SUNLIBB

Sustainable Liquid Biofuels from Biomass Biorefining

Grant Agreement no. 251132

Collaborative Project
EU 7th Framework Programme
ENERGY

Project duration: 1st October 2010 – 30th September 2014

Deliverable 7.5

"Pilot scale fermentation of sugars from maize and Miscanthus biomass optimised"

Authors: **Dr. Namdar Baghaei-Yazdi (Biotech Consultants Ltd., UK)**
Dr. Muhammad Javed (Biotech Consultants Ltd., UK)

Workpackage: **7**
Workpackage Leader: **Prof. Phillip Wright (University of Sheffield, UK)**

Due date: **September 2014 (Month 48)**
Actual date submitted: **October 2014**
Revised version submitted: **December 2014**

Dissemination Level: **PU**

This project has received funding from the European Union's Seventh Programme for research, technological development and demonstration under grant agreement number 251132

Thermophilic Ethanol Fermentation of Miscanthus and Maize Hydrolysates (D7.5)

Dr. Muhammad Javed

&

Dr. Namdar Baghaei-Yazdi

Biotech Consultants Limited

(BTCL)

(This report covers the work carried out from December 2013 to September 2014)

Table of Contents

Executive Summary 3

Characterisation of Received Hydrolysate samples 4

Thermophilic Fermentation of Hydrolysate Samples to Ethanol 6

Preparation of hydrolysate media for shake tube studies 6
Checking the hydrolysate media for thermophilic contamination 6
Preparation of inoculum 6
Fermentation studies of the hydrolysate samples 6
Aerobic growth of BCT-26 in Hydrolysate Samples 7

Fermentation of Hydrolysate Samples with Pelleted Cells 8

Materials and Methods 9

TGP. medium 9
Ethanol Assay with Boehringer Mannheim Kit 10
Measurement of Reducing Sugars by DNS Reagent 11

Conclusions 12

Executive Summary

In the second batch, 16 hydrolysate samples were received from Processum (Sweden) and they were characterised. The samples contained suspended solids and their OD_{600} ranged from 7 to 16. The samples were centrifuged. The supernatants' pH was between 2.1 and 3.6. The wet weights of their pellets were generally between 32 and 50 g/l while their dry weight was 10-fold less, and ranged from 2.3 to 5.5 g/l; however, in one case a dry weight of 7.9 g/l was achieved. The total reducing sugars of the samples were measured and two distinctive kinds of groups were observed in this regard. The genotypes of both maize and Miscanthus with A and B type contained higher reducing sugars while those with genotype W contained lower reducing sugars. The sugar levels in the former genotype samples were generally above 15 g/l and a maximum of 23 g/l reducing sugar was measured in case of sample MIGeA3, while levels in the W genotype were below 3 g/l.

To study the growth in these hydrolysate samples, they were neutralised and buffered. Each sample was buffered with 3 buffer stocks, i.e., HEPES, Bis-Tris and PIPES. Each sample then contained 80% v/v hydrolysate and 20% v/v buffer stock. These samples were inoculated with 10% v/v inoculum which was prepared in a complex medium. The samples were grown semi-aerobically at 65°C in 15 ml Falcon tubes. Results showed that OD_{600}, in most cases, reached over 1. Sugars from both maize and Miscanthus samples were utilised and ethanol was produced. 60 to 90% of the reducing sugars were used and up to 9 g/l sugar was used under these non-optimised growth conditions, and ethanol above 3.3 g/l was produced under these conditions.

The hydrolysate cultures were also grown at 65°C in 15 ml Falcon tubes under aerobic conditions. There was no significant improvement in sugar utilisation under these conditions. However, there was a marginal increase in the sugar utilisation in a few cases and some decrease in other cases.

In the above growth studies, complex nutrients from the inoculum also went into the hydrolysate medium. To test if the strain could grow in the absence of any added nutrients, the inoculum was centrifuged and pellets were re-suspended in the respective hydrolysate before inoculation. The cultures were then grown anaerobically at 65°C. A more than two-fold decrease in sugar utilisation and ethanol production was observed in the absence of any added nutrients.

Furthermore, in most cases, especially in genotype W, ethanol production was higher than the reducing sugars measured in the samples, indicating that these samples contained other non-reducing sugars as well, such as cellobiose which BTCL strains use very easily and ferment to ethanol.

