

Review

# *Miscanthus* as cellulosic biomass for bioethanol production

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The members of the genus *Miscanthus* are potential feedstocks for biofuels because of the promising high yields of biomass per unit of planted area. This review addresses species, cultivation, and lignocellulose composition of *Miscanthus*, as well as pretreatment and enzyme saccharification of *Miscanthus* biomass for ethanol fermentation. The average cellulose contents in dried biomass of *Miscanthus floridulus*, *Miscanthus sinensis*, *Miscanthus sacchariflorus*, and *Miscanthus × giganteus* ( $M \times G$ ) are 37.2, 37.6, 38.9, and 41.1% wt/wt, respectively. A number of pretreatment methods have been applied in order to enhance digestibility of *Miscanthus* biomass for enzymatic saccharification. Pretreatment of *Miscanthus* using liquid hot water or alkaline results in a significant release of glucose; while glucose yields can be 90% or higher if a pretreatment like AFEX that combines both chemical and physical processes is used. As ethanol is produced by yeast fermentation of the hydrolysate from enzymatic hydrolysis of residual solids (pulp) after pretreatment, theoretical ethanol yields are 0.211–0.233 g/g-raw biomass if only cellulose is taken into account. Simultaneous saccharification and fermentation of pretreated  $M \times G$  and *M. lutarioriparius* results in experimental ethanol yields of 0.13 and 0.15 g/g-raw biomass, respectively. Co-production of value-added products can reduce the overall production cost of bioethanol.

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## 1 Introduction

*Miscanthus* is a genus of about 20 species of perennial grasses, distributed widely across Asia and the Pacific Islands. Most native *Miscanthus* species can be found in China, Korea, Japan, Taiwan, and the Philippines [1, 2]. The diversity and population structure of *Miscanthus sinensis* in its native location of East Asia has been recently characterized in order to facilitate the breeding of *Miscanthus* plants [2]. *Miscanthus* plants were used historically as forage, clothing, and shelter. Recently, *Miscanthus* biomass has attracted interest as a potential

feedstock for ethanol production because it is rich in carbohydrates and grows well even in poor soils [3]. *Miscanthus* is a fast-growing C4 rhizomatous grass. When it is established it requires little water or fertilization and can be harvested every year with high biomass yields. As a promising energy crop, *Miscanthus* spp. has a high yield of biomass per unit planted area. For example, a biomass yield up to 41 t/ha/y can be achieved for some *M. sinensis* hybrids [4], and once the crop has been established and with cultivation in European conditions, a dry biomass yield of over 37 t/ha/y over a period of four years can be achieved [5]. A well-known hybrid, *Miscanthus × giganteus* ( $M \times G$ ), has been planted and studied in Europe and the United States for a long time. Other *Miscanthus* species of great potential have also been cultivated and investigated. As candidates of energy crops for biofuel production, *M. floridulus* [6] and *M. lutarioriparius* [7] have received attention recently, in addition to  $M \times G$ .

To produce biofuels like ethanol, the lignocellulosic biomass of *Miscanthus* needs to be pretreated and enzymatically hydrolyzed to become fermentable sugars before fermentation by microorganisms. The fermentable

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**Abbreviations:** AFEX, ammonia fiber explosion; LHW, liquid hot water;  $M \times G$ , *Miscanthus × giganteus*; SHF, separated hydrolysis and fermentation; SSF, simultaneous saccharification and fermentation; XOSs, xylooligosaccharides

sugars come from the carbohydrates in *Miscanthus* biomass. The biomass of *Miscanthus*, regardless of the variety, possesses a carbohydrate content of more than 60%, w/w, in which approximately 40% is cellulose based on dry weight [8]. After pretreatment, the residual solids of *Miscanthus* biomass contain mainly cellulose, which is subjected to enzymatic hydrolysis to yield glucose, which can be fermented to ethanol. Here we provide an overview of *Miscanthus* species, their cultivation, and their lignocellulose composition. We also provide a literature review and discussion of the methods for pretreatment of *Miscanthus* biomass and the process of enzyme saccharification for ethanol fermentation.

## 2 *Miscanthus* species and geographic distribution

Worldwide, *M. sinensis* is the most widely distributed *Miscanthus* species. A study on the evolution of *Miscanthus* species proposes that *M. sinensis* is the origin of *Miscanthus* taxa, which evolved to *M. sinensis* var. *formosana*, *M. sinensis* var. *flavidus*, and *M. sinensis* var. *transmorrisonensis*. Another dominant species, *M. floridulus*, is thought to be an outgroup of the *M. sinensis* complex [1]. The different varieties of *Miscanthus* grow well in their

appropriate geography and climate. In China, for example, each *Miscanthus* species has its unique latitude distribution: *M. lutarioriparius* grows at about 30°N, *M. sacchariflorus* grows between 30 to 45°N, and *M. floridulus* and *M. sinensis* grows below 30°N [8]. In Taiwan, *Miscanthus* plants are widely distributed, from riverbanks to high mountains. *M. floridulus* and *M. sinensis* Anders var. *glaber* are two major native species, while *M. transmorrisonensis* is a native species of *Miscanthus* in high altitudes. *M. floridulus* (also called Giant Chinese silver grass or *Miscanthus japonicus*) is the dominant grass at elevations below 2000 m, whereas *M. transmorrisonensis* dominates habitats above 2400 m [9]. A previous study concludes that growth of *M. floridulus* at high altitude is limited at temperatures lower than 15°C, while *M. transmorrisonensis* is able to grow in cold temperatures at higher altitudes [10].

The most well-known species of *Miscanthus* is *M* × *G*. *M* × *G* originated in Japan and is believed to be a hybrid of *M. sinensis* and *M. sacchariflorus*. This *Miscanthus* has been tested as a biofuel in Europe since the early 1980s, and has been planted widely throughout Europe and even in the U.S. Cultivation of *M* × *G* in Germany yields biomass of 14.8–33.5 t/ha of dry matter [11, 12]. Cultivation of *M. floridulus* in Taiwan results in a slightly higher biomass yield (Table 1), probably due to the hot and humid climate.

**Table 1.** Comparison of *Miscanthus* and other C4 grasses

Species	Biomass yield (t/ha/y)	Location	Composition	Reference
<i>Miscanthus</i> × <i>giganteus</i>	14.8–33.5	Germany	Cellulose: 33.9% Xylan + Arabin: 32.2% Lignin: 26.3%	[11, 12]
<i>Miscanthus floridulus</i>	27.8–38.0	Taiwan	Cellulose: 43.13% Lignin: 22.33%	[6] [19]
<i>Miscanthus lutarioriparius</i>	32.0	China	Cellulose: 43.9% Xylan+Arabin: 21.4% Lignin: 23.2%	[7]
Purple guinea grass ( <i>Panicum maximum</i> cv. TD53)	9.4–25.0	Thailand	Cellulose: 41.7% Hemicellulose: 27.1% Lignin: 10.4% (dried solid)	[16]
<i>Pennisetum purpureum</i> (Napier grass, elephant grass)	30–42	Cerrado region, Brazil	Cellulose: 38.2% Lignin: 33.6% Hemicellulose: 76.3% (cellulose + hemicelluloses)	[14, 15]
<i>Panicum virgatum</i> L. (switchgrass)	15	Rock Springs, PA, USA	Cellulose: 39.5% Hemicellulose: 25.0% Lignin: 21.8%	[17] [18]
<i>Sorghum bicolor</i> (L.) Moench (sweet sorghum)	22.7–30.1°	Illinois, USA	Cellulose: 12.4% Hemicellulose: 10.2% Lignin: 4.8% Sucrose: 55% Glucose: 3.2%	[20] [21]

Three lines of *M. floridulus* collected from different altitudes (260, 1000, 1500 m) in central Taiwan were transplanted and harvested after cultivation for 210 days, resulting in dry biomass yields of 31.2, 38.0, and 29.5 t/ha, respectively, in the first year. In the second year, however, biomass yields following 210 days of cultivation were reduced to 16.7, 27.3, and 24.4 t/ha, respectively, because of too much rainfall. Both the biomass yield and plant height depend on the species as well as growth conditions. The data show that both fresh and dry yields of *M. floridulus* are positively correlated with plant height, and that plant height of *Miscanthus* is positively correlated with cumulative rainfall, temperature, radiation, and sunlight exposure time [6]. These results suggest that climatic conditions significantly affect the growth of *Miscanthus*. Biomass yields can also be influenced by crop age and nitrogen fertilization [5], as well as when the *Miscanthus* is harvested [13].

