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Research Article

# Effect of Fermentation Parameters on Bioethanol Yield from *Miscanthus*

Kouame Fulbert OUSSOU[1], M. Ümit ÜNAL*[1], Aysun Şener GEDÜK[2]

**ABSTRACT**
Dilute sulphuric acid and alkaline pre-treatments (NaOH) followed by enzymatic hydrolysis (Cellic CTec2) were used to release sugars from *Miscanthus giganteus*. In order to determine optimum pre-treatment conditions pyrolysis was carried out using $H_2SO_4$ and NaOH at 0.5 and 1% (w/v) and 120°C and 180°C for 10 and 90 min. Pre-treatments with NaOH (0.5%, w/v), 120°C, 90 min resulted in highest total fermentable sugar concentration (32.78g/L). Ethanolic fermentations were performed at 25°C and 30°C with or without nitrogen source addition using *Saccharomyces cerevisiae*. Both temperature and nitrogen supplementation affected bioethanol yields from *Miscanthus giganteus*. Higher bioethanol yields were obtained with nitrogen addition at temperatures. The fermentation at 30°C with nitrogen addition gave the highest bioethanol yield.
**Keywords:** Nitrogen, temperature, bioethanol, *Miscanthus*, fermentation optimization, *Saccharomyces cerevisiae*

## Fermantasyon Parametrelerinin Miscanthus'tan Elde Edilen Biyoetanol Verimine Etkisi

**ÖZ**
*Miscanthus giganteus'*tan şekerlerin serbest hale getirilmesi için seyreltik asit ve alkali (NaOH) ön muamelelerini takiben enzimatik hidroliz uygulanmıştır. Optimum ön muamele koşullarını belirleyebilmek için % 0.5 ve 1.0 (a/h) $H_2SO_4$ ve NaOH konsantrasyonlarında, 120°C ve 180°C'lerde, 10 ve 90 dk süreyle piroliz işlemleri gerçekleştirilmiştir. 0.5% NaOH konsantrasyonu, 120°C ve 90 dk sürede gerçekleştirilen piroliz sonucu en yüksek fermente edilebilir şeker (32.78 g/L) elde edilmiştir. Etanol fermantasyonu 25°C ve 30°C'lerde *Saccharomyces cerevisiae* ile azot kaynağı ilaveli ve ilavesiz yürütülmüştür. Hem sıcaklık hem de azot kaynağı ilavesi *Miscanthus giganteus*'tan elde edilen etanol verimini etkilemiştir. Her iki sıcaklıkta da azot ilavesinin etanol verimini artırdığı bulunmuştur. En yüksek etanol verimi 30°C'de azot ilavesi ile gerçekleştirilen fermantasyonda elde edilmiştir.

**Anahtar Kelimeler**; Azot, sıcaklık, biyoetanol, *Miscanthus*, fermantasyon optimizasyonu, *Saccharomyces cerevisiae*

ORCID ID (Yazar sırasına göre)
0000-0003-1663-4959, 0000-0002-2686-5286, 0000-0001-5306-6722

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[1]University of Cukurova, Faculty of Agriculture, Department of Food Engineering, Balcali Adana Turkey Sivas Cumhuriyet Üniversitesi, Yıldızeli Meslek Yüksekokulu, Gıda Teknolojisi, Sivas
[2]University of Adana Science and Technology, Faculty of Engineering and Department of Food Engineering, Saricam 01250 Adana, Turkey
*E-posta: muunal@cu.edu.tr

# Effect of Fermentation Parameters on Bioethanol Yield from *Miscanthus*

## Introduction

Due to increasing global energy demand, worldwide population growth, industrialization, urbanization and concerns about decreasing fossil fuels and environmental impact biofuels have become a promising alternative to fossil fuels (Uihlein and Schebek, 2009; Bajpai, 2013; Jambo et al., 2016). The global bioethanol production in 2018 was 110 x $10^9$ L, which is expected to reach 140 x $10^9$ L in 2022 (Sharma et al., 2020). Numerous agricultural crops and residues and waste biomass can be utilized in bioethanol production. United State and Brazil are the leaders in bioethanol production using corn and sugarcane, respectively (Zhang et al., 2011; Raghavi et al., 2016). Bioethanol is a promising renewable energy with low environmental impact. Use of bioethanol instead of fossil fuels can alleviate some of the environmental problems (Galbe and Zacchi, 2012; Domínguez-Bocanegra et al., 2015).

