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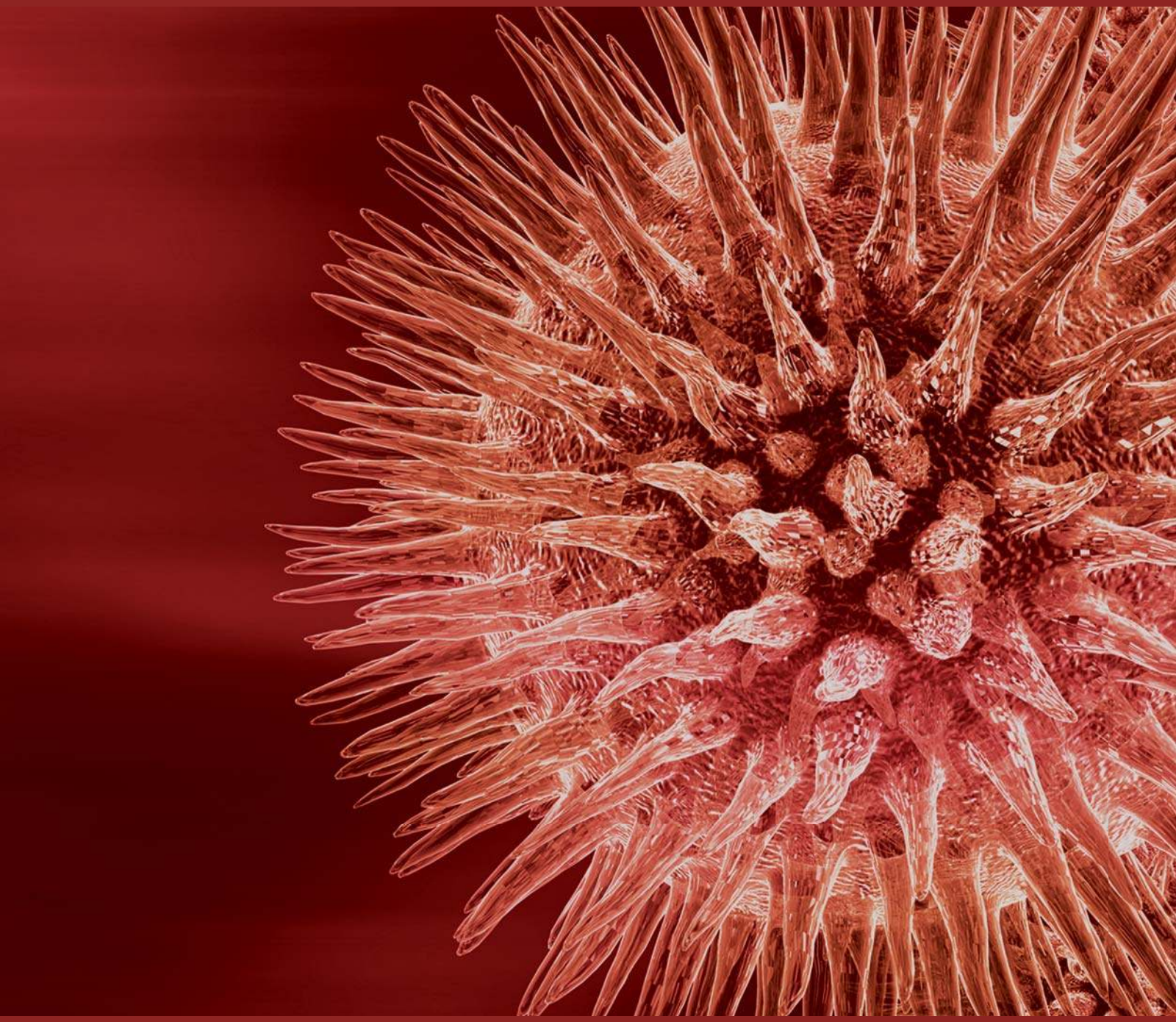
Fermentative Production of Value-Added Products from Lignocellulosic Biomass

Guest Editors: Silvio S. da Silva, Anuj K. Chandel,
S. Ranil Wickramsinghe, and José M. D. González



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Editorial

Fermentative Production of Value-Added Products from Lignocellulosic Biomass

Silvio S. da Silva,[1] Anuj K. Chandel,[1] S. Ranil Wickramasinghe,[2] and José M. G. Domínguez[3]

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Bioconversion of lignocellulosic biomass (agro residues, grasses, wood, weed, dedicated energy crops and others) into biofuels and other value-added products offers numerous geopolitical, environmental, and strategic benefits. Lignocellulosic biomass (LB) is the most abundant renewable organic resources (~200 billion tons annually) on earth that are readily available for conversion to biofuels and other value-added products (industrial enzymes, organic acids, pharmaceuticals, commodity chemicals, and food/feed). However, they have not yet been tapped for the commercial production of these products.

The last three decades witnessed the success made in research and development for the conversion of LB into biofuels and other commercially viable products. Despite the success achieved in the laboratory, there are several challenges for the successful bioconversion of lignocellulosic substrates into value-added products at commercial scale. The future of lignocellulosics conversion is expected to lie in improvement in pretreatment technologies, cellulolytic enzymes producing microorganisms, fullest exploitation of biomass components, and process integration. Further, advancement in system-biology-based "OMICS" approaches may provide new breakthroughs for the development of biocatalysts/enzyme titers for the cost effective production of commodity chemicals and fuels from biomass.

This special issue presents a diverse range of experimental advancements on biomass pretreatment, enzymatic saccharification, production of cellulosic biofuels, polyhydroxyalkanoates (PHA), polyhydroxybutyrate (PHB), and lovastatin and blends of polyacrylonitrile (PAN) and lignin. Authors here report on process variables including feedstock types (grasses and sugarcane-based agro residues), bioprocess engineering simulations, multivisual structural analysis of lignocellulosics after pretreatment, improvements in cellulase-mediated hydrolysis, and modification in fermentation strategies for the production of a range of value-added products.

Selection of suitable lignocellulosic substrates plays a key role for the economic cellulosic ethanol production. Factors such as availability of feedstock and presence of high amount of carbohydrates and lignin amounts are critical for biofuels production at large scale under the biorefinery concept. Of particular interest in this regard is the use of sugarcane residues, corn stover, and grasses as a second-generation feedstock. L. Canilha et al. comprehensively reviewed the technologies (pretreatment methods, detoxification, hydrolysis, fermentation, and end-product distillation) for second-generation (2G) ethanol production from sugarcane residues (sugarcane bagasse and straw). Grasses are another excellent feedstock for 2G ethanol production in countries like Thailand. J. Wongwatanapaiboon et al. collected data on composition of cellulose, hemicellulose, and lignin in 18 types of grasses followed by the alkaline peroxide mediated pretreatment. The pretreated materials were evaluated for ethanol production under simultaneous saccharification and fermentation approach employing yeasts cocultures. J. Lu et al. explored the Reed as a primary source for 2G ethanol production. They presented the data on sugar

recovery after liquid hot water pretreatment followed by enzymatic hydrolysis and conversion of sugars into ethanol under separate hydrolysis and fermentation. J. E. Jessen and J. Orlygsson examined the 2G ethanol production from various feedstocks (hemp stem, grass, wheat straw, newspaper, and cellulose) employing a new thermophilic isolate, *Thermoanaerobacter* J1, from a hot spring in Iceland in addition to the exploration of a mechanism of electron-scavenging systems on end-product formation. For the economization of biofuel or any value-added product formation from lignocellulosic materials, the efficient bioconversion of cellulose and hemicellulose into monomeric sugars is inevitable. In this line, A. D. Eckard et al. demonstrated the mechanism of casein (skimmed milk protein) action to improve the enzymatic saccharification of corn stover.

Pretreatment is the key technology for the direct methanation of agroresidues. Y. Feng et al. investigated the changes in the maize straw characteristics during the methanation process. M. A. K. M. Zahari et al. optimized major influential parameters for poly(3-hydroxybutyrate) production from oil palm frond (OPF) juice by *Cupriavidus necator* CCUG52238T and concluded that OPF juice could be a competitive carbon source for the economic production of PHB. P. Chakraborty et al. presented the data on modified feeding strategies using a mixture of volatile fatty acids and artificial rumen fluid to maximize the PHA production by *Ralstonia eutropha*. Towards the pharmaceuticals intermediate production, M. F. Jahromi et al. investigated the lovastatin production by *Aspergillus terreus* under solid-state fermentation using rice straw and oil palm frond. M. Ö. Seydibeyoğlu examined the properties of blends of polyacrylonitrile (PAN) and lignin followed by the structural analysis employing modern structural tools.

We sincerely hope readers will find these papers helpful to their research pursuits. It has been our pleasure to put together this special issue in Journal of Biomedicine and Biotechnology.

Acknowledgments

We thank all of the contributing authors for sharing their quality research through this special issue. Editors would like to thank the reviewers for their critical comments and suggestions which helped to improve the quality of the papers.

Silvio S. da Silva
Anuj K. Chandel
S. Ranil Wickramasinghe
José M. G. Domínguez

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Review Article

Bioconversion of Sugarcane Biomass into Ethanol: An Overview about Composition, Pretreatment Methods, Detoxification of Hydrolysates, Enzymatic Saccharification, and Ethanol Fermentation

Larissa Canilha, Anuj Kumar Chandel, Thais Suzane dos Santos Milessi, Felipe Antônio Fernandes Antunes, Wagner Luiz da Costa Freitas, Maria das Graças Almeida Felipe, and Silvio Silvério da Silva

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Depleted supplies of fossil fuel, regular price hikes of gasoline, and environmental damage have necessitated the search for economic and eco-benign alternative of gasoline. Ethanol is produced from food/feed-based substrates (grains, sugars, and molasses), and its application as an energy source does not seem fit for long term due to the increasing fuel, food, feed, and other needs. These concerns have enforced to explore the alternative means of cost competitive and sustainable supply of biofuel. Sugarcane residues, sugarcane bagasse (SB), and straw (SS) could be the ideal feedstock for the second-generation (2G) ethanol production. These raw materials are rich in carbohydrates and renewable and do not compete with food/feed demands. However, the efficient bioconversion of SB/SS (efficient pretreatment technology, depolymerization of cellulose, and fermentation of released sugars) remains challenging to commercialize the cellulosic ethanol. Among the technological challenges, robust pretreatment and development of efficient bioconversion process (implicating suitable ethanol producing strains converting pentose and hexose sugars) have a key role to play. This paper aims to review the compositional profile of SB and SS, pretreatment methods of cane biomass, detoxification methods for the purification of hydrolysates, enzymatic hydrolysis, and the fermentation of released sugars for ethanol production.

1. Introduction

Brazil is the biggest producer of sugarcane in the world. In the 2012/13 harvest, for example, it was estimated that more than 602 million tons of sugarcane will be processed by the Brazilian sugar-alcohol mills. The sugarcane is basically consisted of stem and straw. The sugarcane straw (or trash) is divided in three principal components, that is, fresh leaves, dry leaves, and tops. The sugarcane stem are milled to obtain the cane juice, which is subsequent used for sugar (sucrose) or alcohol (ethanol) production. The residual fraction from the sugarcane stem milling is named bagasse. Sugarcane bagasse (SB) and straw (SS) are normally burned in industries to supply all the energy required in the process. If, instead, both were used for ethanol production, much more ethanol would be produced from each hectare of sugarcane processed.

SB and SS are chemically composed of cellulose, hemicellulose and lignin. Cellulose, and hemicellulose fractions are composed of mixture of carbohydrates polymers. A number of different strategies have been envisioned to convert the polysaccharides into fermentable sugars. One of them, the hemicellulose fraction can be hydrolyzed with dilute acids followed by cellulose hydrolysis with enzymes. The cellulosic

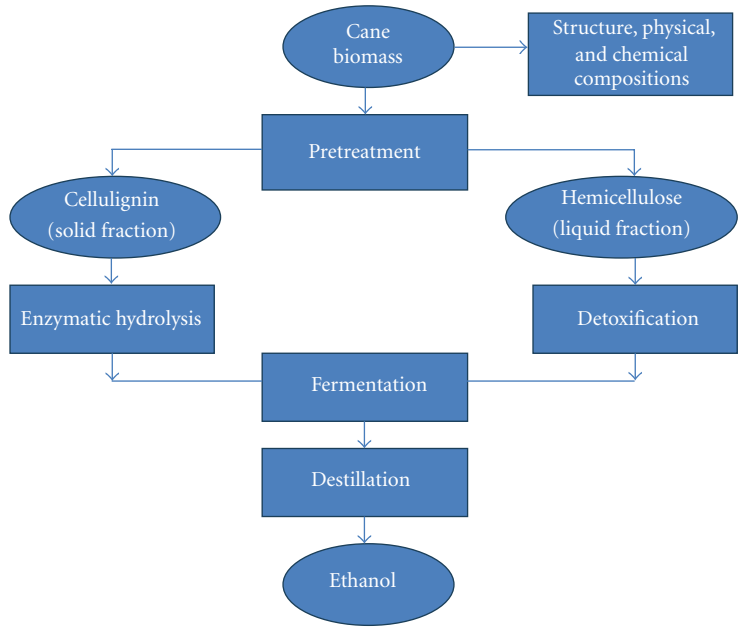


Figure 1: Procedural flow diagram for the bioconversion of cane biomass into 2G ethanol.

fraction is solid rich in glucose, and hemicellulosic fraction is liquid rich in xylose, glucose, and arabinose, where both (solid and liquid) can be fermented to produce ethanol.

In general, the biological process from converting the lignocellulose biomass to fuel ethanol involves: (1) pretreatment either to remove lignin or hemicellulose to liberate cellulose; (2) depolymerization of carbohydrate polymers to produce free sugars by cellulase mediated action; (3) fermentation of hexose and/or pentose sugars to produce ethanol; (4) distillation of ethanol. Ethanol produced from sugarcane residues is one of the most suitable alternatives for partial replacements of fossil fuels because it provides energy that is renewable and less carbon intensive than gasoline. Bioethanol reduces air pollution and also contributes to mitigate climate change by reducing greenhouse gas emissions.

This paper reviews the important information on the structure and chemical composition of sugarcane biomass (SB and SS), pretreatment of biomass, enzymatic hydrolysis of cellulose, conditioning and detoxification of hemicellulosic hydrolysate, bioconversion of sugars into ethanol, and distillation of ethanol (Figure 1).

2. Sugarcane versus Other Feedstock for the Ethanol Production

Sugarcane is the main agricultural crop cultivated in Brazil followed by soybean and corn (Table 1). Among the feasible raw materials for ethanol production, sugarcane shows the most promising results because it has a high planted area in the Brazil territory and presents the higher quantity of biomass generated which could be eventually converted into ethanol. The costs of ethanol production are directly related with the costs of feedstock that represents more than one-third of the production costs. Furthermore, the costs of feedstock may vary considerably, depending of its geographic locations, availability, and price [1, 2].

Table 1: Different feedstock cultivated in the Brazilian territory.

Biomass	Planted area (1000 hectare)	Production of biomass (1000 t)
Wheat	2,166.2	5,788.6
Rice	2,427.1	11,600.3
Sorghum	785.1	2,204.9
Cassava	1,787.5	24,524.3
Soybean	25,042.2	66,383.0
Castor bean	129.6	25.8
Corn	7,596.3	38,861.8
Sugarcane	8,527.8	602,178.8
Barley	88.4	305.1

Source: Conab [3, 4] and Embrapa [5].

According to Conab [3], the Brazilian sugar-alcohol mills will process more than 602 million tons of sugarcane in the 2012/13 harvest, leading the production of roughly 39 million tons of sugar and 24 billion liters of ethanol. Each ton of sugarcane processed by the mills generates approximately 270–280 kg of bagasse [6] and 140 kg of straw [7]; thus, it can be inferred that Brazilian mills will produce around 163–169 million tons of sugarcane bagasse and 84 million tons of straw only in the 2012/13 harvest.

Nowadays, ethanol producing units employ sugarcane efficiently (first generation). However, it is anticipated that in the coming years, SB along with SS will also be used for 2G ethanol production. The deployment of the SB and SS for ethanol production is favored in Brazil because the production process can be annexed to the sugar/ethanol units

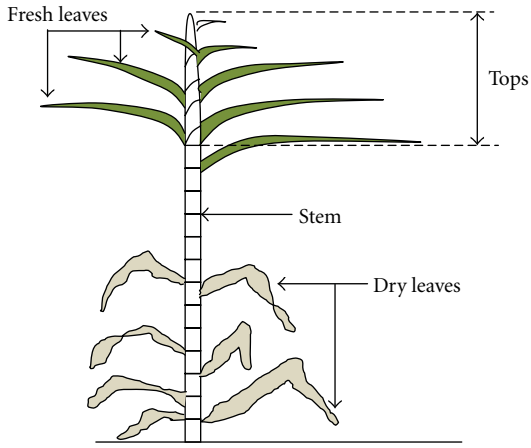


Figure 2: The sugarcane plant morphology. Adapted from [9].

already in place, requiring lower investments, infrastructure, logistics, and energy supply [1]. In such a scenario, more ethanol would be produced from the same amount of sugarcane processed, without increasing the area used to sugarcane cultivation [8]. The yield of ethanol is equivalent to 6,000 L/hectare planted. It is estimated that ethanol production could reach 10,000 L/hectare if only half of the SB generated is harnessed for the production of biofuel [1].

3. Sugarcane Structure

Sugarcane is any of 6 to 37 species (depending on taxonomic interpretation) of tall perennial grasses of the genus *Saccharum* (family Poaceae, tribe Andropogoneae). Sugarcane is native of warm temperate climate, common in the tropical regions as Brazil, India, Africa, and Asia pacific. The sugarcane plant morphology can be seen in Figure 2.

Sugarcane is composed by stem and straw (or trash). Sugarcane stem is the material removed before the milling of cane to obtain a juice which is subsequently used for sugar (sucrose) or alcohol (ethanol) production. SB is the residue from stems after extraction of juice. SS (or trash) is composed by fresh leaves, dry leaves, and tops available before harvesting. Fresh leaves are green and yellow in color, tops are the part of cane plant between the top end and the last stalk node, and dry leaves are normally in brownish color [10]. Potential uses of the leaves include: (1) as a fuel for direct combustion; (2) as a raw material for conversion by pyrolysis to char, oil, and/or gas; (3) as a raw material for conversion by gasification and synthesis to methanol. Potential uses for the tops include: (1) as a ruminant feed, either fresh or dried; (2) as a substrate for anaerobic fermentation to methane; (3) after reduction in water content, for the energy uses listed for cane trash [10, 11]. SB and SS are normally burnt in the open agricultural field after the harvesting of the crop, or in some cases, used as an untapped source of simple sugars that can be utilized for the alcohol production [12].

Characterizing the sugarcane stalk, SS and SB, following observations can be interpreted: the sucrose accumulation is greater at the base of the stem, and the amount of reducing sugars and cellulose contents is superior in the tops; the length of the stem depends on some factors like the variety of the plant and the cultural management given, so that an adult stem may have from less than two meters to over four in size, affecting the length and the number of internodes; the diameter of the stem also varies, oscillating in its middle part from 250 to 350 m; the color depends on the chlorophyll content and that of anthocyanins as well as on aspects of agronomy; there is a large variation in the moisture content of the sugarcane material, varying from 13.5% (in dry leaves) up to 82.3% (in the tops); the values of ash, fixed carbon, and volatile matter have little variation among the three components of the straw, with a lower amount of ash for the bagasse; all material present practically the same composition in carbon (~45%), hydrogen (~6%), nitrogen (0.5–1%), oxygen (~43%), and sulfur (~0.1%); mineral composition for alkalis and phosphorus shows some variation among the three components of the SS, indicating that its content grows from the dry leaves to the tops, and is quite higher than SB [10, 11].

4. Physical and Chemical Compositions of Sugarcane

Physically, sugarcane is constituted by four major fractions, whose relative magnitude depends on the sugar agroindustrial process: fiber, nonsoluble solids, soluble solids, and water (Figure 3). The fiber is composed of the whole organic solid fraction, originally found in the cane's stem, and characterized by its marked heterogeneity. The nonsoluble solids, or the fraction that cannot be dissolved in water, are constituted mainly by inorganic substances (rocks, soil, and extraneous materials), and it is greatly influenced by the conditions of the agricultural cane processing, types of cutting, and harvesting. Soluble solids fraction that can be dissolved in water are composed primarily of sucrose as well as other chemical components such as waxes, in a smaller proportion [11]. SB or SS which are the focus of 2G ethanol production are lignocellulosic materials chemically composed by cellulose, hemicelluloses, and lignin.

Cellulose is a linear polymer of glucose units linked by β $(1 \rightarrow 4)$-glycosidic bonds, forming cellobiose that is repeated several times in its chain. This cellulosic fraction can be converted into glucose by enzymatic hydrolysis, using cellulases, or by chemical way, using acids like sulfuric acid, that subsequently can be fermented to ethanol [14, 15]. Hemicellulose is a heteropolysaccharide composed by hexoses (D-glucose, D-galactose, and D-mannose), pentoses (D-xylose, L-arabinose), acetic acid, D-glucuronic acid, and 4-O-methyl-D-glucuronic acid units. The hemicelluloses are classified basically according to the sugars that are present in the main chain of polymer: xylan, glucomannan, and galactan [16]. The hemicellulose differs substantially of cellulose to be amorphous, which make it easier to be hydrolyzed than cellulose [17]. The hemicellulosic fraction can be removed from lignocellulosic materials by some type of pretreatment, like acid or hydrothermal hydrolysis, and liberating sugars, mainly xylose, that subsequently can also be fermented to ethanol [18, 19].

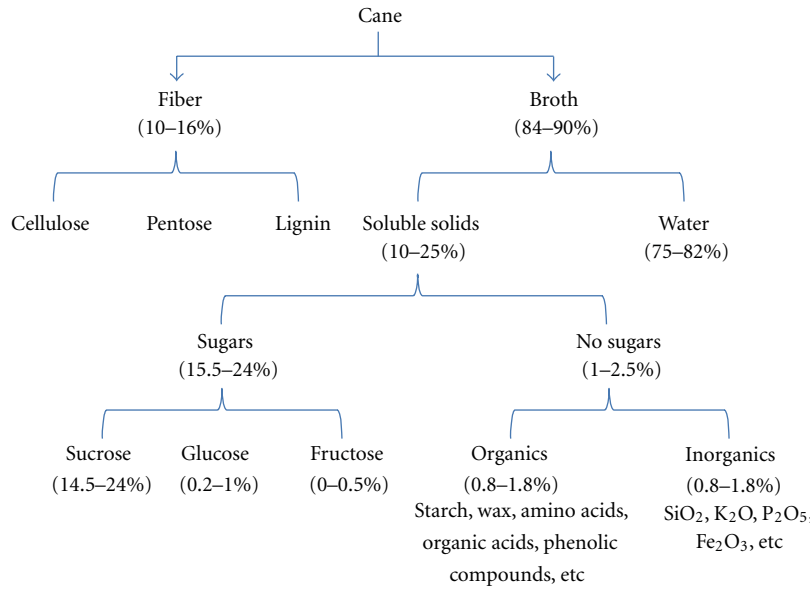


Figure 3: General composition of Sugarcane. Adapted from [13].

Table 2: Chemical composition (% w/w, dry basis) of Brazilian SB reported in the literature.

Component (%)	Reference					
	Pitarelo [27][1*]	da Silva et al. [28][2]	Canilha et al. [19][3*]	Rocha et al. [15][4*]	Brienzo et al. [29][5]	Rabelo et al. [30][6]
Cellulose	41.1	38.8	45.0	45.5	42.4	38.4
Hemicellulose	22.7	26.0	25.8	27.0	25.2	23.2
Lignin	31.4	32.4[#]	19.1	21.1	19.6	25.0
Ash	2.4	2.8	1.0	2.2	1.6	1.5
Extractives	6.8	—	9.1	4.6	—	—
Others	—	—	—	—	—	—

[*] Extractives-free basis.
[#] Lignin and others.
Extracting solvents: [1] dichloromethane, ethanol : toluene (1 : 2), ethanol, and hot water; [2] none; [3] water and ethanol; [4] ethanol; [5] ethanol; [6] none.

Lignin is a complex aromatic macromolecule formed by radical polymerization of three phenyl-propane alcohols, namely *p*-coumarilic, coniferilic, and synapilic. In the plant cell wall, lignin and hemicelluloses involve the cellulose elementary fibrils, providing protection against chemical and/or biological degradation [20]. The content of lignin and its distribution are the responsible factors for the recalcitrance of lignocellulosic materials to enzymatic hydrolysis, limiting the accessibility of enzyme, and therefore, the process of delignification can improve the conversion rates of enzymatic hydrolysis [21]. The lignin is primarily used as a fuel, but it can be chemically modified to be used as chelating agent [22], for removal of heavy metals from wastewater [23], or as precursor material for production of add-value products as activated carbon [24], surfactants [25], and adhesives [26].

SB of the Brazilian territory is quantitatively composed by 38.4–45.5% cellulose, 22.7–27.0% hemicellulose, and 19.1–32.4% lignin (Table 2). Nonstructural components of biomass, namely, ashes (1.0–2.8%) and extractives (4.6–9.1%) are the other substances that compose the chemical compositional of bagasse.

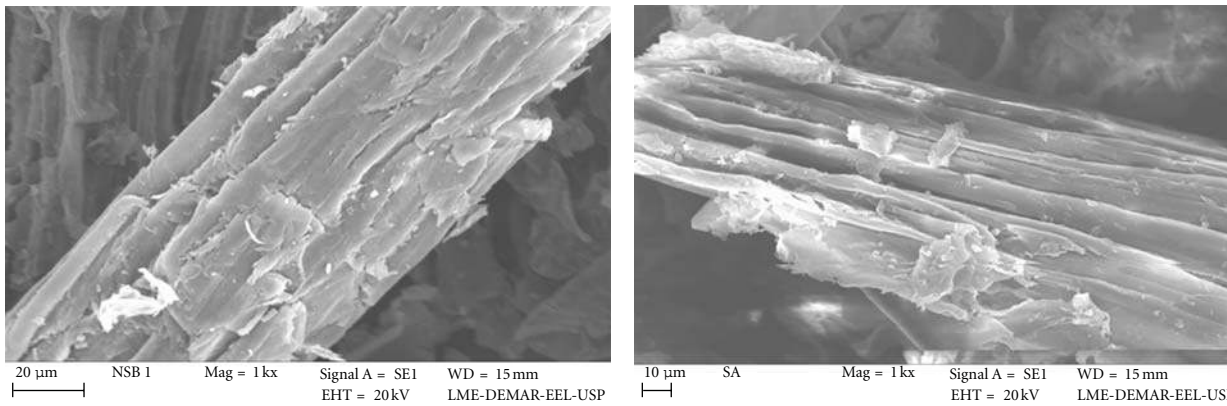
The ash content of SB is lower than the other crop residues like rice straw and wheat straw (with approximately 17.5 and 11.0% of this compound, resp.). SB is also considered a rich solar energy reservoir due to its high yields and annual regeneration capacity (about 80 t/ha) in comparison with agricultural residues like wheat, grasses, and trees (1, 2, and 20 t/ha, resp.) [31]. The bagasse can also be used as a raw material for cultivation of microorganisms and for bioconversion process for the production of industrial enzymes, xylitol, and ethanol production. Due to these advantages the bagasse is considered not only a subproduct of sugar industry, but also a coproduct of high added value [31].

The fact that chemical composition varies for the same type of material did not cause surprise because the major fractions of lignocellulosic materials depend on many factors including plant genetics, growth environment, and

Table 3: Chemical composition (% w/w, dry basis) of Brazilian SS reported in the literature.

Component (%)	Reference					
	Moriya [34][1]*	Pitarelo [27][2]*	Saad et al. [35][3]*	da Silva et al. [28][4]	Luz et al. [36][5]	Costa et al. [37][6]
Cellulose	36.1	34.4	36.1	33.6	33.3	33.5
Hemicellulose	28.3	18.4	26.9	28.9	27.4	27.1
Lignin	26.2	40.7	26.2	31.8#	26.1	25.8
Ash	2.1	11.7	2.1	5.7	2.6	2.5
Extractives	5.3	11.5	5.3	—	—	—
Others	—	—	—	—	10.6	—

* Extractives-free basis.
#Lignin and others.
Extracting solvents: [1]ethanol; [2]dichloromethane, ethanol : toluene (1 : 2), ethanol, and hot water; [3]water; [4]none; [5]none; [6]none.



(a) (b)

Figure 4: SEM of natural SB (a) and cellulignin obtained after dilute sulfuric acid pretreatment (b) [40].

processing conditions as well as methods employed for the compositional analysis [32]. It is impossible to compare the composition of samples of different origins, performed by different laboratories that do not use the same methods.

The large variation in the values of chemical components also is observed for the SS. Generally it is composed of 33.3–36.1% cellulose, 18.4–28.9% hemicellulose, and 25.8–40.7% lignin (Table 3). Ashes (2.1–11.7%) and extractives (5.3–11.5%) are also present in SS. The amount of straw from sugarcane harvesting depends on several factors such as: harvesting system, topping, height, cane variety, age of crop (stage of cut), climate, soil, and others. For example, when mechanically harvested, and depending on the harvesting technology applied, the range of straw that is collected and transported to the mill together with the stems is from 24% to 95% of the total trash available [33].

5. Ethanol Production from Sugarcane Biomass

Ethanol production from lignocellulosic biomass (second generation) includes pretreatment of biomass, enzymatic hydrolysis of cellulose, fermentation of hexose/pentose sugars, and recovery of ethanol. Intensive efforts have been made in recent years to develop efficient technologies for the pretreatment of SB, developments of enzymes for enhanced cellulose/hemicellulose saccharification, and suitable technologies for the fermentation of both C_6 and C_5 sugars [1].

5.1. Pretreatment of Sugarcane Biomass. Ideally, the pretreatment of lignocellulosic biomass should (1) increase the accessible surface area and decrystallize cellulose, (2) depolymerize partially cellulose, (3) solubilize hemicellulose and/or lignin, (4) modify the lignin structure, (5) maximize the enzymatic digestibility of the pretreated material, (6) minimize the loss of sugars, and (7) minimize capital and operating costs [38, 39].

Figure 4 presents scanning electronic microscopy (SEM) of SB before diluted sulfuric acid pretreatment and of cellulignin obtained after pretreatment. A rupture of cellulose-hemicellulose-lignin strong matrix occurred after the pretreatment. In the Figure 4(a), an ordered structure of matrix can be seen, while Figure 4(b) presents a disordered structure of cellulose-lignin complex. It is also possible to find empty spaces between the fibers, as consequence of removal of hemicelluloses and low-crystallinity cellulose flocks [15]. In general, hydrolysate originated after diluted acid pretreatment is rich in the hemicellulose fraction.

Various pretreatment technologies (alone or in combination) have been proposed in the literature. Broadly, pretreatment technologies can be categorized into 4 types: physical (mechanical); physicochemical; chemical; biological pretreatments. Mechanical pretreatment increases the surface area by reducing the size the SB or SS [41]. A high control of operation conditions is required in the physicochemical methods because these reactions occur at high temperature and pressure [21]. Chemical methods degrade hemicellulose or remove lignin and thus, loosening the structural of lignin-holocellulose network. Biological pretreatment methods are used for the delignification of lignocellulosic biomass [42]; however, the longer pretreatment times and loss of a considerable amount of carbohydrates can occur during this pretreatment [43].

Each method has its own specificity in terms of mechanistic application on cell wall components with the applied conditions [42]. Some types of pretreatments (like milling, pyrolysis, steam explosion, ammonia fiber explosion, acid or alkaline cooking, organosolv extraction, and so on) are described as follows.

5.1.1. Physical Pretreatments

(1) Milling. Milling is a mechanical pretreatment that breaks down the structure of lignocellulosic materials and decrease the cellulose crystallinity [44]. Ball milling method is most commonly employed, where the contact of the biomass with balls inside a cycle machine reduces the particles size [28]. This method can be considered environment friendly because it does not required chemicals addition [45] and thus inhibitors are not generated [28]. A disadvantage of milling is the high power required by the machines and consequently high energy costs. For sugarcane bagasse pretreatment is necessary for a lot of cycles and many passes through the miller and the cycles usually have a long time of operation [46].

(2) Pyrolysis. The pyrolysis process is carried out at high temperatures (more than 300°C). This process degrades cellulose rapidly into H_2, CO, and residual char [43]. After the separation of char, the recovered solution is primarily composed by glucose, which can be eventually fermented for ethanol production [47]. This process starts with the heating of the biomass. Primary pyrolysis reactions initiate at high temperatures to release volatiles, followed by condensation of hot volatiles and proceeded with autocatalytic secondary pyrolysis reactions [48].

The yield and quality of products after pyrolysis will depend on several parameters which can be categorized as process parameters (temperature, heating rate, residence time, reaction time, reactor type, type and amount of catalyst, type of sweeping gas, and flow rate) [48] and feedstock properties (particle size, porosity, cellulose, hemicellulose, and lignin content [49].

(3) Microwave. Microwave pretreatment is considered as an alternative process for conventional heating. If compared with conventional heating method that uses superficial transfer of heat, microwave pretreatment uses the direct interaction between a heated object and an applied electromagnetic, generating high heating efficiency and easy operation [50]. The main advantage of this process is the short reactions times and homogeneous heating of the reaction mixture [51]. Microwave assisted pretreatment of SB/SS could be a useful process to save time and energy and minimum generation of inhibitors [52].

It can be considered as one of the most promising pretreatment method to change the native structure of cellulose [53], with the occurrence of the lignin and hemicellulose degradation and thus increasing the enzymatic susceptibility [54]. Microwave can be combined with the chemicals further to improve the sugar yield from the substrate [51].

5.1.2. Physicochemical Pretreatments

(1) Steam Explosion or Hydrothermal. Steam explosion (or hydrothermal) is one of the most common pretreatment methods. This method can be described as a thermochemical process, where lignocellulosic material is exposed with steam [55]. This pretreatment requires minimum, or in some cases, no chemical addition, then it can be seen as a good technology when it is regarding environment concerns [56]. In this process, a mix of biomass and steam is maintained in high temperature in a reactor, promoting the hemicellulose hydrolysis followed by a quickly decompression ending the reaction [57].

Steam explosion treatment yields high solubility of the hemicellulose (producing mainly oligosaccharides) with low lignin solubility [14]. Usually, temperatures between 160 and 240°C and pressure between 0.7 and 4.8 MPa are employed [57]. Steam explosion process followed by enzymatic saccharification is a promising approach to enhance the amount of fermentable sugars.

(2) Ammonia Fiber Explosion (AFEX). AFEX process consists of liquid ammonia and steam explosion. It is a alkaline thermal treatment that exposes the lignocellulosic material to high temperature and pressure followed by fast pressure release. This pretreatment can significantly improve the fermentation rate of various herbaceous crops and grasses [43], and it can be used for the pretreatment of many lignocellulosic materials including alfalfa, wheat straw, wheat chaff, barley straw, corn stover, rice straw, and bagasse [44]. The main advantages of AFEX are the efficient lignin removal and less generation of inhibitors, retaining appreciable amount of carbohydrates in the substrates. Furthermore, it is a simple with short-time process [47]. During the AFEX, structure of the material is changed, resulting in a increase of water holding capacity and of digestibility of substrates (hemicellulose and cellulose) by enzymes, obtaining thus, high sugars recovery [43, 47].

The cost of AFEX process could be minimized if the ammonia is recovered from the lignin-rich solution [43, 47]. The parameters that influence the AFEX process are ammonia loading, temperature, high pressure, moisture content of biomass, and residence time [47, 58].

(3) CO_2 Explosion. The CO_2 explosion occurs similarly to ammonia explosion and is based on the hypothesis that CO_2 would form carbonic acid, increasing the hydrolysis rate of the pretreated material [44]. Carbon dioxide molecules are comparable in size to water, penetrating into the bagasse or straw surfaces, improving the hydrolysis of hemicellulose and cellulose fractions. The increase of the pressure during the explosion helps the penetration of CO_2 molecules into the crystalline structure of lignocellulosics [43].

CO_2 explosion presents conversion yields higher than the steam explosion method, more cost effective than ammonia explosion and does not cause the formation of inhibitors because mild temperature is used during the process, preventing any appreciable decomposition of monosaccharides [43, 59]. This method is nontoxic, noninflammable, and environmental friendly. However, it is a method with hard operation and process complexities [60].

(4) Hot Water. This method employs hot water under high pressure in the biomass hydrating the cellulose and removes a considerable part of hemicellulose fraction. One of the main advantage of this process is the no use of chemicals and consequently not necessary to use corrosion-resistant materials in the hydrolysis reactor. In addition, reduction the size of the raw material is also not required [61].

Usually, in this process the hot water is maintained in contact of the biomass for about 15 minutes at a temperature of 200–230°C. During this process, about 40–60% of the total biomass is dissolved, and all hemicellulose is removed. This process is generally used for pretreatment of corn fibers and herbaceous crops [62].

5.1.3. Chemical Pretreatments

(1) Acid Pretreatment. Among all types of chemical pretreatments of biomass, dilute acid hydrolysis is reported as one of the most used and oldest method. The solubilization of hemicellulose occurs at high temperatures, or at high concentrated acid, releasing pentose sugars [14, 63] and facilitating the enzymatic hydrolysis of remaining substrate (cellulignin) [61]. The most commonly used acid is H_2SO_4, where its contact with biomass promotes hemicellulose breakdown in xylose and other sugars [14]. However, other acids such as HCl [64], phosphoric acid [65], nitric acid [66], and oxalic acid [67] have also shown promising results. The conditions of the process usually can be performed at temperatures among 120–180°C and residence times ranging 15–60 min [63].

One advantage of the acid pretreatment process is the operation at low and medium temperatures and consequently decreasing of energy costs [68]. However, in high concentration of acid problems can occur with equipment corrosion and expensive costs of maintenance [63], and also after this pretreatment, it is necessary to neutralize the hydrolysate before fermentations [14]. Another disadvantage of this process is the possibility of formation of other by-products that are considered inhibitory to microbial fermentation, like furans, furfural, carboxylic acids, formic levulinic and acetic acids, and phenolic compounds. Therefore, a detoxification step is required to remove these inhibitory compounds to increase the fermentability of hydrolysates [69].

To calculate the efficiency of acid hydrolysis, factors like temperature, reaction time and acid concentration must be considered [70]. Overend and Chornet [71] developed an equation that involves the temperature and reaction time, indicating the severity of the pretreatment by combined severity factor (CSF):

$$\mathrm{CSF} = t_{\exp}\left[\frac{(T - T_{\mathrm{ref}})}{14.75}\right], \tag{1}$$

where t is the residence time (min); T is the temperature (°C), and T_{ref} is the reference temperature, usually set to 100°C.

(2) Alkaline Pretreatment. Alkaline pretreatment is a delignification process, in which a significant amount of hemicellulose is also solubilized. It employs various bases, including sodium hydroxide, calcium hydroxide (lime), potassium hydroxide, ammonia hydroxide, and sodium hydroxide in combination with hydrogen peroxide or others [72]. The action mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components [44]. This process utilizes lower temperatures and pressures than other pretreatment technologies; however, pretreatment times are on the order of hours or days [72]. Compared with acid-based pretreatment processes, alkaline processes causes less sugar degradation, and many of the caustic salts can be recovered and/or regenerated [43].

Alkaline pretreatment of lignocellulosic materials causes swelling, leading to an increase in internal surface area, decrease in the degree of polymerization and crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure [72] making cellulose and hemicellulose available for the enzymatic degradation [47].

The effectiveness of alkaline pretreatment varies, depending on the substrate and treatment conditions. In general, alkaline pretreatment is more effective on hardwood, herbaceous crops, and agricultural residues with low lignin content than softwood with high lignin content [72]. The end residue (mainly cellulose) can be used to produce either paper or cellulose derivatives [47] or the sugars generation upon enzymatic hydrolysis which can eventually be used for ethanol production. Reactor costs are lower than those for acid technologies. However, the use of these more expensive salts in high concentrations is a significant disadvantage that raises environmental concerns and may lead to prohibitive recycling, wastewater treatment, and residual handling costs [59, 72].

(3) Oxidative Delignification. Oxidative delignification process causes the delignification and the chemical swelling of the cellulose improving enzymatic saccharification significantly [73]. In this process, the lignin degradation is catalyzed by the peroxidase enzyme with the presence of

H_2O_2 [44]. The oxidative delignification has been successfully operated in a continuous flow operation at high biomass loading (approximately 40% solids) and low H_2O_2 loading; though it is still a relatively less explored method compared to other thermochemical pretreatments [74]. This pretreatment method has been applied to a large variety of biomass such as corn stover, barley straw, wheat straw, bamboo, rice straw, and sugarcane bagasse [74].

(4) Ozonolysis. Ozone can be used to degrade the lignin and hemicellulose fractions from lignocellulosic materials such as wheat straw, bagasse, peanut, pine, cotton straw, and poplar sawdust [43]. Ozone is a powerful oxidant, soluble in water and is readily available. It is highly reactive towards the compounds incorporating conjugated double bonds and functional groups with high electron densities. Therefore, the most likely biomass constituent to be oxidized is lignin due to its high content of C=C bounds [75].

Ozonolysis pretreatment has the advantages of effectively removal of lignin, it does not produce toxic residues for the downstream processes, and the reactions are carried out at ambient temperature and pressure [76]. However, a large amount of ozone is required, making the process expensive [44]. Furthermore, the fact that ozone can be easily decomposed by using a catalytic bed or increasing the temperature means that processes can be designed to minimize environmental pollution [43].

(5) Organosolv. Organosolv process seems one of the most promising methods for the pretreatment of lignocellulosics materials [77]. During this process, strong inorganic acid acts as a catalyst, promoting the breakdown of lignin-lignin and carbohydrates-lignin bonds from the biomass [38]. When the lignin is removed, the superficial area and volume of the material are also increased considerably, facilitating the enzyme accessibility and consequently improving the efficiency of the process to achieve fermentable sugars [78].

The organosolv process uses fewer amounts of chemicals to neutralize the hydrolyzate and generates few amounts of wastes compared with other similar process [61]. Chemicals such as NaOH or Na_2SO_3 could be used as catalyst [79]. High efficiency for lignin removal coupled with the high pressure of carbon dioxide has been observed using this process [80].

(6) Wet Oxidation. The wet oxidation process occurs in the presence of oxygen or catalyzed air, where the most used catalyst is the sodium carbonate [81]. Wet oxidation allows obtaining high yields of biomass conversion into monosaccharides with low formation of furan and phenolic aldehydes. In the wet oxidation process, the delignification is reported with the increasing of aliphatic acids. This pretreatment is considered expensive [81]. The major advantage of this pretreatment is the combination with alkalis where it is possible to achieve released sugars without generation of furfural and 5-hydroxymethylfurfural, undesirable compounds for fermentations [82].

5.1.4. Biological Pretreatment. Biological pretreatment is the alternative to chemical pretreatment to alter the structure of lignocellulosic materials. Generally, wood degrading microorganisms like bacteria and brown rot, white rot, and soft rot fungi are employed in the biological pretreatment [72]. This method provides degradation of lignin and hemicellulose making the biomass more amenable to enzyme digestion [47].

The most effective microorganism for biological pretreatment of lignocellulosic materials is white rot fungi [47]. These microorganisms degrade lignin through the action of lignin-degrading enzymes such as peroxidases and laccase [43]. Brown rot fungi mainly attack cellulose, while white and soft rot fungi attack both cellulose and lignin [44].

This pretreatment is environmental friendly because of its low energy use and mild environmental conditions [59]. The main disadvantages, that is, low efficiency, considerable loss of carbohydrates, long residence time, requirement of careful control of growth conditions, and space restrain its applications. In addition, most ligninolytic microorganisms solubilize/consume not only lignin but also a considerable fraction of hemicellulose and cellulose [72]. To overcome these limitations, biological treatments can be used in combination with other treatments [59]. Wang et al. [83] combined fungal treatment with liquid hot water (LHW) to enhance the enzymatic hydrolysis of *Populus tomentosa.*

5.2. Enzymatic Hydrolysis of Cellulosic Fraction. The general concept of conversion of cellulosic fraction into fermentable sugars involves the pretreatment of the raw material followed by its enzymatic hydrolysis. Enzymatic hydrolysis is an ideal approach for degrading cellulose into reducing sugars because mild reaction conditions (pH between 4.8–5.0 and temperature between 45–50°C) can be used; it does not present corrosion problems in the reactors and result in negligible by-products formation with high sugar yields. However, enzymatic hydrolysis depends on optimized conditions for maximal efficiency (hydrolysis temperature, time, pH, enzyme loading, and substrate concentration) and suffers from end-product inhibition and biomass structural restraints [84, 85]. To overcome the end-product inhibition and reducing the time, hydrolysis and fermentation can be combined, so-called simultaneous saccharification and fermentation (SSF) or simultaneous saccharification and cofermentation (SSCF).

The enzymatic hydrolysis of cellulosic fraction requires three classes of cellulolytic enzymes (cellulases): (1) endo-β-1,4-glucanases (EG, E.C. 3.2.1.4) which attacks regions of low crystallinity in the cellulose fiber, creating free chain ends; (2) cellobiohydrolases or exoglucanase (CBH, E.C. 3.2.1.91) which degrades the molecule further by removing cellobiose units from the free chain-ends; (3) β-glucosidases (E.C. 3.2.1.21) which hydrolyses cellobiose to produce glucose [44]. To breakdown the hemicellulose, several enzymes such as xylanase, b-xylosidase, glucuronidase, acetylesterase, galactomannanase, and glucomannanase are required [84]. Cellulase enzymes when acting together with xylanases on

delignified SB/SS exhibit a better yield due to the synergistic action of enzymes [86].

Both bacteria (*Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora, and Streptomyces*) and fungi (*Sclerotium rolfsii, Phanenerochate chrysosporium and species of Trichoderma, Aspergillus, Schizophyllum and Penicillium*) can produce cellulases for the hydrolysis of cellulosic materials [44, 84]. Amongst the cellulase producing microorganisms, *Aspergillus* and *Trichoderma* genera are the widely studied.

For ethanol production from cellulosic materials, removal of lignin improved significantly the substrate digestibility. Furthermore, due to the lower lignin content, enzyme loadings can be considerably reduced. However, extensive lignin removals by pretreatments add cost to the processes [85].

Although enzyme price has decreased due to intensive research to improve their production, enzymes loading during cellulose hydrolysis should be minimized because it also increases the cellulosic ethanol production costs. Thus, finding paths to reduce cellulase loadings would be particularly effective in lowering the process costs. The enzyme source has also a major effect on the hydrolysis efficiency. Therefore, understanding the interaction between cellulases and pretreated biomass is vital to effectively develop low-cost pretreatment and enzyme properties that can lead to competitive ethanol costs [85].

