University of Abertay Dundee

BIOETHANOL FROM BREWER'S AND DISTILLER'S SPENT GRAINS

Yeast Research Group, School of Contemporary Sciences

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(shortened version of the report)

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1. INTRODUCTION

In the recent years growing attention has been devoted to production of biofuel from biomass, as conventional fuels like petroleum, natural gas, oil etc have started depleting due to there extensive consumption. This has led to pollution in turn contributing to green house effect. Biofuels are known to be the cleanest liquid fuels and thus act as better alternative to any fossil fuel, they are the combustible fuels produced mainly from biomass. These fuels are generally in the form of alcohols, esters, ethers, and other chemicals produced from biomass and known as biodiesel, biomethanol, **bioethanol**, biooil, biohydrogen depending on their source and end product.

The aim of this project is hydrolysis and bioconversion of lignocellulosic byproducts from breweries and distilleries to ethanol. Economically acceptable production of ethanol from lignocellulosic material could be also solution to dilemma: food, feed or biofuel, which has appeared in last years as a result of growing demand for biethanol on world's market. Industrial production of fuel ethanol is predominantly from agricultural crops, which also serve as food or animal feed. In order to meet the increasing demand for alternative biofuels, biomass sources other than those used as food need to be explored. We have identified spent grains from breweries and distilleries as a potential biomass source for bioethanol in Scotland.

2. SHORT DESCRIPTION OF THE PROJECT

Spent grains, consisting of the residues remaining after starch extraction for wort preparation, are low value products and are currently processed as an animal feed or disposed of as waste. They are a rich source of lignocellulose, which may be converted to fermentable sugars for production of bioethanol.

This research focuses on both the **physicochemical** and **enzymatic treatment of spent grains** to maximise recovery of carbohydrates from the cellulose and hemicellulose fractions and the **conversion of these sugars to ethanol by fermentative yeast**. Spent grains from a lager brewery (consisting of 85% malt and 15% wheat) are the raw materials currently being studied, although in the future these will be compared with spent grains from Scotch grain whisky.

Hydrolysis and extraction of sugars from such spent grains yields mainly glucose from cellulose and xylose and arabinose from hemicellulose. Pre-treatments could involve hydrolysis using, for example, pressure-cooking, microwave digestion, acid treatment, ultrasonication, ozonation and enzyme hydrolysis. One of the main aims of this project was to compare the efficacy of a selection of these methods, either individually or in combination, to liberate sugars and to improve the enzyme digestibility of the cellulose and hemicellulose components of spent grains.

Spent grain hydrolysate is complex, consisting of both hexose and pentose sugars. This is a challenge to yeast fermentation. *Saccharomyces cerevisiae*, the yeast traditionally used in the alcohol industry, does not utilise pentose sugars. To address this challenge, xylose-fermenting yeasts (*Pichia stipitis*, *Candida tenuis*, *Pachysolen tannophilus* and *Cryptococcus albidus*) were preliminary screened and their sugar fermentation characteristics assessed. The ability of these yeasts to ferment spent grain hydrolysate individually is being investigated. The aim of comparative trials with yeast strains and/or enzymes is to obtain near-complete utilisation of the hydrolysate sugars with fermentations yielding high levels of ethanol that may be used as a renewable transportation fuel.

The main benefits of bioethanol produced by yeasts are :

- CO_2 neutral,
- low toxicity,
- less GHG emissions (≈65% less)
- biodegradable
- agricultural diversification
- reduced dependence on oil

5. RESULTS AND CONCLUSIONS

5.1. PRELIMINARY CHECKING OF SUITABILITY OF DNS ASSAY FOR ANALYSIS OF REDUCING SUGARS IN SG

Based on the result in following table (Table 5) and some previous considerations, final conclusion is that DNS assay is appropriate for analysing of our samples. SG treated at 121°C and spiked with 1 g/l glucose yielded a similar concentration as the sum of individual SG treated at 121°C and 1 g/l glucose samples, respectively. This confirmed that SG hydrolysate did not interfere with the DNS assay.

sample	reducing sugars in extract (g/l)
SG extract, after 1h, at room temperature	0.56
SG extract, after 1h at 60ºC	0.82
SG extract, after 1h at 121ºC	**1.79**
glucose **1**g/l	0.96
SG(121°C) extract+**1**g/l glucose	**2.07**+0.96

Table 5. Amount of reducing sugars in SG extracts. Amount was also checked after addition of glucose standard.