The yields of other C4 forage grasses can be used for comparison: Cultivation of *Pennisetum purpureum* (Napier grass) in the Cerrado of Brazil results in a similar level of biomass yield (30–42 t/ha/y) [14, 15], but the biomass yields of *Panicum maximum* cv. TD53 (Purple guinea grass) cultivated in Thailand (9.4–25.0 t/ha/y) [16] and of *Panicum virgatum* L. (switchgrass) cultivated in the USA (15 t/ha/y) are lower [17]. Both Napier grass and switchgrass have high cellulose contents [15, 18], like *Miscanthus* [7, 19]. Sweet sorghum is also a C4 plant with a high biomass yield, but its carbohydrate composition, including a high sucrose content, is quite different from those of other plants as shown in Table 1 [20, 21]. Lignocellulosic feedstocks including various agricultural residues and fast-growing plants in general consist of about 70% wt/wt or more cellulose and hemicellulose, which are subjected to saccharification to sugars for producing biofuels and value-added products. In addition to its high biomass yield, the high cellulose and hemicellulose content renders *Miscanthus* a promising energy crop.

### 3 Chemical composition of *Miscanthus*

#### 3.1 Composition of different *Miscanthus* species

*Miscanthus* is a perennial grass, with the whole plant including roots, underground stems, and over ground stems and leaves. Stems and leaves are most of the biomass. Like other lignocellulosic feedstocks, this biomass comprises mainly cellulose, hemicelluloses and lignin. Hemicelluloses are heteropolymers, including xylan and arabinoxylan, present along with cellulose in cell walls of plants. Lignin binds the cellulose fibers, forms a protective covering, and hardens the cell wall. By definition, lignin is an amorphous, polyphenolic material that includes both acid-insoluble lignin (Klason lignin) and acid-soluble lignin. In term of monomers, *Miscanthus*

fiber is composed mainly of glucose and xylose, which account for about 39 and 20% of fiber, respectively, whereas arabinose, galactose, and uronic acid account for 3 or less of fiber content [22, 23]. According to the literature [7, 8, 19, 23–29], the lignocellulose of *Miscanthus* contains 32.7–49.5% w/w cellulose, 21–34.8% w/w hemicellulose, and 17.8–27.7% w/w lignin (Table 2). The data in Table 2 indicate that most of the lignin in *Miscanthus* is acid-insoluble lignin, although some data show a higher level of acid-soluble lignin. For ethanol fermentation, the lignocellulose composition in different *Miscanthus* species should be carefully investigated. Because glucose generated from the hydrolysis of cellulose is the main fermentable sugar for bioethanol production, the cellulose content of *Miscanthus* is a major concern in using this grass as an energy crop.

A composition study of stem samples from *M. floridulus* ( $n = 118$ ), *M. sinensis* ( $n = 217$ ), *M. lutarioriparius* ( $n = 45$ ), and *M. sacchariflorus* ( $n = 130$ ), collected from a plantation site in Hunan, China, shows that the average cellulose content of these four *Miscanthus* species on a dry basis are 32.7, 35.1, 42.1, and 38.5%, respectively. The highest reported cellulose content for these species are 43.6, 47, 53.9, and 51.57%, respectively on a dry basis. The results suggest that *M. lutarioriparius* may be preferred, in terms of cellulose content [8]. Similar research has been performed on samples of *M. sinensis* ( $n = 7$ ) and *M. sacchariflorus* ( $n = 3$ ) collected from a planted site in Korea. The average cellulose contents are 39 and 39.7% in stems and 34.7 and 34.1% in leaves from these two species, respectively [25]. According to these two studies, the order of cellulose content, from greatest to least, is *M. lutarioriparius*, *M. sacchariflorus*, *M. sinensis*, and then *M. floridulus*. Based on the average values of cellulose content taken from the literature, as shown in Table 2, there is no big difference among the four major *Miscanthus* grasses. The average cellulose contents are 37.2, 37.6, 38.9, and 41.1%, w/w, for biomass of *M. floridulus*, *M. sinensis*, *M. sacchariflorus*, and  $M \times G$ , respectively. No significant difference is found in hemicellulose content among species, whereas  $M \times G$  has slightly higher cellulose content than the others. Among these four grasses, *M. sacchariflorus* has a relatively lower level of lignin. However, the data in Table 2 show large variation between samples. For example, the data range and variance for cellulose content are 10.6 and 21.4% for *M. floridulus*, 12.0 and 18.8% for *M. sinensis*, 10.4 and 21.6% for *M. sacchariflorus*, and 11.7 and 27.3% for  $M \times G$ . These statistics suggest that for every species, the extent of cellulose content variation is greater than 10% w/w. Based on their average cellulose contents, the maximum amounts of ethanol that can theoretically be produced are 0.211, 0.213, 0.221 and 0.233 g ethanol, respectively, per gram raw biomass of *M. floridulus*, *M. sinensis*, *M. sacchariflorus*, and  $M \times G$ .