5.3. Detoxification (Treatment) of Hemicellulosic Hydrolysates. The main preoccupations in the pretreatment of lignocellulosic materials are to minimize the sugars degradation and subsequently minimize the formation of inhibitory compounds for microbial metabolism, limit the consumption of chemicals, energy and water, and the production of wastes [39]. The inhibitory compounds could be divided into four groups: (1) substances that are released by the hemicellulosic structure, such as acetic acid, which originates in the deacetylation of xylan; (2) phenolic compounds and other aromatic compounds derived from the partial degradation of lignin; (3) the furan derivatives, furfural and 5-hydroxymethylfurfural, resulting from the degradation of pentoses and hexoses, respectively; (4) metals like chromium, copper, iron, and nickel leached from the equipment [69]. These compounds individually as well as synergistically affect the physiology of fermenting microorganisms, therefore, it is essential to eliminate these inhibitory compounds or reduce their concentration to obtain the satisfactory product yields during microbial fermentation of lignocellulose hydrolysates [67].

A number of methods like evaporation; neutralization; use of membranes, ion exchange resins, and activated charcoal; enzymatic detoxification using laccases and peroxidases have been attempted to detoxify the hydrolysates aiming ethanol production. Considering that different lignocellulosic hydrolysates have different degrees of inhibition and that microorganisms have different inhibitor tolerances, the methods of detoxification change will depend on the source of the lignocellulosic hydrolysate and the microorganism being used [87]. Several detoxification methods are described in this overview and can be divided into physical, physicochemical, chemical, and biological treatments.

5.3.1. Physical Treatments

(1) Evaporation (Concentration). The concentration of hydrolysates by vacuum evaporation process is a physical detoxification method which reduces the volatile compounds concentration, including acetic acid, furfural and vanillin [87]. However, this treatment has the disadvantage of increasing the nonvolatile toxic compounds, as extractives [88].

(2) Use of Membranes. The use of membranes has several advantages over conventional extraction. Membrane adsorption prevents that the aqueous phase (hydrolysate) is mixed with organic phase (solvent) which is likely to be toxic to microorganisms [89]. The membranes have surface functional groups attached to their internal pores, which may eliminate metabolic inhibitors as acetic acid, 5-hydroxymethylfurfural, furfural, formic, levulinic and sulphuric acid [67].

5.3.2. Physicochemical Treatments

(1) Ion Exchange Resins. The ion exchange resins process has been reported as the most efficient detoxification method. It is known that this process remove lignin-derived inhibitors, acetic acid, and furfurals of hydrolysate, improving significantly the yield fermentation [67]. The main advantage of the use of ion exchange resins is that they can be regenerated and reused without affecting the efficiency of the treatment [65, 90]. However, this method presents some disadvantages: the high pressure drop across the bed that tends to increase during operation due to media deformation; long processing time because of the slow pore diffusion; possible degradation of fragile biological product molecules, and it is difficult to scale-up [91]. The ion exchange resins process also leads to a significant loss of fermentable sugars after the process [67].

(2) Neutralization. Considering the low pH of the hydrolysates provided by acid hydrolysis, it is needed that the neutralization of the pH to be close to the fermentations conditions. In this step, phenolics and furfurals are removed due to precipitations [67]. The chemicals employed in the neutralization of hydrolysates are calcium hydroxide and sodium hydroxide. The addition of $Ca(OH)_2$ generates precipitate of $CaSO_4$; therefore, it is desirable to be removed by centrifugation, adding one more stage in the process. During the generation of precipitates it can offer problems in fermentation [92].

(3) Overliming. Among different types of detoxification, overliming is reported as the most used method [93]. This process consists in an increase of the pH of acid hydrolysate followed by reduction until a pH desirable to fermentations. The principle of this process is the precipitation of toxic components and the instability of some inhibitors at high pH [69]. This method showed high efficiency towards the removal of inhibitors and is being widely used [90, 94, 95].

This method has been considered promising and economic, revealing good efficiency for the removal of the furans compounds [67].

(4) Activated Charcoal. Activated charcoal adsorption is a widely used detoxification method which is considered a low cost and efficient for inhibitors compounds removal. This method removes mainly phenolics compounds and does not provide large changes in the fermentable sugars levels [90]. The ratio of charcoal and hydrolysates, pH, time of contact, and temperature are the important factors for the improvement of this method [88, 96].

(5) Extraction with Organic Solvents. Due to large availability of inhibitors such as acetic acid, furfural, vanillin, 4-hydroxybenzoic acid, and low molecular weight phenolics, the solvent extraction has been considered an efficient method of detoxification. The most common solvents used in this process are ethyl acetate, chloroform, and trichloroethylene [97].

5.3.3. Biological Treatment. Biological method uses specific enzymes or microorganisms that act on the inhibitors compounds present in the hydrolysate and change them [87]. In contrast with physical and chemical detoxifications, biological detoxification represents an improvement because little waste is generated and could be performed directly in the fermentation vessel before fermentation [98]. This method is still more feasible, environmental friendly, with fewer side reactions and less energy requirements [99]; however, it presents a long process time [100].

The use of enzymes is a very studied and promising method. Laccase and peroxidase enzymes derived from white rot fungi have been found effective for the removal of phenolics compounds from lignocellulosic hydrolysates [99]. The detoxification mechanism of these enzymes probably involves oxidative polymerization of phenolic compounds of low molecular weight [88]; they catalyze the oxidation of substituted phenols, anilines, and aromatic thiols, at the expense of molecular oxygen [101]. The disadvantages of enzyme detoxification are long incubation time and high costs of enzymes; however it has the advantage that is usually conducted at mild conditions (pH 5.0, mesophilic temperature) [102].

The use of microorganism has also been applied to remove inhibitors compounds from lignocellulosic hydrolysates [87]. There are several microorganisms which can naturally assimilate inhibitory compounds, including yeasts, fungi, and bacteria [102]. Some microorganisms during incubation are able to release cellulase and hemicellulase and degrade only lignin, resulting in a lignocellulosic substrate which can be easily hydrolyzed into fermentable sugars with mild conditions and short time [99].

These microorganisms can effectively degrade lignin while retaining cellulose and hemicellulose in the substrate. This method can also be referred *as in situ* microbial delignification (ISMD). Recently, several microorganisms have shown their preference towards inhibitors by transforming their chemical nature and can be employed for detoxification of lignocellulose hydrolysates [99, 103]. The adaptation of a microorganism to a nondetoxified hydrolysate is another interesting alternative to replace the detoxification step. This method is based on successive fermentations using the microorganism of each experiment as the inoculum of the next one [88]. The use of adapted microorganisms not only reduces the detoxification cost but also avoids loss of fermentable sugars [102].

5.4. Fermentation of Sugars from Sugarcane Biomass into Ethanol

5.4.1. Bioconversion of Hexose Sugars into Ethanol. Ethanol fermentation is a biological process in which sugars are converted by microorganisms to produce ethanol and CO_2. Even though there are the existence of many methods and process to use lignocellulosic materials for ethanol production, however, it is still difficult to obtain economic ethanol from lignocellulosics [86].

The microorganism most commonly used in fermentation process is the yeasts and, among the yeasts, *Saccharomyces cerevisiae* is the preferred choice for ethanol fermentation [104]. This yeast can grow both on simple sugars, such as glucose, and on the disaccharide sucrose. Furthermore, the availability of a robust genetic transformation system of *S. cerevisiae* along with a long history of this microorganism in industrial fermentation processes makes it most desired microorganisms for ethanol production. *S. cerevisiae* has high resistance to ethanol, consumes significant amounts of substrate in adverse conditions, and shows high resistance to inhibitors present in the medium [105]. Unfortunately, xylose metabolism presents a unique challenge for *S. cerevisiae* to assimilate pentose sugars due to the absence of genes required for assimilation of these molecules [105].

There are three kinds of processes to produce ethanol from sugarcane bagasse (SB) and sugarcane straw (SS). The first process is called separate (or sequential) hydrolysis and fermentation (SHF) where hydrolysis of lignocellulosic material and ethanol fermentation is done separately. SB/SS is pretreated, and the pretreated material is enzymatically hydrolyzed separately to recover the sugars. The recovered sugar solution (hexose sugars) is then fermented with appropriate microorganism into ethanol. SHF is a little staggered process [21]. The other two kinds of processes are called simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and cofermentation (SSCF), where both enzymatic hydrolysis and fermentation of released sugars into ethanol occur simultaneously making the overall process short [21, 86]. In the SSF process, the glucose (from cellulose hydrolyzed) is fermented separately of pentoses (from hydrolysate) in a separate reactor, while that in the SSCF process, the fermentation of xylose and glucose occurs together in the same reactor [21, 86].

5.4.2. Bioconversion of Pentose Sugars into Ethanol. The maximum utilization of all sugar fractions is essential to obtain an economic and viable conversion technology for bioethanol production from sugarcane bagasse (SB) and sugarcane

straw (SS). To obtain the desired ethanol yields from SB/SS hydrolysates, it is essential that the hemicellulose fraction should be fermented with same conversion rates as the cellulose fraction [104].

Hemicellulose hydrolysate typically contains primarily pentose sugars (xylose and arabinose) and some amounts of hexose sugars (mannose, glucose and galactose) [106, 107]. A variety of yeast, fungi, and bacteria are capable of assimilating pentose, but only a few are promising candidates for the efficient xylose fermentation into ethanol [106]. In yeasts, the assimilation of D-xylose follows the pathway where the sugar passes through a pool enzymatic to enter in the phosphopentose pathway [108]. There are several microorganisms capable of assimilating pentose sugars, but only few species are capable of assimilating sugars to produce ethanol at industrial scale. Microorganisms, such as *Scheffersomyces stipitis* (*Pichia stipitis*) [109], *Candida guilliermondii*, *Candida shehatae*, and *Pachysolen tannophilus* are able to assimilate pentose sugars by a reduction/oxidation pathway bioconversion of sugarcane bagasse/sugarcane straw hydrolysates under different cultivation conditions [110].

The process to assimilate pentose sugars consists in the xylose being converted to xylitol, by the action of D-xylose reductase (E.C. 1.1.1.21) and immediately oxidized by the action of xylitol dehydrogenase (E.C. 1.1.1.9), producing D-xylose-5-phosphate. Ribolosephosphate-3-epimerase (5.1.3.1), transaldolase (E.C. 2.2.1.2), and transketolase (E.C. 2.2.1.1) sequentially convert D-xylose-5-phosphate into glyceraldehyde-3-phosphate and fructose-6-phoshate by non-oxidative rearrangement resulting into the formation of ethanol by the Emden-Meyorhoff Pathway. NADPH must be regenerated through metabolic routes. The metabolic pathway of the arabinose is similar to the route shown by xylose, where aldose reductase mechanistically converts arabinose into L-arabitol. Through the action of L-arabitol dehydrogenase, L-arabitol is reduced to ethanol [99, 105]. Recently published reviews have competently presented the important progress made for pentose sugars fermentation into ethanol [99, 110].

5.5. Distillation of Ethanol. Despite the downstream process being the highest energy consuming process during the ethanol production [111], the ethanol recovery from the fermented broth is necessary. The final medium is composed by water and ethanol (5–12 wt%) [112]. The ethanol-water cannot be separated by conventional distillation processes because they form a nonideal mixture system [113]. The dehydration is sophisticated method because they form an azeotropic mixture with water (at 95.6 wt% at a temperature of 78.15°C), which makes it impossible to separate in a single distillation column [112]. This way, the ethanol purification occurs in three steps: distillation, rectification, and dehydration. A high concentrated ethanol solution is obtained in the two first steps (about 92.4 wt%), then the mixture is dehydrated in order to obtain ethanol anhydrous by a dehydration method. The dehydration can be realized by azeotropic distillation, extractive distillation, liquid-liquid extraction, adsorption, or some complex hybrid separation methods [112].

6. Conclusion

Sugarcane bagasse (SB) and sugarcane straw (SS) are the attractive second-generation renewable feedstock available in several countries like Brazil. This feedstock if used judiciously may provide the sustainable supply of drop-in ethanol, industrial enzymes, organics acids, single cell proteins, and so forth. However, a significant fraction of this biomass goes to industries for steam and electricity generation. The remaining fraction represents the ideal feedstock for the generation high-value commodities. Last three decades of vigorous developments in pretreatment technologies, microbial biotechnology, and downstream processing have made it reality to harness the sugarcane residues for the production of many products of commercial significance at large scale without jeopardizing the food/feed requirements. Biomass recalcitrance is a main challenge toward the successful exploitation of these residues. To overcome the biomass recalcitrance, pretreatment is an inevitable process to ameliorate the accessibility of carbohydrate for the subsequent enzymatic hydrolysis reaction to generate fermentable sugars. There are several robust pretreatment methods available; however, the ultimate choice for the selection of pretreatment process depends upon the effective delignification or hemicellulose removal, minimum generation of inhibitors, low sugar loss, time savings, being economic and causing less environmental pollution. The released sugars after enzymatic hydrolysis and hemicellulose depolymerization are converted into ethanol by the suitable ethnologic strain. In order to get desired ethanol yields, the ethnologic strains should have ability to utilize pentose and hexose sugars, inhibitor resistance, and high osmotolerance. The following ten requirements are pivotal in order to establish a long-term sustainable second-generation ethanol production process from sugarcane residues.

(1) Fullest utilization of SB and SS generated in the country for the better management.

(2) Selection of right pretreatment and detoxification strategy.

(3) In-house cellulase production and development of cellulolytic strains and ethanol producing strains from pentose and hexose sugars showing inhibitor resistance, ethanol tolerance, and faster sugar conversion rates.

(4) Process intensification: hydrolysis and fermentation together in one place.

(5) Cheap, fast, and effective ethanol distillation.

(6) Integration of bioethanol producing units with sugar/distilleries for the coutilization of machinery, reactors, and other equipment.

(7) Maximum by products utilization (lignin, furans, and yeast cell mass).

(8) Environmental protection.

(9) Government subsidies to promote the renewable energy.

(10) Encouragement of private investments.

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Research Article

Production of Ethanol from Sugars and Lignocellulosic Biomass by *Thermoanaerobacter* J1 Isolated from a Hot Spring in Iceland

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Thermophilic bacteria have gained increased attention as candidates for bioethanol production from lignocellulosic biomass. This study investigated ethanol production by *Thermoanaerobacter* strain J1 from hydrolysates made from lignocellulosic biomass in batch cultures. The effect of increased initial glucose concentration and the partial pressure of hydrogen on end product formation were examined. The strain showed a broad substrate spectrum, and high ethanol yields were observed on glucose (1.70 mol/mol) and xylose (1.25 mol/mol). Ethanol yields were, however, dramatically lowered by adding thiosulfate or by cocultivating strain J1 with a hydrogenotrophic methanogen with acetate becoming the major end product. Ethanol production from 4.5 g/L of lignocellulosic biomass hydrolysates (grass, hemp stem, wheat straw, newspaper, and cellulose) pretreated with acid or alkali and the enzymes Celluclast and Novozymes 188 was investigated. The highest ethanol yields were obtained on cellulose (7.5 $mM \cdot g^{-1}$) but the lowest on straw (0.8 $mM \cdot g^{-1}$). Chemical pretreatment increased ethanol yields substantially from lignocellulosic biomass but not from cellulose. The largest increase was on straw hydrolysates where ethanol production increased from 0.8 $mM \cdot g^{-1}$ to 3.3 $mM \cdot g^{-1}$ using alkali-pretreated biomass. The highest ethanol yields on lignocellulosic hydrolysates were observed with hemp hydrolysates pretreated with acid, 4.2 $mM \cdot g^{-1}$.

1. Background

More than 95% of the ethanol produced today is from simple biomass like mono- and disaccharides and starch [1]. The use of this type of biomass has been increasingly debated due to its impact on food and feed prices as well as for environmental reasons [2]. Therefore, complex (lignocellulosic) biomass has been put forward as a feasible alternative due to its abundance in nature and the large quantities generated as waste from agricultural activities [2, 3]. Lignocellulosic biomass is primarily composed of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are the main substrates used for ethanol production, but lignin is composed of aromatic lignols that need to be separated and removed before enzymatic hydrolysis. Today, expensive pretreatments are the main reason for unsuccessful implementation of complex lignocellulosic biomasses as a starting material for ethanol production [2].

The best-known microorganisms used for ethanol production today are the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis*. Both organisms have very high yields of ethanol (>1.9 mol ethanol/mol hexose) but very narrow substrate spectra and thus are not suitable for ethanol production from complex substrates. Therefore, the use of thermophilic bacteria with broad substrate range and high yields may be a better option for ethanol production from complex biomasses. It has been known for some time now that many thermophilic bacteria are highly efficient ethanol producers [4]. After the oil crisis in the 1980s, there was a peak in investigations on thermophilic ethanol-producing bacteria; bacteria within the genera of *Thermoanaerobacterium*, *Thermoanaerobacter,* and *Clostridium* have demonstrated good ethanol yields and fast growth rates [5–8]. There are several advantages in using these thermophilic bacteria: the increased temperature deters contamination from mesophilic bacteria and fungi,

possible self-distillation of ethanol avoiding the generally low ethanol tolerance problem with those bacteria, and broad substrate spectrum [9, 10]. Some of these strains produce more than 1.5 mol ethanol/mol hexose [11–16], whereas the theoretical maximum yield is 2.0 mol ethanol/mol hexose degraded. The main reasons for low yields are the formation of other end products such as acetate, butyrate, and CO_2 [11–16].

The present study focuses on a recently isolated thermophilic bacterium, strain J1, which is most closely related to species within the genus *Thermoanaerobacter*. Bacteria within this genus seem to be among the most efficient ethanol producers known and show very high yields from simple sugar fermentations [12–14, 16] as well as from complex lignocellulosic biomass [10, 13, 17–19]. These bacteria are Gram-variable rods with broad substrate spectrum (mostly sugars) and produces ethanol, acetate, lactate, hydrogen, and carbon dioxide during anaerobic fermentation [20, 21]. The physiological characteristics of *Thermoanaerobacter* strain J1, isolated from Icelandic hot spring, were investigated in detail with the main aim of exploring the ethanol production capacity both from simple sugars as well as from various lignocellulosic biomass.

2. Methods

2.1. Medium. The composition and preparation of the medium used has been described earlier [12]. This medium, referred to as basal medium (BM) hereafter, contains yeast extract (2 g/L) in addition to glucose or other carbon sources. All experiments were performed at 65°C at pH 7.0 without agitation with the exception of the temperature and pH optimum experiments. The inoculum volume was 2% (v/v) in all experiments which were always performed in duplicates.

2.2. Isolation of Strain J1. The strain was isolated in BM with glucose (20 mM) from a hot spring (69°C, pH 7.5) in Grensdalur in Southwest of Iceland. Samples were enriched on glucose, and positive samples (increase in growth and production of hydrogen) were reinoculated five times. From the final enrichment series, end point dilutions were performed by using BM containing agar ($30\,g\cdot L^{-1}$). Colonies were picked from final positive dilution and reinoculated to liquid BM with glucose. Isolation of the hydrogenotrophic methane producing strain has been described elsewhere [22].

2.3. Optimum pH and Temperature Growth Experiments. To determine the strain's growth characteristics at various pHs and temperatures, the strain was cultivated on glucose (20 mM), and cell concentration was measured by increase in absorbance at 600 nm by a Perkin-Elmer Lambda 25 UV-Vis spectrophotometer. Maximum (specific) growth rate (μ_{max}) for each experiment was derived from absorbance data. For pH optimum experiments, the initial pH was set to various levels in the range from 3.0 to 9.0 with increments of 1.0 pH unit. The experimental bottles were supplemented with acid (HCl) and alkali (NaOH) to set the pH accordingly. To determine the optimum temperature for growth, the incubation temperature varied from 35°C to 80°C. For the pH optimum experiments, the strain was cultivated at 65°C, and for the temperature optimum experiments, the pH was 7.0. Optimal pH and temperature were used in all experiments performed. Experiments were done in 117.5 mL serum bottles with 50 mL liquid medium.

2.4. Phylogenetic Analysis. Full 16S rRNA analysis of 1479-nucleotide long sequence was done according to Orlygsson and Baldursson [23] and references therein. Sequences from 16S rRNA analysis were compared to sequences in the NCBI database using the nucleotide-nucleotide BLAST (BLAST-N) tool. The most similar sequences were aligned with the sequencing results in the programs BioEdit [24] and CLUSTAL_X [25]. Finally, the trees were displayed with the program TreeView. *Caloramator viterbiensis* was used as an outgroup.

2.5. Effect of Initial Glucose Concentration on End Product Formation. The effect of initial glucose concentration on strain J1, by varying the concentration from 5 to 200 mM, was tested. Control samples contained only yeast extract. Glucose, hydrogen, acetate, and ethanol concentrations were measured at the beginning and at the end of incubation time (7 days). Experiments were done in 117.5 mL serum bottles with 60 mL liquid medium, and the pH was measured at the end of incubation time.

2.6. Substrate Utilization Spectrum. The ability of strain J1 to utilize different substrates was tested using the BM medium supplemented with various carbon substrates (xylose, arabinose, glucose, mannose, galactose, fructose, rhamnose, maltose, cellobiose, sucrose, lactose, trehalose, raffinose, starch, cellulose, CMC, avicel, xylan (from oat spelt), glycerol, pyruvate, serine, and threonine). All substrates were added from filter-sterilized (0.45 μm) substrates except for xylan, starch, CMC, cellulose, and avicel which were autoclaved with the medium. In all cases, the concentration of substrates was 20 mM except for xylan, starch, CMC, cellulose, and avicel when $2\,g\cdot L^{-1}$ was used. Hydrogen, acetate, and ethanol concentrations were analysed after one week of incubation. Experiments were performed in 24.5 mL serum bottles with 10 mL liquid medium.

2.7. Pretreatment of Biomass and Hydrolysates Preparation. Hydrolysates (HLs) were made from different biomasses: Whatman no. 1 filter paper, newspaper, hemp stem (*Cannabis sativa*), barley straw (*Hordeum vulgare*), and grass (*Phleum pratense*). Hydrolysates were prepared according to Sveinsdottir et al. [12], and the final concentration of each biomass type was $22.5\,g\cdot L^{-1}$. Biomass was pretreated chemically by using 0.50% (v/v) of acid (H_2SO_4) or alkali (NaOH) (control was without chemical pretreatment) before heating (121°C, 60 min). Two commercial enzyme solutions, Celluclast (Novozyme, $750\,U\cdot g^{-1}$) and Novozyme 188 (Sigma C6105, $200\,U\cdot g^{-1}$), were added to each bottle after chemical pretreatment; the bottles were cooled down to

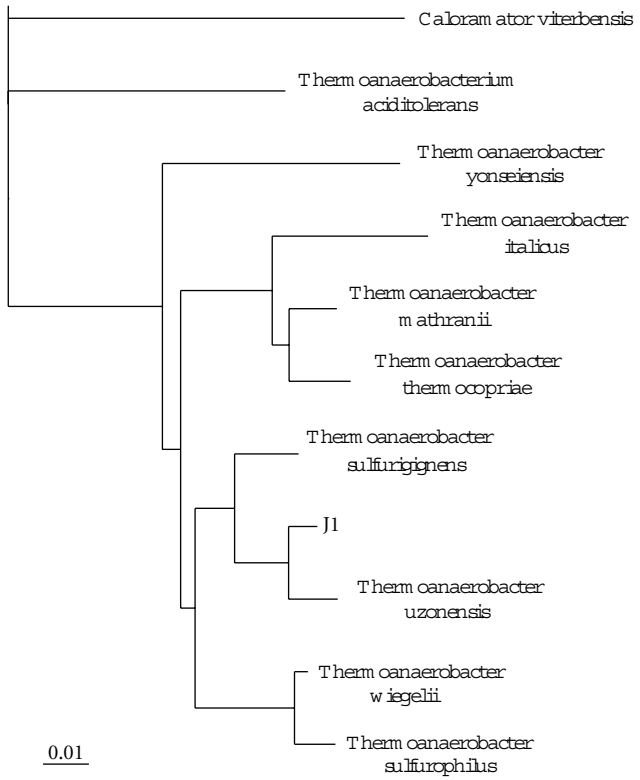

Figure 1: Phylogeny of strain J1 based on partial 16S rRNA sequence analysis. The phylogenetic tree was generated by using distance matrix and neighbor-joining algorithms. *Caloramator viterbensis* was selected as outgroup. The bar indicates 0.01 substitutions per nucleotide position.

room temperature and the pH adjusted to 5.0 before enzymes were added. The hydrolysates were incubated in water bath at 45°C for 68 h. After the enzyme treatment, the pH was adjusted with NaOH or HCl to pH 7.0 which is the pH optimum of the strain. The hydrolysates were then filtered (Whatman-WeiBrand; 0.45 μm) into sterile bottles.

2.8. Fermentation during External Electron-Scavenging Systems. In one set of experiments, strain J1 was incubated on glucose (20 mM) in the presence of sodium thiosulfate (40 mM) and in coculture with a hydrogenotrophic methanogen. The methanogen was precultivated in BM medium with a gas phase consisting of 80% of H_2 and 20% of CO_2 for one week. Then the experimental culture bottles were flushed with nitrogen prior to the addition of glucose (20 mM) and strain J1. The coculture was incubated at 65°C for one week.

2.9. Fermentation of Hydrolysates. Fermentation of carbohydrates present in the hydrolysates after chemical and enzymatic pretreatment was performed in 24.5 mL serum bottles. The BM medium and inoculum (8.0 mL) were supplemented with different hydrolysates (2.0 mL, total liquid volume of 10 mL) giving a final hydrolysate concentration of 4.5 g·L^{-1}. Control samples did not contain hydrolysate; the only carbon source was yeast extract.

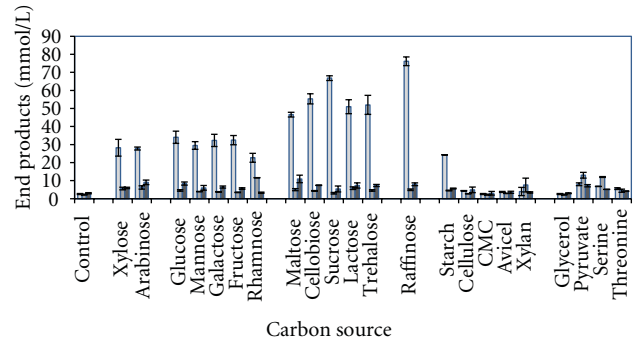

Figure 2: End product formation from various substrates by strain J1. Data represents average of two replicate experiments. Standard deviation are shown as error bars. From left to right; ethanol, acetate and hydrogen.

2.10. Analytical Methods. Hydrogen, ethanol, and volatile fatty acids were measured by gas chromatography as previously described [23]. Glucose was determined by slight modification of the method from Laurentin and Edwards [26]; supernatant broth (400 μL) was mixed with 2 mL of anthrone solution (0.2% (w/v) of anthrone in 72% (v/v) of sulphuric acid). The sample was boiled for 11 minutes and then cooled down on ice. Absorbance was then measured at 600 nm by using Perkin-Elmer Lambda 25 UV-Vis spectrophotometer.

3. Results and Discussion

3.1. Phylogeny. Figure 1 shows that strain J1 belongs to the genus *Thermoanaerobacter* with its closest neighbours being *T. uzonensis* (97.7% homology) and *T. sulfurigenes* (95.5%). The genus *Thermoanaerobacter* falls into clusters V in the phylogenetic interrelationship of *Clostridium* according to Collins and coworkers [27]. All species within the genus are obligate anaerobes and ferment various carbohydrates to ethanol, acetate, lactate, hydrogen, and carbon dioxide [20], while some species can degrade amino acids [28]. Most strains can reduce thiosulfate to hydrogen sulphide [20, 28]. Today, the genus consists of 18 species according to the Euzeby list of prokaryotes.

3.2. Optimum Growth Conditions. The strain was able to grow between 55.0°C and 75.0°C with optimal temperature being 65.0°C (μ_{max}; 0.23 h). The pH optimum was 7.0 (μ_{max}; 0.19 h). No growth was observed below pH 4.0 and above pH 9.0.

3.3. End Product Production from Sugars and Other Substrates. One of the main reasons for increased interest in using thermophilic bacteria for second-generation ethanol production is because of their broad substrate spectrum. Therefore, it was decided to cultivate the strain on the most common sugars present in lignocellulosic biomass as well as pyruvate, glycerol, serine, and threonine (Figure 2). Clearly, the strain is a very powerful ethanol producer; it produces 1.70 mol ethanol/mol glucose and 1.25 mol ethanol/mol

xylose (control values subtracted) or 85.0 and 75.0% of theoretical yields, respectively. The following stoichiometry from glucose and xylose was observed:

$$1.0\ \text{Glucose} \longrightarrow 1.70\ \text{EtOH} + 0.15\ \text{Acetate} + 0.30\ H_2 + 1.85\ CO_2 \quad (1)$$

$$1.0\ \text{Xylose} \longrightarrow 1.25\ \text{EtOH} + 0.20\ \text{Acetate} + 0.20\ H_2 + 1.45\ CO_2 \quad (2)$$

Lactate was not analysed in the present paper, but high carbon recoveries from analysed end products from glucose and xylose (92.5 and 87.4%, resp.) indicate that if it was produced, its significance is very little. The substrate spectrum of the strain shows a broad capacity in degrading pentoses (xylose, arabinose), hexoses (glucose, mannose, galactose, fructose, and rhamnose), disaccharides (maltose, cellobiose, lactose, trehalose, and sucrose) the trisaccharide raffinose, and starch, pyruvate, and serine. In all the cases, the major end product is ethanol except for serine and pyruvate in which acetate is the primary end product. The highest ethanol concentrations were produced from the trisaccharide raffinose (75.2 mM). As earlier mentioned, the strain is most closely related to *T. uzonensis* (strain JW/IW010) which also produces ethanol and acetate as the only volatile end products, but the ratio between ethanol and acetate is 1.35 in that strain [28]. However, *T. uzonensis* has a more narrow sugar degradation spectrum as compared to strain J1; it cannot degrade arabinose and rhamnose. Other well-known ethanol producers within the genus are *T. ethanolicus*, *T. thermohydrosulfuricus*, and *T. finnii* with yields between 1.5 and 1.9 mol ethanol/mol glucose [11, 13, 14, 29].

During growth on serine and pyruvate, the carbon flow was shifted away from ethanol to acetate and hydrogen. This can be explained by the oxidation state of these substrates as compared to sugars; the oxidation state of the carbon in glucose is zero, and during its oxidation to pyruvate, the electrons are transferred to NAD^+ leading to the formation of NADH. Reoxidation of NADH to NAD^+ by the strain occurs most likely through acetaldehyde dehydrogenase and alcohol dehydrogenase rendering ethanol as the main product. However, both pyruvate and serine are more oxidized substrates as compared to sugars (glucose), and there is no need to reoxidize NADH. Instead, the strain deaminates serine directly to pyruvate which is decarboxylated to acetyl phosphate (by phosphotransacetylase) and further to acetate (by acetate kinase) resulting in ATP formation. However, since hydrogen production is less as compared to acetate, it is likely that the strain is also producing formate (not analyzed) instead of hydrogen from these substrates.

3.4. Effect of Initial Glucose Loadings on Ethanol Production. High initial substrate concentrations may inhibit substrate utilization and/or decrease end product yields [5, 10, 30]. In closed systems, such as batch cultures, the limited buffer capacity of the medium may be overloaded by the accumulation of organic acids resulting in a pH drop and the inhibition of substrate fermentation utilization

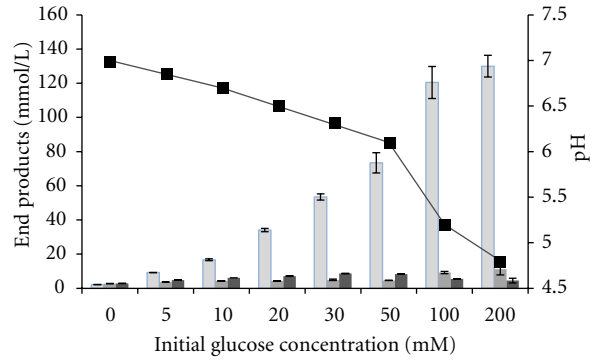

Figure 3: End product formation from different initial glucose concentrations. Also shown are percent of glucose degraded. Values represent means of two replicates and standard deviation are shown as error bars. Columns from left to right; ethanol, acetate, hydrogen. pH measered after fermentation (■).

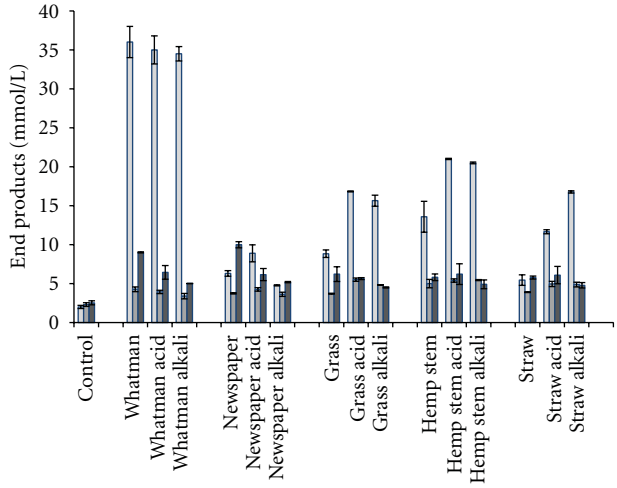

Figure 4: Production of end products from hydrolysates (4.5 g·L^{-1}) from different biomasses. Values represent mean of two replicates (±standard deviation). From left to right: ethanol, acetate, and hydrogen.

[30]. To investigate the influence of initial substrate concentration on end product formation, changes in pH, and substrate degradation, strain J1 was cultivated with different concentration of glucose (0 to 200 mM). The strain completely degraded glucose in all experiments, except for the highest (200 mM) initial glucose loadings, and ethanol yields were between 1.2 and 1.7 mol ethanol/mol glucose (Figure 3). Acetate formation increased from 2.7 mM in control bottles (without glucose) to 9.5 mM at 100 mM glucose concentrations which was directly linked to a decrease from pH 7.0 (control) to 5.2 (100 mM glucose). At 200 mM glucose concentrations, acetate was only slightly higher as compared to 100 mM glucose concentrations, the pH dropped from 5.2 to 4.8, and only 110 mM of glucose was degraded. Thus, the limit of glucose seems to be pH related, because of the formation of acetate, rather than

Table 1: Utilization of glucose by strain J1 in the presence of thiosulfate or a hydrogenotrophic methanogen. Data represents average of two replicate experiments ± standard deviation.

	Concentration ($mmol \cdot L^{-1}$)			
	Ethanol	Acetate	Hydrogen	Methane
Control	3.0 ± 0.1	2.9 ± 0.1	2.0 ± 0.1	0.0 ± 0.0
Control + S_2O_3	1.1 ± 0.1	5.2 ± 0.2	0.3 ± 0.0	0.0 ± 0.0
Control + methanogen	0.9 ± 0.5	4.9 ± 0.4	0.0 ± 0.0	2.4 ± 0.0
Glucose	29.0 ± 1.5	4.2 ± 0.3	7.2 ± 0.5	0.0 ± 0.0
Glucose + S_2O_3	20.0 ± 0.3	15.5 ± 2.1	0.3 ± 0.1	0.0 ± 0.0
Glucose + methanogen	4.1 ± 0.2	29.5 ± 1.2	0.5 ± 0.0	7.4 ± 1.2

substrate inhibition. The strain seems to be more tolerant for initial substrate concentrations as compared to many other thermophilic bacteria where often a concentration between 20 and 30 mM is too high for a complete degradation [7, 8]. In those cases, however, more acetate was produced as compared to ethanol and may be crucial for lowering the pH at lower substrate concentrations.

3.5. Effect of Hydrogen-Scavenging Systems on End Product Formation. It is well known that *Thermoanaerobacter* species are highly flexible concerning end product formation depending on the culture conditions. Fardeau et al. [31] showed a dramatic shift in end product formation by *Thermoanaerobacter finnii* when grown on glucose in the presence and absence of thiosulfate. In that case, both ethanol and lactate decreased during thiosulfate reduction to hydrogen sulphide, whereas the acetate concentration increased. The influence of using biological hydrogen-scavenging systems has also been investigated throughout *Thermoanaerobacter brockii* during amino acid degradation [27]. Both thiosulfate and the presence of a hydrogen-scavenging methanogen were crucial for the oxidative deamination of the branched chain amino acids by this strain. However, degradation of a substrate that is thermodynamically easier to degrade, for example, the amino acid serine, was completely degraded in the presence and absence of thiosulfate and *Methanobacterium* sp. although a shift occurred between ethanol and acetate formation [27]. To investigate the influence of low partial pressure (pH_2) on end product formation, strain J1 was cultivated in the presence of thiosulfate and in coculture with a hydrogenotrophic methanogen. As observed earlier, strain J1 produced ethanol as the main end product during glucose fermentation only (Table 1). The addition of thiosulfate to glucose fermentations resulted in a shift towards acetate from ethanol where the ratio between ethanol and acetate changed from 6.90 to 1.29. Cocultivating strain J1 with a hydrogenotrophic methanogen led even to more dramatic shift towards acetate (and methane), and the ratio of ethanol and acetate was 0.14. This difference in end product formation by using thiosulfate or a hydrogenotrophic methanogen is surprisingly big considering that the concentration of hydrogen is very low at the end of experimental time (0.3 to 0.5 $mmol \cdot L^{-1}$) in both cases. This difference could be caused by more rapid uptake of hydrogen in the coculture experiment, but end products were only analysed at the end of the experimental time.

3.6. Fermentation of Hydrolysates from Lignocellulosic Biomass. The strain is producing maximally 33.9 mM (1.56 g/L) of ethanol from 4.5 g/L of hydrolysates made from cellulose (Figure 4). The yields on cellulose pretreated only with enzymes and heat are 7.5 $mM \cdot g^{-1}$ dry weight (dw) which is considered lower as compared to glucose degradation alone (1.70 mol ethanol/mol glucose; 9.4 $mM \cdot g^{-1}$ glucose). No glucose was analysed in the cellulose hydrolysate after fermentation. Thus, the lower ethanol yields on cellulose as compared to glucose indicate that the cellulose was not completely degraded during enzymatic hydrolysis. Chemical pretreatment of cellulose by the addition of acid or alkali did not increase the end product formation yields on cellulose. The highest ethanol yields on the more complex biomass types (without chemical pretreatment) were observed on hemp (11.6 mM; 2.6 $mM \cdot g^{-1}$ dw) but lowest on straw (3.5 mM; 0.8 $mM \cdot g^{-1}$ dw). Chemical pretreatment by adding either acid or alkali increased yields substantially on most of the lignocellulosic biomasses tested. The increase was most profound on hydrolysates from straw pretreated with alkali where ethanol production was increased from 3.5 to 14.8 mM (controls subtracted). The highest ethanol yields were however observed on hemp, 4.3 $mM \cdot g^{-1}$ dw (19.0 mM). The highest ethanol yields by *Thermoanaerobacter* species have been reported by continuous cultures of *Thermoanaerobacter* strain BG1L1 on wheat straw [17] and corn stover [18], or 8.5–9.2 $mM \cdot g^{-1}$ sugar consumed. *Thermoanaerobacter ethanolicus* has been reported to produce 4.5 and 4.8 mM ethanol $\cdot g^{-1}$ hexose equivalent degraded from wood hydrolysate and beet molasses, respectively [13, 32]. *Thermoanaerobacter mathranii,* isolated from the same geographical area in Iceland [33] as strain J1 produced 5.3 $mM \cdot g^{-1}$ sugar from wheat straw hydrolysate [34]. Recently, a new *Thermoanaerobacter* strain, AK_5 closely related to *T. thermohydrosulfuricus* and *T. ethanolicus,* was isolated from a hot spring in Iceland and has similar yields on cellulose (7.7 $mM \cdot g^{-1}$), hemp (3.1 $mM \cdot g^{-1}$), and grass (4.1 $mM \cdot g^{-1}$) hydrolysates [22].

4. Conclusion

Ethanol production was studied by *Thermoanaerobacter* J1 isolated from hot spring in Iceland. The main aim of the study was to investigate the importance of various factors on ethanol production from both sugars and complex lignocellulosic biomass. The strain produces 1.70 mol ethanol/mol glucose and 1.25 mol ethanol/mol xylose and shows a broad substrate spectrum, degrading various sugars and starch but not cellulosic substrates. High ethanol yields were observed at initial glucose concentrations up to 100 mM. During growth under hydrogen removal, a shift from ethanol to acetate formation occurs. The strain produces up to 7.5 mM ethanol$\cdot g^{-1}$ cellulose and 4.2 mM$\cdot g^{-1}$ hemp hydrolysate.

Authors' Contribution

J. E. Jessen carried out all experimental procedures. J. Orlygsson planned the experimental procedure and drafted the paper. Both authors read and approved the final paper.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

The Potential of Cellulosic Ethanol Production from Grasses in Thailand

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The grasses in Thailand were analyzed for the potentiality as the alternative energy crops for cellulosic ethanol production by biological process. The average percentage composition of cellulose, hemicellulose, and lignin in the samples of 18 types of grasses from various provinces was determined as 31.85–38.51, 31.13–42.61, and 3.10–5.64, respectively. The samples were initially pretreated with alkaline peroxide followed by enzymatic hydrolysis to investigate the enzymatic saccharification. The total reducing sugars in most grasses ranging from 500–600 mg/g grasses (70–80% yield) were obtained. Subsequently, 11 types of grasses were selected as feedstocks for the ethanol production by simultaneous saccharification and cofermentation (SSCF). The enzymes, cellulase and xylanase, were utilized for hydrolysis and the yeasts, *Saccharomyces cerevisiae* and *Pichia stipitis,* were applied for cofermentation at 35°C for 7 days. From the results, the highest yield of ethanol, 1.14 g/L or 0.14 g/g substrate equivalent to 32.72% of the theoretical values was obtained from Sri Lanka ecotype vetiver grass. When the yields of dry matter were included in the calculations, Sri Lanka ecotype vetiver grass gave the yield of ethanol at 1,091.84 L/ha/year, whereas the leaves of dwarf napier grass showed the maximum yield of 2,720.55 L/ha/year (0.98 g/L or 0.12 g/g substrate equivalent to 30.60% of the theoretical values).

1. Introduction

At present, the problem of global warming has been undeniably accepted worldwide. The cause of this situation is the increase of the greenhouse gases. The most important one is carbondioxide gas resulted from the combustion of fossil fuels and deforestation. One of the means to address this problem is the application of alternative energy. Thus, it is necessary to search for the sources of the renewable energy such as solar, wind, hydraulic, geothermal, and tide energy. On the other hand, the biofuels from biomass can also represent the promising type of energy source [1]. Bioethanol is now considered interesting biofuel and has been attracting attention since it can be directly used in place of benzene or diesel. It can be applied in the form of the mixture with benzene called gasohol or blended with diesel called diesohol. Bioethanol can be produced from biomass such as starch, sugar, and lignocellulosic materials. Previous studies revealed that the cheaper and suitable one for the production of ethanol can be derived from corncobs, grasses, wooden spills, and bagasses [2]. However, the use of starch and sugar from cassava, corn, and sugarcane which are basically human-food might possibly lead to the problem of food crisis. Therefore, lignocellulosic biomass

which is abundant and low on production cost obtained from nonfood sources should be considered as an appropriate feedstock for ethanol production.

The energy crops are the types of plants expected to be cultivated as the raw materials for the production of the biofuels such as ethanol. Therefore, the plants with the fast growing rate such as grasses or shrubs should represent the appropriate energy crops for the future. These plants are perennial crops considered suitable as feedstock for lignocellulosic ethanol production because of high yields, low costs, fit for infertile land, and less environmental impacts [3]. Grasses are targeted as potential energy crops because of high productivity per hectare, abundancy, availability, and utilization of the whole plants. Fibres and storage carbohydrates within some species of grass can be used as substrates to produce ethanol whereas the species of grasses that contain high amounts of proteins can be used as nitrogenous waste for biorefineries [4].

Most of the researches in this area have been conducted on bioethanol production from either cellulose or hemicellulose hydrolysis to glucose or xylose for ethanol fermentation. In this research, both cellulose and hemicellulose from grasses were aimed to be used as the plant biomass as much as possible. The reason is from the fact that grasses contain very high percentages of total cellulose and hemicellulose. In general, the average compositions of grass biomass are 25–40% of cellulose, 25–50% of hemicellulose, and 10–30% of lignin [2, 5]. The scientific researches on ethanol production from grasses have been mostly performed in switch grass [6, 7]. Moreover, there were studies carried out in timothy grass, reed canary grass [8], Bermuda grass (*Cynodon dactylon*) [9], and silver grass (*Miscanthus floridulus*) [10], and so forth. These grasses are fast to grow likewise require less nutrient, thus suitable to be used as an energy crops for bioethanol production [1]. In particular, vetiver grass also has a potential for biofuel production by using a raw material in biomass power plant as well as ethanol production [11].

Ethanol production can be achieved from cellulose and hemicellulose hydrolysis to sugar by acid or enzyme catalysts. The use of acid is disadvantageous from the high temperature required, vigorous and nonspecific reactions resulting in the unwanted products. The containers have to be resistant to the acidic corrosion which is rather costly. Moreover, the process of waste elimination is needed for the remained acid contaminant. Therefore, the application of enzymes appears more interesting from the high specificity, neutral reaction without the unwanted by products, and therefore cost reduction in the waste management [12].

Obviously, the production of ethanol from agricultural wastes can only be applied for the short-term purpose since the amount of such waste won't be sufficient in the future. Therefore, in order to maintain long-term and successfully sustainable production of the alternative energy, the cultivation of energy crops together with the utilization of agricultural wastes and the search for the potential plants which can produce ethanol should be implemented. Grasses are considered one of the most suitable energy crops from their longevity, regeneration after the cutoff, and effective capability to withstand the drought. There are so many types of grasses that are popularly grown in Thailand. Hence, the objective of this research was to select the types of grasses in Thailand suitable as the feedstocks for cellulosic ethanol production by biological process. The fungus, *Trichoderma reesei,* has been widely accepted as the organism capable of producing the enzymes, cellulase, and hemicellulase in considerable quantities, appropriate for lignocellulosic biomass digestion [3]. Although the fermentation can be achieved from various processes, the application of simultaneous saccharification and cofermentation (SSCF) presents more advantages than the others. This method allows the raw materials to be hydrolyzed to hexose and pentose sugars for the subsequent fermentation to obtain ethanol in the single step. The obtained glucose gradually released from the hydrolysis will be continuously utilized in the fermentation. This low concentration of the glucose will result in the improved fermentation of xylose sugar [13]. Similarly, the yeasts, *Saccharomyces cerevisiae* and *Pichia stipites,* are widely known organisms that can utilize glucose and xylose for ethanol fermentation, respectively. Although, *P. stipitis* can ferment both glucose and xylose, but ethanol production rate from glucose is at least five times less than obtained with *S. cerevisiae* [14]. They were therefore applied in SSCF for higher efficient ethanol production.