Characterisation of Received Hydrolysate samples

16 hydrolysate samples in volumes of up to 2 to 5 L (each in 2 to 3 plastic bottles) were received from Processum (Sweden). The samples were stored in a cold room. Some of the bottles containing the hydrolysate samples were found bloated and therefore the gas from these bottles had to be released. Most of the samples had pH of around 3 and samples' pH ranged from 2.1 to 3.6. These samples contained suspended solid particles. Their OD was measured and most samples showed an OD_{600} of around 10 but samples' ODs ranged from 7 to 16. The wet and dry weight of the solid contents of the samples was determined as mentioned below:

50 ml of each sample was transferred to Falcon tubes. The hydrolysate samples were centrifuged at 8000 rpm for 20 to 30 minutes at 5C. The supernatants were decanted and pellets were weighed for wet weight.

Each pellet was then dried at 65 °C for 48 hours and dry weight of the pellet was measured and their g/l weight was calculated.

The wet weights were found to be 10 fold higher than the dry weights. Most samples had solid contents with the wet weight between 40 and 50 g/l and dry weight between 4 and 5 g/l. However, 2 to 3 samples showed weights higher or lower than these values.

Total reducing sugars were determined with the DNS method (Dinitrosalicylic acid). Two different kinds of samples were observed, Miscanthus and maize samples which were labelled as Ge A or B (1 to 8 samples) contained higher reducing sugars; more than 10 g/l. The highest level of 23 g/l was noted for sample MI Ge A3. Samples labelled E W (9 to 16) contained lesser amount of reducing sugars, less than 3 g/l.

The results are shown in Table 1.

Table 1: Initial characterisation of hydrolysate samples received for fermentation studies

S. No.	Samples	OD_{600} (sample)	pH	Wet weight (g/l)	Dry weight (g/l)	Total reducing sugars (g/l)
1	MA Ge A1	12.2	3.4	43.94	4.33	14.6
2	MA Ge A3	16.4	2.5	48.64	4.47	5.2
3	MA Ge B1	10.0	3.4	34.10	3.27	3.3
4	MA Ge B2	13.2	2.7	40.12	4.6	15.4
5	MI Ge A1	10.4	3.6	33.44	4.07	18.4
6	MI Ge A3	12.7	2.9	45.27	5.47	23.7
7	MI Ge B1	10.8	3.3	42.27	3.87	17.8
8	MI Ge B3	16.0	3.0	64.41	7.93	21.7
9	MA E W1	9.4	2.1	36.40	3.20	2.3
10	MA E W3	13.4	2.8	50.65	3.93	2.9
11	MA nE W2	9.6	3.1	61.34	4.53	2.4
12	MA nE W3	10.2	2.9	42.94	3.13	2.0
13	MI E W1	9.5	2.4	45.89	3.07	1.1
14	MI E W2	7.4	2.5	32.73	2.47	1.1
15	MI nE W2	10.4	2.4	48.45	2.87	1.6
16	MI nE W3	6.7	2.6	26.01	2.33	1.3

OD_{600} (sample), OD of the sample as it was received. **OD_{600} (supernatant),** OD of the supernatant after centrifugation at 14000 rpm for 5 minutes. **Wet and Dry weight** recorded as g/l samples. **Total sugars** recorded as g/l samples (measured by DNS method).
Prefix **MA** represents Maize samples and **MI** represents Miscanthus samples.

Thermophilic Fermentation of Hydrolysate Samples to Ethanol

Preparation of hydrolysate media for shake tube studies

The hydrolysate samples were centrifuged in 50 ml Falcon tubes at 8000 rpm for 20 to 30 minutes at 5 °C. The supernatants were decanted into new Falcon tubes.

100 ml supernatant was neutralised with 0.5 ml of 4M NaOH.

The neutralised hydrolysate was then buffered by adding 3 buffers [Bis-Tris (1.0M, pH 7.5), PIPES (0.5M, pH 7.5) and HEPES (0.5M, pH 7.5)].

To 80 ml hydrolysate, 8 ml PIPES, 8 ml of HEPES and 4 ml of Bis-Tris were added. Hence, each hydrolysate medium contained 80 % (v/v) hydrolysate supernatant and 20% (v/v) buffer.

Checking the hydrolysate media for thermophilic contamination

All neutralised-buffered hydrolysate samples were incubated at 65 °C for 24 hours. No growth was observed in any of the hydrolysate samples. This shows that these hydrolysate samples did not contain any thermophilic contaminants.