**Table 2.** Lignocellulose composition of different *Miscanthus* species

Growing Regions	% Dry Matter					References
	Cellulose	Hemicellulose	Acid-soluble Lignin	Klason Lignin	Ash	
<b><i>Miscanthus floridulus</i></b>	<b>37.2 ± 6</b>	<b>26.7</b>	–	<b>21.7</b>	–	
Hunan, China	34.8	22.2	3.0	17.8	4.5	[7]
Hunan, China	32.71	34.86	8.9	–	3.75	[8]
Chiayi, Taiwan	43.13	–	1.31	21.02	–	[19]
Torre Garofoli, Italy	38.2	23	–	26.4	2.6	[23]
<b><i>Miscanthus sinensis</i></b>	<b>37.6 ± 4.3</b>	<b>24.6</b>	–	<b>23.4</b>	–	
Hunan, China	35.2	20.5	2.6	18.1	6.0	[7]
Hunan, China	35.1	34.8	9.51	–	4.02	[8]
Galicia, Spain	42.6	21.1	–	19.9	0.7	[24]
CD, Korea	28.1 <sup>L</sup> 36.1 <sup>S</sup>	25.1 <sup>L</sup> 26.6 <sup>S</sup>	0.12 <sup>L</sup> 0.17 <sup>S</sup>	24.9 <sup>L</sup> 27.5 <sup>S</sup>	–	[25]
DR, Korea	34.4 <sup>L</sup> 37.7 <sup>S</sup>	27.5 <sup>L</sup> 28.1 <sup>S</sup>	0.14 <sup>L</sup> 0.13 <sup>S</sup>	17.7 <sup>L</sup> 22.7 <sup>S</sup>	–	
GB, Korea	35.3 <sup>L</sup> 32.9 <sup>S</sup>	25.2 <sup>L</sup> 25.3 <sup>S</sup>	0.34 <sup>L</sup> 0.42 <sup>S</sup>	21.2 <sup>L</sup> 23.5 <sup>S</sup>	–	
HD, Korea	33.9 <sup>L</sup> 37.3 <sup>S</sup>	20.5 <sup>L</sup> 26.3 <sup>S</sup>	0.12 <sup>L</sup> 0.1 <sup>S</sup>	19.1 <sup>L</sup> 22.6 <sup>S</sup>	–	
IB, Korea	38.5 <sup>L</sup> 42.2 <sup>S</sup>	20.2 <sup>L</sup> 22.9 <sup>S</sup>	0.1 <sup>L</sup> 0.12 <sup>S</sup>	26 <sup>L</sup> 26.8 <sup>S</sup>	–	
Illinois, USA	35.5 <sup>L</sup> 44.9 <sup>S</sup>	21.7 <sup>L</sup> 18.5 <sup>S</sup>	0.11 <sup>L</sup> 0.15 <sup>S</sup>	24.8 <sup>L</sup> 27.1 <sup>S</sup>	–	
IS, Korea	37.2 <sup>L</sup> 42.1 <sup>S</sup>	20.8 <sup>L</sup> 21.9 <sup>S</sup>	0.17 <sup>L</sup> 0.18 <sup>S</sup>	26.2 <sup>L</sup> 26.8 <sup>S</sup>	–	
CD, Korea	28.1 <sup>L</sup> 36.1 <sup>S</sup>	25.1 <sup>L</sup> 26.6 <sup>S</sup>	0.12 <sup>L</sup> 0.17 <sup>S</sup>	24.9 <sup>L</sup> 27.5 <sup>S</sup>	–	
DR, Korea	34.4 <sup>L</sup> 37.7 <sup>S</sup>	27.5 <sup>L</sup> 28.1 <sup>S</sup>	0.14 <sup>L</sup> 0.13 <sup>S</sup>	17.7 <sup>L</sup> 22.7 <sup>S</sup>	–	
Germany	41.4 <sup>a)</sup> 44.2 <sup>b)</sup>	29.6 <sup>a)</sup> 30.9 <sup>b)</sup>	7.6 <sup>a)</sup> 8.9 <sup>b)</sup>	–	–	[26]
<b><i>Miscanthus sacchariflorus</i></b>	<b>38.9 ± 4.6</b>	<b>26.4</b>	–	<b>16.7</b>	–	
Hunan, China	38.8	21.4	2.1	22.4	4.1	[7]
Hunan, China	38.5	32.98	11.22	–	5.69	[8]
Andeok-myeon, Korea	35.8 <sup>L</sup> 38 <sup>S</sup>	26.1 <sup>L</sup> 30.5 <sup>S</sup>	0.83 <sup>L</sup> 0.64 <sup>S</sup>	13.8 <sup>L</sup> 12.7 <sup>S</sup>	–	[25]
Hacheonri, Korea	33.9 <sup>L</sup> 43.4 <sup>S</sup>	24.4 <sup>L</sup> 27 <sup>S</sup>	0.84 <sup>L</sup> 0.54 <sup>S</sup>	19.9 <sup>L</sup> 14.2 <sup>S</sup>	–	
Seongsan-eup, Korea	32.7 <sup>L</sup> 37.7 <sup>S</sup>	25.1 <sup>L</sup> 24.5 <sup>S</sup>	0.2 <sup>L</sup> 0.73 <sup>S</sup>	16.1 <sup>L</sup> 17.5 <sup>S</sup>	–	
Germany	42.7 <sup>a)</sup> 48.1 <sup>b)</sup>	25.7 <sup>a)</sup> 26.5 <sup>b)</sup>	9.0 <sup>a)</sup> 10.5 <sup>b)</sup>	–	–	[26]
<b><i>Miscanthus × giganteus</i></b>	<b>41.1 ± 5.0</b>	<b>23.5</b>	–	<b>21.7</b>	–	
Iowa, USA	33.2 <sup>L</sup> 41.6 <sup>S</sup>	17.7 <sup>L</sup> 17.1 <sup>S</sup>	0.1 <sup>L</sup> 0.12 <sup>S</sup>	24.1 <sup>L</sup> 25.6 <sup>S</sup>	–	[25]
Germany	42.3 <sup>a)</sup> 45.6 <sup>b)</sup>	25.3 <sup>a)</sup> 24.8 <sup>b)</sup>	9.2 <sup>a)</sup> 10.4 <sup>b)</sup>	–	–	[26]
Attica, Greece	41.8	–	–	27.7	1.9	[27]
Inra Estrees-Mons, France	37.8	19.8	–	14.4	–	[28]
Bedfordshire, UK	49.5	22.5	–	13.1	16.24	[29]

a) Autumn harvests.

b) Winter harvests.

<sup>L</sup> Leaves; <sup>S</sup> Stalks; CD, Chuncheon Dongsan-myeon; DR, Mt. Daeryong; GB, Mt. Gumbyeong; HD, Hoengseong Dunnae; IB, Inje Bangdong-myeon; IS, Inje Sangnap.

### 3.2 Influence of cultivation conditions on *Miscanthus* composition

For application as a feedstock in producing cellulosic ethanol, breeding and screening of plants with high cellulose content for cultivation is an important concern. In the study by Hodgson et al. [26], different *Miscanthus* genotypes grown in five European locations (Denmark, Sweden, England, Germany, and Portugal) are investigated to find the influence of genetic and environmental factors on cell wall composition. Results show that the cellulose content changes with the location planted, as well as the harvest season. The cellulose content of *Miscanthus* biomass increases 5–7% in the winter harvest as compared to the autumn harvest [26]. When a two-year old *M × G* is harvested between November and April, the cellulose content is increased by 4% and hemicellulose in leaves decreases by 2%. These results demonstrate that cellulose content increases with aging of leaves [29].

In addition, application of fertilizers to the cultivation of *M × G* has an impact on the cell wall composition of *Miscanthus* stems. Hodgson et al. [29] have investigated the effect of fertilizer treatment on lignocellulose composition of *Miscanthus*. The use of nitrogen fertilizer significantly reduces hemicellulose and lignin contents and increases ash content. However, a small amount of potassium chloride can increase the cellulose and hemicellulose content. In summary, the cellulose content of *Miscanthus* varies by species, genotype, climatic conditions, seasonal changes, and harvest time.

## 4 Pretreatment and enzymatic saccharification

### 4.1 Methods of pretreatment on *Miscanthus* biomass

The production of bioethanol and other fermentation products from lignocellulosic feedstocks like *Miscanthus* biomass is a multi-step biochemical conversion process that normally includes pretreatment, enzymatic hydrolysis and yeast fermentation. After proper pretreatment, carbohydrates in *Miscanthus* can be converted by enzyme hydrolysis into simple sugars like glucose and xylose. By saccharification of the cellulose and hemicellulose content in *Miscanthus*, resultant sugars can be fermented to produce fuel ethanol, organic acids, and other chemicals. Pretreatment affords the recovery of cellulosic content from lignocellulosic biomass and renders it digestible to cellulases. Some of the most promising pretreatment technologies, including mechanical, mineral acid, alkali, liquid hot water, organosolv, wet oxidation, ozonolysis, CO<sub>2</sub> explosion, steam explosion, ammonia fiber explosion (AFEX), and ionic liquid, have been summarized and compared for the commercialized produc-

tion of biofuels from lignocellulosic feedstocks [30]. Biological pretreatment also appears to be promising but is less attractive commercially [30]. Most of the aforementioned physical, physico-chemical, and chemical pretreatment methods have been attempted with *Miscanthus* biomass, followed by enzymatic hydrolysis to convert it to fermentable sugars for bioethanol production, as shown in Table 3.

Size reduction, through the mechanical processes of chipping, grinding, or milling, was frequently required before other pretreatments. For example, for bioethanol production, *M × G* was treated using hammer milling for reduction of particle size, followed by a hydrothermal pretreatment. Sugar yields from enzyme hydrolysis of pretreated biomass were found to increase inversely with mean particle size, with the best results observed for all pretreatments with a particle size of 0.08 mm [31, 32]. Simply reducing the size of particles could reduce cellulose crystallinity. In one study, *M. sinensis* was ground by ball-milling, and the resulting powder was separated into four size fractions by passage through a series of sieves. Results of the study indicated that the crystallinity of biomass decreased with particle size [33].

