CONVERSION OF LIGNOCELLULOSIC BIOMASS INTO NANOFIBER BY MICROFLUIDIZATION AND ITS EFFECT ON THE ENZYMATIC HYDROLYSIS

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ABSTRACT

CONVERSION OF LIGNOCELLULOSIC BIOMASS TO NANOFIBER BY MICROFLUIDIZATION AND ITS EFFECT ON THE ENZYMATIC HYDROLYSIS

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Lignocellulosic biomass is under extensive investigation as a bioethanol and bio-based materials feedstock. However, the complex structural and chemical mechanisms of lignocellulosic plant, which cause resistance to deconstruction during saccharification, require a pretreatment process. In this study, raw materials (corn bran, wheat bran and wheat straw) were selected because of their production and consumption in Turkey and also their accessibilities to be used as bioethanol source. Microfluidization pretreatment (high-pressure fluidization), which stands as a new approach for nano-cellulosic fibers production, was studied at 500 bar and 2000 bar to observe the qualitative and quantitative modifications in enzymatic hydrolysis depending on its effects on lignocellulosic structure. Optimum cellulase concentrations were determined for microfluidized samples as 4.5 U/g dry biomass for wheat bran, corn bran and 6.0 U/g dry biomass for wheat straw samples for the

first 150 min interval. Effective usage of solid loads were found as 5.0 %, 2.5 %, and 7.5 % (dw/v) for wheat bran, wheat straw and corn bran, respectively. X-ray diffraction and SEM results of the microfluidized samples have indicated that the pretreatment has increased crystallinity index of all the samples and resulted in a scattered structure. Comparisons with other methods (softening, dilute-acid and lime pretreatments) have shown that microfluidization is advantageous over others by reducing the time required for enzymatic hydrolysis and thus can be a promising alternative pretreatment.

Keywords: Cellulase, enzymatic hydrolysis, lignocellulosic biomass, microfluidization pretreatment

ÖZ

LİGNOSELÜLOZİK BİYOKÜTLEDEN MİKROAKIŞKANLAŞTIRICI İLE NANOLİF ELDE EDİLMESİ VE BUNUN ENZİMATİK HİDROLİZE ETKİSİ

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Lignoselülozik biyokütleler, biyoetanol ve biyokaynaklı malzeme hammaddesi olarak yoğun bir şekilde araştırma altındadır. Ama sakarifikasyon sırasında bozulmaya karşı direnişe neden olan lignoselülozik bitkilerin karmaşık yapısal ve kimyasal mekanizması, önişleme ihtiyaç duymaktadır. Bu çalışmada, hammaddeler (buğday kepeği, saman, mısır küspesi), Türkiye'deki üretim ve tüketimlerinden ve ayrıca biyoetanol kaynağı olarak erişilebilirliklerinden dolayı seçilmişlerdir. Nano-selülozik lif üretiminde yeni bir yaklaşımı temsil eden mikroakışkanlaştırma (yüksek basınçlı akışkanlaştırıcı) önişlemi, lignoselülozik yapıdaki etkinliğe bağlı olarak enzimatik hidroliz sırasında nitel ve nicel değişimleri gözlemlemek amaçlı 500 ve 2000 bar basınçta çalışılmıştır. Mikroakışkanlaştırılmış örneklerin ilk 150 dakika için ideal selülaz konsantrasyonları, kepek ve mısır küspesi için 4.5 U/g kuru biyokütle, saman içinse 6.0 U/g kuru biyokütle olarak belirlenmiştir. Kuru maddenin etkin

kullanım oranları buğday kepeği, saman ve mısır küspesi için sırasıyla % 5.0, 2.5 ve 7.5 (kuru ağırlık/hacim) şeklinde bulunmuştur. X-ışını kırınımı ve TEM sonuçları, bu önişlemin kristallik indisini arttırdığı ve dağınık bir yapıya neden olduğunu göstermişlerdir. Diğer yöntemlerle (yumuşatma, seyreltik-asit ve kireçleme yöntemleri) ile yapılan karşılaştırmalar, mikroakışkanlaştırmanın, enzimatik hidroliz için gerekli süreyi kısaltarak diğerlerine göre avantajlı olduğunu ve dolayısıyla gelecek vadeden alternatif bir teknik olabileceğini göstermiştir.

Anahtar Sözcükler: Biyoetanol, selülaz, enzimatik hidroliz, lignoselülozik biyokütle, mikroakışkanlaştırma önişlemi

TO MY BELOVED FAMILY

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LIST OF ABBREVIATIONS

U: Enzyme activity unit

FPU: Filter paper unit

CrI: Crystallinity index

Dw: Dry weight

Rpm: Revolutions per minute

GPa: Gigapascals

FFV: Flexible fuel vehicles

HMF: Hydroxymethylfurfural

LHV: Lower heating value

LHW: Liquid hot water

AFEX: Ammonia fiber explosion

TRS: Total reducing sugar

DNS: Dinitrosalicylic acid

SL: Solid load

XRD: X-ray diffraction

SEM: Scanning electron microscopy

O: Oxygen

C: Carbon

H_2: Hydrogen

H_2SO_4: Sulfuric acid

H_2O: Water

$Ca(OH)_2$:Calcium hydroxide (lime)

CO_2: Carbon dioxide

CHAPTER 1

INTRODUCTION

1.1 Renewable energy

Energy meaning *activity*, *operation* in Greek (ἐνέργεια - *energeia*) (Roche, 2003) is physically known to be a quantity that can be applied to every particle, object, and / or system as a result of the state of that particle, object or system and it is essential for life consistency. Energy, in terms of socio-economic point of view, plays an important role in the development and growth of economy. Energy supply of the world mostly comes from fossil fuel and nuclear energy. However, since the demand for energy is increasing and the stocks of petroleum sources are unstable, modern lifestyles need a secure supply of energy which will ensure prosperity and mobility of the next generations (Dresselhaus and Thomas, 2001). Thus, a clean energy alternative is needed to be developed when environmental impacts like global climate change are also considered.

1.1.1. Future of renewable energy

The programs including technologies of utilization of solar, wind, hydroelectricity and biomass-derived renewable energies are initiated to be supported by governments all across the world for the last century (Lange, 2007). Biofuels, which are produced from biomass, are now an important topic in research and development programs (Faaij, 2006). For instance, the scenarios from the U.S. Department of Energy Office of the Biomass Program (2006) points out that

biofuels, by the year of 2030, will supply 30% of the motor gasoline demand of the year 2004 (Ragauskas *et al*., 2006). Likewise, European Union has stated that 25% of the transportation fuel will be provided from biomass by 2030s (Biofuels Research Advisory Council, 2006). For the year 2010, European Union has set an overall target of 12% share for renewable energy and similarly USA has formulated nearly the same targets (European Commission, 1997 and Biomass Technical Advisory Committee, 2002). These targets are likely to increase in the future.

1.1.2. Sustainability of renewable energy

The general factors that affect the suitability of the renewable energy production and consumption can be categorized in three groups; being an alternative supply of energy, having an environmental impact on transportation and improving development of economic growth.

Firstly, renewable energies are found to be a steady and reliable solution to the increasing demand for energy, which is thought to be duplicated from 2000 to 2050, as a consequence of approximately 50% rise in world population and correspondingly an increase in the energy consumption per capita (Klass, 1998; World Business Council for Sustainable Development, 2004). Since crude oil can not satisfy this demand due to the estimated depletion of oil-stocks around 2050, the nuclear energy and renewable energy together are to cover about half of the demand by 2050s and two-thirds of it by 2100s (Lange, 2007).

The second driving force for the utilization of renewable energy is the possible threat of global climate change on earth and the increase in average global temperature. The devastation of the forests, combustion of large amounts of coal, oil and natural gas which are used for the production of transportation fuel and electricity have caused CO_2 concentration in the atmosphere to increase by one-forth for the last two centuries. In addition to these, during the last 30 years, annual anthropogenic emission level of CO_2 has raised to the twice of its value

(Sarmiento *et al.*, 1995). As a solution to these problems, for instance, first-generation bioethanol is proved to reduce the emissions by 20-50% when it is compared with petroleum-based fuels. In addition to that, second-generation bioethanol, in which the raw materials are mainly agricultural byproducts, is expected to decrease CO_2 emissions nearly by 90% (Joint Research Centre, 2007). Furthermore, the study of Davis and Pilger (2004) showed that when ethanol content is increased from 10% to 85% in gasoline blends, the particulate matter, hydrocarbons, carbon monoxide and nitrous oxides decreased approximately by 60%, 50%, 35% and 20% respectively (Figure 1.1). Thus, it can be concluded that renewable energy may offer long-term solutions also to environmental problems by reducing emissions, and thereby defending against pollutants.

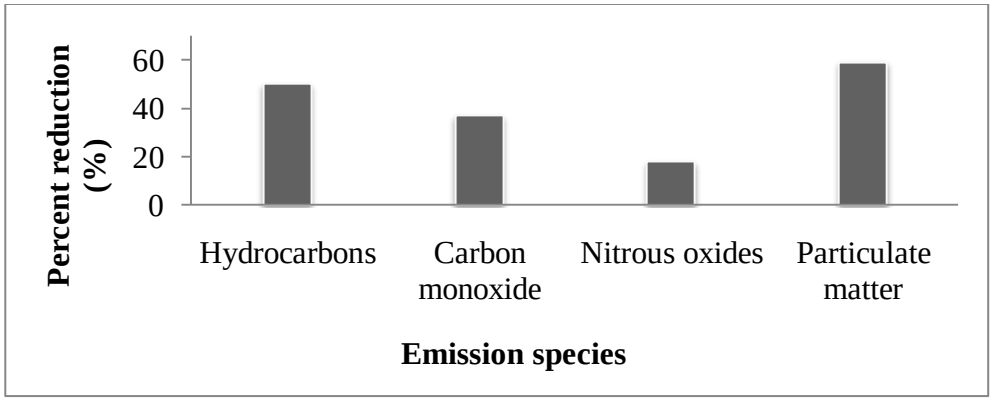


Figure 1.1 Change in the reduction emissions when E85 is used instead of E10 in clean snowmobiles (Davis and Pilger, 2004)

The last driver is the attainable positive effect on the socio-economic growth in rural areas by improving agricultural activities and developing new sectors. For instance, the number of jobs that have been created in rustic areas of Brazil in that sector is figured out to be 700,000 (Macedo, 1995), and also the predicted number from the 2020 target of China is 600,000 for bioethanol sector (Kearney, 2006).

Also, UN-Energy and World Watch Institute emphasizes that the biofuel energy industry can encourage the development of economic growth and reduce the dependency on imported oil in developing countries (UN-Energy, 2007). In addition to having a capacity to hold a high number of jobs, according to the studies of Goldemberg (2002) that are based on the bioethanol industry in Brazil, the number of deployable jobs per one unit of energy produced for ethanol industry is approximately 152, 50 and 38 times higher than oil, hydroelectric and coal industries respectively (Figure, 1.2, left). Besides, the lowest investment for a job employment is in ethanol agroindusty and industry sector, by US$ 11,000 (Figure 1.2, right). It is expected that developments in these renewable technologies will result in more common usage of bio-based sources for fuel and electricity production and also more developed socioeconomic situation in rural areas.,

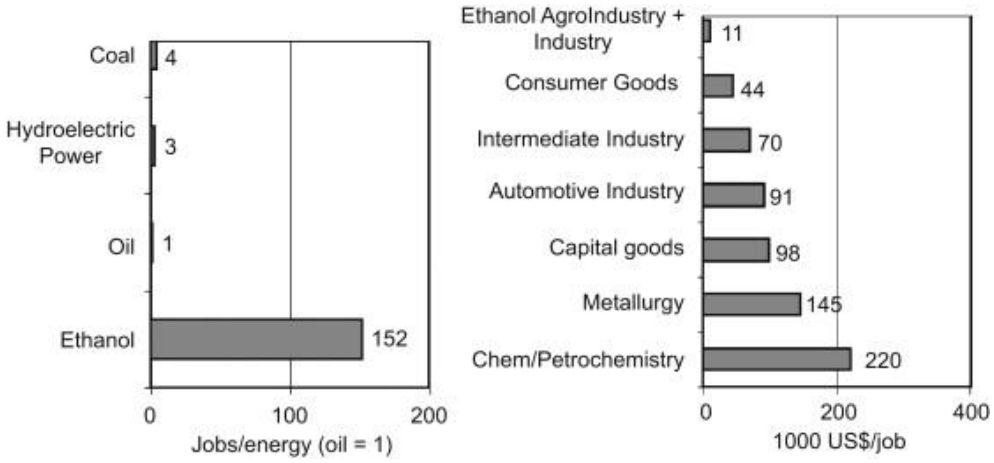


Figure 1.2 Employment data from Proalcool, the Brazilian Ethanol Program; Jobs per unit of energy produced (left) and investment costs for job created (right). (Goldemberg, 2002).

The three advantages of renewable energy fulfill the three conditions of sustainability: profitability, planet and people (3 Ps). Being an applicable solution to the limitations on current energy supplies, that alternative is lucrative from an economic point of view. Besides, by determining the earth's welfare, renewable energy option can be said to be a correct choice for the planet and also by verifying the development of the production and utilization area, this option shows that it meets the needs of people. Thus, when renewable energy is compared with nuclear energy and others, it is obvious that the most prominent option, nuclear energy can not be as satisfying as renewable energies in terms of the 3 Ps.

1.2. Need of biomass conversion

The total energy produced can be divided into two on the basis of requirements; namely, energy to be used as power and energy to be used in transportation. Wind, solar, hydraulicity and biomass are some of the several sources that are utilized in the power sector; nevertheless, for the transportation sector there are not as employable diverse choices as in the power sector yet. In terms of being renewable, the alternatives to fuel oil are biomass (i.e. vegetable oil, bioalcohol and biodiesel), H_2 and electricity. Hydrogen and electricity are promising environmentally-friendly solutions to the energy problem in transportation sector but their production, distribution, storage are not yet satisfactorily accepted to be used as frequently as biomass-based fuels.

The most far-flung and hopeful types of biofuels are bioethanol and biodiesel. Biodiesel, which is a product of vegetable oil, is expected to overcome the diesel produced from oil in the near future. For the case of bioethanol, it has already been used as fuel since 1980s and after the depletion and consequently the price surge on petroleum-based oil, demand has increased and bioethanol is now available for all types of vehicles which can work with gasoline (Balat, 2005). The reasons why ethanol can be utilized in blends with gasoline are its high

octane number and low cetane number. Also its high vaporization heat value is crucial for prevention of self-ignition in diesel vehicles (Kim, 2005). Furthermore, the high quantitative value of oxygen in biomass-based fuels increase the efficiency of combustion and so decrease the possibility of carbon monoxide and hydrocarbon production. When worldwide ethanol consumption profile is considered, it is obvious that 70% of ethanol produced is used in the fuel industry in combustion engines while 15% of it is utilized for the production of strong alcoholic beverages and the rest 15% in the chemical industry. In addition, the trend in bioethanol consumption is continuously becoming widespread (Figure 1.3)

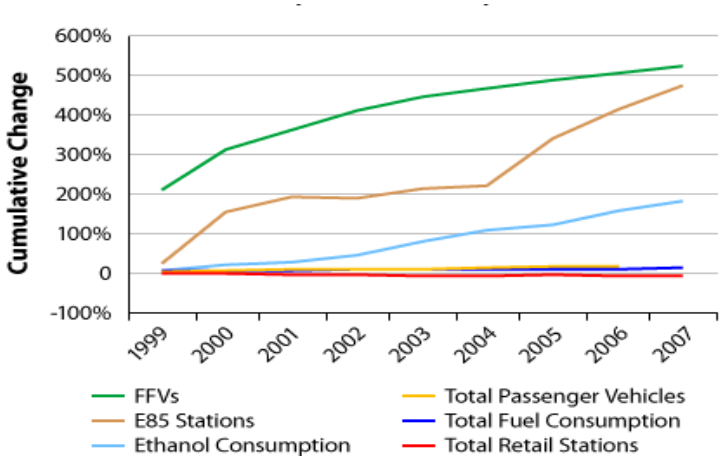


Figure 1.3 Cumulative changes in total fuel and ethanol consumption, vehicles and stations in USA. (US Department of Energy, 2009)

The bioethanol content of these blends may vary, for example in the case of availability of the machine, blends specific to flexible fuel vehicles (FFV) have a ethanol content of 85%- E85 (Malça, Energy, 2006). To illustrate, the blends that are available in United States are E10 (10% ethanol and 90% gasoline) and for FFV E85, Canada are E10 and for FFV E85, Sweden are E5 and for FFV E85, India is E5, Australia is E10, Thailand is E10, China is E10, Columbia is E10,

Peru is E10, Paraguay is E7, and Brazil are E20, E25 and for FFV any blend (Kadiman OK, 2005). Especially in Brazil, pure ethanol from sugar cane is available as a fuel. Moreover, it is obvious that when the ethanol content in the blend increases, the environmental effect of bioethanol is getting more striking (Table 1.1)

The other benefit of using biomass as an energy source, beyond being environmentally-friendly and being already available with a minimum vehicle modification, is its huge feed stock. For instance, 3-5 giga tons of lignocellulosic biomass is produced per year worldwide and that much residue have a potential to provide 50-85 EJ/year of energy (Klass, 1998; Okkerse, 1999; Dale, 2006). Besides, lignocellulosics can be harvested from food lands as in crop residues or on non-agricultural soil.

Table 1.1 Change in emissions with a comparison of low level and high level blends. (Kadam, 2002)

Emission	**Low level blends (eg. E10)**	**High level blends (eg. E85)**
Carbon monoxide (CO)	25-30% decrease	25-30% decrease
Carbon dioxide(CO_2)	10% decrease	Up to 100% decrease (E100)
Nitrogen oxides(NO_x)	5% increase or decrease	Up to 20% decrease
Volatile organic compounds		
Exhaust	7% decrease	30% or more decrease
Evaporative	No change	Decrease
Sulfur dioxide (SO_2) and particulate matter	Decrease	Significant decrease
Aldehydes	30-50% increase (but negligible due to catalytic converter)	Insufficient data
Aromatics (benzene and butadiene)	Decrease	More than 50% decrease

1.3. Bioethanol feedstock

The type of biomass, based on bioethanol production is divided into two: first-generation and second-generation (Figure 1.4). The first generation bioethanol is made from sugar and starch based feed stocks, crops like sugarcane/beet, corn, wheat, barley, rye, soybean, sorghum and cassava. The first generation bioethanol production is readily available and today's mercantile ethanol is basically from these crops. The second-generation bioethanol is made from residues of cellulosics (stalks, leaves and husks of plants) and energy crops (switch grass).

1.3.1. First-generation bioethanol

During the growth of crops i.e. sugarcane/beet, corn, wheat, barley etc., they absorb carbon dioxide, and thus they have an adverse effect on the initiation of carbon dioxide emission. Since fossil fuels are known to have an influential role on global warming (Mabee, 2006), the first-generation bioethanol is considered as a solution.

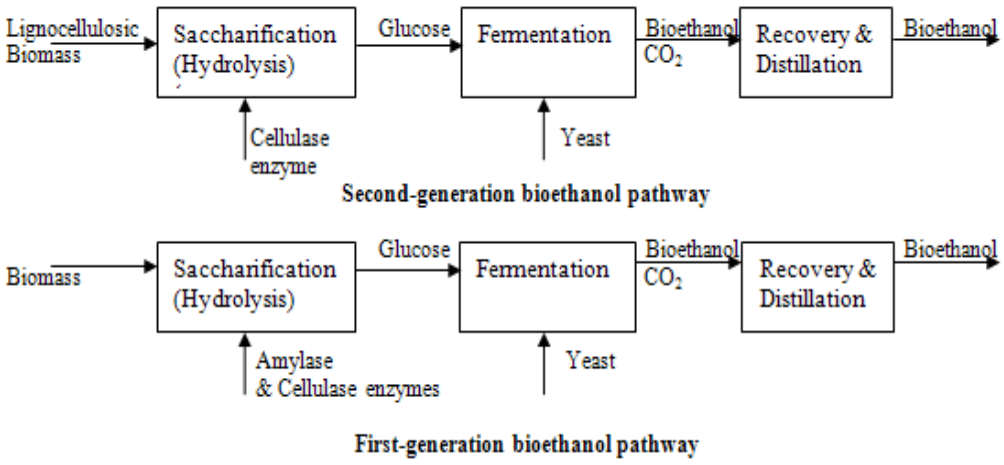


Figure 1.4 The processing steps of first and second generation bioethanol.

On the contrary, the struggle between food and fossil supply (especially for the important food materials corn and sugarcane, which are the raw materials of commercialized bioethanol in the United States and South America, and also for the oil and fat containing crops like rapeseed and soybean) causes a negative effect on using that first-generation bioethanol (Tat Tan, 2008). Instead of the development in marketplace of biofuels, this struggle has already caused the price of those food materials to alter. Recently, there was a case in Mexico, where people protested the price surge on tortilla, a corn product because of corn bioethanol production in USA (Steele, 2007). Furthermore, the available fertile land and the yield/hectare are restricted and also, the energy necessary for growth and conversion to fuel are so high that effective gains of carbon dioxide emission and fossil fuel are restrained (Lange, 2007). For instance, for the ethanol produced from corn, 60%-75% of the accessible energy is lost during production process, which can be detailed as 20-25% during growth and harvesting, and 40-50% during conversion to ethanol, predominantly the fermentation step (Hammerschlag, 2006). Considering all these disadvantages, to overcome the problem, second generation bioethanol has emerged.

1.3.2. Second-generation bioethanol

These lignocellulosic crops, i.e. corn stover, rice hull, wheat straw etc., can be grown with less fertilizer and can be harvested several times a year or they are already the left-after parts of food material plants. So they are seen as a resolution to the problems experienced with the first-generation biomass. However, their structure is so complex that additional steps such as pretreatment and saccharification are necessary to convert lignocellulose to sugars.

Lignocellulosic biomass can be categorized into two: plant-derived and animal derived resources (Figure 1.5) The recent studies aimed at efficient and sustainable ethanol production are mostly about agricultural residues i.e. wheat straw, wheat bran, rice straw, rice bran, barley straw, sugarcane bagasse, corncob and corn stover (Saha *et al.*, 2005; Nigam, 2001; Klinke *et al*., 2003;; Karimi,

2006; Palmarola-Adrados *et al*., 2005; Rossell *et al*., 2005; Laser *et al*.,2002; Zhao and Xia, 2010, Talebnia *et al*., 2010, Zhang *et al*., 2010), energy plantation i.e. switch grass (Jin *et al*., 2010; Keshwani *et al*., 2009) and forestry (Söderström, 2003; Zhu, 2010). Because of the complex structure of lignocellulosics, the studies are generally about pretreatment development, production steps' optimization.

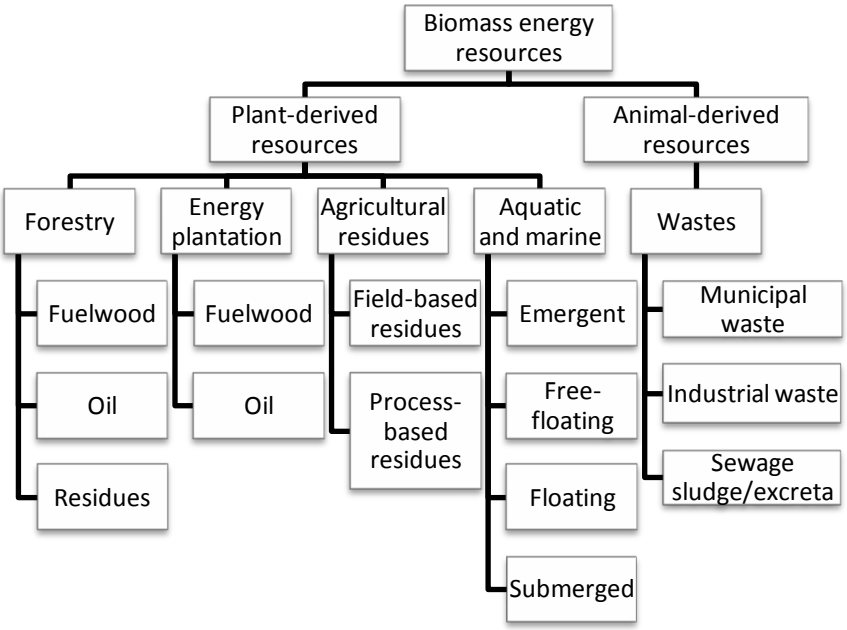
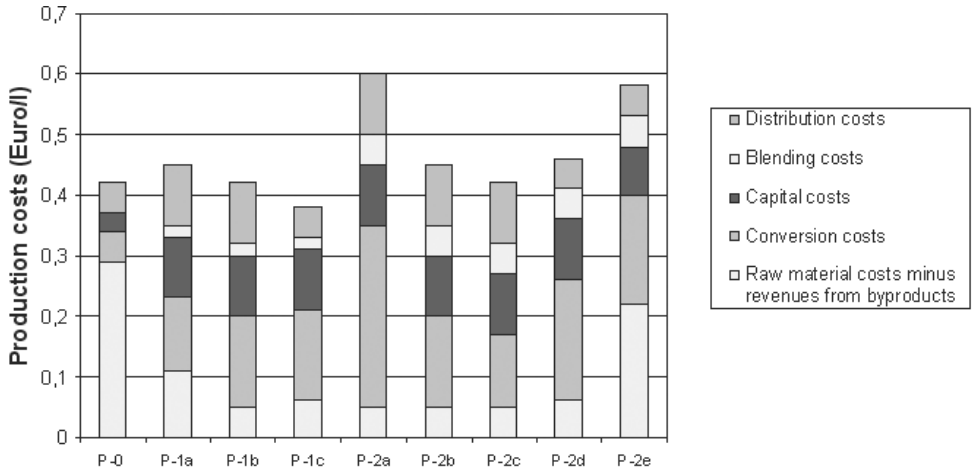


Figure 1.5 Origin based classification of lignocellulosic biomass resources. (Kishore, 2009)

The solution to the complexity of the lignocellulosic biomass is crucial in case of being economical and so being a challenging alternative to petroleum-based fuels. Since European Union has foreseen in 2030 ¼ of transportation fuel will be the biofuels (Biofuels Research Advisory Council, 2006), finding a way to disrupt the complicated structure is getting important. It can be seen from the Figure 1.6 that since the raw material cost is lower than petrol, the most prominent part of the costs affecting profitability is the conversion costs (Festel, 2008). The trend in

improving the bioethanol and biobutanol production costs is to decrease the cost of conversion and distribution (Figure 1.6, P-2c and P-2d).



P-0 Petrol (crude oil, 60 USD/barrel)
P-1a Biobutanol (corn, USA, large scale 2008)
P-1b Biobutanol (lignocelluloses, USA, large scale 2008)
P-1c Biobutanol (lignocelluloses, Europe, large scale 2008)
P-2a Bioethanol lignocelluloses, USA, small scale 2008)
P-2b Bioethanol (lignocelluloses, USA, large scale 2008)
P-2c Bioethanol (lignocelluloses, USA, large scale 2012)
P-2d Bioethanol (lignocelluloses, Europe, large scale 2008)
P-2e Bioethanol (wheat, Europe)

Note: Including mineral oil tax in Germany (0,65 Euro/liter for petrol and 0,47 Euro/liter for diesel)
Source: FESTEL CAPITAL analysis

Figure 1.6 The scenarios showing the competitiveness between alternative fuels and petroleum based fuel (Festel, 2008)

The reasons why there exists a complexity in the structure of lignocelluloses are described by Himmel and its fellows (2007) based on the characteristics of plants (Figure 1.7) as (i) plant epidermal tissue, (ii) the arrangement and density of vascular bundles, (iii) the relative amount of sclerenchymatous tissue, (iv) lignificational degree, (v) the structural diversity and complicity of cell wall ingredients, (vi) an insoluble substrate causing a residence to enzymatic hydrolysis, and (vii) the inhibitory components of cell wall that show their effect during fermentation or the ones formed during preliminary conversion steps. They all affect liquid penetration, accessibility and consequently the conversion costs at production scale.

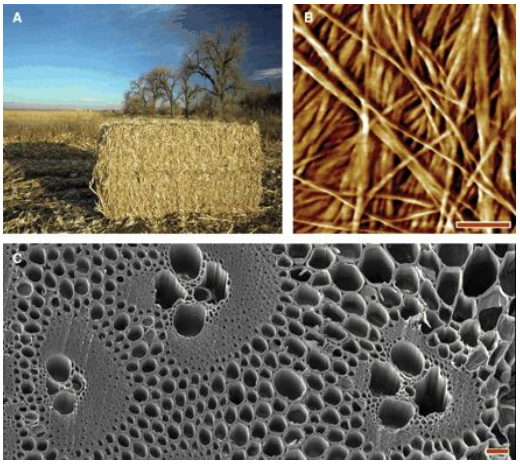


Figure 1.7 Structural and chemical complexity of cell wall biomass. (A) Examples of high density bales of corn stover harvested on the eastern plains of northern Colorado. (B) An atomic force micrograph of the corn parenchyma cell wall surface. Scale bar, 50nm. (C) A scanning electron micrograph of the cross section of a corn stem. Scale bar, 50μm. (Himmel, 2007)

1.4. The composition of lignocellulose

The lignocellulose is mainly a fibrous material and basically composed of three parts, cellulose, hemicellulose and lignin (Klass, 1998).

The cell wall microfibril's structure (Figure 1.8), which has arranged chains of cellodextrins, causes a resistance to chemical and biological hydrolysis. Because there is strong interchain hydrogen bonding between close chains of cellulose sheets, the network of these sheets creates resistant crystalline celluloses, affecting enzymatic hydrolysis negatively, while hemicellulose and amorphous cellulose are not (Nishiyama, 2002). Also, due to the hydrophobic interactions between these cellulose sheets, there occurs a contribution near the hydrated cellulose surface to a formation of a dense layer of water, and so that hydrophobic surface causes acid hydrolysis of the crystalline cellulose to happen slowly and

challengingly (Matthews *et al.*2006). In addition to that in high-structured plants, the hydrolysis of the lignocellulosics gets harder.

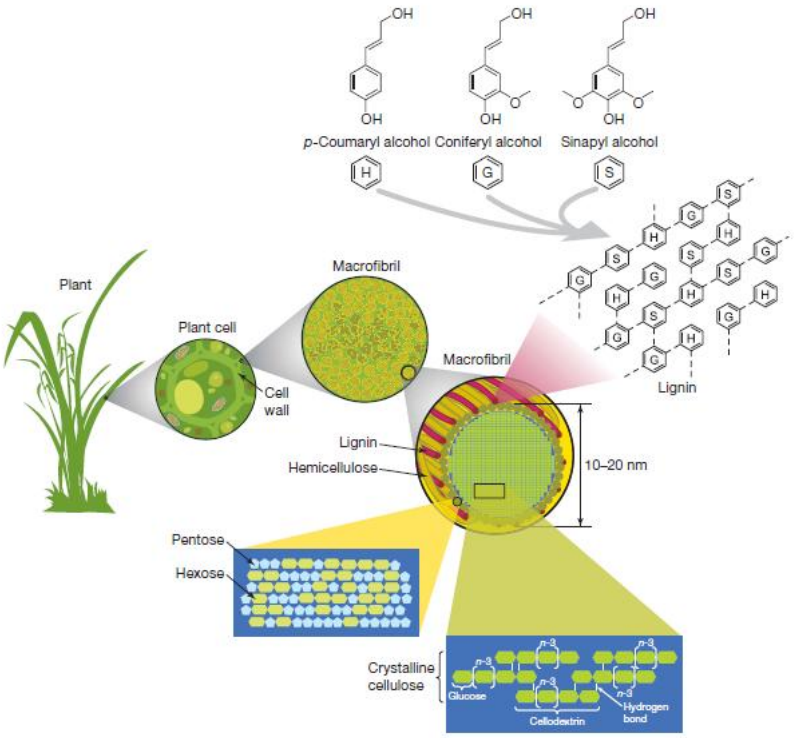


Figure 1.8 The lignocellulose structure. (Rubin, 2008)

1.4.1. Cellulose

Cellulose, the main structural component of plant cell walls, is a long chain of glucose molecules, β-1,4 linked to each other by glycosidic bonds (Figure 1.9). They are held together very tightly as bundles to get intense network (Lange, 2007) which consist of semi crystalline polymer chains of glucose. Cellulose contains only glucose and since it has hydroxyl groups in equatorial bonding, close packaging and strong hydrogen bonding becomes possible.