2. Materials and Methods

2.1. Sampling and Raw Materials. 18 samples of grasses were collected from Chiang Mai, Lampang, Ratchaburi, and Petchburi provinces. All samples were dried at 60°C for 3 days, ground to small particles and later filtered through 0.4 mm mesh. Biomass contents were analyzed according to the method of Ruminant Nutrition Laboratory, University of Nebraska [15]. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and permanganate lignin (PML) were determined. Hemicellulose content of biomass was determined as NDF minus ADF, lignin content of biomass as ADF minus PML, and cellulose content of biomass as PML minus residue after ash.

2.2. Enzyme Production. T. reesei TISTR 3081 (*T. reesei* QM9414) was obtained from Thailand Institute of Scientific and Technological Research (TISTR), Thailand and was later cultured in potato dextrose agar at 30°C for 8 days. A cork borer (7 mm diameter) was used to cut agar plugs from a culture on PDA. Five agar plugs with the filaments of fungi were inoculated into 250 mL flask containing 100 mL of Mandels medium [16] for cellulase production in the presence of 10 g/L α-cellulose as a carbon source. The induction of xylanase production was carried out in xylan medium containing 10 g/L birchwood xylan as a carbon source, 5 g/L corn steep liquor, 5 g/L polypeptone, 1 g/L yeast extract, 4 g/L K_2HPO_4, 0.2 g/L KCl, 1 g/L $Mg_2SO_4 \cdot 7H_2O$, and 0.02 g/L $FeSO_4 \cdot 7H_2O$. The media were sterilized by autoclaving at 121°C for 15 min. After incubation at 30°C and shaken at 150 rpm for 7 days, the mycelia were removed by filtration through Whatman no.1 filter paper. Crude enzyme was collected and determined for the activities of

cellulase and xylanase using Whatman no.1 filter paper and 1% birchwood xylan as the substrates, respectively, according to Ghose [17].

One unit of enzyme was referred as the amount of enzyme which could convert substrates to 1 μmole of reducing sugar in 1 minute. Protein was determined by micro Lowry's assay [18] to obtain the specific activity of enzyme (U/mg protein).

2.3. Pretreatment. Alkaline peroxide pretreatment was performed. The milled grasses were suspended in 7.5% (v/v) H_2O_2 and NaOH was then added to adjust the pH to 11.5. The pretreated samples were then incubated at 35°C, shaken at 250 rpm for 24 hours. Finally, conc. HCl was added to adjust the pH to 4.8 before enzymatic hydrolysis [19]. The filtration was not required after the pretreatment. The liquid phase with solubilized hemicellulose and the solid phase with cellulose of the samples were subsequently hydrolyzed by the enzymes.

2.4. Enzymatic Hydrolysis. Cellulase (60 U/g substrate), xylanase (1200 U/g substrate), and 5 mL of 0.05 M sodium citrate buffer (pH 4.8) were added to each substrate (0.6 g of milled grass) and positive control (0.3 g of α-cellulose mixed with 0.3 g of xylan). After the addition of the enzymes, the samples were incubated at 50°C and shaken at 150 rpm for 72 hours. Total reducing sugar was analyzed by DNS method [20] and subsequently calculated for the percentages of conversion to cellulose and hemicellulose according to the equations below.

$$\%\ \text{conversion of cellulose and hemicellulose to sugars} = \frac{B}{A} \times 100. \quad (1)$$

Total reducing sugar after the hydrolysis (ton/ha/year) was considered for selection of suitable grasses as the substrates for ethanol production by SSCF process.

$$\text{Total reducing sugar after hydrolysis} = \frac{B \times C}{1000}, \quad (2)$$

where A is total cellulose and hemicellulose before hydrolysis (mg/g dry weight of substrate). B is total reducing sugar after hydrolysis (mg/g dry weight of substrate). C is dry matter yield of grass (ton/ha/year).

2.5. Fermentation. *S. cerevisiae* TISTR 5339 was obtained from TISTR, Thailand. *P. stipitis* CBS 5773 was obtained from The Centraalbureau voor Schimmelcultures (CBS), The Netherlands. Yeast strains were maintained at 4°C on YM agar containing 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose, and 20 g/L agar. *S. cerevisiae* and *P. stipitis* were precultured as inocula for fermentation in Erlenmeyer flasks containing 50 mL of 3 g/L yeast extract, 3 g/L malt extract, 5 g/L $(NH_4)_2SO_4$, and 20 g/L glucose for *S. cerevisiae* or 20 g/L xylose for *P. stipitis* [21]. The media were sterilized by autoclaving at 121°C for 15 min. Flasks were incubated at 30°C and shaken at 150 rpm for 9 h (*S. cerevisiae*) and 10 h (*P. stipitis)*.

1.2 g of pretreated substrates was supplemented in fermentation medium containing 0.45 g yeast extract, 0.45 g malt extract, and 0.75 g $(NH_4)_2SO_4$ in 7.5 mL of 0.05 M sodium citrate buffer (pH 5.0) and sterilized by autoclaving at 121°C for 15 min. Cellulase and xylanase were filtered and sterilized through a 0.2 μm filters and the enzymes were added with the activities of 72 U and 1,440 U in each flask, respectively. Then, 7.5 mL (5% v/v) of each yeast strain was added last. Final volume was adjusted to 150 mL by sterilized distilled H_2O. The samples were incubated with shaking at 35°C, 150 rpm for 7 days, and ethanol production was determined by gas chromatography (Shimadzu, Japan)

2.6. Statistical Analysis. Collected data (3 replicates) were analyzed by one-way ANOVA at 95% confidence level. Comparison was performed by Duncan's Multiple Range Test (DMRT) using SPSS for Window version 15.0.

3. Results and Discussion

3.1. Raw Materials. The samples of 18 types of grasses were divided into 2 groups. The first group was composed of 8 types of the forage grasses (napier leaves, dwarf napier leaves, king napier leaves, bana leaves, purple guinea, ruzi, pangola, and atratum) and the second one contained 10 ecotypes of vetiver grasses (Kamphaeng Phet 2, Songkhla 3, Surat Thani, Sri Lanka, Roi Et, Loei, Nakhon Sawan, Prachuap Khiri Khan, Ratchaburi, and Kamphaeng Phet 1) as shown in Figure 1. Since the height of all the grasses in this study is more than 1 metre, and the harvest can be carried out several times per year, considerably huge amount of dry biomass can be obtained from the conversion. Both leaves and stems from the purple guinea grass, ruzi grass, pangola grass, atratum grass, and vetiver grasses were used since their stems are rather small. However, only the leaves were employed from napier grass, dwarf napier grass, king napier grass, and bana grass from their strong, hard stems together with the limitation of the specific types of facilities required for the chopping and grinding. As the compositions of biomass in each type of grass diverse immensely, even the same type possibly differs depending on the environment or the plant ages. The factors that can affect growth, production and composition of grasses can be genetics, soil properties, maintenance, amount, and distribution of rain. Moreover, the cuttings of the stems and leaves of plants for various purposes result in the decrease of photosynthetic activities thereby reducing the accumulation of carbohydrates [22]. On the contrary, the proteins will be increased [23].

From the analysis of the composition of the biomass in 18 types of grasses studied, it was found that all types contained the average quantities of cellulose in the range of 31.85–38.51%, 31.13–42.61% of hemicellulose and 3.10–5.64% of lignin (Table 1). The low level of lignin present in the samples of grasses used in this study appeared more advantageous than the other types, for example, 6.4% in coastal Bermuda grass, 12% in switch grass, 7.3% in S32

Table 1: Biomass composition of grasses.

Grasses	Dry matter yield (ton/ha/year)	Biomass composition (%)				
		Cellulose	Hemicellulose	Lignin	Ash	Others
Forage grasses						
Napier	7.7*	32.92 ± 1.48	36.46 ± 1.19	3.60 ± 0.67	0.33 ± 0.14	26.69 ± 3.04
Dwarf napier	17.5*	35.64 ± 0.21	34.19 ± 1.24	3.66 ± 0.20	0.13 ± 0.12	26.38 ± 1.38
King napier	7.7*	32.01 ± 0.14	31.13 ± 0.57	3.10 ± 0.04	1.65 ± 0.04	32.11 ± 0.53
Bana	7.7*	33.93 ± 2.27	35.12 ± 1.62	3.55 ± 0.34	0.18 ± 0.01	27.21 ± 3.71
Purple guinea	18.8	33.40 ± 0.74	31.26 ± 1.91	4.00 ± 0.54	0.61 ± 0.08	30.73 ± 2.10
Ruzi	14.1	33.64 ± 0.92	34.01 ± 0.81	4.56 ± 0.17	0.27 ± 0.12	27.52 ± 1.67
Pangola	37.5	33.07 ± 0.70	35.46 ± 1.27	4.47 ± 0.61	0.28 ± 0.01	26.72 ± 1.60
Atratum	18.8	34.87 ± 0.61	32.60 ± 0.53	5.64 ± 0.22	0.31 ± 0.14	27.75 ± 0.97
Vetiver grasses						
Kamphaeng Phet 2	6.0	35.54 ± 0.38	39.02 ± 0.89	4.36 ± 0.78	0.07 ± 0.04	19.72 ± 0.61
Songkhla 3	5.8	31.85 ± 1.73	37.87 ± 1.59	4.67 ± 0.49	0.26 ± 0.16	25.67 ± 2.69
Surat Thani	5.5	33.97 ± 1.16	39.12 ± 1.57	3.67 ± 0.70	0.09 ± 0.03	22.16 ± 0.74
Sri Lanka	6.4	37.54 ± 0.45	38.33 ± 2.07	3.99 ± 0.31	0.08 ± 0.01	20.38 ± 2.81
Roi Et	3.5	34.04 ± 0.27	42.61 ± 1.17	4.83 ± 0.24	0.04 ± 0.04	19.32 ± 0.88
Loei	4.9	34.42 ± 0.99	42.43 ± 1.32	5.09 ± 0.56	0.08 ± 0.07	18.23 ± 0.98
Nakhon Sawan	4.2	32.67 ± 1.31	39.60 ± 1.25	4.96 ± 1.18	0.06 ± 0.04	22.57 ± 0.94
Prachuap Khiri Khan	8.5	35.53 ± 1.52	38.87 ± 0.09	4.65 ± 1.52	0.19 ± 0.11	20.44 ± 2.26
Ratchaburi	7.6	38.51 ± 0.25	39.21 ± 0.70	4.79 ± 0.48	0.05 ± 0.01	17.58 ± 0.87
Kamphaeng Phet 1	6.5	34.95 ± 0.58	39.67 ± 0.18	5.06 ± 0.70	0.12 ± 0.10	20.47 ± 1.39

* The yields of napier grass, dwarf napier grass, king napier grass, and bana grass were the values of leaves only.

rye grass (seed setting), 4.7% in orchard grass [2, 5], and 10.7% in *Miscanthus* grass [24]. Moreover, since the free sugars were not detectable from the samples, the hydrolysis was necessary in order to obtain the sugar prior to the further fermentation process. The contents of plant biomass can be used to initially indicate the potentiality of grasses whether they are suitable for the application as the energy crops. All the studied 18 types of plants contained high contents of the total cellulose and hemicellulose of approximately 60–80%. Therefore, the determination of cellulose and hemicellulose can be applied to quantify the theoretical production of ethanol.

3.2. Enzyme Production. The enzymes produced from the fungus *T. reesei* TISTR 3081 were assayed for the activities of cellulase using α-cellulose and xylanase using xylan as the carbon sources, respectively. The results revealed that cellulase and xylanase showed the activities of 0.948 ± 0.05 and 92.13 ± 6.86 U/mL, respectively, whereas the obtained specific activities were 1.09 ± 0.09 and 65.32 ± 1.59/mg protein, respectively.

The various strains of fungus, *Trichoderma*, especially *T. reesei* including the mutants, have been popularly applied from the high capacity of the cellulase production suitable for the hydrolysis [3]. The enzymes have been shown to be tolerant against many inhibitors and show stability at 50°C comparatively higher than other types of fungi [25]. The advantages of the application of *T. reesei* are the fungal capability to produce the mixture of cellulases containing activities of at least 2 types of cellobiohydrolases, 5 types of endoglucanases together with β-glucosidases. In addition, hemicellulases can also be produced which was reported by Zhang and Lynd [26]. The studies on the production of cellulases and hemicellulases from the fungus, *T. reesei* RUT C30 in the presence of 7.5 g/L of Solka Floc as the carbon source were also conducted by Juhász et al. [27]. The results revealed that the obtained specific activities of cellulase and xylanase by the FPU assay were approximately 0.58 and 119 U/mg protein, respectively. It can be clearly seen that the fungus, *T. reesei* can produce both cellulases and hemicellulases. Hence, the organism appears suitable for the production of the hydrolytic enzymes for lignocellulosic material degradation. As a result, the number of the types of enzymes required for the process can be reduced thereby reducing the costs.

3.3. Enzymatic Hydrolysis. In this research, only the glucose and xylose produced from the hydrolysis of cellulose and xylan by cellulase and xylanase were studied, respectively, since the main composition of hemicellulose is generally xylan. The comparison of the efficiency for the hydrolysis of each type of grass was evaluated from the percentages of the conversion of the total reducing sugar since each type contained different quantities of cellulose and hemicellulose. The pretreatment of the raw materials was conducted to disaggregate lignocellulose into the various compositions, namely, cellulose, hemicellulose, and lignin [25]. The objective was to remove lignin, reduce cellulose crystallinity, increase the porosity of the raw materials resulting in cellulose accessibility to hydrolysis for conversion

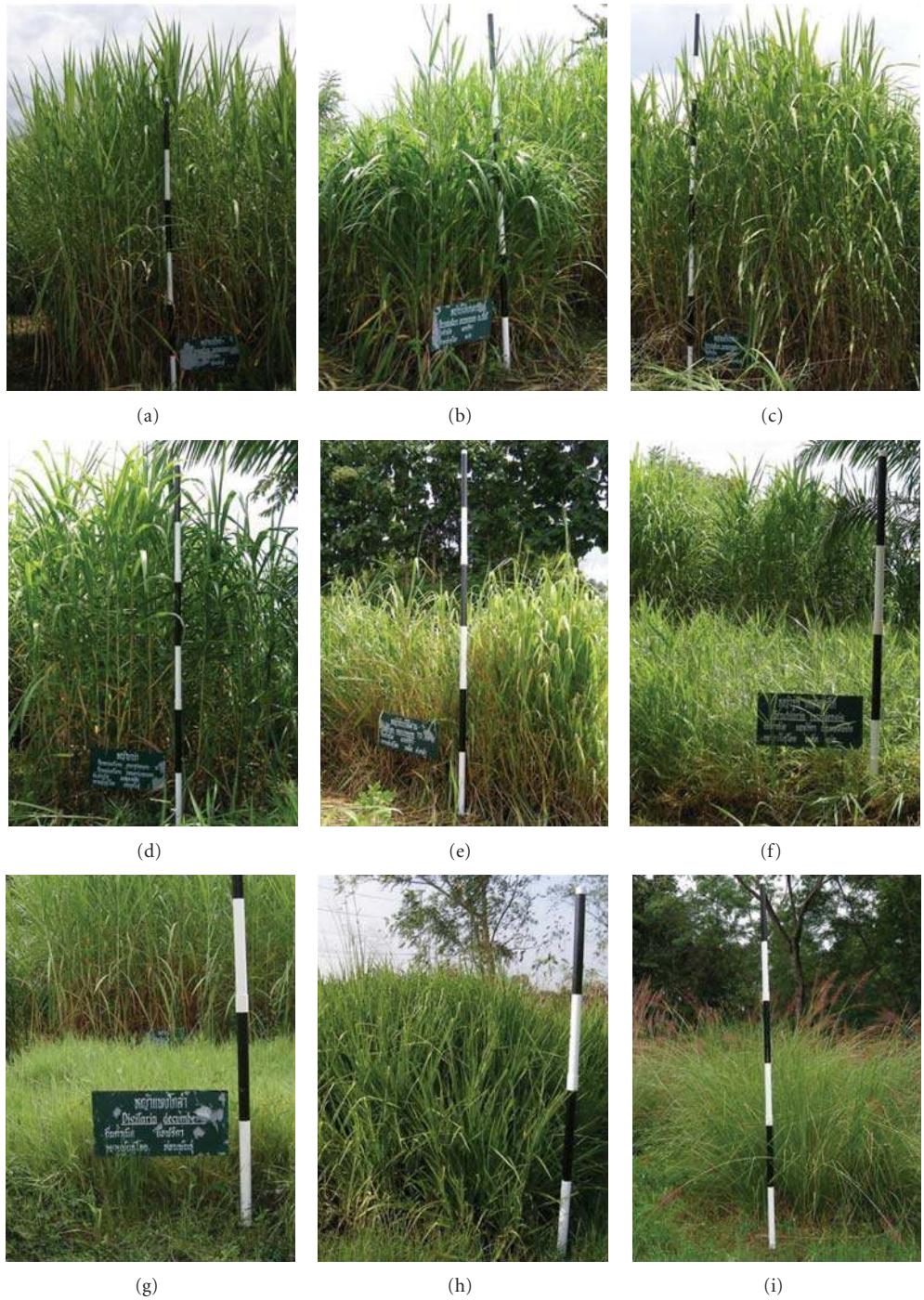

Figure 1: 18 samples of grasses collected from Chiang Mai, Lampang, Ratchaburi, and Petchburi provinces. Forage grasses (a–h), (a) napier grass (*Pennisetum purpureum*), (b) dwarf napier grass (*P. purpureum* cv. *Mott*), (c) king napier grass (*P. purpureum* cv. King Grass), (d) bana grass (*P. purpureum* × *P. americanum*), (e) purple guinea grass (*Panicum maximum* TD 58), (f) ruzi grass (*Brachiaria ruziziensis*), (g) pangola grass (*Digitaria decumbens*), (h) atratum grass (*Paspalum atratum*) and (i) vetiver grasses.

to fuels [2, 25, 28]. The use of alkaline hydrogen peroxide was an effective pretreatment of grass stovers and other plant materials in the context of animal nutrition and ethanol production. In addition, it has been widely applied with various substrates such as wheat straw [29, 30], rice hulls [19], sugarcane bagasse [31], barley straw [32], and *Miscanthus* grass [24]. The advantages of this pretreatment are the use of reagents with low environmental impact and avoidance of special reaction chambers [33]. As a consequence, this pretreatment was selected for this research. From the preliminary results, no reducing sugars were obtained without the raw material pretreatment before the

enzymatic hydrolysis (data not shown). Hence, the necessity of the pretreatment is very imperative.

From the hydrolysis of 18 types of grasses by chemical pretreatment with alkaline peroxide followed by hydrolysis with cellulase and xylanase produced from the fungus *T. reesei* TISTR 3081, the total reducing sugar obtained from the hydrolysis was tabulated in Table 2. Most of the grass samples' total reducing sugar showed the values in the range of 500–600 mg/g of raw materials equivalent to the concentration of 4–5 g/L and the obtained conversion was approximately 70–80%. There was no furfural and hydroxymethylfurfural produced from this method during the pretreatment [19]. These chemicals are always detected after the pretreatment and the hydrolysis by the use of acid and act as the inhibitors for the growth of yeasts used for the ethanol fermentation [34]. Rice hulls and wheat straw pretreated with alkaline peroxide could be converted to fermentable sugars with an excellent yield (96–97%) by enzymatic saccharification [19, 29]. The values were high since the commercial enzymes, namely, Celluclast 1.5 L (cellulase), Novozyme 188 (β-glucosidase), and Viscostar 150 L (xylanase) were applied in their work. These commercial enzymes must absolutely give much higher activities than the enzyme produced from the fungus *T. reesei* in our research. Moreover, the presence of β-glucosidase facilitated the hydrolysis of cellobiose to glucose resulting in the higher percentages of sugar conversion.

Since the sugar content and the value of sugar conversion from each grass obtained by the enzymatic hydrolysis were rather indifferent, the decisive selection of the suitable grass for the subsequent fermentation was not accurately possible. Therefore, the dry mass values of the grass samples were included in the calculations of the obtained reducing sugar contents from the enzymatic hydrolysis and the theoretical production of ethanol could be later calculated from the reducing sugar, assuming that the theoretical ethanol yield for fermenting is 0.511 g per g of hexose or pentose. From the results tabulated in Table 2, 11 types of grassses with the total reducing sugars over 3.9 ton/ha/year or the theoretical values for the ethanol production exceeding 2,550 L/ha/year were selected for the subsequent fermentation in descending order from the maximum, namely, pangola, purple guinea, dwarf napier leaves, atratum, ruzi, Prachuap Khiri Khan, Ratchaburi, bana leaves, napier leaves, king napier leaves and Sri Lanka, respectively.

3.4. Fermentation. Hydrolysis of the main composition, cellulose and hemicellulose, into glucose and xylose as the substrates of fermentation is necessarily required for the production of ethanol. In this research, the mixture of 2 types of yeasts was used, *S. cerevisiae* and *P. stipites,* since the former organism can use only glucose while the latter can catalyze both glucose and xylose as the substrates. Nonetheless, *P. stipitis* showed slower rate of fermentation than *S. cerevisiae* [35]. There are various methods to achieve the fermentation but SSCF has been shown to be more efficient than the others from the fact that the raw materials can be hydrolyzed to hexose and pentose for further ethanol fermentation in single step. The gradual release of glucose from the hydrolysis can be continuously used for the fermentation resulting in the low concentration of glucose which yields better fermentation for xylose [13].

When the initially selected 11 types of grasses were fermented by SSCF by the addition of 2 types of yeasts; *S. cerevisiae* and *P. stipitis* together with 2 enzymes produced from *T. reesei* TISTR 3081; cellulase and xylanase at 35°C for 7 days, the ethanol production from various raw materials was tabulated in Table 3. The highest yield of ethanol, 1.14 g/L or 0.14 g/g substrate equivalent to 32.72% of the theoretical values, was obtained from Sri Lanka ecotype vetiver grass. For purple guinea grass and pangola grass, it was found that the lowest yield of ethanol was obtained at 6.08 and 6.8%, respectively, compared to the theoretical values. This result conflicted with the preliminary results of enzymatic hydrolysis that purple guinea grass and pangola grass gave the highest conversion of sugar of 85.56 ± 3.44 and 76.03 ± 0.84% respectively. This indicated that these 2 types of grasses may contain certain chemicals such as tannin or silica in the quantities that could possibly affect the activities of the enzyme and the growth of the yeasts resulting in the reduction of the ethanol production obtained from fermentation. Moreover, the presence of other substances toxic to the microorganisms in the system, such as the derivatives of lignin which are the phenolic compounds, may reduce the efficiency of the fermentation [36]. Isci et al. [7] studied the SSF fermentation in switchgrass by aqueous ammonia pretreatment and found that lignin could be eliminated by 40–50%. The yield of ethanol at 72% of the theoretical values was obtained from the application of the fermentation mixture between commercial cellulase (Spezyme CP) with the activity of 77 FPU/mL together with *S. cerevisiae* D_5A for the period of 10 days. The differences could be from the fact that hemicellulose and lignin which can act as the inhibitors were preseparated. Only cellulose was used in the ethanol production by cellulase hydrolysis and single microorganism in SSF fermentation resulting in higher amount of ethanol.

In the analysis to determine the potential suitable grasses for the cultivation as the energy crops, the dry mass production of each type of grass (Table 1) was included in the calculation. The quantities of ethanol production were calculated in 3 cases as tabulated in Table 4. For the first case, the theoretical ethanol production was calculated from the determination of cellulose and hemicellulose from each type of grass. The complete hydrolysis of cellulose and hemicellulose and the amount of sugar totally converted to ethanol were used for the calculations. The overall theoretical yield for conversion was 0.581 g of ethanol per g of xylan and 0.568 g of ethanol per g of cellulose, assuming that xylan represents the major sugar source in all the biomass hemicellulose. The theoretical ethanol yield for fermentation is 0.511 g per g of hexose or pentose [37]. The results showed that the highest yield of ethanol was obtained from the pangola grass at 18,706.69 L/ha/year from the highest value of dry biomass at 37.5 ton/ha/year. As a result, it might have been suitable to be applied as the energy crops for the production of ethanol. Nevertheless, there are still

Table 2: Total cellulose and hemicellulose of grasses before pretreatment and hydrolysis and total reducing sugar released after hydrolysis.

Grasses	Total cellulose and hemicellulose before pretreatment and hydrolysis	Total reducing sugar after hydrolysis		% conversion	Total reducing sugar after hydrolysis (ton/ha/year)	Theoretical ethanol yield (L/ha/year)*
	(mg/g substrate)	mg/g substrate	g/L			
Napier	693.76 ± 26.67	528.58 ± 11.76	4.14 ± 0.34	76.19 ± 1.70	4.05	2,621.06
Dwarf napier	698.37 ± 10.78	558.61 ± 15.58	4.13 ± 0.34	79.99 ± 2.23	9.78	6,331.31
King napier	631.40 ± 4.91	516.63 ± 8.82	4.04 ± 0.32	81.82 ± 1.40	3.96	2,561.81
Bana	690.54 ± 36.65	556.27 ± 24.94	4.36 ± 0.46	80.56 ± 3.62	4.26	2,758.31
Purple guinea	646.60 ± 17.78	553.20 ± 22.22	4.34 ± 0.54	85.56 ± 3.44	10.37	6,717.88
Ruzi	676.53 ± 17.24	469.13 ± 19.09	3.97 ± 0.61	69.34 ± 2.82	6.60	4,272.69
Pangola	685.30 ± 19.62	521.03 ± 5.78	4.09 ± 0.39	76.03 ± 0.84	19.54	12,654.31
Atratum	674.70 ± 11.09	505.93 ± 27.58	4.01 ± 0.53	74.99 ± 4.09	9.49	6,143.81
Kamphaeng Phet 2	745.60 ± 7.74	586.55 ± 22.59	4.60 ± 0.56	78.67 ± 3.03	3.54	2,295.91
Songkhla 3	697.13 ± 29.98	510.94 ± 37.41	3.98 ± 0.20	73.29 ± 5.37	2.97	1,921.38
Surat Thani	730.90 ± 4.13	563.81 ± 18.02	4.42 ± 0.50	77.14 ± 2.47	3.12	2,017.50
Sri Lanka	758.70 ± 25.11	619.31 ± 6.38	4.85 ± 0.42	81.63 ± 0.84	3.95	2,557.00
Roi Et	766.50 ± 9.15	516.95 ± 16.35	3.81 ± 0.33	67.44 ± 2.13	1.83	1,184.36
Loei	768.53 ± 6.05	550.89 ± 22.62	4.31 ± 0.27	71.68 ± 2.94	2.71	1,757.17
Nakhon Sawan	722.70 ± 4.20	532.25 ± 17.80	4.37 ± 0.58	73.64 ± 2.46	2.25	1,456.41
Prachuap Khiri Khan	744.05 ± 14.42	573.62 ± 4.92	4.31 ± 0.19	77.09 ± 0.66	4.85	3,139.24
Ratchaburi	777.20 ± 4.50	616.34 ± 18.36	4.54 ± 0.25	79.30 ± 2.36	4.68	3,033.76
Kamphaeng Phet 1	746.17 ± 7.01	578.10 ± 16.80	4.52 ± 0.28	77.48 ± 2.25	3.73	2,414.94
α-cellulose + xylan	1,000.00 ± 0.00	753.94 ± 6.89	8.76 ± 0.06	75.39 ± 0.69	—	—

Grasses (0.6 g) were pretreated with alkaline peroxide (7.5% (v/v) H_2O_2; pH 11.5; 35°C, 24 h) followed by enzymatic hydrolysis at 50°C for 72 h. Positive control was 0.3 g of α-cellulose mixed with 0.3 g of xylan.

*Theoretical ethanol yield (L/ha/year) was calculated from total reducing sugars after enzymatic hydrolysis, assuming that the theoretical ethanol yield for fermenting is 0.511 g per g of hexose or pentose.

Table 3: Ethanol production from grasses by SSCF process.

Grasses	Ethanol		% of the theoretical values
	g/L	g/g substrate	
Sri Lanka	1.14 ± 0.09^{a}	0.14 ± 0.01^{a}	32.72 ± 2.69^{a}
Ratchaburi	1.10 ± 0.10^{a}	0.14 ± 0.01^{a}	$30.95 \pm 2.81^{a,b}$
α-cellulose + xylan	$1.06 \pm 0.30^{a,b}$	$0.13 \pm 0.04^{a,b}$	23.01 ± 6.47^{b}
Dwarf napier	$0.98 \pm 0.16^{a,b}$	$0.12 \pm 0.02^{a,b}$	$30.60 \pm 4.90^{a,b}$
Napier	$0.97 \pm 0.31^{a,b}$	$0.12 \pm 0.04^{a,b}$	$30.30 \pm 9.78^{a,b}$
Bana	$0.87 \pm 0.14^{a,b}$	$0.11 \pm 0.02^{a,b}$	$27.28 \pm 4.56^{a,b}$
Prachuap Khiri Khan	0.80 ± 0.12^{b}	0.10 ± 0.01^{b}	23.31 ± 3.48^{b}
King napier	0.40 ± 0.13^{c}	0.05 ± 0.02^{c}	13.93 ± 4.53^{c}
Atratum	0.36 ± 0.05^{c}	0.05 ± 0.01^{c}	11.64 ± 1.60^{c}
Ruzi	0.30 ± 0.06^{c}	0.04 ± 0.01^{c}	9.56 ± 1.79^{c}
Pangola	0.21 ± 0.05^{c}	0.03 ± 0.01^{c}	6.80 ± 1.58^{c}
Purple guinea	0.18 ± 0.07^{c}	0.02 ± 0.01^{c}	6.08 ± 2.49^{c}

Grasses (1.2 g) were pretreated with alkaline peroxide (7.5% (v/v) H_2O_2; pH 11.5; 35°C, 24 h). SSCF process was performed using cellulase and xylanase (72 and 1440 U, resp.) from *T. reesei* for hydrolysis and *S. cerevisiae* and *P. stipitis* for cofermentation at 35°C for 7 days. Positive control was 0.6 g of α-cellulose mixed with 0.6 g of xylan. Values not sharing a common superscript (a, b, c, and d) differ significantly (Duncan's Multiple Range Test).

Table 4: Comparison of the theoretical ethanol yield and ethanol produced from SSCF process of 11 types of grasses.

Rank	Theoretical ethanol yield (L/ha/year)					
	Calculated from total cellulose and hemicellulose		Calculated from total reducing sugars after enzymatic hydrolysis		Ethanol yield from SSCF (L/ha/year)	
1	Pangola	18,706.69	Pangola	12,654.31	Dwarf napier	2,720.56
2	Atratum	9,201.69	Purple guinea	6,717.88	Ratchaburi	1,330.06
3	Dwarf napier	8,889.88	Dwarf napier	6,331.31	Pangola	1,272.00
4	Purple guinea	8,818.50	Atratum	6,143.81	Napier	1,171.69
5	Ruzi	6,922.69	Ruzi	4,272.69	Sri Lanka	1,151.69
6	Prachuap Khiri Khan	4,576.81	Prachuap Khiri Khan	3,139.25	Atratum	1,071.19
7	Ratchaburi	4,297.88	Ratchaburi	3,033.75	Prachuap Khiri Khan	1,066.75
8	Napier	3,867.38	Bana	2,758.31	Bana	1,045.81
9	Bana	3,847.50	Napier	2,621.06	Ruzi	661.56
10	Sri Lanka	3,519.81	King napier	2,561.81	Purple guinea	536.44
11	King napier	3,517.00	Sri Lanka	2,557.00	King napier	489.81

The theoretical ethanol yield for fermenting is 0.511 g per g of hexose or pentose.

many necessary factors required for the analysis such as the plantation areas for the cultivation, maintenance, and the composition in various grasses which may contain inhibitors affecting the activities of certain enzymes and the growth of the microorganisms in the fermentation process. Apparently, the obtained values of the sugar contents from the hydrolysis and the obtained ethanol from the fermentation were inconsistent with the theory. Hence, further studies on the hydrolysis of the biomass composition and the ethanol production should be conducted to determine the real quantity of the ethanol production. When the analysis has been performed in more details, other appropriate types of grasses may also have been discovered.

For the second case, when the theoretical values of ethanol production were calculated from the total reducing sugars obtained after the enzymatic hydrolysis, it was found that pangola grass still produced the highest theoretical values of ethanol production of 12,654.31 L/ha/yr despite the rather similar values of reducing sugars obtained from each type of grass after the enzymatic hydrolysis. Therefore, the highest yield of dry mass obtained from pangola grass consistently resulted in the highest value of theoretical ethanol production.

For the final third case, the ethanol production was calculated from the fermentation process, SSFC. It was found that dwarf napier grass leaves showed the highest yield of 2,720.56 L/ha/year. Therefore, this grass should have potential to be developed as the energy crop for the production of ethanol by this SSCF fermentation. Further studies can be carried out on the optimal conditions of fermentation to increase the production of ethanol approximately closed to the theoretical values. In this research, since the samples of rather aged grasses with the very strong and hard stems were collected, only the leaves were selectively applied for the experiments. Nevertheless, if the dwarf napier grass leaves were to be utilized for the application in the future, they should be harvested at the earlier stage so that both the stems and the leaves can be used.

When the theoretical ethanol production from pangola grass obtained from the second case was comparatively considered with the values obtained from the process of SSFC, it can be seen that the theoretical ethanol production from pangola grass was 5-folds higher than what obtained from dwarf napier grass from the process of SSFC. On the contrary, the ethanol production from pangola grass obtained from SSFC was much lower than the theoretical values since the concentration of ethanol obtained was considerably low when compared with the others. This result suggested that the fermentation by the method in this study might not have been suitable for this grass. There might be some substances that inhibit the process of fermentation. Pangola grass may not be appropriate for the ethanol production by SSCF fermentation. However, pangola grass yielded the highest quantities of ethanol when the analysis was derived from both theoretical calculations. More importantly, it seems appropriately applicable as the hay from the tremendously high contents of dry mass in this grass. Hence, pangola grass may also present potentiality as the energy crop in Thailand. In case this type of grass would be further developed for the production of ethanol that would yield the closest values to the theory, the fermentation process should be further investigated with some alterations. In addition, the fermentation by SHF can represent the good alternative process since the high level of sugar can be produced from the enzymatic hydrolysis of this type of grass.

Recently, Bank of Thailand, in January 2012 reported the consumption of ethanol in Thailand at approximately 1.3 million litres per day or 457 million litres per year [38]. In case that the dwarf napier grass is to be applied for the production of ethanol by the process of SSFC, at least 0.17 million hectares of the plantation area are necessarily required. When the production of ethanol from other types of energy crops was comparatively studied, it has been found that the yield from sugar cane was equal to 4,900 kg/ha/year or 6,210 L/ha/year, whereas from cassava was 6,000 kg/ha/year or 7,604 L/ha/year [39]. Although the

production of ethanol from the SSCF process derived from dwarf napier grass was found to be 2,720.56 L/ha/year or 2.3 and 2.8-folds less than what obtained from the sugar cane and cassava, respectively, both of the mentioned energy crops are mostly utilized for human food consumption. Hence, the search for the alternative nonfood energy crops for the production of ethanol appears undeniably and continuously imperative in order to sustain the availability of the food energy crops for the prevention of food shortage problem in the future. This research particularly focused on the SSCF process that unfortunately had not been optimized. After the process, the total raw materials did not appear to be completely hydrolyzed. This might be from the fact that the optimal temperature for the activity of the enzymes should be in the range of 45–50°C. However, the temperature applied in this process was at 35°C. For the fermentation by yeast, the optimal temperature required has been known at 30°C [14]. Furthermore, the other conditions for the fermentation by these 2 microorganisms might not be optimal resulting in less ethanol production than it should have been. Therefore, the investigation on the optimal conditions about the various factors such as pH, temperature, oxygen content, and the medium ingredients affecting the fermentation is considerably imperative. This will enhance the efficiency of the sugar cofermentation from both *S. cerevisiae* and *P. stipitis*. The method to produce high concentration of ethanol can be accomplished by producing the enzyme with high hydrolytic activities to increase the efficiency of the hydrolysis. Moreover, the obtained enzymes should exhibit high activities at low concentration. Since the enzyme applied in this research was totally isolated from the fungi in our lab, the activities were therefore considerably low compared to the commercials. As a consequence, the large quantities of the enzyme were necessarily applied for the process. This might be the reason why the obtained ethanol concentration was rather low. The improvement of the production media such as the sources of carbon and the growth conditions for the culture should greatly enhance the production of the enzyme with higher activities. In addition, the development of the better strain by genetic manipulation also represents the interesting method to increase the fungal capacity for the production of the better enzyme with higher activities. This can be achieved through the higher efficiency of the fermentation in various aspects, for example, the strains that can possibly use various types of sugars as the substrates, the capability of the organisms to tolerate the high temperature, and high concentration of ethanol. In particular, in case that cellulosic ethanol will be further produced for the industries in the future, the development of the strains with the capability of simultaneous hydrolysis and fermentation of lignocellulose should be more advantageous.

4. Conclusions

Lignocellulosic biomass, like grasses, could be successfully used in cellulosic ethanol production by biological process. Pangola grass has potential for saccharification before fermentation process, whereas dwarf napier grass has potential for fermentation by SSCF process. Therefore, they are potential alternative energy crops to serve as feedstocks for cellulosic ethanol production in the future for Thailand.

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Research Article

Changes in the Material Characteristics of Maize Straw during the Pretreatment Process of Methanation

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Pretreatment technology is important to the direct methanation of straw. This study used fresh water, four bacterium agents (stem rot agent, "result" microbe decomposition agent, straw pretreatment composite bacterium agent, and complex microorganism agent), biogas slurry, and two chemical reagents (sodium hydroxide and urea) as pretreatment promoters. Different treatments were performed, and the changes in the straw pH value, temperature, total solid (TS), volatile solid (VS), and carbon-nitrogen ratio (C/N ratio) under different pretreatment conditions were analyzed. The results showed that chemical promoters were more efficient than biological promoters in straw maturity. Pretreatment using sodium hydroxide induced the highest degree of straw maturity. However, its C/N ratio had to be reduced during fermentation. In contrast, the C/N ratio of the urea-pretreated straw was low and was easy to regulate when used as anaerobic digestion material. The biogas slurry pretreatment was followed by pretreatments using four different bacterium agents, among which the effect of the complex microorganism agent (BA4) was more efficient than the others. The current study is significant to the direct and efficient methanation of straw.

1. Introduction

The rural household biogas in China has rapidly developed in recent years. By 2010, the number of rural household biogas users had reached 41.8 million in China [1, 2]. However, the rural household biogas industry has been facing a fermented material shortage due to the changes in agricultural structure. Moreover, the use of anaerobic digesters has been discontinued due to material shortage [3–5]. In China, the straw yield is nearly 7×10^9 t every year [6, 7] (of which rice, corn, and wheat straw account for 79.5%) [8]. Aside from the small portions used as animal feeds or returned to the field, most straws are either used as fuels or burned directly in the fields, which cause a huge waste [9–11]. However, straw as fermented material possesses many problems, such as long run-up time, low gas output, low material utilization ratio, and material crusting [12, 13]. Crop straw has high contents of lignin, cellulose, and hemicellulose, and the degradation is difficult. These problems seriously affect the fermentation process and material processing of straw [14]. Therefore, straw pretreatment is essential for an efficient direct methanation.

Maize straw is a lignocellulosic biomass which contains components such as cellulose (34.0%), hemicellulose (37.5%), and lignin (22%). The carbon-nitrogen ratio (C/N ratio) for maize straw is about 66.31%, while the proper C/N ratio for anaerobic digester should be within the range of 25–35 [15]. At present, straw pretreatment studies focus on adjusting the nutritional value of straw and improving its characteristics [16, 17]. Adjusting the nutritional value

is usually achieved by regulating the C/N ratio by mixing straw with fermented materials having different carbon and nitrogen contents. Therefore, exogenous nitrogen needs to be supplemented to increase the nitrogen fraction for more efficient anaerobic digestion of maize straw. Nitrogen can be added in the form of inorganic form (e.g., ammonium bicarbonate) or organic form (e.g., urea or animal manure). Animal manure and other organic wastes are additional nutrient sources, provided they are readily available for anaerobic digestion. Nitrogen fertilizer (e.g., ammonia or urea) is another nitrogen source that can be easily added to the maize straw if nitrogenous wastes are not available [18]. On the other hand, straw characteristics are improved by using physical, chemical, or biological pretreatment, which improve the straw's utilization rate [16]. Lignocellulose is difficult to degrade biologically. Pretreatment of straw by mechanical size reduction, heat treatment, and/or chemical treatment usually improves its digestibility. Chemical pretreatment methods that have been explored in previous research include bicarbonate treatment [19], radiation [20], alkaline peroxide treatment [21], and ammonia treatment [22]. Among them, ammonia treatment has several advantages over the other ones, since ammonia itself is a nitrogen source for biodegradation.

Based on the theory and practice of straw anaerobic digestion and rural methane fermentation, this study investigated pH, temperature, total solid (TS), volatile solid (VS), and carbon-nitrogen ratio (C/N ratio) of maize straw during biological and chemical pretreatment processes. Moreover, the changes in the material characteristics during the process were compared, and efficient straw pretreatment agents were chosen according to the degree of straw maturity. The current study also provides theoretical reference for practical methanation.

2. Materials and Methods

2.1. Raw Material. Air-dried maize straw used in this study was collected from the experimental field of Northwest Agriculture and Forestry University in Yangling, China. Before the pretreatment, the maize straw was chopped into 2-3 cm pieces [23]. The raw material contained 79.50±0.42% of TS, and there were about 89.20% VS in the dry matter. The C/N ratio of the raw material was 66.31.

2.2. Biological Pretreatment. The biological pretreatment promoters were mixed microorganism, which were bacterium agent 1(BA1) [24] (stem rot agent, main composition: *Bacillus polymyxa, Bacillus subtilis, Bacillus brevis, Bacillus licheniformis, Brevibacterium sulphureum,* and so on.), bacterium agent 2(BA2) [25] ("result" microbe decomposition agent, main composition: *Saccharomyces cerevisiae, Coccidioides, H. anomala, S. cerevisiae, Bacillus licheniformis, Pseudomonas, Leucothrix, Lactobacillus delhi* and so on.), bacterium agent 3(BA3) [26] (straw pretreatment composite bacterium agent, main composition: *Bacillus subtilis, Streptomyces microflavus, Trichoderma koningii, Chaetomium globosum,* and so on.), and bacterium agent 4(BA4) [27] (complex microorganism agent, main composition: *Bacillus subtilis, Bacillus natto, Streptoverticillium baldaccii, Thermoactinomyces vulgaris, Saccharomyces cerevisiae, Candida utilis, Candida tropicalis, Aspergillus niger, Aspergillus oryzae, Rhizopus nigricans,* and so on.).

These four kinds of microorganism were weighed 0.04, 8.00, 8.00, and 8.00 g, respectively, according to their different number of viable bacteria. Then these pretreatment promoters were put into four different 2000 mL beakers, which contain 2000 g fresh water. After that, the mixer was put into a constant temperature incubator for a 24 h cultivation in 37°C [28]. The activated promoters were then added into the pretreatment reactor, which contained 800 g maize straw. The pretreatment process was 10 days.

Biogas slurry was obtained from an anaerobic digester that produced gas normally in Yangling, China. 2000 g biogas slurry was added in the pretreatment reactor with 800 g maize straw, and the pretreatment process was 10 days.

2.3. Chemical Pretreatment. Sodium hydroxide and urea were used in this study. The amount of chemicals added was referred to in the previous researches [29, 30]. This study added 800 g maize straw, 2000 g fresh water, and then 160 g sodium hydroxide and 160 g urea, respectively. The pretreatment process lasted for 10 days.

In this study, fresh water was used as the control group, and 2000 g was added into 800 g maize straw for a ten-day pretreatment. All operations were of unified management, and the experiment was repeated three times.

2.4. Tested Indexes and Methods. The pH value was measured by intelligent pH meter (pHs-3CT, China) every day. Temperatures at the center of each pile as well as environmental temperature were recorded manually by a thermometer every day. The untreated and treated maize straw samples were analyzed for TS and VS, according to the APHA standard methods [31]. The total organic carbon (TOC) was determined using the $K_2Cr_2O_7$ volumetric and outside heating methods [32]. The total organic nitrogen (TON) was analyzed by Kjeldahl method (Model KDN-08C, Shanghai, China) as recommended by Cottenie et al. [33], while the carbon-nitrogen (C/N) ratio was calculated using values of the TOC and TON.

3. Results

3.1. Changes in the Physical and Chemical Characteristics of Maize Straw before and after Pretreatment. The characteristics of maize straw between before pretreatment and after ten-day pretreatment were compared in Table 1. After pretreatment, TS of maize straw was dramatically decreased, which was between 11.07 ± 1.33% and 20.00 ± 0.30%. Significant differences were observed between control group (11.53 ± 0.55%) and experimental groups, except those treated with BA1 (11.07 ± 1.33%) and BA2 (11.83 ± 0.21%). An extremely significant difference was also observed between the control group and the specimens pretreated with BA4 (14.31 ± 0.37%),

Table 1: Basic characteristics of maize straw before and after ten-day pretreatment.

Pretreatment promoters		TS[a] (%)	VS[b] (%)	TOC[c] (%)	TON[d] (%)	C/N ratio
Raw material		79.50 ± 0.42	89.20 ± 0.31	36.95 ± 0.28	0.56 ± 0.02	66.31
After ten-day pretreatment	BA 1	11.07 ± 1.33	92.33 ± 0.45	33.00 ± 0.43	0.54 ± 0.00	61.05
	BA 2	11.83 ± 0.21	91.65 ± 0.64	33.78 ± 0.41	0.72 ± 0.05	46.68
	BA 3	13.67 ± 0.23	90.36 ± 0.37	34.69 ± 0.55	0.68 ± 0.05	50.90
	BA 4	14.31 ± 0.37	89.64 ± 0.27	32.50 ± 0.30	0.70 ± 0.03	46.53
	Urea	14.64 ± 0.60	91.24 ± 0.32	32.43 ± 0.43	1.45 ± 0.02	22.36
	Sodium hydroxide	20.00 ± 0.30	44.63 ± 0.45	15.03 ± 0.24	0.25 ± 0.02	60.25
	Biogas slurry	13.71 ± 0.34	86.11 ± 0.39	28.00 ± 0.08	0.84 ± 0.00	33.23
	Fresh water	11.53 ± 0.55	93.19 ± 0.38	33.37 ± 0.54	0.59 ± 0.01	56.26

[a]TS: total solid.
[b]VS: volatile solid, dry basis.
[c]TOC: total organic carbon, dry basis.
[d]TON: total organic nitrogen, dry basis.

ure(14.64 ± 0.60%),andsodiumhydroxide (20.00 ± 0.30%). The VS content of the sodium hydroxide-treated specimen (44.63 ± 0.45%) was significantly different from that of the raw material (89.20% ± 0.31), while VS of the other treatments fluctuated between 86.11 ± 0.39% and 93.19 ± 0.38% without substantial change. Compared with raw material, TOC of all experimental groups decreased. TOC of the sodium hydroxide-treated specimen was only 15.03 ± 0.24%, indicating that majority of water-insoluble carbon was decomposed and transformed during the pretreatment process. TON of the groups treated with BA1 (0.54%) and sodium hydroxide (0.25 ± 0.02%) both decreased, while others increased. The C/N ratios of the pretreated straws were lower than that of the raw material, implying that pretreatment reduces the C/N ratio of fermented materials, which is significant to the life activities of methanogenic bacteria.