Autohydrolysis (also called hydrothermal pretreatment) is a physico-chemical process performed by treating lignocellulosic feedstock with liquid hot water (LHW) at temperatures of 160 to 240°C. Hot water disrupts hydrogen bonds between cellulose microfibrils and swells the cellulose structure. At high temperatures, hemicellulose dissolves, lignin is loosened, and water dissociates to form a weak acid, leading to deacetylation of xylan. Acetic acid produced during pretreatment further decreases pH and increases xylan hydrolysis [31]. In summary, autohydrolysis causes hydrolysis of the hemicellulose component, without significantly modifying lignin, and leaving cellulose in the solid residue [23]. LHW pretreatment with a severity of 4.71 can completely degrade the hemicellulose in *M. lutarioriparius* [34]. When milled *M × G* (particle size of 0.08 mm) is treated with LHW, the water-pretreated biomass results in low xylose yield (0.030 g/g-pretreated biomass). The glucose yield, defined as the ratio of the glucose released to the glucose in the pretreated biomass, can reach 93.4% [31].

Physico-chemical pretreatment methods also include steam explosion, AFEX, and other kinds of explosion processes. To yield fermentable sugars, AFEX has been used for the pretreatment of *M × G* [35]. In the AFEX process, lignocellulosic materials are exposed to liquid ammonia at temperatures of 70–180°C and pressure ranging between 200 and 1000 psi for a brief period of time, and then the pressure is swiftly reduced. As pressure is explosively released, the structure of the biomass can be effectively disrupted. AFEX can decrystallize cellulose, partially hydrolyze hemicellulose, and depolymerize lignin [36], but the AFEX process is not very effective for biomass with high lignin content [37]. In the pretreatment of



**Table 3.** Pretreatment and enzyme hydrolysis of *Miscanthus* biomass for ethanol production

Species	Pretreatment method and conditions	Size reduction prior to pretreatment	Enzyme hydrolysis (Enzyme loading)	Yield of sugars (based on raw biomass)	Ethanol yield	Estimated cost of production (US \$/L) <sup>a)</sup>	Ref.
<i>Miscanthus × giganteus</i>	<b>Aqueous-ethanol organosolv</b> with dilute-acid presoaking (1.4% sulfuric acid): 180°C, 60 min, 1.25% H <sub>2</sub> SO <sub>4</sub> , and 60% ethanol	Hammer-milled to 1–3 mm	20 FPU of cellulase mixture (Celluclast 1.5 L) supplemented with 40 IU of β-glucosidase (Novozym 188)	95% glucans yielded in solid residue, from which 98% was recovered after 48 h of enzymatic hydrolysis	70% from glucose at 48 h		[12]
<i>Miscanthus × giganteus</i>	<b>Liquid hot water:</b> Hot water treatment at 200°C for 10 min	Hammer-milled to 0.08 mm	0.25 mL/g-biomass of Accellerase 1500, 0.05 mL/g-biomass of Accellerase XY, 0.125 mL/g-biomass of Accellerase XC at 0.09 mL/g-biomass of Accellerase BG	76.7% glucan, 11.8% xylan. If pretreatment liquor included 78.1% glucan, 51.3% xylan	SSF: 70% ethanol yield, 1.3 g/100 g-raw biomass	0.75	[31]
<i>Miscanthus lutarioriparius</i>	<b>Liquid hot water:</b> Liquid–solid ratio of 100:15 (V:W), severity of 2.56–4.71	Cut to 1–2 cm	15 FPU/g-biomass and 1 CBU/g-biomass Cellulase from Sukahan (Weifang) BioTechnology Co.		SSF: 15 g/100 g-raw biomass		[34]
<i>Miscanthus × giganteus</i>	<b>Ammonia fiber expansion (AFEX):</b> 160°C, 2:1 w/w ammonia to biomass loading, 233% moisture (dry weight basis), and 5 min reaction time	Hammer-milled to 3.175 mm screen	15 FPU/g-glucan of cellulase and 64 p-NPGU/g-glucan of β-glucosidase, supplemented with xylanase and tween-80	96% glucan and 81% xylan	n.d. <sup>b)</sup>	0.61	[35]
<i>Miscanthus</i> harvested in Denmark	<b>Wet explosion</b> with dilute acid presoaking: Hammermilled, presoaked in 0.75% v/v sulphuric acid solution (10% TS) for 14 h at 100°C, then wet exploded in air/H <sub>2</sub> O <sub>2</sub>	Hammer-milled to 1–3 mm	45 FPU/g-TS of Cellulase (Celluclast 1.5 L) and 55 FBC/g-TS of β-glucosidase (Novozym 188)	94.9% xylose, 61.3% glucose (in air); 82.4% xylose, 63.7% glucose (in hydrogen peroxide).	n.d. <sup>b)</sup>	0.77	[39]

Table 3. Pretreatment and enzyme hydrolysis of *Miscanthus* biomass for ethanol production (continue)

Species	Pretreatment method and conditions	Size reduction prior to pretreatment	Enzyme hydrolysis (Enzyme loading)	Yield of sugars (based on raw biomass)	Ethanol yield	Estimated cost of production (US \$/L) <sup>a)</sup>	Ref.
<i>Miscanthus floridulus</i>	<b>Dilute acid pretreatment:</b> 3% sulfuric acid at 121 °C for 180 min	Hammer-milled to 0.5 cm	6.5 FPU/g-DM cellulase (Spezyme CP from Genencor)	Hydrolysate: 50–60% glucose. In pretreatment liquor: 70–75% xylose, 80% arabinose, 10% glucose	64–66% g/g-xylose (fermentation on pretreatment liquor)	0.93	[41]
<i>Miscanthus × giganteus</i>	<b>Combined acid catalysis:</b> 0.73 wt% sulfuric acid blended with 50:50 trifluoroacetic acid (TFA)/maleic acid (MA) at 150 °C for 6.1 min <b>Dilute acid pretreatment:</b> 0.73 wt% sulfuric acid	Hammer-milled to 6.35 mm	60 FPU/g-biomass of cellulase (Spezyme CP, Genencor), 2 CBU/FPU of $\beta$ -glucosidase (Novozym 188) supplemented with xylanase (Multifect Xylanase, Genencor).	<b>Combined acid catalysis:</b> 90% of cellulose recovered in residue, 54–75% hydrolyzed. Up to 81% xylose yield after pretreatment <b>Dilute acid pretreatment:</b> 69.7% glucose yield, 57% xylose yield	67% from xylose  84.69% and 73.71% from glucose (10 and 24% pretreated biomass loading, respectively)	0.75 (Combined acid catalysis)  0.86 (Dilute acid pretreatment)	[42]
<i>Miscanthus</i> harvested in Korea	<b>NaOH pretreatment:</b> 1.49 M NaOH at 145.29 °C for 28.97 min (optimal conditions)	Hammer-milled to 1–3 mm	50 FPU/g-cellulose of cellulase complex (Novozymes NS50013) and 30 cellobiase unit/g $\beta$ -glucosidase (Novozymes NS50010)	83.92% glucose (10%, w/v biomass loading); 55.07% hemicellulose loss		0.89	[43]

a) Estimated production cost was calculated on the basis of ethanol yield and product value (ethanol unit cost) for ethanol processes using each pretreatment as reported in the literature [59, 60]. The detail calculation is shown in Supporting information, Table S1.

b) n.d.: No fermentation was performed.

$M \times G$  at optimal AFEX conditions, approximately 96% glucan and 81% xylan conversions are achieved after enzyme hydrolysis [35].