Of the raw materials which can be used in bioethanol production lignocellulosic biomass is the most abundant biomass on earth. It is made up of cellulose, hemicellulose and lignin (Hahn-Hägerda et al., 2006; Han et al., 2011). However, its recalcitrant nature is the main bottleneck to overcome in the enzymatic hydrolysis of lignocellulosic biomass, necessitating the pre-treatment prior to fermentation to make it more accessible to cellulolytic enzymes (Zoghlami and Paës, 2019). There are numerous pre-treatment techniques available, of which pre-treatment with NaOH and $H_2SO_4$ have been widely investigated (Nashiruddin et al., 2020).

Distinctive features of *Miscanthus giganteus*, such as high yield, high cellulose content and low input requirements as well as the possibility to harvest twice a year make it an ideal energy crop for bioethanol production. *Miscanthus giganteus* has a low moisture content and does not require irrigation systems and soils for its production since it grows in swampy soils in most tropical countries and its production per hectare has been estimated at 20-25 tons based on dry matter. Last but not least it does not compete with food or feed production (Brosse et al., 2009; Dubis et al., 2017; Alam et al., 2020). Despite its potential for biofuel production, swamps with large quantities of *Miscanthus giganteus* in West Africa, and particularly in the Ivory Coast, are burnt during the rice-growing season. Although use of Miscanthus as a feedstock for bioethanol production has been the subject of numerous studies, the effect of fermentation temperature and nitrogen supplementation on bioethanol yield is scarce. In the present study, we studied the optimization of pre-treatment conditions of *Miscanthus giganteus* followed by enzymatic hydrolysis as well as bioethanol yield as affected by fermentation parameters (temperature and nitrogen addition).

## Materials and Methods

### Materials and equipment

Miscanthus was obtained in Ivory Coast from swamps. Cellic® CTec2 enzyme was kindly provided by Novozymes. The reagents used in the present study were purchased from Sigma (Sigma chemical company, MO, USA) and Merck (Germany). *S. cerevisiae* Lalvin (Lallemand, Canada) was used in ethanolic fermentation.

Pre-treatments were carried out in a reactor with temperature control (Parr 4590, USA). Ethanolic fermentations were conducted in Thermo scientific MAXQ 500 and Memmert UNB 400 (Germany) incubator. Sugar and fermentation products were determined using an HPLC with a refractive index detector (Shimadzu Prominence I-series LC 2030, Japan). Aminex HPX-87H (300 x 7.8 mm) column was purchased from Bio-Rad (USA). Calibration curves for glucose, sucrose, fructose and ethanol were prepared using standard solutions at different concentrations. The mobile phase contained 5 mM $H_2SO_4$ (HPLC grade) and ultra-pure water solution. The separation was performed using isocratic elution at a flow rate of 0.5 mL/min. The eluent was vacuum filtered through a 0.45-µm membrane and degassed by sonication. The column temperature was maintained at 30 °C and the elution was monitored by RI (Refractive index) detector. All samples were filtered through 0.22-µm membrane filters before injection. A sample of 20 µL was injected into the HPLC. Two replications of each sample were performed and chromatogram data were

analyzed using the LC solution software package (Ünal et al., 2020).

**Feedstock preparation**

Miscanthus feedstock which was dried at 50°C for 4 days was homogenized using Waring blender into fine particles and sifted with a sieve of 0.4 mm diameter. The powder was transported in a moisture-proof plastic bag to Çukurova University in Turkey.

**Pyrolysis**

Samples were pre-treated at 120 and 180°C for 10 and 90 min and using $H_2SO_2$ and NaOH at 0.5 and 1% in Parr reactor. The amount of sample was 10 g of feedstock in 200 mL sulphuric acid or NaOH solution. After pyrolysis, the samples were placed in airtight jars and stored at -20°C until fermentation trials.