3.2. Changes in pH. pH plays a crucial role in the growth metabolism of microbes. Microorganism used in this study was a mixture which contained bacterium, fungus, saccharomycetes, and actinomycetes, with its suitable pH varying from 4.5 to 6.5. pH of BA1 treated decreased to the range of 4.1 to 4.8 from the 5th day, which is unfavorable for bacterium growth. pH of BA2 treated fell to 4.4 on the 4th day. However, it gradually increased later, and hence, the bacterium activity significantly decreased. pH of BA3 treated was stable between 4.7 and 6.2 before the 8th day. However, pH was then decreased to 4.3 at the 9th day and kept at the same level at the 10th day. pH of BA4 treated remained between 4.7 and 5.9. Thus, the bacterium grew well under the moderate conditions. Compared with that of control group, pH of biogas slurry-treated group was higher at the first two days. Then, it started to decline three days later and stabilized between 6.15 and 7.75, maintaining a neutral condition, which fell in the scope of the suitable pH.

pH of two chemically treated specimens was both higher than the others, particularly between 8.9 and 9.2 and between 11.3 and 11.8 for the urea and sodium hydroxide-treated groups, respectively. pH of control group was kept falling down and then fluctuated near 4.4 since the 4th day (Figure 1).

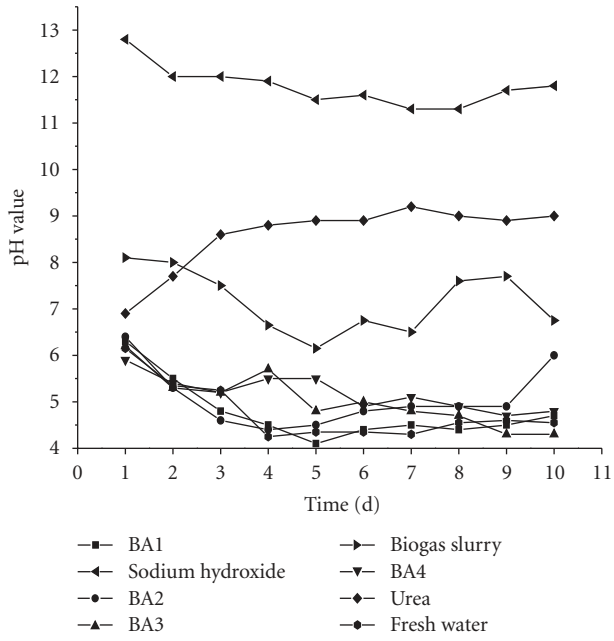

Figure 1: Changes in pH level during the pretreatment process.

3.3. Changes in Temperature. Temperatures of the environment and every treated group were shown in Figure 2. The temperature of the environment significantly changed during the 6th and 8th days, whereas those of the treated groups did not. The temperature of the group treated with BA1 was slightly lower than that of environment during the 3rd and 5th days, whereas the other bacterium agents groups had higher temperatures than that of environment. This result can be attributed to the restrained growth of BA1 due to the interaction of the pH and temperature during the process, which thus causes the decline of the decomposition characteristics of the material. The temperatures

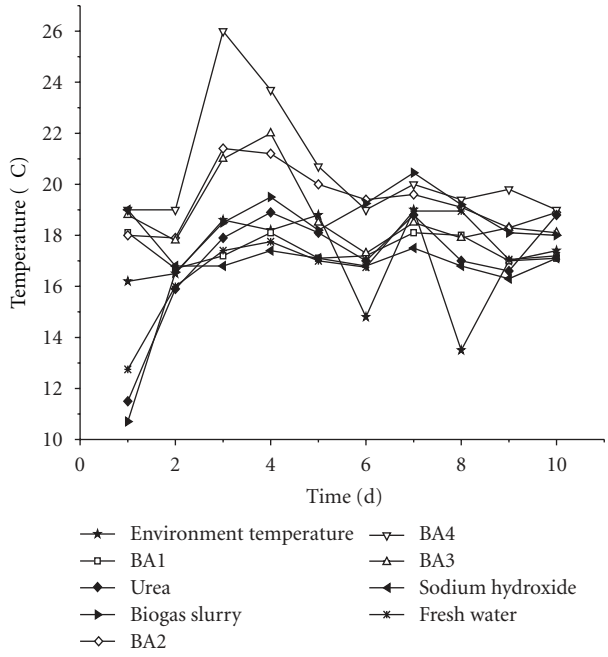

Figure 2: Changes in the temperature during the pretreatment process.

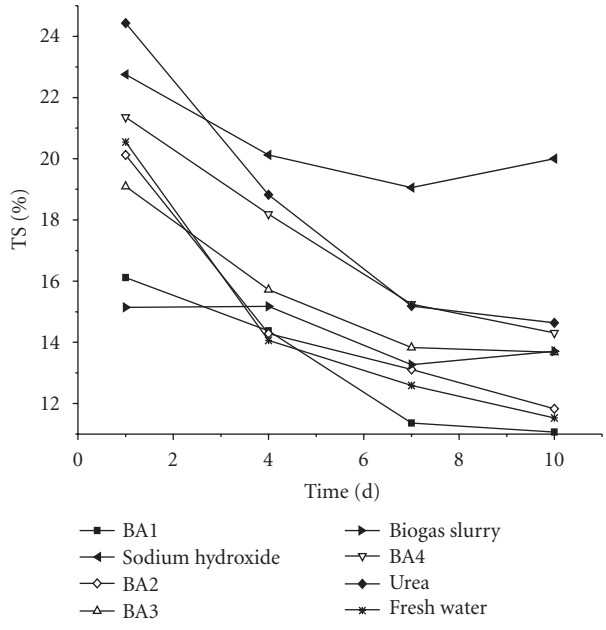

Figure 3: Changes in the TS content during the pretreatment process.

of the groups treated with BA3 and BA4 were high and thus beneficial to the straw decomposition. Moreover, the temperatures of the two chemically treated groups slightly fluctuated along the temperature of environment. During the 1st day, the temperature of the urea-treated group was quite low (only 11.5°C) and that of the sodium hydroxide-treated group was higher (19.0°C). Both seemed to stabilize after that and, hence, were beneficial for the organic decomposition

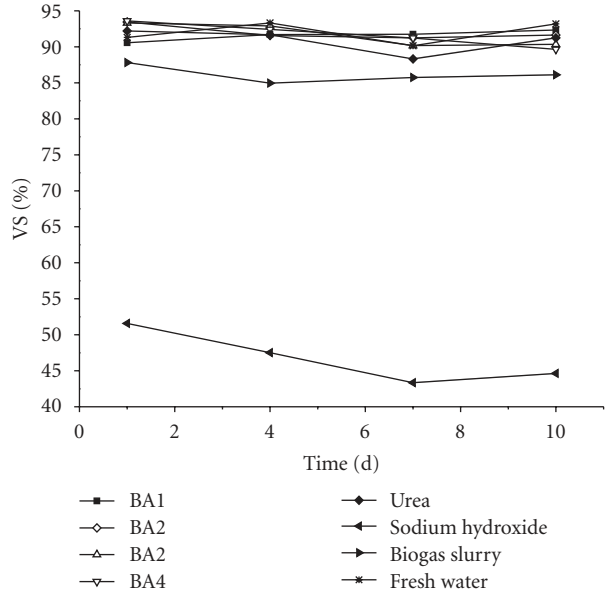

Figure 4: Changes in the VS content during the pretreatment process.

in straw. The temperatures of the biogas-slurry-treated and control groups were subject to the environment.

3.4. Changes in TS and VS. TS and VS are two indexes of the degree of decomposition maturity and are important parameters of the fermented substrate concentration. Figure 3 showed that the TS contents of all experimental groups were higher than that of the control group since the 4th day, indicating the effect of the promoters on straw decomposition. However, these TS contents all decreased as time elapsed. During the 1st day, the TS content of the urea-treated group was the highest, followed by that of the sodium hydroxide-treated group. However, the former declined by 9.79%, whereas the latter decreased by only 2.1%. Among the biological promoters, TS of the group treated with BA4 was higher than those of the others, whereas those of the groups treated with BA1 and biogas slurry stayed at low levels without significant changes. In summary, during the pretreatment process, the two chemical promoters induced the highest degrees of straw decomposition maturity, whereas BA4 and BA1 and biogas slurry had lesser effects.

Figure 4 showed the changes in VS. VS of all groups stabilized between 88.31% and 93.61% without significant fluctuations and differences, except those of the groups treated with sodium hydroxide and biogas slurry. VS of the sodium hydroxide-treated group was the lowest (43.35% to 51.18%), followed by that of the group with biogas slurry (84.95% to 87.80%), with slight fluctuations.

3.5. Changes in C/N Ratio. C/N ratio (Figure 5(a)) of pretreated straw increased firstly and decreased subsequently, and had the same trend as TOC (Figure 5(b)). However, TON (Figure 5(c)) varied slightly except urea-treated group. Urea as a rich nitrogen promoter added exogenous nitrate in

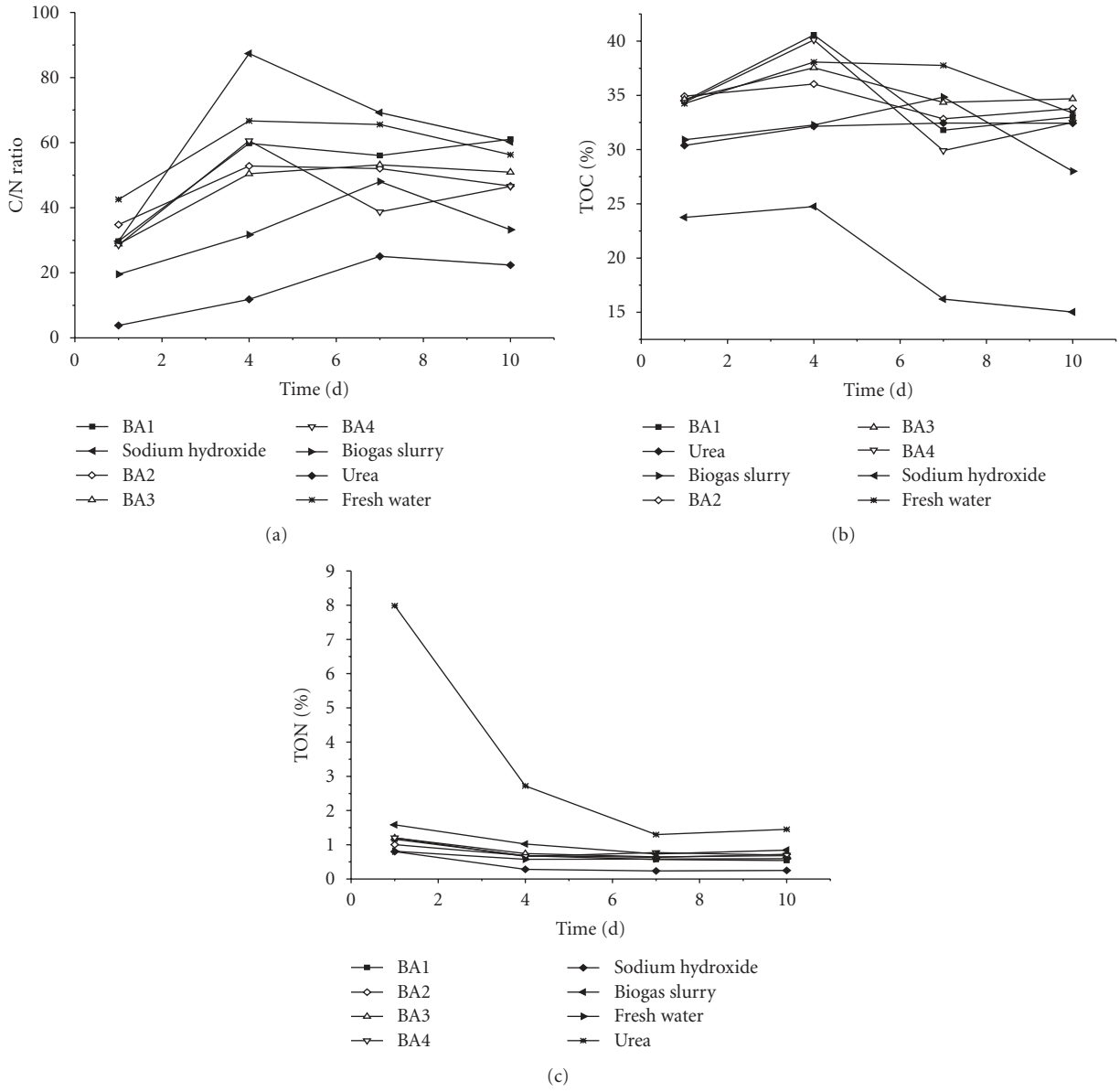

Figure 5: Changes in C/N ratio, TOC, and TON during the pretreatment process: (a) changes in C/N ratio, (b) changes in TOC, (c) changes in TON.

material. So C/N ratio of urea-treated group was the lowest from beginning to end. Its C/N ratio was only 3.80 on the 1st day. However, it gradually increased with time and remained between 3.80 and 22.36. C/N ratios of BA1-treated, sodium hydroxide-treated, and control groups were very high during and after pretreatment, which were 51.65, 61.64, and 57.76 in average, respectively. In biological pretreatment, BA2, BA3, and BA4 treated had the same value in TOC and TON, and the C/N ratio was very close to 46.58, 45.86, and 43.59 in average, respectively. For its lower TOC and higher TON, C/N ratio of biogas slurry-treated group was kept at a lower level (33.12 in average). And the same results were achieved by Zhong et al. [34].

4. Discussions

Straw maturity is a key factor of the pretreatment. Several authors have concluded that using a single parameter as a maturity index is insufficient and that amalgamation of several parameters is usually needed. Various physical, biological, and chemical parameters have been used to monitor the quality and maturity of compost [35–37]. The effects of different promoters on the changes of the indexes during the pretreatment process varied, as well as the requirements of the different promoters on the pretreatment external conditions. Only in suitable temperatures, pH levels, and other environment conditions can bacterium agents

obtain good effects. Too high or low temperatures and unsuitable pH levels can impede the normal life metabolism of microorganisms and thus influence the degree of straw decomposition maturity during the pretreatment process [38]. For the group treated with the stem rot agent (BA1), pH level of the material was lower during the early stages and was even as low as 4.1 during the 5th day. The temperatures on the 3rd and 5th days were lower than the environmental temperature, thus restraining the growth of microorganism and negatively affecting the straw organic degradation. Complex microorganism agent- (BA4-) treated group could maintain a better living condition for microbe, and its treatment effect was superior to other bacterium agent treatments. Urea and sodium hydroxide are both alkaline and, hence, beneficial to the degradation of lignocellulose and hemicellulose. Studies results showed that sodium hydroxide treatment can improve the conversion rate of lignocelluloses [39–47]. Moreover, the results of the studies by Chandra and Jackson [29] and Chesson [48] suggested that the degradation of lignocellulose was optimal upon the addition of 10% of sodium hydroxide. During the pretreatment process, the TS contents of the urea and sodium hydroxide-treated groups were both high. These results indicate that, under alkaline conditions, the lignocellulose degradation rate is improved, macromolecular substances are decomposed, the water-holding capacity of straw is decreased, and the water content of straw is lower than those of other samples of equivalent weight. The sodium hydroxide-treated group had an obviously lower VS content than those of the other groups. However, its C/N ratio was higher. Thus, some restrictions were encountered when the group was used as fermented materials, and better effects could have been achieved by adding nitrogen to regulate the C/N ratio to a suitable value. The biogas-slurry-treated group was more suitable for use in microorganism anaerobic fermentation, with only modest changes in the material's characteristics and a suitable C/N ratio of 33.23. The control group had a lower pH level, temperature, and TS content. Its C/N ratio was higher, and the straw degradation was bad. Hence, its treatment effect was worse than those of the promoters.

5. Conclusions

Synthesizing each index of pretreatment material, it can be summarized that the effect of sodium hydroxide-pretreated group was better than any others, followed by urea-treated group. In biological pretreatment, biogas slurry was the best promoter, for its good corrosion effect, and more economic. BA4 also had a good effect on straw maturity, and the next is BA3 treated. BA1 treated was the worst group, because its microorganisms' survival conditions were limited by unsuited pH and temperature.

As compared with biological pretreatment, chemical treatment is easier to operate and has good effect. However, it will be a difficult and expensive task to recycle the chemicals used for the hydrolysis to avoid the environmental pollution. On the other hand, although the biological pretreatment was less effective than the sodium hydroxide and urea treatment, there exists a big room for improvement of the microbial degradation of cellulosic biomass by optimization of the fungal growth conditions and manipulation of the process parameters such as pH and temperature. However, in order to approach the biomass-to-fuels issue in a more environmentally friendly way, we will continue to improve the efficiency of biological treatment of maize straw to optimize the biogas production.

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Research Article

PHA Productivity and Yield of *Ralstonia eutropha* When Intermittently or Continuously Fed a Mixture of Short Chain Fatty Acids

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The research described in this present study was part of a larger effort focused on developing a dual substrate, dual fermentation process to produce Polyhydroxyalkanoate (PHA). The focus of this study was developing and optimizing a strategy for feeding a mixture of SCFAs (simulated ARF) and maximizing PHA production in a cost-effective way. Three different feeding strategies were examined in this study. The substrate evaluated in this study for the growth phase of *R. eutropha* was condensed corn solubles, a low-value byproduct of the dry-mill, corn ethanol industry. The culture was grown to high cell densities in nitrogen-supplemented condensed corn solubles media in 5 L bioreactors. The overall growth rate of *R. eutropha* was 0.2 h^{-1}. The 20 mL ARF feeding every 3 h from 48 to 109 h strategy gave the best results in terms of PHA production. PHA productivity (0.0697 g L^{-1} h^{-1}), PHA concentration (8.37 g L^{-1}), and PHA content (39.52%) were the highest when ARF was fed every 3 h for 61 h. This study proved that condensed corn solubles can be potentially used as a growth medium to boost PHA production by *R. eutropha* thus reducing the overall cost of biopolymer production.

1. Introduction

Biodegradable polymers made from renewable resources such as agricultural wastes, corn, cassava, tapioca, whey, and so forth, do not lead to depletion of finite resources. The most studied of the biodegradable polymers include polyesters, polylactides, aliphatic polyesters, polysaccharides, and various copolymers [1]. These biopolymers have many of the desirable physical and chemical properties of conventional synthetic polymers [2]. To this point, high production costs have limited the use of biopolymers. However if these costs can be reduced, there would be widespread economic interest [3].

The focus of this project was production of the biopolymer Polyhydroxyalkanoate (PHA) at a low cost. PHA is actually a term used to describe a diverse family of polymers that are composed of 3-hydroxy fatty acid monomers. The carboxyl group of one monomer forms an ester bond with hydroxyl group of the neighboring monomer. Polyhydroxybutyrate (PHB) has been studied in most detail. PHB has good oxygen impermeability, moisture resistance, water insolubility, and optical purity [4, 5]. Young's modulus and tensile strength of PHB are similar to polypropylene, but elongation at break is 6% as opposed to that of 400% for polypropylene [6]. It has good UV resistance, but poor resistance to acids and bases [7]. The oxygen permeability is very low, making PHB a suitable material for use in packaging oxygen-sensitive products. PHB has low water vapor permeability compared to other bio-based polymers but higher than most standard polyolefins and synthetic

polyesters [5, 8]. Since PHB is toxicologically safe, it can be used for articles which come into contact with skin, feed, or food [9].

In the food industry, PHA has a wide application as edible packaging material, coating agent, flavor delivery agent, and as dairy cream substitute [10, 11]. It can also be used for making bottles, cosmetics, containers, pens, golf tees, films, adhesives and nonwoven fabrics, toner, and developer compositions, ion-conducting polymers, and as latex for paper coating applications [12, 13]. It can be used to make laminates with other polymers such as polyvinyl alcohol. The degradation products of PHB are found in large concentrations in human-blood plasma, so is not toxic for human use [14].

PHA production by *Ralstonia eutropha (R. eutropha)* generally occurs during stationary phase. Hence cells are first grown to high density, after which a key nutrient is limited to trigger PHA synthesis [14]. Because of this dual phase process, PHA production lends itself to fed-batch, as well as, continuous operation. This follows Pontryagin's maximum principle, which is an optimal feeding strategy for fed-batch fermentation [15]. The key to this principle is determining the optimum switching time (t_c). The maximum growth rate ($\mu_{\max}$) should be initially maintained, then switched to the critical growth rate (μ_c) at t_c to maximize the specific product production rate ($\rho_{\max}$). Since growth rate is affected by Carbon : Nitrogen (C : N) ratio, it should also be changed at t_c.

A variety of carbon sources have been used for production of PHA using different fermentation strategies. Carbohydrates, oils, alcohols, fatty acids, and hydrocarbons are potential carbon sources for PHA production. Ethanol byproducts, cane and beet molasses, cheese whey, plant oils, hydrolysates of corn, cellulose, hemicellulose, palm oil, soybean oil, tallow, corn steep liquor, casamino acids, and food scraps had been used as substrates to produce PHA using different organisms [16–21].

The substrate evaluated in this study for the growth phase of *R. eutropha* was condensed corn solubles (CCS), which is a low-value byproduct of the dry-mill, corn ethanol industry. In the dry mill process, the whole corn is milled, mixed with water, and enzymatically hydrolyzed to convert starch to glucose, which is converted to ethanol by fermentation. After distillation to remove ethanol, the larger corn particles are recovered by centrifugation as distiller's wet grains. The supernatant is condensed in multiple-effect evaporators to give condensed corn solubles [22].

The composition of CCS is shown in Table 1. Corn-milling byproducts are typically marketed as animal feed because of their protein content. However, these byproducts may also contain residual carbohydrates, which might be utilized by microbial fermentation to produce industrial biopolymers. CCS is an excellent source of vitamins and minerals, including phosphorous and potassium.

The objective of this study was to determine the effects of adding artificial rumen fluid (ARF) on cell viability, growth as well as PHA production to a 48 h culture of *R. eutropha*. Different strategies of feeding simulated ARF into the bioreactors were assessed to maximize PHA production.

Table 1: CCS composition from a dry mill ethanol plant.

Components	CCS
Dry matter %	34.9
Crude protein %	13.7
Crude fat (ether extract) %	16.2
Ash %	9.5
Crude fiber %	0.8
Copper ppm	8.3
Sodium ppm	5,620
Calcium ppm	487
Magnesium ppm	6,850
Zinc ppm	49
Phosphorus ppm	15,400
Potassium ppm	22,900

Composition is on dry matter basis.

2. Materials and Methods

2.1. Culture, Maintenance, and Inoculum Propagation. The ATCC 17699 type strain of *R. eutropha* was used. The culture was routinely transferred to nutrient broth and incubated on a reciprocating shaker (250 rpm) at 30°C for 24 h. For short-term maintenance, the culture was stored on Tryptic Soy Agar (TSA) slants covered with mineral oil and stored in the refrigerator. Inoculum for all trials was prepared in a stepwise manner, by transferring the culture from TSA plates into 100 ml of the CCS medium (described below), then incubating for 24 h on a rotary shaker (250 rpm) at 30°C. The inoculum rate for all bioreactor trials was 1% ($v\,v^{-1}$) from a 24 h grown culture to an average OD of 1.04.

2.2. Medium. A low-cost medium based on CCS was developed in a prior study [23]. This medium, containing 240 g CCS L^{-1}, with a C : N ratio of 50 : 1 was the best medium for the growth of *R. eutropha*. The medium was prepared by mixing 1,370 mL CCS with 4,630 mL deionized water, adjusting the pH to 6.5 using 10 M sodium hydroxide (NaOH), then centrifuging at 11,000 rpm for 7 min at 15–25°C. The supernatant was then filtered through Whatman filter paper #113 and autoclaved. A filter sterilized 178 g L^{-1} ammonium bicarbonate (NH_4HCO_3) stock solution was prepared and then 20.4 mL of this solution was added to each liter of CCS medium to adjust the C : N ratio to 50 : 1. The pH was further adjusted to 7.0 by adding 10 N sulfuric acid (H_2SO_4) before inoculation.

2.3. Fermentation Conditions for Cultivation of R. eutropha in Bioreactor. Experimental trials were conducted in 5 L New Brunswick, Bioflo III, Edison, NJ bioreactor that contained 4 L of CCS medium. Filter sterilized air (1 L L^{-1} min^{-1}) was sparged into the bioreactor, and 2-3 mL of antifoam (Cognis Clerol FBA 5059, Cognis, Cincinnati, OH) were added to the medium before inoculation. Fermentation medium was incubated at 30°C and 500 rpm for 48 h, since prior research had shown *R. eutropha* to reach its

maximum population by this time/temperature combination [23].

At 48 h we began feeding the fermenter a mixed short-chain fatty acid (SCFA) solution, using any of the three different strategies. This SCFA solution, referred to as ARF, contained 10 parts acetic, 2 parts butyric, 15 parts lactic, and 20 parts propionic acids ($v\,v^{-1}$). The composition of ARF was based on a separate study evaluating fermentation of biomass with rumen consortia. A total volume of 372 mL of ARF was fed to the culture over a period of 48 h. In the first feeding strategy (24 h feeding), 124 mL of ARF was added at 48, 72, and 96 h. In the second feeding strategy (3 h feeding), 20 mL ARF was added at 48 h and then every 3 h until 109 h. In the third feeding strategy (continuous feeding), ARF was continuously added from 48 to 96 h at a rate of 7.75 mL h^{-1}. All incubations were continued until 144 h. Two replications were performed for each feeding strategy to determine the effect of mixed fatty acids on cell viability, acid utilization, and PHA production.

2.4. Analytical Methods

2.4.1. Viable Counts, Cell Dry Weights, pH, HPLC, and Ammonium/Phosphate Analysis. Samples were collected every 12 h and viable cell counts were done with TSA. At 72, 96, and 144 h, 50 mL samples were collected to determine cell dry weights. Samples were centrifuged and the precipitate was dried in the hot air oven at 80°C for 2 days. pH was measured using an Acumet 950 pH meter (Thermo Fisher Scientific of Waltham, MA). Samples were also analyzed via a Waters HPLC system (Milford, MA) for sugars, organic acids and glycerol. These samples were first filtered through a nonsterile 0.2 μm filter to remove solids, and then frozen until analysis. An Aminex HPX 87H column (Bio-Rad Laboratories, Hercules, CA), operated at 65°C with a helium-degassed, 4 mM H_2SO_4 mobile phase at a flow rate of 0.6 mL min^{-1} was used. Peaks were detected using a refractive index detector. Standard solutions of maltose, glucose, lactic acid, butyric acid, acetic acid, propionic acid, succinic acid, and glycerol (at 3 and 30 g L^{-1}) were used to calibrate the integrator. Samples collected at 0, 72, and 120 h were also tested for ammonia and phosphate using Hach Ammonium and Phosphate Unicell tests (Hach Company, Loveland, Colorado).

2.4.2. PHA Analysis. To measure PHA, 50 mL samples of broth were collected at 72, 96, and 144 h and centrifuged. The pellets were then lyophilized and ground using a mortar and pestle. The method developed by Braunegg et al. [24] was used to simultaneously extract and derivatize PHA to the 3-hydroxyalkanoate methyl esters of the monomers. In this method, 20–30 mg of ground cells were digested by adding 5 mL of digest solution and incubating at 90–100°C for 4 h. The digest solution contained 50% chloroform, 42.5% methanol, 7.5% H_2SO_4 ($v\,v^{-1}$). After cooling, sample was washed with 2 mL of water, and the bottom layer (containing the chloroform and methyl esters of PHA) was collected and placed in a Gas Chromatography (GC) vial with anhydrous sodium sulfate (to remove residual water). Vials were frozen until analysis.

PHA was quantified using a Hewlett-Packard 5890 Series II (Palo Alto, CA) GC with a flame ionization detector (GC-FID) [24, 25]. Split injection was used onto a Supelco SSP-2380 (Park Bellefonte, PA) capillary column (30 m × 0.25 mm I.D. with 0.20 um film). The inlet head pressure was maintained at 28 psi, and the temperature program started at 50°C for 4 min, then increased by 3°C min^{-1} to a final temperature of 146°C for 4 min. The injector and detector temperatures were 230°C and 240°C, respectively. Purified poly (3-hydroxybutyric acid co-3-hydroxyvaleric acid) (P(HB-HV)) obtained from Sigma-Aldrich was used for a standard calibration. The copolymer consisted of 88% 3-hydroxybutyric acid (3-HB) and 12% 3-hydroxyvaleric acid (3-HV). Copolymer concentrations of 2–10 mg mL^{-1} were digested as above, and then analyzed by GC-FID. Retention times were 14.9 min for methylated 3-HB, and 17.8 min for methylated 3-HV.

2.5. Statistical Analysis. All trials were performed in duplicates. Various fermentation parameters (maximum cell populations, SCFA utilization rates, PHA productivity, etc.) were analyzed to determine least significant differences between treatments using randomized complete block design. Fermentation efficiency (FE) was calculated as the percentage of substrate supplied to that was consumed. PHA productivity was calculated as the concentration of PHA produced per hour whereas PHA content was determined as a percentage of PHA concentration over cell dry weight. Data were analyzed using the PROC GLM procedure of SAS software to determine F values and least squares (LS) means. Exponential regression equations were used to determine growth rates and acid utilization rates for each replication, from which means were calculated. They were statistically analyzed by ANCOVA to test homogeneity of slopes. Statistical data were analyzed at the significant level of $P < 0.05$.

3. Results

In all bioreactor trials, the organism was incubated under similar conditions (30°C, 500 rpm, and aeration at 1 L L^{-1} min^{-1}) for the first 48 h, and this data was relatively uniform. Figure 1 and Table 2 show the average cell population, growth rate, acid utilization, and ammonia and phosphate uptake rates during this growth period.

Compared to shake flasks trials (Table 3), the maximum cell population in bioreactor trials was almost 10-fold higher (2.3×10^{10} cfu mL^{-1}). Likewise, the growth rate of *R. eutropha* was also higher in the bioreactor (0.20 h^{-1}) compared to the aerated shake flasks (0.13 h^{-1}). In the shake flask trials, lactic acid was consumed at the fastest rate followed by acetic, butyric, succinic, and propionic acids. In the bioreactors, the organic acid utilization rates in the growth phase were generally similar to shake flask trials except for lactic acid. It was consumed slower than acetic acid. Overall acid utilization rates were higher in the bioreactors than in shake flasks probably due to the higher cell population. Percentage

Table 2: Average growth and nutrient utilization rates of *R. eutropha* in CCS medium through 48 h in biorector.

Maximum cell population (cfu mL^{-1})		Maximum growth rate (h^{-1})	Ammonia utilization rate (mg L^{-1} h^{-1})	Phosphate utilization rate (mg L^{-1} h^{-1})
2.3×10^{10}		0.20	2.3	0.023
		SCFA utilization rate (g L^{-1} h^{-1})		
Acetic	Butyric	Lactic	Propionic	Succinic
0.051	0.026	0.046	0.023	0.027
		SCFA FE (%)		
Acetic	Butyric	Lactic	Propionic	Succinic
100	100	81.25	65.5	71.2

Table 3: Average growth and nutrient utilization rates of *R. eutropha* in CCS medium through 48 h in shake flasks.

Maximum cell population (cfu mL^{-1})		Maximum growth rate (h^{-1})	Ammonia utilization rate (mg L^{-1} h^{-1})	Phosphate utilization rate (mg L^{-1} h^{-1})
2.6×10^{9}		0.13	1.7	0.022
		SCFA utilization rate (g L^{-1} h^{-1})		
Acetic	Butyric	Lactic	Propionic	Succinic
0.033	0.026	0.054	0.010	0.021
		SCFA FE (%)		
Acetic	Butyric	Lactic	Propionic	Succinic
77.6	76.2	94.2	35.6	62.7

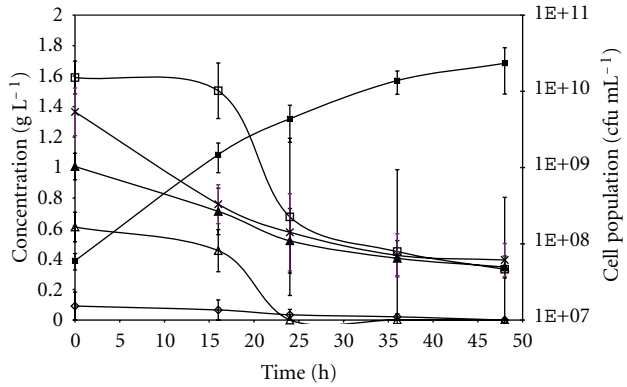

Figure 1: Average growth rate and organic acid utilization during the initial 48 h incubation in the CCS medium. The values for average cell count (■), acetic acid utilization (△), butyric acid utilization (◊), lactic acid utilization (□) propionic acid utilization, (▲) and succinic acid utilization (×) are indicated. Values are the means of two replications with standard deviation showed by error bars.

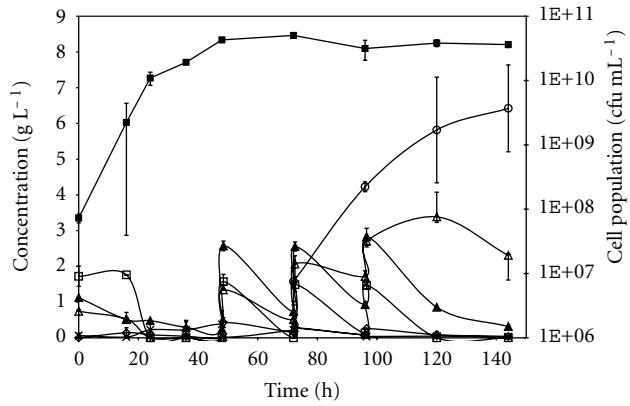

Figure 2: Acid utilization and growth of *Ralstonia eutropha* with 24 h interval additions of ARF. The values for cell count (■), acetic acid utilization (△), butyric acid utilization (◊), lactic acid utilization (□), propionic acid utilization (▲), succinic acid utilization (×), and PHA concentration (O) are indicated. Values are the means of two replications with standard deviation showed by error bars.

acid consumptions were also higher in the bioreactor with the exception of lactic acid. Ammonia and phosphate usage rates were higher in bioreactor trials again due to the higher cell population.

Three ARF-feeding strategies were compared in this study. Figures 2, 3, and 4 show that ARF feeding resulted in a slight increase in cell populations. The effects of different feeding strategies on maximum cell population, and ammonia and phosphate utilization rates are shown in Table 4. The maximum cell populations were not significantly different between the three treatments. In all cases, the cell population continued to rise after ARF additions began, suggesting that the acid levels were not inhibitory. The rates of ammonia utilization and phosphate utilization were similar for all feeding strategies.

As expected, the 24 h addition method (Figure 2) resulted in the highest spikes in SCFA concentration, with acid levels then falling as *R. eutropha* metabolized the acids to

Table 4: Comparison of all the key parameters under different ARF feeding strategies.

Key parameters	Feeding strategies		
	24 h addition	3 h addition	Continuous addition
Cell population at 48 h (CFU^{-1} mL)	4.28×10^{10a}	1.70×10^{10b}	1.01×10^{10b}
Maximum cell population (CFU^{-1} mL)	5.03×10^{10a}	5.68×10^{10a}	5.47×10^{10a}
Ammonia utilization rate (g^{-1} L^{-1} h)	1.2^a	1.0^a	1.1^a
Phosphate utilization rate (g^{-1} L^{-1} h)	0.010^a	0.013^a	0.011^a
Acid utilization (g^{-1} L^{-1} h)			
Acetic	0.029^a	0.052^b	0.047^b
Butyric	0.009^a	0.010^a	0.014^a
Lactic	0.074^a	0.080^a	0.078^a
Propionic	0.077^a	0.083^a	0.080^a
Combined	0.20^a	0.25^b	0.24^b
Fermentation efficiency (%)			
Acetic	58.8^a	100^b	98.7^b
Butyric	94.7^a	100^b	100^b
Lactic	100^a	100^a	100^a
Propionic	95.7^a	100^b	95^a
Combined	82.7^a	100^b	97.8^b
PHA production			
Cell dry weight (g^{-1} L)	17.6^a	21.13^a	17.3^a
PHA concentration (g^{-1} L)	6.42^a	8.37^a	6.67^a
PHA productivity (g^{-1} L^{-1} h)	0.0537^a	0.0697^b	0.056^a
PHA content (%)	36.23^a	39.52^a	37.78^a

[a,b,c] Means within column not sharing common superscript differ significantly ($P < 0.05$).

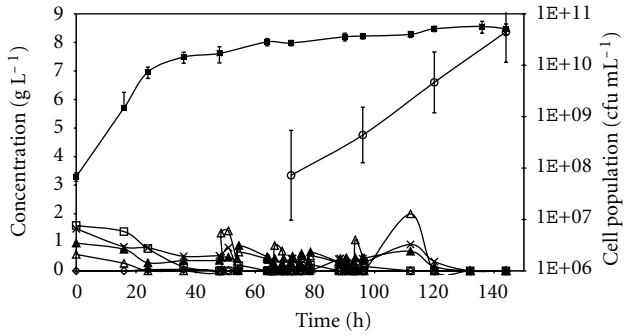

Figure 3: Acid utilization and growth of *Ralstonia eutropha* with 3 h interval additions of ARF. The values for cell count (■), acetic acid utilization (△), butyric acid utilization (◊), lactic acid utilization (□), propionic acid utilization (▲), succinic acid utilization (×), and PHA concentration (O) are indicated. Values are the means of two replications with standard deviation showed by error bars.

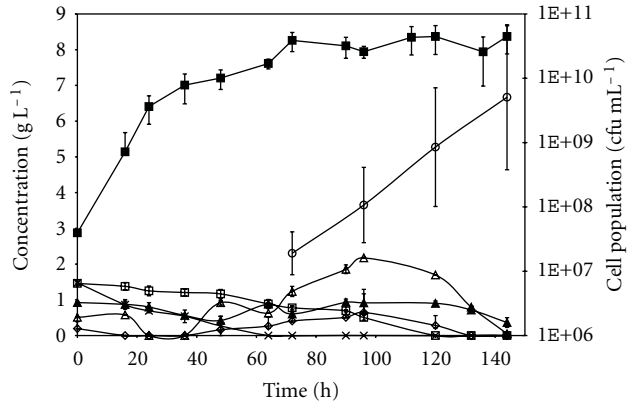

Figure 4: Acid utilization and growth of *Ralstonia eutropha* with continuous ARF addition. The values for cell count (■), acetic acid utilization (△), butyric acid utilization (◊), lactic acid utilization (□), propionic acid utilization (▲), succinic acid utilization (×), and PHA concentration (O) are indicated. Values are the means of two replications with standard deviation shown by error bars.

PHA. There was no apparent accumulation of lactic, butyric, or succinic acids for the 24 h feeding strategy. However, acetic acid (2.3 g L^{-1}), and to a lesser extent propionic acid (0.5 g L^{-1}), had accumulated over time. All the SCFAs were completely utilized by 144 h for the 3h feeding strategy. For the continuous-feeding strategy, all the SCFAs were consumed by 144 h with the exception of propionic acid (0.5 g L^{-1}).

Utilization rates of the individual SCFAs during the final 96 h of fermentation (48 to 144 h), along with the combined acid utilization rates, are shown in Table 4. The combined utilization rate also included consumption of succinic acid that was already present in the CCS medium. The highest combined acid utilization rates were observed in the 3 h and

Table 5: Comparison of combined short fatty acid feeding of *Ralstonia eutropha* in shake flasks.

Parameters	Volatile fatty acid			
	Acetic (5 g L^{-1})	Butyric (5 g L^{-1})	Lactic (8 g L^{-1})	Propionic (5 g L^{-1})
Maximum cell population (cfu mL^{-1})	5.70×10^{9a}	6.17×10^{9ab}	5.32×10^{9a}	6.67×10^{9b}
Fermentation efficiency (%)	70.6^{a}	95.6^{b}	70.7^{a}	68.6^{a}
Acid utilization rate (g L^{-1} h^{-1})	0.048^{a}	0.041^{a}	0.080^{b}	0.046^{a}
PHA concentration (g L^{-1})	2.9^{ab}	4.6^{a}	2.4^{b}	4.3^{a}
Cell dry weight (g L^{-1})	9.9^{ab}	14.5^{b}	6.0^{a}	14.7^{b}
PHA productivity (g L^{-1} h^{-1})	0.024^{ab}	0.037^{a}	0.020^{b}	0.036^{a}
PHA content (%)	29.2^{a}	31.9^{a}	30.7^{a}	29.3^{a}

[a,b] Means within column not sharing common superscript differ significantly ($P < 0.05$).

continuous feeding strategies, with the lowest rate in the 24 h feeding method.

The trend of higher acid utilization rates with the 3 h and continuous feeding methods also evident for the individual acids. However, the difference was only significant for acetic acid. Propionic and lactic acids were used most rapidly followed by acetic and butyric acid.

Table 4 also shows the FEs of the four SCFAs, along with the combined FE. The 3 h and continuous-feeding strategies resulted in the highest combined FE, while the lowest occurred with the 24 h feeding method. This is consistent with the lower acid utilization rates observed with the 24 h feeding strategy.

In comparing the individual acids, lactic acid was consumed completely, with greater than 95% utilization of propionic and butyric acids. FEs were higher for all the acids in the bioreactor trials compared to the prior shake-flask trials (Table 5).

Cell dry weights and PHA production parameters for the different ARF-feeding strategies are provided in Table 4. The 3 h feeding strategy resulted in the highest cell dry weight, PHA concentration, and PHA content, but the values were not significantly different from the other feeding strategies. Only the PHA productivity for the 3 h feeding strategy was significantly higher. Cell dry weight and PHA production were much higher than that obtained during the shake-flask trials. The highest PHA concentration in the cells in the shake-flask trials was 4.6 g L^{-1} (Table 5), whereas in the fermenter trials, the PHA concentration of the cells was about 1.82 times higher [23].

4. Discussion

Typically, PHA production occurs during stationary phase. Hence cells are first grown through exponential phase in a balanced medium to maximize growth rate. This medium is formulated to run out of a key nutrient when the maximum cell population is achieved, then additional carbon is added by fed-batch or continuous mode to maximize PHA production [26].

In this study, nitrogen deficiency was used to trigger PHA production in the presence of excess carbon. Nitrogen-supplemented CCS medium resulted in better growth of *R. eutropha* in the bioreactors as compared to the shake flasks due to the improved aeration and agitation provided in the bioreactor, along with more consistent pH control. The organism continued to grow after the SCFAs were added at 48 h. This growth was supported by the residual ammonia and phosphorus present in the medium. The organism reached its stationary phase by 96 h when most of the ammonia and phosphorous present in the medium were consumed. Ideally, one or both of these nutrients becomes limiting at the end of exponential phase, to trigger the shift from reproductive metabolism to PHA synthesis [26]. Because nitrogen and phosphate were not depleted until 72 h, this could have contributed to the continued increase in cell numbers observed after 48 h. It is likely that at least some of the SCFAs fed at 48 h were utilized for growth, until the point at which nitrogen became limiting. Researchers have found that the complete lack of nitrogen may suppress enzyme activity in PHA synthesis [27]. Thus, a small amount of ammonia in the media might be necessary to trigger PHA synthesis.

The utilization rate of lactic acid in the initial 48 h was lower than that of acetic acid probably due to higher concentration of lactic acid (1.6 g L^{-1}) in the bioreactor media compared to the shake-flask media (1 g L^{-1}). This variation was due to the different batches of CCS obtained from the ethanol plant.

In this study, fed-batch (24 h feeding) and continuous-feeding strategies were chosen to determine their effect on PHA production. The lowest acid utilization rates and FEs were observed for the 24 h feeding strategy due to the periodic spikes in SCFA concentrations, that might have disrupted the acid utilization and decreased cell activity. At high SCFA concentration the pH of the medium can reach below the pKas for SCFAs (lactic 3.86, acetic 4.76, butyric 4.83, and propionic 4.87). At low pHs the undissociated form predominates, and the SCFAs readily cross the cell membrane. Once inside, they rapidly dissociate and acidify the cytoplasm [28]. As a result, the proton gradient cannot be maintained as desired, and energy generation and transport systems dependent on proton gradient are disrupted [29]. This can also cause an increase in osmotic pressure due to the accumulation of anions [30]. At pH levels closer to the optimum for *R eutropha* (~7.0), SCFAs would be in the dissociated form in the medium. While the anions wouldn't

be transported as readily, once inside the cell they would not cause the adverse effects of the undissociated form. Therefore, SCFAs can only be effective carbon sources when pH and SCFA concentrations are carefully regulated. Acid utilization rates might have also been reduced by depletion of certain acids at the end of each 24 h phase.

For the continuous strategy, small volumes of the SCFAs were fed continuously. Though FEs of the SCFAs were higher than that of the 24 h feeding strategy, there were slight accumulations in SCFAs towards the end of the fermentation.

It was thus necessary to develop an optimal feeding strategy which could potentially increase acid utilizations and FEs. The 3 h feeding strategy was thus chosen. Since the SCFAs were added in smaller doses at shorter intervals, pH of the medium was more efficiently regulated, which resulted in better utilization and 100% FE of the organic acids.

In comparison to the prior aerated shake-flasks trials (Table 5), which were fed with individual SCFAs, the addition of mixed acids to the bioreactor resulted in more rapid uptake of propionic acid and slower utilization of butyric acid. Propionate utilization rate rose from $0.046\,g\,L^{-1}\,h^{-1}$ in aerated shake flasks [23] to approximately $0.08\,g\,L^{-1}\,h^{-1}$ when added as a part of the ARF mixture in the bioreactor trials. This was likely due to the higher cell populations achieved in the bioreactor trials coupled with the fact that proprionate utilization by *R. eutropha* is energetically favorable [31]. Moreover, addition of propionic acid in small doses might have controlled the change in pH, and thus resulted in better utilization. The decline in the utilization rate of butyric acid, from $0.041\,g\,L^{-1}\,h^{-1}$ when fed individually in aerated shake flasks to $0.011\,g\,L^{-1}\,h^{-1}$ in bioreactor trials, may have been due to additional ATP needed to transport this acid [32, 33]. Thus utilization of other acids was preferred over butyric acid when fed as a mixture for growth.