Steam explosion is a commonly used physico-chemical method that can also cause autohydrolysis. Steam explosion has been used to produce Steam-Exploded Biomass of  $M \times G$  for the study of its suppression against soil-borne plant pathogens [38]. As a pretreatment in steam explosion, chipped biomass is treated with high-pressure saturated steam and then the pressure is swiftly reduced, leading to an explosive decompression of the materials [37]. A wet explosion process combining steam explosion and wet-oxidation has been employed for the pretreatment of *Miscanthus* biomass [39]. Because of the presence of air or  $H_2O_2$  in the steam explosion process, there are oxidation reactions that cause the chemical degradation of biomass in addition to physical rupture. With wet explosion pretreatment combined with dilute acid presoaking of *Miscanthus*, higher sugar yields can be obtained after enzyme hydrolysis. Yields of 94.9% xylose and 61.3% glucose are obtained when the steam explosion occurred in the presence of air, while yields of 82.4% xylose and 63.7% glucose are obtained in the presence of hydrogen peroxide [39].

Microwave-chemical pretreatments are a class of processes in which microwave heating is used during the chemical pretreatment process. Microwave heating has been employed to digest *M. sinensis* during dilute ammonium hydroxide pretreatment, dilute acid pretreatment, and two-stage pretreatment (dilute ammonium hydroxide followed by dilute phosphoric acid pretreatment). Results show that the microwave-assisted 1.0% w/v  $NH_4OH$  treatment liberates 2.9 g of monomeric sugars per 100 g of dried biomass, whereas the corresponding yield for treatment with 1.78% v/v  $H_3PO_4$  is 62.3 g/100 g. The microwave-assisted two-stage pretreatment results in the highest total monomeric sugar yield of 71.6 g/100 g dried biomass [40].

Chemical pretreatment is a category of pretreatment methods using acids, alkalis, alcohols, organic acids, pH-controlled LHW, or ionic liquids. Acid hydrolysis using dilute sulfuric acid ( $H_2SO_4$ ) is the most common option, among the chemical pretreatments. During dilute acid pretreatment, carbohydrates (mainly hemicelluloses) are depolymerized in the liquid into oligosaccharides and monosaccharides, as well as furfural, acetic acid, and 5-hydroxymethylfurfural (HMF) as degradation products that are toxic for the following fermentation. Dilute acid has been used to treat silvergrass (*M. floridulus*) with 3% sulfuric acid at 121°C for 180 min. In addition to xylose (70–75% yield), arabinose (80% yield), and glucose (10% yield), the resultant pretreatment liquor contains furfural and acetic acid [41].

Combined acid catalysts, blending sulfuric acid with one of two biomimetic acids, trifluoroacetic acid (TFA) and maleic acid (MA), have been used for the pretreat-

ment of  $M \times G$  [42]. Positive synergistic effects in hemicellulose decomposition, which help increase xylose yield and reduce phenol production, are observed in the combined TFA pretreatment. Combined acid catalysis (sulfuric acid with TFA or MA) increases overall ethanol yield by 27–54% compared to  $H_2SO_4$  pretreatment alone [42].

Incubation of lignocellulosic feedstock with alkaline solutions, such as NaOH,  $Ca(OH)_2$ , or ammonia, could be useful to remove lignin and hemicelluloses, further increasing the accessibility of enzymes to the cellulose. In comparison with acidic solutions, hemicellulose could exhibit higher solubility in alkaline solutions with less degradation. For pretreatment of *Miscanthus* collected in the Netherlands, extrusion or milling was used for size reduction, followed by incubation with 12% NaOH at a moderate temperature (70°C) for 4 h. This pretreatment resulted in 77% delignification, 44% of hemicelluloses removed and a cellulose yield of more than 95%. After enzyme hydrolysis, 69 and 38% of the initial cellulose and hemicellulose fractions, respectively, were converted into monosaccharides for hydrogen production [22]. In another study, pretreatment with NaOH at optimal conditions yielded 83.92% glucose from *Miscanthus* harvested in Korea [43].

Pretreatment of *Miscanthus* with aqueous ammonia was performed by treating with 33% aqueous ammonia at a *Miscanthus*/ammonia ratio of 15 g/100 mL for three days at room temperature. A significant enhancement in enzyme digestibility was observed. With the delayed harvest of  $M \times G$ , the saccharification yield increased by a factor of four for cellulose after pretreatment with aqueous ammonia [28].

Organosolv pretreatment is similar to organosolv pulping, the process to extract lignin from lignocellulosic feedstocks with organic solvents or their aqueous solutions, but the degree of delignification is relatively lower for pretreatment [44]. Organosolv pretreatment with the use of alcohol, organic acid, organic peracid, and acetone has been frequently used to obtain cellulose pulp from lignocellulose materials. Fractionation of raw materials to yield their main components (cellulose, hemicelluloses, and lignin) in a less degraded form can be achieved by the organosolv process. Organosolv treatment with phenol as the solvent is first applied to  $M \times G$ . A delignification efficiency of 83% can be achieved using organosolv with phenol. However, because of its toxicity, phenol in the aqueous phase needs to be removed after the organosolv treatment [45]. An ethanol organosolv process has been used for the pretreatment of  $M \times G$ , presoaked with dilute acid. This combined dilute acid presoaking and aqueous-ethanol organosolv treatment process results in efficient fractionation of *Miscanthus* biomass into a cellulose-rich residue, a precipitate of ethanol organosolv lignin, and a water-soluble fraction mainly containing hemicellulose sugars. The optimized conditions yield a solid residue containing about 95% of the initial glucans, from which



98% are recovered after enzymatic hydrolysis [12]. The improved enzymatic digestibility is due to a presoaking step at low severity, prior to the organosolv process, which enhances the removal of lignin and hemicelluloses [46].

Autohydrolysis of *Miscanthus* is induced in the aqueous-ethanol organosolv treatment with 0.5–1.2% w/w with sulfuric acid as a catalyst. Acetic acid is produced from xylans and a large part of hemicellulose is hydrolyzed and dissolved by the action of autohydrolysis. In addition, the cleavage of lignin-carbohydrate bonds and the lignin depolymerization during autohydrolysis enhance the solubility of lignin in a subsequent extraction [47, 48]. In the presence of 2-naphthol, autohydrolysis can even lead to a comprehensive depolymerization of lignin and the production of low molecular weight lignin with higher solubility in ethanol [48]. The cleavage of aryl ether bonds,  $\beta$ -O-4-linkages, and ester bonds (acetyl and coumaryl residues) are responsible for lignin depolymerization in the ethanol organosolv process [49, 50].

Organic acids (acetic acid and formic acid) have also been used as the organosolv media. The organic acid pretreatment of lignocellulosic biomass can cause not only the solvation of lignin fragments, but also the delignification and hydrolysis of hemicelluloses by the action of hydrogen ions generated from partial dissociation of the organic acid [44]. An organosolv process using a mixture of water and acetic acid/formic acid in the presence of hydrochloric acid has been used for the pretreatment of *M × G* [51]. Treatment with acetic acid and formic acid can lead to the breakdown of the lignin-carbohydrate structure and effectively remove lignin from the lignocellulosic biomass. Glucan digestibility is highly dependent on the degree of delignification. The optimum conditions for maximum enzymatic digestion of glucan (75.3%) can be obtained with an incubation time of 3 h, at 107°C, and with a formic acid/acetic acid/water ratio of 40/40/20%. In those conditions, a solid (pulp) yield of 56.4% with delignification of 79.6% can be achieved [52].

Perorganic acids such as performic acid have also been used as organosolv media. Performic acid is generated from aqueous formic acid in combination with the more strongly oxidizing  $H_2O_2$  [53]. An autothermal, single-stage, performic acid pretreatment has been used for the rapid fractionation of *M × G* biomass components into a lignin/hemicellulose-rich liquor and a cellulase-digestible pulp. Lignin and hemicellulose removal (89% and 68%, respectively) yields a pulp with higher enzymatic digestibility than untreated *Miscanthus* (up to 20-fold) [53].