The rigidity of the cellulose depends on the intramolecular and intermolecular hydrogen bonds. Besides, the worldwide production of cellulose is about 180 billion tons/year, which makes cellulose the largest organic carbon store (Fentucci-Buselli *et al*., 2007). And it makes up 40-50 wt% of the lignocellulosic biomass (Lange, 2007). It can be degraded to cellobioses and glucoses by enzyme hydrolysis.

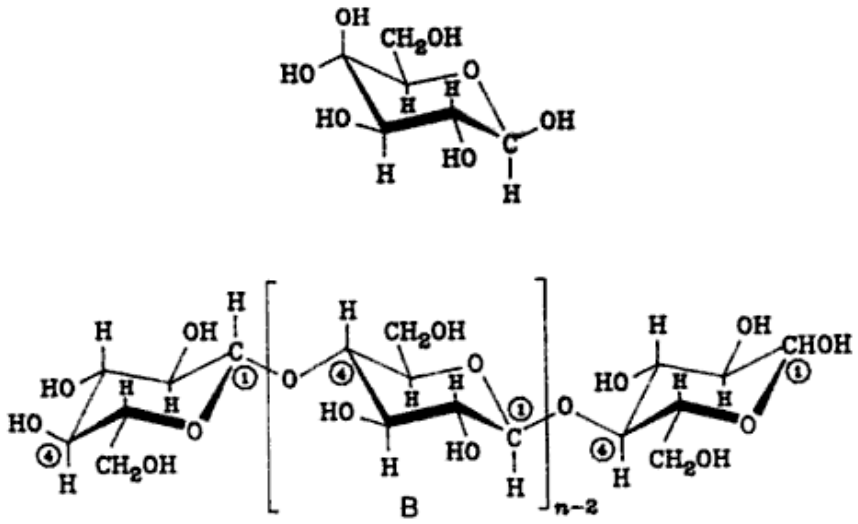


Figure 1.9 The conformational structure of D-glucose and the cellulose (Krassing, 1993)

1.4.2. Hemicellulose

Hemicellulose, on the other hand, does not have a homogenous structure as cellulose. It composed of various different sugar units as pentoses (D-xylose, L-arabinose), hexoses (D-galactose, D-glucose, L-galactose, L-rhamnose and L-fucose) and sugar acids (glucuronic acid). Hemicellulose has many types such as xylan and galactoglucomannan. The most abundant type of hemicelluloses is xylan (Figure 1.10), which has a 1-4 linked β-D-xylopyranose units containing homopolymeric backbone chain (Polizeli *et al*., 2005). On the branches of xylose

backbone, xylans may have arabinose, glucuronic acid, acetic acid, ferulic acid and ρ-coumaric acid depending on the plant species (Saha, 2003). The type of residue that the xylan has may change according to the plant species. For instance, softwood has 4-O-methyl-α-glucuronyl and α-L-arabinofuranosyl residues but not the acetyl residue, on the other hand, hardwood has 4-O-methyl-α-glucuronyl and acetyl residues but not the α-L-arabinofuranosyl residue (Sinnott, 2007).

The whole hemicellulosic layer coats the cellulose microfibrils with xyloglucans and it is the second abundant polysaccharide (Collins *et al*, 2005), makes up of nearly 20-40% of plant-cell wall. Hemicellulose does not have a rigid structure like cellulose because of the side-chains and the axial hydroxyl groups which inhibit the formation of semi-crystallinity. But, again because of that structure, hemicellulose is a known to be a good linker (Lange, 2007).

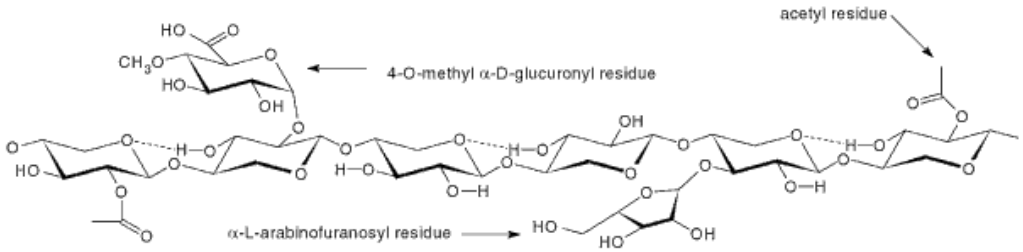


Figure 1.10 The conformational structure of xylan found on grasses. (Sinnott, 2007)

1.4.3. Lignin

Lignin, the third abundant natural polymer in the earth after cellulose and hemicellulose, has an amorphous structure. It is composed of phenylpropane units, namely guaiacyl, syringyl and ρ-hydroxyphenyl units. Figure 1.11 shows

the three aromatic alcohols (ρ-coumaryl, coniferyl and sinapyl alcohols) which make up the precursors of these units (Buranov and Mazza, 2008).

β-O-4 (β-aryl ether) linkage is the most frequently seen inter-unit linkage in the structure of lignin. This bond is weak and its chemical cleavage is less problematic. The other linkages, which are β-5, β- β, 5-5, 5-O-4 and β-1, are very resistant to chemical hydrolysis and that makes the difference between lignin and cellulose and hemicellulose (Boerjan *et al.*, 2003). Depending on the source of lignin i.e. wood or herbaceous lignin, the relative composition of guaiacyl, syringyl and ρ-hydroxyphenyl unit differs and thus the resistivity of the lignin structure changes (Billa *et al*, 1998). The cellulose and xylose and lignin is known to be linked to each other with ester, phenyl and covalent bonds, so the whole structure of lignin plays a crucial role in protection from environmental stresses such as humidity changes (Falkehag, 1975), prevention of pathogens and insects invasions (Mosier *et al.*, 2005) and also gives strength and rigidity to the plants. Lignin makes up approximately 20-30 dry wt% of plants cell wall (Kaplan, 1998).

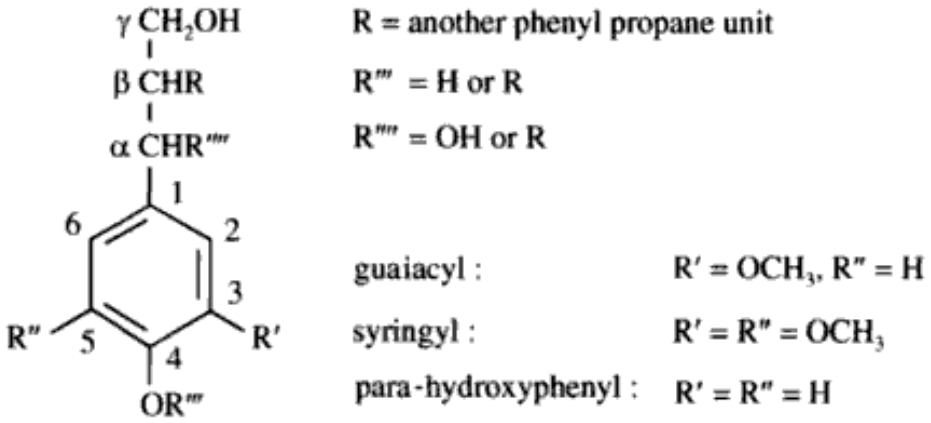


Figure 1.11 The conformational structure of lignin, showing the elementary phenylpropane building blocks (Kaplan, 1998)

1.5. Processing of lignocellulosic biomass and the possible products

Lignocellulose is found in all plants and is seen as the most valuable part for renewable products (Malherbe and Cloete, 2003). However, it is known that most of the biomass is not used efficiently and is generally burned (Levine, 1996). Since the biomass can be regained from forests, agricultural lands and also from paper, timber and agro-industries, finding a sustainable way to use them becomes a necessity.

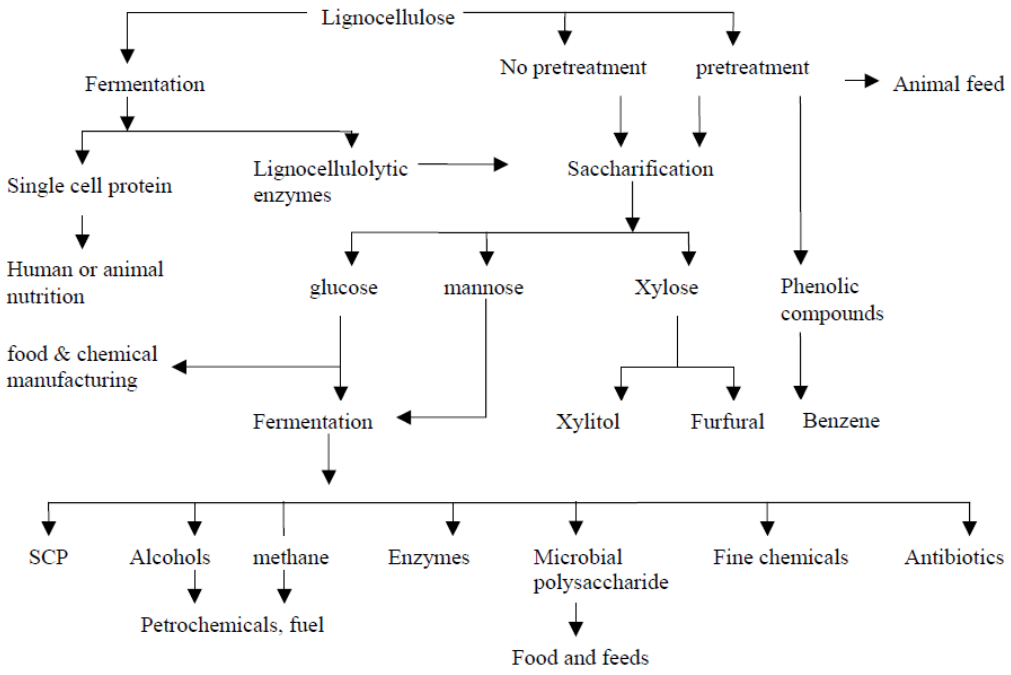


Figure 1.12 Possible products, obtainable from lignocellulosic biomass (Howard, 2003)

In terms of a biotechnological point of view, lignocellulosic biomass, has a various varieties such as plants and their parts (e.g. seeds, stalks, stovers), processing byproducts (e.g. distillers grains, soluble parts), animal and marine-based byproducts and also municipal wastes (Smith *et al.*, 1987). Thus they are

needed to be studied in detail to achieve efficient hydrolysis and high productivity. The main possible products gained from the biomass are chemicals, high-value bioproducts, biofuels and enzymes (Figure 1.12).

1.5.1. Chemicals and biochemicals

The possible products attainable from lignocelluloses can be categorized into four groups, chemicals, biofuels, high-value added products and enzymes. Since the organic chemicals such as ethylene, propylene, benzene etc. can be gained from the biomass; Coombs (1987) mentions that they can also be used for further production of resin and also other chemicals.

1.5.2. Biofuels

Crude oil production is estimated to decrease by 80% from the year 1998 to 2050 (Campbell and Laherrere, 1998); so there needs to be a solution to the problem. While in USA corn meal and in Brazil sugar cane are used to produce bioethanol, as a result of the corresponding needs, the debate between using them as a food or energy source leads to a requirement for finding another raw material, e.g. the lignocellulosic biomass. Since the lignocellulosic biomass has the potential to supply the energy gap, its production technology is being studied nowadays to make it more compatible with crude oil.

1.5.3. High-value bioproducts

High-value bioproducts are composed of organic acids, vitamins, amino acids which can be produced after glucose fermentation (Howard, 2003). Other than these fermentation products, there are also vanillin and furfural.

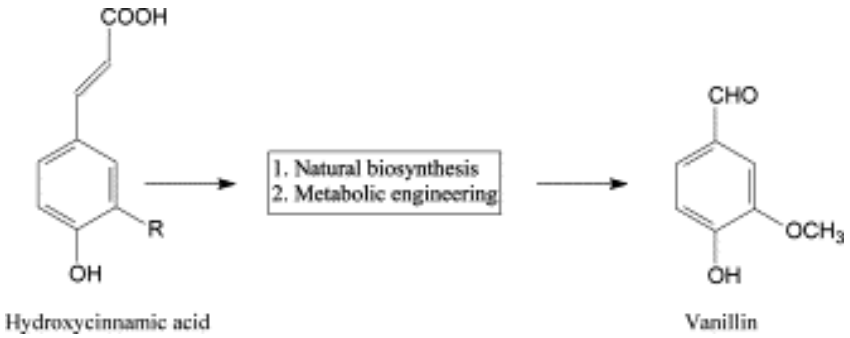


Figure 1.13 Possible production techniques to produce vanillin (Watson *et al.*, 2003)

Vanillin (4-hydroxy-3-methoxybenzaldehyde) for instance can be used in different areas such as, nearly 60% in foods, confectionery and beverages industry, 33% in perfumes and cosmetics industry, 7% in pharmaceuticals industry e.g. herbicides, anti-foaming agents and drugs (papaverine, L-dopa) (Priefert *et al.*, 2001). But its only small portion is produced from its original source, the plant vanilla pods (Krings and Berger, 1998). Mainly, it is produced by chemical synthetic reactions involving lignin and guaiacol (Clark, 1990) (Figure 1.13). This is because of the price of produced vanillin. While the price of synthetic vanillin is about US$ 12 kg^{-1}, the price of vanilla pods are between US$ 30-120 kg^{-1}, in addition to that, vanilla pods contain very limited amount of vanillin, 2 (w/w)% (Priefert *et al.*, 2001). On the other hand, due to the difference in prices and also the demand of costumers towards natural and healthy products, producing natural vanillin via biotransformations has aroused interest. The biotransformation may be via lignin, ferulic acid, vanillic acid, phenolic stilbenes, aromatic amino acid or eugenol/isoeugenol (Walton *et al.*, 2003) in most of which can be attained from lignocellulosic biomass.

The other examples to the high-value added products are xylitol and furfural which are the hemicellulose-originated chemicals. Xylitol has area of usage in food industry (sweeteners), odontology (remineralization, teeth hardening,

anticariogenicity) and pharmaceutics (Roberto *et al*., 2003), whereas furfural can be utilized in plastics, varnishes and pesticides production (Montané *et al*.,2002).

1.5.4. Enzymes

Enzymes production is an increasing field of biotechnology. The main enzymes that can be attainable from lignocellulosic biomasses are cellulases and hemicellulases. They have important application areas such as chemical, fuel, brewery, food, animal feed, textile, agriculture, pulp and paper industries (Sun and Cheng, 2002).

Table 1.2 Examples of lignocellulose degrading enzymes produced from different substrates

Ligninolytic enzyme	Substrare	Microorganism	Reference
Laccase	Ryegrass seed	*Botryosphaeria sp.*	Barbosa *et al*., 1996
	Bagasse	*Pleurotus ostreatus*	Pradeep *et al*., 2002
		Phanerochaete chrysosporium	
	Corn	*Lentinus edodes strain* CS-495	D'Annibale *et al*., 1996
	Cotton	*Pleurotus ostreatus*	Jaszek et. al, 1998
	Wheat bran	*P. pulmonarius*	Marques de Souza *et al*., 2002
	Wheat straw	*Pleurotus ostreatus*	Baldrian *et al*., 2002
		P. pulmonarius	Marques de Souza *et al*., 2002
		Pleurotus sp.	Lang *et al*., 1996
	Wood	*Pleurotus ostreatus*	Pradeep, 2002
		Phanerochaete chrysosporium	
Cellulase	Wood	*Ceriporiopsis subvermispora*	Ferraz *et al*., 2003
Ligninase	Bagasse	*Polyporus* BH1	Nigam *et al*., 1987
		Polyporus BW1	
	Wheat straw	*C. versicolor*	Golovleva *et al*., 1987
		P. tigrinus	
		Phanerochaete chrysosporium	

The others are laccase, xylanase, ligninase which have usage in wastewater treatment, in baking industry, and in the delignification of woods and degradation of some aromatic pollutants, respectively.

It is apparent that in the conversion of lignocellulose to glucose and xylose, the main problem is the hydrolysis step. At that stage, the necessity of using enzymes, cellulase and hemicellulase, becomes crucial. However, due to the type and compositional difference of lignocellulose, low yields of the enzymes and the economy of the degradation step, there needs a specific and costly available enzyme. Thus, there is a high variety of studies done on lignocellulose-hydrolyzing microbial strains which are able to use inexpensive substrate, lignocellulosic biomass again (Table 1.2).

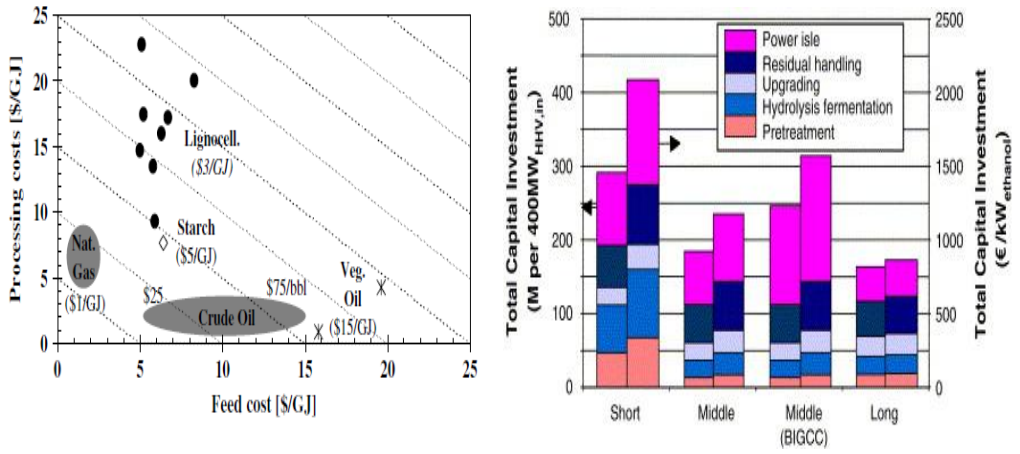
1.5.5. Economy of processing lignocellulosic biomass

The cost of all these categorized groups of lignocellulosic-based products should be considered in a detailed view in terms of applicability to the industry. The economy of processing lignocellulosics can be studied in three steps: plant cost, feed cost, manufacturing cost and by-products (chemicals) cost. Plant cost is dependent on the energy efficiency which is the ratio of lower heating value of products to lower heating value of feed plus energy ($LHV_{products}/LHV_{feed+energy}$). As the energy efficiency increases from 20% to 80%, the capital cost, the division of plant cost to product cost, decreases by approximately 70% when the intake of lignocellulose to the plant is kept constant. That shows why efficient utilization of feed and energy is essential in production (Lange, 2007).

Lignocellulose has a value of energy changing from $ 2 to $ 4 GJ^{-1} (Klass, 1998) and the difference is caused because of the variability in source, quality and transportation. The overall feed cost is related with the energy value of the cost, which depends on the energy efficiency like plant cost and also with the price of the feed, Furthermore, the price of the raw material may differ according to production facilities and feed type (Table 1.3).

Table 1.3 Comparison of costs based on the sources (Mousdale, 2008)

Source of ethanol	Production cost (€/GJ)	Production cost ($/liter)	Production cost ($/gallon)
Sugarcane (Brazil)	10-12	0.24-0.29	0.91-1.10
Starch and sugar (US and Europe)	16.2-23	0.39-0.55	1.48-2.08
Lignocellulosics (US)	15-19	0.36-0.46	1.36-1.74
Lignocellulosics (Europe)	34-45	0.82-1.08	3.10-4.09



(a) (b)

Figure 1.14 (a) Comparison of lignocellulosic biomass-based fuel with other alternatives (Lange, 2007), (b) the development in total capital investment of ethanol made from lignocellulosics (Hamelinck, 2005)

The manufacturing cost is the sum of direct costs (e.g. costs of raw material, operational labor, utilities etc), fixed costs (e.g. costs of depreciation, insurance etc) and general expenses (e.g. costs of administrative, research and development etc). If the manufacturing cost is estimated to be only capital and fixed costs, it is observed that lignocellulosic fuel has a favorable feed cost, its production cost has

the highest value compared to starch, crude oil and vegetable oil-based fuel and natural gas (Lange, 2007) (Figure 1.14.a) The difference in processing cost between lignocellulosic fuels and others shows the necessity to work on the improvements on handling, pretreatment, hydrolysis, fermentation and purification steps during production. Studies of Hamelinck (2005) demonstrates that in near future, total capital investment will decrease with a sharp fall in the pretreatment costs (Figure 1.14.b)

The chemical intermediates that can be obtained during ethanol production affect the overall cost positively because of their valuableness; their price is 2-20 times higher than fuels. In addition, their production is applicable to be input into fuel production. Their O/C atomic ratio is between the one of ethanol and biomass (0-1) and their production capacity are parallel to the capacity of fuel ethanol production. According to Zhang (2008), if all sugars are converted to ethanol and lignin as burning fuels, the potential revenue is about 150 $/ton lignocellulose, whereas if all sugar is converted to ethanol while half of lignin is converted to burning fuel and the other half to polymeric materials and acetic, the revenue rises to approximately 300 $/ton. Moreover, when only glucose is converted to ethanol while xylose and other minor sugars are exchanged to high value-added products (e.g. vanillin, furfural, xylitol etc.), and the whole lignin is turned to polymeric materials and acetic acid, then the revenue becomes nearly 700 $/ton of lignocellulosic biomass.

1.6. Pretreatment methods for lignocellulosic biomass

Lignocellulosic biomass, as mentioned before, is the most abundant source of fermentable sugar and it should be pretreated because of its recalcitrance to hydrolysis. The recalcitrance is caused by mainly the crystallinity degree of cellulose, lignin that protects the cellulose from environmental effects, accessibility of cellulose which is dependent on surface area and porosity of the material and the heterogeneous chemistry of biomass. Thus the ideal pretreatment method should break down the cellulose-hemicellulose matrix, hydrolyze the

hemicellulose, reduce the crystallinity degree of cellulose thus increase the amount of amorphous cellulose, release lignin from the environment, increase the porosity of lignocellulosic structure and also it should prevent the formation of inhibiting compounds such as furfural, hydroxymethylfurfural (HMF) and luvilinic acid; plus, it should be costly-available. In addition to these main goals, the sub-goals of the pretreatment process are having an agent or catalyst with a minimum cost, making recycle possible so that the agents or catalysts are to be used more than one, generating lignin as a by-product, being applicable to high amount of biomass and also having methods that are already reached to a degree of maturity in terms of technological point of view.

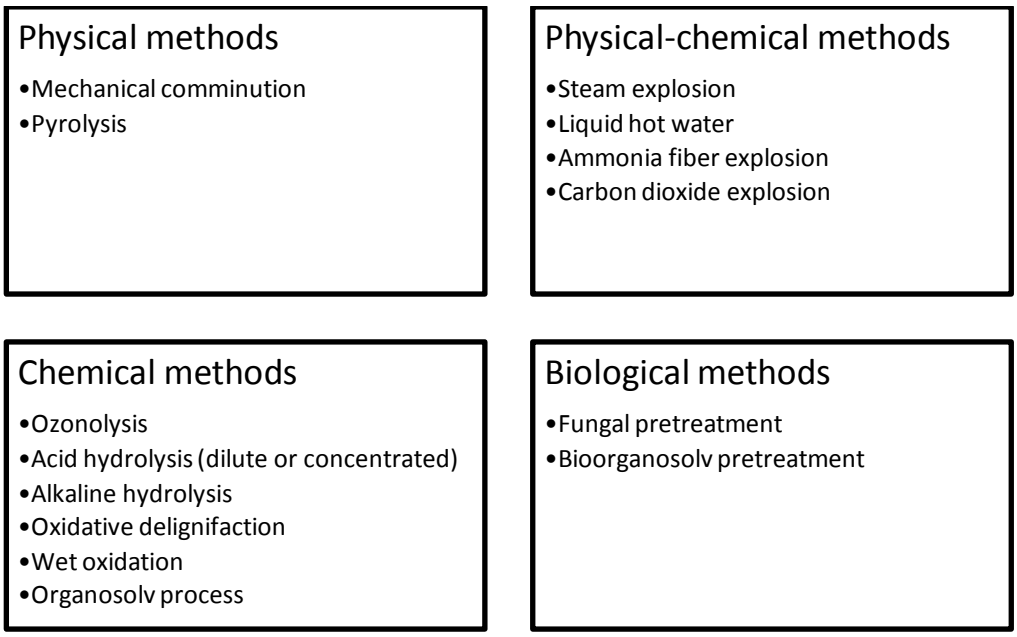


Figure 1.15 Classification of lignocellulose-pretreatment methods

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.

The pretreatments studied so far are summarized in four groups: physical methods, physical-chemical methods, chemical methods and biological methods (Figure 1.15).

1.6.1. Physical methods

Physical methods consist of two types, mechanical comminution (grinding, chipping and milling) and pyrolysis. By mechanical comminution, the cellulose crystallinity is destructed and thus cellulase can penetrate to the biomass surface in a more efficient way. The size of the material is generally 10-30 mm after chipping while it is much more decreased after milling or grinding, gaining a value between 0.2-2.mm (Sun and Cheng, 2002). Millet *et al*. (1976) observed that vibratory ball mill gives more impressive results for the breakdown of cellulose crystallinity rather than other milling methods. The study of Alvo and Belkacemi (1997) proved that when alfalfa is pretreated with roller mills and compared with non-milled materials, the hydrolysis yield of the samples increases from 38% to 62.5% and when the same procedure is applied to timothy grass, the change in hydrolysis yield is only 5% (from 51.4% to 56.4%). The final size of the samples of Alvo and Belkacemi (1997) are recorded between 53-106 μm.

On the other hands, since the reduction in particle size is effective in enzymatic hydrolysis of the samples, the energy required is also related with the final size of the sample and the properties of lignocellulosic biomass (Sun and Cheng, 2002). The study on final particle structure and energy consumption (Cadoche and López, 1989) proves that as the size of lignocellulosic material decreases, the energy used in the system increases (Table 1.4).

Table 1.4 The effect of final particle size and type of mechanical comminution on the energy consumption (Cadoche and López, 1989)

Lignocellulosic materials	Final size (mm)	Energy consumption (kWh/ton)	
		Knife mill	Hammer mill
Hardwood	6.35	25	95
	3.20	50	115
	2.54	80	120
	1.60	130	130
Straw	2.54	6.4	29
	1.60	7.5	42
Corn stover	9.50	3.2	NA
	6.35	15	NA
	3.20	20	9.6
	1.60	NA	14

NA: not applicable

Pyrolysis is experienced as a physical pretreatment method as well as mechanical comminution. The procedure includes firstly increasing the temperature more than 300°C and then cooling and condensing. It is studied on timothy grass, alfalfa (Boateng, *et al*., 2006), wood (Prosen *et al*., 1993), waste cotton (Yu and Zhang, 2003) and corn stover (Khiyami *et al*., 2005).

1.6.2. Physical-chemical methods

The second group, physical-chemical pretreatment, is composed of studied methods such as steam explosion, liquid hot water (LHW), ammonia fiber explosion (AFEX) and carbon dioxide explosion. Steam explosion, also known as autohydrolysis, is the most frequently used one among them (McMillan, 1994), and it is applicable in industry. It is performed with a saturated steam, with a temperature range of 160-290°C and pressure range of 0.69-4.85 MPa and it ends after several seconds or minutes. Afterwards, decompression is done to reach atmospheric pressure. The advantages of steam explosion are summarized by Sanchez (2008). It can work at high solid contents, the energy to reduce the size of lignocellulosic biomass is much less than mechanical comminution, hemicellulose hydrolysis can be reached up to 80-100% with a degradation of

some xylan molecules. On the other hand, there are some negative effects of steam explosion on the all production procedure. For instance, lignin-complex matrix can be disrupted completely (Mackie *et al.*, 1985) and some inhibitory compounds form from the degradation products of the process. These inhibitors affect the microbial growth, enzymatic hydrolysis and fermentation. Thus the pretreated biomass is washed to get rid of the inhibitors but the washing causes some of soluble sugars to be lost since 20-25% of the first dry matter is withdrawn by water (Mes-Hartree *et al.*, 1988 and McMillan, 1994).

The other promising physical-chemical pretreatment methods are liquid hot water (LHW, thermohydrolysis) and ammonia fiber explosion (AFEX). For LHW, pressurized hot water is used with a process pressure of more than 5 MPa, and the temperatures are between 170-230°C. The pro of the LHW pretreatment is having high pentose recovery rates without production of inhibitors as in steam explosion (Nguyen *et al.*, 1999). On the contrary, the applicable solid concentration is lower than 20%, while the solid load exceeds 50% in steam explosion technique.

Another prominent and developing physical-chemical method is the ammonia-fiber explosion technique. Liquid ammonia is loaded to the system with a range of 1-2 kg/ kg dry biomass and lignocellulose is soaked with high pressure such as 10-52 atm at mild temperatures (90°C) (Holtzapple, 1994). AFEX pretreatment is very similar to steam explosion whereas it has some advantages and disadvantages. First of all, in AFEX pretreatment, there is no necessity to decrease the particle size before the pretreatment application and secondly, there is no formation of inhibitory compounds. Contrarily, the ammonia added to the system needs a recovery process to decrease the cost and the process is dependent on the moisture content of the sample (Sanchéz and Cardona, 2008). AFEX is applied to a various kind of lignocellulosic biomasses such as aspen wood chips (Tengerdy and Nagy, 1988), bagasse (Holtzapple, 1991), wheat straw (Mes-Hartree *et al.*,1988), barley straw, rice hulls, corn stover, switchgrass (Alizadeh, 2005), coastal Bermuda grass (Holtzapple, 1994), and newspapers (Holtzapple,

1992).The study of Holtzapple (1991) showed that AFEX is not very effective for high-lignin containing samples as newspapers (18-30%) and the composition of pretreated biomasses does not change as great as in steam explosion or dilute acid pretreatment (Sun and Cheng, 2002). Closely to AFEX, carbon dioxide explosion works with a similar procedure. But the yields are lower compared to other techniques (Sanchéz and Cardona, 2008).

1.6.3. Chemical methods

Chemical pretreatment methods can be investigated in six categories: ozonolysis, acid hydrolysis, alkaline hydrolysis, oxidative delignification, wet oxidation and organosolv process. In ozonolysis, the aim is to degrade lignin and hemicellulose by using pressurizing ozone to the lignocellulosic sample at room temperature (Sun and Cheng, 2002). Vidal and Molinier (1988) investigated that while untreated poplar sawdust has a lignin content of 29%, the treated sample has a lignin of 8%. Besides, the removal of lignin, inhibitory compound formation is not observed in ozonolysis and the pretreatment can be hold under mild conditions- at room temperature and pressure. On the other hand, since the price of ozone is really high, there are not so many studies about ozonolysis.

