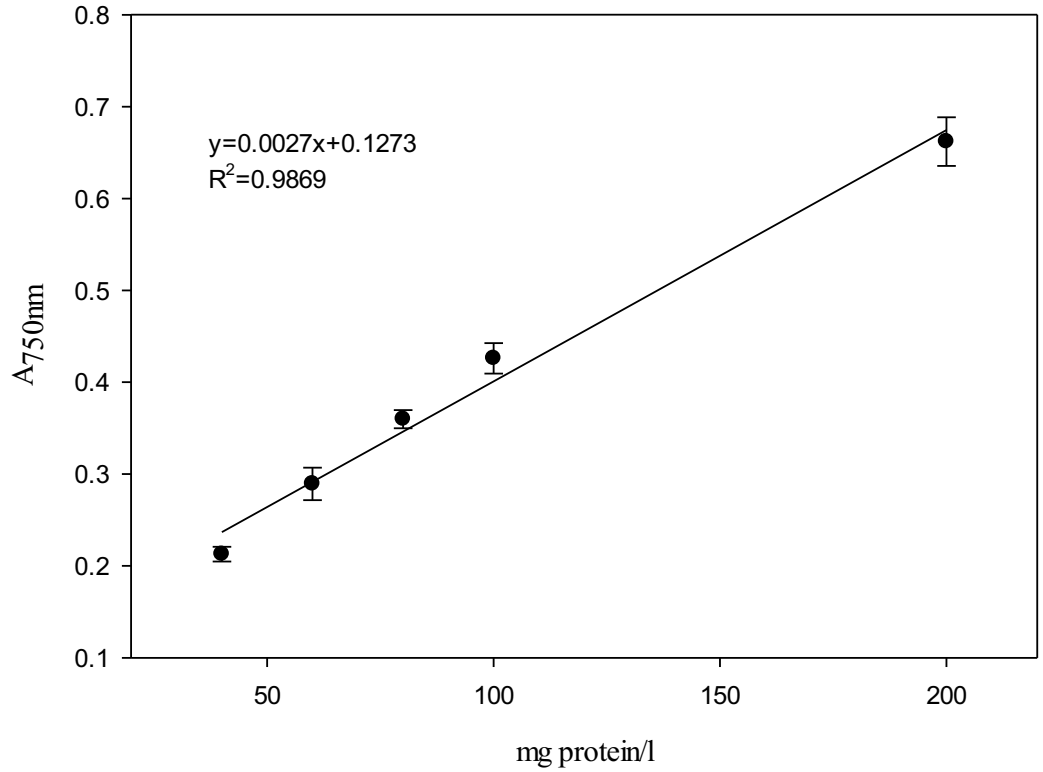


Figure G.1 BSA standard curve used in Lowry Method for the determination of total protein concentration

APPENDIX H

SAMPLE HPLC CHROMATOGRAM

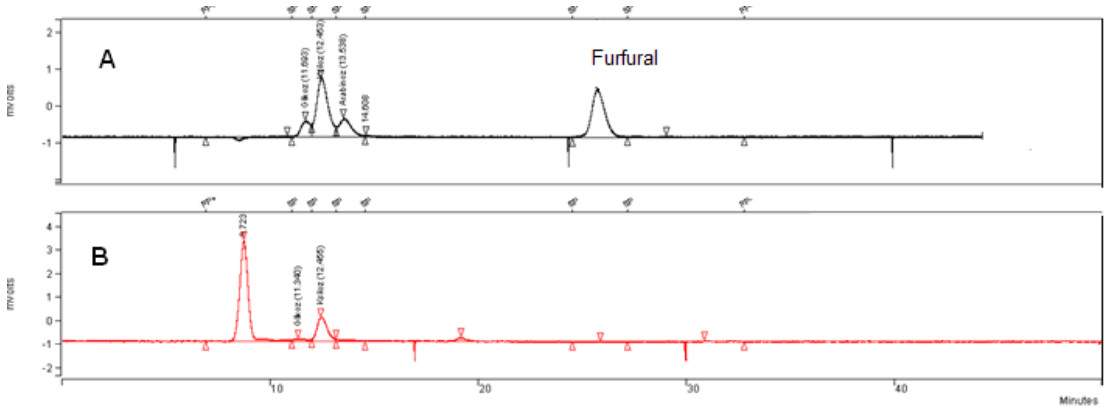


Figure H.1 Chromatograms: A: furfural standard, B: After acid hydrolysis.