Chemical pretreatments also include oxidative treatments that use  $O_2$ , ozone,  $H_2O_2$ , or other peroxide compounds. Combined alkaline peroxide (pH 11.5,  $H_2O_2$ ) and electrolyzed water has been used to deconstruct *M × G* [54]. Hemicellulose and lignin are partially removed during the first pretreatment of 1.0–4.0% alkaline peroxide

solution at 50°C, and then the residual solids are subjected to a second pretreatment at 121°C with electrolyzed water. A digestibility of up to 95% can be achieved by this two-stage method, which is higher than that obtained from dilute acid pretreatment. After the first pretreatment with an alkaline peroxide solution, 63% of hemicellulose and 64% of lignin are removed from the *Miscanthus* materials and cellulose is enriched from 46 to 70% [54]. This hemicellulose removal rate is higher than that (44%) achieved with 12% NaOH pretreatment [22]. Gaseous ozone has also been used in different flow configurations for the pretreatment of *M × G*, *M. sinensis* 'Gracillimus', and other energy grasses. Ozonolysis is found effective in removing up to 59.9% of lignin without cellulose degradation [55].

Sub-critical water processing that combines the use of ethanol and carbon dioxide was used as a pretreatment method for delignification of the *M × G* biomass. This use of sub-critical water:ethanol:CO<sub>2</sub> can mediate hydrolysis of the lignocellulosic biomass and drive the delignification of biomass without destroying cellulose fibers [56].

## 4.2 Comparison of pretreatment methods on *Miscanthus*

As shown in Table 3, both AFEX and aqueous-ethanol organosolv processes leads to very high glucose yields (>95%). The use of NaOH results in lower glucose yields. The glucose yield is even lower (about 60%) when dilute sulfuric acid is used. However, the dilute acid is favored for the degradation of hemicellulose to release xylose. Approximately 70% of the total xylose in *Miscanthus* can be extracted by using 0.75% sulfuric acid at 100°C [39]. The highest xylose yield (94.9%) can be achieved by combining wet explosion and dilute acid presoaking. Table 3 suggests that AFEX is most promising for the pretreatment of *Miscanthus* in terms of total glucose and xylose yields.

Dilute sulfuric acid more easily renders a yield of xylose than glucose from the biomass, while pretreatment in alkaline solutions like NaOH and ammonia causes solubilization and removal of hemicelluloses. On the other hand, organosolv is a promising approach for solubilizing lignin in an organic medium, e.g. ethanol. Solvent can be recycled by evaporation or distillation, rendering the recovery of sugars and lignin from the liquid phase. Organosolv fractionation of *Miscanthus* biomass can thus make the grass into a valuable source of lignin. Both chemical and physico-chemical pretreatment methods involve the use of chemicals. The repeated use or recycling of these chemicals can reduce not only the cost of pretreatment, but also the impact on the environment. For example, the alkaline solution can be reused several times, until the solubility of hemicellulose and lignin reaches saturation [57]. Combining the use of chemicals and physical methods, such as explosion and microwave

heating, for pretreatment can more effectively destroy *Miscanthus* biomass, releasing lignin and hemicelluloses and decreasing cellulose crystallinity, which promotes the efficiency of enzyme hydrolysis. However, more energy input is necessary for these combined methods.

A comprehensive techno-economic analysis has been performed on the production of cellulosic ethanol by using some of the common pretreatment technologies on corn stover [58, 59] or tall fescue (*Festuca arundinacea* Schreb) as a model feedstock [60]. Each pretreatment process is embedded in a full bioethanol facility model in which the systemic effects of variations in pretreatment are accounted for in the overall process. Both five- and six-carbon sugars released during pretreatment and following enzymatic saccharification are taken into account for ethanol fermentation. Using the data on sugar yields summarized in Table 3, an economic comparison of ethanol production costs for *Miscanthus* pretreated by different methods is thus possible. The following data are taken from the literature for calculations of the estimated ethanol costs for *Miscanthus*: projected ethanol yields of 250.0 [59], 252.62, 255.80, 255.27, and 230.23 L/dry metric ton biomass [60] and corresponding ethanol costs of 0.891 [59], 0.83, 0.88, 0.81, and 0.85 \$/L of ethanol for ethanol processes using AFEX, dilute acid, alkali, hot water, and steam explosion pretreatment technologies, respectively. The ethanol cost is defined as the product value (PV), which includes the ethanol production cost and a 10% return on investment, as reported in the literature [59, 60]. Details of the calculations are shown in Supporting information, Table S1. The results indicate that among liquid hot water, AFEX, wet explosion, NaOH, and dilute acid (including combined dilute acid) pretreatments for cellulosic ethanol processes, AFEX leads to the lowest ethanol cost (0.61 \$/L). The costs of ethanol produced via other pretreatments are in the range of 0.75 to 0.93 \$/L, suggesting that these pretreatments are economically competitive with ethanol production from *Miscanthus* biomass. AFEX results in the lowest process cost for ethanol production from *Miscanthus* because of the very high sugar yields (96% glucose yield), as well as the very high cellulose content in *Miscanthus* biomass. These results suggest that the cost driver is strongly influenced by sugar yields, which depend on the pretreatment method.

### 4.3 Enzymatic saccharification of pretreated *Miscanthus* biomass

Enzymatic saccharification of *Miscanthus* biomass is normally performed on the residual solid (pulp) after the pretreatment process. Carbohydrate content (cellulose and a relatively small amount of hemicellulose) in the pretreated materials is converted to monosaccharides by using cellulolytic enzymes. As shown in Table 3, almost all of the enzymes used for cellulose hydrolysis come from two major enzyme companies, Novozymes and Genencor.

Typical cellulase preparations are made of cellulase complexes, Celluclast 1.5 L (Novozymes) or Spezyme CP (Genencor), that contribute to FPU activity, and Novozyme 188 (Novozymes), that contributes mainly to  $\beta$ -glucosidase activity. The cellulase complex is composed of three classes of enzymes:  $\beta$ -1,4-cellobiohydrolases (CBH),  $\beta$ -1,4-glucan endoglucanase (EG) and  $\beta$ -glucosidase (cellulobiase). Either Celluclast 1.5 L or Spezyme alone cannot completely digest cellulose into monosaccharides (glucose). Due to the relatively weak hydrolytic activity of disaccharides (cellobiose) generated from cellulose hydrolysis and to the inhibition of end-product glucose, there is a significant fraction of cellobiose present in the reaction mixture. Supplying Novozyme 188 with strong  $\beta$ -glucosidase activity fully converts cellobiose to glucose. New generations of commercial cellulase complexes, such as Accellerase 1500 (Genencor) and Cellic CTec2 (Novozymes), have already blended enzymes with high levels of FPU and  $\beta$ -glucosidase activities together. For the hydrolysis of hemicelluloses in pretreated solids, xylanases must be supplemented to convert xylan into xylose [31, 35, 42]. The multi-enzyme cocktail Cellic CTec3 (Novozymes) is a cellulase and hemicellulase complex that contains xylanase activity, and that allows for the conversion of pretreated lignocellulosic materials to fermentable sugars.