**Enzymatic hydrolysis**

Cellulose contents of the pre-treated samples of Miscanthus were hydrolysed using 0.09 mL Cellic® CTec2 enzymes per gram sample at pH 5.0 in Erlenmeyer flasks. Chloramphenicol (0.5g/l) was added to the mixture in order to prevent any microbial growth. Enzymatic hydrolysis was carried out in an incubator equipped with a shaker stirred at 150 rpm at 50°C for 3 days. The sample with the highest fermentable sugar content was used in ethanolic fermentation.

**Preparation of inoculum**

The composition of the culture medium was given elsewhere (Ünal et al., 2020). The culture was incubated at 30°C in a shaker at 150 rpm for 24 hours. 10% of the culture broth containing approximately 6.0 x 10 7 cell/mL was centrifuged at 4000 rpm for 10 min at 4°C. After carefully decanting the supernatant *S. cerevisiae* cells were mixed with sterile 50 mL 0.1% peptone solution and used as inoculum (Cheng et al., 2007; Laopaiboon et al., 2009).

**Fermentation process and analytical method**

The ethanolic fermentation was carried out in in a 250 mL shake flask containing 90 mL Miscanthus hydrolysate and 10 mL inoculum. The experiments were conducted in duplicate at two temperatures (25°C and 30°C) with or without nitrogen addition in an incubator at 150 rpm. Samples were taken in vials every 24 hours for sugar and ethanol analysis. Sugar utilization and ethanol formation were monitored by HPLC analysis as described above.

**Statistical analysis**

Represented data were expressed as mean of three replicates, and independent t-test analysis was used to test the significant effect between two different factors during fermentation. Plackett-Burman design was performed to test the effect of some pretreatment factors on total fermentable sugar. All the data analysis was performed using two software programs of statistics (SPSS, version 10.0 for Windows, SPSS Inc., Chicago, USA and Design of experiment version 12.0.3.0 for Windows).

**Results and Discussion**

**Pyrolysis and Hydrolysis of miscanthus**

The miscanthus samples were pre-treated to reduce the recalcitrance of lignocellulosic biomass and make it more accessible to cellulolytic enzymes. Pre-treatments were conducted at 120°C and 180°C for 10 and 90 min using NaOH and $H_2SO_4$ at 0.5% and 1.0% (w/v). Cellulolytic (Cellic CTec2) enzymes were used to hydrolyse and to saccharify the complex polysaccharides in the pre-treated miscanthus samples. The efficiency of pre-treatments depends on the formation of fermentable sugar concentration (glucose, fructose and sucrose) after enzymatic hydrolysis (Cha et al., 2015). The highest total fermentable sugar with 32.78 g/L was obtained at 120°C for 90 minutes using NaOH at 0.5 % (w/v), followed by the pre-treatment with 1.0 % (w/v) $H_2SO_4$ at 120°C for 10 min that yielded 26.03 g/L total fermentable sugar.

Nlewem and Thrash (2010) compared three pre-treatments (NaOH, dilute $H_2SO_4$ and hot water) in terms of glucose yield from switchgrass. They reported that 0.5% NaOH gave a higher glucose yield compared with $H_2SO_4$ dilute and hot water treatment. Nashiruddin et al. (2020) conducted a research to determine the effect of pyrolysis parameters (NaOH (0.5%, 80°C, 60 min), $H_2SO_4$ (0.5 %, 80°C, 60 min) and hot water (100°C for 90 min) on the formation of reducing sugar from

pineapple leaves fiber. The authors reported that treatment with NaOH yielded the highest reducing sugar.

The temperature, duration of pre-treatment, reagent type and its concentration had a pronounced effect on the yield of total fermentable sugars in the pre-treatment of the feedstock (Table 1). These four variables associated with reducing sugars concentration released in enzymatic hydrolysis were initially screened for significant effect while ignoring other nonsignificant effects. Considering the linear regression model built, a P-value of 0.025 was observed. Moreover, the linear regression coefficient ($R^2$) is found to be 0.908, meaning that 90.8% of the variation could be explained by the model. All the factors showed significant effects on reducing sugars concentration at $P<0.05$.

