

Complete Cycle from Feedstock *Miscanthus Giganteus* to Target Product Bacterial Nanocellulose

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Abstract. Valuable bacterial nanocellulose (BNC) was produced herein from the abundant, cheap, lignocellulosic feedstock *Miscanthus giganteus* of the variety Kamis from the Russian selection. Four methods using dilute solutions of nitric acid and sodium hydroxide were examined for pretreating *M. giganteus* for the first time. Further, the stages involving enzymatic hydrolysis of the resultant pulps and biosynthesis of BNC using symbiotic culture Kombucha were carried out under the same conditions. Through comparing the BNC yield using four pretreatment methods on the conversion of *M. giganteus*, and one-step nitric acid pretreatment was the best method. And the yield of BNC was 1.7 - 1.9 times higher than other pretreatment methods. Unlike the majority of similar works, the present study performed a complete conversion cycle from the *Miscanthus* into BNC and made a full calculation of the yield of intermediates and the target product.

Keywords: *Miscanthus giganteus*, pretreatment, bacterial nanocellulose, enzymatic hydrolysis, Kombucha, yield.

1. Introduction

Bacterial nanocellulose (BNC) is a unique microbial polymer, which, thanks to its nanoscale dimension and chemical purity, possesses properties distinct from any plant-based cellulose and has a huge range of applications. Due to the metabolic peculiarities of BNC-producing microorganisms, the BNC yield is not high and its production cost is significant; therefore, one of the ways to reduce the production cost is by using cheap raw materials. Among the significant directions towards reducing the cost of raw materials is the concept of the conversion of cheap cellulosic raw materials into high-value BNC, and this concept is widely developing across the world [1-3].

This direction is of particular significance for northern countries where the growing season is very short and it is impossible to gather a harvest of sugary feedstocks within this time span; the more so, even harvesting starchy feedstocks is questioned.[4] Under the said conditions and given the global agenda, the competition for food raw materials between people and industries is unacceptable. Therefore, the concept of the conversion of cheap cellulosic feedstocks into high-value BNC has significance not only for circular economy [1-3, 5], but also has critical social significance.

An important aspect is the adequate choice of a cellulosic feedstock. *Miscanthus giganteus* is an energy crop that holds one of the world's leading positions among cellulosic raw materials. Because of its high growth rate, cellulose content superior to that of wood (50-55% vs. 35-50%) and low-cost cultivation, the scale of *Miscanthus* plantings is increasing annually worldwide.

Miscanthus is processed into paper, cardboard, bio-concrete, cellulose chemical derivatives, and microbiological synthesis products [6-8].

Like any other type of cellulosic feedstock, Miscanthus is composed of cellulose, hemicelluloses, lignin, fatty fraction and mineral constituents that are all tightly bound to each other to form a composite matrix. According to the world experts' unanimous opinion, pretreatment of cellulosic feedstocks is a key stage that determines the success of the subsequent stages of enzymatic hydrolysis and microbial biosynthesis of any biotechnology product [9-11].

The present study is the first to calculate the yield of intermediates and the target product with the use of four chemical pretreatment methods for Miscanthus and evaluate the efficiency of the complete cycle of BNC biosynthesis from Miscanthus giganteus starting from the feedstock through to the target product.

2. Materials and Methods

Miscanthus giganteus var. Kamis from the Russian selection was raised in the Marushkino village, the Moscow region in 2021, harvested in February 2022, and kindly provided by Master Brand LLC (Moscow). Symbiotic Medusomyces gisevii Sa-12 was acquired from the Scientific Center 'Kurchatov Institute' – Research Institute for Genetics and Selection of Industrial Microorganisms, Russia.

Herein, we examined four Miscanthus pretreatment methods using dilute 4 wt.% solutions of HNO₃ and NaOH at atmospheric pressure, and the processes were carried out in laboratory setting. The procedures are described in detail in [12, 13]. These procedures are authors' proprietary methods, which have been reproduced under laboratory and pilot conditions multiple times using feedstocks such as oat hulls and Miscanthus sacchariflorus var. Soranovskii. The success of the Miscanthus giganteus conversion had been achieved only after a thorough sample handling that involved the five-fold grinding on a KR-02 fodder grinder (Miass city, Russia) to a size of at most 12 mm, with 50% of the feedstock ground to a size of at most 4 mm. The chemical composition of the feedstock and pulps was quantified by the standard wet methods and expressed on a dry matter basis; the methods are set forth in [12].