Apart from the pretreatment method, efficiency of enzyme hydrolysis is influenced by the dose of enzyme, biomass loading, and reaction time. The amount of enzyme complexes loaded and their combination depends strongly on their specific activities. Of course, the higher the amount of enzyme loaded, the greater the enzymatic digestion of pretreated *Miscanthus* [43]. Normally, 15–20 FPU per gram of cellulose (glucan) combined with sufficient  $\beta$ -glucosidase activity can effectively convert cellulose to glucose with a yield of greater than 95% [12, 35]. In addition, under the same conditions of enzyme loading, the longer the reaction time the higher the yield of sugars produced by enzymatic hydrolysis [41]. For enzymatic saccharification, the pretreated biomass load, i.e. the ratio of residual solids from pretreatment to the volume of reaction mixture, is a crucial factor. Enzymatic saccharification of NaOH-pretreated *Miscanthus* at various biomass loads (1–24%, w/v) has been studied [43]. The glucose concentration released from pretreated biomass increases with biomass loading but enzymatic digestibility decreases with biomass loading. Enzyme digestion of 85% of biomass can be achieved using 10% w/v biomass loading, whereas 20% w/v biomass loading results in about 80% digestion, probably due to glucose inhibition. In addition, higher biomass loading (>24%, w/v) is impractical because of difficulty in stirring [43].

#### 4.4 Influence of *Miscanthus* composition on the efficiency of enzymatic saccharification

Both the lignin and hemicellulose contents of *Miscanthus* are important factors influencing the efficiency of enzyme hydrolysis of pretreated biomass. The enzymatic saccharification of biomass after dilute acid or alkali pretreatment is taken as an example. Results from NaOH pretreatment show an inverse relationship between lignin content and the efficiency of enzymatic hydrolysis of polysaccharides [22]. Similar results have been obtained from experiments of NaOH and H<sub>2</sub>SO<sub>4</sub> pretreatment of wild *Miscanthus* collected in China. A *Miscanthus* sample with a high cellulose or lignin level shows an increased crystallinity and low biomass saccharification, particularly after H<sub>2</sub>SO<sub>4</sub> pretreatment [61]. Results also show that *Miscanthus* with high hemicelluloses levels has a relatively low cellulose crystallinity index and enhanced biomass digestibility after pretreatments with NaOH and H<sub>2</sub>SO<sub>4</sub>. In sum, hemicelluloses play a dominant, positive role, whereas cellulose and lignin (mainly acid-insoluble lignin) have synergistic, negative effects on biomass digestibility [61].

Recently, a series of papers have been published by Pang's lab; the researchers reported on the cell wall composition and cellulose structural features that can distinctively affect biomass enzymatic digestibility with various chemical pretreatments of *Miscanthus* [62–66]. Three lignocellulose features, i.e. cellulose crystallinity, degree of polymerization, and mole number, display significant influences on the enzymatic digestibility of pretreated *Miscanthus* biomass [63]. In addition, the xylose/arabinose ratio in xylan is a key factor that positively affects biomass saccharification. Increasing the degree of arabinose substitution for xylan reduces the cellulose crystallinity for high biomass digestibility [64]. Regarding lignin composition, three major monolignols, including p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), and the S/G ratio are found to negatively influence biomass digestibility upon NaOH and H<sub>2</sub>SO<sub>4</sub> pretreatments [65]. A slight genetic modification of the proportions of the three monolignols significantly enhances the biomass enzymatic digestibility [66]. These findings provide some suggestions for genetic modifications of plant cell walls in *Miscanthus* towards high biomass enzymatic digestibility after a cost-effective pretreatment. New varieties of *Miscanthus* are expected to be developed by the use of a newly developed gene transfer technology [67].

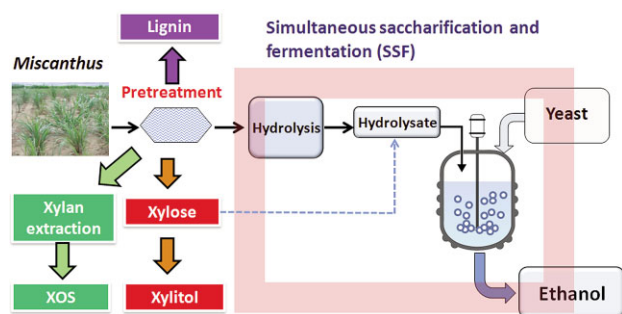
## 5 Production of bioethanol and value-added byproducts

Monosaccharides released from *Miscanthus* biomass in pretreatment and enzymatic hydrolysis are subjected to fermentation for bio-ethanol production. As described in

the previous section, some pretreatments, such as diluted acid-based technologies, result in a significant concentration of xylose in pretreated liquor. This xylose-containing liquor can be fermented to ethanol by using C5-fermenting microorganisms. Using the xylose-fermentation yeast *Candida shehatae*, an ethanol yield of 64–66% g/g-xylose can be achieved from the fermentation of pretreated liquor of *Miscanthus* by dilute acid pretreatment [41]. This lower ethanol yield is due to the presence of fermentation inhibitors, acetic acid and furfural, in the pretreated liquor. Similar results have been reported from fermentation by an engineered *Saccharomyces cerevisiae* of liquor from pretreatment with combined sulfuric acid and trifluoroacetic acid/maleic acid. When maleic acid pretreatment is used, an ethanol yield of 0.67 g/g-xylose can be obtained from the sugar solution. The highest overall ethanol yield, 0.162 g/g-hemicellulose, can be achieved when *Miscanthus* is treated with 75:25 H<sub>2</sub>SO<sub>4</sub>-MA [42].

Separated hydrolysis and fermentation (SHF) is a bioethanol production process with ethanol fermentation of the hydrolysate from enzymatic hydrolysis. Most of the pretreatments result in residual solids containing mainly cellulose (pulp). Glucose is thus the major fermentable sugar in the hydrolysate after enzymatic saccharification. *S. cerevisiae* is a commonly used microorganism for ethanol production from glucose in hydrolysate. A theoretical ethanol yield of 70% can be obtained in 48 h fermentation using ordinary baker's yeast (*S. cerevisiae*) on the hydrolysate of *M* × *G* after pretreatment using a two-step procedure with a dilute acid presoaking step followed by an aqueous-ethanol organosolv treatment [12]. In the SHF process, the final ethanol concentration depends on the glucose concentration in the hydrolysate. Higher biomass loading in the enzymatic hydrolysis step leads to a higher glucose concentration in the hydrolysate, and, consequently, a higher ethanol concentration in the product. However, increased biomass loading decreases the ethanol yield, which is calculated by dividing the experimental ethanol concentration by the theoretical amount of ethanol produced from glucose conversion from pretreated biomass. A final ethanol concentration of 28.34 g/L, corresponding to an ethanol yield of 84.69%, can be achieved by fermentation of the hydrolysate from enzymatic saccharification with 10% biomass loading of NaOH-pretreated *Miscanthus*.

Ethanol fermentation can be performed directly on the pretreated *Miscanthus* biomass. The simultaneous saccharification and fermentation (SSF) process combines the enzymatic hydrolysis of cellulosic matters and ethanol fermentation of hydrolysate within a single stage (Fig. 1). By the SSF process, *M* × *G* pretreated with LHW is converted to ethanol using a native industrial *S. cerevisiae* strain D5A (nonrecombinant yeast). Ethanol yields of 70% based on glucan content in pretreated biomass are obtained after 72 h fermentation. This experimental result suggests that 1 g of *Miscanthus* can generate 0.13 g of



**Figure 1.** Production of bioethanol and value-added by-products from *Miscanthus* biomass. After pretreatment, the cellulosic fraction of *Miscanthus* biomass is converted by enzyme hydrolysis into simple sugars in the hydrolysate which are then fermented to yield ethanol. Value-added products including xylitol, xylooligosaccharides and lignin can also be co-produced from the matter removed in the pretreatment step.

ethanol [31]. In another study, SSF of *M. lutarioriparius* pretreated with LHW yields 0.15 g ethanol from 1 g raw biomass [34]. This ethanol yield is higher than that (11.1 g ethanol/100 g biomass) obtained from fermentation of enzymatic hydrolysate of *Miscanthus* after a two-step ozone pretreatment [68].