Table 1. Statistical analysis of pre-treatment factors on sugars yields

| Variable | Coefficient | F-value | P-value |
|---|---|---|---|
| Model | - | 2.804 | 0.025 |
| Constant | 11.825 | - | 0.008 |
| Temperature | -2.536 | 3.468 | 0.036 |
| Time | 2.407 | 2.626 | 0.019 |
| Reagent concentration | 3.104 | 0.564 | 0.023 |
| Reagent type | 1.695 | 9.763 | 0.009 |

$R^2$=0.908; Adj $R^2$=0.884; Pred $R^2$=0.498

Time, reagent type and its concentration exhibited a positive effect on sugar yield while temperature showed a negative effect on fermentable sugar yield which is attributed to the conversion and condensation of reducing sugars degradation into furans compounds during pre-treatment at high temperature (Hu and Ragauskas, 2014). Bio-products such as furfural and 5-hydroxymethylfurfural from pyrolysis have a considerable effect on enzymatic hydrolysis and consequently an influence on sugars yields (Li et al., 2010).

**Bioethanol yield as affected by fermentation temperature and nitrogen supplementation**

Like other organisms, yeasts need sufficient nutrients to grow and multiply during fermentation (Valdes et al., 2011). Nitrogen is an important element required for the synthesis of DNA, RNA, and proteins in cells. Nitrogen deficiency can cause stuck and sluggish fermentations. During fermentation, assimilable nitrogenous compounds not only affect yeast growth but also ethanol yield and fermentation rate and duration (Valdes et al., 2011; Bely et al., 2003; Gutiérrez et al., 2012; Zhaofen et al., 2017). Fermentation temperature is another important parameter that affects yeast growth, fermentation rate and bioethanol yield (Zabed et al., 2014). Extreme temperatures significantly affect yeast growth and cause a drop in bioethanol yield. The optimum temperature of *S. cerevisiae* is between 25-35°C (Alvira et al., 2010; Zhao et al., 2011; Moon et al., 2012).

Bioethanol yield ($Y_{P/S}$) is expressed as g ethanol produced per g total fermentable sugar (glucose, fructose and sucrose) consumed. The mean bioethanol yields ranged between 0.301-0.473 (Table 2). Nitrogen supplementation and temperature affected the bioethanol yields obtained. Higher bioethanol yields were obtained with nitrogen addition at both 25°C and 30°C. The fermentation at 30°C with nitrogen source supplementation gave the highest bioethanol yield (0.473), being 92.75% of the theoretical bioethanol yield (0.510). Moreover, higher bioethanol yields were observed at 30°C compared to 25°C. At both temperatures (25°C and 30°C) bioethanol yields showed significant differences ($p< 0.05$) between the control (no nitrogen supplementation) experiment and the experiments with nitrogen supplementation.

Table 2. Bioethanol yields from Miscanthus as affected by fermentation temperature and nitrogen supplementation

| 25°C | | 30°C | |
|---|---|---|---|
| Control | Nitrogen supplementation | Control | Nitrogen supplementation |
| $0.301 \pm 0.025^{cD}$ | $0.386 \pm 0.035^{bB}$ | $0.460 \pm 0.014^{aC}$ | $0.473 \pm 0.098^{aA}$ |

*****Values are means with standard deviations (±SD). Small letters show supplementation with (+) and without (-) nitrogen within the same fermentation temperature and capital letters in the same line show temperature effects. Small letters (addition with or without nitrogen) and capital letters (fermentation temperature) indicate statistical difference at $p<0.05$.

Sturgeon et al. (2013) who investigated the effect of nitrogen supplementation on ethanol fermentation by *S. cerevisiae* reported that nitrogen supplementation resulted in faster fermentation. Schwarz et al. (2020) who investigated wine production from honey by *S. cerevisiae* reported that nitrogen and mineral addition had a great impact on fermentation rate and ethanol yield. Tan et al. (2019) conducted a research on the effects of nitrogen supplementation and pH on bioethanol production from banana frond juice by *S. cerevisiae*. The authors reported that a higher ethanol yield was observed by the addition of yeast extract as a nitrogen source at optimum pH Reddy et al. (2020) investigated the effect of temperature on ethanol production from molasses by *S. cerevisiae*. They observed that ethanol production increased with an increase in temperature from 25-35°C, thereafter it decreased. Sivamani et al. (2015) reported similar results, in that ethanol concentration increased with increasing temperature from 27°C to 37°C, and thereafter the ethanol concentration decreased. Ünal et al. (2020) obtained a bioethanol yield of 0.502 from muskmelon juice at 30°C with nitrogen supplementation.