The resultant pulps were subjected to enzymatic hydrolysis with cellulolytic enzymes in line with the procedure reported in [12]. The peculiar feature of the present study is the use of a 0.05 M acetate buffer, as we have discovered that this concentration is not inhibitory to the BNC biosynthesis.

The derived enzymatic hydrolyzates were standardized against reducing sugars (RS) and black tea extractives, and then used as a nutrient medium for the biosynthesis of BNC using symbiotic culture Medusomyces gisevii Sa-12, also known as Kombucha, under static conditions; the procedure details are set forth in [13]. After being washed, the BNC was freeze-dried in a HR7000-M freeze-drier (Harvest Right LLC, USA).

This study was performed using the equipment provided by the Biysk Regional Center for Shared Use of Scientific Equipment of the Siberian Branch of the Russian Academy of Sciences (IPCET SB RAS, Biysk city, Russia). The experimental results were obtained in triplicate and statistically processed by standard methods using Microsoft Office Excel 2019. The yields of intermediates and the product (BNC) were calculated on an oven-dry basis.

3. Results and Discussion

3.1 Chemical pretreatment of *Miscanthus giganteus*

The chemical compositions of *Miscanthus giganteus* and its pulps are illustrated in Figure 1.

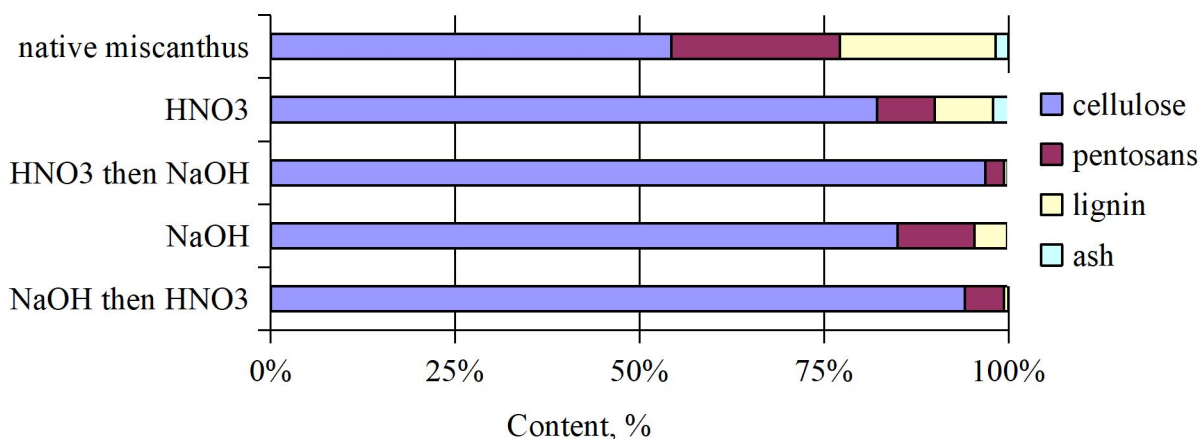


Fig. 1 Component composition of miscanthus and its pulps

The nitric-acid pretreatment led to a 1.7-fold increase in the cellulose content relative to the feedstock (87.3 % vs. 50.2 %) and to a decrease in the content of the non-cellulosics: pentosans down by 2.5 times (8.3 % vs. 21.2 %), lignin down by 2.3 times (8.4 % vs. 19.5 %), while the proportion of the mineral constituent increased from 1.63 % to 2.21 %, which is a peculiar feature of the nitric-acid treatment [11, 13].

The pretreatment with nitric acid at the first stage and with sodium hydroxide at the second stage furnished a chemically pure pulp—namely, the cellulose content showed a 1.9-fold increase compared to the feedstock (95.7 % vs. 50.2 %), the pentosan content showed a 8.4-fold decline (2.5 % vs. 21.2 %), the lignin content showed a 39-fold decrease (0.5 % vs. 19.5 %), and the ash content dropped by 14.8 times (0.11 % vs. 1.63 %). This is a very efficient method for cellulose extraction.