The high FEs for lactic and propionic acids were consistent with the metabolic preference of *R. eutropha*, especially considering that the ARF contained 15 and $20\,g\,L^{-1}$ of these acids, respectively. The high FE of butyric acid may be due to the fact that ARF contained only $2\,g\,L^{-1}$ of this acid. While conducting the shake-flask trials, we had previously noted that *R. eutropha* did not prefer acetic acid, therefore its lower FE was not surprising.

Thus, it can be concluded that in the 24 h strategy the sudden rise in the SCFA concentrations must have lowered the pH by accumulation of acid. This might have caused decrease in acid consumption, FE, and PHA productivity. Though the dosages of acids were small for the continuous feeding, continuous addition might have lowered the efficiency of the process. The smaller dosage and the fed-batch mode of the 3 h feeding strategy might have resulted in better control of pH and of catabolite repression. So the optimized growth conditions (as discussed before) for the organism and 3h feeding strategy of ARF addition was considered the best to obtain optimum PHA production by the organism.

Yu et al. suggested [31] in their study that an average yield of PHA was $0.39\,g\,g^{-1}$ of carbon sources (acetic, butyric, and propionic acids). In the current study the highest yield of about $0.2\,g\,g^{-1}$ was observed when the 3 h feeding strategy was used. Investigators have reported that inexpensive carbon sources lead to low PHA productivity due to inefficient utilization of nutrients by organisms [34–36]. A majority of carbon sources was probably utilized to generate energy for cell maintenance.

Wild-type strain of *R. eutropha* used in this study was not able to use glucose and did not completely utilize the high amount of glycerol present in the CCS medium. Recombinant microorganisms can be used to increase growth rate and nutrient utilization. PHA biosynthesis genes can be inserted in organisms that have wider range of utilizable substrates to increase PHA productivity and PHA content. For example, the recombinant strains of *R. eutropha* (H16) containing glucose-utilizing genes of *Escherichia coli (E. coli)*, and *E. coli* harboring the *R. eutropha* genes had higher PHA productivity and concentration as compared to the wild-type strain [14, 34].

5. Conclusion

Since the carbon source is a major contributor to PHA cost, inexpensive sources of carbon are important. From an economical point of view, the use of purified media to increase PHA yield will significantly increase the production cost. This study showed that *R. eutropha* is capable of growing in CCS medium, a low-value byproduct of ethanol industry. We also concluded that mixture of SCFAs in the same compositional ratios of ARF was not inhibitory when added at stationary phase of growth. SCFAs can be diverted toward PHA production by *R. eutropha*. As the use of purified SCFAs is cost prohibitive, developing a mixed culture system to produce a mixture of SCFAs from another ethanol industry byproduct is highly cost-effective. Thus utilization of inexpensive carbon sources may lead to economically viable PHA production in future when superior genetics and fermentation strategies are applied together.

Acknowledgments

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Research Article

Analysis of Casein Biopolymers Adsorption to Lignocellulosic Biomass as a Potential Cellulase Stabilizer

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Although lignocellulosic materials have a good potential to substitute current feedstocks used for ethanol production, conversion of these materials to fermentable sugars is still not economical through enzymatic hydrolysis. High cost of cellulase has prompted research to explore techniques that can prevent from enzyme deactivation. Colloidal proteins of casein can form monolayers on hydrophobic surfaces that alleviate the de-activation of protein of interest. Scanning electron microscope (SEM), fourier transform infrared spectroscopy (FT-IR), capillary electrophoresis (CE), and Kjeldahl and BSA protein assays were used to investigate the unknown mechanism of action of induced cellulase activity during hydrolysis of casein-treated biomass. Adsorption of casein to biomass was observed with all of the analytical techniques used and varied depending on the pretreatment techniques of biomass. FT-IR analysis of amides I and II suggested that the substructure of protein from casein or skim milk were deformed at the time of contact with biomass. With no additive, the majority of one of the cellulase mono-component, 97.1 ± 1.1, was adsorbed to CS within 24 h, this adsorption was irreversible and increased by 2% after 72 h. However, biomass treatment with skim-milk and casein reduced the adsorption to 32.9% ± 6.0 and 82.8% ± 6.0, respectively.

1. Introduction

Production of ethanol from residual lignocellulosic biomass may serve as a promising clean fuel substitute that can reduce the greenhouse gases, ease the resource limitations of fossil fuel, eliminate the concerns of using food for fuel production, progress the rural economy, and create direct and indirect jobs. The market of ethanol grew from less than a billion liters in 1975 [1], and it is expected to reach more than 22 million gallons by 2022 [2].

Carbohydrates of cellulose and hemicellulose are hydrolyzed with two routes of acid or enzymatic hydrolysis to their subunits that can be fermented to ethanol by, for example, baker's yeast [3]. Despite extensive research, the production of ethanol from lignocellulosic biomass in a manner that can economically compete with that of corn has not yet been achieved.

The cost of cellulolytic enzymes remains one of the key challenges for second-generation biofuel production. In a recent study, the price of cellulytic enzymes has been estimated to be \$0.68/gal ethanol [4]. Considering that the price of enzyme in dry-grind corn ethanol is only 0.03–0.04 \$/gal ethanol [5], an extensive price gap must be diminished before lignocellulosic ethanol can compete with corn ethanol process. Enzymes have been found to be deactivated by a variety of reasons such as thermal effects imposed in a longer process [6], shearing effect [7], air-liquid contact [8], irreversible adsorption to active (e.g., cellulose) and nonactive sites (e.g., lignin) [9–12] and high concentrations of monomer sugars of hemicellulose (i.e., xylose, mannose, and galactose) [13, 14], xylo-oligomers [13], soluble lignin or lignin degradation products [15, 16], polymeric phenol tannic acids, and to a lesser extent monomeric phenolic compounds [17].

Cellulase deactivation can reach to as low as 16% of the initial activity within the first 24 h of hydrolysis [9]. Deactivation of cellulase is certainly a negative property that impacts the process cost by eliminating the chance of enzyme recycling or requiring more enzymes to maintain acceptable conversion rates.

In addition to efforts to genetically engineer new types of enzymes and enzyme producing microbes, application of surfactants (especially the nonionic) showed to be an effective approach to improve enzyme activity and hence reduce their application rate or increase the possibility for recycling. When surfactants are present in solution at levels beyond the critical micelle concentration (CMC), core-shell nanoparticles are formed. The interactions between enzymes and these micelle particles can result in a strong positive modification of the catalytic properties of the enzyme, such that "superactivity" of enzymes can be observed. Thus, an enhanced catalytic reaction can occur at the interface of micelles enzyme compared to that in aqueous phase [18]. It was suggested that the electrostatic interaction between micelle and enzyme, such as lipase, activates the key amino acids of enzyme (e.g., lysine and arginine) resulting in increased catalytic activity [19]. Also it was suggested that surfactants adsorb in the monomer form [20] to the surface of lignocellulosic biomass and prevent irreversible enzyme adsorption by increasing entropy at the time of contact with enzyme, thus increasing the amount of free enzyme in solution [21, 22]. Disruption effect of biomass (e.g., removal of lignin, disruption of H-bonding in cellulose, and removal of amorphous cellulose) was reported to be another potential effect of surfactants [14, 20, 23].

Although surfactants have demonstrated these potential advantages, amphiphilic polymers of proteins and biopolymers are better choices in improvement of enzyme activity. This is because surfactants might have disadvantages such as foaming property and environmental pollution [24], and in some cases even small quantities of Tween 80 have been shown to be inhibitory to some strains of yeast [25]. For instance, application of 2.5 g/L Tween 20 helped to reduce the enzyme loading by 50%, while retaining cellulose conversion [11]. However, 1 g/L of Tween 20 was found to be an inhibitor to *D. claussenii* [26].

Alternative lignin-blocking polypeptides that were reported to enhance the catalytic reaction of cellulase by several folds include soybean meal, corn steep liquor, bovine serum albumin (BSA), amylase, chicken egg albumin, and combinations thereof [12]. The high cost of some of these proteins, such as BSA, has prompted us to further investigate more cost-efficient protein sources to be used as an enzyme activator.

Recently, we found for the first time that casein can be a good alternative stabilizer for cellulase, depending on the type of casein used (e.g., ultrafiltered liquid, lyophilized acid casein [27], and complete casein (gluten free)). In our recent study, it was found that casein can increase the ethanol yield from corn stover by as much as 8.48%–33.7% through enhancement of enzyme activity. However, the mechanism of action behind the effectiveness of casein during hydrolysis and fermentation of lignocellulosic biomass remained a question. Application of casein as a stabilizer for protein structure or even as a chaperone in promoting proper protein folding is well established [28–30]. It has been found that the casein aggregates into complexes ranging from a few nanometers to hundreds of nanometers in diameter [31, 32] and that surface binding may also alter the structure of casein. Casein has been used in the past on the surface of SiO_2 for immobilization and induced activity of kinesin [33]. It was predicted that above a minimum casein concentration, an irreversible monolayer of casein is formed on the surface with a thickness corresponding to the size of the casein in solution [33]. Reduced adsorption of microbes (*Listeria monocytogenes*) due to the protection provided by surface preadsorption with milk protein has also been reported [34].

Therefore, the aim of this study was set to investigate one of the potential mechanisms of action behind the casein and whey protein effectiveness that would reduce the cellulase irreversible adsorption to lignocellulosic biomass. Analytical techniques such as Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), and Kjeldahl and BSA protein analyses were used to determine the adsorption of whey and casein protein to biomass, and capillary electrophoresis (CE) was used to analyze the modifications in enzyme solubilization in the presence of additives.

2. Materials and Methods

2.1. Pretreatment of Corn Stover. Ground corn stover (8 mm screen, Speedy King, Winona Attrition Mill Co., MN, USA) was pretreateds with different techniques as described in Table 1. Lime and dilute acid pretreatments of corn stover were performed according to optimized conditions reported previously by Kaar and Holtzapple [35] and Lloyd and Wyman [36], respectively. These pretreatments were performed in a 1-L Parr reactor (Parr Instrument Company, Moline, IL, USA) equipped with Rushton disc impeller rotating at 100 rpm, with pressure and temperature control. For dilute sulfuric acid pretreatment, ground corn stover was soaked in 0.98% sulfuric acid overnight at a solid loading rate of 5%. The soaked corn stover in acid solution was then loaded into the reactor and treated for 40 min at 140°C. The reaction was terminated by immersing the reactor in a cold water bath. Vacuum filtration with Whatman no. 2 filter paper was used to separate pretreated biomass from the liquid, and the solids were then washed with DI water until a neutral pH was achieved. For lime pretreatment, the ground corn stover was mixed with 0.075 g/g $Ca(OH)_2$ at a solid loading rate of 19.5% and then heated to 120°C for 4 h. The solid fraction was recovered, rinsed, and filtered as described earlier.

Alkali pretreatment was conducted according to Gupta and Lee [37]. In brief, 1% w/v NaOH was mixed with ground corn stover to achieve a solid loading rate of 8.3% and then was heated in sealed Erlenmeyer flasks at 60°C for 24 h. Solids were filtered and washed until a neutral pH was obtained. Extrusion pretreatment was conducted according to Karunanithy and Muthukumarappan [38] using a single screw extruder (Brabender Plasti-corder Extruder Model

Table 1: Pretreatment condition of corn stover and the resulting corn stover composition.

Pretreatment	Condition	Pretreatment severity ($\log R_0$)[f]	Yield of original components left in pretreated solids (%)		
			Glucan	Xylan	Lignin
Untreated	NA	NA	34.6	14.9	20.2
Acid	0.98% $H_2(SO_4)$, 5% SL, 140°C, 40 min	2.77	26.7–28.9	1.0	16.4
Lime	0.075 g/g[a] $Ca(OH)_2$, 19.5% SL, 120°C, 5 h	2.96	33.5	10.6	12.3
Alkali	1% NaOH, 8.3% SL[b], 60°C, 24 h	3.31	31.1–33.0	11.9	7.3
Extrusion	90°C[c], 180°C[d], 180°C[e], 45–90 sec, 1 : 5 SL	2.20	33.5–33.9	14.7	20.2
AFEX	50% SL, 140°C, 15 min	2.34	31.1	14.9	15.8

[a]g $Ca(OH)_2$/g Biomass, [b]SL: solid loading (biomass: H_2O), [c]feed zone temperature = 90°C, [d]transition zone temperature = 180°C, [e]die zone temperature = 180°C, [f]$\log R_0 = \log[\text{time} \exp(H–R)/14.75]$, where H is pretreatment temperature and R is a reference temperature of 100°C.

PL2000, Hackensack, NJ, USA) with a barrel length to screw diameter ratio (L/D) of 20 : 1 and a compression ratio of 3 : 1. The moisture content of ground corn stover was adjusted to 20% wb and held overnight before being manually fed into the extruder at an average rate of 16.5 g/min. While the residence time of the material in the barrel varied slightly due to the nature of the manual feeding, a mean reaction time of 45–90 s was estimated. The temperatures of feed, barrel, and die zone of the extruder were held at 90, 180, and 180°C, respectively. AFEX-pretreated biomass was provided by Michigan State University (see conditions in Table 1).

2.2. Enzymes. Celluclast 1.5 L, with a cellulase activity of 71.7 FPU/mL, and Novozyme 188, with a β-glucosidase activity of 422.14 CBU/mL obtained from Sigma Aldrich were used as the cellulytic enzymes. Celluclast 1.5 L and Novozyme 188 were used at dosages of 25 FPU/g glucan and CBU : FPU ratio ~2.5, respectively.

2.3. Adsorption of Casein and Whey Proteins to Corn Stover Determined by FT-IR. Fourier transform infrared spectroscopy (FT-IR) was initially used to determine the casein biopolymers physical adsorption onto the corn stover. Corn stover containing 1% (w/v) glucan prepared with different techniques according to Section 2.1 was blended in 50% (v/v) citrate buffer (pH 4.85), 2.5 g/g glucan of casein or skim milk, and sufficient deionized water (DI) for a total volume of 10 mL to achieve a 3% solid loading. Prepared test tubes (in duplicate) were incubated for 72 h in a shaker incubator at 50°C and 150 rpm. After treatment, biomass was subsequently collected with vacuum filtration, and washed with 2 times the sample volume with DI water. Collected solid residues of biomass were then scanned with Fourier transform infrared spectroscopy (Nicolet 380) with an ATR (attenuated total reflectance) accessory as described in more detail elsewhere [39]. Samples were uniformly pressed against the diamond surface with the swivel pressure tower accessory; then for each spectrum, a 150-scan interferogram was collected using single beam mode with 4 cm^{-1} resolution for the region of 4000 to 500 cm^{-1}. Prior to each analysis, a background spectrum (air) was collected and automatically corrected from the sample spectrum. Reference spectra consisted of biomass that had been incubated under similar conditions as treatment samples, with the absence of additives. Wavenumber assignments brought in Table 2 demonstrate the regions of protein (amides I–II) that could be used to evaluate the protein adsorption on biomass.

Table 2: Band frequencies and assignment for protein in aqueous solution.

Designation	Bandwidth (cm^{-1})	Assignments	Ref
H_2O	1500–1800	C=O stretching	[40]
Amide I	1617–1692	C=O stretching	[41]
Amide II	1510–1580	N–H bending vibration C–N stretching vibration	[41]
Amide III	1229–1301	Mix of several displacement	[41]

2.4. Adsorption of Casein and Whey Proteins to Corn Stover Determined by SEM. Scanning electron microscopy (SEM) was used to provide a more in-depth qualitative analysis of the casein adsorption onto the pretreated corn stover. To prevent redundant imaging, only pure casein (with minor whey) and extrusion pretreated biomass were used in this analysis. Extruded corn stover (5% w/v) was solubilized in a 50 mL solution of citrate buffer (pH 4.85) with 4.1% (w/v) casein. After 72 h of incubation at 50°C and 150 rpm, biomass was separated from the solution by vacuum filtration using Whatman filter paper no.2 after being washed with 2 times the sample volume with DI water. Collected biomass was then lyophilized at −48°C for 48 h prior to SEM analysis. The samples were gold coated for 180 s to help reduce sample charging typically observed on non-conductive samples. All samples were imaged under high vacuum conditions, utilizing the secondary electron detector (SED). This detector is ideal for observing fine surface morphology. Images were acquired at various areas

throughout the samples, at a variety of magnifications from 42x to 19,000x.

2.5. Adsorption of Casein and Whey Proteins to Corn Stover Determined by BSA and Kjeldahl. To quantify the amount of adsorbed protein, Kjeldahl digestion [42] and Bovine serum albumin (BSA) assays were used to determine the amount of soluble protein (casein and whey). The difference between the applied protein through casein or milk and the remaining level of protein in solution after incubation would represent the amount of bonded protein. Corn stover containing 1% (w/v) glucan prepared with different techniques according to Section 2.1 was solubilized in a total volume of 80 and 30 mL solution for Kjeldahl and BSA assays, respectively. The solution was comprised of 50% (v/v) citrate buffer (pH 4.85), 2.5% (w/v) commercial casein or skim milk, and DI water. Samples were incubated for 30 min at 60°C to maximize binding of the casein or milk proteins to lignin; this was because it was reported that the elevated temperatures enhanced the adsorption activity [43]. The temperature was then reduced to 50°C for 72 h of incubation similar to the enzymatic hydrolysis condition according to NREL protocol (With no enzymes added) [44]. Samples were collected at 24, 48, and 72 h, centrifuged at 3,000 rpm for 5 min, and the supernatant (biomass free) was subjected to Kjeldahl or BSA analysis in duplicates.

2.6. Cellulase Solubilization Determined by CE. To estimate the modifications in cellulase solubilization during hydrolysis of corn stover with and without preincubation with casein polymers, CE (Beckman PACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA) equipped with a UV detector set at 214 nm) was applied as the analytical tool. Samples of extruded corn stover (1% w/v glucan) were prepared in a total volume of 80 mL consisting of 50% (v/v) citrate buffer (pH 4.85), DI water, and either 2.5% w/v casein or skim milk. Each reaction vial was incubated in a shaker bath set at 150 rpm and 60°C for 30 min to maximize protein binding [9]. The temperature was then reduced to 50°C, and 25 FPU cellulase with 2.5 CBU : FPU of β-glucosidase was added to each vial for 72 h enzymatic hydrolysis. Samples were withdrawn after 24, 48, and 72 h of incubation and then centrifuged at 3,000 rpm for 5 min, and the biomass-free supernatants were prepared for CE analysis as described in the following.

Samples were processed via CE according to the method defined by Salunke et al. [45]. In brief, pure skim milk, casein, enzymes cocktail, or hydrolyzate supernatants were diluted to 10 mg/mL protein using HPLC grade water. Separation was obtained via a 50 μm bare fused silica capillary with the length of 30.2 cm. Gel formulation in a sieving range of 10–225 KDa was used. For estimation of protein molecular weights in the sample, the SDS-MW size standard (recombinant proteins 10–225 kDa supplied with the ProteomeLab SDS-MW Analysis Kit) was used to calibrate the gel. β-mercaptoethanol (5 μL) was added to each microfuge vial containing diluted SDS-MW size standard (10 μL in 85 μL of sample buffer). Prepared vials were heated in a water bath for 10 min at 90°C. A separation at constant voltage of 15 KV (25°C temperature and 20 bar pressure) was performed with reverse polarity in SDS-MW gel buffer. Sample was electro kinetically introduced at 5 kV for 20 s. A capillary preconditioning method was run every six samples. The area of each peak and identification of each protein were found and calculated from the electropherogram.

3. Results and Discussion

3.1. Adsorption of Casein and Whey Proteins to Corn Stover Determined by FT-IR. FT-IR was used to demonstrate the physical adsorption of casein or skim milk proteins on biomass after 72 h of incubation. Figures 1(a)–1(e) show the IR spectra of corn stover pretreated by various methods that have been incubated with skim milk or casein. Although the protein secondary structure can be obtained from the IR spectra to quantify the modifications in enzyme structure, the IR spectra obtained in this study were used without any extra manipulations (i.e., subtraction, smoothing, or convolution) to compare the amides I and II profiles visually. It is known that water and amide I and II demonstrate IR absorption at the same regions, with water peaks appearing at 1500–1800 cm^{-1} and amides I and II absorbing at 1617–1692 cm^{-1} and 1510–1580 cm^{-1}, respectively (Table 2). The differences observed in peak shapes from samples treated with casein or milk compared to those without additives can be associated to adsorbed protein (Figures 1(a)–1(e)). IR spectra collected from aqueous buffer were included in each of Figures 1(a)–1(e) to demonstrate the portion of each spectrum belonging to water. FT-IR technique was also applied before to successfully estimate the protein content of the milk [46].

As it is demonstrated in Figure 1, the sign of protein adsorption can be simply observed in almost all samples. Lime-pretreated corn stover (Figure 1(b)) and AFEX-pretreated CS (Figure 1(e)) showed the highest profile of amide II compared to adsorbed protein to other pretreated CSs, while alkali-pretreated samples did not show a significant increase in amides I or II regions compared to control (Figure 1(a)). These results suggest that either a lower amount of protein was adsorbed to some biomass (e.g., alkali pretreated) or the casein substructure was deformed when it adsorbed to biomass.

Ozeki et al. [33] reported that when 0.2 mg/mL of casein was introduced to SiO_2, most of the casein was tightly adsorbed to the surface of SiO_2, and when it was washed with casein-free buffer, only some part of the casein released from the surface. Repeated introduction of casein solution to the surface of SiO_2 resulted in readsorption of casein to the surface. As a result, the author suggested that casein adsorption to SiO_2 surfaces has two modes of a tightly and a weakly bound layer.

3.2. Adsorption of Casein and Whey Proteins to Corn Stover Determined by SEM. Scanning electron microscopy (SEM) was used as another surface analysis technique to demonstrate whether casein and milk proteins have any affinity to

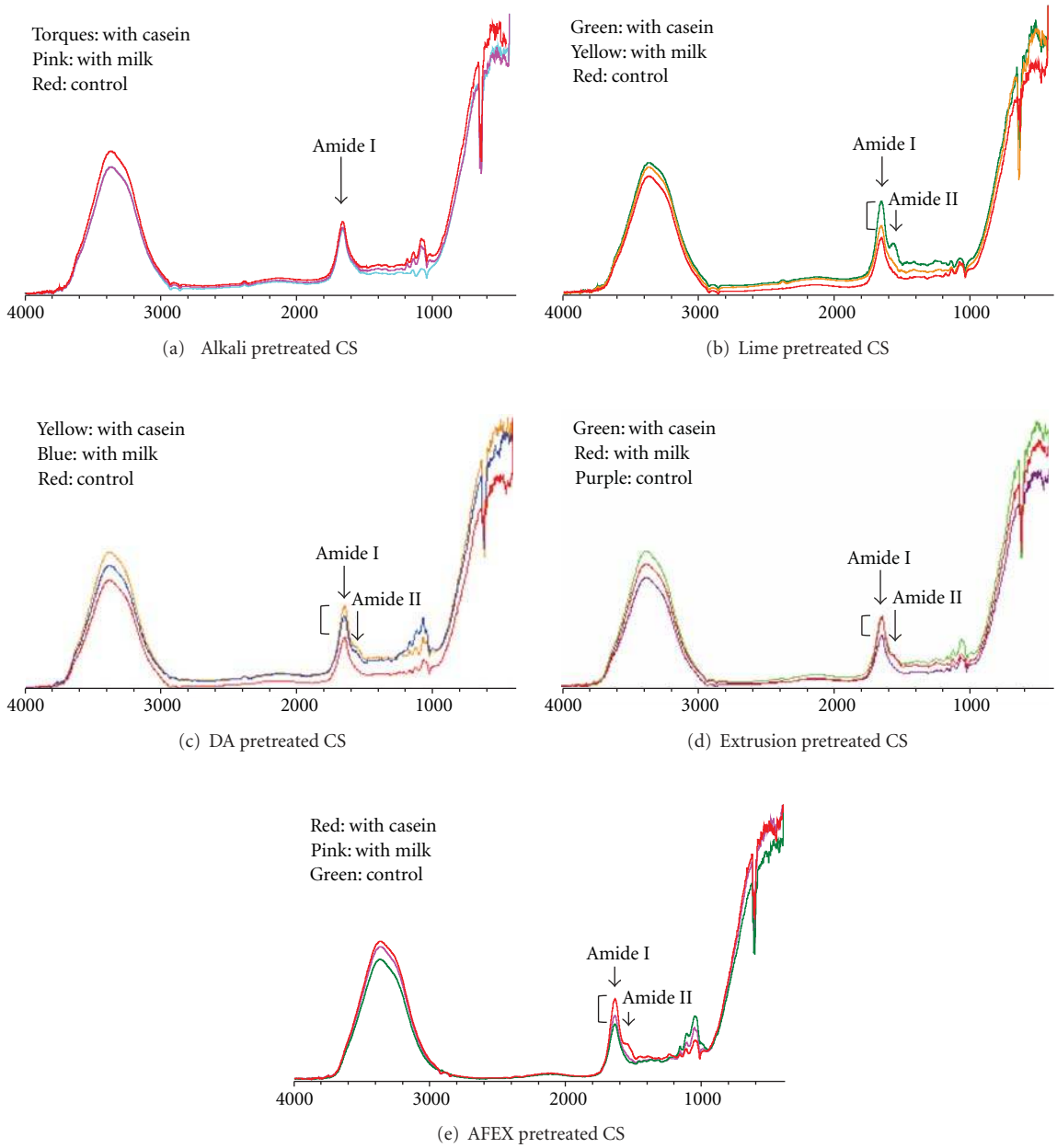

Figure 1: FTIR plots of alkali-pretreated corn stover incubated with casein and skim milk in solution of aqueous citrate buffer for 72 h (a); FTIR plots of lime-pretreated corn stover incubated with casein and skim milk in solution of aqueous citrate buffer for 72 h (b); FTIR plots of dilute acid-pretreated corn stover incubated with casein and skim milk in solution of aqueous citrate buffer for 72 h (c); FTIR plots of extrusion-pretreated corn stover incubated with casein and skim milk in solution of aqueous citrate buffer for 72 h (d); FTIR plots of AFEX-pretreated corn stover incubated with casein and skim milk in solution of aqueous citrate buffer for 72 h (e). Bracket sign demonstrates the amount of protein adsorbed on biomass.

corn stover and the degree to which they are able to adsorb to biomass after a certain period of incubation (Figure 2). As can be observed in Figure 2, when 4.5% w/v of casein in citrate buffer solution of biomass was lyophilized, the casein formed a white cake with a substantial number of perforations and globules on the surface of biomass. Due to the magnification limitations of SEM, the casein micelles themselves were not shown in this study. However single casein micelles images have been taken by field-emission scanning electron microscopy and can be found in the paper of Dalgleish et al. [47]. According to their imaging results, the size of the casein micelles varies between 200 nm and 350 nm, and the surface of each micelle at this magnification has been shown to conform to cylindrical or tubular structures that vary between 10 and 20 nm [47]. The size of the lyophilized casein globules on biomass varied between 5 and 50 μm

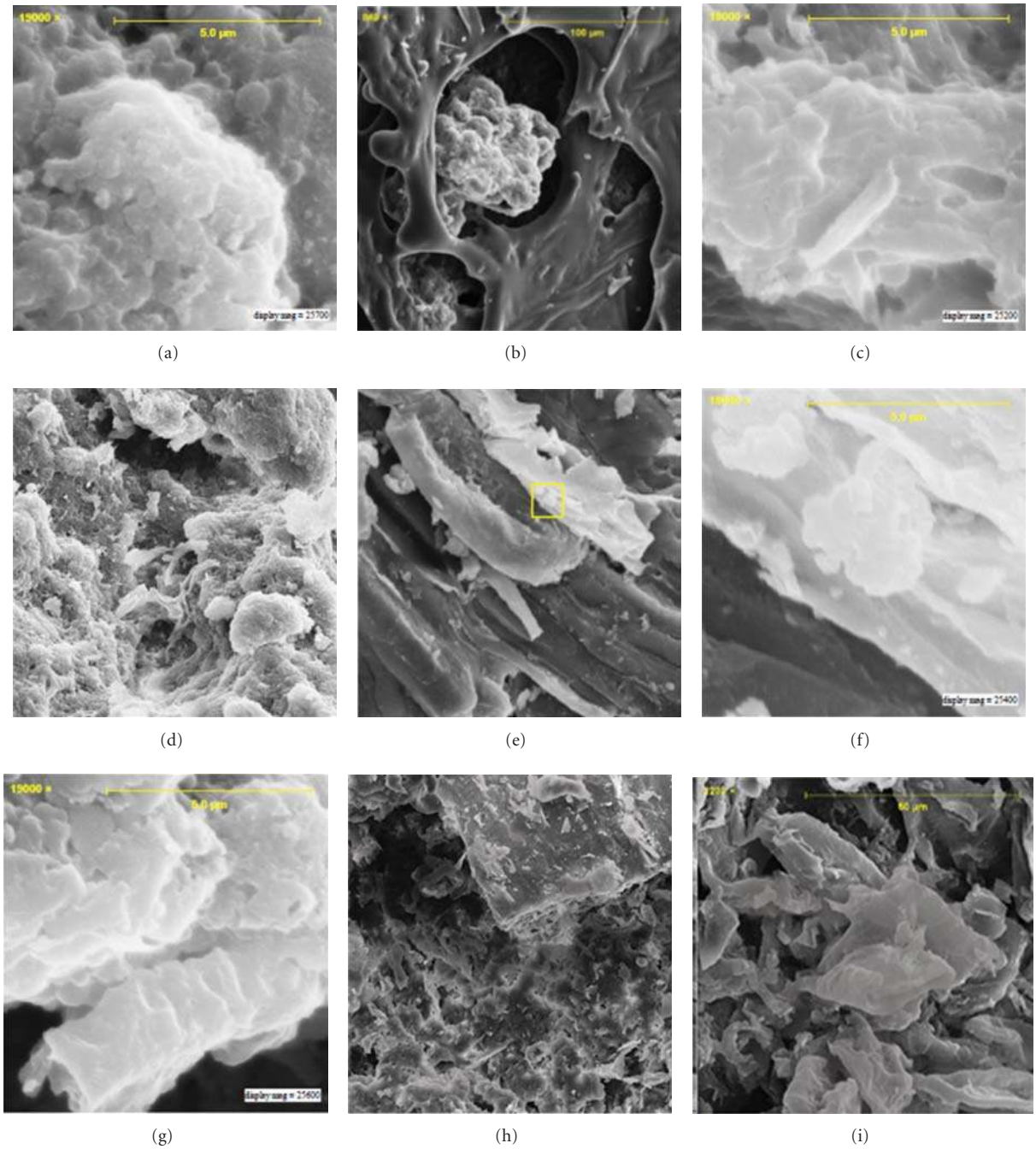

Figure 2: SEM analysis of lyophilized: 4% w/v casein solution in citrate buffer((a), (b), and (c)); extrusion-pretreated corn stover (8 mm) incubated for 24 h with 4% w/v casein solution of citrate buffer ((d), (e), (f), and (g)); extrusion-pretreated corn stover incubated for 24 h in citrate buffer only ((h), and (i)).

(Figures 2(a), 2(b) and 2(c)). Although the structure and position of casein at the time of reaction are best captured by imaging from a liquid sample, the aggregation of casein micelles at pH 4.85 was previously reported to be observed even by naked eye [48].

Observed contrast between the two Figures 2(d) and 2(h), associated with casein treated biomass and control, respectively, can clearly demonstrate the adsorption of casein polymers to the surface of corn stover. It appears that casein initially created several layers of coating in some areas, while in other regions it adhered to the strands of biomass in a discontinuous coagulation form (Figures 2(e) and 2(f)). Interfacial studies on casein-hydrophobic surfaces for protein activations have shown that casein binds to the SiO_2 surfaces by forming a tightly bound monolayer of β-casein, followed by a second loosely bound layer [49]. Tiberg et al. [50] found similar subunit interactions for casein adsorbed to silicon oxide. It is noteworthy to indicate that

Table 3: Comparison of soluble casein or skim milk proteins after 72 h of incubation with lime-, alkali-, dilute acid-, and extrusion-pretreated corn stover determined with BSA assay.

Pretreatment	Soluble skim milk proteins (%)			Soluble casein proteins (%)		
	24 h	48 h	72 h	24 h	48 h	72 h
Dilute acid	29	<1	<1	21	<1	<1
Lime	55	15	18	5.6	5.2	<1
Alkali	90	22	14	65	3.1	<1
Extrusion	92	25	10	55	8.6	<1
AFEX	97	28	15	30	3.5	<1
Raw	91	<1	15	49	2.5	<1

we used double the casein dose that we usually apply for hydrolysis in order to clearly show the coating effect of the casein on cellulosic biomass.

3.3. Adsorption of Casein and Whey Proteins to Corn Stover Determined by BSA and Kjeldahl. In addition to the two surface analysis techniques (SEM and FT-IR) that were applied to illustrate the adsorption of casein or milk proteins onto corn stover, Kjeldahl and BSA protein assays were used for quantitative evaluation of the same phenomenon. Solution of aqueous citrate buffer comprised of 2.5% w/v casein polymer or skim milk and 1% w/v of corn stover (8 mm) were incubated together at 60°C for 30 min followed by 72 h of incubation at 50°C. The amount of soluble proteins was obtained by measuring the protein left in supernatant of samples drawn after 24, 48, and 72 h of incubation of biomass with casein or milk and comparing that to control (aqueous buffer of casein or milk with no biomass). The difference in protein content would represent the amount of protein adsorbed onto the biomass.

The results of Kjeldahl analysis demonstrated that after 24 h of incubation of casein with biomass, 68.37% of casein proteins (1.17 to 0.37% (w/v)) were adsorbed to the surface of biomass. Based on the compositional analysis of the milk used in the study, 2.5% (w/v) of milk solution was projected to contain 0.87% w/v protein; this was also confirmed by Kjeldahl assay that was indicated to be 0.89% w/v protein. After 24 h of incubation of 1% w/v corn stover in milk solution, the results demonstrated that 15.7% of the milk protein was adsorbed to the biomass. According to the amount of the biomass used, an adsorption rate of 0.80 and 0.14 g of protein/g of biomass can be estimated for casein and milk proteins, respectively.

Another method used to evaluate the adsorption of casein or milk proteins to pretreated corn stover was BSA assay. In this assay, BSA protein of 2 mg/mL was used as reference, and the reactivity of the protein being evaluated was assumed to be comparable to that of BSA. Table 3 shows the percentage of soluble protein found after 24, 48 and 72 h of incubation of 1% w/v glucan equivalent of pretreated corn stover with 2.5% w/v casein polymers or skim milk.

The greater adsorption of soluble casein proteins to biomass compared to milk proteins was in agreement with Kjeldahl analysis. However, Kjeldahl analysis demonstrated a slightly higher protein adsorption compared to what was obtained with BSA analysis. The adsorption of milk protein to biomass was found to vary between 1.12 and 73.9% whereas casein adsorption varied between 37.5 and 93.4% after 24 h of incubation. These results suggest that casein proteins have a much higher affinity for corn stover compared to proteins in milk. Moreover, the affinity of proteins varies depending on the pretreated corn stover used. This might have been due to the differences in particle size, surface area, and chemical structure originating from different pretreatment techniques.

While casein has shown a stronger earlier affinity to pretreated corn stover compared to milk protein, adsorption of both milk and casein approached 100% by 72 h of incubation. Recently, Zhang et al. [51] reported that increasing the incubation time of lignocellulosic substrate with PEG 4000 from 0–2 h increased the amount of adsorbed PEG. They suggested that increasing the incubation time provided PEG with additional opportunity to interweave into the biomass structure and create a denser hydration layer on the exterior surface. As a result, the denser layer of polymer can provide a greater steric hindrance for the enzyme from the nonspecific sites.

The extensive adsorption of casein onto biomass observed in this research study was not unanticipated in light of the widespread application of casein as glue for adhesion of wood particles. Moreover, the adsorption of β-casein to a silica-aqueous solution interface or bimodal PEG brushes and many other supports has been reported in the past [32, 52, 53]. Based on the prior arts, β-casein creates a densely packed monolayer on surfaces via hydrophobic interaction and adsorption of its highly charged N-terminal to the pseudophases [52].

3.4. Cellulase Solubilization Determined by CE. Evaluating a specific protein in a mixture of proteins has always been challenging, since methods that use the total nitrogen value cannot distinguish between specific proteins. Analytical methods that can distinguish between proteins include CE, SDS-Page gel electrophoresis, and size exclusion chromatography. However, one of the concerns with these methods is that the peak associated with the protein of interest may overlap with other proteins in the mixture. We selected CE to evaluate the modifications in enzyme adsorption under the effect of casein and milk's preadsorption to biomass. In this trial, the relative amount of enzyme substructure

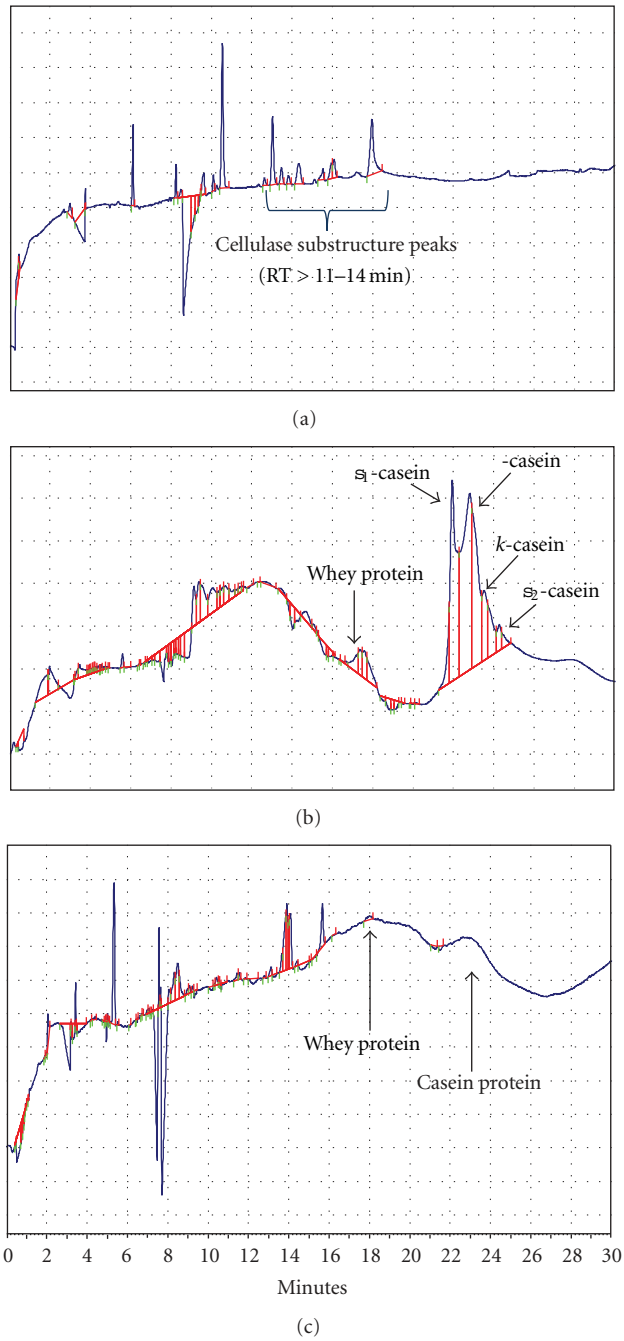

Figure 3: Capillary electrophoresis plots of pure cellulase diluted in HPLC grade water (a); commercial casein diluted in HPLC grade water (b);skim milk powder diluted in HPLC grade water (c).

in control (enzymatic hydrolysis without additive) and treatment samples (with casein and milk) were compared with each other. Using CE, we were able to differentiate between peaks associated with enzyme and those associated with casein and whey proteins. To locate the characteristic peaks for each of these compounds, separate solutions of HPLC-grade water containing each of these compounds were prepared and processed via capillary electrophoresis.

Table 4: Reduction in one of the mono-component of soluble cellulase in hydrolyzate of corn stover preadsorbed with casein or skim milk compared to control (no additive) determined by CE.

Sample condition	Reduction in soluble cellulase (%)
No additive 24 h	97.1 ± 1.1
No additive 72 h	99.5 ± 0.0
With casein 24 h	32.8 ± 6.0
With casein 72 h	0.0 ± 0.0
With skim milk 24 h	82.8 ± 6.0
With skim milk 72 h	74.8 ± 0.8

*Standard errors of the mean reported after ±.

As can be observed in Figures 3(a)–3(c), the CE analysis of individual samples of cellulase, casein, and milk proteins resulted in characteristic peaks for each material. The cellulase sample (Figure 3(a)) contained peaks at 10.5, 13, 14.5, 16, and 18 min. Commercial casein (Figure 3(b)) consisted of casein substructures (as labeled), along with minute amounts of whey protein. As can be observed, peaks for k-casein appeared at 24.2 min, αs_2-casein at 23.2 min, β-casein at 23.8 min, αs_1-casein at 22.0 min, and whey protein at 16–18 min. As it is apparent, the peaks of casein and whey proteins associated with ~20–24 and ~18 min (Figures 3(b) and 3(c)) did not overlap with peaks of cellulase. Therefore, retention times of >11–14, 20–28, and 16–18 min were used to track the cellulase, casein, and skim milk proteins, respectively.

According to the results (Figure 4(a)), the relative cellulase concentration in corn stover samples that were enzymatically hydrolyzed without the use of any polypeptide additives (casein or skim milk) was smaller than that in samples preincubated with casein or milk. This suggests that cellulase was either adsorbed to corn stover or other hydrophobic surfaces in reaction site or has been degraded. However, when corn stover was treated with casein or skim milk, the specific subunit of cellulase at 12.8 min was significantly increased compared to control (Figures 4(b) and 4(c)).

In the first 24 h of reaction, the majority of cellulase was adsorbed to biomass, during which the amount of one of the cellulase mono-component was reduced in solution by 97.1% ± 1.1. Application of casein and milk reduced the adsorption to 32.9% ± 6.0 and 82.8% ± 6.0, respectively (Table 4). After 72 h of hydrolysis the amount of soluble cellulase adsorption was further reduced to 74.9% ± 0.8 for milk-treated samples and to less than a quantifiable amount for casein-treated corn stover.

It was found that the reduction in casein and whey proteins after 72 h compared to that of 24 h was correlated with the increase in cellulase solubilization. These results suggest the steric barrier role for casein and milk, which prevents from the cellulase nonproductive adsorption to biomass. Similar effects were obtained from the application of nonionic surfactants and polymers such as Triton X-100, Tween 20 and 80, PEG 4000 and 6000, and many others [9–11, 54] in which the adsorption of surfactants to surface of

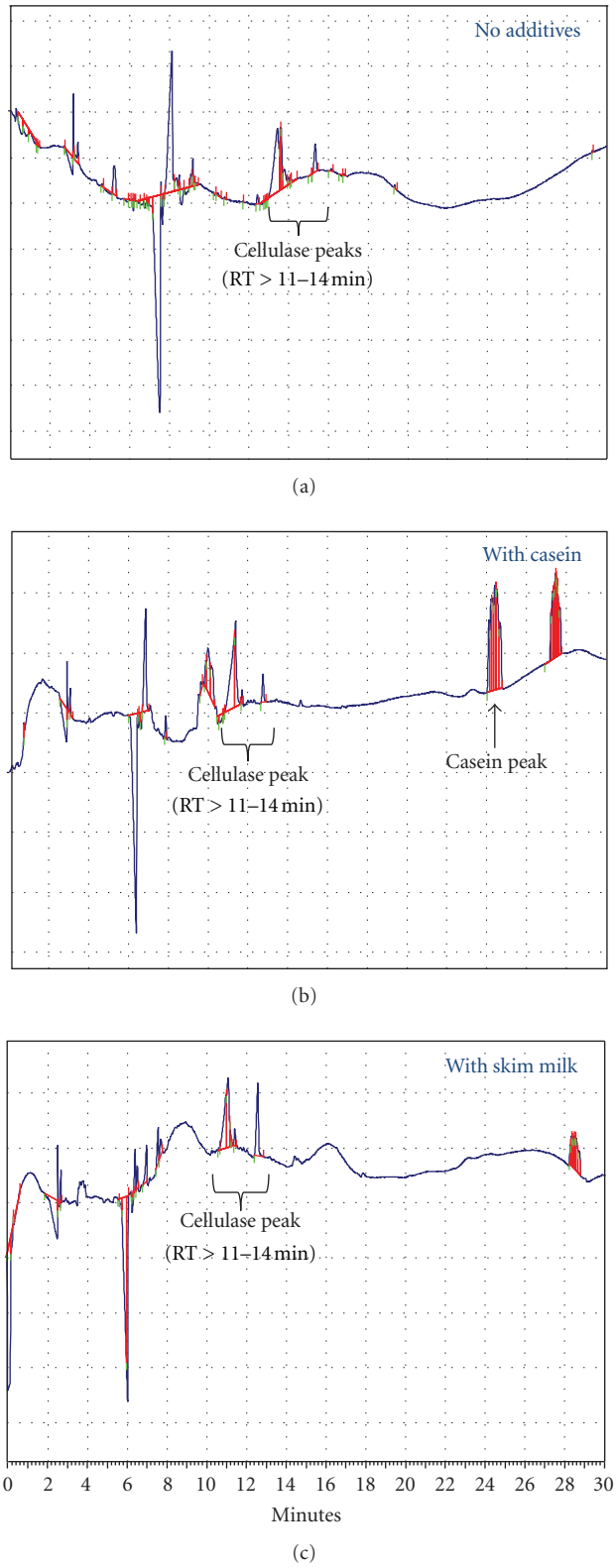

Figure 4: Capillary electrophoresis plots of supernatant from the solution of corn stover hydrolyzed for 24 h with cellulase (no additives) (a); supernatant from the solution of casein-treated corn stover hydrolyzed for 24 h with cellulase (b); supernatant from the solution of skim milk-treated corn stover hydrolyzed for 24 h with cellulase (c).

biomass was demonstrated to improve the cellulase activity and increase the enzyme solubilization [20, 43, 55].

It was reported that polymers adsorbed to surfaces can effectively use the relationship between electrostatic and steric interactions in order to control the adsorption and desorption of proteins of interest [56]. These fundamental findings have been applied in pharmaceutical, surface chemistry, and many other fields to serve as the basis for the design of controlled-release devices [57, 58]. Whole casein or the substructures of casein were used onto microtubule motility assays to reserve the kinesin functionality. It was found that the adsorbed casein bilayer improves the activity of kinesin, by one of the tightly bound casein layer anchoring the kinesin, while the second loosely bound layer of casein improves the position of kinesin for interaction with microtubules [59].