Preparation of inoculum

10 ml TGP medium in a 50 ml Falcon tube were inoculated with 50 µl of BT-26 frozen stock and incubated at 65°C for about 20 hours. The culture was then used to inoculate the hydrolysate samples.

Fermentation studies of the hydrolysate samples

The 3 – 4 ml neutralised-buffered hydrolysate samples added to 15 ml Falcon tubes were inoculated with 0.34 ml BCT-26 inoculum (grown in TGP medium) and grown semi-aerobically at 65ºC for 24 hours. The samples were analysed for OD_{600}, pH, sugar utilisation and product formation.

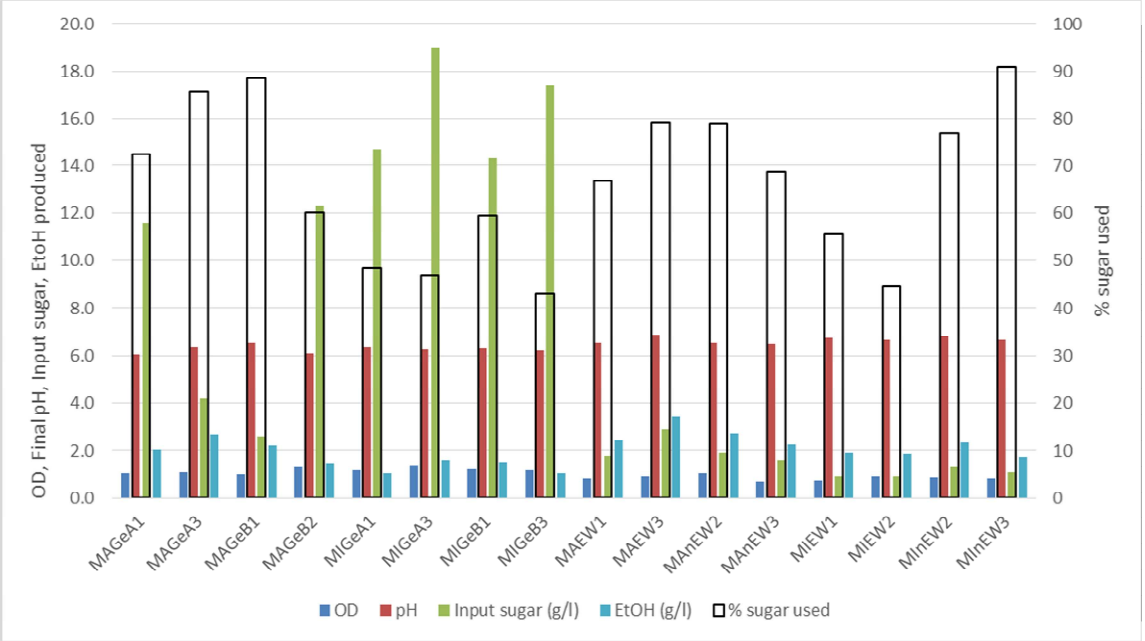

Figure 1: OD, final pH, ethanol and sugar levels after 24 hour fermentation of the hydrolysate samples by BCT-26 at 65C. OD was measured at 600nm, sugar represents total reducing sugar measured by DNS method, and ethanol was measured by a Boehringer Mannheim ethanol kit.

NB In some cases it appears that the concentration of ethanol produced is greater than the starting concentration of sugars (e.g MAEW1, MAEW3 etc). This point was queried at the SUNLIBB Final Review meeting in Brussels in November 2014. A possible explanation was that there was already some ethanol in the hydrolysates, that had accumulated during transport. Another explanation was that the sugar levels mentioned above only refer to the reducing sugars. Non-reducing sugars and di- and polysaccharides were not measured, but may have been present in the hydrolysates. These could have contributed to ethanol production, especially from cellobiose, as the *Geobacillus* strain used is capable of hydrolysing cellobiose into glucose and fermenting this into ethanol.

Aerobic growth of BCT-26 in Hydrolysate Samples

5 ml of neutralised-buffered hydrolysate samples in 15 ml Falcon tubes were inoculated with 0.3 ml thermophilic strain (prepared in TGP medium) and grown aerobically (by keeping the lid slightly loose) at 65 °C for 24 hours. The OD_{600} of the culture was measured and 1.5 ml of the culture was added to clean Eppendorf tubes and centrifuged at 14,000 rpm for 10 minutes. The supernatants were analysed for sugar utilisation and product formation. The cultures were incubated again for a further 24 hours and then analysed for sugar levels, OD_{600} and final pH values. The results are shown in Figure 2.