Acid pretreatment is the leading method in all of the pretreatments. It is applied in two different types, as concentrated or dilute. In all of them, the acid used is generally sulfuric acid or hydrochloric acid. Since concentrated acid pretreatment is founded to be a harmful and expensive application because of the toxicity and corrosivity of the chemical (Sivers and Zacchi, 1995), instead of concentrated acid pretreatment, dilute acid is well-developed and an influential method. After the pretreatment, nearly 80-100% of hemicellulose is hydrolysable and depolymerization of cellulose is achieved at a certain level (Sanchéz and Cardona, 2008). It is observed that when temperature is increased (McMillan, 1994) more cellulose is hydrolyzed. The amount of dilute acid added to the system changes between 0.75 and 5 (v/v) % (Hamelinck *et al*., 2005; Saha *et al.*, 2005 and Schell

et al., 2003). Besides having an application area in industry, the cost of the process, which also contains neutralization of pH after the treatment and acid itself, makes the cost higher than steam explosion and AFEX.

Alkali pretreatment looks similar to acid pretreatment whereas in that method, dilute bases (sodium hydroxide or calcium hydroxide) are added. The bases cause an increase in surface swelling, decrease in crystallinity, cleavage of the links between lignin and cellulose/hemicellulose and lastly a collapse of lignin structure. The advantage of alkali addition over acid addition is the 50% lower cost of reactors. This pretreatment is shown to be efficient for lignin removal in corn stovers with delignification efficiency of 60-80% and in switch grass with a delignification efficiency of 65-85% by Iyer *et al*. (1996). But it is known that small amount of inhibitors form during alkali pretreatment. The other chemical pretreatments are oxidative delignification and wet oxidation, for which peroxidase with 2% hydrogen peroxide are used as 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 an opponent to other methods (Lynd *et al*., 1999) because of the economical disadvantage of solvent prices.

1.6.4. Biological methods

The fourth group of pretreatment type is biological pretreatment. These methods are favorable in terms of the needs for low energy and being able to work under mild conditions. However their processes take longer times than other pretreatments. For instance the study of Hatakka (1983) records that *Pleurotus ostreatus* is able to convert 35% of wheat straw into reducing sugars in 5 weeks.The enzyme producers, fungi are the mostly-used organisms. As Sun and Cheng (2002) mentioned while white-rot fungi are able to break down cellulose and lignin, brown-rot fungi usually destruct only cellulose.

Evans *et al*. (1994) point out that *Phanerochaete chrysosporium* and *Phlebia radiata* are the most feasible fungi to produce the enzymes hemicellulases, cellulases and lignin-degrading enzymes (e.g. ligninases, lignin peroxidases, polyphenoloxidases, laccase and quinine-reducing enzymes). The recent work of Tengerdy and Szakacs (2003) comment on that cellulase and hemicellulase can also be produced by solid state fermentation rather than submerged fermentation which is commonly used in production of enzymes.

The cost of submerged fermented enzymes is still high, thus production of enzymes with solid state fermentation, which allows using host-specific native fungi and producing the most suitable enzyme complex for a specific lignocelluloytic biomass by mixing culturing, may offer a solution to economical disadvantages of biological treatments in near future.

Table 1.5 The relative compositional and structural influences of recent pretreatment types on lignocellulose model

Pretreatment	**The parameters affecting the lignocellulose model**				
	Accessibility of surface area	Cellulose decrystallization	Structural change in lignin	Removal of lignin	Removal of hemicellulose
Steam explosion	+ +	N	+	N	+ +
Dilute-acid pretreatment	+ +	N	+ +	N	+ +
Ammonia fiber explosion	+ +	+ +	+ +	+ +	+
Liquid hot water	+ +	ND	+	N	+ +
Lime pretreatment	+ +	ND	+ +	+ +	+

ND: not determined

N: no effect determined yet

Among all the pretreatment methods, steam explosion, dilute-acid pretreatment, lime (alkali) pretreatment, LHW and AFEX are seen to be the most cost-effective and most-powerful to disrupt the structural lignocellulose model (Table 1.5). But the pretreatment issue needs to be developed by improving the existing methods or finding novel technologies that find a solution to all needs described above.

1.7. Lignocellulose Hydrolysis

After pretreatment process, the partially delignified and depolymerized lignocellulosic biomass needs to be hydrolyzed to release glucose molecules-the feed material used in fermentation. The hydrolysis can be performed in two different ways: enzymatic and acid hydrolysis. As Hamelinck *et al*. (2005) mentioned 50% glucose conversion increases to approximately 90% when concentrated acid is applied rather than two-step dilute-acid hydrolysis. But the cost of the hydrolysis process exceeds the feasibility because of the necessity to recover hydrolysis, and also the operational need of corrosive-resistant equipments.

Enzymatic hydrolysis, on the other hand, is able to handle the handicaps of acid hydrolysis. Enzymatic hydrolysis makes the process easier by being specific, not forming inhibitory compounds, and working under mild environment. On the contrary, the reaction rates of enzymatic hydrolysis are really short that results in longer times.

The factors that affect the efficiency of enzymatic hydrolysis should be controlled and also improved to get more industrially-producible results. The factors are summarized by Cardona, Sanchéz and Gutiérrez (2010) intimately: (i) adsorptivity of enzyme on the substrate, (ii) reduction in rate caused by lignin-enzyme complex formation, (iii) cellulase inhibition as a result of increase in cellobiose concentration, (iv) β-glucosidase inhibition owing to glucose formation (v) reduction in cellulose hydrolysis rate since substrate conversion continues,

(vi) co-operative work of cellobiohydrolases and endoglucanases and (vii) optimized enzyme-load and solid-loads.

The studies investigating the conversion of cellulose to glucose usually emphasize on the parameters such as reaction time, temperature, pH, enzyme load and substrate load. For instance, Hari Krishna *et al*. (1998) determined that optimized conditions for hydrolysis of sugarcane leaves are 50°C and pH of 4.5. Although 100 FPU/g cellulose is observed to result in 100% hydrolysis, 40 FPU/g is advised with a conversion of 87% due to the cost of enzymes. And it is mentioned that solid load should not exceed 10%, otherwise, there would be mixing problems that will affect the accessibility of enzyme and the inhibitors would form a mass in the reaction volume.

1.7.1. Cellulase

The enzymes that can degrade lignocelluloytic structure are cellulases, hemicellulases and lignin-degrading enzymes. Bhat (2000) describes that among them; cellulases have a high variety of application from textile and food industries to paper pulp and paper production. Some anaerobic bacteria (e.g. *Clostridium thermocellum*), aerobic bacteria and mostly fungi (e.g. *Trichoderma reesei, Aspergillium niger*) are the microorganisms that can convert cellulose to glucose. Generally for the production of the industrial cellulase enzymes, *Trichoderma reesei*, whose cellulase consists of five enzymes: two cellobiohydrolases (EC 3.2.1.91), two endoglucanases (EC 3.2.1.4) and cellobiase (β-glucosidase) (EC 3.2.1.21), is used. Rabinovich *et al*. (2002) records that cellobiohydrolases and endoglucanases posses identical sequence of thirty-five amino acid residues, which is responsible for binding of crystal cellulose to fibrils. Zhang and Lynd (2004) mention that nearly 92% of total cellulase produced by *T. reesei* composed of endoglucanases and cellobiohydrolases.

Cellobiohydrolases are responsible for the breakage of β(1,4) linkages of cellulose from its reducing and nonreducing ends resulting in cellobiose.

Although endoglucanases breaks the same linkages of cellulose, its action is observed inside the chain. Since endoglucanase produces smaller units in short time, the reduction in polymerization degree happens more quickly rather than the reduction seen in the action of cellobiohydrolases. But as Lynd *et al*. (2002) shows, while cellobiohydrolases are able to progress on crystal and amorphous cellulose together, endoglucanases can only work on amorphous cellulose.

β-glucosidase is cellobiase which is capable of hydrolyzing cellobiose into two glucose molecules. In spite of being produced by T. reesei, the activity of it is not as required. As a result, β-glucosidase utilization additionally to the industrial cellulase complex of T. reesei during lignocellulose hydrolysis is studied and found out there is a synergic effect on hydrolysis by the work of Walker *et al*. (1993).

The rate of enzymatic hydrolysis of lignocelluloytic structure by cellulases is lower than the hydrolysis rate of other substrates such as starch saccharification. The reason mostly lies in accessibility of cellulases to the lignocellulosic substrate and also in structural hindrance. Firstly, β(1,4) linkages are known to be stronger than α(1,4) linkages. Secondly, the presence of lignin, which surrounds hemicellulose and cellulose, causes a barrier. And thirdly, the 3-D structure of lignocellulose structure is more complex than starch structure.

Using surfactants, making different kind of enzyme blends or producing multicellulase plasmid, and trying new methods before or after the enzymatic hydrolysis generate possible solutions to the slowness of lignocellulose hydrolysis. For example, Duff *et al*. (1995) tried sophorolipid as a surfactant and recorded a 67% increase in the hydrolysis of steam-exploded wood. Kim and his colleges (1998) made a factorial enzyme optimization to find the best cellulase composition.

1.8. Nanofibers

Nanofibers are defined as fibers with a diameter smaller than 100 nm and they have gained great interest because of their wide application. Automotive, packaging, construction industries have already started to use nanofibers due to their characteristic behaviors such as large surface to volume ratio and highly porous structure. In spite of the increasing demand of synthetic nanofibers, they have some environmental negative drawbacks like non-recyclability and partial combustibility. At that point, natural nanofibers become more of an issue. In addition to biodegradability, the natural nanofibers, which can be produced from wood, starch or lignocellulosics, have the featured properties of having low cost, low density, well-developed thermal properties.

1.8.1. Cellulose nanofibrils

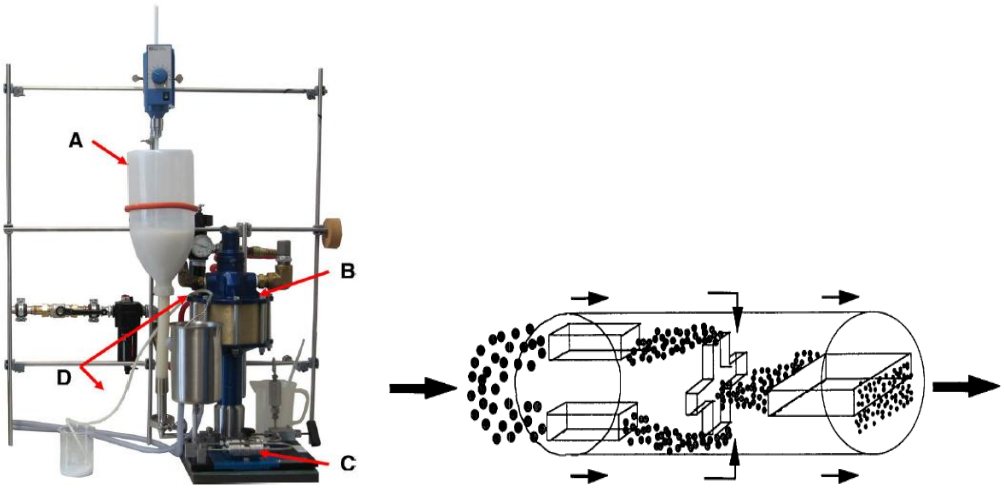
The most known and most abundant natural nanofibril, cellulose nanofibril, can be excluded from the plant cell wall or be produced by some bacteria i.e. *Acetobacter xylinum* (Yano, 2005). Somerville (2006) shows that their width is around 5 to 30 nm and the author mentions about the structure of celluloses, parallel long fibers bound by hydrogen bonds and Van der Waals forces, which is the reason of high-crystallinity. Nishino *et al.* (2004) record that the elasticity modulus of the crystalline parts of cellulose is 138 GPa and they point out that the value is really competitive with other important synthetic fibers (e.g. Vectran with 126 GPa, Technora with 88 GPa and Ekonol with 130 GPa). Also, Page (1971) determines the maximum macroscopic Young-modulus of wood-origin cellulose as 128 GPa which is more than the that of aluminum (70GPa) and glass fibers (76 GPa). In addition the ultimate tensile strength of cellulose fiber is found to be seven times higher than steel by Bledzki *et al.* (1999). These all data ensure that cellulose nanofibrils are available to be used as reinforcement material and thus in biofilm industry.

1.8.2. Production of cellulose nanofibrils

Due to the recalcitrance of cellulose, which is embedded in lignocellulose in plant cell walls, the extraction of it becomes an important issue. The studies about nano-fibrillation process show that the processes can be mechanical, ultrasonic or enzymatic. The mechanical treatments consist of grinder, cryocrushing and high-pressure fluidizer (microfluidizer). Grinding application is studied recently by Iwamoto *et al*.(2005) and it is said that grinding is successful in nano-fibrillation process of wood pulp fibers and the authors claimed that it is possible to produce nanofibers similar to bacterial celluloses by that application. Bhatnagar and Sain (2005) applied cryocrushing to remove lignin from chemically treated flax bast fibers, hemp fibers, kraft pulp and rutabaga samples by liquid nitrogen. Liquid nitrogen is used to freeze the samples and then high-shear mechanical treatment is performed to set the nano-scaled fibrils free. As a conclusion, it is described that since the nanofibrils produced by cryocrushing method are very resistant to reinforcement and their weight is so low, the fibrils can be used in aerospace and automotive sector. The other example is the ultrasonic application (Zhao, Feng and Gao, 2005), which is an environmentally-friendly, multifunctional, economic and practical method. It is found out that ultrasonic method can be used without self-assembly and electrospinning to produce bionanofibrils. Pääkkö *et al*. (2007) insert mild enzymatic hydrolysis process into the nanofibrils production from softwood cellulose pulp with a combination of mechanical shearing and high-pressure homogenization. They observe that enzymatic hydrolysis is very effective in improving the strength of the gel because enzymatic hydrolysis is seen to increase aspect ratio and thus it conserves junction zones of cellulosic structure. When acid hydrolysis is compared with enzyme hydrolysis at the study of Pääkkö *et al*. (2007), it is highlighted that using enzymes is more feasible since it increases elastic modulus of the gels more than acids, and also it is found out to be more economic. It is conclusive to say that, the bionanofibrils production technique is a developing issue because of their wide-range of applications and their prominence on synthetic nanofibers.

1.8.3. Microfluidization

Microfluidization is a developing technique since the early 1980s and it has emerged as a type of high pressure homogenization. Nowadays, microfluidizers (Figure 1.16.a) are used for cell disruption, production of fine emulsions, microcapsules and other high pressure applications. Cook and Lagace (1985) mention about the working principles of the microfluidizer. It is said that the reaction chamber allows the fluid to be divided into two microstreams and then the streams are coming across each other face to face (Figure 1.16.b). The microfluidizer has a capacity to get high shear rates with a fluid flow range of 100 to 600 ml/min.



(a) (b)

Figure 1.16 (a) Lab-scale microfluidizer (M-110Y Microfluidizer® Materials Processor) showing the parts of equipment; A: Container for suspension equipped with stirrer, B: Air pump, generating high pressure, C: Patented air chamber, D: Cooling loop and product outlet; (b) The diagram illustrating the air chamber in the microfluidizer (Lagoueyte and Paquin, 1998).

Microfluidization has been experienced in different areas, such as pharmaceutical, cosmetic, polymer, ceramic and food (e.g. milk, xanthan gum) applications. Zimmerman *et al*. (2010) study microfluidized wheat straw and wood fibers to observe their reinforcement characteristics. It is stated that fibrillated materials have lower degree of polymerization and higher tensile strength, modulus of elasticity compared to commercial fibrous materials. Lagoueyte and Paquin (1998) investigate the effect of microfluidization on thickening and stabilizing properties of xanthan gum, and mention that microfluidized gum has minimized viscosity and pseudoplastic behavior, plus, these gums have shown less hydration rate and water uptake compared to untreated xanthan gum.

Recently, Ahola *et al*. (2008) have investigated the enzymatic properties of nanofibrillated cellulose produced by microfluidization technique. The study demonstrates that nanofibrillated celluloses have higher enzymatic hydrolysis rates compared to Langmuir-Schaefer cellulose films, spin-coated cellulose films and cellulose nanocrystals. The nanofibrillated celluloses are found to have smaller thickness (10 nm) than others and have such a three-dimensional network that affects the accessible surface area.

1.9. Wheat structure, by-products and usage

Wheat is an agricultural product that is produced worldwide since it stands for one of the most used human food. The chemical composition of the wheat bran may change due the species of the grain, the weather during harvest, the type of milling method and drying conditions. According to the compositional analysis results of Neutral Detergent Fiber method, an accurate method introduced by Van Soest, dietary fibers makes up more than half of the bran (53%), and the mineral content of bran is relatively high (Table 1.6).

Table 1.6 The chemical composition of the grain and its components given on a dry matter basis. (Belderok, 2000)

	Whole grain (%)	Mealy endosperm (%)	Bran (%)	Germ (%)
Proteins	16	13	16	22
Fats	2	1.5	5	7
Carbohydrates	68	82	16	40
Dietary fibers	11	1.5	53	25
Minerals (ash)	1.8	0.5	7.2	4.5
Other components	1.2	1.5	2.8	1.5
Total	100	100	100	100

1.9.1. Wheat bran

Wheat bran is the first part separated from the mealy endosperm of the wheat grain (*Triticum* spp.) during the milling process. It contains the fused pericarp plus the seed coat, the aleurone layer and the remnants of the starchy endosperm (Pomeranz, 1987). Industrial wheat bran accounts for 13-17% of the grain while germ consists of 2-3% and mealy endosperm 80-85%. Pericarp and seed coat, which are the two external layers of the grain (Figure 1.17), are composed of empty and dead cells.

On the other hand, living protoplasts are stored in the cells of the inner layer, i.e. aleurone layer. Thus, bran has relatively high amounts of protein, carbohydrates and lignin. According to the study of Lequart *et al*. (1999) wheat bran is comprised of 42.5% cellulose, 21.2% hemicellulose and 3.4% lignin.

Maes and Dalcous (2001) mentions that wheat bran is composed of mainly starch, glucurono-arabinoxylans, cellulose, β-glucan, protein and mixed-linked (1-3), (1-4)- β-D glucan. Because of its low cost, that feed stock is getting interest due to the pressure to ensure total utilization of them, and to address economic and environmental concerns.

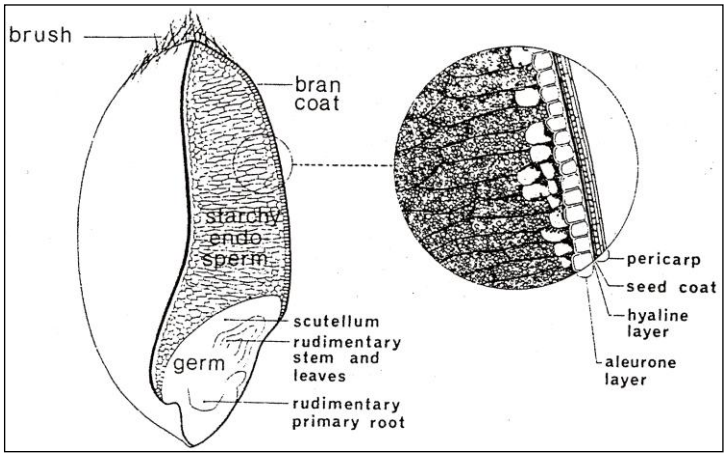


Figure 1.17 The anatomic structure of the wheat grain (Belderok, 2000)

1.9.2. Wheat straw

Wheat straw is the stem and leaf part of wheat which is left after harvesting of grains. Its composition is mainly divided into three: 39.6 % cellulose, 26.4 % hemicellulose and 21 % lignin (Lequart *et al*., 1999). The production of dry wheat straw is nearly 11 million MT per year (Perlack *et al*., 2005).

1.9.3. Processing of wheat byproducts

The waste material can be processed by the biotechnology industry such as for bioethanol production, enzyme production and biodegradable film production. For instance, Chotěborská *et al*. (2004) have shown that wheat bran needs a pretreatment process before enzyme hydrolysis. Palmarola-Adrados *et al*. (2005) have studied the production of ethanol from the non-starch part of wheat bran and they have observed that enzymatic hydrolysis step after microwave pretreatment (170°C, 20min) has a crucial influence on the production of fermentable sugars. The enzymatic hydrolysis is performed with a pH 5.0 and temperature 50°C with the aid of industrial enzymes Celluclast (mainly cellulase) and Ultraflo (β-

glucanase and xylanase). At the end of the study it is recorded that Ultraflo has no significant effect on the total sugar yield. Tabka *et al*. (2006), on the other hand, have investigated the pretreatment and enzymatic saccharification of wheat straw. In that study, steam explosion was used as a pretreatment and enzymatic hydrolysis was done at 50˚C, pH 4.8 and a solid load of 10% (w/v, dry biomass). The enzymes and the adjusted concentrations are: xylanase, 5 U/g (dw); cellulase, 10 U/g (dw), feruloyl-esterase, 20 U/g (dw); laccase, 20 U/g (dw). As a conclusion, addition of xylanase and feruloyl-esterase was found to yield a 1.4-fold increase in glucose recovery but since there is a barrier not to exceed enzymatic hydrolysis cost, it is said that there is a need for the optimization of enzyme loads.

1.9.4. Production and consumption of wheat

According to the study of Kim and Dale (2004) nearly all the wheat (71% in total) is known to be consumed as a food (Figure 1.18). The consumption profile of wheat grain changes according to the regions. For instance, 47% of wheat produced is used as food in Europe, while the percentage increases to 84% in Asia. Also, waste percentage increase from 2.5% to 4.5% when the production profile of Europe is compared with Asia. Almost 8% of wheat grain is left as waste in Central America.

Turkey has an important production scale in wheat production. According to FAOSTAT, wheat production has exceeded 17 million mega tones in 2007. Turkey is the third largest producer of wheat in Europe, following France and Germany, and wheat bran is the second most produced agricultural residue after sugar beet bagasse in accordance with 1999 data recorded in TUIK (2001).

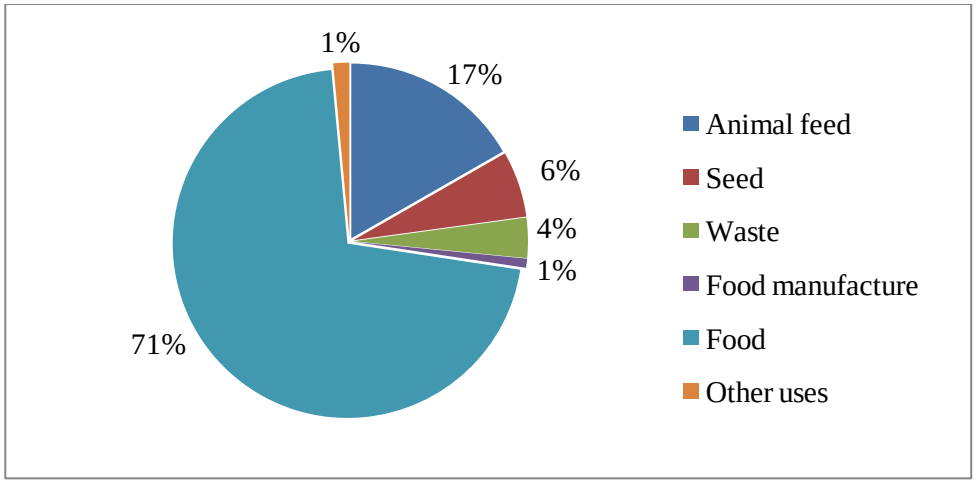


Figure 1.18 The worldwide wheat grain consumption profile (Kim and Dale, 2004)

1.10. Corn structure, by-products and usage

Corn grain is a caryopsis and thus it is a one seeded plant. The composition, size and structure of corn grain may change due to the different species (Inglett GE, 1970). It is known that the upper part of the plant contains less lignin and it is more digestible compared to other parts.

1.10.1. Corn bran

The by-products of corn industry include stalks, leaves, husks and cobs (which are named totally as corn stover) which remain after harvesting and also corn bran which is left after corn starch process (Figure 1.19). The composition of corn by-products differ depending on the time and method of harvesting, the collected fraction and genetic factors coming from the whole corn.

The compositional study of corn bran that is the separated part of corn kernel from starch and protein was performed and reported that 67.5% of bran is arabinoxylan, cellulose 22.5% and protein 2.4% by Saulnier *et al.* (1995). The

pericarp, layer outside the corn kennel, makes up the major component of the corn bran.

Doner and Hicks (1997) have mentioned about the abundance of corn bran and reported that about 4 millions tons/year corn bran was producible as a byproduct from corn wet milling industry, which makes corn bran as the most plentiful by-product of corn industry.

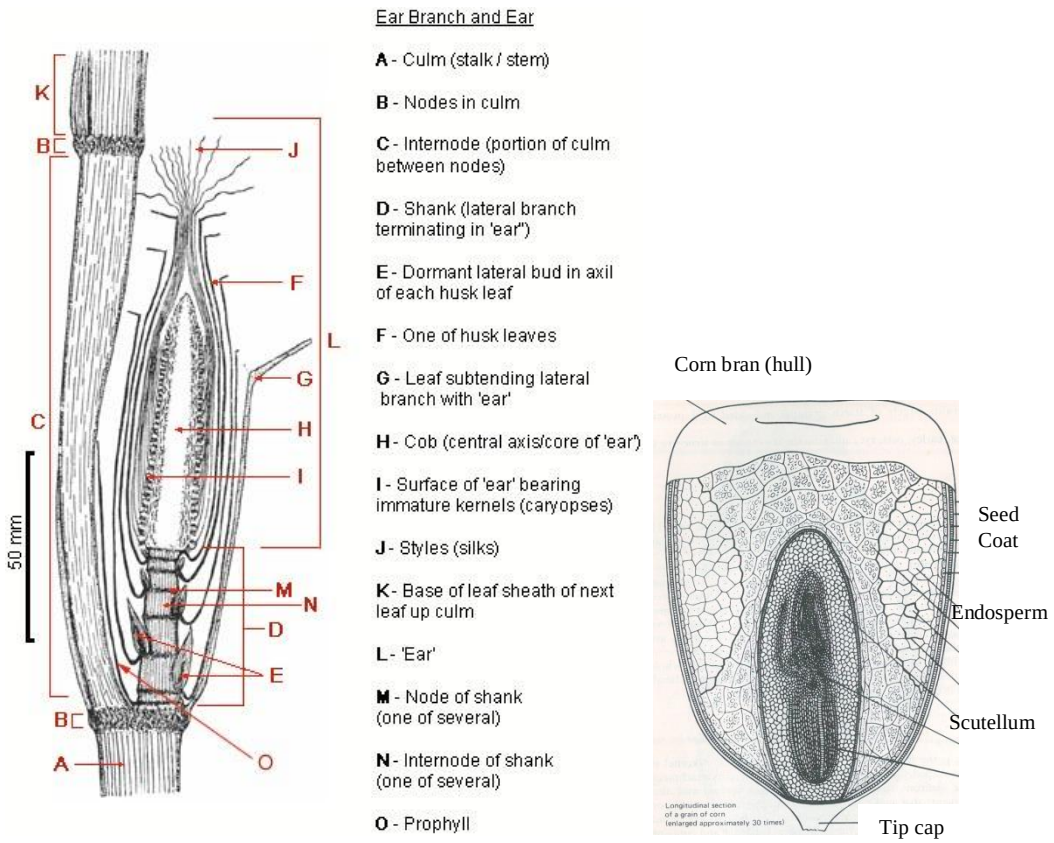


Figure.1.19 The anatomic structure of (a) corn plant (Weatherwax, 1955) and (b)corn kernel (Pomeranz, 1987)

1.10.2. Processing of corn byproducts

Corn by-products usage areas are mainly, animal feed, bio-based products such as paper-pulp, composites and chemicals (e.g. furfural, rayon, cellulose acetates and cellulose nitrates) and fuels.

Corn bran is mostly utilized in animal feed industry. In terms of finding new technologies to use the bran efficiently as a lignocellulosic biomass, liquefaction was developed recently. For instance, Lee *et al*. (2000) study the corn bran to produce resin with liquefying method by sulfuric acid at high temperature and pressures.

Most of the studies on corn by-products are concentrated on corn stover, especially in fuel-industry. It is known that there are two ways to get use of stover. One is burning the residues of hammer-milling of corns instantly in a boiler furnace and the other is producing fermentable sugars from stover and using them in bioethanol production. For instance, Öhgren *et al*. (2007) investigate the effects of steam explosion, delignifaction and additional xylanase addition on the enzymatic hydrolysis of corn stover. They observe that adding sulfur dioxide and increasing temperature from 170°C to 190°C increases the fermentable sugar at the end of enzymatic hydrolysis. It is seen that delignification with ethanol decreases lignin content of pretreated stover approximately by 40% so increases the glucose content obtained from enzymatic hydrolysis but it also decreases xylose content at that unit, thus the need of delignification is still questionable. Moreover, it is seen that xylanase addition has a significant effect on the total sugar hydrolyzed.

A recent study, performed by Yang *et al*. (2010), tries a new method named as biopretreatment with white-rot fungi (*Pleurotus ostreatus* BP2, *Echinodontium taxodii* 2538 and *Irpex lacteus* CD2). It is recorded that the hemicellulose content of the samples decreases after the construction of integrity and complexity of the stover structure. In addition, sulfur removal, which shows the environmentalist

effect of biopretreatment, is observed thus it is suggested that less sulfur dioxide will be formed after the biofuel produced by bio-treated stover is burned.

1.10.3. Production and consumption of corn

Kim and Dale (2004) recorded that corn is generally produced in North America (42%), then Asia (26%) and Europe (12%). Most of the corn produced is used in animal feed application and almost 5% of it is left as a waste (Figure 1.20). Corn waste is defined by FAOSTAT as a material lost in the farm and also during handling, storage and transport.

Turkey has produced 3.5 million mega tones of corn and its value is calculated to be $4x10^5$ million $ which puts Turkey to the third order in Europe after France and Ukraine in Europe according to the results of FAOSTAT (2007). Thus it can be said Turkey has a capacity to work on corn by-products to make use of it in biotechnological advances.