5.2. pH VALUE OF SG EXTRACTS

In the following table it is obvious that pH value of SG extract after subjecting to different temperature is in range that is suitable for using of mentioned enzymes and yeast cultures and that adjusting of pH value is not necessary in trials where sulphuric acid was not used.

sample	pH
SG extract, after 1h, at room temperature	6.03
SG extract, after 1h at 60ºC	6.05
SG extract, after 1h at 121ºC	**5.49**

Table 6. pH values of SG water extracts treated with different temperatures

5.3. ACTIVITY OF LAB (HEMICELLULASE AND CELLULASE) ENZYMES

The cellulase and hemicellulase activities of the laboratory enzymes were not specified by the suppliers. Activity of the enzymes, in terms of liberating reducing sugars, on cellulose filter paper and xylan (from oat spelts) was compared.

conditions	**cellulase on cellulose**	**cellulase on xylan**	**hemicellulase on cellulose**	**hemicellulase on xylan**
	released reducing sugars g/l			
30ºC, 1 hour	0.51-1.0	**1.04-2.3**	0.02	0.07
30ºC, 14 hours	0.85	0.59-0.78	-	0.44
40ºC, 14 hours	-	**1.3-2-4**	-	**0.48-0.56**

Table 7. Amount of reducing sugars after treatment of standard substrates with enzymes

Conclusions:

- Significant amount of reducing sugars was already present in cellulase (approximately 10%).
- Cellulase on cellulose showed better activity at 30ºC than 40, and that prolonged incubation did not increase amount of sugar. On xylan, cellulase can be used at 30 ºC, 1 hour. For this case, there is no need for prolonging incubation or temperature increasing.
- Hemicellulase did not show any activity on cellulose, for any used conditions. Actually, it did not brake cellulase chains, but it could influence solubility of chains.
- Hemicellulase on xylan showed the best activity for prolonged fermentation, both at 30 and 40ºC.

5.4. ENZYMATIC HYDROLYSIS OF SG BY LAB ENZYMES (HEMICELLULASE AND CELLULASE)

Temperature treatment for 1hr at 136 ºC doubled solubility in comparison to 121 ºC treatment, amount of reducing sugars was increased but in very small amount (table 8).

sample	solubility (%)	red. sug. (g/l)
SUSPENDED, WITHOUT ANY TREATMENT	**2.35**	
JUST TEMP. TREATMENT (121ºC)	**6.81**	**2.25**
JUST TEMP. TREATMENT (136ºC)	**15.22**	**3.12**

Table 8.influence of increased temperature without using of enzymes

In figure 1 we can see changing of solubility and amount of released reducing sugars depending of days of incubation with 1000U of hemicellulase, at 30°C, for SG suspension after treatment with 121°C. In figure 2 there is changing of the same parameters during 3 days of incubation depending on amount of used hemicellulase.

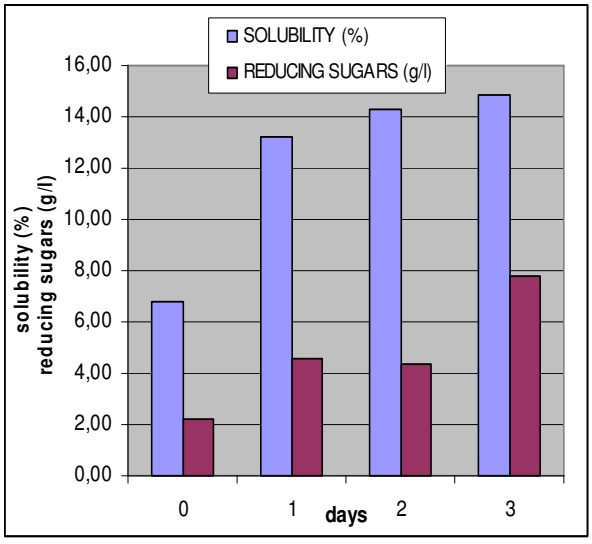


Figure 1. Changing of solubility and released reducing sugars during 0,1,2,3 days incubation with 1000U of hemicellulase