4. Conclusion

The cost of cellulase is a major barrier in biomass conversion process, necessitating new techniques to maintain cellulase activity for an extended period of time to reduce cellulase utilization and facilitate cellulase recycling. Casein was found to be an effective biopolymer that can reduce enzyme deactivation. One of the mechanisms of action associated with casein effectiveness on the induction of cellulase activity during conversion of lignocellulosic biomass was investigated using several techniques. As a result, it was found that with no additive, the majority of one of the cellulase monocomponent, 97.1 ± 1.1, was irreversibly adsorbed to corn stover within the first 24 h of hydrolysis. However application of casein or skim milk reduced the cellulase adsorption to 32.9% ± 6.0 and 82.8% ± 6.0, respectively. The preadsorption of casein proteins to biomass was demonstrated to be much higher than skim milk protein, and the adsorption of either varied based on the pretreatment of biomass used. Amide profile of the adsorbed casein or skim milk proteins to biomass suggested that perhaps some of the proteins substructures are deformed at the time of adsorption. The results of this study suggest that steric barrier provided by adsorbed casein and whey proteins on lignocellulosic biomass may induce the cellulase activity by prohibition of cellulase adsorption to nonproductive sites of the biomass. Other impacts of biomass-adsorbed protein on cellulase should be further studied.

Conflict of Interests

The authors of this paper have no conflict of interest to declare.

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Research Article

Factors Affecting Poly(3-hydroxybutyrate) Production from Oil Palm Frond Juice by *Cupriavidus necator* (CCUG52238^T)

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Factors influencing poly(3-hydroxybutyrate) P(3HB) production by *Cupriavidus necator* CCUG52238^T utilizing oil palm frond (OPF) juice were clarified in this study. Effects of initial medium pH, agitation speed, and ammonium sulfate $(NH_4)_2SO_4$ concentration on the production of P(3HB) were investigated in shake flasks experiments using OPF juice as the sole carbon source. The highest P(3HB) content was recorded at pH 7.0, agitation speed of 220 rpm, and $(NH_4)_2SO_4$ concentration at 0.5 g/L. By culturing the wild-type strain of *C. necator* under the aforementioned conditions, the cell dry weight (CDW) and P(3HB) content obtained were 9.31 ± 0.13 g/L and 45 ± 1.5 wt.%, respectively. This accounted for 40% increment of P(3HB) content compared to the nonoptimized condition. In the meanwhile, the effect of dissolved oxygen tension (DOT) on P(3HB) production was investigated in a 2-L bioreactor. Highest CDW (11.37 g/L) and P(3HB) content (44 wt.%) were achieved when DOT level was set at 30%. P(3HB) produced from OPF juice had a tensile strength of 40 MPa and elongation at break of 8% demonstrated that P(3HB) produced from renewable and cheap carbon source is comparable to those produced from commercial substrate.

1. Introduction

Poly(3-hydroxybutyrate), P(3HB) is a biodegradable thermoplastic polyester accumulated intracellularly by many microorganisms under unfavorable growth conditions [1]. The high production cost of P(3HB) can be decreased by strain development, improving fermentation and separation processes [2–4], and/or using a cheap carbon source [5]. In P(3HB) production, about 40% of the total production cost is contributed by the raw material, whereby the cost of carbon feedstock alone accounts for 70 to 80% of the total raw material cost [6, 7]. Therefore, the utilization of renewable and sustainable substrates for the production of P(3HB) has become an important objective for the commercialization of bioplastics. A lot of research have been carried out to discuss and propose the utilization of renewable biomass to replace commercial sugars as carbon source in order to reduce the production cost of P(3HB) [8–12].

Recently, we reported on the use of oil palm frond (OPF) juice as the novel and renewable feedstock for the production of P(3HB) [13]. We demonstrated that OPF juice is a good substrate for the production of P(3HB) from wild-type *Cupriavidus necator* (CCUG52238^T), with better yield of product formation in comparison to technical grade sugars. This can be explained by the presence of minerals and nutrients in the OPF juice which are essential for bacterial growth

during fermentation. Apart from contributing to higher product formation and microbial growth, the use of OPF juice is advantageous compared to the other lignocellulose-based sugars due to the ease in its processing wherein no harsh pretreatment steps and enzymatic treatment will be needed in order to obtain the sugars.

In our report, 32 wt.% of P(3HB) accumulation was successfully obtained under nonoptimized fermentation condition [13]. *C. necator* is well known as polyhydroxyalkanoate (PHA) producer and its ability to accumulate PHA more than 50 wt.% has been previously reported [8, 14]. In general, P(3HB) accumulation is favored by an excess carbon source and inadequate supply of macrocomponents such as nitrogen, phosphate, and dissolved oxygen or microcomponents such as magnesium, sulphate, iron, potassium, manganese, copper, sodium, cobalt, tin, and calcium [7, 15]. Moreover, it was also reported that the accumulation of P(3HB) in microorganisms were influenced by several physical parameters including pH and agitation speed [16–19].

In order to make the production of P(3HB) feasible for industrial application, it is crucial to have high P(3HB) production yield. In this study, we investigated the effect of initial medium pH, agitation speed, and ammonium sulfate $(NH_4)_2SO_4$ concentration on P(3HB) production from *C. necator* (CCUG52238^T) utilizing OPF juice in shake flasks fermentation with the aim to clarify the effect of each fermentation parameter on the microbial growth and P(3HB) formation. The effect of dissolved oxygen tension (DOT) level on cell growth and P(3HB) production was investigated by conducting batch fermentation in 2-L-bioreactor. P(3HB) produced from this study was then characterized for its thermal and mechanical properties.

2. Materials and Methods

2.1. Bacterial Strain. In this study, *C. necator* (CCUG52238^T) was obtained from the Culture Collection, University of Goteborg, Sweden and used for the production of P(3HB). The culture was maintained on slants of nutrient agar at 4°C. The inoculum preparation, media, and cultivation conditions for *C. necator* (CCUG52238^T) are similar to those reported by Zahari et al. [13], unless otherwise stated.

2.2. Biosynthesis of P(3HB) in Shake Flask. P(3HB) biosynthesis was carried out through one-stage cultivation fermentation in shake flasks. OPF juice in this study was obtained by pressing fresh OPF following the method described earlier [13]. OPF juice which comprises fructose, glucose and sucrose was diluted from stock (55 g/L) to 16-17 g/L of total initial sugars and used as carbon sources throughout the study period. In order to study the effect of culture medium initial pH on biosynthesis of P(3HB), the initial pH value of each MSM and OPF juice was adjusted to pH 6.0–8.0 using 2 M NaOH prior to autoclaving. Another set of experiment was conducted to study the effect of agitation on P(3HB) production by testing several agitation speed at 180, 200, 220, 240, and 260 rpm. For the effect of ammonium sulfate concentration, various concentrations of $(NH_4)_2SO_4$ in the range of 0–2.0 g/L were tested. The cultures were incubated at 30°C under aerobic condition, and all experiments were conducted in duplicates.

2.3. Biosynthesis of P(3HB) in 2-L-Bioreactor. In order to study the effect of dissolved oxygen tension (DOT) on cell growth and P(3HB) production profile under the optimized condition obtained from the shake flask study, batch experiment was conducted in 2-L-bioreactor (1 L working volume) at different DOT levels of 20, 30, 40, and 50%. 100 mL of pregrown cells from growing stage were transferred into 900 mL MSM in 2L bioreactors (Sartorius, Germany) supplemented with OPF juice at 30% (v/v) dilution. The stock of OPF juice with 55 g/L of initial total sugars concentration was autoclaved separately prior to addition with the MSM medium. The MSM compositions were prepared as previously reported by Zahari et al. [13], except that 0.5 g/L of $(NH_4)_2SO_4$ was used in this study. The temperature inside the bioreactor was set at 30°C, while DOT level was set at various concentrations of saturation throughout the fermentation using cascade mode and supplied with air at 1.0 vvm. The pH value during fermentation was controlled at pH 7.0 ± 0.05 by 2 M $NaOH/H_2SO_4$. Samples were withdrawn every 5 h for the period of 50 h for the determination of CDW, P(3HB) concentration, residual sugars, and ammoniacal nitrogen (NH_3-N) content.

2.4. Analytical Procedures

2.4.1. Biomass and Culture Medium Separation. Residual sugars concentration, cell dry weight measurement, and P(3HB) analysis were done as previously described by Zahari et al. [13]. The samples from the bacterial fermentations were taken at the end of the cultivation period to measure the total dry weight and P(3HB) content. Each sample was centrifuged at 11,000 × g for 5 min at 4°C (Thermo Fisher Scientific, NC, USA) and the solids were washed with distilled water and centrifuged for two consecutive times.

2.4.2. Determination of Cell Dry Weight and P(3HB) Content. Dry weight measurements were carried out by drying the solids at 50°C and cooling in a desiccator to constant weight. The P(3HB) content and composition in the lyophilized cell were determined using the gas chromatography (Shimadzu GC-2014). Approximately, 20 mg of lyophilized cells were subjected to methanolysis in the presence of methanol and sulfuric acid [85% : 15% (v/v)]. The organic layer containing the reaction products was separated, dried over Na_2SO_4, and analyzed by GC according to the standard method [20] using an ID-BP1 capillary column, 30 m × 0.25 mm × 0.25 μm film thickness (SGE).

2.4.3. Determination of Residual Ammoniacal Nitrogen (NH_3-N) Content. The supernatant was then analyzed for residual sugars and ammoniacal nitrogen content. Residual ammoniacal nitrogen (NH_3-N) content analysis was done using

Table 1: Effect of initial pH value on the biosynthesis of P(3HB)[a].

Initial pH	CDW (g/L)	Total P(3HB) (g/L)	P(3HB) content (wt.%)[b]
6.0	6.42	1.28	20
6.5	7.12	1.99	28
7.0	8.57	2.91	34
7.5	6.89	1.72	25
8.0	4.02	0.40	10

[a]MSM containing 16 g/L of total sugars in OPF juice and supplied with 1.0 g/L of $(NH_4)_2SO_4$, incubated at 30°C for 48 h with agitation at 200 rpm.
[b]Determination by GC from freeze dried samples.
*Values obtained herewith are means of two independent experiments.

Nessler method according to standard procedures (HACH, USA) which was previously described by Zakaria [21]. Samples with appropriate dilution factor were filled to 25 mL in the sampling bottles. Three drops of mineral stabilizer were added into solution and the bottle was inverted for several times. Three drops of polyvinyl alcohol also were added into the solution and mixed well with inversion several times. Lastly, 1.0 mL of Nessler reagent was added to the mixtures and mix thoroughly by inversion. The standard solution was prepared by replacing the samples with deionised water as blank sample. The sample solution was determined at the wavelength (λ) 425 nm using DR/4000 spectrophotometer by following the manufacturer, instructions (HACH, USA).

2.4.4. Determination of Residual Sugars. Residual sugars were determined by a high performance liquid chromatography (HPLC) (Agilent Series 1200, USA) using the Supelcosil LC-NH2 column (Sigma Aldrich) (25 cm × 4.6 mm ID, 5 μm particles) with a RI detector operated at 30°C. The mobile phase was acetonitrile : water (75% : 25%) at a flow rate of 1.0 mL/min. The components were identified by comparing their retention times with those of authentic standards under analytical conditions and quantified by external standard method [22].

2.5. Extraction of P(3HB). Solvent extraction method as described by Zakaria et al. [23] was carried out in order to extract the P(3HB) produced from fermentation. P(3HB) film was then prepared by solvent casting using chloroform.

2.6. Characterization of P(3HB). Thermal properties of the polymer were determined by differential scanning colorimetry (DSC) (TA Instruments). For DSC analysis, 5–7 mg of homopolymer samples were weighed and heated from 20 to 200°C at heating rates 10°C/min and held for 1 min. The first scan was conducted to eliminate the polymer history. The samples were then fast cooled from 200°C to −30°C. The second scan was used in reheating the samples at the same heating rates and was used in evaluating the thermal properties of the biopolymer. The tensile strength, Young's modulus and elongation to break were determined by using Instron Universal Testing Machine (Model 4301) at 5 mm/min of crosshead speed [21]. Mechanical tensile data were calculated from the stress-strain curves on average of five specimens.

3. Results and Discussion

3.1. Biosynthesis of P(3HB) in Shake Flask Experiment

3.1.1. Effect of Initial Medium pH. The effect of initial medium pH on biosynthesis of P(3HB) from OPF juice was studied by varying the pH between pH 6.0 and 8.0 due to the fact that *C. necator* can tolerate and produce PHA at the aforementioned pH range [24]. Suitable initial medium pH is crucial for the cell growth and P(3HB) accumulation by *C. necator* (CCUG52238^T). As shown in Table 1, increasing the initial medium pH value at intervals of 0.5 units affected both the cell growth and P(3HB) production. Both the cell growth and P(3HB) content were increased when the initial medium pH was increased from pH 6.0 to pH 7.0, that is, from 6.42 g/L to 8.57 g/L for CDW and 20 wt.% to 34 wt.% for P(3HB) content, respectively. However, further increase of initial medium pH above pH 7.0 decreased both the CDW and P(3HB) content. From the results, it can be concluded that pH 7.0 was the optimum initial medium pH for the growth and biosynthesis of P(3HB) by *C. necator* (CCUG52238^T) in which, 8.57 g/L of CDW and 34 wt.% of P(3HB) accumulation was recorded. The optimal pH for the cell growth and P(3HB) accumulation in this study was similar to those reported in the literature. It was reported that the optimum pH for growth and P(3HB) production by *A. eutrophus* was pH 6.9 and that a pH of 5.4 inhibited its growth [16].

On the other hand, lowest CDW and P(3HB) content, 4.02 g/L and 10 wt.%, respectively, were obtained at pH 8.0. Lowest cell growth and P(3HB) accumulation at this initial pH value were obtained might due to alkaline condition which could affect the P(3HB) production. These results corroborate with other be previous findings. For instance, N. J. Palleroni and A. V Palleroni. [25], recommended a pH range of between 6.0 to 7.5 for microbial growth and P(3HB) production. Although P(3HB) production can be controlled by precisely manipulating the medium pH, it has been reported that pH values other than 7.0 affected P(3HB) production [26]. These results suggested that P(3HB) production is sensitive to the pH of cultivation.

3.1.2. Effect of Agitation Speed. Table 2 displays the effect of agitation speed on biosynthesis of P(3HB) using OPF juice as substrate in shake flasks experiment. It is interesting to note that both cell growth and P(3HB) production

Table 2: Effect of agitation speed on the biosynthesis of P(3HB)[a].

Agitation speed (rpm)	CDW (g/L)	Total P(HB) (g/L)	P(3HB) content (wt.%)[b]
180	7.37	1.62	22
200	8.30	2.66	32
220	9.42	3.77	40
240	6.37	1.72	27
260	5.19	1.09	21

[a]MSM containing 16 g/L of total sugars in OPF juice and supplied with 1.0 g/L of $(NH_4)_2SO_4$, incubated at 30°C for 48 h (initial pH medium adjusted at 7.0 ± 0.1).
[b]Determination by GC from freeze dried samples.
*Values obtained herewith are means of two independent experiments.

Table 3: Effect of $(NH_4)_2SO_4$ concentration on biosynthesis of P(3HB)[a]

$(NH_4)_2SO_4$ concentration (g/L)	CDW (g/L)	Total P(3HB) (g/L)	P(3HB) content (wt.%)[b]
0.0	5.25	2.31	44
0.5	8.31	3.49	42
1.0	8.65	2.94	34
1.5	9.05	2.62	29
2.0	10.15	2.33	23

[a]MSM containing 16 g/L of total sugars in OPF juice, incubated at 30°C for 48 h with agitation at 200 rpm (initial pH medium adjusted at 7.0 ± 0.1).
[b]Determination by GC from freeze dried samples.
*Values obtained herewith are means of two independent experiments.

showed an increasing trend with the agitation speed up to 220 rpm. For the agitation speed of more than 220 rpm, the cell biomass and P(3HB) content was decreased. This result suggests that agitation speed plays an important role in the fermentation process. Agitation not only provides mixing and homogeneous cell and heat dispersion in the fermentation broth, but also better aeration for the cells by increasing the oxygen transfer rate throughout the fermentation medium. Generally, slower agitation speed may cause the possibilities of cells aggregation, making the culture medium more heterogeneous. This may cause the cell growth to be decreased and thus affecting the production of P(3HB). On the other hand, increasing agitation speed higher than its optimal level may reduce the P(3HB) formation, and hence, the CDW. This is due to the fact that PHA is only produced and stored as granules in the cell cytoplasm by microorganisms when they are under stress conditions, for example when there is limitation of nutrient or electron acceptor such as oxygen [27].

In our study, the best condition for the biosynthesis of P(3HB) is at moderate agitation speed which is at 220 rpm with the highest CDW and P(3HB) content reaching up to 9.42 g/L and 40 wt.%, respectively.

3.1.3. Effect of $(NH_4)_2SO_4$ Concentration. Nitrogen is an essential element for cell growth and P(3HB) accumulation. $(NH_4)_2SO_4$ has been widely used as the inorganic nitrogen source for the biosynthesis of P(3HB) by *C. necator*. It is important to optimize nitrogen content in fermentation medium as P(3HB) accumulation in the microorganisms can be triggered when one of the nutrients (N, P, Mg, and O_2) in the mineral salt is limited in the presence of excess carbon source [14, 15].

The effect of different $(NH_4)_2SO_4$ concentrations on biosynthesis of P(3HB) by *C. necator* (CCUG52338^T) from OPF juice is summarized in Table 3. In overall, it was observed that CDW was increased when $(NH_4)_2SO_4$ concentration increased from 0 to 2.0 g/L. On the other hand, P(3HB) accumulation decreased with the increase of $(NH_4)_2SO_4$ concentration. Highest P(3HB) accumulation at 44 wt.% was achieved when there was no addition of $(NH_4)_2SO_4$, in the culture medium. However, unsatisfactory cell growth that is, 5.25 g/L of CDW was observed in the experiment.

Based on the results, it was also found that $(NH_4)_2SO_4$ concentration at 0.5 g/L was the optimal concentration for P(3HB) accumulation and CDW formation, giving 42 wt.% and 8.31 g/L, respectively. Further increasing nitrogen concentration slightly improved the cells growth; however the accumulation of P(3HB) was restricted. This may be due to excess nitrogen concentration that limited the P(3HB) accumulation. These results corroborate to the literature, which reported that P(3HB) formation predominantly occurs under-nitrogen and oxygen-limited conditions [14, 15, 28, 29]. It was discussed that excess nitrogen source may restrict acetyl-CoA from entering P(3HB) production pathways and otherwise channelling into TCA cycle for biomass production [15, 21, 28].

3.1.4. Biosynthesis of P(3HB) under Optimized Condition in Shake Flask. Biosynthesis of P(3HB) was then carried out in shake flask under the optimized conditions: initial pH medium, 7.0; agitation speed, 220 rpm and $(NH_4)_2SO_4$ concentration, 0.5 g/L. Under these conditions, the maximum cell dry weight obtained was 9.31±0.13 g/L with 45±1.5 wt.% of P(3HB) accumulation. The P(3HB) produced from this

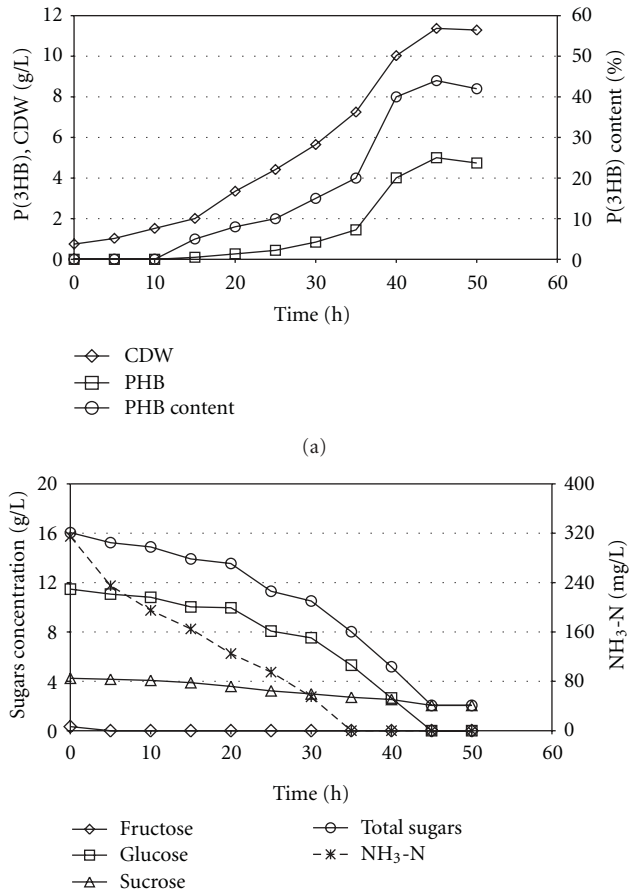

Figure 1: (a) Time profile of cell growth and P(3HB) production by *C. necator* (CCUG52238^T) using OPF juice in 2-L bioreactor at 30% DOT level. (b)Time profile of sugars and ammonical nitrogen utilization by *C. necator* (CCUG52238^T) using OPF juice in 2-L bioreactor at 30% DOT level.

study is 40% higher compared to the P(3HB) produced under nonoptimized condition as shown in our previous study [13]. The results presented herewith demonstrated that pH, agitation speed, and nitrogen concentration indeed plays an important role in the P(3HB) production by *C. necator* (CCUG52338^T) utilizing OPF juice.

3.2. Biosynthesis of P(3HB) in 2-L Bioreactor

3.2.1. Effect of Dissolved Oxygen Tension (DOT). Biosynthesis of P(3HB) from OPF juice by *C. necator* (CCUG52338^T) was carried out through batch cultivation process in 2-L bioreactor. The effect of DOT level in the bioreactor was studied for DO concentrations of 20 to 50% and the results are shown in Table 4. It was observed that CDW was increased when DOT level increased from 20 to 50%. On the other hand, P(3HB) accumulation was decreased with the increase in DOT level. Highest CDW (12.81 g/L) and P(3HB) content (46 wt.%) were achieved at 50 and 20% DOT level, respectively. Based on the result, it was found that dissolved oxygen concentration in the fermentation medium improved the cell growth; however, P(3HB) accumulation was found to be increased towards oxygen limitation. This result suggested that appropriate level of oxygen is needed for cell development, and oxygen depletion was favorable for P(3HB) accumulation.

As shown in Table 4, P(3HB) accumulation was tripled at lower dissolved oxygen concentration (20%) compared to the higher ones (50%). This might be due to the fact that insufficient supply of oxygen to the bacteria may decrease oxidation of NADH and lead to P(3HB) biosynthesis [15, 21, 28]. A similar observation was obtained in our previous study on the effect of different $(NH_4)_2SO_4$ concentration on P(3HB) production using OPF juice in shake flask experiment. These results indicate that both nitrogen and oxygen limitation do not improve cell biomass development, but markedly improve the P(3HB) accumulation. Therefore, it can be suggested that besides nitrogen depletion, oxygen limitation is also important in getting the optimal level of P(3HB) accumulation.

3.2.2. Cell Biomass and P(3HB) Production Profile. In order to study cell biomass and P(3HB) production profile by *C. necator* (CCUG52238^T), batch cultivation process was carried out using OPF juice in 2-L bioreactor with aeration supplied at 30% DOT level and the results were depicted in Figures 1(a) and 1(b). It was observed that the culture entered the exponential phase after a lag of 15 h, and nitrogen was completely consumed within 35 h. Highest CDW (11.37 g/L) and P(3HB) content (44 wt.%) were achieved at 45 hr cultivation period. The biomass yield ($Y_{x/s}$) and P(3HB) yield ($Y_{p/s}$) were 0.81 g biomass/g sugars consumed and 0.36 g P(3HB)/g sugars consumed, respectively. The maximum P(3HB) productivity was 0.11 g/L/h.

Almost similar P(3HB) content with some improvement in cell growth was obtained in this study compared to the shake flask experiment under optimal condition. Higher CDW (11.37 g/L) and biomass yield (0.81 g biomass/g sugars consumed) obtained in fermentor compared to shake flask were due to different conditions which prevail in the shake flasks and fermentor; some of these conditions include aeration, agitation, and temperature. In fermentor, aeration was supplied via air sparging, and agitation is provided by an impeller or by the motion imparted to the broth (liquid phase) by rising gas bubbles [30]. Temperature is maintained at a constant and uniform value by circulation of cooling water through coils in the vessel or in a jacket surrounding the vessel [31]. Compared to our previous studies in shake flasks using technical grade sugars [13], batch studies in 2-L bioreactor using renewable sugars from OPF juice showed superior results in *C. necator* CCUG52238^T probably due to the additional components in the OPF juice that improve the fermentation performance. An almost similar observation was reported by Koutinas et al. [12] when WH and FE were used as renewable feedstock for P(3HB) production. It was reported that the consumption of various carbon sources (carbohydrates,

Table 4: Effect of DOT (%) level on biosynthesis of P(3HB)[a].

DOT (%)	Maximum CDW (g/L)	Maximum total P(3HB) (g/L)	Maximum P(3HB) content (wt.%)[b]
20	9.55	3.93	46
30	11.37	4.78	44
40	12.52	3.38	25
50	12.81	2.37	15

[a]Experiments were conducted in 2-L bioreactor (1 L working volume) by batch mode using OPF juice with initial total sugars of 16 g/L as substrates.
[b]Determination by GC from freeze dried samples.
*Values obtained herewith are means of duplicate sample.

Table 5: Comparison of thermal and mechanical properties of P(3HB) obtained in this study with literature.

Microorganisms	Carbon sources	T_m	Tensile strength (MPa)	Elongation to break (%)	References
C. necator CCUG52238^T	OPF juice	162.2	40	8	This study
R. eutropha	Fructose	177	43	5	Doi, 1990 [28]
A. latus	Maple sap	177	—	—	Yezza et al., 2007 [10]

amino acids, peptides) presented in the feedstock resulted in high growth yields (up to 1.07 g cells/g glucose) as related to glucose.

As shown in Figures 1(a) and 1(b), the microbial growth is mainly associated with ammoniacal nitrogen consumption. For the first 35 h, lower sugars consumption by *C. necator* was observed. The sugars consumption within the time range was only 8.03 g/L which is half of the total sugars in the culture broth. On the other hand, the NH_3-N was found to be decreased drastically from initial and completely exhausted after 35 h of cultivation period. This result indicates that at initial, the microbial growth was mainly attributed by the consumption of nitrogen sources from $(NH_4)_2SO_4$ supplied earlier as one of the medium composition in bioreactor. In addition to that, other organic compounds such as amino acids, carbohydrates, and other minerals which were previously characterized in the OPF juice could be used as supplementary growth substrates by the bacterium [13]. After that, the cell growth was mainly contributed by the cell expansion due to P(3HB) accumulation inside the cells. It can be seen that the P(3HB) accumulation was doubled that is, 20 wt.% to 40 wt.%, from 35 h to 40 h of cultivation period. From sugars consumption and P(3HB) profiles, it can be observed that the detectable depletion of sugars in the medium from 35 h onwards can be associated with P(3HB) accumulation. These results are in agreement with the findings of other researchers that reported P(3HB) accumulation is favored by an excess of carbon source and limited supply of macrocomponents such as nitrogen and dissolved oxygen [7, 16, 29]. It is interesting to note that *C. necator* CCUG52238^T completely utilized the glucose in the OPF juice. Regardless of fructose (due to too low concentration of fructose in the medium), it seems like the bacterium preferred to consume glucose compared to sucrose. Glucose consumption rate by *C. necator* CCUG52238^T was much higher at 0.33 g/L/h, compared to that of sucrose that is, 0.049 g/L/h. This shows that *C. necator* CCUG52238^T prefers monosaccharide than disaccharide as its carbon source.

3.2.3. Characterization of Homopolymer P(3HB). The mechanical and thermal properties of the homopolymer produced in 2-L bioreactor are shown in Table 5. The mechanical and thermal properties of P(3HB) obtained in this study showed an almost similar properties to those reported in the literature. For instance, the tensile strength and elongation to break for P(3HB) produced in this study were 40 MPa and 8%, respectively, and it was comparable to the P(3HB) produced from pure fructose [28]. The melting temperature, T_m of P(3HB) obtained from OPF juice (T_m = 162.2°C), was slightly lower compared to the melting point 177°C reported for P(3HB) produced from pure fructose [28] and other renewable sugars such as maple sap [10]. This could be influenced by other properties of the P(3HB) such as molecular weight. It has been reported that the molecular weight of P(3HB) produced is mainly influenced by the type of bacterial strain, substrate, growth rate, and production temperature [15, 29, 32].

4. Conclusions

This study demonstrated that higher cell growth and P(3HB) accumulation can be obtained by culturing *Cupriavidus necator* strain CCUG52238^T at optimized condition using OPF juice as the sole renewable carbon source. Under the optimal conditions, the highest cell weight was 9.31 ± 0.13 g/L with 45 ± 1.5 wt.% of P(3HB) contained in the cells, accounts of 40% increment for P(3HB) content compared to the nonoptimized condition. Cultivation in a 2-L bioreactor with 30% DOT yielded CDW of 11.37 g/L and P(3HB) content of 44 wt.%. In the meanwhile, thermal and mechanical characterization of the P(3HB) obtained from OPF juice showed almost similar properties to those reported in the literature. It is worth to mention that this study may contribute to the process development for P(3HB) production from renewable OPF juice in pilot and industrial scale. Furthermore, since OPF is an abundant solid waste at oil palm plantation and is currently underutilized, it has a great potential to be used as sustainable, renewable, and cheap fermentation feedstock for the production of P(3HB).

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Research Article

Lovastatin Production by *Aspergillus terreus* Using Agro-Biomass as Substrate in Solid State Fermentation

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Ability of two strains of *Aspergillus terreus* (ATCC 74135 and ATCC 20542) for production of lovastatin in solid state fermentation (SSF) using rice straw (RS) and oil palm frond (OPF) was investigated. Results showed that RS is a better substrate for production of lovastatin in SSF. Maximum production of lovastatin has been obtained using *A. terreus* ATCC 74135 and RS as substrate without additional nitrogen source (157.07 mg/kg dry matter (DM)). Although additional nitrogen source has no benefit effect on enhancing the lovastatin production using RS substrate, it improved the lovastatin production using OPF with maximum production of 70.17 and 63.76 mg/kg DM for *A. terreus* ATCC 20542 and *A. terreus* ATCC 74135, respectively (soybean meal as nitrogen source). Incubation temperature, moisture content, and particle size had shown significant effect on lovastatin production ($P < 0.01$) and inoculums size and pH had no significant effect on lovastatin production ($P > 0.05$). Results also have shown that pH 6, 25°C incubation temperature, 1.4 to 2 mm particle size, 50% initial moisture content, and 8 days fermentation time are the best conditions for lovastatin production in SSF. Maximum production of lovastatin using optimized condition was 175.85 and 260.85 mg/kg DM for *A. terreus* ATCC 20542 and ATCC 74135, respectively, using RS as substrate.

1. Introduction

Lovastatin is a potent drug for lowering the blood cholesterol and it was the first statin accepted by United States Food and Drug Administration (USFDA) in 1987 as a hypercholesterolemic drug [1]. It is a competitive inhibitor of HMG-CoA reductase, which is a key enzyme in the cholesterol production pathway [2]. Lovastatin is a secondary metabolite during the secondary phase (idiophase) of fungi growth [3]. This product can be produced by cultures of *Penicillium* species [4], *A. terreus* [5–7], *Monascus* species [8, 9], *Hypomyces*, *Doratomyces*, *Phoma*, *Eupenicillium*, *Gymnoascus*, and *Trichoderma* [10]. Although the ability of different groups of fungi for production of lovastatin was reported in many studies, only production of this compound by *A. terreus* was commercialized (for manufacture of high quantity of lovastatin for used as anticholesterol drag) [11]. Microorganisms are able to produce lovastatin in SSF or submerged culture [5, 7, 12–15]. Experiment showed that quantity of lovastatin production in SSF is significantly higher than submerged culture [5]. Different substrates were used for lovastatin production in SSF, including sorghum grain, wheat bran, rice, and corn [5, 7]. These substrate materials are normally expensive and are competing with food or feed ingredients for human and livestock. On the other hand, large quantity of agro-industrial biomass such as RS and OPF are produced globally particularly in the tropical countries. These agro-biomass are often burned away for disposal, causing huge environmental concerns, with only some remaining being used as roughage feed for ruminant livestock. These

biomasses are, however, potential substrates for growth of microorganisms and production of biomaterials.

Over the last 250 years, the concentration of atmospheric methane (CH_4) increased by approximately 150% [16], with agricultural activities contributing 40% of the total anthropogenic source, of which 15 to 20% is from enteric fermentation in ruminants [17]. On the other hand, ruminal CH_4 production accounts for between 2 to 15% of dietary energy loss for the host animals [18]. Because of the negative effects on environment and the host animal nutrition, mitigation of enteric CH_4 emission in ruminant livestock had been extensively researched, including the use of various mitigating agents such as ionophores [19], organic acids [20], fatty acids [21], methyl coenzyme M reductase inhibitors [22], vaccine [23], and oil [24]. However, these technologies have limited application primarily because they, besides suppressing CH_4 also, decrease nutrients digestibility (such as oil and fatty acids), have negative effect on human and animal health (antibiotics), or are not economically acceptable (methyl coenzyme M reductase inhibitors and vaccine).

Wolin and Miller [25] showed significantly reduction in growth and activity of methanogenic Archaea using lovastatin without any negative effect on cellulolytic bacteria that was due to the effect of this drug on inhibition the activity of HMG-CoA reductases in the archaeal microorganisms. It is uneconomical to use pure lovastatin as a feed additive for the mitigation of CH_4 production in ruminants. Production of this component using low-cost substrate and process for being used as animal feed additive were the main objective of present study.

Thus, the primary objective of this study was to investigate the efficacy of two strains of *A. terreus* (ATCC 20542 and ATCC 74135) for production of lovastatin using RS and OPF as substrates. In addition, the effects on nitrogen source, mineral solution, moisture, incubation time, pH, inoculum size, particle size, and incubation time on lovastatin production were investigated.

2. Materials and Methods

2.1. Substrate. RS and OPF were collected from the local fields in the state of Selangor, Malaysia. The materials were ground and sieved through mesh size 6 to obtain particles size of about 3.4 mm and dried in oven at 60°C for 48 h and used in SSF studies.

2.2. Microorganism and Preparation of Spore Suspension. *A. terreus,* ATCC 20542 and ATCC 74135, used in this study was obtained from the American Type Culture Collection (ATCC). They were maintained on potato dextrose agar (PDA) slants at 32°C for 7 days, stored at 4°C, and subcultured every two weeks. For the preparation of spore suspension, 10 mL of sterilized 0.1% Tween-80 solution was added to the 7-day old culture slants of the fungi at the end of incubation, surface of the culture was scratched with sterilized loop, and the Tween-80 solution containing spores was transferred into 100 mL Schott bottle containing the same solution and agitated thoroughly using a shaker to suspend the spores. The number of spores was measured using a hemocytometer and adjusted to approximately 10^7 spores/mL for use as inoculum throughout the study.

2.3. Solid State Fermentation. This study consisted of two subexperiments. In the first, the efficacy of lovastatin production by two strains of *A. terreus* (ATCC 20542 and ATCC 74135*)* using two types of agro-biomass (RS and OPF) as substrate was examined. In addition, soybean meal, urea and ammonium sulphate were used as nitrogen sources and the need to supplement mineral to enhance the fermentation process was studied. In the next subexperiment, fermentation conditions were optimized for maximum lovastatin production in SSF. The procedure of SSF for each subexperiments was described below. Both subexperiments were conducted in triplicate. Presented data are Mean ± Standard Deviation.

Subexperiment 1: Effect of Substrate, Nitrogen, and Mineral Solution. Solid state fermentation was carried out in 500 mL Erlenmeyer flasks containing 20 g of the respective substrate (RS or OPF). The moisture content of the substrate were adjusted with mineral solution (KH_2PO_4: 2.1 g/L, $MgSO_4$: 0.3 g/L, $CaCl_2$: 0.3 g/L, $FeSO_4$: 0.11 g/L, $ZnSO_4$: 0.3 g/L) or distilled water to produce a moisture content of approximately 75%. pH of all the solutions were adjusted to 6 before adding into the solid substrate. For the study on effect of nitrogen source on lovastatin production, 1% urea, 1% ammonium sulphate, or 10% of soybean meal was added in to the solid culture. The contents in the flasks were autoclaved for 15 min at 121°C and after cooling the flasks to room temperature, 10% inoculum were added and the contents of the flasks were thoroughly mixed. The flasks were incubated at 32°C for ten days.

Subexperiment 2: Optimization the Fermentation Condition. Since the production of lovastatin using RS as substrate was significantly higher than that for OPF, a follow-up experiment was conducted to optimize several factors (pH, temperature, particle size, inoculums size, and initial moisture content) known to affect SSF process for production of lovastatin by both strains of *A. terreus* using only RS as substrate. The second experiment consisted of five subexperiments, each evaluating the effect of one of the above five factors on the SSF process with the remaining factors being constant. For study on the effect of pH, because of the difficulty of adjusting the pH of solid sample in SSF, pH of solution was adjusted using 1 M Sodium hydroxide and 1 M hydrogen chloride to pH 5, 6, 7, and 8 (before adding in the substrate). Incubation temperatures between 25 to 42°C, five different particle sizes: <425 μm (mesh no. 40), 425–600 μm (mesh no. 30), 600–1400 μm (mesh no. 14), 1.4–2 mm (mesh no. 10), and 2–3.35 mm (mesh no. 6), inoculums size of 5, 10, and 15% and initial moisture contents at 50, 66, and 75% were investigated.

2.4. Extraction and Determination of Lovastatin. At the end of fermentation, the solid culture was dried at 60°C for 48 h and 0.5 g of the dry culture was extracted with 15 mL methanol and shaking in a shaker for 60 min at 220 rpm [5].

Table 1: Effect of nitrogen source and mineral solution on lovastatin production in solid state fermentation by *A. terreus* using rice straw (RS) and oil palm frond (OPF) as substrates.

Treatments	Lovastatin production (mg/kg DM)	
	ATCC 20542	ATCC 74135
RS	154.48 ± 22.88^{a}	157.07 ± 1.92
RS plus mineral	119.35 ± 16.59^{ab}	146.09 ± 7.49
RS plus mineral and urea	96.3 ± 3.02^{bc}	118.26 ± 6.38
RS plus mineral and soybean meal	126.36 ± 22.84^{ab}	139.63 ± 25.45
RS plus mineral and ammonium sulphate	66.19 ± 3.39^{c}	129.39 ± 21.90
Significant	**	NS
OPF	7.84 ± 0.14^{b}	13.09 ± 2.22^{b}
OPF plus mineral	9.66 ± 0.03^{b}	7.2 ± 1.55^{b}
OPF plus mineral and urea	21.25 ± 4.66^{b}	17.58 ± 3.03^{b}
OPF plus mineral and soybean meal	70.17 ± 4.84^{a}	63.76 ± 14.00^{a}
OPF plus mineral and ammonium sulphate	10.06 ± 0.31^{b}	11.16 ± 3.41^{b}
Significant	**	**

NS: not significantly different.
**Significantly different at 1% level.
[a,b,c] indicating that means for each substrate within column are significantly different.

After filtration with membrane filter (0.2 μm), the concentration of lovastatin in the filtrate was assayed using HPLC (Waters, USA, 2690) attached with an ODS column (Agilent, 250 × 4.6 mm i.d., 5 μm). The mobile phase consisted of acetonitrile and water (70 : 30 by volume) contained 0.5% acetic acid. The flow rate was 1 mL/min. The photo diode array (PDA) detection range was set from 210 to 400 nm and lovastatin was detected at 237 nm. The sample injection volume was 20 μL, and the run time was 12 min. Since two forms of lovastatin (lactone and β-hydroxyl) are normally present in the fermented culture, they were separately determined in the HPLC. Commercial lovastatin (mevinolin K, 98%, HPLC grade, M2147, Sigma, USA) used as standard is in the lactone form. And β-hydroxyl lovastatin was produced from the lactone form using the method of Friedrich et al. [26]. Briefly, to prepare β-hydroxyl acid, lactone lovastatin was suspended in 0.1 M NaOH and heated at 50°C for 1 h in a shaking incubator. Subsequently, the mixture was adjusted to pH 7.7 with 1 M HCl, filtered through 0.2 μm filters and used as standard for HPLC. The retention times of the hydroxyl and lactone forms of lovastatin were 6.668 and 10.898 min, respectively. Different concentrations ranged from 0.5 to 500 ppm of lovastatin were used as standard and standard curve of lovastatin.

2.5. Scanning Electron Microscope. Microscopy analysis was conducted using a Scanning Electron Microscope (SEM) to determine the morphological growth of *A. terreus* on the surface of RS. The samples were dried (60°C for 48 h), cut into 1 mm size, and affixed to a metal SEM stub and sputter coated in gold using SEM coating unit (BAL-TEC SCD 005 Spotter coater). The coated specimens were viewed using Environmental Scanning Electron Microscope (Phillips XL30, Germany) at accelerating voltage of 15–25 KV.

2.6. Chemical Analysis. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the detergent system [27]. Acid detergent lignin (ADL), crude protein (CP), and ash were determined using the method described by AOAC [28]. Hemicelluloses content was estimated as the difference between NDF and ADF, while cellulose content was the difference between ADF and ADL.

2.7. Statistical Analysis. All of the experiments were done with 3 replicate. Individual culture flasks were considered as experimental units. Data were analyzed as a completely randomized design (CRD) using the general linear model (GLM) procedure of SAS 9.2 [29] with a model that included treatment effects and experimental error. All multiple comparisons among means were performed using Duncan's new multiple range test ($\alpha = 0.05$).

3. Results and Discussion

3.1. Lovastatin Determination. Lovastatin was quantified as its β-hydroxyl and lactone forms (Figure 1). The β-hydroxyl of lovastatin is the more active form of this drug but is unstable [5], thus preparation for its standard solution was prepared freshly from the lactone form according to Friedrich et al. [26]. Because the hydroxyl form of lovastatin is not stable, lactone form is normally the primary lovastatin detected in the fermented products. The quantities of lovastatin reported in Table 1 were the combination of the two forms, but results of this study show that β-hydroxyl form is the dominant lovastatin in the solid cultures.

It was reported that the conditions needed for the conversion of lactone into the β-hydroxyl form are high pH (e.g., by addition of NaOH), heating to 50°C, and naturalization by acid. Since none of the above conditions was applied in this study, the β-hydroxyl form of lovastatin present in the extract in this study is believed to be a direct product of SSF and not due to conversion from the lactone form. Hydroxyl lovastatin has been reported to be the more active form of this drug [26], and its efficacy for inhibition of HMG-CoA reductase will be validated in subsequent experiment.

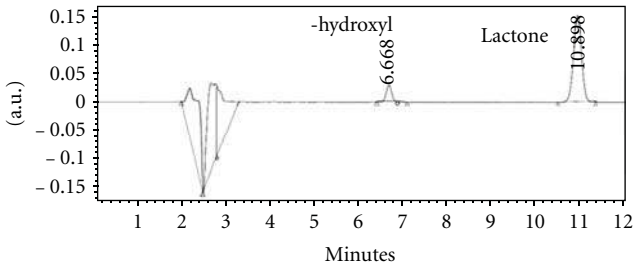


Figure 1: HPLC chromatogram of lovastatin in β-hydroxyl and lactone forms.

3.2. Effect of Substrate. In the first experiment, the efficacy of two strains of *A. terreus* on the production of lovastatin in SSF using RS and OPF as substrate was investigated. Lovastatin productions by both strains using RS and OPF as substrates are shown in Table 1. Quantity of lovastatin production by both strains of *A. terreus* in the RS samples was higher than OPF. The highest lovastatin production was 154.48 and 157.07 mg/kg DM, respectively, for *A. Terreus* ATCC 20542 and ATCC 74135 using RS as substrate without additional nitrogen and mineral supplementation.

Although the two strains of *A. terreus* tested were capable to produce lovastatin from RS and OPF, results of this study showed that RS is a more suitable substrate, producing significantly higher lovastatin than OPF.

Lovastatin can be produced from 9 molecules of acetyl-CoA during the fermentation (Figure 2) [30]. Since acetate production by microbial activity is correlated with carbohydrate fermentation, source of carbohydrate is important for production of this product. Lignocelluloses including cellulose, hemicelluloses, and lignin are the main components of agricultural biomass, therefore, for production of acetate, these macromolecules must first be hydrolyzed into their subunits such as glucose, xylose, in the presence of the appropriate enzymes, such as cellulase, hemicellulase, pectinase, and cellulobiase. The resulted monomers can then be used during the fermentation process of fungi and production of acetyl-CoA which can be used as substrate for lovastatin production (Figure 2). Therefore, the microorganisms selected for SSF must be able to degrade the lignocelluloses by producing sufficient amount of the appropriate enzymes to hydrolyze the respective lignocelluloses fractions. Production of cellulase (such as betaglucosidase, endoglucanase, and cellobiohydrolase) and hemicellulases (mainly xylanase) enzymes by *A. terreus* and their effects on lignocelluloses degradation have been well documented [31–33]. However, there is no known data on production of lignin degradation enzyme by *A. terreus.*

RS has two main advantages over OPF as substrate for *A. terreus* in SSF. Lignin content of RS is half that of OPF and its hemicelluloses content is higher than OPF (Figure 3). The lower production of lovastatin recorded for OPF using the two *A. terreus* could be partly due to the absence of enzyme to degrade the lignin component of the biomass. Reports also showed that *A. terreus* has high ability to produce hemicellulase enzymes such as xylanase [34], making RS which contains high hemicelluloses more appropriate as substrate for lovastatin production.

3.3. Effect of Mineral Solution and Nitrogen Source. Effects of mineral solution and nitrogen on lovastatin production by *A. terreus* are shown in Table 1. Addition of mineral solution has no significant effect on quantity of lovastatin production by both strains of *A. terreus*. Although addition of nitrogen source has no significant effect on lovastatin production by *A. terreus* ATCC 74135 ($P > 0.05$), it negatively affected lovastatin production by *A. terreus* ATCC 20542 ($P < 0.01$) mainly in the ammonium sulphate treatment. Supplementation of minerals and nitrogen sources (especially soybean meal) increased the of total lovastatin to about 64 mg/kg DM in *A. terreus* ATCC 74135 using OPF, but it is still half of that compared to RS (Table 1).