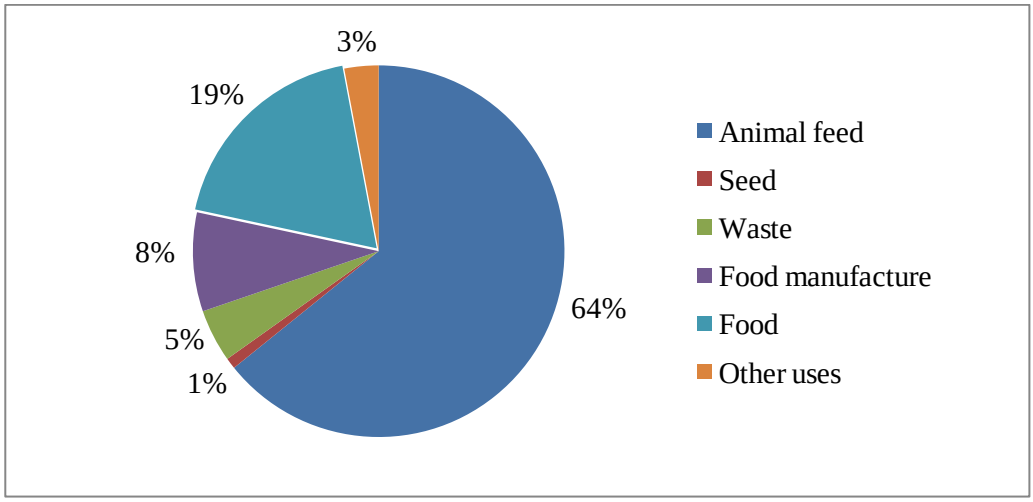


Figure 1.20 The worldwide corn consumption profile (Kim and Dale, 2004)

1.11. Usability of wheat and corn by-products as a lignocellulosic biomass

The cultivated wheat area in Turkey has exceeded 9 million hectares in 2000s and the amount of production is recorded as 21.5 million tons in 2005 (FAOSTAT). Wheat is known to have species specific for all types of soils. In poor-quality soils, wheat species for bread making, in high-quality soils, wheat species for pasta making are generally grown (T.C. Tarım ve Köy İşleri Bakanlığı, 2004). The total by-product left after harvesting wheat kernels is calculated to be nearly 35% (Meyers Norris Penny LLP, 2004). Rice (1999) records that the bioethanol yield is about 369 l bioethanol per 1 ton of wheat, while the yield for sugar beet is 101 l bioethanol per 1 ton of beet. Thus, wheat is known to be available for bioethanol and bio-based materials production due its properties. In addition to these, it can be stored in good condition, it is found all over the world, and it has high rural production compared to other grains. Thus, wheat has been studied extensively as a bioresource and there are already some applications in wheat-based bioethanol production in Canada (Iogen Corporation), France (Tate & Lyle, BENP, BCE, Tereos), Australia (Grainol), Germany (Südzucker), Spain (Abengoa) and Sweden (Agroethanol).

The records about corn production show that Turkey has nearly 800 thousand hectares of cultivated corn area and the production of corn is calculated as approximately 3.5 millions ton in 2005 (FAOSTAT). Corn is very valuable for the production of bioethanol, especially in the USA. The by-product of corn, corn stover is not collected thus, for every 1 kg of corn harvested; nearly 1 kg of stover is produced. And the bioethanol yield is calculated as 400 L bioethanol/ton corn by Shapouri *et al*. (2002).

Bulut (2006) has studied the possible biomass resources in Turkey, including wheat, barley, corn, sugar-beet and potato. Agricultural properties (production capacity, storage time, production yield/land, production energy), bioethanol-processing properties (bioethanol yield, by-product availability, experience gained in processing), and financial properties (raw material cost, energy

consumption, offer-demand situation) were taken as the criteria. The most advantageous biomass, in terms of agricultural properties was wheat. And, in terms of bioethanol-processing properties, corn was selected. On the other hand, according to financial properties, sugar-beet appeared to be better than others. When all these properties were taken into consideration, wheat was selected to be the most suitable biomass to produce bioethanol in Turkey.

1.12. Aim of the study

The aim of this study was to determine the effect of microfluidization on the efficiency of enzymatic cellulose hydrolysis by reducing the size of wheat bran, wheat straw and corn bran lignocellulose into nano-fibrils. Microfluidization was performed at two different pressures: 500 and 2000 bar. Comparison was made with softening process, and also with some outstanding processes such as dilute acid and lime pretreatments. The effect of microfluidization on wheat bran, wheat straw and corn bran was investigated with X-ray diffraction and scanned electron microscopy equipments to estimate the change in the crystalline structure and surface characteristics before and after processing.

For enzymatic hydrolysis Celluclast and β-glucosidase addition was used. Comparisons were made between substrates and enzyme loads.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Lignocellulosic biomass

Wheat straw, wheat bran and corn bran samples were obtained from local producers in Ankara.

2.1.2. Chemicals

Chemicals used in this study were analytical grade, either purchased from Sigma-Aldrich (N.Y., USA) or Merck Chemical Companies (Deisenhofen, Germany). The list of the chemicals and their suppliers is given in Appendix A.

2.1.3. Enzymes

Commercial cellulase, Celluclast (specific activity ≥700 U/g) is a mixture of enzymes which are cellobiohydrolase, endoglucanase and cellobiase (β-glucosidase) and is produced by *Trichoderma reesei*. In this study the commercial enzyme was obtained from Novozymes Corporation. β-glucosidase (5.2 units/mg solid specific activity) from almonds was obtained from Sigma-Aldrich. The enzymes were kept at 4°C until their usage.

2.1.4. Buffers and Solutions

The preparation of buffers and solutions used are given in Appendix B.

2.2. Methods

2.2.1. Sample preparation

Milling was accomplished separately by a hammer mill (Thomas-Wiley Laboratory Mill, Model 4, Arthur H. Thomas Company, Philadelphia, PA, USA) to get homogenous uniformly-sized samples from wheat bran, wheat straw and corn bran until it becomes possible to pass 2.0 mm round screen. They were kept at room temperature in a closed container until use.

2.2.2. Microfluidization

Microfluidization was carried out with a Microfluidizer (model M-110Y, Microfluidics, Newton, USA) at two different pressures: 500 bar (120 ml/min) and 2000 bar (180 ml/min). The principle of the equipment is based on three subjects. Firstly the liquid is divided into two jet microstreams. Secondly they are allowed to enter microchannels where high rate of shear rates are applied. Lastly the microstreams enter an area where there is liquid to liquid contact and then fluidized liquid leaves the chamber by the formation of a cavity and turbulence since there is a pressure drop afterwards.

After the samples were processed by microfluidization, washing was done. Microfibrillated samples were centrifugated at 16,400 g for 10 min (Sorvall Instruments, RC5C, DuPont Co., Wilmington, DE, USA) followed by moisture content determination and storage at 4°C up to one month.

2.2.3. Softening pretreatment

The wheat bran, wheat straw and corn bran samples were kept at 50°C for a one day in a water bath at pH 8.0, adjusted using 10 M NaOH. Afterwards, washing was performed by centrifugation at 16,400 g for 10 min (Sorvall Instruments, RC5C, DuPont Co., Wilmington, DE, USA). Moisture content of the samples

were measured, then stored at 4°C until enzymatic saccharification and kept there for maximum one month.

2.2.4. Dilute-acid pretreatment

Milled samples (5%, dw/v) were suspended in dilute sulfuric acid (H_2SO_4) solution which was adjusted to 0.75% (v/v). That sulfuric acid concentration of the mixture was selected since it was determined to give the highest yield (Saha *et al.*, 2005). Then they were pretreated in an autoclave at 121°C for 1 h. The pH of the pretreated samples was adjusted to 4.8 using10 M NaOH, before enzymatic hydrolysis.

2.2.5. Lime pretreatment

Samples were pretreated with lime (calcium hydroxide) in the presence of water. The pretreatment conditions were: 45°C or 60°C, 16h or 24 h, 0.075g $Ca(OH)_2.g^{-1}$ dry biomass, and 5 ml $H_2O.g^{-1}$ dry biomass was added to the system. After the treatment, washing procedure was followed up by centrifugating the samples four times at 3000 rpm for 10 min using an Eppendorf, Minispin Plus (Westbury, NY, USA) . Finally, pH was adjusted to 4.8 by adding acetic acid before saccharification.

2.2.6. Enzymatic hydrolysis

For lignocellulose hydrolysis, Celluclast, which consists of three enzymes namely cellobiohydrolase, endoglucanase and cellobiase (β-glucosidase), was used. Substrate mixtures (pretreated wheat bran, wheat straw and corn bran) with different substrate loads (2.5%, 5.0%, 7.5% dw/v) were brought to pH 4.8 using 50 mM citrate buffer. Cellulase at various concentrations (1.5 U/g-9.0 U/g dry biomass) was added to initiate the reaction. In some cases, β-glucosidase (25 U/g) was added together with Celluclast. Enzymatic saccharification was performed at 50°C in a rotary shaker at 100 rpm (Infors HT, Aerotron, Bottmingen,

Switzerland). Duplicate samples were taken periodically and were kept at 100°C for 3 min to stop the hydrolysis reaction. Reducing sugar concentration in the samples was analyzed by the DNS method.

2.2.7. Determination of reducing sugar

Dinitrosalicylic acid method was used for the determination of reducing sugar content released during enzymatic reaction (Miller, 1959). DNS reagent is prepared as described in Appendix B. In DNS method, reducing sugar within the samples was determined by using glucose as standard (Appendix C).

For the optimization experiments, solid load of the hydrolysis medium was adjusted to 5% (dw/v) unless otherwise stated. The cellulase activity of the medium was kept at 4.5 U/g, if enzyme concentration optimization was not performed. The pH of the medium was adjusted to 4.8, and the hydrolysis was performed for five days at 50°C, which is the optimum temperature of cellulase. During hydrolysis, duplicates of samples were taken from the medium at every 30 min for the first 150 min, and afterwards, daily for five days and their reducing sugar content was estimated by the DNS method. To determine the efficient usage of solid load during hydrolysis, relative TRS ratio was estimated as:

$$Relative\ TRS\ ratio(\%) = \frac{\left(\frac{TRS}{SL}\right)_i}{\left(\frac{TRS}{SL}\right)_{max}} \times 100\%$$

$(TRS/SL)_i$ = Total reducing sugar (TRS) content to solid load (SL) ratio of the samples of interest

$(TRS/SL)_{max}$=The maximum total reducing sugar (TRS) content to solid load (SL) ratio reached

2.2.8. Cellulase assay

The assay was done with carboxymethyl cellulose to determine the endoglucanase activity of Celluclast (Ghose, 1987). The reaction was performed by adding Celluclast (250 µl) to 50 mM citrate buffer, pH 4.8 at 50°C (Appendix D). Total reducing sugar content liberated during the enzymatic reaction was assayed by the DNS assay (Miller, 1959). One unit of cellulase is defined as the amount of enzyme that releases 1 µmole of glucose equivalents per minute under assay conditions. It was found that Celluclast mixture exhibits 915 U/ml CMCase activity and this value was used in preparation of enzyme dosages.

2.2.9. Protein determination

Protein concentration was determined to be used in activity calculations according to Lowry Method by using Bovine Serum Albumin (BSA) as standard (Lowry, 1951), (Appendix D).

2.2.10. Extraction of samples

Extraction was applied by using the method of Tappi T 264 om 88. Triplicate samples of approximately 5 grams oven dry material were extracted using 100 mL of water for extracting water soluble solutes, 70 mL of ethanol and 30 mL of water for extracting ethanol soluble solutes. The mixtures were allowed to stand 1 hour and then they were attached to a reflux condenser for 1 hour. The hot mixtures were cooled to room temperature, weighed and readjusted to original initial weight. They were filtered through a dry filter and 25 mL of the filtrates were transferred to dishes which were dried to a constant weight before. After they were evaporated to dryness, they were kept at 105°C for 3 hours, followed by cooling in a desiccator for 30 min, and weighing.

Water soluble extracts and ethanol soluble extracts were determined in terms of their percentage with respect to the weight of the dried samples. Extract solutes free samples were used for further analyses.

2.2.11. Determination of klason lignin content

The standard Tappi method (Tappi 222 om 88) was used to determine the lignin concentration. Triplicate samples of 1.5 g of extractive free samples were mixed with 15 mL of 72% sulfuric acid and they were stirred continuously for 2 h in a 20°C water bath. After the initial hydrolysis of Klason lignin, the acid in the mixtures were diluted to 3% by addition of 345 mL distilled water. The mixtures were refluxed at 100°C for 4 h and then they were cooled to room temperature, filtered through pre-weighed dry filters. The filtered samples were afterwards dried at 105°C overnight and reweighed. Klason lignin content (acid-insoluble lignin) was calculated in terms of percentage with reference to initial dried samples.

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

W_1= Initial sample weight

W_2 = Weight of filter paper, acid insoluble lignin

W_3= Weight of filter paper

T_{final}= % total solids content of the prepared sample used in the analysis, on a 105°C dry weight basis.

2.2.12. Determination of hemicellulose content

Holocellulose portion of the lignocellulose biomass was extracted firstly to determine hemicellulose content. Holocellulose content, the whole carbohydrate

fraction, was calculated by sodium chlorite method (Browning, 1967). Approximately 2 grams of triplicate extractive free samples were mixed with 320 ml distilled water, 1 ml of acetic acid and 20 ml 15% sodium chlorite solution. The reaction was maintained at 75℃ for four hours. The samples were placed on ice and brought to room temperature and then the holocellulose fraction was filtered. The samples were washed with 25 ml acetone and 200 ml ethanol, and afterwards, they were dried in an oven (50℃) overnight.

Hemicellulose, later, was extracted from the holocellulose preparations. Oven dried holocellulose samples (nearly 1.5 g) were mixed with 100 ml of 17.5% sodium hydroxide. The reaction was carried out at 20℃ for 2 hours in a shaker incubator. Later, the samples were filtered and; the filtrate was taken and brought to pH 7.0 with acetic acid. Next, 200 ml of ethanol was added to precipitate the hemicellulose. The precipitate was washed and dried in an oven (50℃) overnight and reweighed. The hemicellulose content was estimated as a percentage of the oven dried initial sample.

2.2.13. Determination of moisture content

The milled and/or pretreated samples were weighed initially and then put into an oven (Şimşek Laborteknik Oven, 120, Ankara, Turkey) at 105˚C and kept for 24 hours. Afterwards, the final weight of the samples was measured. The moisture content was calculated as follows:

$$Moisture\ content = \frac{initial\ weight - final\ weight}{initial\ weight} \times 100$$

2.2.14. X-ray diffraction (XRD) analysis

XRD was performed to study the crystallinity properties of the samples. A Rigaku Ultima-IV X-ray diffractometer instrument (National Nanotechnology Research Center, Bilkent University, Ankara) was used with a Cu-Kα radiation at 40 kV/40

mA and the samples were scanned from 5° to 50° in a 2θ angles with a scan speed of 2.0°/min. Sampling width was adjusted to 0.02°. The crystallinity index (CrI) was calculated according to the method of Segal *et al*. (1959) with the diffraction intensities of I_{002} at (002) peak position ($2\theta=22°$) and I_{am} at $2\theta=18°$. The I_{002} peak corresponds to the crystalline fraction and I_{am} corresponds to the amorphous fraction.

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100$$

2.2.15. Scanning Electron Microscopy (SEM) analysis

Differences in surface film morphology were observed using a scanning electron microscope (Nova NanaSEM 430, METU Central Laboratory, METU, Ankara). The samples were coated with gold before examination. The micrographs were taken at magnifications ranging from 300X to 1200X for the all three type of samples.

2.2.16. Statistical analysis

Statistical analysis was performed by using Minitab-15.0 software. The comparisons were made by Tukey's test with a significance level of 0.05.

CHAPTER 3

RESULTS AND DISCUSSION

The aim of this study was to determine the effect of generating nanofibrils of lignocellulose on the efficiency of cellulose hydrolysis using cellulase. Wheat bran, wheat straw and corn bran were selected as samples for determination of their enzymatic hydrolysis, because of their amount of production, usage area and processing abilities determined by Bulut (2006) and described in section 1.11. In order to obtain nanofibrils, microfluidization (high pressure fluidization) was employed at two different pressures, 500 bar and 2000 bar. Softening, lime and dilute-acid pretreatments were also applied for the sake of comparison.

3.1. Effect of microfluidization on microcrystalline cellulose hydrolysis

Microcrystalline cellulose, Avicel (PH-101), was firstly analyzed as a control to determine the effect of nanofibrillation on the hydrolysis of pure cellulose.

Avicel (10% dw/v) was pretreated with microfluidization equipment (high pressure fluidizer) at 500 bar, and then the substrate was prepared for enzymatic hydrolysis. Celluclast was added to the medium at different concentrations (18 U/g and 36 U/g dry biomass). The medium was then incubated at 50°C for 2 days and samples were taken at 18, 24, 30 and 42 h and total reducing sugar content was determined using the DNS method (Section 2.2.7).

It was observed that after one day, there is no difference in reducing sugar content between the microfluidized and untreated samples (Figure 3.1). However after 18 hours, the effect of pretreatment was clearly observed. Namely, the

reducing sugar concentration was approximately 1.8-fold higher for the case of cellulase with 18 U/g activity is and it was nearly 1.5-fold for the case of 36 U/g application. For the 36 U/g cellulase application, reducing sugar content reached 95% of its maximum after 18 hours of treatment while, in the absence of pretreatment, only 60% of the maximum was reached. The results showed that microfluidization enhances the rate of enzymatic hydrolysis of cellulose within the first 24 hours.

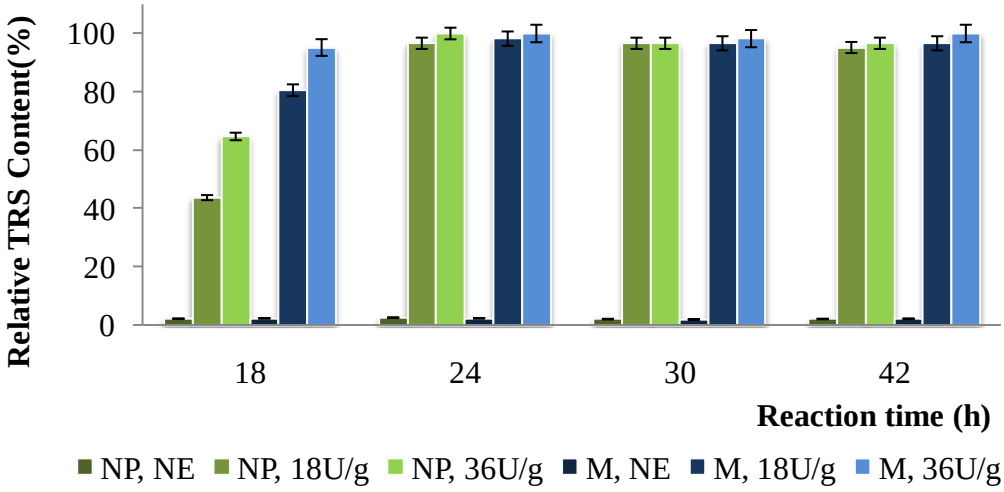


Figure 3.1 Effect of microfluidization on the enzymatic hydrolysis (50°C and 4.8 pH) of Avicel, microcrystalline cellulose (10% dw/v) in a total volume of 10 ml with mixing (100 rpm). NP, no pretreatment; NE, no enzyme addition; M, microfluidization.

3.2. Establishment of a time-interval for enzymatic hydrolysis

Wheat bran (5% dw/v) was studied to determine an optimum experimental time-interval for enzymatic hydrolysis and reaction was carried out for 3 days (Figure 3.2). Accordingly it was seen that there is a sharp increase at the second hour of the reaction and then insignificant differences are observed (Appendix H).

As for the time interval, it was decided to study the first 150 min interval under the same conditions (50°C, 4.8 pH). It was concluded from these data (Figure 3.3) that, the first 150 min interval is adequate to analyze the change in reducing sugar content.

Dourado *et al.* (2002) have shown that after 7 days, approximately 60% of the initial cellulase activity of Celluclast was lost at 50°C and thus, it was supposed that the lost of activity for the first 150 min interval is ignorable.

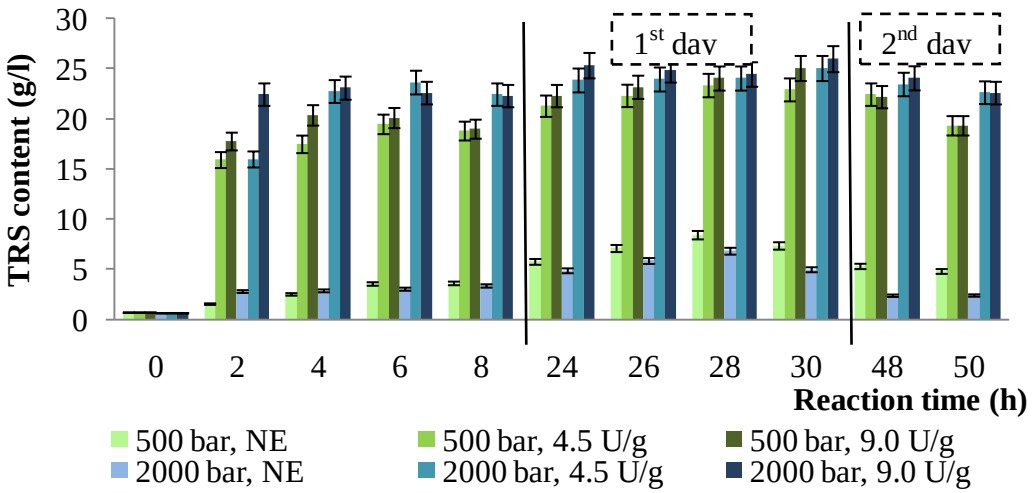


Figure 3.2 Effect of microfluidization at 500 bar and 2000 bar on the enzymatic hydrolysis (50°C and 4.8 pH) of wheat bran (5% dw/v) using Celluclast (4.5 U/g or 9.0 U/g dry biomass) in a total volume of 10 ml with mixing (100 rpm). NE, no enzyme addition

According to results presented in Figure 3.3, the initial enzymatic hydrolysis data have shown that wheat bran samples, which are microfluidized at 2000 bar, give a reducing sugar content 2.5 times higher than the untreated ones. The ratio decreases approximately to 1.8 for the 500 bar-treated samples.

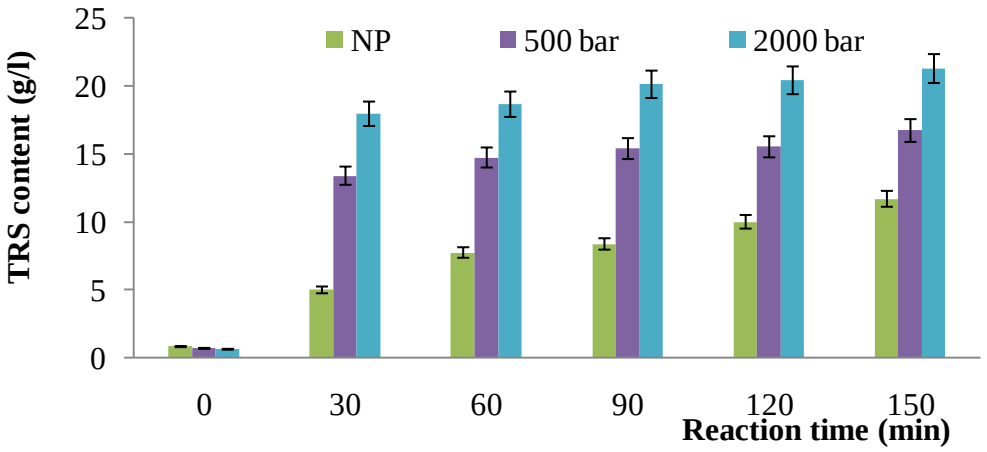


Figure 3.3 Effect of microfluidization at 500 bar and 2000 bar on the enzymatic hydrolysis (50°C and 4.8 pH) of wheat bran (5% dw/v) using Celluclast (9.0 U/g dry biomass) in a total volume of 10 ml with mixing (100 rpm). NP, no pretreatment

3.3. Optimization of enzymatic hydrolysis conditions for microfluidized lignocellulosic biomass

Microfluidized samples were used to determine optimum hydrolysis conditions. The effect of β-glucosidase, solid load (2.5, 5.0 and 7.5% dry wt) and cellulase load (1.5 U/g, 3.0 U/g, 4.5 U/g and 9.0 U/g dry biomass) during hydrolysis were investigated.

Prior to optimization, the moisture content of microfluidized samples was determined. The moisture contents of the treated and untreated samples are given in Table 3.1. The chemical compositions of the lignocellulosic samples were also analyzed before optimization experiments. It is known that a typical lignocellulosic biomass has 10-30% lignin, 15-35% hemicellulose and 30-50% cellulose (Costa Sousa *et al*., 2009). The results obtained were close to the values

in the literature (Estaghlalian *et al.*, 1997; Montané *et al.*, 1998 and Kim *et al.*, 2003) (Table 3.2).

Table 3.1 The moisture content of the lignocellulosic samples (expressed as weight percentage, % dry weight)

Sample	Moisture content (%)
Wheat straw	
Untreated	10.22±0.01
Softened	91.10±0.08
Microfluidized-500bar	88.60±0.02
Microfluidized-2000bar	88.01±0.04
Wheat bran	
Untreated	4.00±0.02
Softened	84.48±0.06
Microfluidized-500bar	90.05±0.01
Microfluidized-2000bar	92.58±0.02
Corn bran	
Untreated	1.25±0.01
Softened	91.48±0.04
Microfluidized-500bar	94.38±0.01
Microfluidized-2000bar	97.14±0.01

Table 3.2 The hemicellulose, klason lignin and cellulose content of the samples (expressed as weight percentage, % dry weight)

	Wheat straw	Wheat bran	Corn bran
Hemicellulose, % dry wt	22.55±1.30	29.45±1.50	16.25±1.80
Klason lignin, % dry wt	24.13±0.15	20.12±0.20	11.03±0.10
Cellulose, % dry wt	41.32±1.70	13.90*	14.99**

*: Data is taken from literature (Mongeau and Brassard, 1982)
**:Data is taken from literature (Jeltema, 1979)

3.3.1. Effect of β-glucosidase on the hydrolysis of lignocellulosic samples

Saccharification of lignocellulosics with cellulase ends up generally with the end products of glucose and cellobiose. Celluclast, which includes endoglucanases, cellobiohydrolases as major and β-glucosidases as minor, is able to solubilize

crystalline cellulose. While endoglucanases cuts randomly the internal β-1,4-linkages, and cellobiohydrolases release cellobiose units from non-reducing ends of glucan; β-glucosidases hydrolyze cellobiose to glucose. The activity of enzymes, found in the cellulase mixtures, differ depending on the usage area. The activities of the enzymes as an alternative to the Celluclast were studied by Saha *et al.,* 2005, and it was observed that β-glucosidase activity of the Celluclast is almost 5% of its carboxymethyl cellulase (CMCase) activity. Moreover, the glucose to cellobiose ratio is important in terms of application requirements. It is known that excess cellobiose inhibits the activity of cellobiohydrolases and endoglucanases, thus it should be removed from the system if maximum cellulase activity is important and thus maximum glucose yield is the crucial point in hydrolysis process (Emert, 1974). Thus the effect of β-glucosidase addition was investigated in enzymatic hydrolysis of lignocellulosic samples.

Table 3.3 Comparison of the enzyme activities of commercial cellulase mixture alternatives (Saha *et al.* 2005)

Enzyme	**Activity (U/ml)**		
	Celluclast	Novozyme 188	Viscostar
CMCase	1513	39	986
β-glucosidase	74	330	3
Xylanase	905	605	32956
β-xylosidase	15	8	68
α-L-arabino-furanosidase	8	29	58

3.3.1.1. Effect of β-glucosidase on the hydrolysis of wheat bran

According to the results shown in Figure 3.4, it was observed that treated samples have 3.2-fold more reducing sugar than untreated ones and the addition of β-glucosidase increases the ratio to 3.8 fold. On the other hand, the effect of β-

glucosidase addition was not detected in untreated samples. This result is probably because of the deficiency of cellobiose at 150 min. The hydrolysis results also have indicated that sole-β-glucosidase treatment does not differ with the control medium. This is likely due to the highly-packaged and crystalline structure of cellulose, and the lack of sufficient hydrolysis and cellobiose generation (Appendix G).

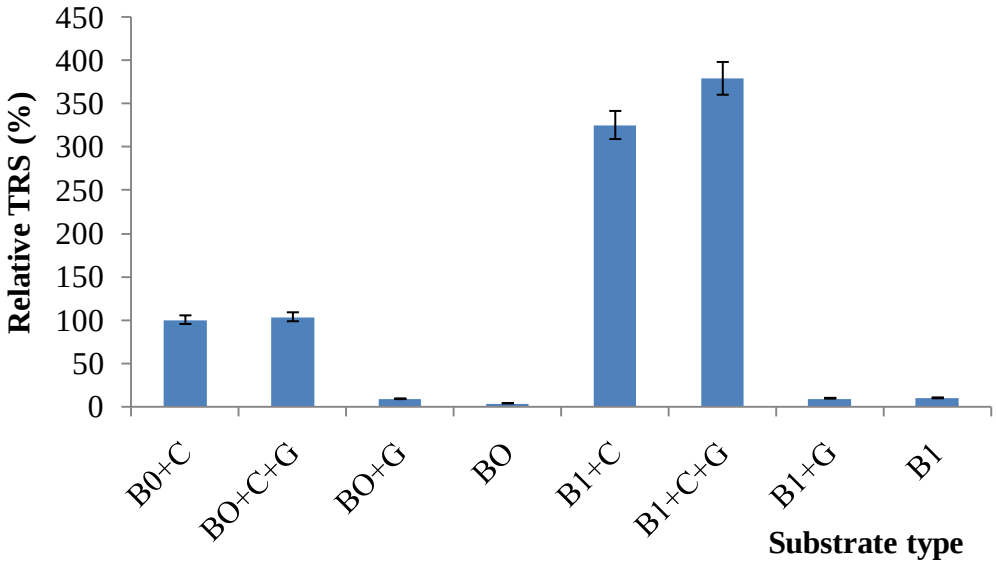


Figure 3.4 The effect of β-glucosidase addition on the hydrolysis (50°C and 4.8 pH) of wheat bran at 150 min where B0+C is taken as basis; (B0+C) untreated bran hydrolyzed with 4.5 Ug^{-1} Celluclast, (B0+C+G) untreated bran hydrolyzed with 4.5 Ug^{-1} Celluclast and 25 Ug^{-1} β-glucosidase, (B0+G) untreated bran hydrolyzed with 25 Ug^{-1} β-glucosidase, (B0) untreated bran, control, (B1+C) 500 bar-treated bran hydrolyzed with 4.5 Ug^{-1} Celluclast, (B1+C+G) 500 bar-treated bran hydrolyzed with 4.5 Ug^{-1} Celluclast and 25 Ug^{-1} β-glucosidase, (B1+G) 500 bar-treated bran hydrolyzed with 25 Ug^{-1} β-glucosidase, (B1) 500 bar-treated bran, control.

3.3.1.2. Effect of β-glucosidase on the hydrolysis of wheat straw

A similar trend was observed for wheat straw as in the case of wheat bran (Figure 3.5). Namely, there was not a significant increase in TRS % in the presence of additional β-glucosidase for untreated samples, while a slight increase was observed for samples microfluidized at 500 bar.