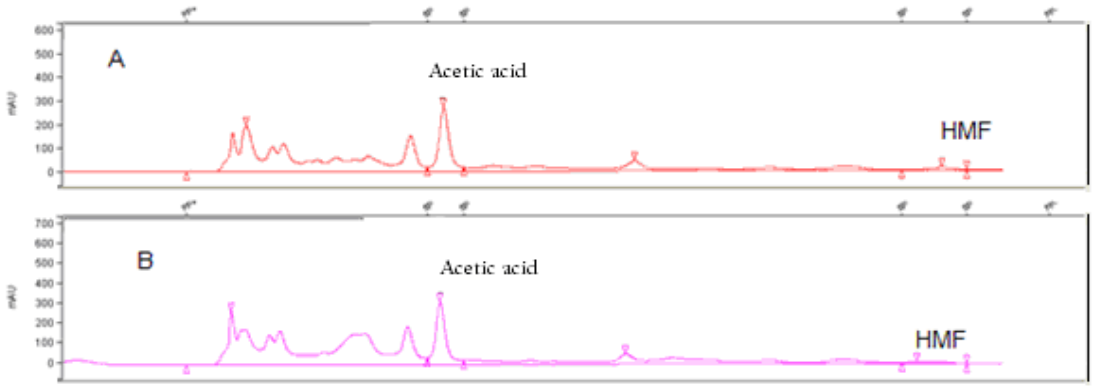


Figure H.2 Chromatograms: A: HMF and acetic acid, B: After acid hydrolysis

APPENDIX I

ANOVA RESULTS OF ALKALI PRETREATMENT

Table I.1 Revised ANOVA table of alkali pretreatment for solid recovery

Term	Coefficient	P Value
Regression		0.000
Linear		0.000
Square		0.000
Interaction		0.008
Lack-of-fit		0.447
Constant	74.0124	0.000
X_1 (NaOH Conc.,%)	-3.0790	0.000
X_2 (SLR, %)	3.1163	0.000
X_3 (Time, min)	4.5590	0.000
X_1^2	4.1440	0.000
X_2* X_3	-2.0358	0.008

Table I.2 Revised ANOVA table of alkali pretreatment for lignin reduction

Term	Coefficient	P Value
Regression		0.000
Linear		0.000
Square		0.000
Interaction		0.837
Lack-of-fit		0.086
Constant	19.3611	0.000
X_3	2.5204	0.000
X_1^2	-2.0679	0.000
X_2^2	-3.2957	0.000
X_3^2	-6.8648	0.000