**Sugar consumption and ethanol production**

Sugar (expressed as total fermentable sugar, TFS) utilization and ethanol formation during ethanolic fermentation are shown in Figure 1 and b. Sugar utilization was faster in the trials without nitrogen addition. However, almost all sugar was depleted in 48 hours in all fermentations. Ethanol concentration reached the maximum in 48 hours. Of the fermentable sugars, *S. cerevisiae* utilized first glucose, whereas only a fraction of fructose was used during the entire fermentation (data not shown), meaning that high residual fructose concentration lowered bioethanol yield.

Berthels et al. (2004) studied effect of addition of ethanol and nitrogen on sugar (glucose and fructose) utilization during ethanolic fermentation by 17 *S. cerevisiae* strains. Results showed that all strains preferred glucose to varying degrees. Ethanol addition inhibited fructose utilization more than glucose utilization while nitrogen addition stimulated fructose utilization more than glucose utilization.

Zinnai et al. (2013) stated that glucose utilization by *S. cerevisiae* is faster than fructose utilization, which is influenced by temperature and composition of fermentation media. It was found by the same authors that an increase in ethanol concentration during fermentation decreased fructose utilization.

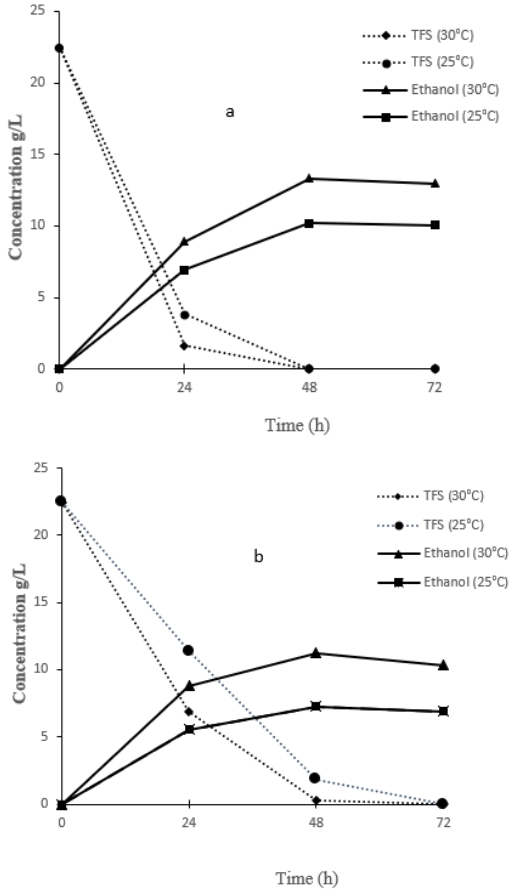


Figure 1. Sugar utilization and ethanol formation with nitrogen (a) without nitrogen (b) during the fermentation of miscanthus hydrolysate. TFS stands for total fermentable sugar

## Conclusions

In this study, Miscanthus (*Miscanthus giganteus*) feedstock was investigated for its potential utilization for bioethanol production. Alkaline and dilute acid pre-treatments followed by enzymatic hydrolysis were studied to compare sugar release. It was found that the highest total fermentable sugar was obtained with NaOH (0.5%) at 120°C for 90 min. The hydrolysate was fermented using *S. cerevisiae* at two different temperatures (25°C and 30°C) with or without nitrogen addition. Nitrogen supplementation and temperature affected the bioethanol yields obtained. Bioethanol yields at both temperatures were significantly different ($p<0.05$) between the control (no nitrogen addition) experiment and the experiments without nitrogen supplementation. As a result, the use of miscanthus biomass for bioethanol production could be a good alternative to first generation feeds without causing food vs feed dilemma and endangering food security, and also alleviate human effect on climate change by producing clean and renewable energy.

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