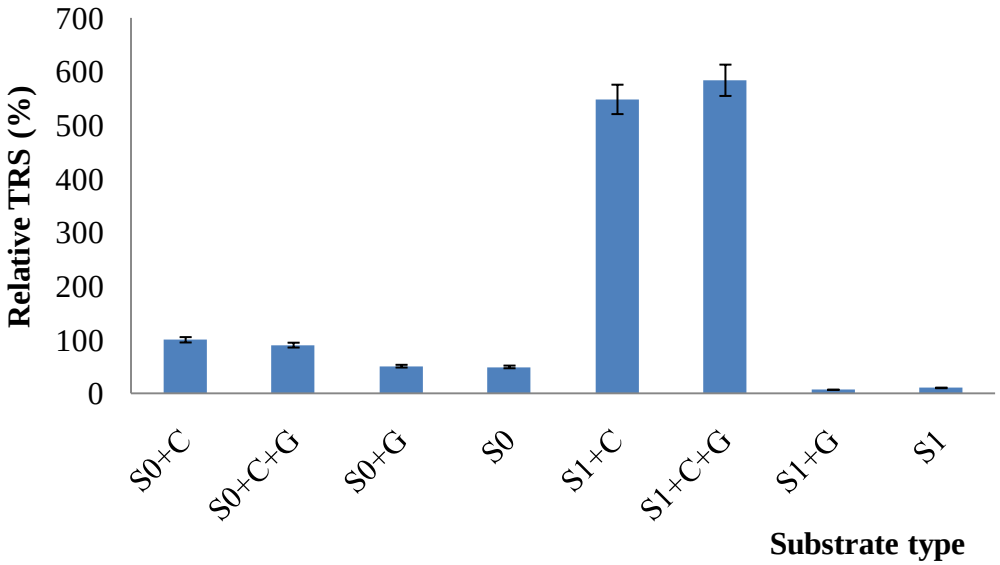


Figure 3.5 The effect of β-glucosidase addition on the hydrolysis (50°C and 4.8 pH) of wheat straw at 150 min where S0+C is taken as basis; (S0+C) untreated bran hydrolyzed with 4.5 Ug^{-1} Celluclast, (S0+C+G) untreated bran hydrolyzed with 4.5 Ug^{-1} Celluclast and 25 Ug^{-1} β-glucosidase, (S0+G) untreated bran hydrolyzed with 25 Ug^{-1} β-glucosidase, (S0) untreated bran, control, (S1+C) 500 bar-treated bran hydrolyzed with 4.5 Ug^{-1} Celluclast, (S1+C+G) 500 bar-treated bran hydrolyzed with 4.5 Ug^{-1} Celluclast and 25 Ug^{-1} β-glucosidase, (S1+G) 500 bar-treated bran hydrolyzed with 25 Ug^{-1} β-glucosidase, (S1) 500 bar-treated bran, control

It was seen that microfluidization process has yielded 5.5 times more reducing sugar than the untreated samples under the stated conditions. This increase in reducing sugar content has reached 5.8 fold when β-glucosidase was also incorporated.

For the following days, up to 5th day, it was experienced that the ratio between β-glucosidase added and not added microfluidized samples cellulase hydrolysis was kept nearly constant (Appendix F). Effect of sole β-glucosidase treatment resembled with the observed results in bran samples, namely there was no influence compared to control hydrolysis treatments. However, the gained reducing sugar (TRS) content was reduced for the situations of S1+G and S1 samples.

3.3.1.3. Effect of β-glucosidase on the hydrolysis of corn bran

The results were found to be alike with those obtained for wheat bran and straw samples (Figure 3.6). While there was not a significant change by the addition of β-glucosidase for untreated samples, the effect of β-glucosidase activity was clearly experienced for microfluidized samples (1.2-fold increase).

According to these results, cellobiose is possibly more accessible after the lignocellulosic biomass is treated by the microfluidization technique. The influence of β-glucosidase loses its effect after approximately one day of treatment (Appendix G). This may be caused by the loss of activity of the enzyme. Since the increase in reducing sugar content was 1.1-1.2-fold for all samples (wheat bran, wheat straw and corn bran) and because additional enzyme means extra processing costs, it may be suggested that Celluclast from *Trichoderma reesei* is sufficient by itself in lignocellulose saccharification without additional β-glucosidase.

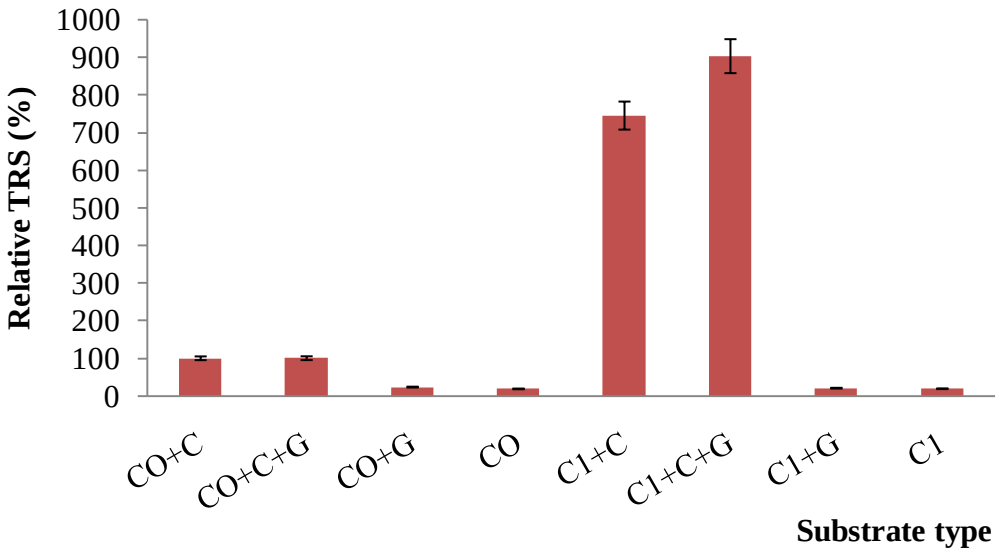


Figure 3.6 The effect of β-glucosidase addition on the hydrolysis (50°C and 4.8 pH) of corn bran at 150 min where C0+C is taken as basis; (C0+C) untreated corn bran hydrolyzed with 4.5 Ug^{-1} Celluclast, (C0+C+G) untreated corn bran hydrolyzed with 4.5 Ug^{-1} Celluclast and 25 Ug^{-1} β-glucosidase, (C0+G) untreated corn bran hydrolyzed with 25 Ug^{-1} β-glucosidase, (C0) untreated corn bran, control, (C1+C) 500 bar-treated corn bran hydrolyzed with 4.5 Ug^{-1} Celluclast, (C1+C+G) 500 bar-treated corn bran hydrolyzed with 4.5 Ug^{-1} Celluclast and 25 Ug^{-1} β-glucosidase, (C1+G) 500 bar-treated corn bran hydrolyzed with 25 Ug^{-1} β-glucosidase, (C1) 500 bar-treated corn bran, control.

3.3.2. Effect of solid load on the hydrolysis of lignocellulosic biomass

In this work, the substrate load was investigated at 2.5%, 5.0% and 7.5% (w/v, dry weight) based on the literature. Palmora-Adrodas *et al.* (2005) have studied wheat bran, which is made free of starch, to determine the saccharification conditions with different pretreatment methods. They have investigated the dilute acid pretreatment with or without enzymatic hydrolysis and also applied the microwave treatment. The solid load that they have applied is 5% (dry wt). And the study of Lequart *et al.* (1999) has used a solid ratio of 3% w/v for wheat bran.

Hydrolysis was performed with Celluclast (9.0 U/g dry biomass) and at 50°C, pH 4.8. Relative TRS/SL ratio (%) was used (Section 2.2.7) to make comparison.

3.3.2.1. Effect of solid load on the hydrolysis of wheat bran

When solid load (SL) increases, the attained total reducing sugar (TRS) content also increases. However, in order to determine the efficiency of hydrolysis, TRS/SL ratio was calculated to observe at which solid load, the maximum reducing sugar content was reachable for 1 gram of solid. For wheat bran samples, although 2.5% solid loaded medium showed the highest ratio at 30 min, after 30 min (Figure 3.7), 5% solid loaded medium was shown to yield the highest TRS/SL ratios for the 150 min period. The lowest yields were obtained with 7.5 % SL, which may be due to the recalcitrancy of mass transfer. Based on these results, it was concluded that 5% solid load is the optimum condition for wheat bran hydrolysis.

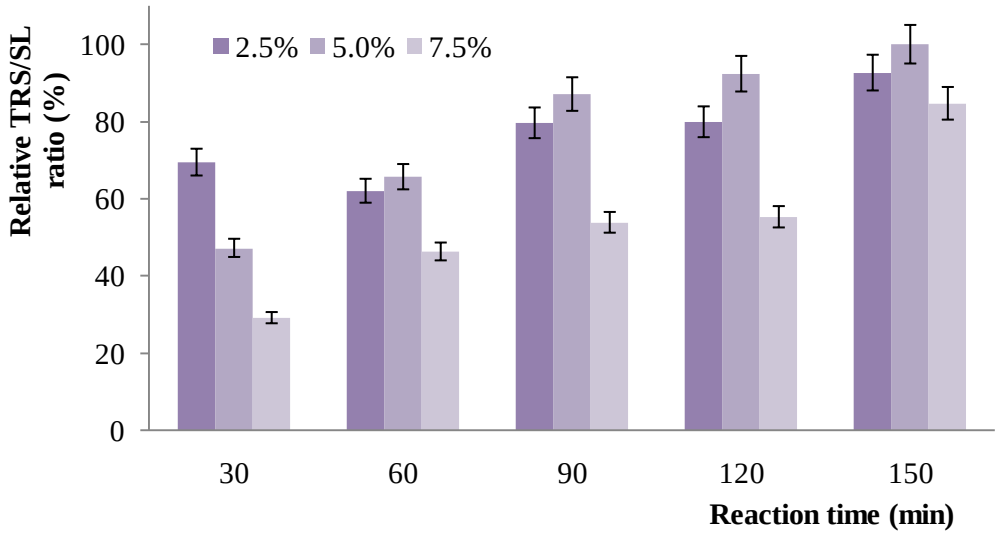


Figure 3.7 The effect of solid load (% dw) on the hydrolysis (50°C and 4.8 pH) of wheat bran using Celluclast (9.0 U/g dry biomass) in a total volume of 10 ml with mixing (100 rpm)

3.3.2.1. Effect of solid load on the hydrolysis of wheat straw

When the solid load for wheat straw was examined, it was seen that 2.5% solid loaded medium had the highest TRS/SL ratio throughout the 150 min hydrolysis period. 5.0% and 7.5% solid loaded samples yielded nearly 1.5 and 1.7 times lower results (Figure 3.8). Considering the results, it was concluded that the most efficient hydrolysis was performed at 2.5% solid load, and at higher solid loaded samples the ratios were reduced. As the time has passed, the differences among them got smaller slightly.

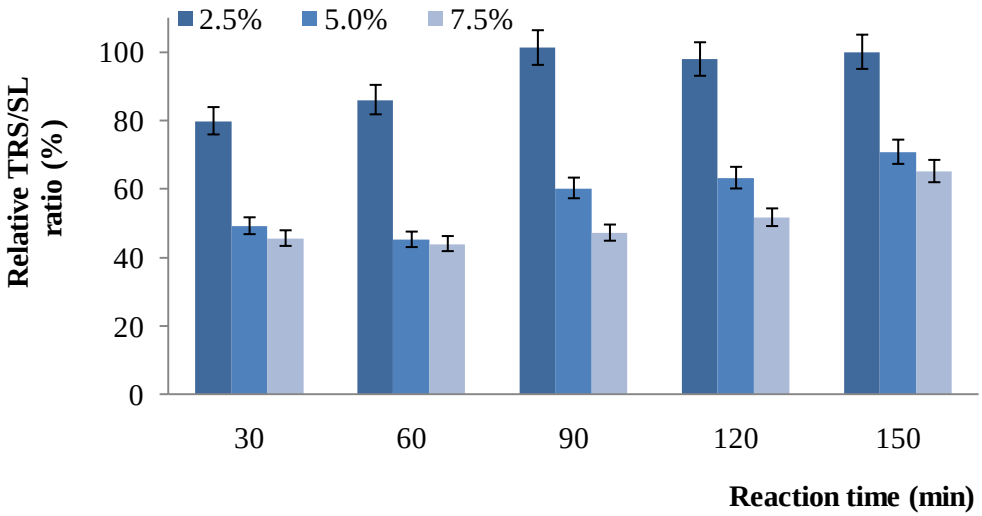


Figure 3.8 The effect of solid load (% dw) on the hydrolysis (50°C and 4.8 pH) of wheat straw (50°C and 4.8 pH) using Celluclast (9.0 U/g dry biomass) in a total volume of 10 ml with mixing (100 rpm)

3.3.2.3. Effect of solid load on the hydrolysis of corn bran

For the case of corn bran, interestingly, 7.5% solid loaded sample yielded the highest TRS/SL ratios throughout the reaction (Figure 3.9).

The change in TRS/SL ratios of different samples is thought to occur because of their compositional characteristics, possible inhibitions caused by products or variations in cellulase adsorption. Rosgaard and his co-workers (2007) suggested that the high viscosity of lignocellulosic biomass affects the mass transfer during enzymatic hydrolysis, especially due to mixing problems.

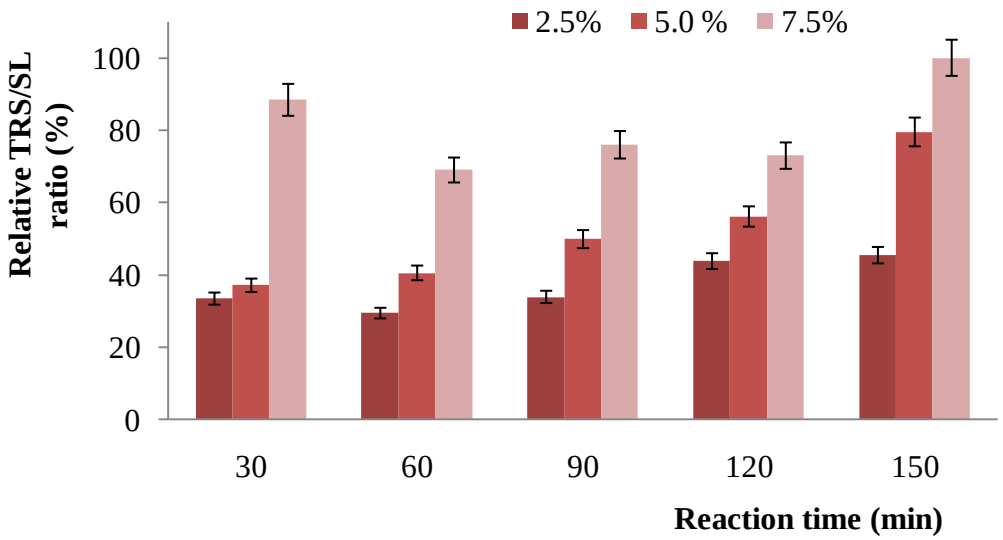


Figure 3.9 The effect of solid load (% dw) on the hydrolysis (50°C and 4.8 pH) of corn bran using Celluclast (9.0 U/g dry biomass) in a total volume of 10 ml with mixing (100 rpm)

The substrate-specific limitations in the hydrolysis, especially cellulose-degradations, were observed to affect the rate and maximum attainable reducing sugar content of saccharification. These are summarized by Mansfield (1999) such as degree of polymerization, crystallinity, accessible surface area, and average particle area and lignin distribution of the lignocellulosic substrate. The limitations show their influence mostly after the initial rapid hydrolysis period,

thus slow down the rate and sometimes end in an incomplete saccharification. Solid load higher than 10% is known to cause inhibition due to the accessible surface area limitation (Krishna *et al*., 1998)

End-product inhibition is also an important issue during hydrolysis. The formation of glucose, cellobiose and ethanol are known to suppress the activity of endoglucanases, cellobiohydrolases, and β-glucosidases (Bezerra *et al*., 2005). But since the substrates are insoluble in the medium and thus do not obey Michelis-Menten kinetics, the inhibition mechanism could not be solved out yet. Gruno *et al.* (2004) have studied Cel 7A (cellobiohydrolase-I) inhibition, and figured out that there happens nonproductive enzyme-substrate complexes due to long-channel shaped active sites which can be in interaction with cellulose. Hodge *et al.* (2008) have shown that when the substrate concentration increases, a deficiency on the enzyme activities occurs and thus inhibits their operation during hydrolysis.

As the solid load increases, water content of the medium decreases, and thus the interaction between enzyme and substrate is affected negatively. Cellulase accessibility and cellulase adsorption is also influenced by solid concentrations. Stutzenberger *et al.* (1986) have mentioned some possible hydrolysis products to affect the inhibition. In addition to all lignin content of the biomass (Pan, 2008) is also known to influence the optimum hydrolysis of lignocellulosic biomass

3.3.3. Effect of enzyme load on the hydrolysis of lignocellulosic biomass

There have been studies to determine the optimum amount of enzyme for the hydrolysis of several lignocellulosics, treated with different methods. Detroy (1981) has used 10 IU cellulase per gram of residue for wheat straw, pretreated by sulfuric acid and sodium hydroxide solutions. Saha *et al*. (2005) have utilized Celluclast 1.5 L, Novozyme 188, laccase from *Trametes versicolor* and lipase from *Candida rugosa* with a concentration of 2 ml/100 g wheat straw (on dry basis) to study on the saccharification. Kristensen *et al*. (2009) have practiced

lignocellulose hydrolysis with enzyme loadings changing from 2 to 20 FPU/g of dry material for the enzyme mixture Celluclast to deconstruct model cellulose, filter paper. The enzyme concentrations were determined as 15 FPU/g glucan for Celluclast and 17 IU/g glucan for β-glucosidase (Novozyme 188) to study ethanol production from wheat meal and wheat straw by Erdei *et al*. (2010). Hoyer *et al*. (2008), in different manner, have used 15 FPU/g and 23 IU/g water insoluble solid for Celluclast and Novozyme 188 respectively. According to all these studies, in this work, the lignocellulose hydrolysis was performed with cellulase concentrations ranging from 1.5 to 9.0 U/g dry biomass.

3.3.3.1. Effect of enzyme load on the hydrolysis of wheat bran

Wheat bran at 5.0% (dw/v) solid concentration was hydrolyzed with different enzyme loadings (Figure 3.10). It was observed that 4.5 U/g enzyme loading shows similar degree of hydrolysis performed by 9.0 U/g enzyme loading (Appendix I) after 150 min.

The contour plot, which is used for optimization studies, was drawn to predict the optimum enzyme dosage. Two-dimensional plot shows different contours of constant response. In this study, the variables were time and enzyme load, while the response was total reducing sugar content and the goal was to determine optimum values for the variables such that the response was maximized.

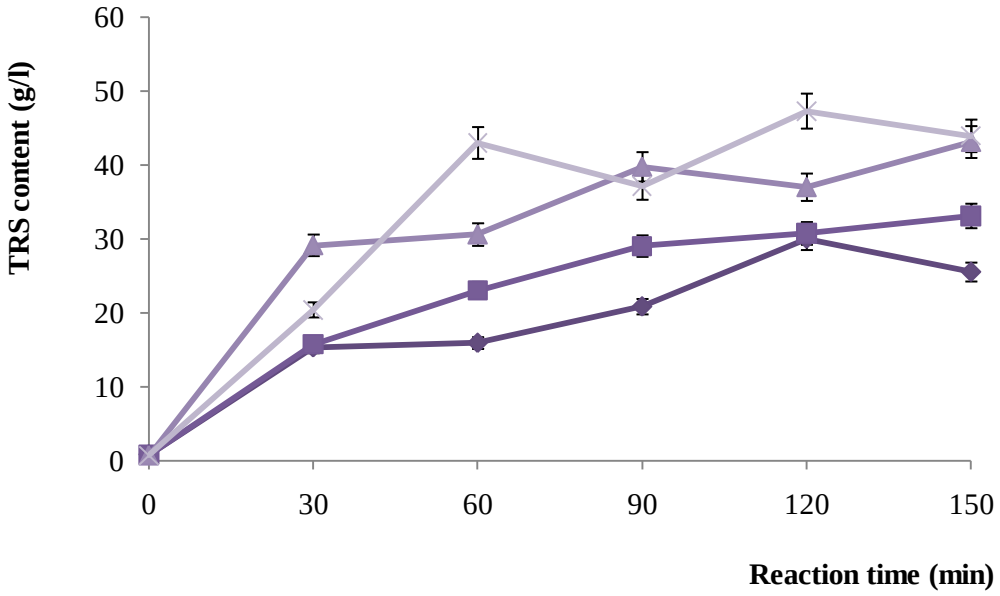


Figure 3.10 The effect of enzyme dosage on the hydrolysis (50°C and 4.8 pH) of wheat bran (5% dw/v) using (♦) 1.5 Ug^{-1} Celluclast, (■) 3.0 Ug^{-1} Celluclast, (▲) 4.5 Ug^{-1} Celluclast, (x) 9.0 Ug^{-1} Celluclast, in a total volume of 10 ml with mixing (100 rpm)

The plot (Figure 3.11) mapped out for wheat bran hydrolysis demonstrates a region that is formed between 4.5 U/g and 9.0 U/g after 75 min of hydrolysis. The amount of enzyme lower than 4.5U/g could not give the results obtained for 4.5U/g and 9.0 U/g for the time interval of 150 min. On the other hand, there was not an important variation in total reducing sugar content between 4.5 U/g and 9.0 U/g enzyme loads and it was shown by statistical studies in Appendix I.

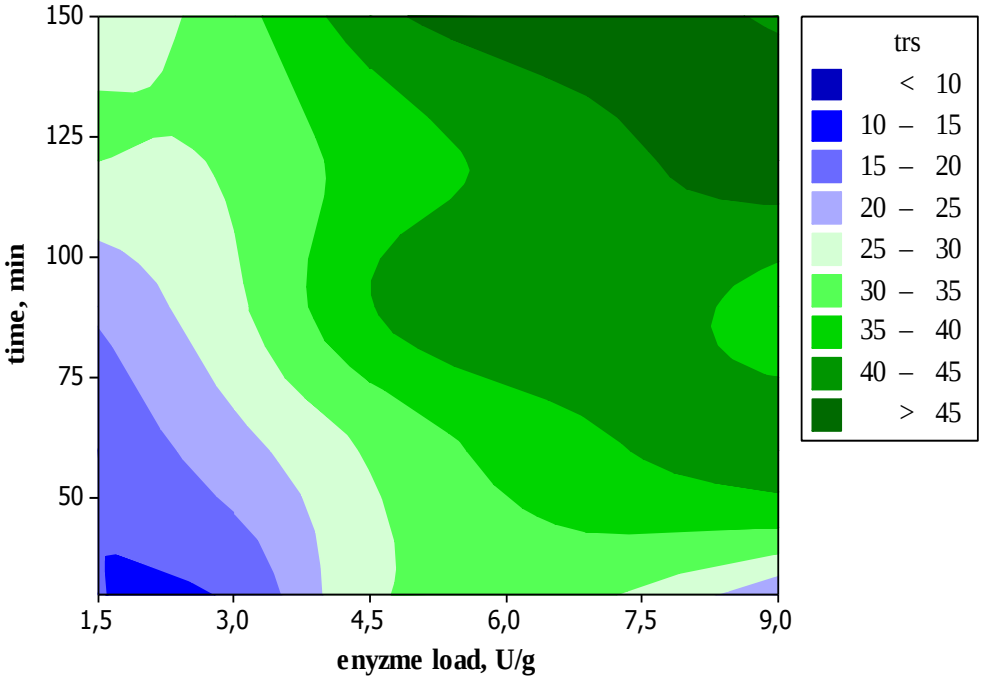


Figure 3.11 The contour plot of the wheat bran hydrolysis showing how total reducing sugar content (trs) changes with time and enzyme load.

3.3.3.2. Effect of enzyme load on the hydrolysis of wheat straw

The same procedure was applied for wheat straw samples, but as opposed to wheat bran, it was seen that there is a significant difference between 4.5U/g and 9.0U/g treated samples (Appendix J). Thus, additionally 6.0 U/g and 7.5U/g cellulase hydrolysis experiments for microfluidized straw samples were therefore performed (Figure 3.12).

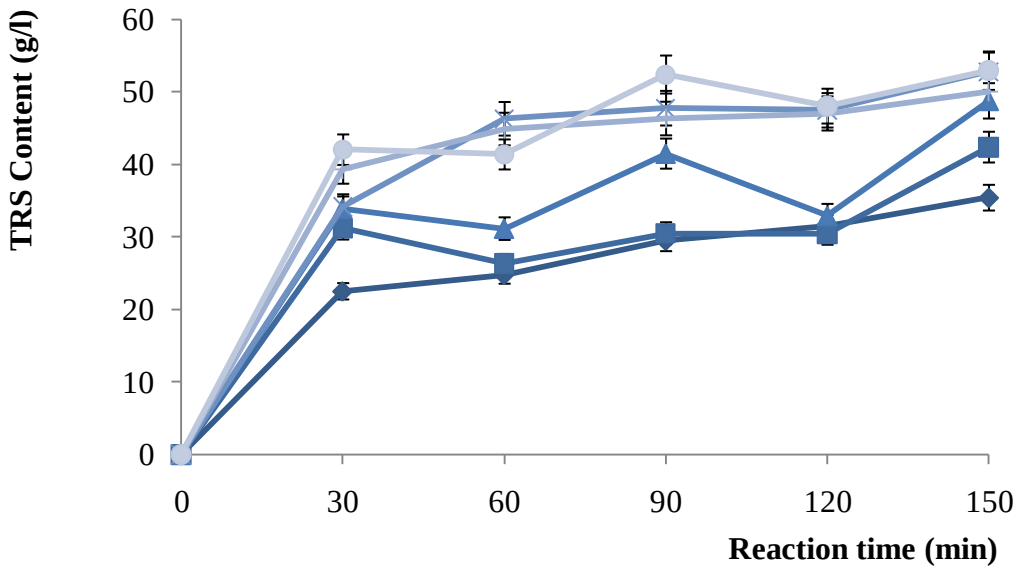


Figure 3.12 The effect of enzyme dosage on the hydrolysis (50°C and 4.8 pH) of wheat straw (5% dw/v) using (♦) 1.5 Ug^{-1} Celluclast, (■) 3.0 Ug^{-1} Celluclast, (▲) 4.5 Ug^{-1} celluclast, (x) 6.0 Ug^{-1} Celluclast, (+) 7.5 Ug^{-1} Celluclast, (●) 9.0 Ug^{-1} Celluclast, in a total volume of 10 ml with mixing (100 rpm)

The contour plot (Figure 3.13) for wheat straw shows that the maximum response was between 6.0 U/g cellulase loading and the higher cellulase loadings- 7.5 U/g and 9.0 U/g after 1 hour of hydrolysis. According to the statistical results (Appendix J), 6.0 U/g was selected as the optimum cellulase content for microfluidized wheat straw samples for the first 150 min interval.

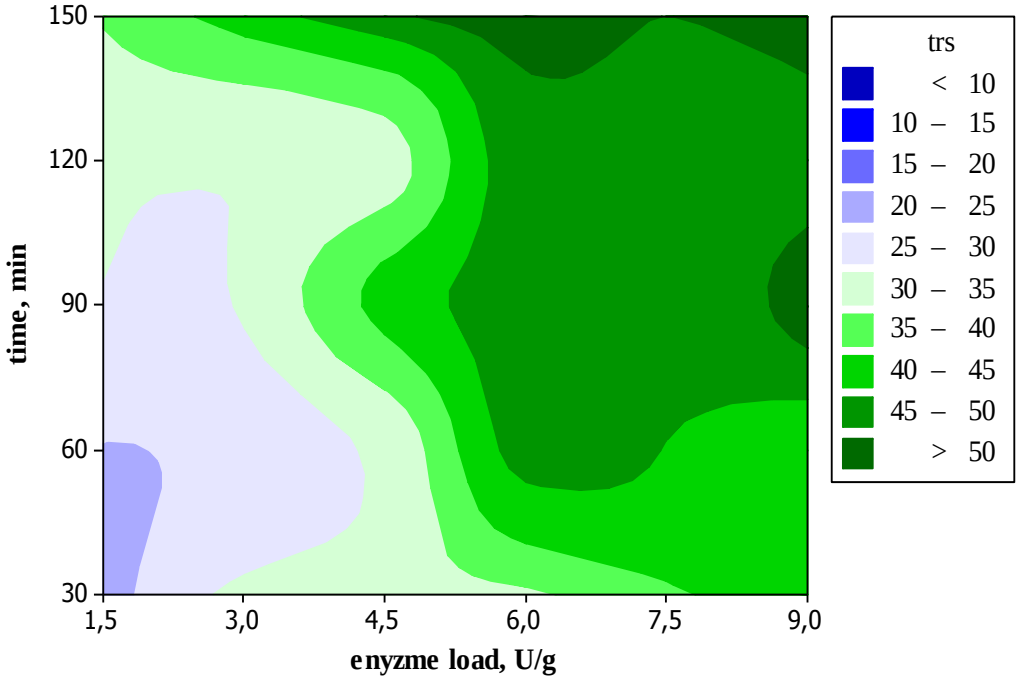


Figure 3.13 The contour plot of the wheat straw hydrolysis showing how total reducing sugar content (trs) changes with time and enzyme load.

3.3.3.3. Effect of enzyme load on the hydrolysis of corn bran

For the case of microfluidized corn bran samples, the results were similar with the ones of wheat bran (Figure 3.14). It was found that after 4.5U/g enzyme loadings there was not a significant change in the released total reducing sugar (Appendix K). But the case was the opposite for the enzyme loadings lower than 4.5 U/g - 1.5 U/g and 3.0 U/g- as shown in the Figure 3.15. As a result, it was recorded that 4.5 U/g cellulase loading is the best condition for microfluidized corn bran samples.

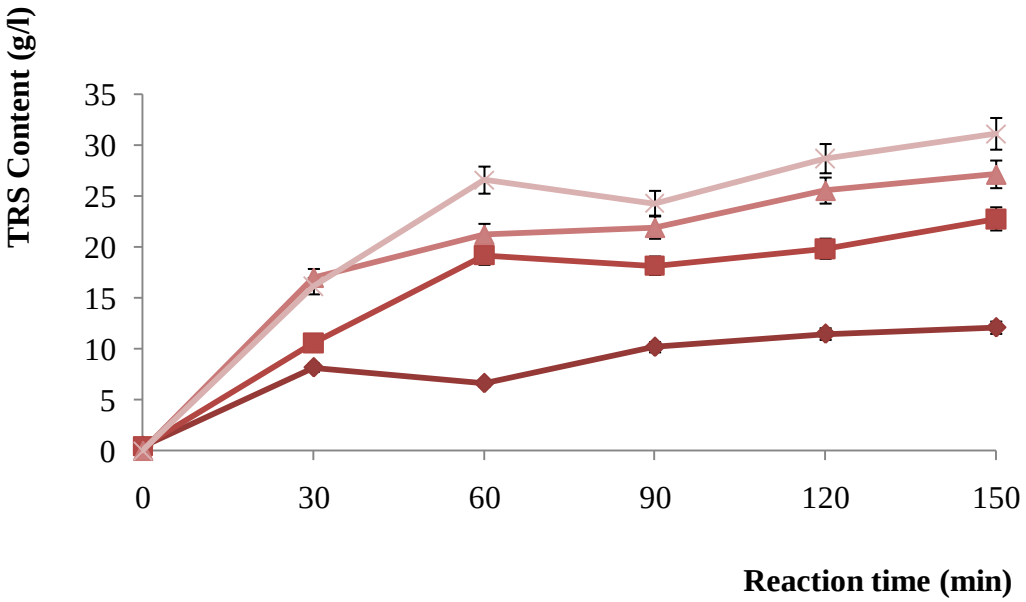


Figure 3.14 The effect of enzyme dosage on the hydrolysis (50°C and 4.8 pH) of corn bran (5% dw/v) using (♦) 1.5 Ug^{-1} Celluclast, (■) 3.0 Ug^{-1} Celluclast, (▲) 4.5 Ug^{-1} Celluclast, (x) 9.0 Ug^{-1} Celluclast, in a total volume of 10 ml with mixing (100 rpm)

The reasons for different optimum enzyme loadings of various lignocellulosic substrates can be described by specific compositional and structural characteristics of the samples. The lignin content is a well-known obstacle at the hydrolysis, and since the chemical compositions of the initial samples are different, this can be the cause of relative higher need of enzyme concentration for wheat straw, which has a the highest lignin content (Table 3.2). As well as the lignin inhibition, it is thought that the crystallinity of the lignocellulose can affect the results. Since the origin of the lignocellulosic biomass differs, the molecular arrangement, capillary structure of the cellulose and also the water swelling degree vary likewise, and it is attainable for all of these parameters to influence the optimum enzyme loadings for the three different microfluidized biomass types.