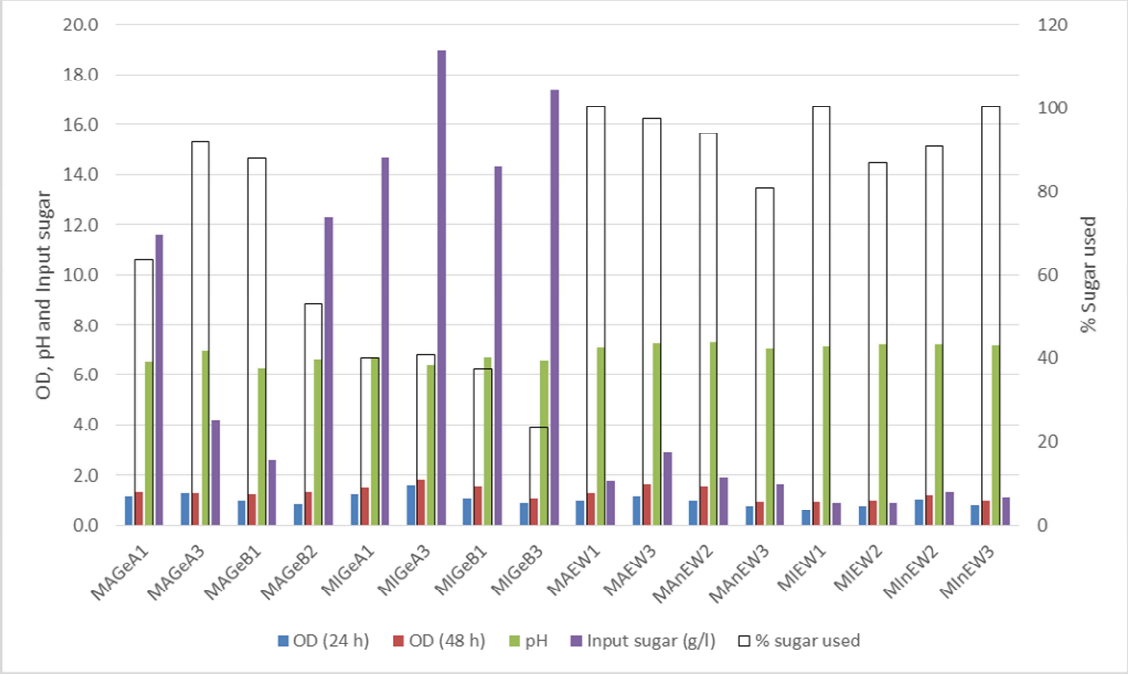

Figure 2: OD after 24 hour, and OD, final pH and sugar levels after 48 hours aerobic growth BCT-26 at 65ºC in the hydrolysate samples. OD was measured at 600nm and sugar represents total reducing sugar, which was measured by DNS method.

Fermentation of Hydrolysate Samples with Pelleted Cells

The neutralised-buffered hydrolysate samples were inoculated with thermophilic strain and grown semi-aerobically at 65ºC for 24 hours and the samples were analysed for OD, pH, sugar utilisation and product formation. The results are shown in Figure 3.