Minerals and nitrogen are essential growth nutrients for microorganisms [15]. In submerged fermentation, these nutrients must be added into the medium to sustain the growth of the microorganisms [34]. Agricultural biomass such as RS contain mineral components [35, 36]. Ash content of RS and OPF which is indicator of mineral content of these by products was shown in Figure 3. This quantity can supply the request of *A. terreus* for growth and lovastatin production and additional mineral solution has no effect on enhancement of the lovastatin production by this fungus.

Although nitrogen source is important for fermentation, but carbon : nitrogen (C : N) ratio is more important for lovastatin production. Negative effect of additional nitrogen source on lovastatin production was reported in previous study [37]. In the study of growth of *A. terreus*, and lovastatin production, the above authors reported that type of carbon and nitrogen sources and C : N ratio in the medium has important effect on lovastatin production and by increased the C : N ratio (from 14.4 to 23.4 and 41.3) the ability of *A. Terreus* for lovastatin production was increased. Use of a slowly metabolized carbon source (lactose) in combination with either soybean meal or yeast extract under nitrogen-limited conditions gave the highest lovastatin production. Effect of C : N ratio on lovastatin production was also studied by Bizukojc and Ledakowicz [6] who reported that higher C : N ratio can provide better fermentation condition for lovastatin (mevinolinic acid) production (Figure 4). Results of the chemical analysis showed that the availability of N in RS (CP = 4.2%) is higher than OPF (CP = 3.2%) (Figure 3). On the other hand, stranger bands in the cell wall polymers of OPF in comparison to the RS due to the higher lignin (Figure 3) can suppress the availability of nitrogen for fungal activity in OPF. It can be concluded that RS without additional nitrogen has better C : N ratio in comparison to the samples that contain additional nitrogen sources. In contrast, to achieve the suitable C : N ratio in the OPF culture, additional nitrogen source is requested.

3.4. Effect on Lignocellulose Reduction. Although the main objective of this study was to investigate the ability of *A. terreus* to produce lovastatin using biomass as substrate, ability of this fungus to reduce the lignocelluloses content

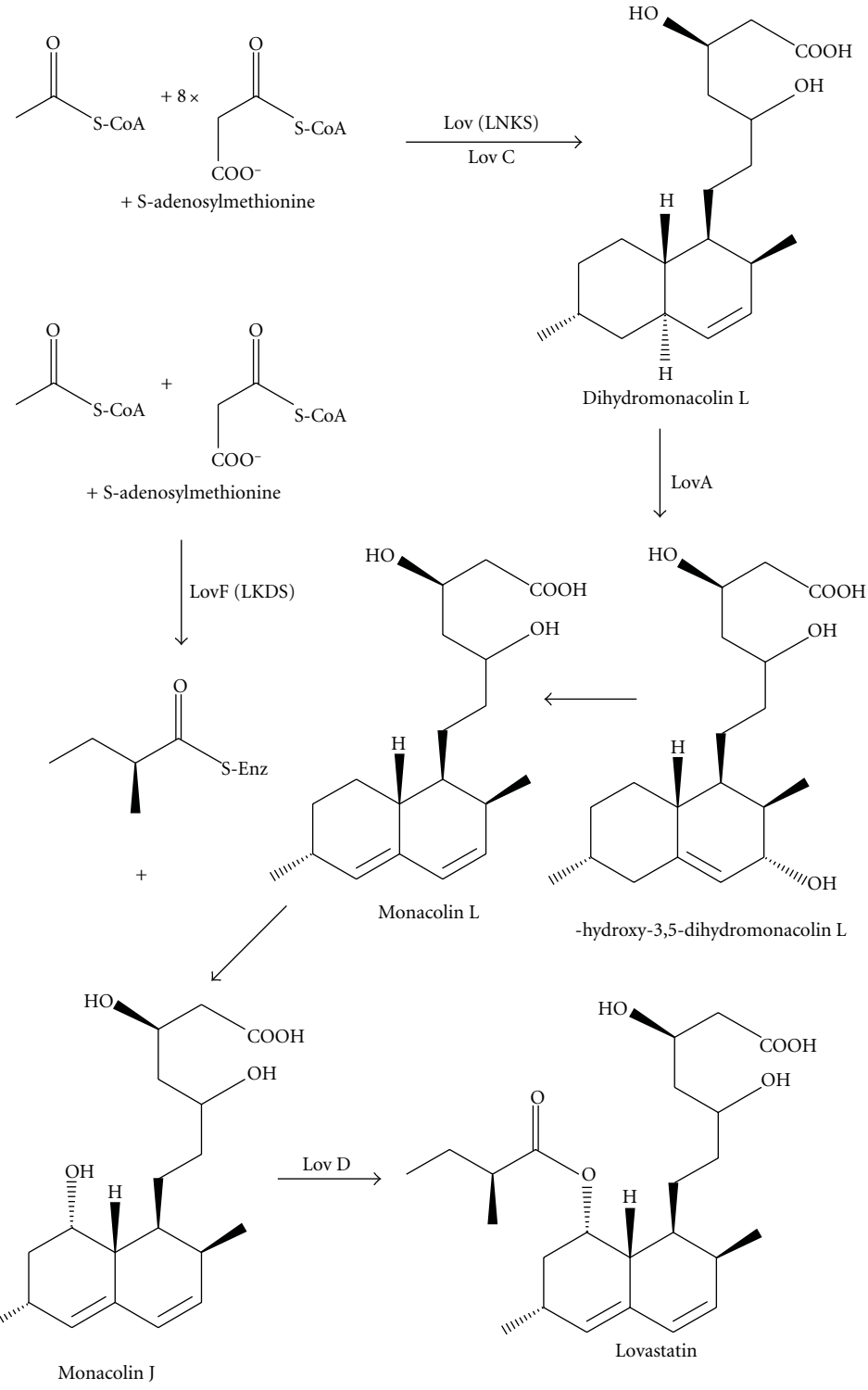


Figure 2: Lovastatin production pathway [30].

of RS and OPF was also important, particularly when these materials would be considered as feed for ruminants. Effect of the two strains of *A. terreus* on reduction of lignocelluloses content of RS and OPF is shown in Table 2. Results show that (i) additional mineral solution has no effect ($P > 0.05$) to enhancing the ability of *A. terreus* to reduce lignocelluloses content of RS and OPF but (ii) nitrogen supplement increases the ability of the fungi to degrade cellulosic materials. Both nitrogen solution, urea and ammonium sulphate significantly increased lignocellulosic components reduction. *A. terreus* has higher ability for reduction of lignocelluloses content of RS compared to OPF.

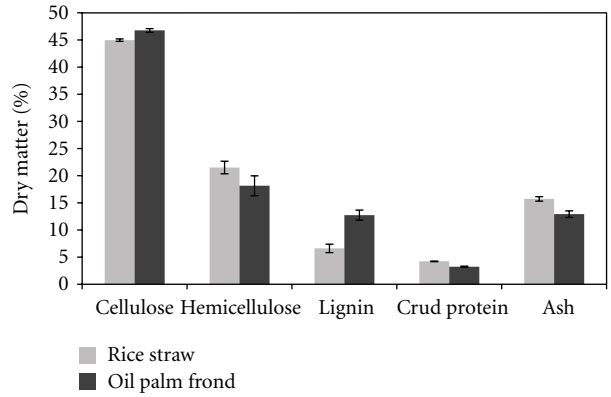


Figure 3: Lignocellulose, crude protein, and ash content of RS and OPF.

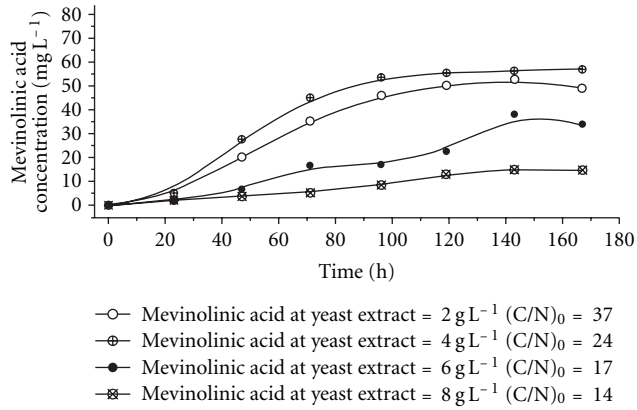


Figure 4: The evolution of mevinolinic acid (lovastatin) production in the batch culture at different initial yeast extract concentrations and C:N ratio content [6].

Although the effect of ammonium sulphate on lignocelluloses content reduction was higher than urea, this difference was not significant (Table 2). Because of its affordable price, urea is the most widely used nonprotein nitrogen in ruminant feed [38]. Thus, it is used as the nitrogen source in the following experiment for *A. terreus*.

Based on the higher yield of lovastatin using RS as substrate, the follow-up study for optimization of influencing factors (pH, temperature, particular size, inoculum size and initial moisture content and incubation time) was examined using the two strains of fungi on RS alone with addition of urea.

3.5. Effect of Initial pH. Study on initial pH (Figure 5) indicated that this factor has no significant effect on lovastatin production by both strains of *A. Terreus* ($P > 0.05$); however, pH 6 produced the highest lovastatin; 67.88 and 85.49 mg/kg DM by *A. terreus* ATCC 20542 and *A. terreus* ATCC 74135, respectively. There was no significant difference in lovastatin production for pH of 5 to 8; however, pH higher than 7 or lower than 6 had negative effect on lovastatin production. Kumar et al. [39] showed that pH 5.8–6.3 provided optimum conditions for lovastatin production by *A. terreus* in the batch process. Other researchers [40–42] also reported optimum pH of within the range of 5–7 using various fungi for statin production. The above information thus suggested that optimum initial pH for lovastatin production in SSF is near neutral pH with small variations which could be due to the types of substrate and microorganism used in the fermentation process. On the other hand, [43] reported that by controlling pH and slowly adding the carbon source lovastatin yield could increase five folds.

3.6. Effect of Temperature. One of the important factors effecting microbial activity and thus biomaterial production is incubation temperature. Results of this study suggest that 25°C is optimum temperature for lovastatin production in SSF by the two strains of *A. terreus* with maximum production of 171.61 and 202.93 mg/kg DM for *A. terreus* ATCC 20542 and *A. terreus* ATCC 74135, respectively (Figure 6). Increasing the incubation temperature to higher than 25°C has negative effect on lovastatin production and *A. terreus* ATCC 74135 being more sensitive to the temperature change. Similar incubation temperature (25°C) was also reported previously [43, 44] for lovastatin production. Optimum temperature of 29.5°C for lovastatin production had been reported by Panda et al. [40] using *Monascus purpureus* and *Monascus Ruber* while Kumar et al. [39] showed that 28°C is

Table 2: Effect of SSF on cellulose and hemicellulose contents of RS and OPF (% of dry mater).

Treatments	*A. terreus* ATCC 20542		*A. terreus* ATCC 74135	
	Cellulose	H-cellulose	Cellulose	H-cellulose
Nonfermented RS	44.97 ± 0.22^{ab}	21.50 ± 1.16^{a}	44.97 ± 0.22^{a}	21.50 ± 1.16^{a}
Fermented RS (FRS)	46.83 ± 2.12^{a}	18.06 ± 1.20^{bc}	45.12 ± 0.23^{a}	13.13 ± 0.79^{b}
FRS plus mineral	45.80 ± 0.67^{ab}	19.63 ± 0.80^{ab}	44.91 ± 0.72^{a}	12.38 ± 0.76^{b}
FRS plus mineral and urea	40.70 ± 2.52^{c}	16.96 ± 1.78^{dc}	41.97 ± 0.61^{b}	9.21 ± 1.05^{c}
FRS plus mineral and ammonium sulphate	42.72 ± 3.69^{bc}	15.11 ± 1.65^{d}	40.97 ± 0.51^{c}	7.79 ± 2.50^{c}
Significant	*	**	**	**
Nonfermented OPF	46.76 ± 0.34^{a}	18.13 ± 1.83	46.76 ± 0.34^{a}	18.13 ± 1.83
Fermented OPF (FOPF)	43.22 ± 2.59^{b}	19.36 ± 0.31	46.23 ± 1.17^{a}	15.66 ± 1.05
FOPF plus mineral	43.04 ± 1.28^{b}	15.82 ± 2.92	45.34 ± 0.95^{ab}	15.01 ± 0.82
FOPF plus mineral and urea	42.37 ± 1.92^{b}	16.90 ± 0.15	43.40 ± 0.89^{b}	15.40 ± 0.62
FOPF plus mineral and ammonium sulphate	44.66 ± 0.54^{ab}	18.30 ± 1.80	43.59 ± 1.69^{b}	15.00 ± 1.51
Significant	*	NS	*	NS

NS: not significantly different.
*Significantly different at 5% level.
**Significantly different at 1% level.
[a,b,c]indicating that means within column are significantly different.

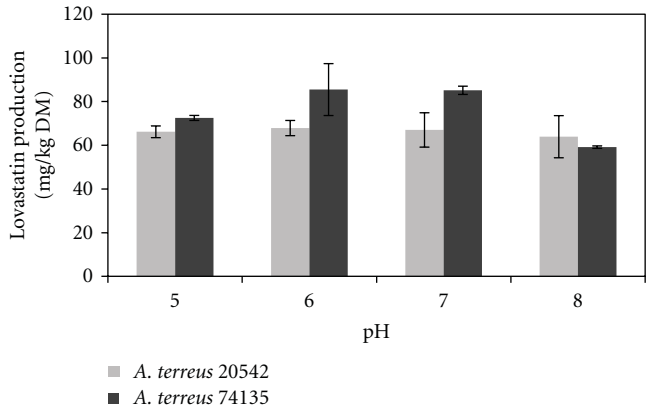


Figure 5: Effect of pH on lovastatin production by two strains of *A. terreus* ($P > 0.05$).

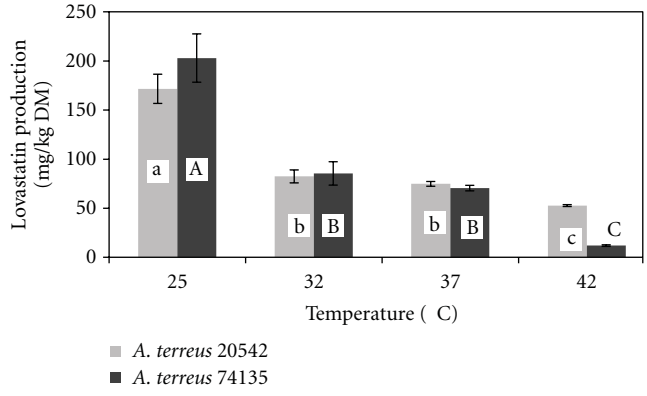


Figure 6: Effect of incubation temperature on lovastatin production by *A. terreus* ($P < 0.01$). (a, b, and c) indicate differences among means between samples for *A. terreus* ATCC 20542. (A, B, and C) indicate differences among means between samples for *A. terreus* ATCC 20542.

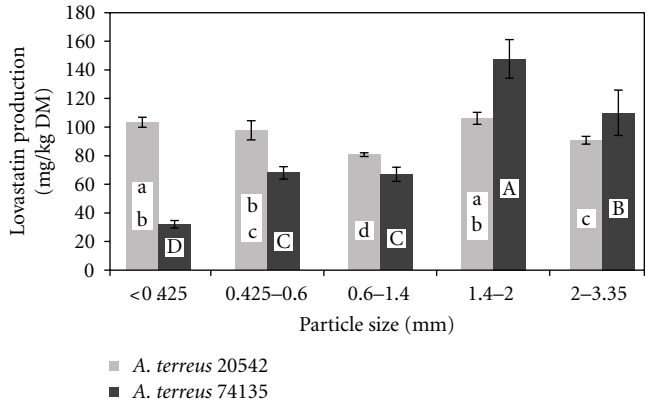
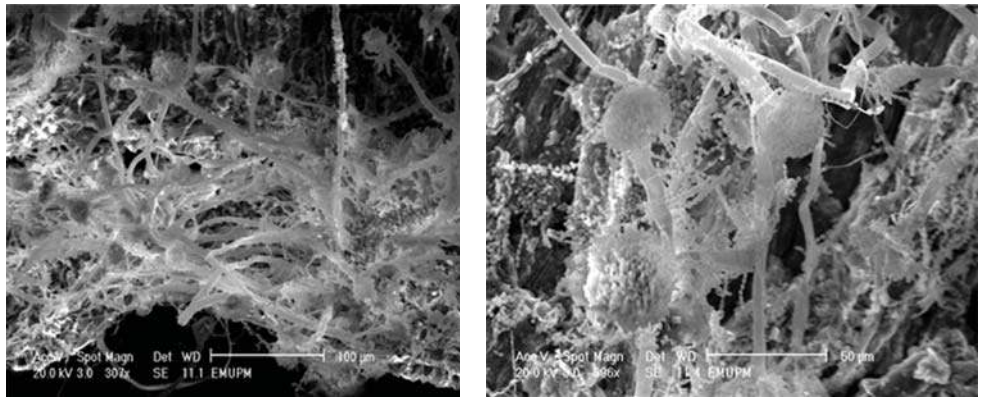


Figure 7: Effect of particle size on lovastatin production by two strains of *A. terreus* ($P < 0.01$). (a, b, c, and d) indicate differences among means between samples for *A. terreus* ATCC 20542. (A, B, C, and D) indicate differences among means between samples for *A. terreus* ATCC 20542.



(a) (b)

Figure 8: Scanning electron micrographs of *A. Terreus* ATCC 20542 (a) and *A. Terreus* ATCC 75135 (b) on the surface of RS. High concentration of mycelium and spore could have negative effects on air flow in the solid culture.

optimum incubation temperature for lovastatin production by *A. terreus*.

3.7. Effect of Particle Size. Five different particle sizes of RS were used to study the effect of this factor on lovastatin production. The results suggest optimum particle size of RS for lovastatin production was between 1.4 to 2 mm and *A. terreus* ATCC 74135 is more sensitive to changes in particle size (Figure 7). The above results are differed with that of Valera et al. [41], who found increasing particle size of wheat bran as substrate (from 0.4 to 1.1 mm) in SSF resulted in reduction of lovastatin production. They further reported an interaction effect between particle size and moisture content of solid material on lovastatin production.

There are two opposing effects of particle size on SSF process at any given moisture content. The first is small particle size that increases surface area of solid materials for the attachment and growth of the fungi. The second is smaller particle size that reduces interspace between particles and gas phase oxygen transfer and thus reduces the growth potential of the aerobic microorganisms. In addition, growth and multiplication of microorganisms on the surface of solid materials further reduce the gas phase space and make it even more difficult for air transfer between solid particles. Effect of high micellium content on reduction of the gas phase space of RS culture are shown in Figure 8. High concentration of mycelium and spores present between the solid particles reduce the flow of air in the culture and could reduce the available oxygen for growth of *A. terreus*.

On the other hand, some reports that indicate reduction of particle size enhanced production of lovastatin in SSF [15]. Wei et al. [7] reported that grounding rice through 20 mesh size (840 μm) has positive effect but further reduction using 40 mesh size (420 μm) reduced lovastatin production compared with the natural size of rice grain. The formation of lovastatin is strictly dependent on the oxygen supplied; however, Bizukojc and Ledakowicz [6] reported that aeration rate up to 0.308 vvm is preferred for lovastatin biosynthesis as higher aeration has negative effect on lovastatin production.

3.8. Effect of Moisture. Moisture content had significant effect on lovastatin production ($P < 0.01$) (Figure 9). Results

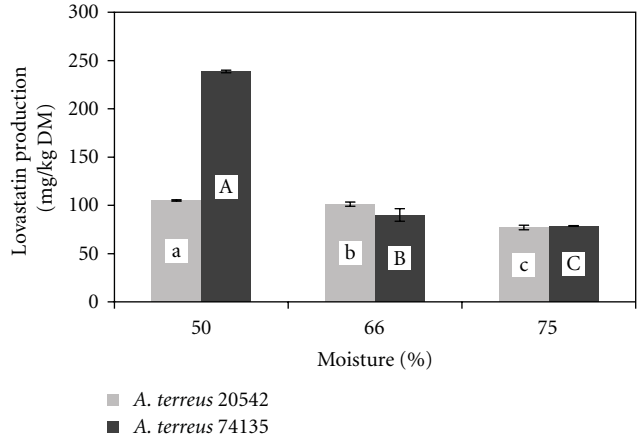


Figure 9: effect of moisture content on lovastatin production by two strains of *A. terreus* ($P < 0.01$). (a, b, and c) indicate differences among means between samples for *A. terreus* ATCC 20542. (A, B, and C) indicate differences among means between samples for *A. terreus* ATCC 20542.

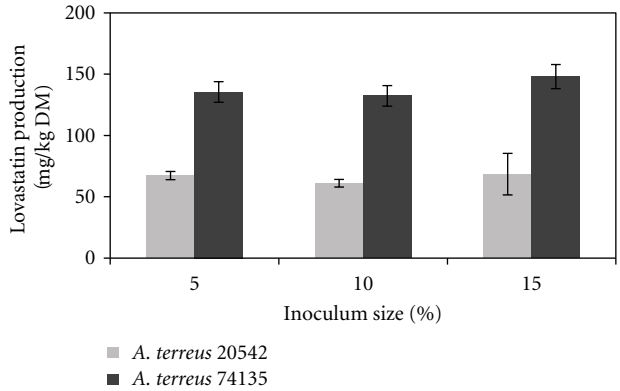


Figure 10: Effect of inoculums size on lovastatin production by *A terreus* ($P > 0.05$).

of this study suggest that 50% moisture content is optimum for lovastatin production by two strains of *A. Terreus*. Results also showed that *A. Terreus* ATCC 74135 is more sensitive to the moisture and at 50% moisture production of lovastatin by this strain can be up to 238.74 mg/kg DM. Other studies [41, 42] reported that slightly higher (58 to 60%) initial moisture produced maximum yield of statin using other fungi. High moisture content resulted in aggregation of substrate particles, reduction of aeration and leading to anaerobic conditions [45].

3.9. Effect of Inoculum Size. Figure 10 shows that different inoculum sizes did not affect ($P > 0.05$) lovastatin production by the two strains of *A. Terreus*. Previous study showed no significant different in lovastatin production using 5×10^7 to 10×10^7 spores/mL (wihin the ranged used in this study), but use of lower than 5×10^7 spores/mL can depress the production of lovastatin [15].

3.10. Effect of Incubation Time. To study the effect of incubation time on lovastatin production, optimal conditions for all the other factors (pH = 6, temperature = 25°C, inoculums size = 10% and moisture = 50%) obtained earlier were applied. Results of the study suggested that maximum production of lovastatin was achieved on day 12 with lovastatin production of 175.85 mg/kg DM for *A. terreus* ATCC 20542 but day 8 with lovastatin production of 260.85 mg/kg DM for *A. terreus* ATCC 74135 (Figure 11). In both fungi cultures, the concentration of lovastatin increased until day 8 with no significant effect on lovastatin production thereafter. Pansuriya and Singhal [15] reported that lovastatin production by *A. terreus* in SSF using wheat bran increased until day 3 of fermentation with no further enhancement thereafter. The shorter duration of the above study compared to the present study could be due to higher quality of substrate (rice bran versus RS). The yields of lovastatin obtained in this study were about 20% of those reported [5, 42] using wheat bran and groundnuts oil cake as substrates. The lower lovastatin yield recorded in this study, using agro-biomass as compared to those using high-energy grains and oil seeds, was acceptable.

4. Conclusion

Results of this study suggest that RS is the better substrate than OPF for lovastatin production in SSF. Although

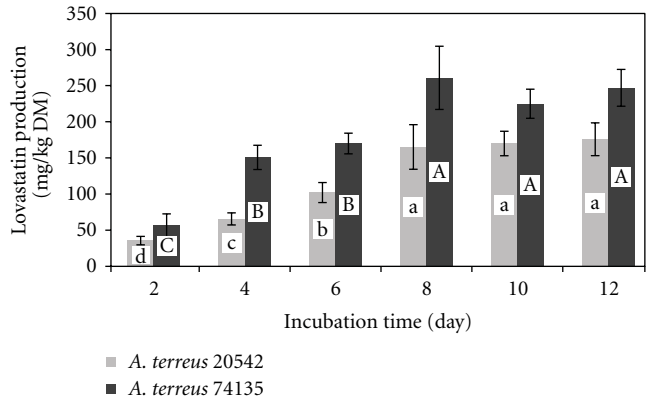


Figure 11: Lovastatin production by *A. terreus* in optimum condition at different time of incubation ($P < 0.01$). (a, b, c, and d) indicate differences among means between samples for *A. terreus* ATCC 20542. (A, B, and C) indicate differences among means between samples for *A. terreus* ATCC 20542.

additional nitrogen source has no benefit for improvement the lovastatin production, it enhances lignocelluloses degradation by the fungi. Since one of the main objectives of this study was to evaluate the use of the fermented RS as ruminant feed, urea was supplemented as nitrogen source in the fermentation process. Results of optimization experiment indicate that pH 6, 25°C incubation temperature, 10% inoculums size, 50% moisture content, and 8 days fermentation are the best conditions for maximum lovastatin production in SSF using RS as substrate with *A. terreus* ATCC 74135 recorded higher lovastatin production of 260.85 mg/kg DM after 8 days fermentation.

The present study provides a new insight for production of lovastatin and/or other similar biomaterials of high value from agro-biomass, which otherwise may be sources of pollutant to the environment. Furthermore, the lovastatin enriched fermented RS has the potential to be used as antimethanogenesis feed supplement for reduction of enteric methane production in the ruminant animals. The above suggestion needs further investigations.

Acknowledgment

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Research Article

A Novel Partially Biobased PAN-Lignin Blend as a Potential Carbon Fiber Precursor

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Blends of polyacrylonitrile (PAN) and lignin were prepared with three different lignin types by solution blending and solution casting. Among three types of lignin, one type was chosen and different blend concentrations were prepared and casted. The casted blend films were characterized chemically with fourier transform infrared spectroscopy (FTIR), and thermally with thermogravimetric analysis (TGA). The mechanical properties of the blends were measured using dynamic mechanical analysis (DMA). FTIR analysis shows an excellent interaction of PAN and lignin. The interaction of the lignins and PAN was confirmed by TGA analysis. The DMA results reveal that the lignin enhance the mechanical properties of PAN at room temperature and elevated temperatures. The blend structure and morphology were observed using scanning electron microscopy (SEM). SEM images show that excellent polymer blends were prepared. The results show that it is possible to develop a new precursor material with a blend of lignin and PAN. These studies show that the side product of paper and cellulosic bioethanol industries, namely, lignin can be used for new application areas.

1. Introduction

Carbon fibers attract attention throughout the world as a strong and light material in the composites industry for applications such as aerospace, automotive, and renewable energy resources [1]. For the fiber reinforced composites, carbon fibers are excellent reinforcing materials with mechanical strength of 5000 MPa, modulus value of 250 GPa, and density of 1.76 g/cm^3. Carbon fiber was first invented in 1871 by using cellulose as the precursor but the industrial applicable carbon fiber was developed in the 1960's by Union Carbide. In the beginning of 1970's, the use of carbon fibers for aerospace and military applications started [1]. Besides superior specific strength (strength/density), carbon fibers exhibit excellent properties in electrical conductivity, shielding effects, and heat resistance [1].

For the production of carbon fibers, there are three main raw materials, precursors. The first one is the cellulose which was first used in 1871. Pitch and polyacrylonitrile (PAN) are two other materials to produce carbon fibers. Due to the final fiber properties obtained, carbon fiber produced from PAN is commonly used which contributes 90% of the total carbon fibers available in the market [2, 3].

The production capacity of carbon fiber is increasing with new composite manufacturing techniques in the world, thus the need for composites and carbon fibers is increasing. However the price of carbon fiber is not declining which limits the widespread use of carbon fibers [4]. One of the reasons for the high cost of the carbon fiber is the precursor material. The research for precursor material is limited in the academy and the research findings from PAN producers are not available in the public press. In the current carbon fiber industry, still the price of the precursor is one of the obstacles to get a widespread use of carbon fiber [5].

The precursor material, PAN is a terpolymer of acrylonitrile, vinyl acetate and itaconic acid. The precursor is prepared with different processing stages including polymerization, PAN-solvent (dimethylacetamide) mixture preparation, and fiber spinning. After obtaining PAN precursor, the fibers are transformed to carbon fiber via oxidation

and carbonization processes followed by certain surface treatments [1].

To reduce the cost of precursor material, PAN polymer is blended with lignin in this study. Lignin is the third most abundant polymer on earth that is available from all the plants. The amount of lignin that is available is 300 billion tones [6]. Lignin constitutes 25–35% of the plants depending on the plant type [7]. With so many different plants there is much lignin resource.

On the other hand, lignin is generally removed during paper manufacturing and lignocellulosic bioethanol production. These industries are trying to find ways to obtain pure cellulose to achieve the highest quality by a process called delignification. Thus, lignin has been undervalued in these processes and it is not even considered as a coproduct [8]. Lignin is used as a fuel by burning at elevated temperatures [9]. However, the efficiency of lignin burning is limited. There are other studies conducted to prepare new materials with lignin. There are many studies reported on the polymer blend manufacturing with lignin and some other polymers [8]. There are studies to synthesize polyurethanes with lignin as the polyol [10]. The lignin finds applications as surfactant, ultraviolet stabilizers, dyes, and colorants [8].

Lignin was used to prepare carbon fibers as well. The research on carbon fibers with lignin is very limited due to the poor properties obtained with neat lignin material [11, 12]. The tensile strength values obtained are in the range of 600–700 MPa which is very low compared to a conventional carbon fiber, thus it cannot be used as the reinforcing fiber.

In this study a novel biobased precursor material was prepared via blending PAN and lignin in order to reduce the cost of precursor, to improve the mechanical properties of precursor and to find new applications of lignin which is undervalued product and moreover to create a sustainable, renewable biobased material. Due to price of carbon fiber, the demand for carbon fiber is not that high. This study demonstrates that it is feasible to reduce the cost of the carbon fiber precursor with the use of side product from cellulosic ethanol and paper industries. The lignin materials and blend morphology were investigated with scanning electron microscopy. Mechanical performance of the blends was measured to understand the effects of lignin for the PAN polymer. Furthermore, the interaction of PAN and lignin was analyzed using FTIR and TGA. SEM images were obtained to understand the blend morphology.

2. Experimental

2.1. Materials. PAN (polyacrylonitrile) was purchased from Acordis Kelheim GmBH as Homo-PAN in the form powder. Three types of lignin (Protobind 1000, Protobind 5000, and Protobind 2400) were obtained from Greenvalue as a gift. Detailed chemical and thermal analyses of the lignin from Greenvalue were done in a previous publication [6]. Dimethyl acetamide (DMAc) solvent was analytical grade.

2.2. Sample Preparation. Blends of PAN and lignin were prepared with solution casting technique. The lignin and PAN were dissolved in DMAc at certain weight percentages. The PAN-lignin solution was stirred overnight and the solution was cast onto glass. The solution was placed in an oven at 120°C for 6 hours to evaporate DMAc. After removing the solvent, films of the PAN-lignin blends were peeled from the glass surface. Table 1 shows the weight percentages of the blends and the sample codes.

Table 1: Sample codes.

Sample code	PAN wt%	Lignin type, wt%
PAN	100	0
PL1020	80	Protobind 1000, 20
PL2420	80	Protobind 2400, 20
PL5020	80	Protobind 5000, 20
PL2410	90	Protobind 2400, 10
PL2430	70	Protobind 2400, 30

2.3. Characterization. Optical microscopy image was obtained using Leica DM 1750 M with 500x magnification. Scanning electron microscopy (SEM) was used to observe the microstructure of the polymer blends. JEOL 640 was used as SEM. The samples were coated with gold and the images were taken at different magnifications.

Fourier transform infrared analysis (FTIR) was done with Perkin Elmer, model spectrum 2000 with ATR mode. The spectrometer was used with transmission mode and the resolution was 4 cm^{-1} with a range of 400–4000 cm^{-1}. Thermogravimetric analysis (TGA) was carried with Perkin Elmer Diamond TG/DTA by heating from room temperature to 600°C under nitrogen atmosphere. Dynamic mechanical analysis (DMA) was carried with Dynatest Metravib with tensile clamps heating from room temperature to 200°C with a frequency of 1 Hz.

3. Results and Discussion

3.1. Microscopy. Microscopy is the essential part of materials science research showing the particles, polymers and their interaction. The microscopy studies demonstrate the particle size, morphology and their change after polymer blending and composite preparation. Optical microscopy enables researchers to observe the preliminary images easily with no sample preparation needed [13]. Moreover, OM provides the actual images with colors. Scanning electron microscopy gives more detailed information by the electron emission and collecting the image via back scattering of the electrons [14]. In this study, lignin particles were investigated with optical microscopy (OM) and scanning electron microscopy (SEM). Blends of PAN and lignin were investigated with SEM.

Lignin Particles. The OM was used to get the images of lignin particles (Protobind 2400) as shown in Figure 1. The OM images of lignin particles have not been published before. Lignin particles are brown in color and they are semitransparent by their nature. This is highly important for many composites which will be made with lignin especially

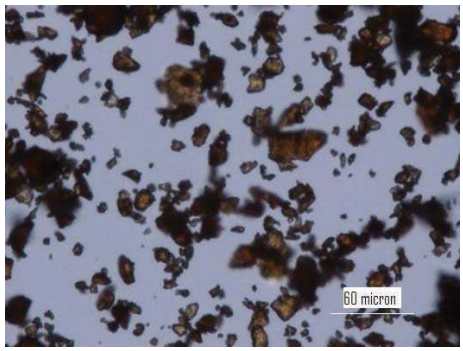

Figure 1: Optical microscopy image of Protobind 2400.

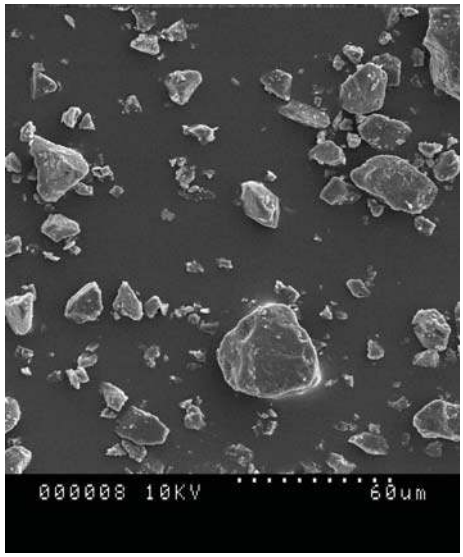

Figure 2: SEM image of Protobind 2400.

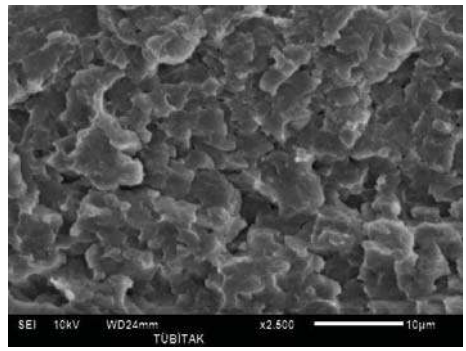

Figure 3: SEM image of PAN.

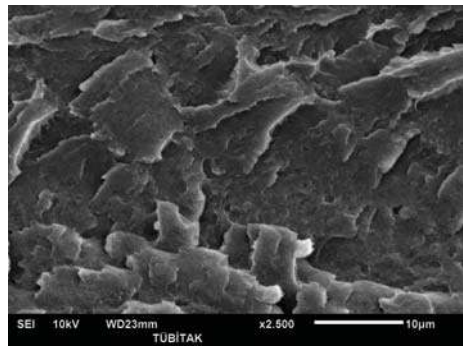

Figure 4: SEM image of PL2420.

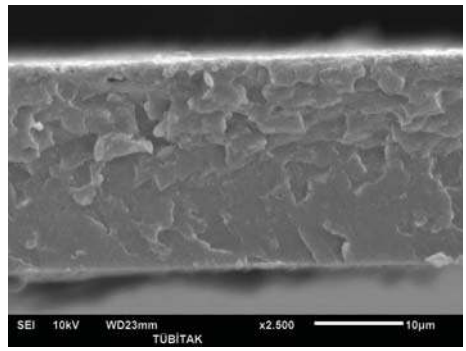

Figure 5: SEM image of PL5020.

polymeric films made with lignin would be very interesting. The average particle size is around 10–15 micron meter. The SEM image of lignin (Figure 2) confirmed the OM image. The particle size is in the same range. The optical properties of lignin cannot be judged with scanning electron microscopy but the image is clearer with higher resolution.

PAN and PAN-Lignin Blends. Figure 3 shows the SEM image PAN polymer. The image was taken at 2500x magnification. The image is a typical polymer SEM figure. There is no void or failure point in the polymer. The neat polymer structure is well demonstrated in this SEM image. Rahman et al. [15] presents similar PAN structures in their SEM images. Rahman et al. [15] tried to make a new fiber without any pore using a new coagulation bath. In this study presented in this paper, it was also possible to obtain PAN materials without holes. Chen and Harrison [16] reports new carbon fibers using PAN and they made PAN fibers with holes as presented in their paper. This is another important aspect of this scientific paper to show that the author was successful in obtaining hole and void free PAN materials.

Figures 4–6 show PAN and lignin blends with 20 wt% lignin. Figure 4 shows PAN blend with lignin Protobind 2400. Figure 5 shows PAN and lignin Protobind 5000 blend and Figure 6 shows PAN and lignin Protobind 1000. All the images are very important in terms of the compatibility and blend morphology. It is clearly observed that PAN and lignin blends form miscible blends with smooth surface and one phase figure. The lignin particles were dissolved in DMAc solvent with PAN so well that the particles of lignin are not visible any more like the OM and SEM images of the lignin. The blend morphologies are also very critical in the performance and compatibility of polymers. The blend morphologies are very homogenous and finely prepared. The similar observations were done by Zhang

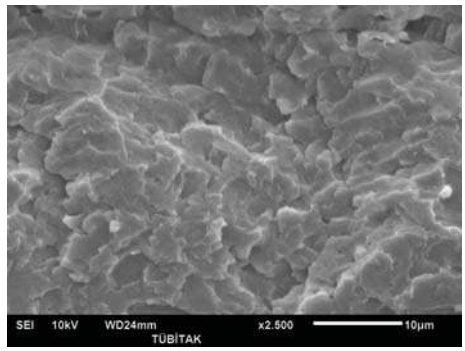

Figure 6: SEM image of PL1020.

Table 2: Modulus values.

Sample code	Storage modulus values, E'	Loss modulus values, E''
PAN	$9.2 * 10^8$	$5.1 * 10^7$
PL1020	$8.3 * 10^8$	$4.2 * 10^7$
PL2420	$1.5 * 10^9$	$1.1 * 10^8$
PL5020	$7.8 * 10^8$	$6.3 * 10^7$
PL2410	$1.2 * 10^9$	$8.2 * 10^7$
PL2430	$1.7 * 10^9$	$3.1 * 10^8$

et al. [17]. Though every blend looks very homogenous in shape, there are differences in the appearance of the lignin blends which arise from the lignin chemistry. Lignin blend with Protobind 2400 shows more brittle failure with sharper images. The lignin-PAN blend with Protobind 5000 looks smoother. The final blend morphology with Protobind 1000 looks more uniform which is very close to the image of the neat PAN. Protobind 2400 with higher purity leads to a sharp image whereas Protobind with certain functionalities creates a smooth PAN blend due to the chemical interaction of PAN and the nitrogen groups in lignin. Protobind 1000 series is less pure than Protobind 2400 and it does not have the functional groups. That is why the image is closer to neat PAN structure.

3.2. DMA Analysis. Dynamic mechanical analysis helps to understand the mechanical performance of the materials with regards to change in temperature [18–21]. The modulus values are plotted against increasing temperature with dynamic testing at 1 Hz frequency. The storage modulus value can be related to Young's Modulus measured during the static testing. The loss modulus value is related to modulus value of the material in the plastic deformation zone of tensile testing. Tan d is the ratio of loss modulus over storage modulus. A typical DMA curve is presented in Figure 7. This figure shows DMA graph of neat PAN material.

When the mechanical properties of neat PAN and PAN-lignin blends are investigated, it was observed that lignin does not deteriorate PAN properties (Table 2). When with Protobind 5000 and Protobind 1000, modulus values decrease slightly. The decrease for storage modulus values were 10% for Protobind 1000 and 15% for Protobind 5000. On the other hand, when Protobind 2400 is used for PAN blending, the modulus values increase due to purity difference of the lignins. 2400 lignin is sulfur free lignin with better properties. The increase is linearly proportional as the lignin content increases. The rule of mixture can be applied here as stated in the following:

$$E_b = E_m * V_m + E_f * V_f \text{ [22, 23]}$$

E_b is the modulus of the blend

E_m is the modulus of matrix polymer (PAN)

V_m is the volume fraction of matrix polymer

E_f is the modulus of the fiber (lignin)

V_f is the volume fraction of the fiber (lignin).

When the data is calculated from each concentration of lignin, the modulus value of lignin is calculated as 3.25 GPa which is slightly higher than PAN and the value found in this study is confirming the previous studies done with lignin [24, 25]. The previous studies showed that lignin has a modulus value of 3–5 GPa. Thus, the lignin material does not only lower the cost of PAN, it also improves the mechanical properties of PAN which will enable to increase the carbon fiber properties as well.

The loss modulus values of the neat PAN and blends follow the same trend as storage modulus values. As Protobind 1000 and Protobind 5000 are blended with PAN, the properties do not alter much but as Protobind 2400 is blended, the loss modulus values also increase parallel with storage modulus values.

Another important finding of this study was to utilize any kind of lignin for PAN studies in terms of mechanical properties. The properties of PAN do not alter much. This study can be even extended for lignin produced as the coproduct of paper industries and other industries.

3.3. FTIR. FTIR is a very powerful tool to understand the interactions of polymers and organic materials. Figure 8 shows FTIR analysis of PAN and PAN-Lignin blends. One of the important peak to mention is the band at 2243 cm^{-1} which corresponds to C≡N bond [26]. There are three other important peaks for these analyses. The first peak is the 1430 cm^{-1} peak which disappears as the lignin is introduced and blends are formed. This peak corresponds to CH_2 scissoring of PAN [27]. This shows the excellent interaction of PAN and lignin forming new bonds and altering the other bonds in the PAN structure.

The other two peaks that are noteworthy are the 1512 cm^{-1} and 1115 cm^{-1}peaks that are apparent as the PAN lignin blends are formed. 1512 cm^{-1} peak corresponds to N–H bending of the lignins whereas 1115 cm^{-1} peak corresponds to C–H stretching bond of the lignins [6]. As Protobind 2400 content increases, the intensity of peak at 1512 cm^{-1} increases. This shows that another important finding of this research was to find similar peaks even though the chemical content of these lignin differ. This shows that the major peaks of the lignin structure appeared in the PAN blends. Though, in literature, the exact structure of lignin

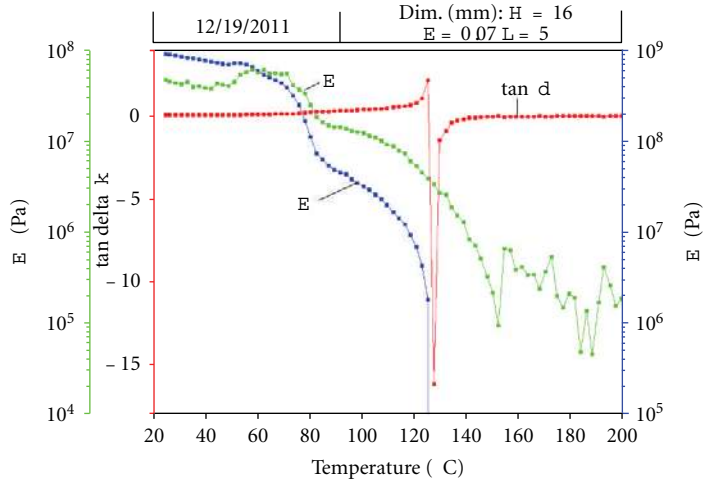

Figure 7: DMA curve.

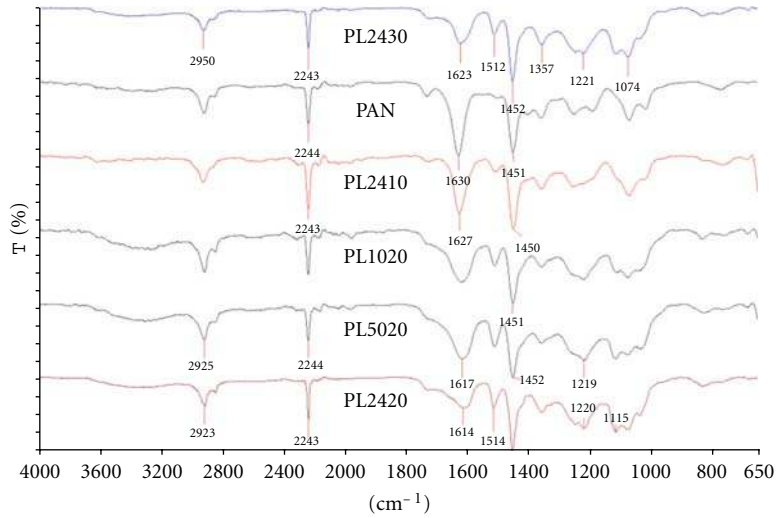

Figure 8: FTIR analysis of PAN and PAN-lignin blends.

could not be identified completely, there are studies showing important bonds of lignin [6, 8].

3.4. TGA. The thermal property of polymers is another important parameter. The thermal degradation occurs in polymeric materials at elevated temperatures. The polymer degrades at certain temperature. The degradation temperature is determined using the derivative of thermogravimetric analysis curve. At 300°C, PAN started to decompose which can be observed from the thermal transition curves. Figure 9 shows the TGA curve without calculation and Figure 10 shows the calculated values. The PAN starts to decompose at 300°C with the first and major decomposition. In this degradation part, C–N bonds degrade [28, 29]. Around 426°C, there is a second minor degradation temperature which would arise from C–C bond breakage.

The influence of lignin for these transitions was investigated during this research. The previous publication on lignin characterization well documents the thermal stability of lignins [6]. The lignin material is not stable for high temperatures. The thermal stability of PAN decreases as the lignin content increases. TGA curve and derivative for the PAN2410 is demonstrated as an example of the study (Figure 11). The figures of all the blends are not shown to avoid too many graphs in the paper. The major degradation temperature of each blend is summarized in Table 3. The onset temperature for lignin decomposition of each lignin differs so the thermal stability of the PAN changes according to the lignin stability. Protobind 5000 was the least thermal stable lignin among others due to some alcohol and acid groups present in the material. The onset temperature of PAN with 5000 (20 wt%) is lower than PL2430 which has 30 wt% of lignin 2400. The finding of the thermal stability is also consistent with the data published in the author's previous paper [6].