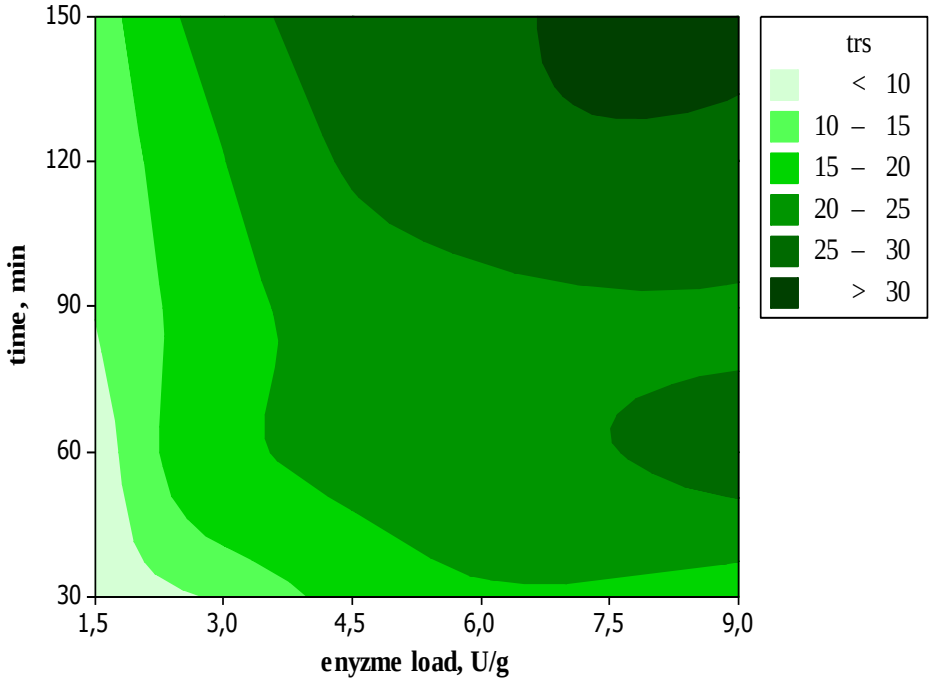


Figure 3.15 The contour plot of the corn bran hydrolysis showing how total reducing sugar concentration (trs) changes with time and enzyme load.

Enzyme dosage in hydrolysis is very crucial because of the economy and also efficiency of the process. As mentioned before, pretreatment and saccharification cost of lignocellulosic biomass-based bioethanol should be reduced to be able to compete with other energy resources.

It is expected that as enzyme dosage increases in the hydrolysis medium, the reducing sugar content will increase. However, there is a limitation caused by the adsorption of cellulase onto the substrate. Furthermore, based on the amount of substrate, the amount of enzyme is expected to reach a level of saturation eventually. The enzyme based-limitations of cellulose hydrolysis are described by Saddler (1986) and Mansfield *et al.* (1999). These are basically, synergistic work, adsorption and thermal inactivation of cellulases. Adsorption of cellulases is mostly aided by the cellulose binding domain (CBD) and also by the catalytic domain. The working principle of CBD is explained by two different theories.

First one proposes that CBD causes an increase in the cellulase concentration at that domain, which is in interaction with cellulose, and the second one describes that these domains play a role in the modifications in the structure of celluloses.

Adsorption of cellulase to the lignocellulosic substrate has some parameters depending on the substrate characteristics. The chemical composition, especially lignin content, crystallinity of the substrate, the degree of water swelling, molecular arrangement and the capillary structure of cellulose fibers make up the main features (Fan, 1980). Mansfield *et al*. (1999) mention that amorphous cellulose is more susceptible to enzymatic hydrolysis since the cellulase activity is dependent on cellulose binding domains when the cellulose is fully crystalline, but not dependent when it is amorphous.

3.4. Comparison of microfluidization with softening process during enzymatic saccharification

Softening process, in which the untreated samples were kept at 50°C in a water bath (pH 8.0), was performed to investigate its effect on the enzymatic hydrolysis step, as compared to the microfluidization process. In the experiments, 5.0% (dw/v) solid loaded lignocellulosic samples were treated with Celluclast and also with β-glucosidase to observe the change in the influence of the that enzyme which was already performed for microfluidized samples.

3.4.1. Comparison of microfluidization with softening process for wheat bran

The results showed that the softening process considerably increases (nearly 3.0-fold) the total reducing sugar (TRS) content within 150 min of hydrolysis as compared to untreated samples (Figure 3.16). As also experienced in former experiments within untreated samples, the effect of β-glucosidase could not be observed for the softened bran samples. It was supposed that, during the softening process, the re-crystallization of cellulose, which results in the formation of high

crystalline structure, may be responsible for this outcome. On the other hand, softening process was observed to be less effective than microfluidization since the increase was 3.5-fold for 500 bar-treated and 5.5-fold for 2000 bar-treated samples. While the TRS content of 500 bar-treated samples have reached to the value of 2000 bar-treated samples at the 5th day of hydrolysis, the softened samples could never reach those quantities (Appendix E).

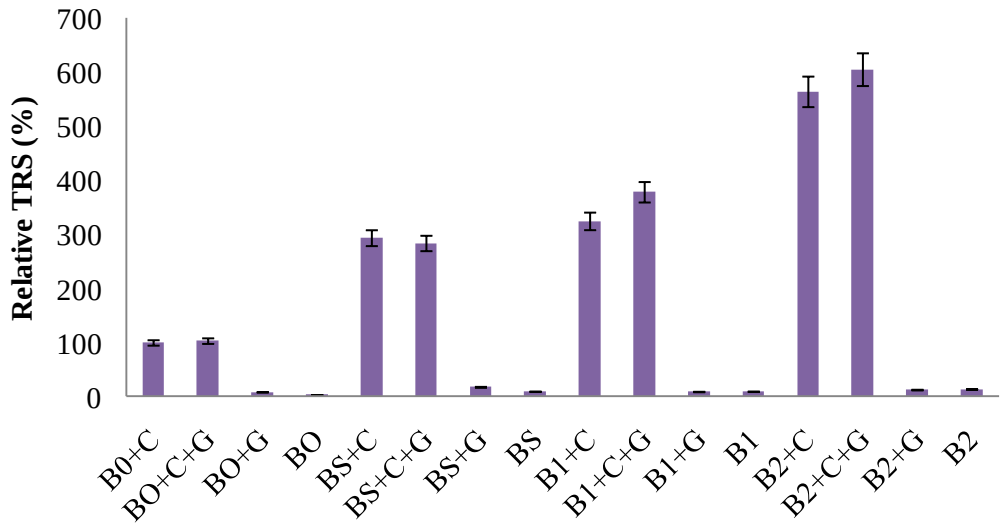


Figure 3.16 Comparison of the effect of softening process and microfluidization on the hydrolysis (50°C and 4.8 pH) of wheat bran (5% dw/v) using (C) Celluclast (4.5 U/g) and/or (G) β-glucosidase (25 U/g) at 150 min. (B0), untreated bran; (BS), softened bran; (B1), 500 bar-treated bran; (B2) 2000 bar-treated bran. B0+C is taken as basis

3.4.2. Comparison of microfluidization with softening process for wheat straw

The effect of the softening process on enzymatic hydrolysis of wheat straw samples was similar to that of wheat bran samples (Figure 3.17). The softening process was found to increase TRS content by approximately 4.8-fold. But,

microfluidization results were higher, namely 5.5 and 7.5-fold for 500 bar-treated and 2000 bar-treated samples respectively. Again, the influence of β-glucosidase addition was not apparent for softened samples, whereas, there was about 1.1 times increase for the microfluidized straw samples.

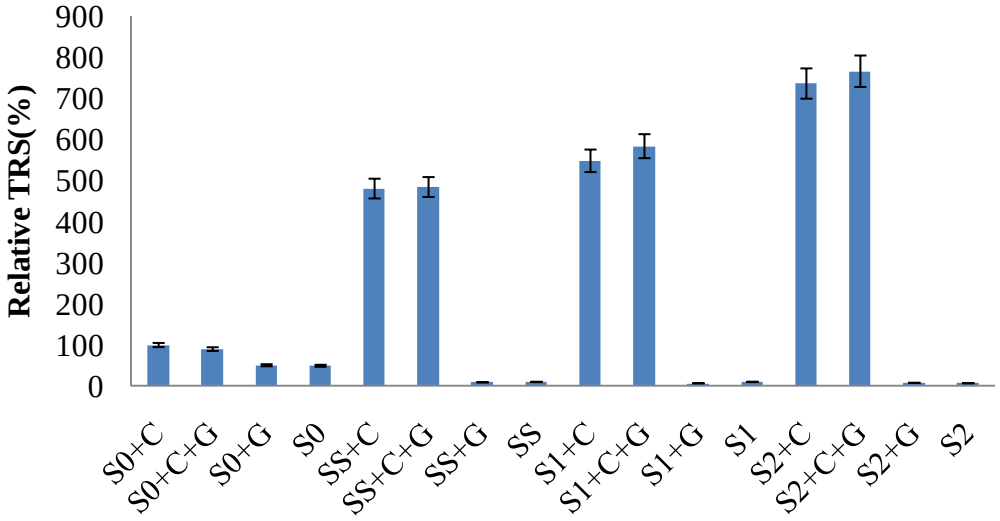


Figure 3.17 Comparison of the effect of softening process and microfluidization on the hydrolysis (50°C and 4.8 pH) of wheat straw (5% dw/v) using (C) Celluclast (4.5 U/g) and/or (G) β-glucosidase (25 U/g) at 150 min. (S0), untreated bran; (SS), softened bran; (S1), 500 bar-treated bran; (S2) 2000 bar-treated bran. S0+C is taken as basis

3.4.2. Comparison of softening process with microfluidization for corn bran

In the ease of corn bran, as opposed to wheat bran and wheat straw, the softening process was more effective than the microfluidization process over 150 min of hydrolysis. Furthermore, the influence of β-glucosidase addition was significant for all types of pretreated samples (Figure 3.18). For instance, β-glucosidase addition has resulted in approximately 1.21-fold increase for both of the

microfluidized samples (500 bar and 2000 bar), whereas it was 1.26-fold for softened ones. The cause of the higher yield with the softening process in corn bran can be described according to the structural and compositional characteristics of the material. Klason lignin content for corn bran was calculated to be roughly 50% less than the others (Table 3.2). During softening process, due to the lower amount of lignin content, it is likely that the deformation of internal surface area occurred more efficiently.

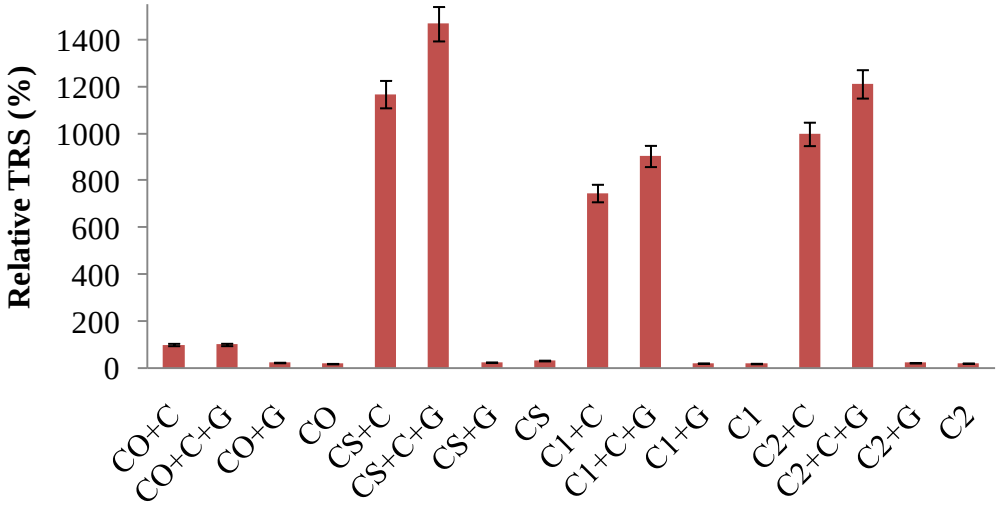


Figure 3.18 Comparison of the effect of softening process and microfluidization on the hydrolysis (50°C and 4.8 pH) of corn bran (5% dw/v) using (C), Celluclast (4.5 U/g), and/or (G), β-glucosidase (25 U/g), at 150 min. (C0), untreated bran; (CS), softened bran; (C1), 500 bar-treated bran; (C2) 2000 bar-treated bran. C0+C is taken as basis

Lignocellulosic biomass is known to have two forms of surface area. The first one, which is the external surface area, contingents on the shape and size of the particle, on the other hand, the second one, internal surface area, turns on capillary structure. Taherzadeh and Karimi (2008) describes that dry cellulosic

fiber have an external surface area about 0.6 to 1.6 m^2/g, which plays a significant role in enzymatic hydrolysis. However, the internal surface area of these fibers are said to have more small-scale areas than external areas. At that point, the study of Fan *et al*. (1980) shows that the internal surface area can be enhanced by swelling the lignocellulosic biomass in water and also, existence of water is found to have an important role on the specific surface area. It has been mentioned that, the specific surface area becomes bigger during wetting process. But then, due to re-crystallization of highly amorphous cellulose, the crystallinity of the cellulose increases during the process.

There are some obstructions that affect the efficiency of the swelling process. For instance, lignin content of the biomass is experienced to prevent swelling of lignocelluloses since it behaves as a linker between cellulose and hemicellulose (Mooney, 1998).

3.5. Comparison of microfluidization with some prominent pretreatment methods

The novel usage of microfluidization technique in enzymatic saccharification was compared with lime and dilute acid methods which are among the most widely used pretreatment techniques. This is mainly because of their simplicity, applicability and their advantage in the accessibility of surface area. Ammonia fiber explosion and steam explosion methods are less preferred in these respects.

Compared to their alternatives, lime and dilute acid pretreatments have an important influence on structural change in lignin, thus wheat by-products which have higher lignin contents (Table 3.2) were studied. Differently from lime pretreatment, dilute acid method is a well-studied pretreatment with wheat straw (Saha *et al.,* 2005, Schell *et al.,*1991, Gonzalez *et al,* 1986), thus in this study, wheat straw was chosen to be treated with dilute acid, whereas, wheat bran was processed with the alkali treatment.

Due to the change in the concentration of dry biomass as a result of microfluidization, the solid load was adjusted to 5.0 % dw/v before enzymatic hydrolysis.

3.5.1. Comparison of microfluidization with lime pretreatment for wheat bran

Lime pretreatment was performed in the presence of water and lime at two different temperatures 45°C and 60°C. Then, wheat bran (5.0 % dw/v) was hydrolyzed with Celluclast (4.5 U/g dry biomass) following microfluidization or lime pretreatment, and the TRS contents were compared. Lime pretreated samples (Figure 3.19) were found to be as effective as the 500 bar microfluidized wheat bran samples (Appendix L) for 150 min interval. However, at 2000 bar, microfluidization proved to be the most effective treatment.

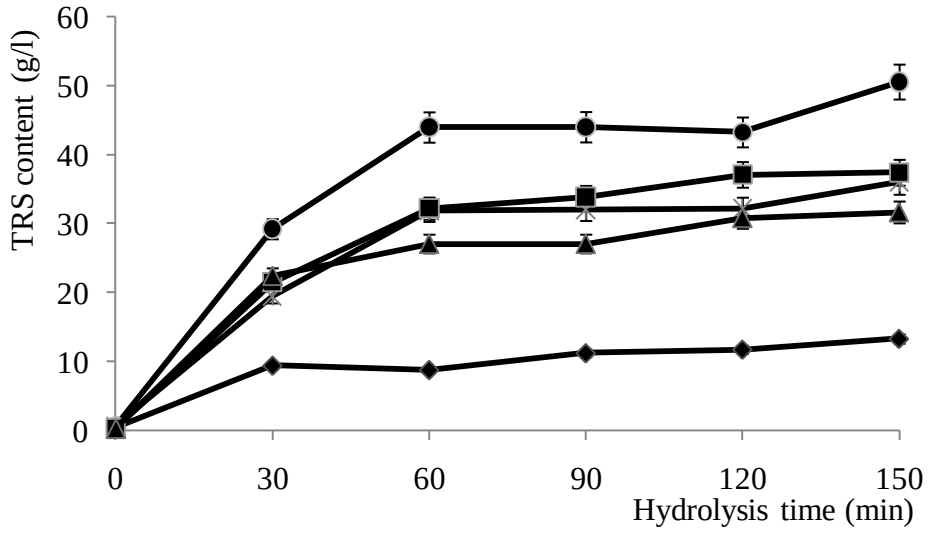


Figure 3.19 The change in the reducing sugar content for microfluidized and lime-treated wheat bran samples (5.0% dw/v). (♦) control: no pretreatment, (▲) lime pretreated (45°C), (■) lime pretreated (60°C), (X) microfluidized (500 bar), and (●) microfluidized (2000 bar)

3.5.2. Comparison of microfluidization with dilute-acid pretreatment for wheat straw

To analyze and compare the effect of dilute acid pretreatment method, wheat straw (5.0% dw/v) was used. Dilute sulfuric acid pretreatment was carried out according to the method which Saha *et al.* (2005) have used for wheat straw. As shown in Figure 3.20, the dilute acid pretreatment was as effective as 2000 bar-microfluidized pretreated samples. Interestingly, it was observed that there was retardation in hydrolysis in the first 30 min for dilute-acid pretreated samples compared to microfluidized samples.

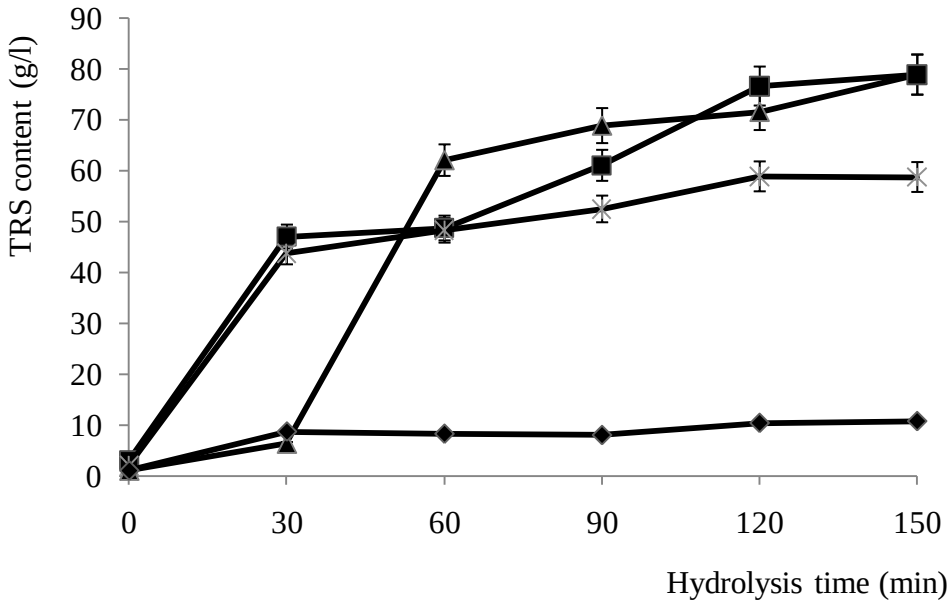


Figure 3.20 The change in reducing sugar content for microfluidized and dilute-acid pretreated wheat straw samples (5.0% dw/v). (♦) control: no pretreatment, (X) microfluidized (500 bar) straw, (■) microfluidized (2000 bar) straw, (▲) dilute acid pretreated straw.

While, dilute acid and lime pretreatments have the disadvantages of adding chemicals, microfluidization does not require any chemical addition. The chemicals are further needed to be recycled and also they cause a threat to environment because of their toxicity. In addition, because of their toxicity and corrosivity, damages occur during production, and thus it results in an increase in the production cost (Sivers and Zacchi, 1995).

3.6. Effect of microfluidization on crystallinity

The ordered form of cellulose, in which hydrogen bonds are present between cellulose molecules, has crystal-like characteristics. Long ordered regions of crystallites are cut off by completely disordered forms. This monoclinic structure of cellulose is influenced by treatments, i.e. chemical, mechanical, biological treatments. For instance, the study of Fengel and Wegener (1984) has shown that while amorphous regions are deconstructed during dilute acid treatment, the crystalline portions remains firm.

According to the findings in this study, microfluidization is a highly efficient pretreatment method. However, its effect on the crystallinity of cellulose and thus how it enhances enzyme hydrolysis is not known. Thus, the objective here was to investigate the influence of microfluidization pretreatment method on crystallinity.

3.6.1. Effect of microfluidization on the crystallinity of microcrystalline cellulose, Avicel

In order to examine the influence of microfluidization on crystalline structure of cellulose, Avicel (microcrystalline cellulose) was used. According to Figure 3.21, it was apparently observable that microfluidization changes the crystalline structure of microcrystalline cellulose. The sharper peak was observed at $2\theta=22°$ for the untreated and treated cellulose. The decrease in intensity is known to result from reduction in the size of crystalline parts and also divergence of crystals from

each other. On the other hand, the crystallinity index (CrI) was estimated as 74% and 78% for untreated and treated (500 bar) microcrystalline cellulose respectively.

The X-ray diffraction curve has demonstrated (101), (10i) and (002) patterns at *2θ=14.7°, 2θ=16.8°, 2θ=22°*, and *2θ=34°*. Thus, these characteristic peaks of native cellulose and pretreated cellulose have shown that that microcrystalline cellulose has cellulose-I lattice formation.

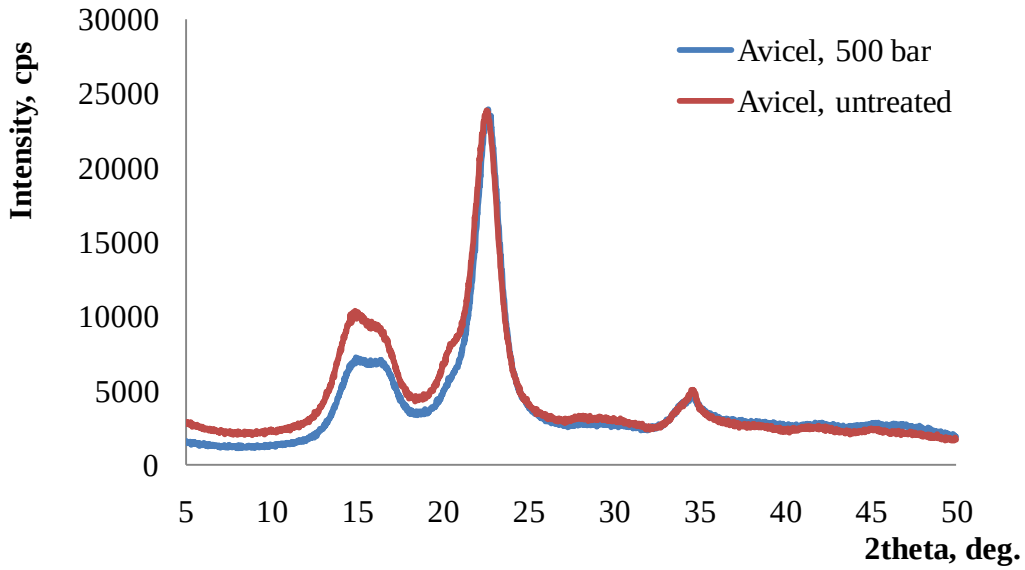


Figure 3.21 The X-ray diffraction pattern of the microcrystalline cellulose, Avicel

3.6.2. Effect of microfluidization on the crystallinity of wheat bran

The diffraction results of untreated wheat bran gave an amorphous pattern while the microfluidized bran showed a more crystalline structure. Moreover, a decrease in the intensity was observed when applied pressure was increased from 500 bar to 2000 bar (Figure 3.22).

The crystallinity degrees (CrI) for the three samples were estimated as 6%, 26% and 40% for untreated, treated at 500 bar and 2000 bar respectively. The decrease in intensity from 500 bar to 2000 bar shows that the highly packaged structure of cellulose in wheat bran was somewhat lost.

The crystallinity peak was seen at $2\theta=22°$ but compared to microcrystalline cellulose, bran was found to have more amorphous cellulose. The peaks of (101) and (10i) planes were merged together and the diffraction peak for (040) plane was almost absent.

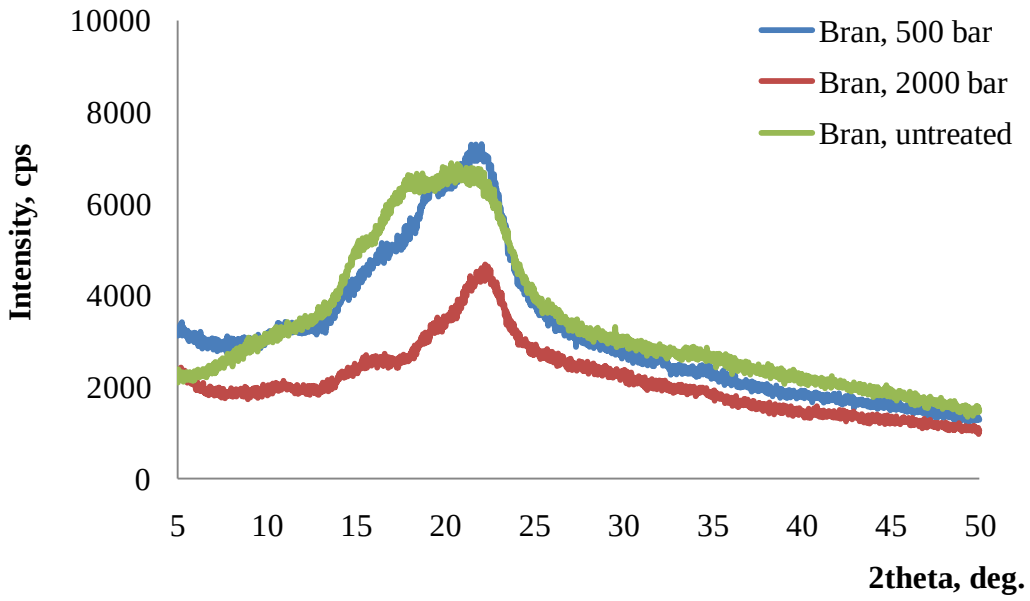


Figure 3.22 The X-ray diffraction pattern of wheat bran

3.6.3. Effect of microfluidization on crystallinity of wheat straw

The diffraction pattern of untreated wheat straw was found to be similar to the microfluidized samples but the intensity of this pattern was higher than microfluidized straw (Figure 3.23). The decrease in intensity in the 500 bar versus 2000 bar samples, could not be observed as in the case of wheat bran and corn bran.

The crystallinity indexes (CrI) of the straw samples were determined as 50%, 52% and 53% for untreated, treated at 500 bar and 2000 bar straw samples respectively. In addition to the peak at $2\theta=22°$, an additional peak was seen at $2\theta=29.5°$ for only microfluidization applied straw samples. Also, at $2\theta=34°$, the peak of (040) plane appeared weakly for the sample microfluidized at 500 bar and 2000 bar which was not present for untreated bran samples.

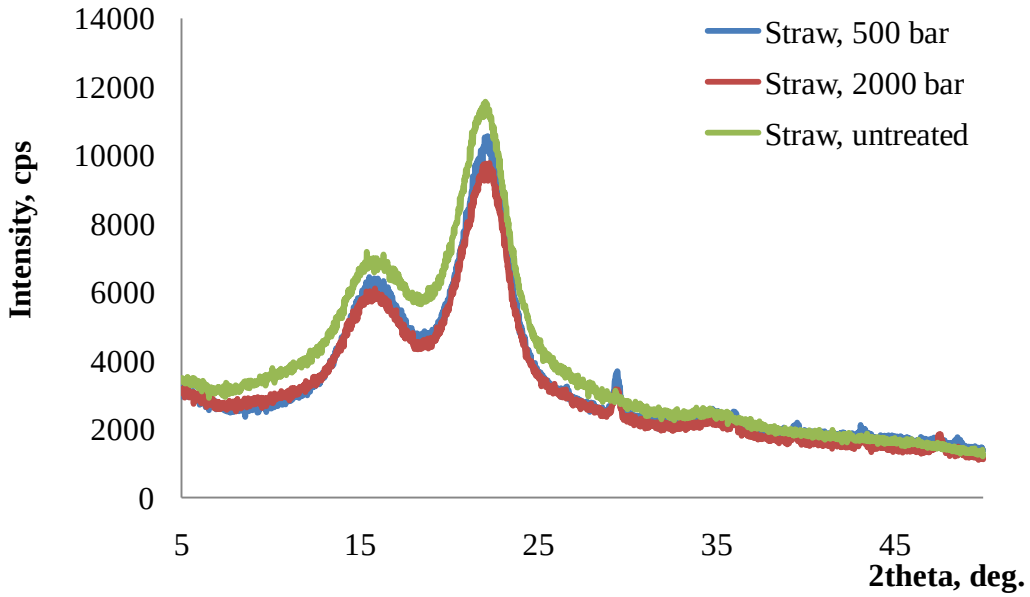


Figure 3.23 The X-ray diffraction pattern of wheat straw

3.6.4. Effect of microfluidization on crystallinity of corn bran

The diffraction pattern obtained for corn bran samples resembled those of wheat bran samples. Untreated bran exhibited an amorphous structure while for treated ones, crystalline structure was observed since the peaks were more uniform (Figure 3.24).

The crystallinity index (CrI) was estimated as 4%, 37% and 62% for untreated, treated at 500 bar and 2000 bar corn bran samples respectively. The additional peak at $2\theta=29.5°$ was present only at 2000-bar treated corn bran samples.

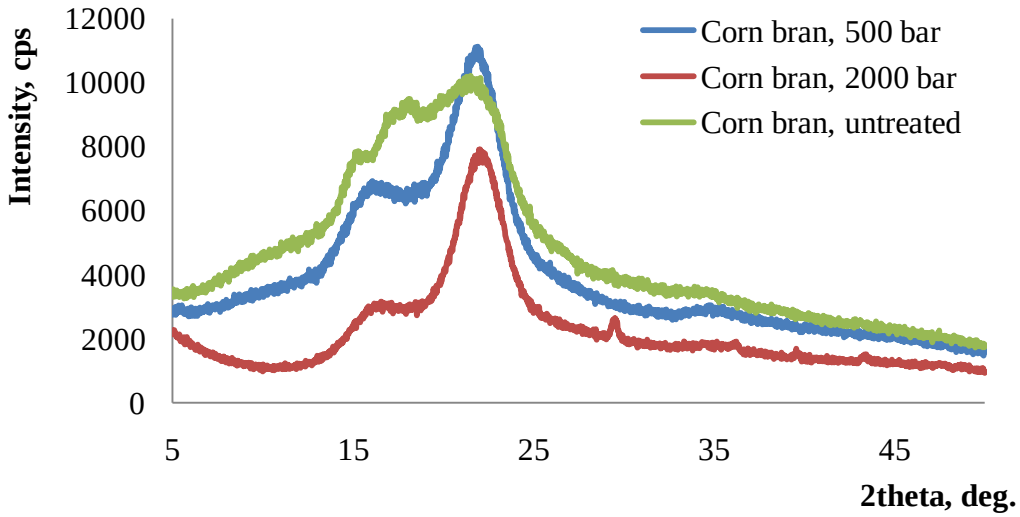


Figure 3.24 The X-ray diffraction pattern of corn bran

The increase in CrI results was similar for aqueous ammonia treated corn stover by Kim *et al.* (2003), sulfuric acid hydrolyzed wheat straw by Liu *et al.* (2005). Kim *et al.* (2003) relates the increase with the removal of xylan-like amorphous substances in biomass. Likewise, Liu *et al.* (2005) describes this increase in

crystallinity with the hydrolysis of amorphous cellulose during the sulfuric acid pretreatment process. It is also likely that the removal of amorphous polymers, namely lignin and xylan, may also cause an increase in crystallinity index in microfluidized lignocellulosic biomass.