The production of bioethanol from *Miscanthus* biomass is a lignocellulose-to-ethanol process. Currently, the total cost of bioethanol production from lignocellulose feedstock is still much higher than production from starch or sugar-based feedstocks. Efficient depolymerization of the carbohydrate content to fermentable sugars by pretreatment, enzyme saccharification, and efficient fermentation of mixed six-carbon (glucose) and five-carbon (mainly xylose) sugars in hydrolysate is a techno-economic challenge [69]. The predominant sugars released from enzymatic hydrolysis are glucose, xylose, and a lesser amount of arabinose and other sugars, depending on the pretreatment method. Enhancing ethanol yield by co-fermentation of six-carbon (glucose) and five-carbon (mainly xylose) sugars in hydrolysate is helpful for reducing production costs. Several recombinant *S. cerevisiae* and *Escherichia coli* strains that can ferment mixed glucose and xylose sugars to produce ethanol have been developed for this co-fermentation purpose.

Other challenges of the cellulosic ethanol process include finding the optimal integration process to minimize energy demands and maximize cost-efficient utilization of the by-product lignin [69]. Lignin is a commercial product with applications as a polymer modifier, an adhesive, a resin, and more. As demonstrated in Fig. 1, not only lignin, but also hemicellulose extracts can be used to produce other value-added products such as xylitol and xylooligosaccharides (XOSs). *Miscanthus* having a xylan content of  $\geq 20\%$  (dry weight) provides a source of xylan after certain pretreatments, like alkaline solution pretreatment or alkaline peroxide pretreatment. XOSs are oligosaccharides containing 2–7 molecules of xylose with

$\beta$ -(1,4) linkages and are regarded as prebiotics. Xylan extracts obtained from pretreatment can be degraded into XOSs by the enzymatic action of endo-xylanases. Sometimes, XOSs can be produced during the pretreatment process itself. XOSs are produced from *Miscanthus* biomass by mixing the biomass with 0.1%  $\text{H}_2\text{SO}_4$ , incubating it in an oven at 60°C for 12 h, and then following with dry steam thermal treatment. The resultant liquor contains xylooligomers with a yield of 65.0% [70]. Furthermore, xylan obtained in the pretreatment step can be totally hydrolyzed into xylose by using xylanases. Xylose can also be obtained from acid catalysis pretreatments. Besides ethanol, xylitol can be produced by fermentation of xylose by microorganisms. Co-production of these value-added products renders the production of *Miscanthus* bioethanol economically competitive with the first generation of bioethanol.

## 6 Concluding remarks

*Miscanthus* biomass has been shown to be a promising raw material for cellulosic ethanol because of its high cellulose content. Once the perennial *Miscanthus* plant, regardless of species, is planted and established, it can be harvested with high biomass yields yearly. Cultivation studies indicate that species, genotype, climatic conditions, seasonal changes, and harvest time, all can influence the cellulose content of *Miscanthus*. To break down the cellulose content in this grass into fermentable sugars requires a series of steps including pretreatment and enzymatic hydrolysis. A number of pretreatment compresses using LHW, NaOH treatment, combined acid catalysis, ammonia fiber expansion, wet explosion with dilute acid presoaking, and aqueous-ethanol organosolv processing, have been tried and found effective for making *Miscanthus* more digestible for enzymatic saccharification. High glucose yields are obtained in the hydrolysate. A pretreatment that results in a high lignin removal rate favors enzymatic hydrolysis. In addition to the effect of pretreatment, the enzymatic hydrolysis of cellulose (glucan) depends on the hemicelluloses and lignin content. Higher hemicelluloses content in *Miscanthus* has a positive effect on the biomass digestibility.

In the SHF process, bioethanol is produced by yeast (normally, *S. cerevisiae*) fermentation of hydrolysate from enzymatic saccharification of pretreated biomass. An ethanol yield of 84.69% is achieved from the fermentation of hydrolysate from NaOH-pretreated *Miscanthus*. The higher recovered yield of cellulose and xylan can lead to a higher ethanol yield. Furthermore, as *M. × G* and *M. lutarioriparius* pretreated by LHW is used as the substrate for SSF, experimental ethanol yields of 0.13 and 0.15 g/g-raw biomass, respectively, are obtained. This can be compared with theoretical ethanol yields calculated from cellulose contents of *Miscanthus* ranging from



0.209–0.231 g/g raw biomass (taking the average value of 0.217 g/g raw biomass from four *Miscanthus* species), where the efficiency of ethanol production is about 70%. The production of bioethanol from *Miscanthus* is thus technologically promising. In addition to lignin, which has many applications, hemicellulose removed in the pre-treatment step can be used as starting material for the production of xylitol and xylooligosaccharides. Because of the co-production of ethanol with lignin and xylan-based value-added products, the total cost of producing bioethanol can be reduced, making bioethanol production from *Miscanthus* economically competitive.

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#### Cover illustration

**Special Issue: Bioenergy and biorefinery.** This special issue, in collaboration with the Asian Federation of Biotechnology, is edited by Yinbo Qu and Wen-Teng Wu. It includes contributions on the improvement of enzymes or organisms and pretreatment methods for increasing the refinery efficiency of plant biomass. Furthermore, articles on the use of microalgae not only for  $\alpha$ -glucan and protein production but also for the removal of different substances from flue gas are part of this special issue. Image: © Olaf Wandruschka-Fotolia.com.

### *Biotechnology Journal* – list of articles published in the June 2015 issue.

#### Editorial: Bioenergy and biorefinery – biological solution for sustainable development of human society

Yinbo Qu and Wen-Teng Wu

<http://dx.doi.org/10.1002/biot.201500291>

#### Review

##### **CO<sub>2</sub>, NO<sub>x</sub> and SO<sub>x</sub> removal from flue gas via microalgae cultivation: A critical review**

Hong-Wei Yen, Shih-Hsin Ho, Chun-Yen Chen and Jo-Shu Chang

<http://dx.doi.org/10.1002/biot.201400707>

#### Review

##### **Miscanthus as cellulosic biomass for bioethanol production**

Wen-Chien Lee and Wei-Chih Kuan

<http://dx.doi.org/10.1002/biot.201400704>

#### Review

##### **Current progress of targetron technology: Development, improvement and application in metabolic engineering**

Ya-Jun Liu, Jie Zhang, Gu-Zhen Cui, Qiu Cui

<http://dx.doi.org/10.1002/biot.201400716>

#### Review

##### **Steam explosion and its combinatorial pretreatment refining technology of plant biomass to bio-based products**

Hong-Zhang Chen and Zhi-Hua Liu

<http://dx.doi.org/10.1002/biot.201400705>

#### Review

##### **Improving polyglucan production in cyanobacteria and microalgae via cultivation design and metabolic engineering**

Shimpei Aikawa, Shih-Hsin Ho, Akihito Nakanishi, Jo-Shu Chang, Tomohisa Hasunuma and Akihiko Kondo

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#### Rapid Communication

##### **Linker length and flexibility induces new cellobiohydrolase activity of PoCel6A from *Penicillium oxalicum***

Le Gao, Lushan Wang, Xukai Jiang and Yinbo Qu

<http://dx.doi.org/10.1002/biot.201400734>

#### Research Article

##### **Improving protein production of indigenous microalga *Chlorella vulgaris* FSP-E by photobioreactor design and cultivation strategies**

Chun-Yen Chen, Po-Jen Lee, Chung Hong Tan, Yung-Chung Lo, Chieh-Chen Huang, Pau Loke Show, Chih-Hung Lin and Jo-Shu Chang

<http://dx.doi.org/10.1002/biot.201400594>

#### Research Article

##### **Bisulfite pretreatment changes the structure and properties of oil palm empty fruit bunch to improve enzymatic hydrolysis and bioethanol production**

Liping Tan, Wan Sun, Xuezhi Li, Jian Zhao, Yinbo Qu, Yuen May Choo and Soh Kheang Loh

<http://dx.doi.org/10.1002/biot.201400733>