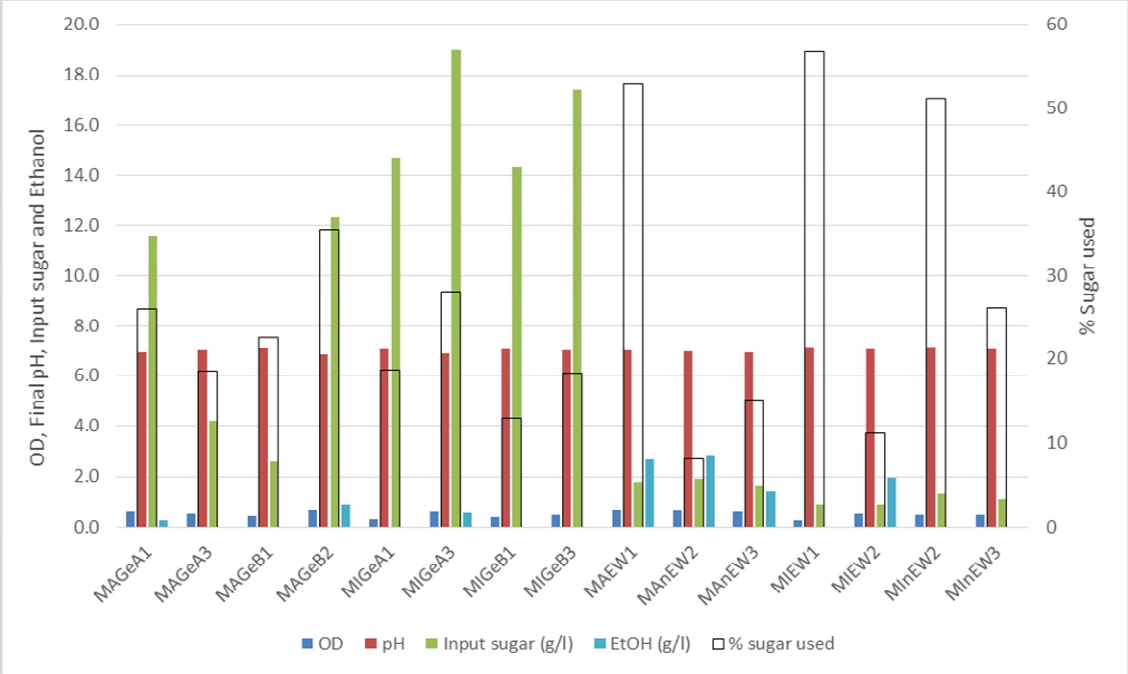

Figure 3: OD, final pH, ethanol and sugar levels after 24 hour fermentation of the hydrolysate samples by BCT-26 at 65ºC in medium without any added nutrients. OD was measured at 600nm, sugar represents total reducing sugar measured by DNS method, and ethanol was measured by a Boehringer Mannheim ethanol kit.

Materials and Methods

TGP. medium

Tryptone		17 g
Soya peptone		3 g
K_2HPO_4		2.5 g
NaCl		5 g
Pyruvate (Na salt)		4 g
Glycerol		4 ml
Deionised water	to	1000 ml

pH of the TGP medium adjusted to 7.2.

Sterilised by autoclaving at 121C for 15 minutes

Ethanol Assay with Boehringer Mannheim Kit

Before you start:

1) Read the instructions in the assay kit
2) Switch on the UV bulb on the spectrophotometer and allow to warm up (approx. 10min)
3) Set the spectrophotometer to 340nm
4) Blank the spectrophotometer with DIW (in a UV grade cuvette) – do not confuse this with the assay water blank which has ADH added and is part of the assay)
5) Cut strips of parafilm to cuvette size and remove the paper backing
6) Make up assay buffer by dissolving the buffer tablets in the buffer provided – each tablet makes 3ml buffer and each sample requires 1ml buffer (use the forceps provided in the kit when handling the buffer tablets)
7) Make appropriate dilutions of your samples – usually 1:10 will suffice but occasionally 1:100 is required
8) Use only UV grade cuvettes and for each batch of samples include a water blank and the 0.061g standard included in the kit (the maximum number of samples that can be assayed at any one time is 10 due to the time limitation (3min) of the first step)

Method:

Step 1 (3min):

1) Aliquot 1ml buffer into enough UV grade cuvettes to include blank, standard and 1 cuvette per sample
2) Start a timer and aliquot 33µl of water into the blank cuvette; seal with parafilm and shake
3) Aliquot 33µl of standard into the standard cuvette, seal and shake
4) Aliquot 33µl of each sample sequentially to the remaining cuvettes, sealing and shaking as before
5) When 3min are up read the absorbance of each cuvette at 340nm and record the results as A1 (ensuring that the spectrophotometer has been water blanked)

Step 2 (5min):

1) Re-start the timer and sequentially add 17µl of ADH (kit solution 3) to each cuvette – reseal and shake each one
2) Re-calibrate the spectrophotometer with the DIW blank
3) When 5min are up read the absorbance of each cuvette and record as A2

Results:

The results may be calculated using the ETOH Assay template or the following formula:

EtOH(g/l) in sample = $(0.7256/\epsilon) \times \Delta A$

Where :

$\epsilon = 340$

$\Delta A = (A2\text{-}A1)_{sample} - (A2\text{-}A1)_{blank}$

NB: If the samples have been diluted then the final figure (ΔA) should be multiplied by the dilution factor.