Although the well-studied size reduction technique, dry ball milling, decreases crystallinity index and cause a loss of crystallinity with an increase of amorphous properties (Puri, 1984; Kelsey and Shafizadeh, 1980), microfluidization has caused an increase in crystallinity index. The reasons of this can be due to the partial reversibility of the change in crystalline structure. When the dry-milled samples were subjected to the effects of highly-moisturized environment, they regain crystallinity depending on the exposure time-interval. Thus, it is perceivable to get an increase in crystallinity index from 500 bar to 2000 bar treatment.

Compared to original cellulose of lignocellulosic biomass, the intensity of the diffraction peaks of the microfluidized cellulose films reduced and this behavior might arise from the deformation of the highly packaged structure of cellulose.

The X-ray diffraction results show that microfluidization is a potential pretreatment for developing cellulose nanofibrils and cellulose whiskers. Dufresne *et al.* (2000) have claimed that these structures can be used in biodegradable composites and nanocomposites.

3.7. Effect of pretreatment on surface characteristics of cellulose fibers

Scanned electron microscopy pictures were produced to examine the physical changes in the structure of biomass by the microfluidization pretreatment and the softening process. Significant morphological differences occurred when untreated and treated samples were compared.

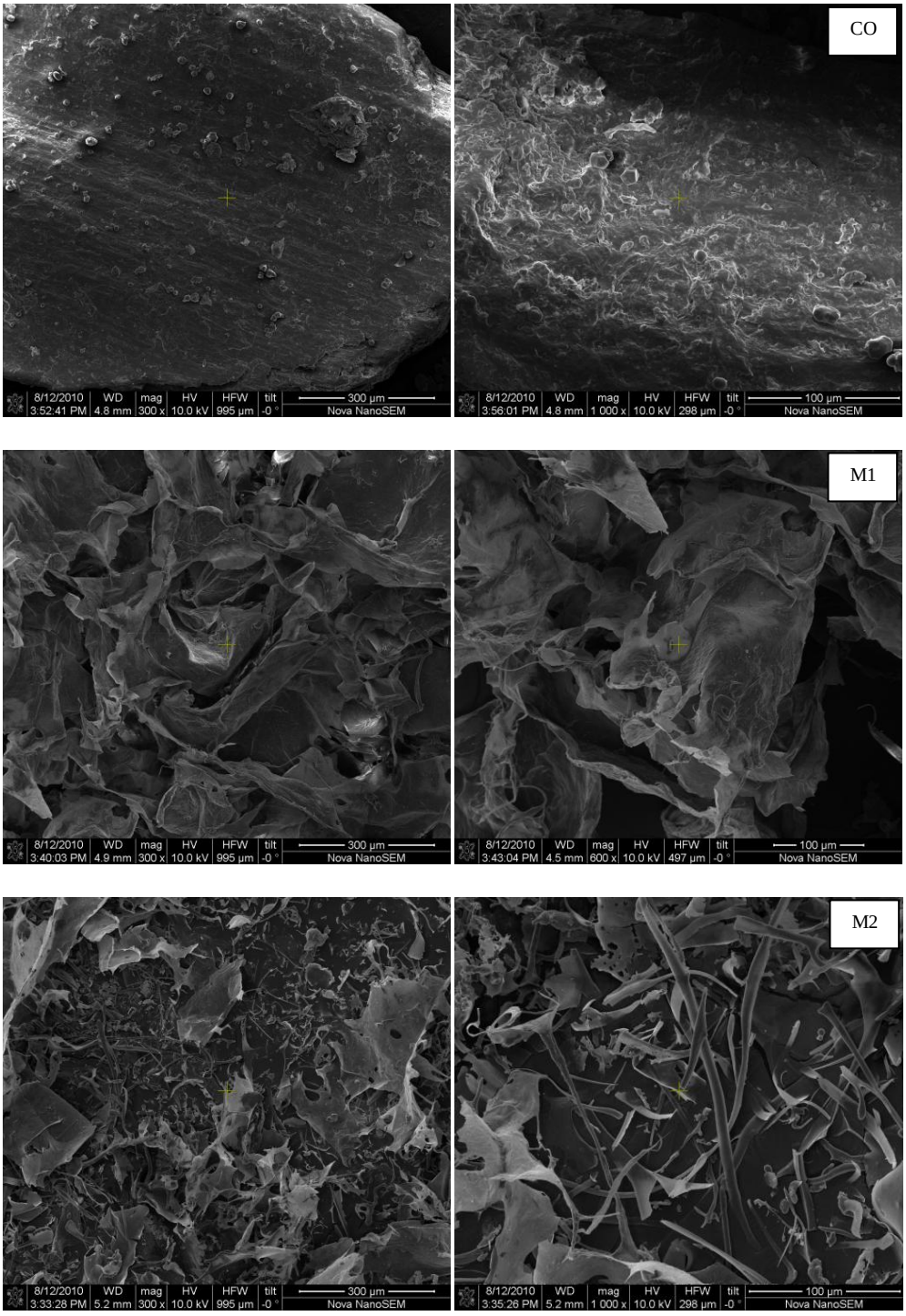
3.7.1. Effect of microfluidization pretreatment on surface characteristics of cellulose fibers

For all untreated samples (Figure 3.25, Figure 3.26, and Figure 3.27), rigid, more-packaged and highly ordered fibrils were exhibited. When microfluidization was performed, the surface of the lignocellulosic samples was deconstructed due to the high shear rate and fibrils were separated from their initial linked structure (Figure 3.25, Figure 3.26, and Figure 3.27).

As the pressure applied during microfluidization was increased from 500 bar to 2000 bar, more exposed structures were obtained (Figure 3.25, Figure 3.26, and Figure 3.27). Thus, SEM pictures proved that external surface area and the porosity of the samples increase with microfluidization pretreatment. It is clear from the pictures that the average diameters of the microfluidized particles were reduced to 10-15 μm.

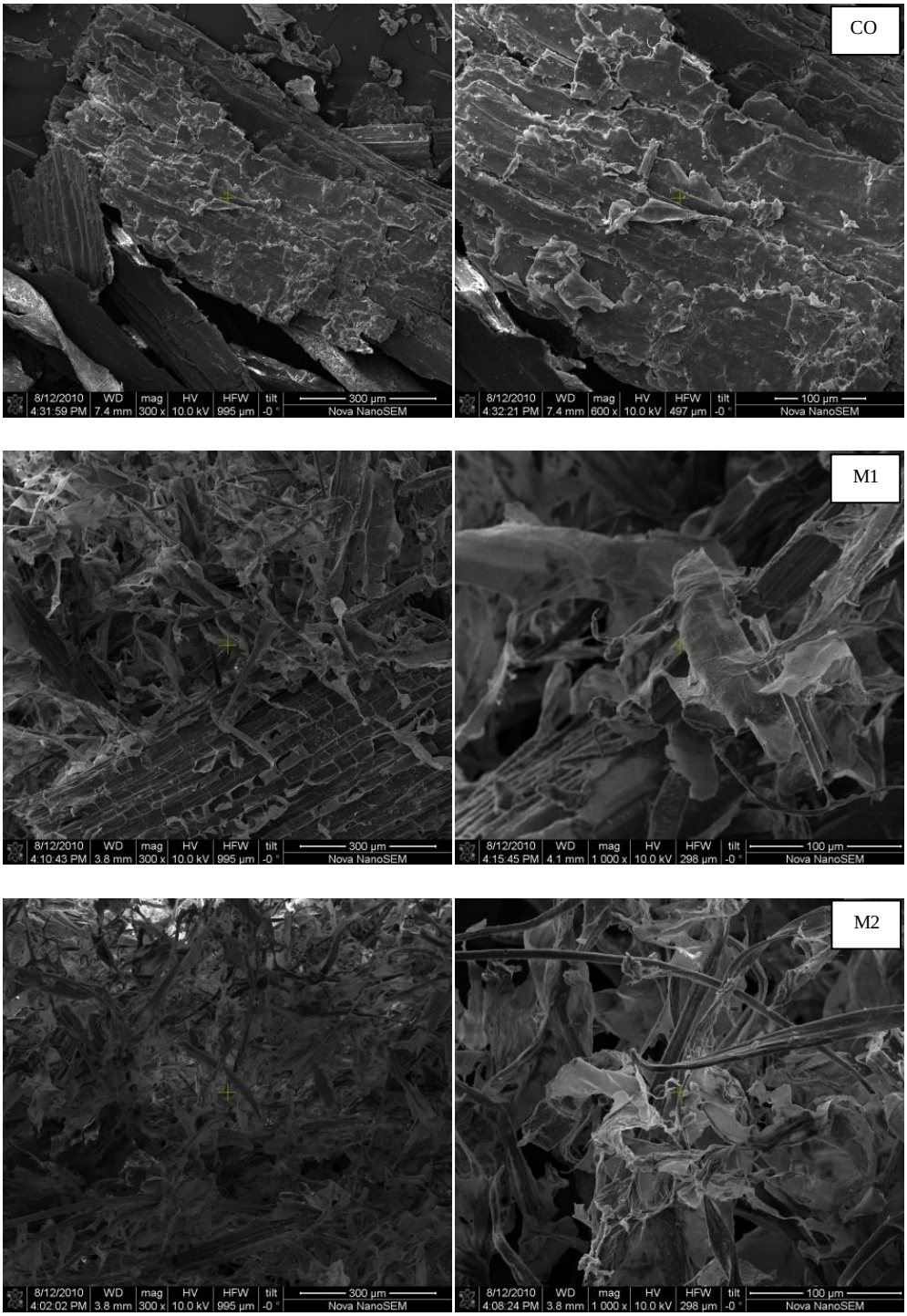
3.7.2. Effect of softening pretreatment on surface characteristics of cellulose fibers

SEM pictures of softened wheat straw and corn bran have illustrated that softening had an effect on the surface characteristic of cellulose but not to the extent reached by microfluidization. The differences on the structures of the samples demonstrate them to be mostly external, as shown in the Figure 3.28. There were not obvious cracks as the ones observed on the surface of microfluidized lignocellulosic samples.



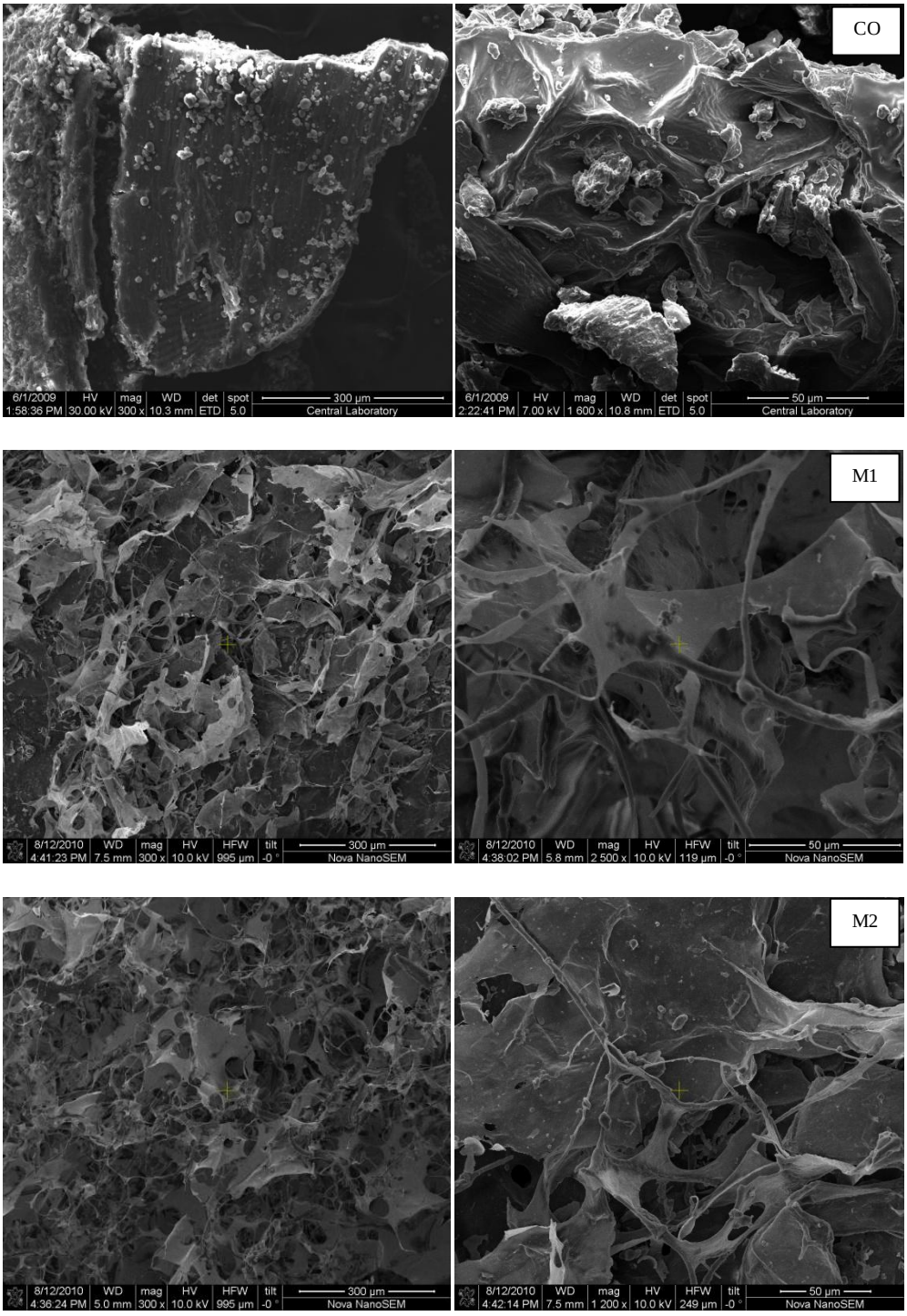
(a) (b)

Figure 3.25 Scanning electron micrographs of microfluidized wheat straw samples, CO: Untreated, M1: Microfluidized at 500 bar, M2: Microfluidized at 2000 bar; (a) Scale bar: 300 µm, (b) Scale bar: 100 µm



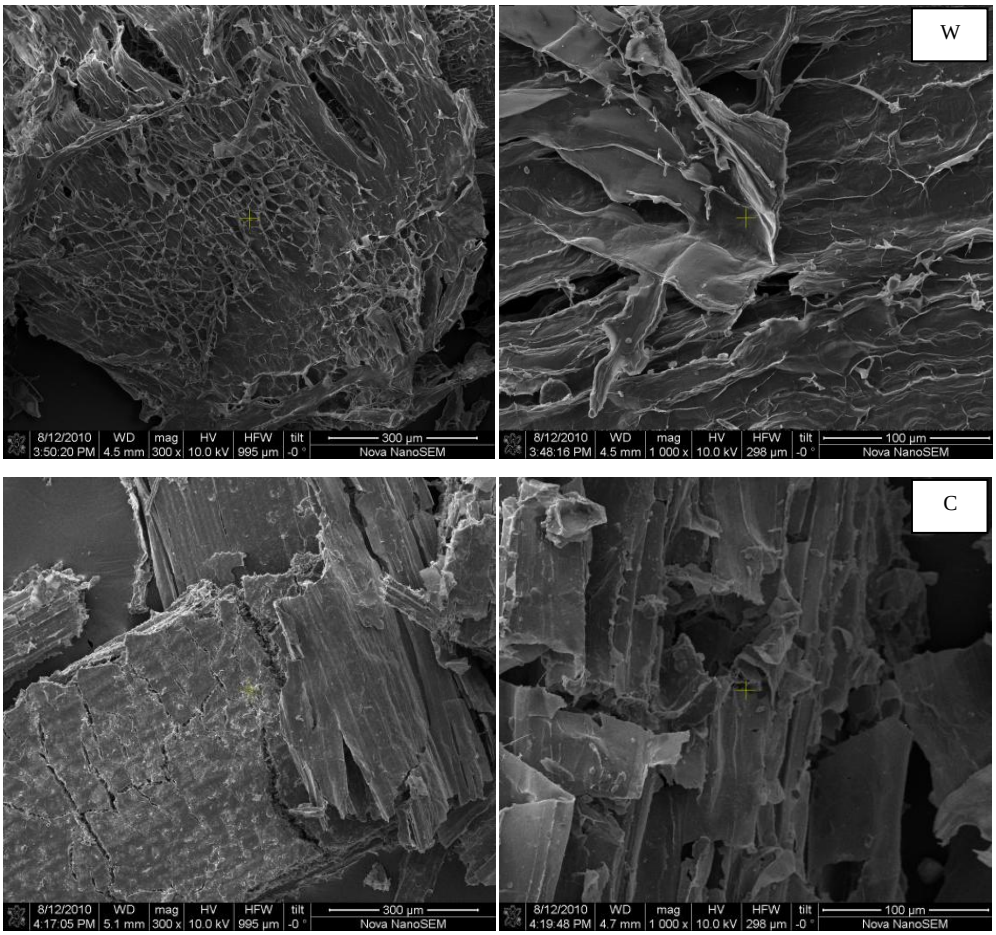
(a) (b)

Figure 3.26 Scanning electron micrographs of microfluidized corn bran samples, CO: Untreated, M1: Microfluidized at 500 bar, M2: Microfluidized at 2000 bar; (a) Scale bar: 300 µm, (b) Scale bar: 100 µm



(a) (b)

Figure 3.27 Scanning electron micrographs of microfluidized wheat bran samples, CO: Untreated, M1: Microfluidized at 500 bar, M2: Microfluidized at 2000 bar; (a) Scale bar: 300 µm, (b) Scale bar: 50 µm



(a) (b)

Figure 3.28 Scanning electron micrographs of the softening process applied samples, W: Wheat straw, C: Corn bran; (a) Scale bar: 300 µm, (b) Scale bar: 100 µm

CHAPTER 4

CONCLUSIONS

- Microfluidization enhances the production of total reducing sugar during enzymatic hydrolysis as compared to untreated samples.

- Efficiency increases by increasing the applied pressure from 500 bar to 2000 bar.

- Microfluidization enhances the total reducing sugar production during hydrolysis of microcrystalline cellulose, Avicel-PH101, during the first 18 hours of application.

- Addition of β-glucosidase results in approximately 10%-20% increase in the TRS over a 150 min reaction period.

- Optimum Celluclast dosage was determined as 4.5 U/g for wheat bran and corn bran and 6.0 U/g for wheat straw samples.

- Optimum solid load was different for different samples. While 5.0% (dw/v) solid load was the best for wheat bran, it was 2.5 % and 7.5 % (dw/v) for wheat straw and corn bran, respectively.

- Microfluidization was more effective on wheat straw and wheat bran samples, while for corn bran softening was better.

- Microfluidization is a method comparable to the widely used lime pretreatment and dilute-acid pretreatment processes.

- The process economics of the microfluidization pretreatment should be analyzed in further studies to determine the availability and sustainability of the method.

- X-ray diffraction (XRD) results of the microfluidized samples show that the pretreatment has increased crystallinity index (CrI) of the samples. Basically, for the case of microcrystalline cellulose, the crystallinity index was increased from 74% to 78%. The increase of pressure during microfluidization has also caused an increase in the CrI. For instance, CrI of bran has increased from 26% to 40% and from 37% to 62% for corn bran.

- SEM pictures of the microfluidized lignocellulosics have demonstrated that the treatment has a significant role in destruction of the highly-packaged structure of cellulose.

- The changes in XRD patterns and the alterations in the surface characteristics of microfluidized lignocellulosics are likely to be responsible from the rise in the TRS production during enzymatic hydrolysis.

- Microfluidized lignocellulosics may also be employed in the production of biobased materials and nanocomposites.

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APPENDIX A

CHEMICALS AND THEIR SUPPLIERS

Chemical	**Supplier**
Avicel, PH101	Fluka Analytical
Acetic acid, glacial	Merck
Ammonium acetate	Applichem
3'-5'- Dinitrosalicylic acid (DNS powder)	Sigma-Aldrich
Ethanol, absolute suitable for use as excipient	Merck
Magnesium sulfate-heptahydrate	Applichem
Potassium-sodium tartrate	Sigma
Sodium acetate trihydrate, extra pure, food grade	Merck
Sodium chloride	Merck
Sodium hydroxide, pellets pure	Merck
Sodium citrate monobasic	Sigma
Sodium sulfite	Merck
Sulfuric acid, 95-98% extra pure	Merck
Phenol	Fluka
Toluene	Merck

APPENDIX B

COMPOSITION OF BUFFERS AND SOLUTIONS

1. Composition of DNS Reagent:

5.00 g Dinitrosalicylic acid

1.00 ml Phenol

0.25 g Sodium sulfite (Na_2SO_3)

5.00 g Sodium hydroxide

180.0 g Potassium-sodium tartrate ($C_4H_4KNaO_6.4H_2O$)

2. Composition of Sodium Citrate Buffer

For 50.0 mM, 4.8 pH citrate buffer solution:

4.39 g/l citric acid and 8.56 g/l sodium citrate solutions are mixed, autoclaved and kept at room temperature for one month.

APPENDIX C

STANDARD CURVE FOR DNS TOTAL REDUCING SUGAR ESTIMATION

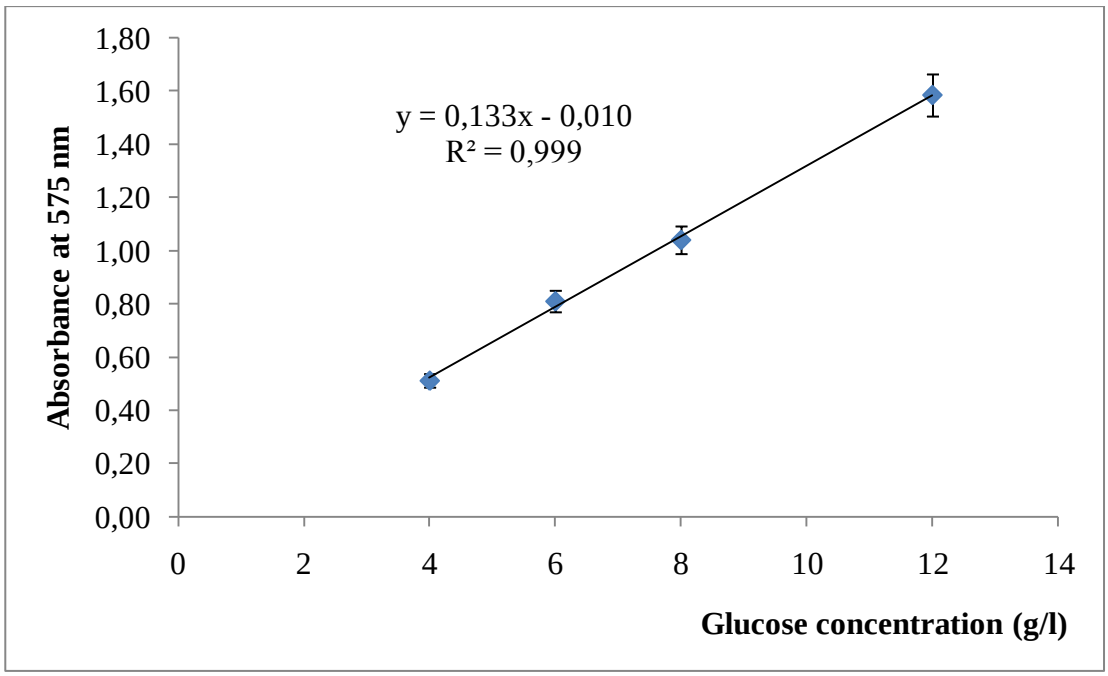

Figure C.1 The standard curve for DNS Method

According to the standard curve, the total reducing sugar concentration was calculated as below:

$$\boldsymbol{Total\ reducing\ sugar\ concentration}(g/L) = \frac{\boldsymbol{Absorbance} + \mathbf{0.01}}{\mathbf{0.133}} \times \boldsymbol{Dilution\ rate}$$

APPENDIX D

CELLUCLAST ACTIVITY DETERMINATION CURVES

The activitiy of cellulase (Celluclast) was estimated with sodium citrate buffer (at pH 4.8) and carboxymethyl cellulose solution. The activity was calculated as 915 U/ml CMCase.

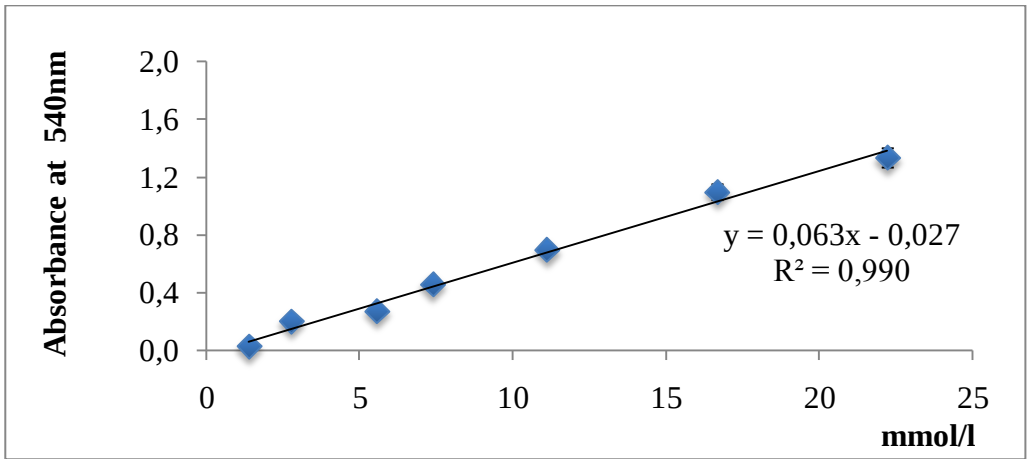

Figure D.1 The standard curve of carboxymethyl cellulose concentration vs absorbance at 540 nm

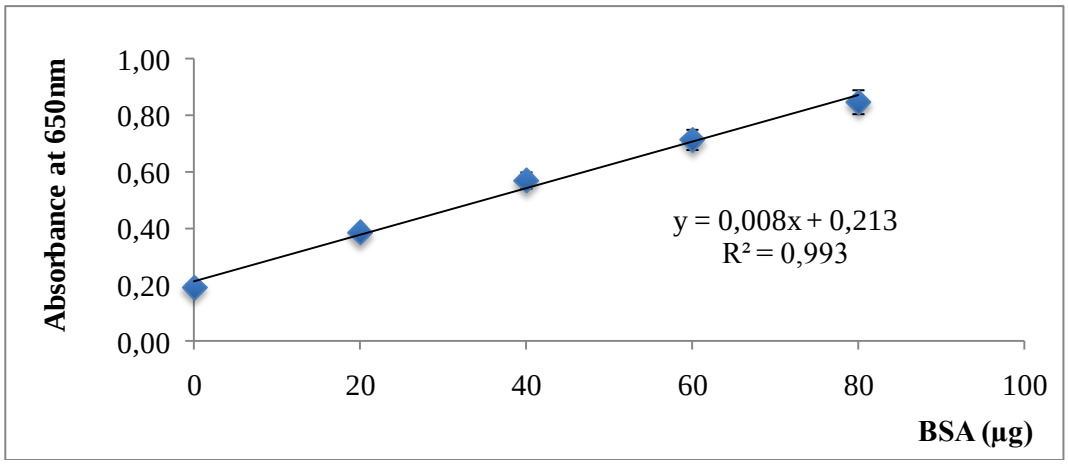

Figure D.2 The standard curve for Lowry protein content determination
(BSA: bovine serum albumin)

APPENDIX E

HYDROLYSIS RESULTS FOR WHEAT BRAN

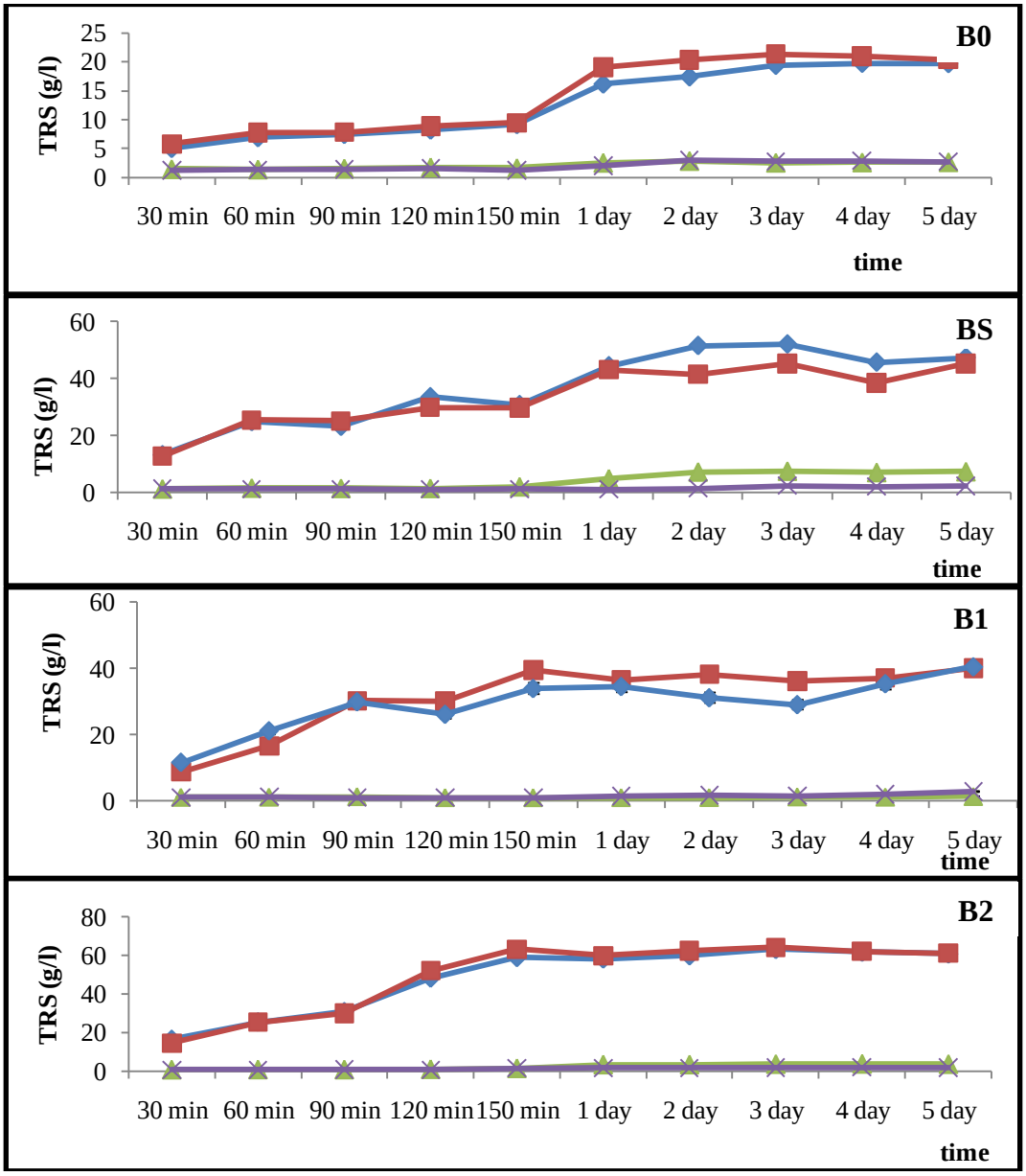

Figure E.1 Total reducing sugar content variation during the first five days for wheat bran , B0:untreated, BS: softened, B1: Microfluidized (500bars), B2: Microfluidized (2000bars), Hydrolysis conditions: (■)Cellulase(4.5U/g), β-glucosidase (25U/g), (♦) Cellulase (4.5U/g), (▲)β-glucosidase(25U/g), (x) No enzyme