The NaOH pretreatment was employed as a referee method in this work, since it is thought by the global community to be a classical pretreatment method for non-woody cellulosic raw materials [8, 9]. Match the results of the alkaline delignification against those of the nitric-acid treatment. The alkaline delignification is less effective at cellulose extraction than the nitric-acid pretreatment because the cellulose content is increased 1.6-fold by the alkaline treatment and 1.7-fold by the nitric-acid treatment. Pentosans are also less effectively removed: their content is decreased 2.1-fold by the alkaline delignification but 2.3-fold by the nitric-acid treatment. It is reasonable to expect that the alkaline delignification would result in a more effective reduction in the pentosan content by 4.5 times, while the nitric-acid treatment only by 2.3 times. The alkaline delignification reduces the content of ash components by 7 times, while the nitric-acid treatment by 1.3 times.

The pretreatment with NaOH at the first stage and with HNO₃ at the second stage afforded a pulp with a low ash content and low lignin content, while yet preserving a 5.2 % residual pentosan content. This cannot be considered a drawback if this pulp is intended for hydrolysis into reducing sugars (RS).

The chemical composition of the *M. giganteus* pulps obtained in this study is very close to that of *Miscanthus sacchariflorus* pulps obtained in [12]. The reproducibility of the results evidences the stability of the pretreatment methods employed for non-woody cellulosic raw materials and validates their efficiency.

3.2 Enzymatic hydrolysis of pulps subject to *Miscanthus giganteus* pretreatment method

Table 1 summarizes the enzymatic hydrolysis results for four pulps as compared to the untreated native feedstock. All the pretreatments appeared to be extremely effective for *M. giganteus*, as they increased the hydrolyzable substrate components (the sum of cellulose and hemicellulose) by 25-27 times compared to the untreated feedstock, that is, the reducing sugar (RS) yield was 63.0 % to 68.4 % for pulps versus 2.5 % for untreated *Miscanthus*. In the literature, pretreatment is considered successful if it leads to a 5-fold or higher increase in the reactivity of a pulp compared to a feedstock. This way, the outcomes of the pretreatments used herein that led to a 25-27-fold increase in the reactivity to enzymatic hydrolysis can be reckoned outstanding.

Table 1. Enzymatic hydrolysis of *Miscanthus giganteus* pulps

Miscanthus pretreatment method	Native <i>Miscanthus</i>	HNO ₃	HNO ₃ , then NaOH	NaOH	NaOH, then HNO ₃
RS concentration in hydrolyzate, g/L	0.6±0.1	21.8±0.2	21.0±0.2	18.5±0.2	21.0±0.2
RS yield based on the sum of cellulose and hemicellulose contents in substrate, %	2.5±0.2	68.4±0.3	64.2±0.3	63.0±0.3	68.3±0.3
Xylose concentration in hydrolyzate, g/L	0.0	2.0±0.1	0.5±0.1	1.8±0.1	1.3±0.1
Xylose yield on a pentosan content basis in substrate, %	0.0	21.7±0.2	18.0±0.2	31.1±0.2	22.5±0.2
Xylose proportion in total reducing sugars, %	0.0	9.2±0.2	2.4±0.2	9.7±0.2	6.2±0.2

However, in the pursuit of scientific objectivity, regard must be paid to the extreme recalcitrance of native *Miscanthus giganteus* to enzymatic hydrolysis: the RS yield was only 2.5 % of the total hydrolyzables, which is 4.4 times lower than the digestibility of native *Miscanthus sacchariflorus*, for which the RS yield is 17 % [12]. This can be explained by the morphology features of *Miscanthus giganteus*. *Miscanthus giganteus* is a powerful, firm and strong plant whose height is 2 times that of *Miscanthus sacchariflorus* and stem thickness 2-3 times that of *Miscanthus sacchariflorus*. These morphology features also had an effect on the behavior of pulps when enzymatically hydrolyzed: despite the fact that the reactivity to enzymatic hydrolysis is greatly increased by the pretreatment, it is 13-25 % lower for *Miscanthus giganteus* pulps than for *Miscanthus sacchariflorus* pulps. This is a very important fact. The results obtained herein are in good agreement with the literature sources [15, 16] that note an exceptional recalcitrance of *Miscanthus* to enzymatic hydrolysis [15], and the behavior of natural rather than model substrates during enzymatic hydrolysis is explained, most notably, by the morphology of the substrate [16].

Besides the low performance of enzymatic hydrolysis in general, the *Miscanthus giganteus* pulps exhibited a low-efficiency hydrolysis of hemicelluloses. The xylose yield on a pentosan content basis in the pulps ranged from 22 % to 31 % (Table 1), which are very low values. That said, the proportion of xylose in the total reducing sugars is only 2.4-9.5 %; thus, the resultant hydrolyzates were chiefly glucosic.