APPENDIX J

PARETO CHART OF PLACKETT BURMAN DESIGN

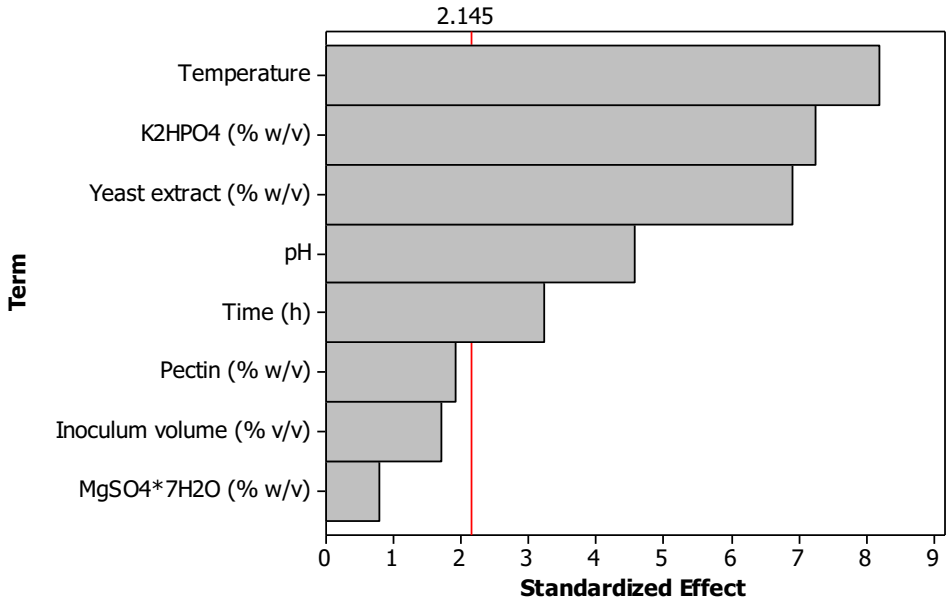


Figure J.1 Pareto chart of Plackett Burman Design for *B.subtilis*

APPENDIX K

ANOVA RESULTS OF PECTINASE ACTIVITY

Table K.1 Revised ANOVA table of pectinase activity

Term	Coefficient	Standard Error Coefficient	T Value	P Value
Constant	4.271	0.025	173.769	0.000
A	-0.075	0.019	-3.960	0.000
B	-0.233	0.019	-12.323	0.000
C	0.162	0.019	8.561	0.000
D	0.137	0.019	7.252	0.000
E	0.137	0.019	7.278	0.000
A*B	0.096	0.038	2.537	0.013
A*C	-0.111	0.038	-2.941	0.004
A*D	-0.093	0.038	-2.460	0.016
A*E	-0.379	0.038	-10.031	0.000
B*C	-0.296	0.038	-7.846	0.000
B*D	-0.218	0.038	-5.763	0.000
B*E	0.218	0.038	5.765	0.000
C*D	0.478	0.038	12.674	0.000
D*E	-0.381	0.038	-10.094	0.000
A^2	-0.221	0.025	-8.990	0.000
B^2	-0.231	0.025	-9.390	0.000
C^2	-0.166	0.025	-6.755	0.000
D^2	-0.258	0.025	-10.498	0.000

APPENDIX L

CONTOUR PLOTS OF PECTINASE PRODUCTION

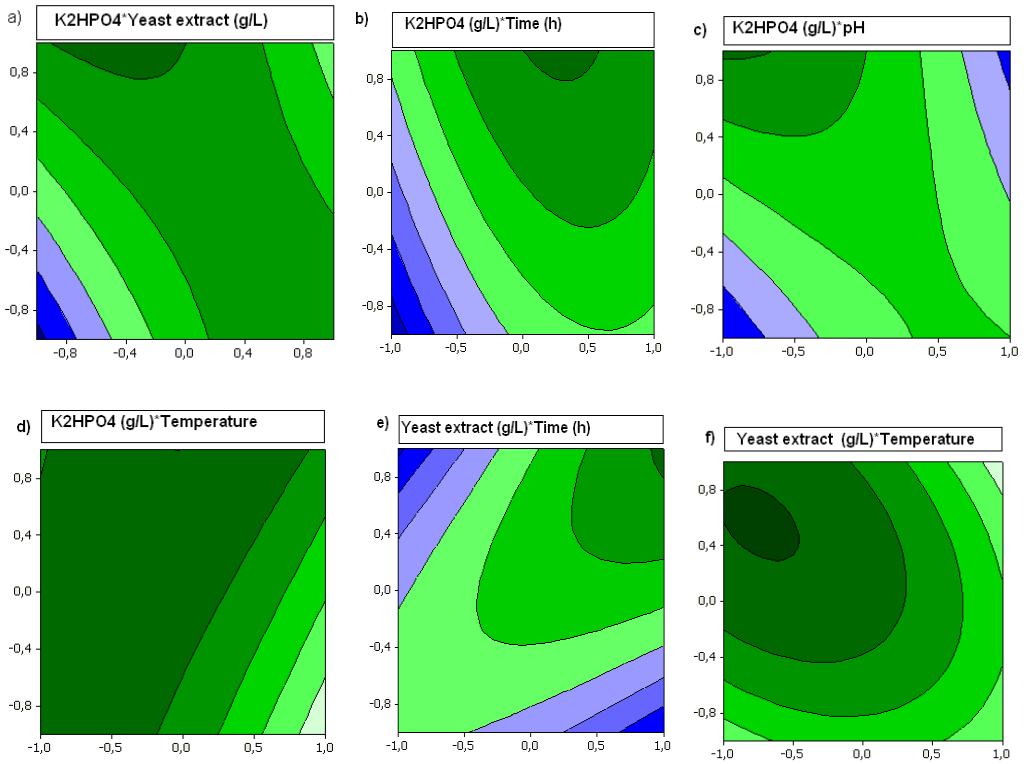


Figure L.1 Contour plots showing the effect of a) [K_2HPO_4] and yeast extract concentration, b) [K_2HPO_4] and time, c) [K_2HPO_4] and pH, d) [K_2HPO_4] and temperature, e) yeast extract concentration and time, f) yeast extract concentration and temperature, g) yeast extract concentration and pH, h) time and temperature, i) time and pH and j) temperature and pH on pectinase production