APPENDIX F

HYDROLYSIS RESULTS FOR WHEAT STRAW

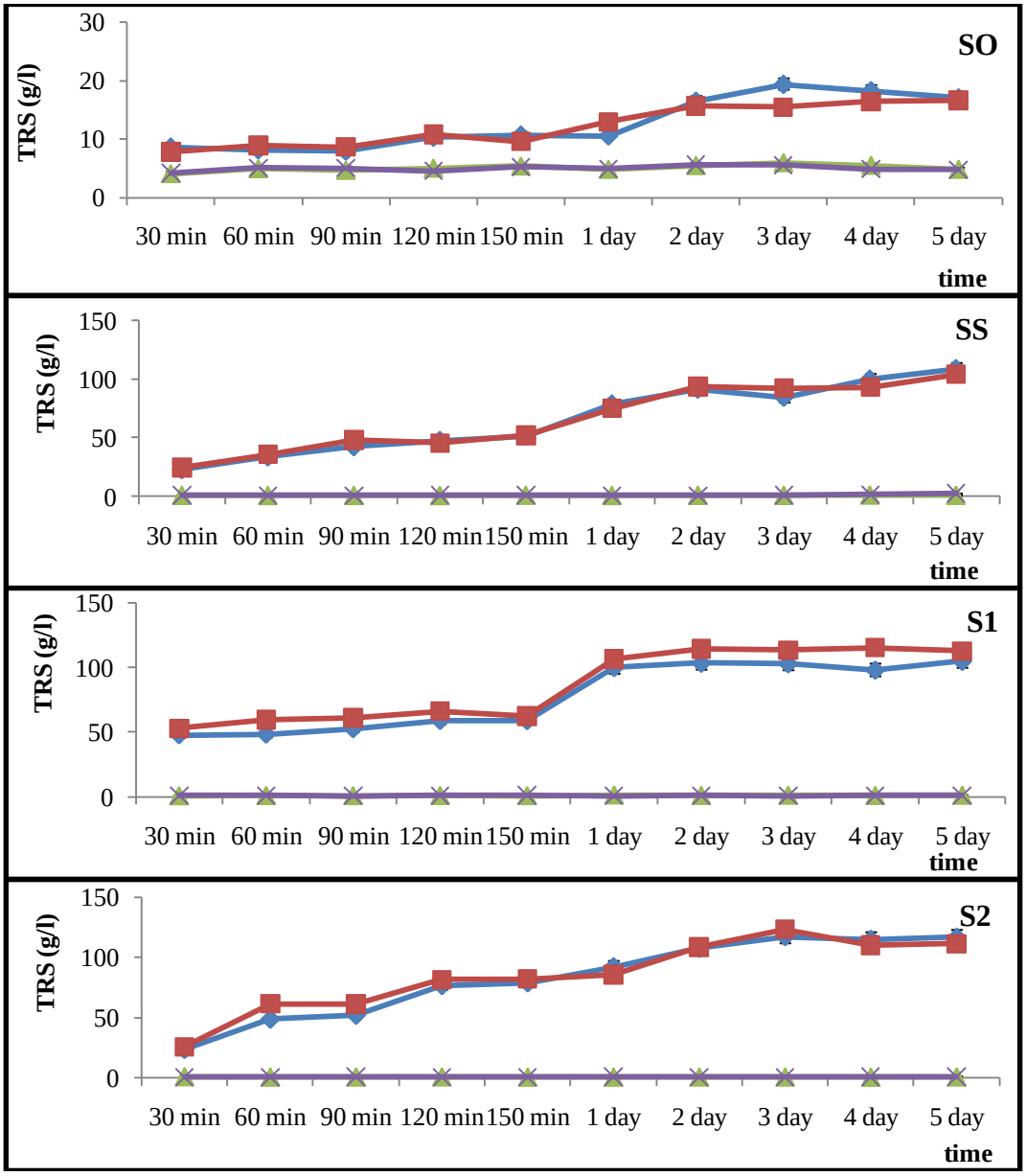

Figure F.1 Total reducing sugar content variation during the first five days for wheat straw , S0:untreated, SS: softened, S1: Microfluidized (500bars), S2: Microfluidized (2000bars), Hydrolysis conditions: (■)Cellulase(4.5U/g) + β-glucosidase (25U/g), (♦) Cellulase (4.5U/g), (▲)β-glucosidase(25U/g), (x) No enzyme

APPENDIX G

HYDROLYSIS RESULTS FOR CORN BRAN

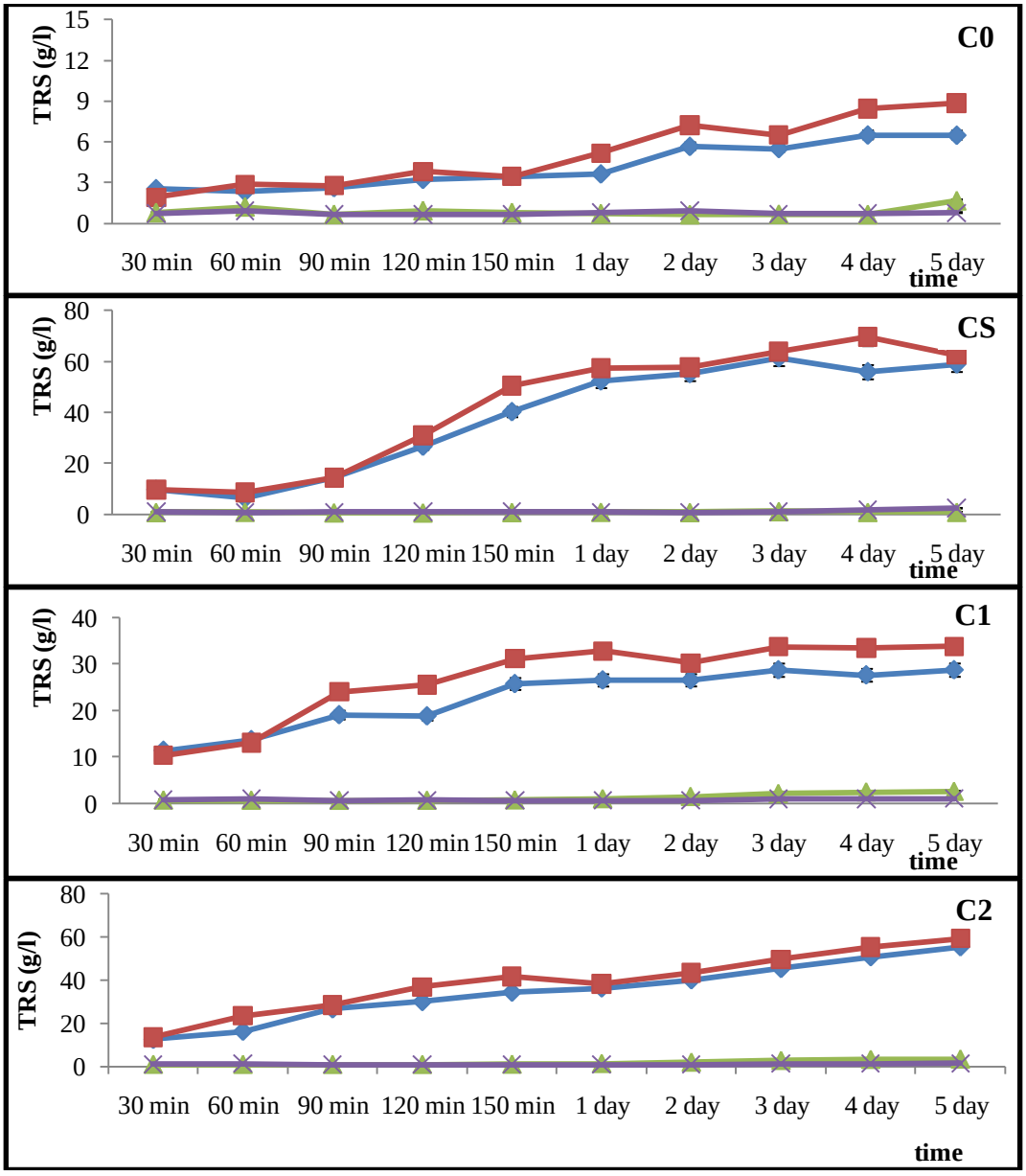

Figure G.1 Total reducing sugar content variation during the first five days for corn bran, C0:untreated, CS: softened, C1: Microfluidized (500bars), C2: Microfluidized (2000bars), Hydrolysis conditions: (■)Cellulase(4.5U/g) + β-glucosidase (25U/g), (♦) Cellulase (4.5U/g), (▲)β-glucosidase(25U/g), (x) No enzyme

APPENDIX H

RESULTS OF ANOVA AND MULTIPLE COMPARISONS FOR DETERMINATION OF TIME INTERVAL FOR MICROFLUIDIZED WHEAT BRAN

Table H.1 Results for Tukey's mean comparison test for the effect of time on long-term wheat bran hydrolysis

```
Factor       Type   Levels  Values
pressure     fixed       2  500; 2000
time         fixed      11  0; 2; 4; 6; 8; 24; 26; 28; 30; 48; 50
enzyme load  fixed       3  0,0; 4,5; 9,0


Analysis of Variance for TRS, using Adjusted SS for Tests

Source       DF   Seq SS   Adj SS   Adj MS       F      P
pressure      1    22,40    22,40    22,40    2,48  0,121
time         10  1508,42  1508,42   150,84   16,69  0,000
enzyme load   2  3745,78  3745,78  1872,89  207,19  0,000
Error        52   470,05   470,05     9,04
Total        65  5746,65


S = 3,00655   R-Sq = 91,82%   R-Sq(adj) = 89,78%


Unusual Observations for TRS

Obs     TRS       Fit  SE Fit  Residual  St Resid
  1  0,6968  -10,5593  1,3847   11,2561      4,22 R
 34  0,6300   -9,3941  1,3847   10,0241      3,76 R
 45  0,6300    6,1143  1,3847   -5,4843     -2,06 R
 56  0,6300    7,0211  1,3847   -6,3911     -2,39 R

R denotes an observation with a large standardized residual.
```

Tukey 95,0% Simultaneous Confidence Intervals
Response Variable TRS
All Pairwise Comparisons among Levels of time
time = 0 subtracted from:

time	Lower	Center	Upper	---------+---------+---------+-------
2	6,230	12,07	17,91	(-----*-----)
4	8,335	14,18	20,02	(-----*-----)
6	8,890	14,73	20,57	(-----*-----)
8	8,406	14,25	20,09	(-----*-----)
24	10,725	16,57	22,41	(-----*----)
26	11,363	17,21	23,05	(-----*-----)
28	12,010	17,85	23,69	(-----*-----)
30	12,040	17,88	23,72	(-----*-----)
48	10,200	16,04	21,88	(-----*-----)
50	8,670	14,51	20,35	(-----*----)
				---------+---------+---------+-------
				0 10 20

time = 2 subtracted from:

time	Lower	Center	Upper	---------+---------+---------+-------
4	-3,738	2,105	7,948	(-----*-----)
6	-3,183	2,660	8,503	(-----*-----)
8	-3,666	2,177	8,019	(-----*-----)
24	-1,348	4,495	10,338	(----*-----)
26	-0,709	5,133	10,976	(-----*-----)
28	-0,063	5,780	11,623	(-----*-----)
30	-0,033	5,810	11,653	(-----*-----)
48	-1,873	3,970	9,813	(-----*-----)
50	-3,403	2,440	8,283	(----*-----)
				---------+---------+---------+-------
				0 10 20

time = 4 subtracted from:

time	Lower	Center	Upper	---------+---------+---------+-------
6	-5,288	0,55500	6,398	(-----*----)
8	-5,771	0,07167	5,914	(-----*-----)
24	-3,453	2,39000	8,233	(----*-----)
26	-2,814	3,02833	8,871	(-----*-----)
28	-2,168	3,67500	9,518	(-----*-----)
30	-2,138	3,70500	9,548	(-----*-----)
48	-3,978	1,86500	7,708	(-----*-----)
50	-5,508	0,33500	6,178	(-----*-----)
				---------+---------+---------+-------
				0 10 20

time = 6 subtracted from:

time	Lower	Center	Upper	---------+---------+---------+-------
8	-6,326	-0,4833	5,359	(-----*----)
24	-4,008	1,8350	7,678	(-----*-----)
26	-3,369	2,4733	8,316	(----*-----)
28	-2,723	3,1200	8,963	(-----*-----)
30	-2,693	3,1500	8,993	(-----*-----)
48	-4,533	1,3100	7,153	(-----*-----)
50	-6,063	-0,2200	5,623	(-----*-----)
				---------+---------+---------+-------
				0 10 20

```
time =  8  subtracted from:

time   Lower  Center  Upper  ---------+---------+---------+-------
24    -3,524  2,3183  8,161       (-----*-----)
26    -2,886  2,9567  8,799        (-----*-----)
28    -2,239  3,6033  9,446         (-----*----)
30    -2,209  3,6333  9,476         (-----*----)
48    -4,049  1,7933  7,636       (-----*-----)
50    -5,579  0,2633  6,106    (-----*-----)
                              ---------+---------+---------+-------
                                       0        10        20


time = 24  subtracted from:

time   Lower  Center  Upper  ---------+---------+---------+-------
26    -5,204   0,638  6,481     (-----*----)
28    -4,558   1,285  7,128     (-----*-----)
30    -4,528   1,315  7,158     (-----*-----)
48    -6,368  -0,525  5,318    (----*-----)
50    -7,898  -2,055  3,788  (-----*-----)
                              ---------+---------+---------+-------
                                       0        10        20


time = 26  subtracted from:

time   Lower  Center  Upper  ---------+---------+---------+-------
28    -5,196   0,647  6,489     (-----*----)
30    -5,166   0,677  6,519     (-----*-----)
48    -7,006  -1,163  4,679   (-----*-----)
50    -8,536  -2,693  3,149 (-----*-----)
                              ---------+---------+---------+-------
                                       0        10        20


time = 28  subtracted from:

time   Lower  Center  Upper  ---------+---------+---------+-------
30    -5,813   0,030  5,873    (-----*-----)
48    -7,653  -1,810  4,033  (-----*-----)
50    -9,183  -3,340  2,503 (-----*-----)
                              ---------+---------+---------+-------
                                       0        10        20


time = 30  subtracted from:

time   Lower  Center  Upper  ---------+---------+---------+-------
48    -7,683  -1,840  4,003  (-----*-----)
50    -9,213  -3,370  2,473 (-----*----)
                              ---------+---------+---------+-------
                                       0        10        20


time = 48  subtracted from:

time   Lower  Center  Upper  ---------+---------+---------+-------
50    -7,373  -1,530  4,313   (----*-----)
                              ---------+---------+---------+-------
                                       0        10        20
```

Tukey Simultaneous Tests
Response Variable TRS
All Pairwise Comparisons among Levels of time
time = 0 subtracted from:

time	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
2	12,07	1,736	6,955	0,0000
4	14,18	1,736	8,167	0,0000
6	14,73	1,736	8,487	0,0000
8	14,25	1,736	8,209	0,0000
24	16,57	1,736	9,544	0,0000
26	17,21	1,736	9,912	0,0000
28	17,85	1,736	10,285	0,0000
30	17,88	1,736	10,302	0,0000
48	16,04	1,736	9,242	0,0000
50	14,51	1,736	8,360	0,0000

time = 2 subtracted from:

time	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
4	2,105	1,736	1,213	0,9782
6	2,660	1,736	1,532	0,9018
8	2,177	1,736	1,254	0,9724
24	4,495	1,736	2,590	0,2806
26	5,133	1,736	2,957	0,1336
28	5,780	1,736	3,330	0,0549
30	5,810	1,736	3,347	0,0525
48	3,970	1,736	2,287	0,4576
50	2,440	1,736	1,406	0,9415

time = 4 subtracted from:

time	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
6	0,55500	1,736	0,31973	1,0000
8	0,07167	1,736	0,04129	1,0000
24	2,39000	1,736	1,37686	0,9487
26	3,02833	1,736	1,74460	0,8055
28	3,67500	1,736	2,11714	0,5710
30	3,70500	1,736	2,13442	0,5593
48	1,86500	1,736	1,07441	0,9910
50	0,33500	1,736	0,19299	1,0000

time = 6 subtracted from:

time	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
8	-0,4833	1,736	-0,2784	1,0000
24	1,8350	1,736	1,0571	0,9921
26	2,4733	1,736	1,4249	0,9363
28	3,1200	1,736	1,7974	0,7761
30	3,1500	1,736	1,8147	0,7661
48	1,3100	1,736	0,7547	0,9995
50	-0,2200	1,736	-0,1267	1,0000

time = 8 subtracted from:

time	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
24	2,3183	1,736	1,3356	0,9578
26	2,9567	1,736	1,7033	0,8270
28	3,6033	1,736	2,0759	0,5989
30	3,6333	1,736	2,0931	0,5872
48	1,7933	1,736	1,0331	0,9934
50	0,2633	1,736	0,1517	1,0000

time = 24 subtracted from:

time	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
26	0,638	1,736	0,368	1,0000
28	1,285	1,736	0,740	0,9996
30	1,315	1,736	0,758	0,9995
48	-0,525	1,736	-0,302	1,0000
50	-2,055	1,736	-1,184	0,9816

time = 26 subtracted from:

time	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
28	0,647	1,736	0,373	1,0000
30	0,677	1,736	0,390	1,0000
48	-1,163	1,736	-0,670	0,9998
50	-2,693	1,736	-1,552	0,8946

time = 28 subtracted from:

time	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
30	0,030	1,736	0,017	1,0000
48	-1,810	1,736	-1,043	0,9929
50	-3,340	1,736	-1,924	0,6991

time = 30 subtracted from:

time	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
48	-1,840	1,736	-1,060	0,9919
50	-3,370	1,736	-1,941	0,6880

time = 48 subtracted from:

time	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
50	-1,530	1,736	-0,8814	0,9982

APPENDIX I

RESULTS OF ANOVA AND TUKEY'S COMPARISONS FOR DETERMINATION OF OPTIMUM CELLULASE CONCENTRATION OF WHEAT BRAN

Table I.1 Results for Tukey's mean comparison test for enzyme load optimization of wheat bran

```
Factor       Type   Levels  Values
enyzme load  fixed       4  1,5; 3,0; 4,5; 9,0
time         fixed       5  30; 60; 90; 120; 150


Analysis of Variance for trs, using Adjusted SS for Tests

Source       DF   Seq SS  Adj SS  Adj MS      F      P
enyzme load   3   943,38  943,38  314,46  16,99  0,000
time          4   727,65  727,65  181,91   9,83  0,001
Error        12   222,11  222,11   18,51
Total        19  1893,14


S = 4,30225   R-Sq = 88,27%   R-Sq(adj) = 81,42%


Unusual Observations for trs

Obs      trs      Fit  SE Fit  Residual  St Resid
 16  20,4035  27,9691  2,7210   -7,5656     -2,27 R
 17  42,9587  35,9384  2,7210    7,0203      2,11 R

R denotes an observation with a large standardized residual.


Tukey 95,0% Simultaneous Confidence Intervals
Response Variable trs
All Pairwise Comparisons among Levels of enyzme load
enyzme load = 1,5  subtracted from:

enyzme
load     Lower  Center  Upper  ------+---------+---------+---------+
3,0     -3,263   4,817  12,90     (-------*-------)
4,5      6,304  14,384  22,47               (-------*-------)
9,0      8,742  16,822  24,90                  (-------*-------)
                                ------+---------+---------+---------+
                                      0        10        20        30
```

enyzme load = 3,0 subtracted from:

```
enyzme
load    Lower  Center  Upper  ------+---------+---------+---------+
4,5     1,486   9,567  17,65         (--------*-------)
9,0     3,924  12,005  20,09            (-------*-------)
                               ------+---------+---------+---------+
                                     0        10        20        30
```

enyzme load = 4,5 subtracted from:

```
enyzme
load     Lower  Center  Upper  ------+---------+---------+---------+
9,0     -5,643   2,438  10,52  (-------*--------)
                               ------+---------+---------+---------+
                                     0        10        20        30
```

Tukey Simultaneous Tests
Response Variable trs
All Pairwise Comparisons among Levels of enyzme load
enyzme load = 1,5 subtracted from:

enyzme load	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
3,0	4,817	2,721	1,770	0,3328
4,5	14,384	2,721	5,286	0,0010
9,0	16,822	2,721	6,182	0,0002

enyzme load = 3,0 subtracted from:

enyzme load	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
4,5	9,567	2,721	3,516	0,0193
9,0	12,005	2,721	4,412	0,0041

enyzme load = 4,5 subtracted from:

enyzme load	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
9,0	2,438	2,721	0,8960	0,8071

APPENDIX J

RESULTS OF ANOVA AND TUKEY'S COMPARISONS FOR DETERMINATION OF OPTIMUM CELLULASE CONCENTRATION OF WHEAT STRAW

Table J.1 Results for Tukey's mean comparison test for enzyme load optimization of wheat straw

```
Factor       Type   Levels  Values
enyzme load  fixed       6  1,5; 3,0; 4,5; 6,0; 7,5; 9,0
time         fixed       5  30; 60; 90; 120; 150


Analysis of Variance for trs, using Adjusted SS for Tests

Source       DF   Seq SS   Adj SS  Adj MS      F      P
enyzme load   5  1543,22  1543,22  308,64  29,76  0,000
time          4   634,53   634,53  158,63  15,29  0,000
Error        20   207,45   207,45   10,37
Total        29  2385,20


S = 3,22063   R-Sq = 91,30%   R-Sq(adj) = 87,39%


Unusual Observations for trs

Obs      trs      Fit  SE Fit  Residual  St Resid
 16  34,1667  40,0157  1,8594   -5,8490     -2,22 R

R denotes an observation with a large standardized residual.


Tukey 95,0% Simultaneous Confidence Intervals
Response Variable trs
All Pairwise Comparisons among Levels of enyzme load
enyzme load = 1,5  subtracted from:

enyzme
load     Lower  Center   Upper  -------+---------+---------+---------
3,0     -2,977   3,433   9,842      (-----*------)
4,5      2,486   8,895  15,305           (------*-----)
6,0     10,532  16,941  23,351                    (-----*-----)
7,5     10,351  16,761  23,170                   (------*-----)
9,0     12,192  18,601  25,010                     (------*-----)
                                -------+---------+---------+---------
                                       0        10        20
```

enyzme load = 3,0 subtracted from:

enyzme load	Lower	Center	Upper	-------+---------+---------+---------
4,5	-0,9466	5,463	11,87	(-----*------)
6,0	7,0995	13,509	19,92	(------*-----)
7,5	6,9186	13,328	19,74	(-----*------)
9,0	8,7591	15,168	21,58	(-----*------)
				-------+---------+---------+---------
				0 10 20

enyzme load = 4,5 subtracted from:

enyzme load	Lower	Center	Upper	-------+---------+---------+---------
6,0	1,637	8,046	14,46	(-----*-----)
7,5	1,456	7,865	14,27	(------*-----)
9,0	3,296	9,706	16,12	(------*-----)
				-------+---------+---------+---------
				0 10 20

enyzme load = 6,0 subtracted from:

enyzme load	Lower	Center	Upper	-------+---------+---------+---------
7,5	-6,590	-0,1809	6,229	(------*-----)
9,0	-4,750	1,6596	8,069	(------*-----)
				-------+---------+---------+---------
				0 10 20

enyzme load = 7,5 subtracted from:

enyzme load	Lower	Center	Upper	-------+---------+---------+---------
9,0	-4,569	1,840	8,250	(------*-----)
				-------+---------+---------+---------
				0 10 20

Tukey Simultaneous Tests
Response Variable trs
All Pairwise Comparisons among Levels of enyzme load
enyzme load = 1,5 subtracted from:

enyzme load	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
3,0	3,433	2,037	1,685	0,5561
4,5	8,895	2,037	4,367	0,0035
6,0	16,941	2,037	8,317	0,0000
7,5	16,761	2,037	8,228	0,0000
9,0	18,601	2,037	9,132	0,0000

enyzme load = 3,0 subtracted from:

enyzme load	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
4,5	5,463	2,037	2,682	0,1232
6,0	13,509	2,037	6,632	0,0000
7,5	13,328	2,037	6,543	0,0000
9,0	15,168	2,037	7,447	0,0000

enyzme load = 4,5 subtracted from:

enyzme load	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
6,0	8,046	2,037	3,950	0,0089
7,5	7,865	2,037	3,861	0,0108
9,0	9,706	2,037	4,765	0,0014

enyzme load = 6,0 subtracted from:

enyzme load	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
7,5	-0,1809	2,037	-0,08879	1,0000
9,0	1,6596	2,037	0,81475	0,9614

enyzme load = 7,5 subtracted from:

enyzme load	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
9,0	1,840	2,037	0,9035	0,9411

APPENDIX K

RESULTS OF ANOVA AND TUKEY'S COMPARISONS FOR DETERMINATION OF OPTIMUM CELLULASE CONCENTRATION OF CORN BRAN

Table K.1 Results for Tukey's mean comparison test for enzyme load optimization of corn bran

```
Factor       Type   Levels  Values
enyzme load  fixed       4  1,5; 3,0; 4,5; 9,0
time         fixed       5  30; 60; 90; 120; 150


Analysis of Variance for trs, using Adjusted SS for Tests

Source       DF   Seq SS  Adj SS  Adj MS      F      P
enyzme load   3   702,93  702,93  234,31  51,11  0,000
time          4   242,87  242,87   60,72  13,25  0,000
Error        12    55,01   55,01    4,58
Total        19  1000,81


S = 2,14107   R-Sq = 94,50%   R-Sq(adj) = 91,30%


Unusual Observations for trs

Obs     trs     Fit  SE Fit  Residual  St Resid
  1  8,1738  3,7505  1,3541    4,4232      2,67 R

R denotes an observation with a large standardized residual.


Tukey 95,0% Simultaneous Confidence Intervals
Response Variable trs
All Pairwise Comparisons among Levels of enyzme load
enyzme load = 1,5  subtracted from:

enyzme
load     Lower  Center  Upper  --+---------+---------+---------+----
3,0      4,396   8,418  12,44           (------*------)
4,5      8,845  12,866  16,89                   (-----*------)
9,0     11,651  15,673  19,69                       (------*------)
                                --+---------+---------+---------+----
                                0,0       6,0      12,0      18,0
```

```
enyzme load = 3,0  subtracted from:

enyzme
load     Lower  Center   Upper  --+---------+---------+---------+----
4,5     0,4267   4,448   8,470     (-----*------)
9,0     3,2334   7,255  11,277          (------*------)
                                --+---------+---------+---------+----
                                 0,0       6,0      12,0      18,0


enyzme load = 4,5  subtracted from:

enyzme
load     Lower  Center  Upper  --+---------+---------+---------+----
9,0     -1,215   2,807  6,828  (------*-----)
                               --+---------+---------+---------+----
                               0,0       6,0      12,0      18,0


Tukey Simultaneous Tests
Response Variable trs
All Pairwise Comparisons among Levels of enyzme load
enyzme load = 1,5  subtracted from:

enyzme  Difference       SE of           Adjusted
load      of Means  Difference  T-Value   P-Value
3,0          8,418       1,354    6,217    0,0002
4,5         12,866       1,354    9,501    0,0000
9,0         15,673       1,354   11,574    0,0000


enyzme load = 3,0  subtracted from:

enyzme  Difference       SE of           Adjusted
load      of Means  Difference  T-Value   P-Value
4,5          4,448       1,354    3,285    0,0289
9,0          7,255       1,354    5,358    0,0009


enyzme load = 4,5  subtracted from:

enyzme  Difference       SE of           Adjusted
load      of Means  Difference  T-Value   P-Value
9,0          2,807       1,354    2,073    0,2166
```

APPENDIX L

RESULTS OF ANOVA AND TUKEY'S COMPARISONS FOR LIME PRETREATMENT AND MICROFLUIDIZATION

Table L.1 Results for Tukey's mean comparison test for lime pretreatment and microfluidization

```
Factor             Type   Levels  Values
pretreatment type  fixed       4  L-45; L-60; M-2000; M-500
time               fixed       6  0; 30; 60; 90; 120; 150


Analysis of Variance for TRS, using Adjusted SS for Tests

Source             DF   Seq SS   Adj SS  Adj MS      F      P
pretreatment type   3   497,89   497,89  165,96  16,07  0,000
time                5  4126,35  4126,35  825,27  79,92  0,000
Error              15   154,90   154,90   10,33
Total              23  4779,14


S = 3,21353   R-Sq = 96,76%   R-Sq(adj) = 95,03%


Unusual Observations for TRS

Obs     TRS     Fit  SE Fit  Residual  St Resid
  7  0,6330  8,0176  1,9679   -7,3846     -2,91 R

R denotes an observation with a large standardized residual.


Tukey 95,0% Simultaneous Confidence Intervals
Response Variable TRS
All Pairwise Comparisons among Levels of pretreatment type
pretreatment type = L-45  subtracted from:

pretreatment
type           Lower  Center   Upper  -----+---------+---------+---------
+-
L-60          -1,520   3,832   9,185               (-----*----)
M-2000         6,680  12,033  17,385                         (----*----)
M-500         -3,207   2,146   7,499              (----*----)
                                     -----+---------+---------+---------
+-
                                        -10         0        10
20
```

pretreatment type = L-60 subtracted from:

pretreatment type	Lower	Center	Upper	-----+---------+---------+--------- +-
M-2000	2,848	8,200	13,553	(----*-----)
M-500	-7,039	-1,686	3,666	(----*-----)

-----+---------+---------+---------
+-
-10 0 10
20

pretreatment type = M-2000 subtracted from:

pretreatment type	Lower	Center	Upper	-----+---------+---------+--------- +-
M-500	-15,24	-9,887	-4,534	(----*----)

-----+---------+---------+---------
+-
-10 0 10
20

Tukey Simultaneous Tests
Response Variable TRS
All Pairwise Comparisons among Levels of pretreatment type
pretreatment type = L-45 subtracted from:

pretreatment type	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
L-60	3,832	1,855	2,066	0,2088
M-2000	12,033	1,855	6,486	0,0001
M-500	2,146	1,855	1,157	0,6618

pretreatment type = L-60 subtracted from:

pretreatment type	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
M-2000	8,200	1,855	4,4199	0,0025
M-500	-1,686	1,855	-0,9090	0,8004

pretreatment type = M-2000 subtracted from:

pretreatment type	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
M-500	-9,887	1,855	-5,329	0,0004