3.3 Biosynthesis of BNC on nutrient media representing *Miscanthus giganteus* enzymatic hydrolyzates

The calculation of the yield of intermediates and BNC from *Miscanthus giganteus* is outlined in Table 2. This is an engineering calculation; therefore, the RS yield for the enzymatic hydrolysis stage is expressed on a substrate weight basis rather than on a total hydrolyzables basis in order to objectively assess what the product yield in real production will be. Also, the BNC yield is given on

a total reducing sugar basis rather than on a consumed reducing sugar basis in order to prevent a mathematical overestimation of the yield. The contemporary researchers investigating the BNC biosynthesis often mathematically overestimate the yield [17, 18], as opposed to the classical works that adhere to real calculations [19, 20].

The analysis of Table 2 shows that the most significant contribution is made by the yield of pulps on a *Miscanthus* weight basis. This is a key technology stage that has the greatest impact on the overall yield of BNC. The enzymatic hydrolysis stage gave approximately equal yields of reducing sugars on a substrate weight basis, except only for the pulp obtained by the NaOH treatment. The BNC biosynthesis stage also provided approximately equal yields of BNC on a reducing sugar basis, with the control medium yielding 11.8%. Only the cumulative analysis helped to identify the leader. It is obvious that the most effective was the nitric-acid pretreatment that yielded 3.78% of absolutely dry BNC on a *Miscanthus* weight basis, which is 1.7-1.9 times higher than that for the other pretreatments.

Table 2. Yield calculation for intermediates and BNC from *Miscanthus giganteus*

Miscanthus pretreatment method	HNO ₃	HNO ₃ , then NaOH	NaOH	NaOH, then HNO ₃
Pulp yield on a <i>Miscanthus</i> weight basis	49±2	32±2	42±2	38±2
RS yield on a substrate weight basis, %	66.0±0.3	63.6±0.3	56.1±0.3	63.3±0.3
BNC yield on a RS basis,%	11.7±0.1	10.7±0.1	8.3±0.1	9.4±0.1
BNC yield on a <i>Miscanthus</i> weight basis, %	3.78±0.05	2.18±0.05	1.96±0.05	2.26±0.05

In the study [21], *Miscanthus* (the species not specified) was hydrothermally treated in the presence of sulfuric acid, and the resultant pulp was subjected to enzymatic hydrolysis with Celic CTec2 enzyme complex (Novozymes). The hydrolyzate was standardized against reducing sugars (50 g/L). Nutrient salts and vitamins were also added. Further, BNC was synthesized using *Gluconacetobacter xylinus* ATCC 53524, with a yield of 16.7 g/L. Thus, the biosynthesis stage was carried out with a high yield, but unfortunately, no material balance for BNC production from *Miscanthus* was reported in that study; therefore, it proves impossible to compare the yields.

4. Conclusions

Herein, we demonstrated a conceptual feasibility of biosynthesizing BNC from an available cellulosic feedstock, the biomass of *Miscanthus giganteus* from the Russian selection. The *Miscanthus* conversion involved three stages. The first stage was the chemical pretreatment with dilute HNO₃ and NaOH solutions. The second stage was the enzymatic hydrolysis of the resultant pulps, and the third stage was the biosynthesis of BNC using symbiotic Kombucha. The second and the third stages were identical for all pretreatment options. The native *Miscanthus* was found to be recalcitrant to enzymatic hydrolysis. The chemical pretreatment with dilute HNO₃ and NaOH solutions was discovered to be extremely efficient, and allowed the reactivity towards enzymatic hydrolysis to be enhanced 25-27-fold compared to the untreated *Miscanthus*. The nitric-acid pretreatment of *Miscanthus giganteus* was the most efficient, with the BNC yield being 3.38 % on a *Miscanthus* weight basis. The merit of the present study is that the complete cycle of the *Miscanthus* conversion into BNC was implemented and the full calculation of the yield of intermediates and the target product on a feedstock weight basis was made.

As an energy crop, *Miscanthus* has been widely recognized for its high yield, low price and rich cellulose, and is a crop with great application potential. Compared with other studies on the production of BNC from *Miscanthus*, the yield of BNC in this study is 1.6-1.8 times higher, indicating that one-step nitric acid method is more suitable for the pretreatment of *Miscanthus*. In

this study, four kinds of pre-treated intermediates were tested and compared in detail, which has practical significance for promoting cost-effective industrial production of BNC.

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