Notes:

1) Mix each sample well before addition to the buffer
2) Make sure each cuvette is properly sealed with parafilm

Measurement of Reducing Sugars by DNS Reagent

The standard curve prepared by this method showed a linear relationship between OD_{549} and glucose concentrations within the range of 0-2 g/L (see Figure 1). The following method was used to determine residual concentrations of glucose and other reducing sugars in the fermentation broth samples. The same method was used to determine the amount of reducing sugars in wheat crude hydrolysate (WCH), wheat solubles (WS), and beet pulp hydrolysate (BPH).

Preparation of DNS reagent

A - Boil 10g of dinitrosalicylic acid (DNS) in 200 ml of 2N NaOH until dissolved.
B - Dissolve 300g of sodium potassium tartarate in 500 ml of hot deionised water.
Mix A & B solutions and cool slightly and make up to 1L with deionised water.

Procedure

- Dilute the samples to sugar concentrations between 0 and 2 g/L. Also, use same sugar concentration range for standards.
- Add 400 µl of each sample in an Eppendorf tube and then add 400 µl of DNS reagent (Also, see note below).
- Place the Eppendorf tubes at 100°C [in heated blocks] for 5 minutes.
- Cool the tubes for 5 to 10 minutes and dilute each reaction mixture 10 folds in spec. cuvettes, by adding 100 µl of the mixture and 900 µl of deionised water.

- Take OD_{549} against the assay blank and calculate the sugar concentrations from the standard curve. (See Figure 4 below).

Note: With sample(s), also include one assay blank by using 400 µl of deionised water and minimum 3 standards, e.g; glucose standards: 2.0 g/L, 1.0 g/L and 0.5 g/L.

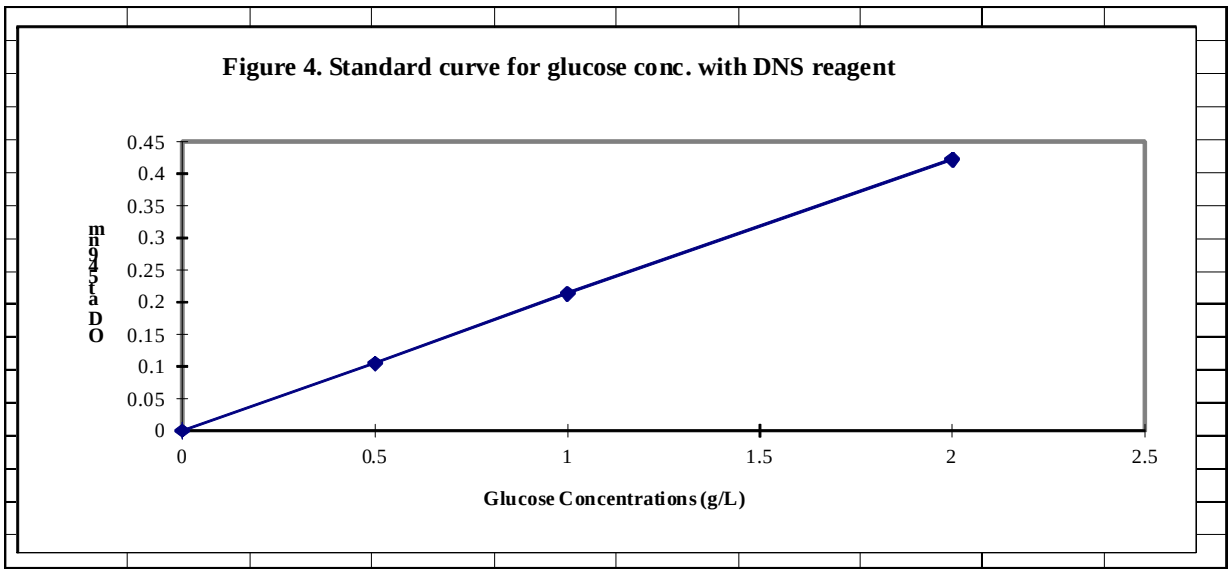

Figure 4. Standard curve for glucose conc. with DNS reagent

Conclusions

The BTCL strain used sugars from both maize and Miscanthus hydrolysate samples. In the absence of added nutrients, only up to 3 g/l sugar could be used. With addition of nutrients, sugar utilisation increased 2- to 3-fold. Indirect results showed that both reducing and non-reducing sugars were present in the hydrolysate samples.

It is expected that with an optimised medium, the BTCL thermophilic microorganism will use all of the sugars present in the hydrolysate samples and will produce ethanol with a minimum yield of 0.4 g ethanol / g sugar consumed. This is required to be performed in a fermenter under optimised conditions.