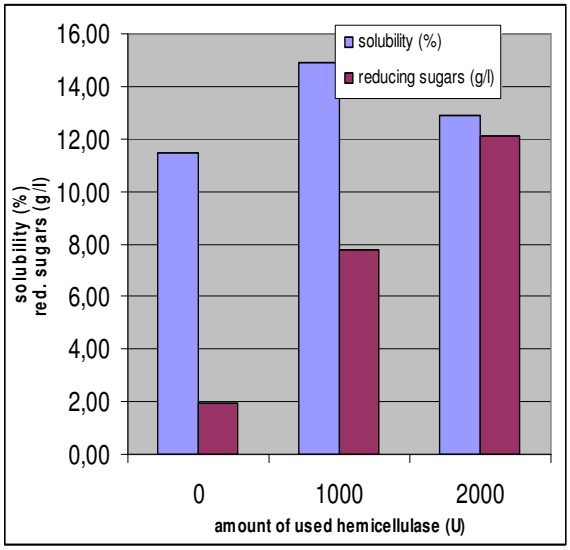


Figure 2. Changing of sulubility and released reducing sugars depending of used amount of hemicellulase.

Incubation at 30 ºC for 1, 2 and 3 days increased solubility in similar amount. Increasing of used amount of **hemicellulase** considerably influenced **amount of released reducing sugars,** while, in this case, solubility was not significantly influenced (Figure 2).

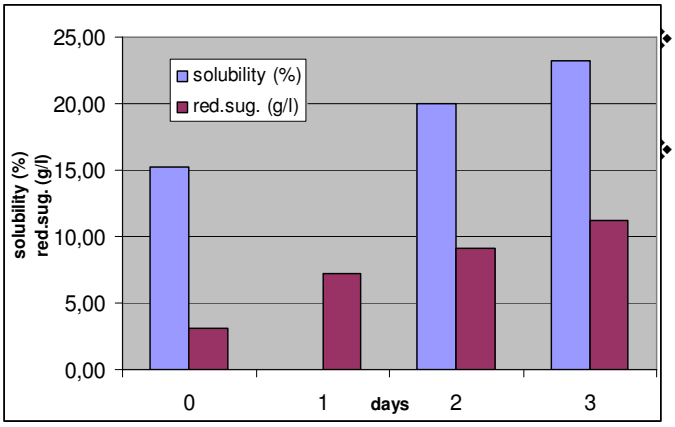
In the following table we can see amount of reducing sugars released after treating of SG **supernatant** with hemicellulase for 3 days at 30 ºC (after 121ºC treatment of suspended SG).

sample	**red sug (g/l)**
SUPERNATANT +1000U HEM	**1.38**
SUPERNATANT +2000U HEM	**5.18**
SUPERNATANT +BUFFER	**2.39**

Table 9. SG supernatants with hemicellulase

It could be concluded that SG supernatant contains carbohydrate polymers soluted but not digested to reducing sugars.

Solubility and amount of red. sugars in samples treated with 136ºC and incubated with hemicellulase (1000U) during 0,1,2 and 3 days are presented in figure 3:



For treatment with 136 ºC, solubility was increased with prolonged incubation and addition of hemicellulase.

On the other hand, the same prolonging of **incubation** (for 3 days instead 1 or 2 days) and particularly increasing of used amount of **hemicellulase** considerably influenced **amount of released reducing sugars** for 136ºC similar to treatment with 121°C. Still, this amount is low and should be increased.

Figure 3. SG Treated with 136ºC

In the following table results of using of cellulase and combination of cellulase and hemicellulase for increasing of solubility and liberating of reducing sugars are presented. Time of incubation was 3 days, temperature was 30 and 40°C.

Incubation	SAMPLE	SOLUBILITY (%)	REDUCING SUGARS (g/100g of dry spent grains)
3 DAYS AT 30ºC	121ºC + 10U CELL.	**13.24**	**5.19**
	121ºC + 20U CELL.	**14.06**	**8.09**
	121ºC + 10U CELL. + 1000U HEM.	**12.35**	**12.54**
	121ºC + 20U CELL + 1000U HEM.	**21.52**	**13.68**
	121ºC, BUFFER - CONTROL	**10.78**	**0.86**
	136ºC, 10U CELL.	**23.93**	**9.09**
	136ºC, 20U CELL.	**24.79**	**9.64**
	136ºC, 10U CELL. + 1000U HEM.	**25.89**	**14.92**
	136ºC, 20U CELL. + 1000U HEM.	**24.56**	**16.12**
3 DAYS AT 40ºC	121ºC, 10U CELL.	**19.47**	**7.83**
	121ºC, 10U CELL. + 1000U HEM.	**21.13**	**16.05**
	136ºC, BUFFER	**19.51**	**2.86**
	136ºC, 10U CELL. + 1000U HEM.	**26.22**	**17.62**

Table 10. Using of lab cellulase in combination with hemicellulase

Conclusions:

- Addition of **cellulase** only, increases solubility and amount of released reducing sugars in similar way to addition of **hemicellulase**. However, it is obviously that doubling of cellulase did not change these parameters very much.
- **Incubation** at 40ºC did not give much changes in solubility but could be useful for increasing of amount of released reducing sugars.
- Using of 20U cellulase with 1000U (even 2000U) of hemicellulase for SG pretreated with 136 ºC is good combination and could be good way for obtaining significant amount of ethanol from spent grains.
- Amount of released reducing sugars could be increased with further increasing of temperature, and using of different concentrations of diluted H_2SO_4.