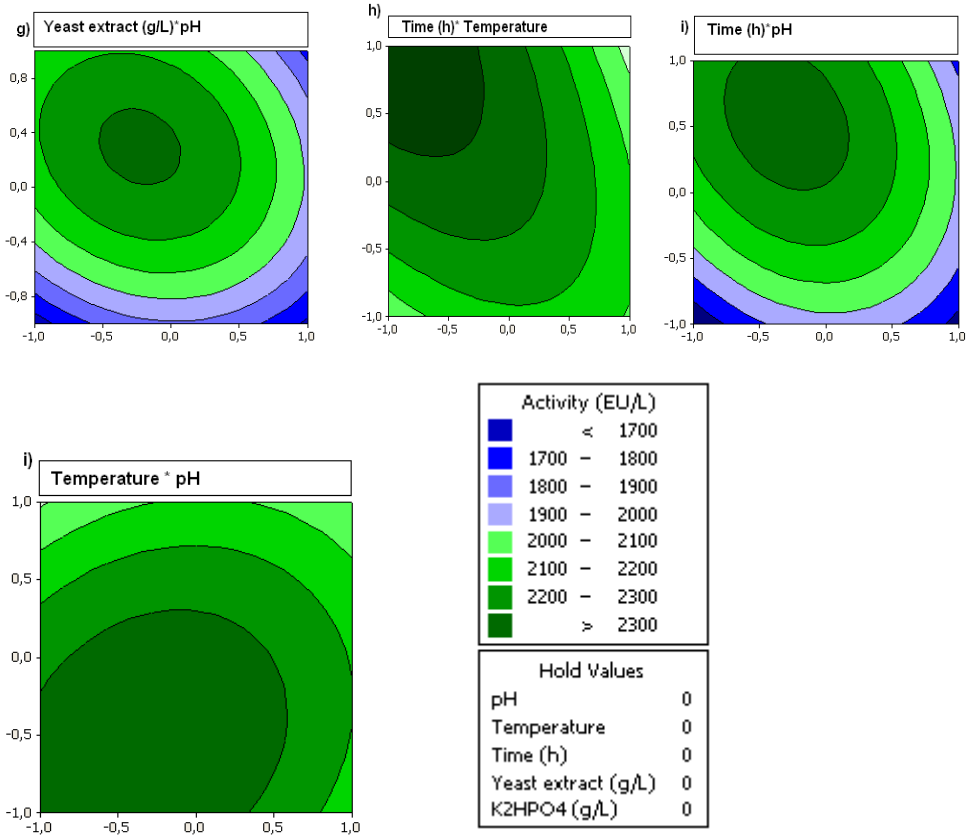


Figure L.1 (continued**)**

APPENDIX M

ANOVA RESULTS OF CLARIFICATION OF CARROT JUICE

Table M.1 Revised ANOVA table of clarification of carrot juice

Term	Coefficient	P Value
Regression		0.000
Linear		0.000
Square		0.000
Interaction		0.000
Lack-of-fit		0.132
Constant	85.62	0.000
pH	3.66	0.000
Time (h)	5.34	0.000
Enzyme con. (%)	1.77	0.001
pH*pH	2.55	0.000
Time*Time	2.83	0.000
Enzyme conc.*pH	1.98	0.000
pH*Time	-2.75	0.000

VITA

Surname, Name	**:**	Uzuner, Sibel
Nationality	**:**	Turkish
Date of Birth	**:**	06/28/1981
Place of Birth	**:**	Istanbul
Marital Status	**:**	Single
Telephone	**:**	+90 312 210 5623
Fax	**:**	+90312 210 2767
e-mail	**:**	suzuner@metu.edu.tr, siuzuners@gmail.com
Address	**:**	METU, Food Engineering Department, Office AZ-16, Lab: Bioprocess Laboratory 06800, Ankara, TURKEY

EDUCATION

2009-Present	Middle East Technical University, Ankara /Turkey PhD in Department of Food Engineering
2006-2008	Hacettepe University, Ankara /Turkey MSc in Department of Food Engineering
2001-2005	Hacettepe University, Ankara /Turkey BSc in Department of Food Engineering

ACADEMICAL EXPERIENCES

01/10/2014-07/10/2014	Visiting Scholar, Department of Agricultural and Biological Engineering, North Carolina State University, USA
09/2008-Present	Research Assistant, Department of Food Engineering, Middle East Technical University, TURKEY
01/2008-09/2008	Research Assistant, Department of Food Engineering, Abant Izzet Baysal University, TURKEY

Publications

Published within this PhD thesis are indicated as **[bold].**

A. Full paper published in International Journals (SCI expanded)

[1] Uzuner, S., & Cekmecelioglu, D. (2014). Hydrolysis of hazelnut shells as a carbon source for bioprocessing applications and fermentation. *International Journal of Food Engineering*, 10 (4), 799-808.