More studies can be conducted at higher temperatures using TGA to understand the carbon fiber formation but

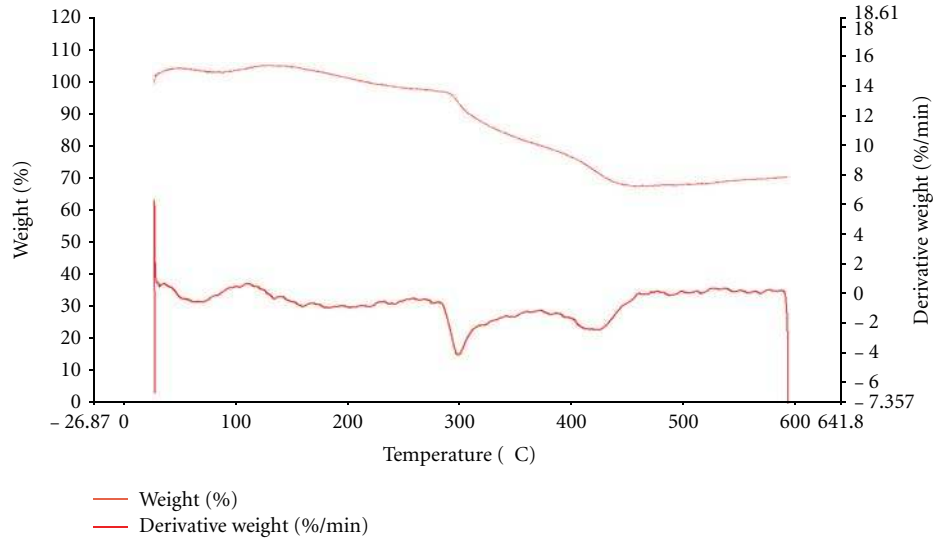

Figure 9: TGA-PAN.

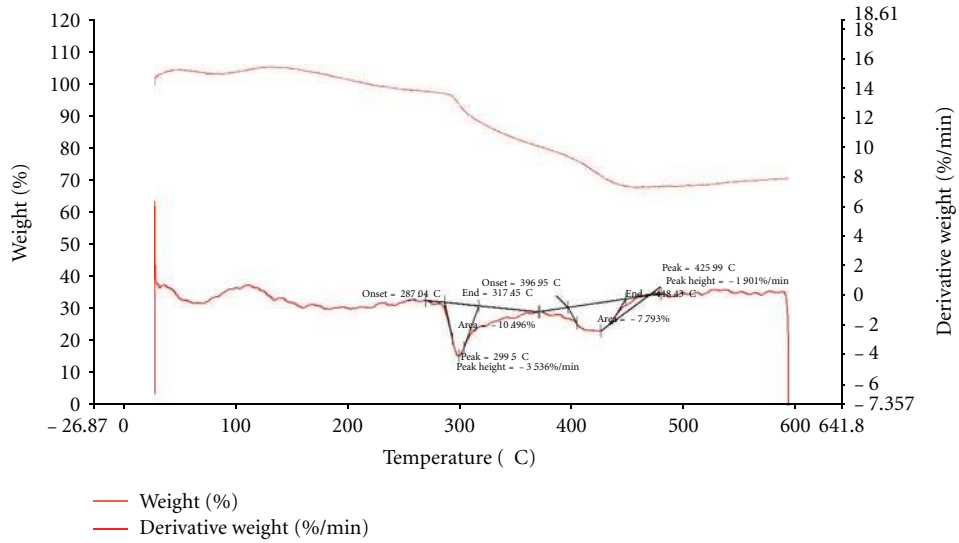

Figure 10: TGA-PAN, values determined.

Table 3: Degradation temperatures.

Sample code	Degradation temperature
PAN	299.50
PL1020	289.60
PL2420	288.01
PL5020	283.91
PL2410	290.06
PL2430	285.41

this is beyond the limits of this study. During the next study, carbonization studies will be performed using the similar blends and at higher temperatures. This study is the first attempt to make polymer lignin blends to be used for carbon fiber precursor. These new findings will open new ways to understand the carbonization phenomena with lignin materials.

4. Conclusion

In this study, a very novel polymer was developed with a blend of PAN and a renewable resource material lignin. To meet the increased need for carbon fiber, there was a very limited research conducted to replace the precursor. There were two studies that prepared carbon fibers from lignin but the mechanical properties of the carbon fibers that were published could not be satisfactory to meet the requirements of the composite industry. In this preliminary study, it is observed that PAN can be replaced with lignin forming

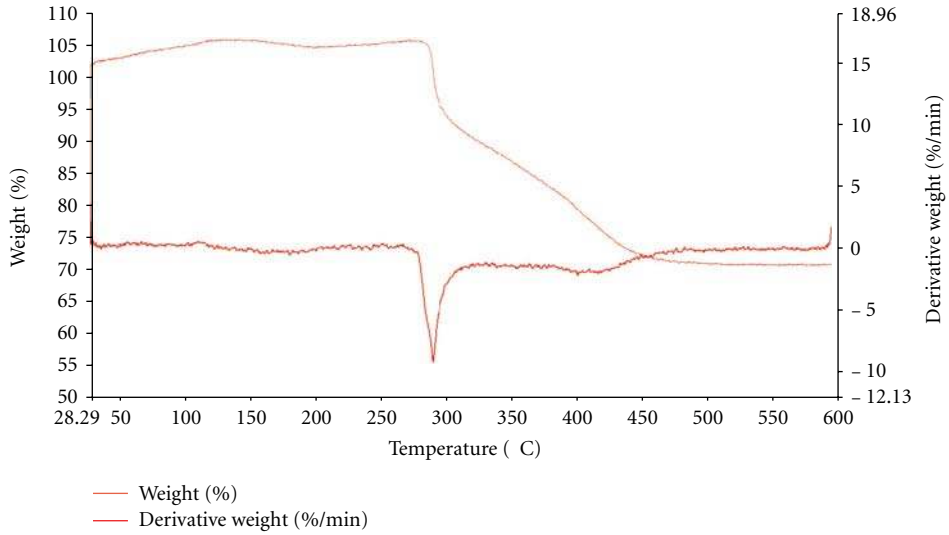

Figure 11: TGA-PL2410.

an excellent polymer blend without sacrificing the mechanical properties of PAN. The interaction of the PAN and lignin was observed with SEM, FTIR, and TGA analysis. This work with a homo-PAN demonstrated the initial steps for the PAN and carbon fiber industry. This study can be further carried with carbon fiber precursor, PAN and moreover that polymer blend can be spun to fiber and formed into carbon fiber via the carbonization processes. Most important part of the research was to utilize the lignin material which is an undervalued co-product of many different industries. This study demonstrated a new application area of lignin which is the second abundant matter on Earth after cellulose material. The most important finding of this research was to find a partial replacement of PAN with a renewable resource and making the PAN precursor greener technology to improve the world environment.

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Research Article

Enzymatic Saccharification and Ethanol Fermentation of Reed Pretreated with Liquid Hot Water

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Reed is a widespread-growing, inexpensive, and readily available lignocellulosic material source in northeast China. The objective of this study is to evaluate the liquid hot water (LHW) pretreatment efficiency of reed based on the enzymatic digestibility and ethanol fermentability of water-insoluble solids (WISs) from reed after the LHW pretreatment. Several variables in the LHW pretreatment and enzymatic hydrolysis process were optimized. The conversion of glucan to glucose and glucose concentrations are considered as response variables in different conditions. The optimum conditions for the LHW pretreatment of reed area temperature of 180°C for 20min and a solid-to-liquid ratio of 1 : 10. These optimum conditions for the LHW pretreatment of reed resulted in a cellulose conversion rate of 82.59% in the subsequent enzymatic hydrolysis at 50°C for 72 h with a cellulase loading of 30 filter paper unit per gram of oven-dried WIS. Increasing the pretreatment temperature resulted in a higher enzymatic digestibility of the WIS from reed. Separate hydrolysis and fermentation of WIS showed that the conversion of glucan to ethanol reached 99.5% of the theoretical yield. The LHW pretreatment of reed is a suitable method to acquire a high recovery of fermentable sugars and high ethanol conversion yield.

1. Introduction

Lignocellulosic material (LCM) is an abundant, natural, and renewable carbon source for biofuel production. For a long time, studies have been performed to enhance the LCM enzymatic hydrolysis for the efficient conversion of cellulose to ethanol [1]. LCM has very strong resistance to enzymatic degradation such that LCM must be pretreated first to make cellulose degradation by cellulase easier and to improve the cellulose conversion in LCMs. In LCM bioconversion, pretreatment is one of the most expensive processing operations. Pretreatment can improve efficiency and reduce costs through research and development [2–7]. One of the most promising pretreatment processes for LCM is liquid hot water (LHW) pretreatment. LHW pretreatment enhances LCM digestibility, sugar extraction, and pentosan recovery. LHW pretreatment can remove up to 80% of the hemicellulose and enhances the enzymatic digestibility of pretreated material in herbaceous feedstocks [8], sugarcane bagasse [9], and wheat straw [10–16].

Reed is an abundant and inexpensive lignocellulosic raw material that can be found throughout northeastern China. According to statistics, the reed output all over the world was about >70 million tons, especially in Asia and Europe. In China, the planting areas for reed are over 10 million mu, and the reed output reaches 3 million tons. The region with the greatest reed output is Panjin, Liaoning province, China. The Panjin reed field, which covers an area of 1.2 million mu, is the largest reed-producing region in the world today. Five hundred thousand tons of reed are produced in the fields every year. Reed has been used in the papermaking industry for years as a good raw material because of its high cellulose content and good fiber properties. Nevertheless, studies on bioethanol production are few. Reed may be used as an alternative raw material for ethanol production. In this study, the LHW pretreatment of reed and the enzymatic hydrolysis

of pretreated reed were investigated to determine its potential application for ethanol production by a bioconversion process. The pretreatment and enzymatic hydrolysis conditions were optimized to obtain a high conversion of cellulose to ethanol, and to enhance the enzymatic digestibility of reed, to obtain a high glucose yield. Ethanol fermentation was also conducted by separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes using the pretreated reed as a glucose source to determine the feasibility of using reed in bioethanol production.

2. Materials and Methods

2.1. Materials. Reed, which has a moisture content of 11.67%, was provided by the Yingkou papermaking mill, Yingkou, Liaoning province, China. The reed was milled to a particle size of 40 mesh to 60 mesh by using a laboratory ball mill (Taijihuan Nanometer Limited Company, Qinhuangdao, China) and was stored in a plastic bag until it was used in the experiments. Before the LHW pretreatment, the chemical compositions of reed were determined.

The commercial cellulase used in the enzymatic hydrolysis was purchased from the Imperial JADE Biotechnology Co., Ltd., Ningxia, China. The cellulase derived from *Trichoderma longibrachiatum* and the filter paper activity was assayed [17].

The yeast *Saccharomyces cerevisiae* was purchased from the Angel Yeast Co., Ltd., China. Before using it for fermentation, the yeast was activated. About 1 g of dry yeast was added to 20 mL of 5% sterilized glucose solution, activated at 38°C for 1 h, cooled from 28°C to 30°C, and then used in the experiment. The yeast concentration was approximately 10^8 cells/mL.

2.2. LHW Pretreatment. The LHW pretreatment was conducted in a 15 L digester with four small tanks (mechanical mill of the Shanxi University of Science and Technology, China). About 40 g of reed and a given volume deionized water were loaded in the small tanks. The pretreatment temperature was controlled at 170°C, 180°C, 190°C, 200°C, and 210°C. The pretreatment time was set at either 20 min or 40 min. After pretreatment, the water-insoluble solids (WISs) and the reed prehydrolyzates were separated by filtration with the Büchner funnel. The WISs were washed with deionized water to obtain a pH of approximately 7. The WISs were used for subsequent enzymatic hydrolysis and ethanol fermentation.

2.3. Enzymatic Hydrolysis. Enzymatic hydrolysis of the washed WISs was performed at 36°C or 50°C for 72 h in 100 mL Erlenmeyer flasks. Each flask contained 20 mL to 50 mL of 0.05 M sodium citrate buffer (pH 4.8) and had a solid-to-liquid ratio of 1 : 50 weight per volume (W/V) of WIS. The enzyme loading was 10 to 30 filter paper unit (FPU) per gram of oven-dried WIS. The samples were collected at 1, 5, 9, 12, 24, 36, 48, and 72 h for glucose concentration determination. All enzymatic hydrolysis experiments were performed in duplicates, and the average results were determined.

2.4. Ethanol Fermentation. Separate hydrolysis and fermentation (SHF) was performed to check the fermentability of pretreated reed. The WIS from the pretreatment experiments (at 180°C and 210°C for 20 min each) was used as the substrate. About 100 mL of the enzymatic hydrolysis liquor and some nutrients, namely, 3 g/L yeast extract, 5 g/L peptone, 25 g/L KH_2PO_4, 0.3 g/L $MgCl_2$, and 0.25 g/L $CaCl_2$, were added into 250 mL Erlenmeyer flasks. The total liquid volume was 100 mL. The solid concentration was 6% (by WIS weight) during the hydrolysis. The resulting slurry was inoculated with 1 mL of activated yeast. The flasks were autoclaved at 121°C for 20 min. The experiments were performed in duplicates in a constant-temperature incubator at 36°C for 72 h. The flasks were sealed with rubber stoppers and equipped with cannulas to remove the generated carbon dioxide. The cannulas were inserted into a container filled with water. The simultaneous saccharification and fermentation (SSF) experiment was conducted based on the SSF protocol by the National Renewable Energy Laboratory (NREL) LAP-008 [18]. The initial WIS concentration was 8%.

All experiments were performed in duplicates in the same conditions, and the average values were reported.

2.5. Analysis Methods. The contents of xylan, Klason lignin, ash, and benzene-alcohol (2 : 1) extractives were determined using the Chinese National Standard methods, namely, the GB/T2677.9-1994, GB/T2677.8-1994, GB/T2677.3-1993, and GB/T2677.6-1994, respectively. The acid-soluble lignin content was determined using the method described in GB/T10337-1989. The glucan content was determined according to NREL methods [19]. The glucose content and ethanol were determined using the SBA-40E Biological Sensing Analyzer (Biology Institute of the Shandong Academy of Sciences, Jinan, China). The glucan content was calculated using formula (1):

$$\text{Glucan content } (\%) = \frac{(\text{glucose} * 0.087 * 0.9)}{m} * 100\%, \quad (1)$$

where glucose is glucose concentration (g/L); m is mass of oven-dried WIS (g); 0.087 iss volume of acid hydrolysis liquid (L); 0.9 is conversion factor for glucose to glucan.

The conversion of cellulose to glucose in the enzymatic hydrolysis was determined by the ratio of the glucose concentration that was released during enzymatic hydrolysis to the total glucose in the substrate and was calculated using formula:

$$\text{Conversion of cellulose to glucose } (\%) = \frac{\text{glucose} * V * 0.9}{\text{glucan content} * m} * 100\%, \quad (2)$$

where glucose is glucose concentration in the enzymatic hydrolysis liquor (g/L); V is volume of enzymatic hydrolysis liquor (L); m is mass of oven-dried WIS (g).

Table 1: Chemical compositions of reed and other biomass sources (%, by dry weight).

Compositions	Reed	Rice Straw	Corn stover	Wheat straw
Glucan	40.5	34.6	36.1	37.8
Xylan	25.9	21.3	21.4	22.8
Klason lignin	16.2	9.6	17.2	16.3
Acid-soluble lignin	2.0	3.4	—	1.8
Ash	3.6	14.5	7.1	6.3

For SHF and SSF of WIS, the conversion of cellulose to ethanol was calculated using formula (3):

$$\text{Conversion of cellulose to ethanol (\%)} = \frac{[\text{EtOH}]}{(f * \text{biomass} * 1.111 * 0.51)} * 100\%, \quad (3)$$

where [EtOH] is ethanol concentration at the end of the fermentation minus any ethanol produced from the enzyme and medium (g/L); f is cellulose fraction of dry biomass (g/g); Biomass is dry biomass concentration at the beginning of the fermentation (g/L); 0.51 is conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast; 1.111 is conversion factor of cellulose to equivalent glucose.

3. Results and Discussion

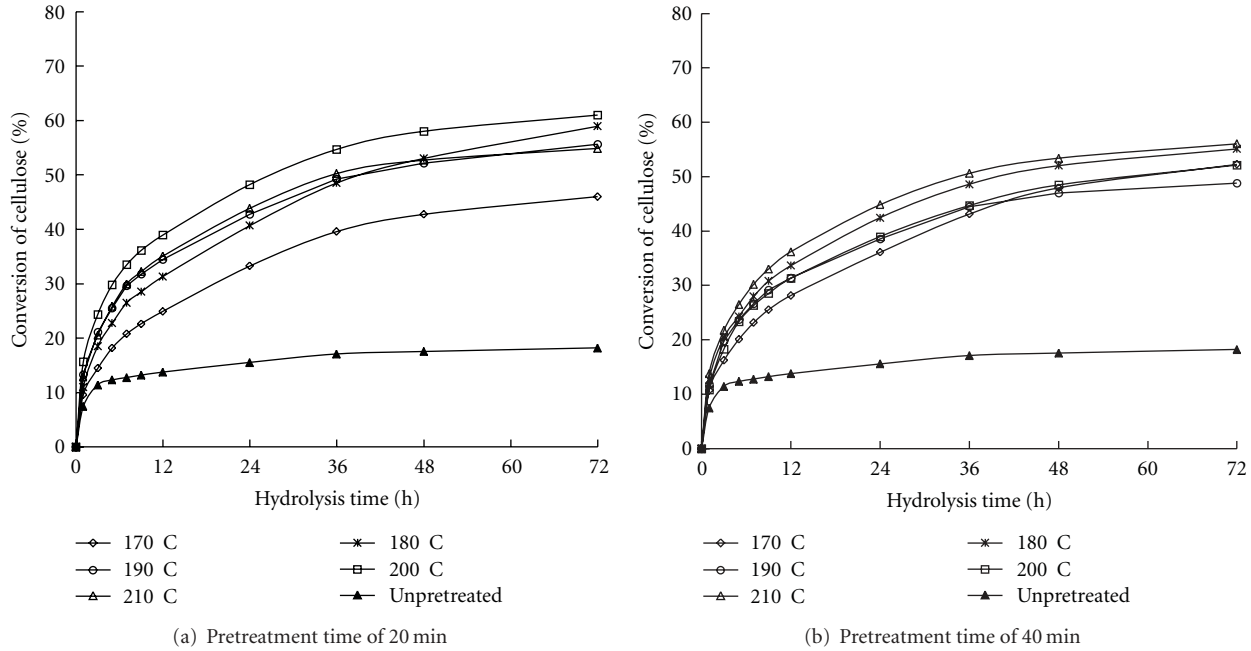
3.1. Chemical Composition of Reed. The chemical compositions of reed used in this study were determined and shown in Table 1. Table 1 also shows the chemical components of other agricultural residues, such as rice straw [20], corn stover [21], and wheat straw [10], which were used in the bioethanol investigation in the present study. Glucan and xylan are the main components of reed, both of which accounted for 66.4% of dry weight of the raw material. Compared with the other raw materials that were generally used in bioethanol investigations, the reed contained more glucan and xylan, which indicated that more fermentable sugars could be produced via enzymatic hydrolysis and that reed can be used in bioethanol production. The lignin component of the raw materials had a significant function in the enzymatic saccharification process [22]. This lignin component can inhibit the enzymatic hydrolysis of cellulose by adsorbing cellulase in the system and/or limiting the enzyme accessibility to cellulose. Klason lignin accounts for 16.2% of the reed. Similar to that of corn stover and wheat straw, this value is higher than that of rice straw, which indicated that the lignin effect on enzymatic hydrolysis might be similar to reed, corn stover, and wheat straw (only for their lignin content). The reed contains less ash than other raw materials, which indicates that it has less effect on cellulase activity in the hydrolysis system and produces more pure products such as fermentable sugars. Thus, reed is a promising source for bioethanol production.

Table 2: Glucan contents of WIS in different pretreatment temperature and time (%, by weight of WIS).

Temperature (°C)	Pretreatment time	
	20 min	40 min
170	45.47	51.16
180	53.98	55.55
190	54.31	57.46
200	54.29	56.81
210	56.42	57.82

3.2. Effect of Pretreatment Conditions on Enzymatic Hydrolysis of Reed WIS

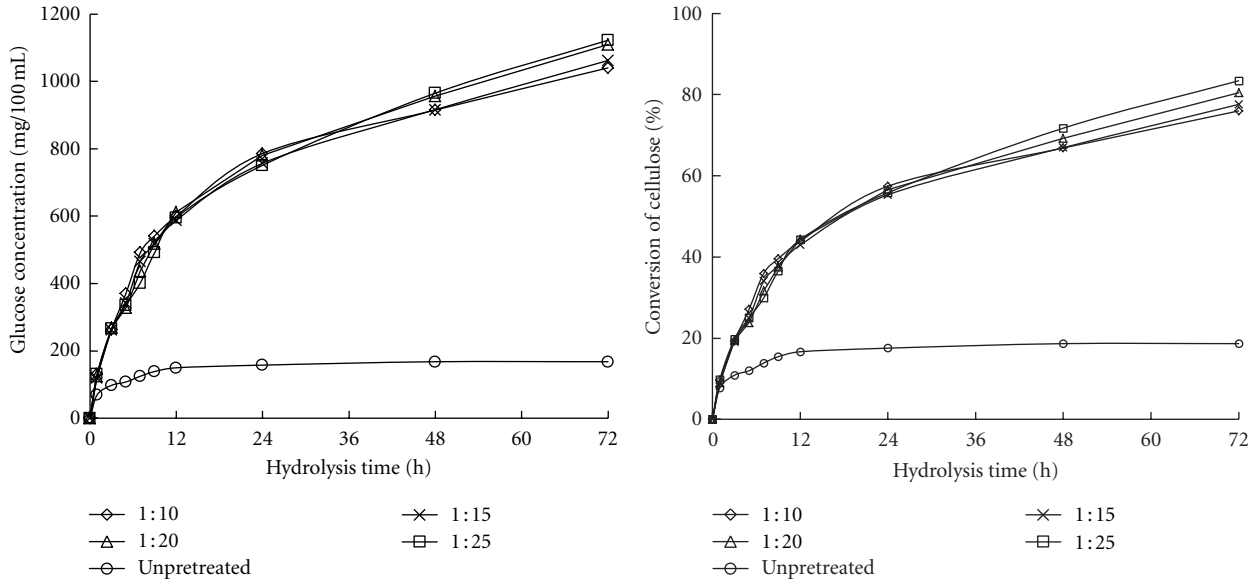
3.2.1. Effect of Pretreatment on Temperature and Time. The pretreatment conditions were selected based on whether they could modify the structural and chemical characteristics of the biomass that limited the enzyme availability to cellulose in cell-wall microfibrils [23]. Several reports have shown that sample pretreatment at higher temperatures resulted in a higher enzymatic digestibility than the untreated sample and could yield a higher glucose concentration upon enzymatic hydrolysis [24]. In the study, enzymatic hydrolysis was also conducted on unpretreated raw material for comparison purposes. The glucan contents of each WIS after LHW pretreatment at different temperatures and time points are summarized in Table 2. The glucan contents of all WISs were higher than that of the raw reed at 40.5% (Table 1). The glucan contents of all WISs ranged from 45% to 58% of WIS, and this value depends on the pretreatment temperature and time. The glucan content increased with increasing pretreatment time. Meanwhile, the glucan content also increased with increasing pretreatment temperature. The highest glucose content was obtained at 210°C for 40 min because the water-soluble components in the raw reed were solubilized. For example, lignin with low molecular weight and extractives resulted in a fraction of glucan-enriched WIS production with increasing temperature and time. The reed hemicellulose component was also partly degraded and dissolved, which also led to the increase in WIS glucan content. The enzymatic hydrolysis profile of WIS-pretreated reed at different pretreatment temperature and time is shown in Figure 1. The enzymatic digestibility of each WIS was different. The conversion of cellulose to glucose rapidly increased at the beginning of the enzymatic hydrolysis process and then slowly declined, as previously reported [25, 26]. The untreated raw reed was difficult



(a) Pretreatment time of 20 min

(b) Pretreatment time of 40 min

Figure 1: Conversion of cellulose to glucose in the enzymatic hydrolysis of WIS from LHW-pretreated reed, with different pretreatment temperatures and times. *Enzymatic hydrolysis conditions: 15 FPU/g oven-dried WIS, pH 4.8, solid-to-liquid ratio 1 : 50 (w/v), and 50°C for 72 h.

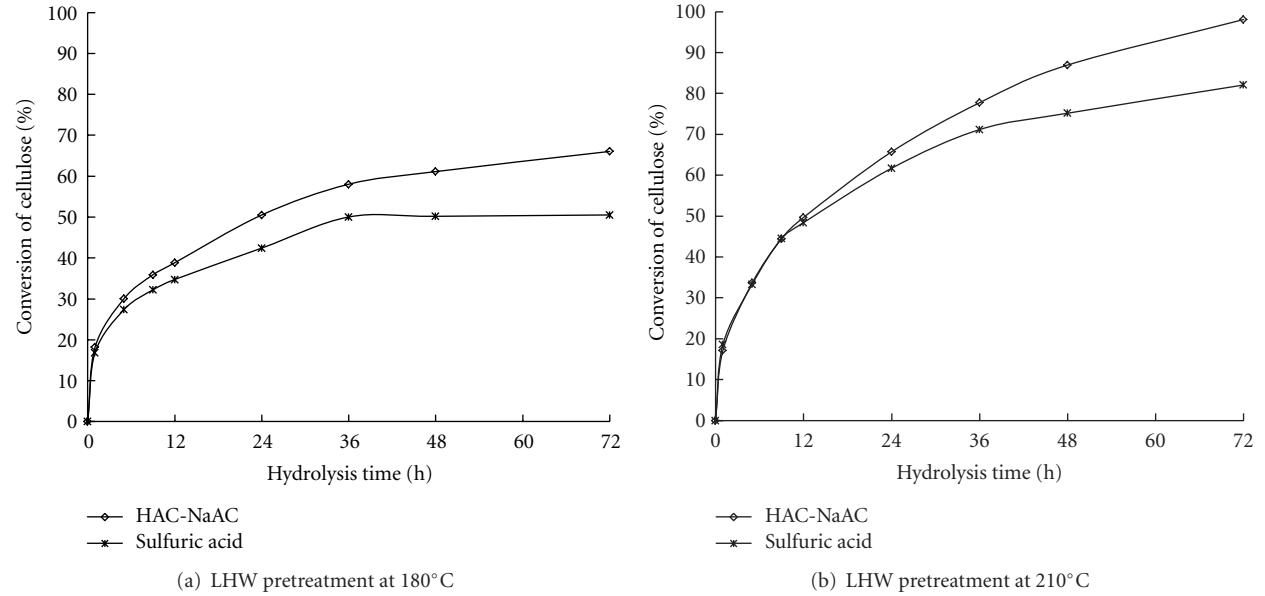


(a) Glucose release from WIS in the enzymatic hydrolysis

(b) Conversion of cellulose of WIS

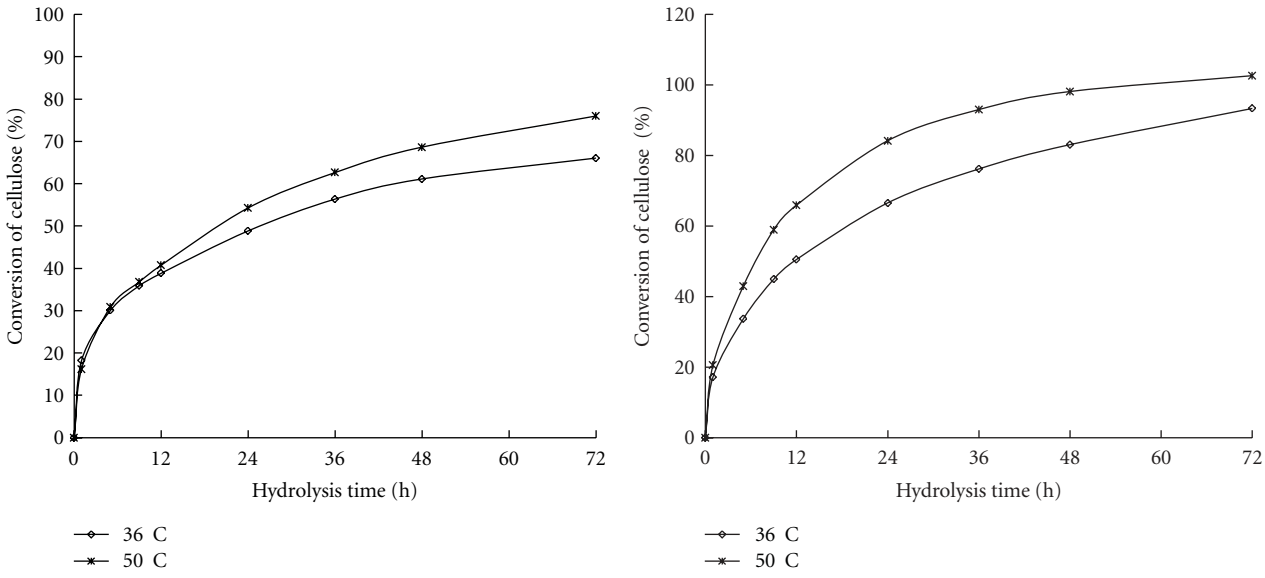
Figure 2: Effect of solid-to-liquid ratio on enzymatic hydrolysis of the WIS from the pretreated reed. *LHW pretreatment: 180°C for 20 min. **Enzymatic hydrolysis: 30 FPU/g oven-dried WIS, pH 4.8, at 50°C.

to hydrolyze enzymatically. The maximum conversion of cellulose to glucose was only 18.22% at 72 h of enzymatic hydrolysis. However, in WIS from the pretreated samples, the cellulose conversion in the enzymatic hydrolysis ranged from 47% to 64% based on the pretreatment severity. The increase in the WIS enzymatic digestibility may be due to the ability of LHW pretreatment to increase the surface area of the raw material samples, which leads to sufficient contact between the cellulase and the substrate. Thus, the glucose release increases. The cellulose conversion decreased from 82.59% to 56.70% at 72 h when the pretreatment time was prolonged from 20 min to 40 min at 180°C. This decrease in cellulose conversion indicated that a prolonged pretreatment time does not improve WIS enzymatic hydrolysis. Figure 1



(a) LHW pretreatment at 180°C (b) LHW pretreatment at 210°C

Figure 3: Enzymatic hydrolysis of WIS from LWH-pretreated reed at 180°C and 210°C by using two types of pH adjustment in the hydrolysis system. *Enzymatic hydrolysis: 30 FPU/g oven-dried WIS at 36°C.

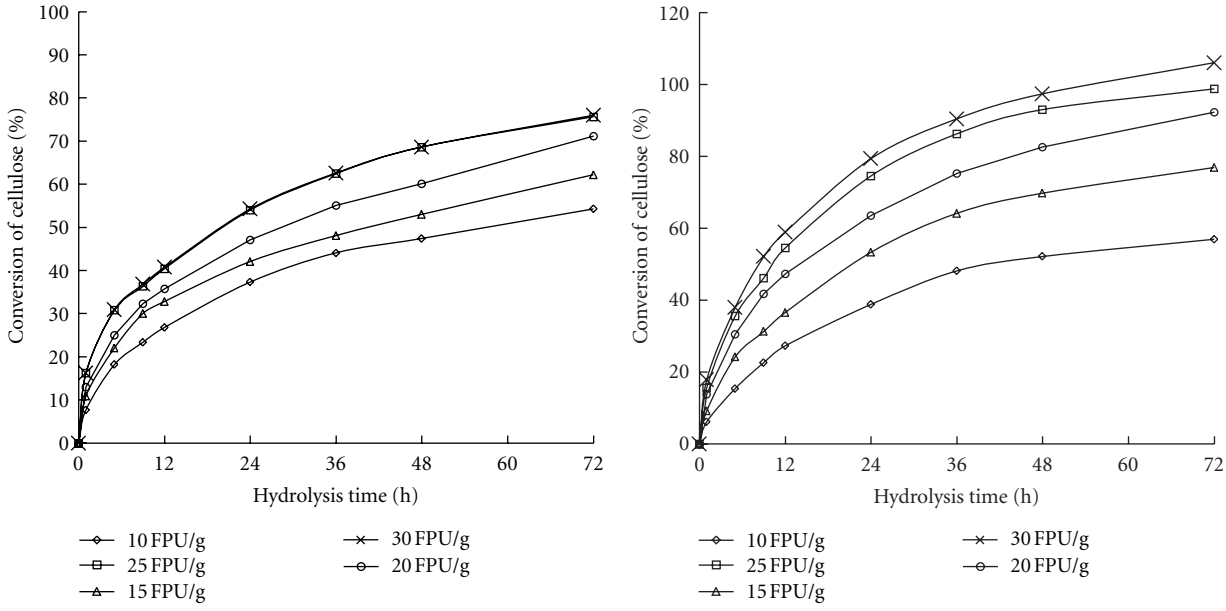


(a) Conversion of WIS from reed pretreated at 180°C (b) Conversion of WIS from reed pretreated at 210°C

Figure 4: Temperature effects on the WIS conversion from reed in the LHW pretreatment at 180°C and 210°C for 20 min. *Enzymatic hydrolysis: 30 FPU/g oven-dried WIS at pH 4.8.

also shows that temperature had no significant effects on WIS enzymatic hydrolysis at a temperature of >180°C. High-temperature and prolonged LHW pretreatment also led to more polysaccharide degradation and loss, which decreased cellulose yield. Therefore, LHW pretreatment at 180°C for 20 min was suitable for the reed. Moreover, a cellulose conversion of 82.59% at 72 h of enzymatic hydrolysis was obtained in the LHW pretreatment in the same conditions.

3.2.2. Effect of Solid-to-Liquid Ratio in LHW Pretreatment. The solid-to-liquid ratio is the ratio between the oven-dried WIS quality and the entire liquid volume in the LHW pretreatment. When the reed quantity was kept constant, a higher solid-to-liquid ratio led to a lower substrate concentration, which further decreased the end-product inhibition. Reducing the solid-to-liquid ratio would decrease process cost by lowering the reactor size and the amount



(a) Conversion of WIS from reed pretreated at 180°C

(b) Conversion of WIS from reed pretreated at 210°C

Figure 5: Enzymatic hydrolysis of WIS from reed in the LHW pretreatment at 180°C and 210°C with respect to different cellulase loadings.

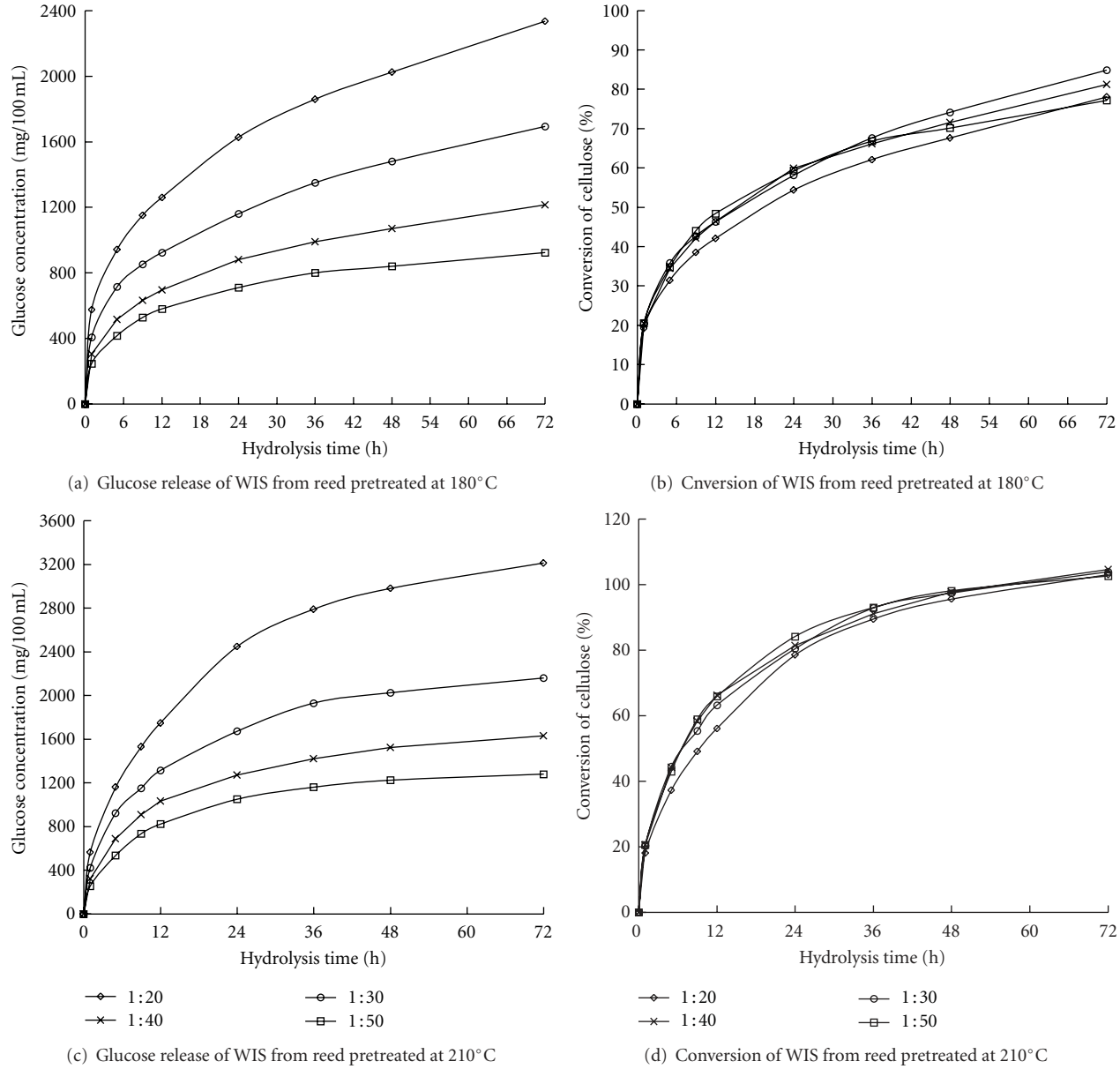
of heat requirement during the pretreatment. When the solid-to-liquid ratio was increased from 1 : 10 to 1 : 25 (w/v) in the LHW pretreatment at 180°C, the glucose release slightly increases in the later enzymatic hydrolysis period. For example, the conversion of cellulose to glucose at 72 h of enzymatic hydrolysis increased from 75.97% to 83.39% with only a difference of 7.42% after 48 h (Figure 2). This increase in the conversion of cellulose to glucose showed that the solid-to-liquid ratio had little effect on the enzymatic digestibility of WIS from reed. However, keeping the reaction system homogeneous is difficult because the liquid present in the reaction system is less when the solid-to-liquid ratio is lower than 1 : 10. Therefore, a solid-to-liquid ratio of 1 : 10 was used for the LHW pretreatment of reed.

3.3. Optimization of Enzymatic Hydrolysis of the Reed WIS

3.3.1. Effect of Adjusting pH on the Enzymatic Hydrolysis of WIS. A suitable pH in the enzymatic hydrolysis system is beneficial to the cellulase function on the substrate. The systemic pH has to be adjusted to improve cellulase effectiveness in hydrolysis. In this study, two different methods were used for adjusting and controlling the pH. One method involves the use of sodium citrate buffer (pH 4.8, 0.05 M), and the other method involves the use of H_2SO_4. Figure 3 shows that the use of sodium citrate buffer to adjust the pH was better than using H_2SO_4 for the cellulase function on the WIS in the LHW-pretreated reed at 180°C and 210°C. A higher cellulose conversion was obtained when the sodium citrate buffer was used to adjust the pH, which indicated that this buffer was effective in controlling the pH of the enzymatic hydrolysis process and in improving the cellulase effectiveness.

3.3.2. Effect of Enzymatic Temperature on the Enzymatic Hydrolysis of Reed WIS. The enzymatic temperature affects cellulase activity and effectiveness in enzymatic hydrolysis. The temperature in the enzymatic hydrolysis is generally kept at 50°C, which is a suitable temperature for the cellulase activity. However, the yeast fermentation temperature is 36°C. Two different enzymatic temperatures were used for the enzymatic hydrolysis of WIS to compare the effects of different enzymatic temperatures on WIS enzymatic digestibility. Figure 4 shows the comparisons of the cellulose conversion after enzymatic hydrolysis between two WISs from LWH-pretreated reed at 180°C and 210°C, respectively. The cellulose conversion after enzymatic hydrolysis at 36°C was slightly lower than that at 50°C. Therefore, a hydrolysis temperature of 50°C may be used in the bioethanol enzymatic hydrolysis via the SHF process. Nevertheless, a temperature of 36°C was considered suitable for yeast fermentation, which was beneficial to the succeeding SSF.

3.3.3. Effect of Cellulase Loadings on the Enzymatic Hydrolysis of Reed WIS. Aside from the enzymatic temperature and pH, cellulase loading is also an important factor for the enzymatic hydrolysis of cellulosic substrates. Increasing the cellulase loading generally resulted in an increase in glucose release and cellulose conversion. The enzymatic hydrolysis curves of the WIS in reed after the LHW pretreatment with respect to several cellulase loadings are shown in Figure 5. Cellulase loading had a significant effect on cellulose conversion in WIS by enzymatic hydrolysis. When the cellulase loading was increased from 10 FPU/g oven-dried WIS to 25 FPU/g oven-dried WIS on pretreated reed at 180°C or 210°C for 20 min, the enzymatic digestibility of reed WIS was



(a) Glucose release of WIS from reed pretreated at 180°C

(b) Cnversion of WIS from reed pretreated at 180°C

(c) Glucose release of WIS from reed pretreated at 210°C

(d) Conversion of WIS from reed pretreated at 210°C

Figure 6: Enzymatic hydrolysis of WIS from reed pretreated at 180°C and 210°C with respect to different solid-to-liquid ratios in enzymatic hydrolysis.

greatly improved. However, the increase in cellulase loading from 25 FPU/g to 30 FPU/g slightly affected the cellulose conversion in the WIS in reed. Figure 5 also shows that cellulose conversion in WIS from reed pretreated at 210°C was higher than that in WIS from reed pretreated at 180°C in the same enzymatic hydrolysis conditions. A higher cellulose conversion in WIS from reed pretreated at 210°C indicated that a higher pretreatment temperature improves the enzymatic digestibility of reed and increases the glucose release during enzymatic hydrolysis.

3.3.4. Effect of Solid-to-Liquid Ratio on the Enzymatic Hydrolysis. A high glucose concentration is necessary to obtain high ethanol concentration by fermentation, which results in a decrease in the production cost, such as ethanol distillation cost. Decreasing the solid-to-liquid ratios during the enzymatic hydrolysis of a cellulosic substrate may increase glucose consistency. Nevertheless, very low solid-to-liquid ratio indicates a very high initial solid concentration, which causes difficulty in uniformly mixing the enzyme liquid and the WIS because the amount of liquid in the system is reduced. The trends of change in the cellulose conversion are illustrated in Figure 6.The glucose concentration in the hydrolyzates increased when the solid-to-liquid ratio decreased in the enzymatic hydrolysis of WIS from reed that was pretreated at both 180°C and 210°C (i.e., $1:50 < 1:40 < 1:30 < 1:20$; Figures 6(a) and 6(c)). The highest glucose concentrations (2326 mg/100 mL at 180°C and 3213 mg/100 mL at 210°C)

Table 3: Ethanol fermentation of WIS after LHW pretreatment.

Substrates	SHF			SSF		
	Ethanol concentrations (g/L)	Residual glucose (g/L)	Conversion of cellulose to ethanol (%)	Ethanol concentrations (g/L)	Residual glucose (g/L)	Conversion of cellulose to ethanol (%)
Reed	5.07	0.06	36.9	4.30	0.39	23.4
Reed at 180°C for 20 min	17.69	0.06	96.6	17.60	0.34	72.1
Reed at 210°C for 20 min	18.99	0.06	99.5	21.75	0.36	85.5

were obtained at a solid-to-liquid ratio of 1 : 20 after 72 h of enzymatic hydrolysis. The lowest glucose concentrations (924 mg/100 mL at 180°C and 1280 mg/100 mL at 210°C) were obtained at a solid-to-liquid ratio of 1 : 50 after 72 h of enzymatic hydrolysis. The difference between the highest and lowest glucose concentrations showed an increase of 60.3% at 180°C and 60.2% at 210°C, respectively. However, the change in the solid-to-liquid ratio did not obviously affect the cellulose bioconversion (Figures 6(b) and 6(d)). Thus, the increase in the glucose concentration by decreasing the solid-to-liquid ratio in the enzymatic hydrolysis process was beneficial in the subsequent ethanol fermentation.

3.4. Ethanol Fermentation of Reed WIS after LHW Pretreatment. Ethanol production depends on the sugar yield and the mixture fermentability. The WIS obtained from different pretreatment conditions, namely, 180°C at 20 min and 210°C at 20 min, were fermented by SHF and SSF methods by using special ethanol yeast to investigate the fermentability of the WIS from LWH-pretreated reed. Table 3 shows the ethanol fermentation results of WIS by SHF and SSF and the cellulose conversion to ethanol expressed as theoretical yield percentages based on (3). In SHF, the obtained ethanol concentrations reached 17.69 g/L during pretreatment at 180°C and 18.99 g/L during pretreatment at 210°C after 72 h of fermentation. The calculated ethanol conversion yield reached 96.6% at 180°C and 99.5% at 210°C of the theoretical yield. These calculated ethanol conversion yields indicated that the SHF process was a very efficient method for producing ethanol from LHW-pretreated reed. In SSF, the pretreated WIS fermentation was performed at an initial pH of 4.8 at 36°C. The yeast strain at this temperature produced a maximum ethanol concentration from glucose, which was consistent with the results of previous SSF studies [27]. With 10% (volume per volume) inoculum, the highest conversion of cellulose to ethanol reached 85.5% for WIS from pretreated reed at 210°C for 20 min. The experiments showed that WIS from LWH-pretreated reed has good fermentability, and LHW pretreatment can be used in bioethanol production.

4. Conclusion

Reed can be used as a substrate for ethanol production in northeast China. The LHW pretreatment significantly enhanced the enzymatic digestibility of reed. The optimum conditions of LHW pretreatment for reed were a temperature of 180°C for 20 min and a solid-to-liquid ratio of 1 : 10, which resulted in a cellulose conversion rate of 82.59% in the subsequent enzymatic hydrolysis at 50°C for 72 h with a cellulase loading of 30 FPU/g oven-dried WIS. After the LHW pretreatment, the conversion of glucan to ethanol in WIS from LWH-pretreated reed reached 99.5% of theoretical yield by the SHF process.

Acknowledgments

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