5.5. ACID AND HIGH TEMPERATURE TREATMENT OF SG

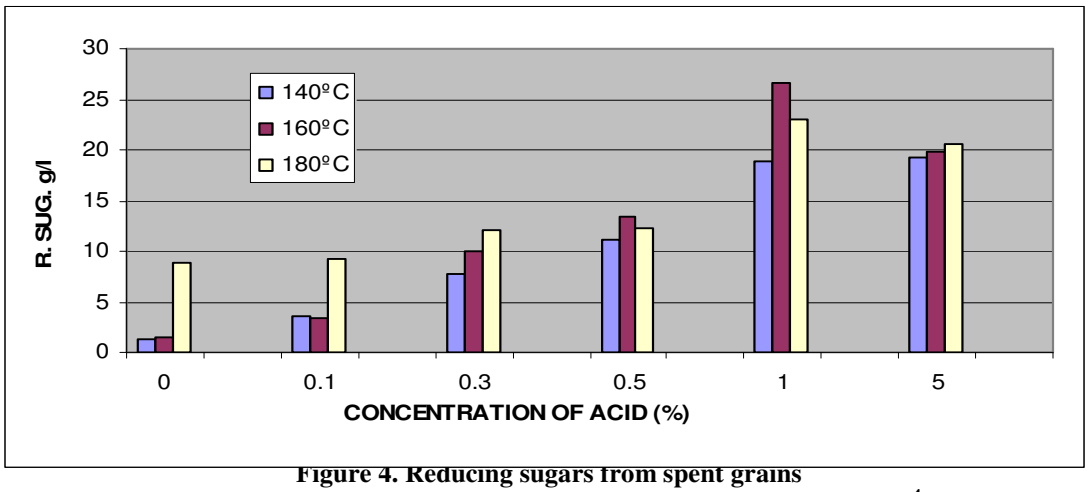


Figure 4. Reducing sugars from spent grains treated with sulphuric acid and different temperatures in MARS[4]

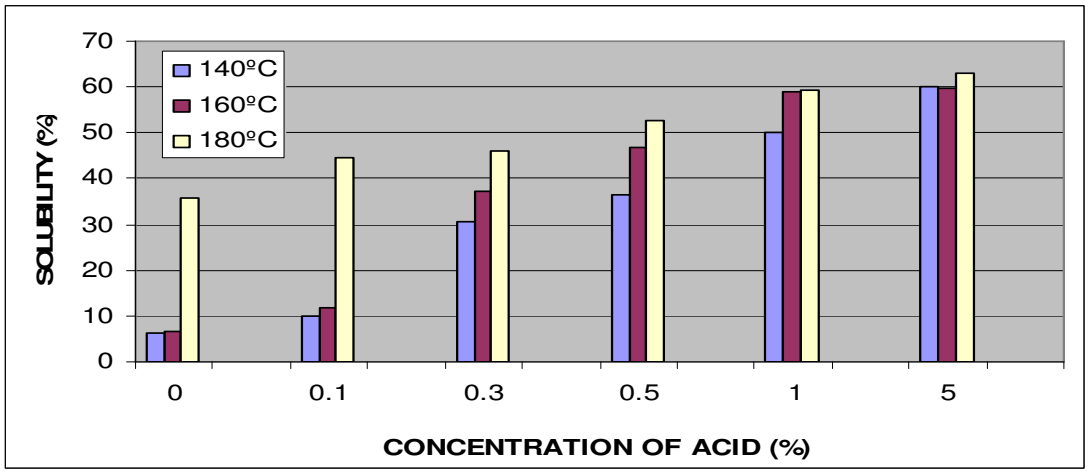


Figure 5. Solubility of spent grains treated with sulphuric acid and different temperatures in MARS

[4] **Microwave Accelerated Reaction System**

Conclusions:

- 1% of acid showed the best increasing of released reducing sugars and solubility for all temperatures used in comparison to control sample.
- solubility at 140 and 160°C for every concentration of acid higher than 0.1% was significantly increased in comparison to experiment No. 2 in which enzymes were used without previous treatment with acid (max ≈26% with enzymes, here: ≈30 to ≈60%).
- the highest result of released reducing sugars is also obviously higher here than in experiment 2 (max 26.5%, comparing to 17.5%).
- the best treatment is 160ºC with 1% acid.
- with further increasing of temperature above 160ºC and/or acid concentration above 1% solubility was increased, but amount of reducing sugars was decreased. It obviously shows that acid "burns" the reducing sugars (which could be concluded based on colour of the samples). In all other cases increasing of solubility was followed by increased releasing of reducing sugars.

5.6. HIGH TEMPERATURE PRE-TREATMENT AND ENZYMATIC DIGESTION (CRUDE INDUSTRIAL ENZYMES) OF SG

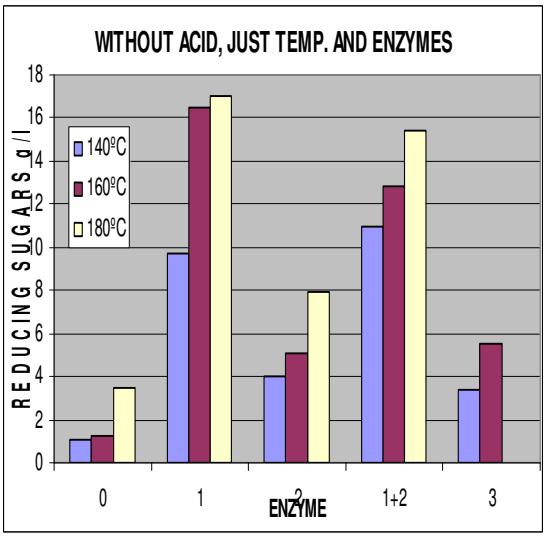


Figure 6. Reducing sugars from spent grains treated with MARS and enzymes

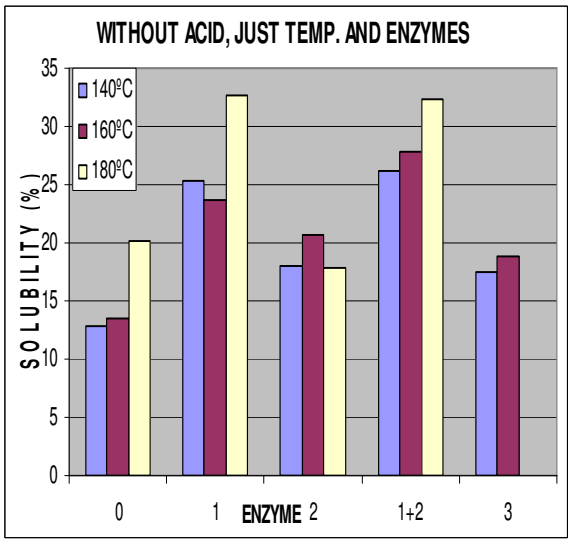


Figure 7. Solubility of spent grains treated with MARS and enzymes

0=without enzymes; 1= Cellulase + β-glucosidase (cellulase should be always used with cellulase to brake cellobiose units); 2=xylanase (=hemicellulase); 1+2= Cellulase + β-glucosidase+ xylanase; 3= enzyme complex.

Conclusions:

- With increasing of temperature used, amount of released reducing sugars after incubation is increasing. This increasing is much higher between 160 and 180 ºC than between 140 and 160 ºC.
- Used cellulase showed very good activity, especially in samples pretreated with 180ºC (more than 60% higher solubility and fivefold increasing of released reducing sugars - comparing to sample without enzymes).
- Hemicellulase did not showed any expected result in this case, same as the enzyme complex.