[2] Uzuner, S., & Cekmecelioglu, D**.** Enhanced pectinase production by optimizing fermentation conditions of *Bacillus subtilis* growing on hazelnut shell hydrolyzate. (under review in *Journal of Molecular Catalysis. B, Enzymatic*, December, 2014).

B. Full paper published in Proceedings of Conferences

[1] Uzuner, S., & Cekmecelioglu, D. (2013). A Rapid screening approach to factors affecting dilute acid hydrolysis of hazelnut shells. *International proceedings of chemical, Biological & Enviromental Enginnering*, Vol 50. 30-33 (Cited by 1).

[2] **Uzuner, S**., & Acar, J. (2012). Hydrolytic properties of ellagic acid in commercial pomegranate juices. *World Academy of Science, Engineering and Technology*, 67.

C. Full paper published in National Journals

[1] Uzuner, S., Onsekizoglu, P., Acar, J. (2011). Effects of processing techniques and cold storage on ellagic acid concentration and some quality parameters of pomegranate juice. GIDA, 36 (5), 263-269 (Cited by 1).

D. Oral Presentations

[1] Uzuner, S., & Cekmecelioglu, D. (2013). A Rapid screening approach to factors affecting dilute acid hydrolysis of hazelnut shells. 4th International Conference on Food Engineering and Biotechnology, 19-20 May, Copenhang, DENMARK (Full paper).

[2] Uzuner, S., & Acar, J. (2012). Hydrolytic properties of ellagic acid in commercial pomegranate juices. World Academy of Science, Engineering and Technology (WASET), 25-26 July, Amsterdam, HOLLAND (Full paper).

E. Poster Presentations

[1] Uzuner, S., & Cekmecelioglu, D. (2014). Exo-polygalacturonase production by *B. subtilis* and its use in clarification of carrot juice. Institute of Food Technologists Annual Meeting (IFT), New Orleans, USA.

[2] Uzuner, S., & Cekmecelioglu, D. (2013). Response Surface Modelling to Optimize Acid Concentration, Time and Temperature during Hydrolysis of Hazelnut Shells. Poster presentation in Northeast Agricultural and Biological Engineering Conference, 16-19 June, Pennsylvania, USA.

[3] Uzuner, S., & Acar, J. (2013). Effect of processing techniques on some quality properties of pomegranate juice. Poster presentation in Eurofoodchem XVII, 7-10 May, Istanbul, TURKEY (Abstract).

[4] Uzuner, S., Onsekizoglu,P., Acar, J. (2011). Effects of cold storage on the total phenolic content and antioxidant activity of pomegranate juice, Poster presentation in 6th International CIGR Technical Symposium 'Towards a sustainable food chain', 18-20 April, Nantes, FRANCE (Full paper).

F. Conference Papers (National)

[1] Uzuner, S., & Cekmecelioglu, D. (2013). Çeşitli *bacillus* türlerinden elde edilen pektinaz enzimini etkileyen dış faktörler, 8.Gıda Mühendisliği Kongresi, 7-9 Kasım, Ankara, Türkiye (Poster).

[2] Uysal, E.E., Uzuner, S., Evrendilek, G.A. (2008). Kırmızı şarapların atımlı elektrik akımı ile proses edilmesi, 1.Ulusal Bağcılık ve Şarap Sempozyumu ve Sergisi, 6-9 Kasım, Denizli (Poster).

PROJECT WORK

[1] Modelling and optimization of alkaline pectinase production using food waste, METU Research Fund Project, BAP-2011-03-14-002, 2011-2012, Researcher.

[2] Modelling and optimization of alkaline pectinase production using food waste, METU Research Fund Project, BAP-2011-03-14-002, 2012-2013, Researcher (Additional support).

SCHOLARSHIPS/AWARDS

TUBITAK (The Scientific and Technological Research Council of Turkey), International Research Fellowship Programme (2214/a) (January-July, 2014, 6 months)

TUBITAK (The Scientific and Technological Research Council of Turkey), International Scientific Events Participation Support (May, 2013)