5.7. Enzymatic digestion of high temperature and 1% acid pre-treated SG.

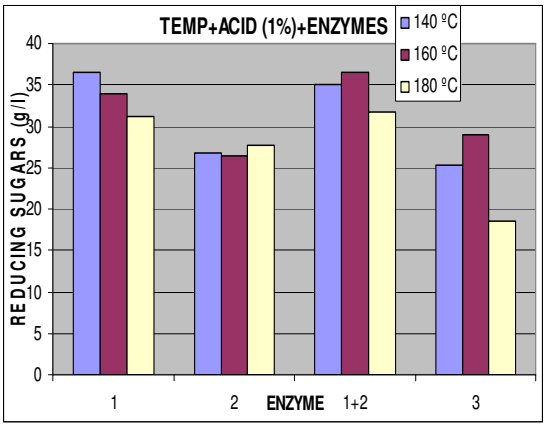


Figure 8. Reducing sugars from SG treated with MARS, 1% sulphuric acid and enzymes

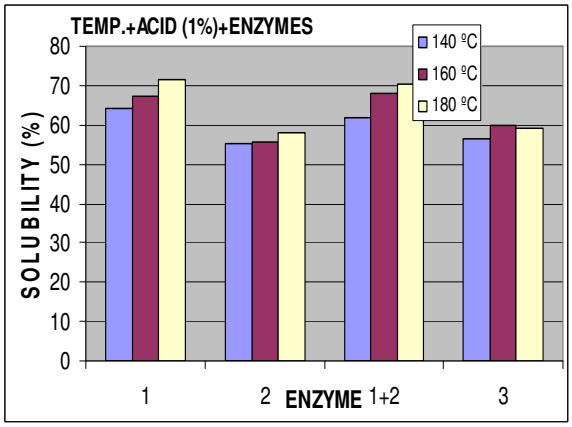


Figure 9. Solubility of SG treated with MARS, 1% sulphuric acid and enzymes

Conclusions**:**

- Undoubtedly, cellulose showed the best results between all enzymes used
- Pretreatment with 1% sulphuric acid doubled amount of released reducing sugars in sample with highest amount in comparison to control sample (31 comparing to 17g/l, for 180ºC in combination with cellulose). Similar situation is with solubility, increasing of solubility is even higher.
- It is very important to notice that when 1% sulphuric. acid was used, 180 ºC is too high temperature, because there is decreasing of concentration of reducing sugars, even when solubility is increasing. Obviously the reason is oxidation of sugars in these conditions. Even at 160 ºC comparing to 140 there is some decreasing in some cases.
- Next steps should be finding of optimum for used sulph. acid concentration and required temperature. Optimum could be 0.5% sulph. acid and 160 ºC or 1% acid with 140 ºC. The second combination could save a lot of energy, but there is more possibility for generating of some inhibitors that could affect yeast during fermentation. First combination requires more energy, but it is better for fermentation.

5.8. RESULTS OF SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF SG HYDROLYSATES

yeast	TREATMENT 1 (15% SG 30min at 136°C)				TREATMENT 2 (15% SG, **1% H_2SO_4,** 30min at 136°)	TREATMENT 3 (10% SG microwaved 30min at 160°C)
	enzymes added					
	0[5]	A[6], B[7]	A,B,C[8]	C	A,B,C	A,B,C
	ETHANOL (%)					
P. stipitis	0.013	**0.114**	0.092	0.027	0.0	**0.15**
Pa. tannophilus	0.0	**0.273**	0.173	0.008	0.0	**0.301**
C. tennuis	0.005	.0.0	0.019	0.006	0.008	0.0
Cry. albidus	0.0	0.0	0.0	0.0	0.0	0.0

Table 11. Percents of produced ethanol after simultaneous saccharification and fermentation in samples of pretreated SG hydrolysates, with different yeasts and enzymes.

- All samples treated with 1% H_2SO_4 did not produce ethanol. Reason for this are inhibitors for yeasts in samples. Inhibitors appeared because of treatment with 1% acid. Lower concentration of acid should be used.
- *Cryptococcus albidus* did not produce any amount of ethanol.
- *Candida tenuis* also showed very low yield of ethanol in all samples.
- *Pachysolen tannophilus* and *Pichia stipitis* produced ethanol, still it was very small amount. Maximum was 0.3% of ethanol (*P. tannophilus*). *P. stipitis* produced. max 0.15% ethanol. The best combination regarding enzymes is using all three enzymes together (cellulase, cellobiase and hemicellulase). Samples with 10% of SG concentration gave higher concentration of ethanol than samples with 15% of SG (max 0.3% comparing to max 0.27% for *P. tannophilus* or 0.15% comparing to 0.114 for *P. stipitis*).

[5] 0 - control samples, just inoculated, without enzymes addition
[6] A - samples with 110µL of NS50013 (cellulase complex)
[7] B - 11µL of NS50010 (β-glucosidase=cellobiase)
[8] C - 10µL of NS50014 (xylanase for insoluble xylan = hemicellulase)

8. ACKNOWLEDGEMENT

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