Title: Recycle residues of sugar beet for ethanol production by enzymes from plant-pathogenic fungi

1. Brief Background

Residues of sugar pulp has potential for use as a renewable biomass for ethanol production due to its high concentration of sugar content, i.e., about 20–24% cellulose, 25–36% hemicellulose, 20–25% pectin, 1 to 2% lignin, and 7 to 8% protein (Foster et al., 2001). Although engineered ethanologenic bacterium, such as *Escherichia coli*, has been applied to converting sugar beet residues to ethanol by simultaneous saccharification and fermentation (Doran et al., 2000), the unsuccessful production of a cadre of enzymes necessary to degrade cellulose, hemicellulose, and pectin to their simple sugar units limits its application. While utilizing fungal enzymes seems to be a promising option, the costs for purchasing required commercial fungal enzymes make it economically infeasible. Thus, effective enzyme sources are needed for economically hydrolyzing the sugar beet residuals and achieving significant ethanol yields. The plant pathogenic fungi have been reported to be able to synthesize pectinase and cellulase that participate in and enhance plant pathogenesis (Babalola, 2007), such as assisting pathogen movement through tissue, hydrolyze cellulose or indirectly stimulate plant cell wall extension by reducing the size and viscosity of matrix polymers (Chanliaud et al., 2004). Therefore, the plant pathogenic fungi can be good sources for hydrolytic enzymes especially for sugar beet residuals of high pectin content.

Objectives

The idea for the proposed investigation stems from the intention of effectively transforming carbohydrate biomass residues into biofuel in an economic way, which can facilitate the potential for industrial application to release the energy crisis. The main goals of the proposed work are to (a) select the appropriate enzyme sources from various plant-pathogenic fungi and compare qualitatively and quantitatively the yields of enzyme, including pectinase, cellulase and hemicellulase; (b) investigate practical benefits of the enzyme mixtures on the hydrolysis of sugar beet residues; and (c) optimize the enzymatic conversion process and evaluate its economic feasibility. After the workability of the above objectives is proved, the scope will be broadened to cover the improvement of enzyme properties (such as stability and tolerance) and apply the enzyme mixtures to yield monosaccharides from various biomass residues for biofuels.

2. Materials and Methods

2.1 Fungus cultivation

Each fungus (*A.rofsii, S.rofsii*) was cultivated in 300 ml Erlenmeyer flasks containing 150 ml of the following medium: SBP 10 g/l, peptone from meat 5 g/l, $MgSO_4.7H_2O$ 1.5 g/l and trace elements in citrate buffer 5.0. After 11 day cultivation at 110 r/min and 25 ^oC, the mycelia was separated from the culture broth by squeezing hard through the sieve. The supernatant was further purified to remove the tiny particles of mycelia by vaccum filtration. The harvested crude extracts were stored at -30 ^oC.

2.2 SBP hydrolysis

The hydrolysis of SBP was performed in 300 ml flasks containing 150 ml of 66 g/L SBP dissolved in citrate buffer 5.0. The SBP broth was sterilized at 120 ^{o}C for 15 minutes for pretreatment and then inoculated with the crude extract at different levels of inoculums at 1 ul/ml, 5 ul/ml 10 ul/ml and 20 ul/ml, respectively. The incubation condition was performed at 40 ^{o}C for 110 r/min. Duplicates of each run were conducted and samples were collected at the 1st, 2nd, 3rd and 7th day.

2.3 Enzyme cocktail setup

To determine the cellulolytic activity of crude extract and cellulase from Sigma, 5-10 mg α-cellulose in a 1,500 ul reaction tube and add 1,000 ul properly diluted sample solution (citrate buffer 5.0). Incubate for 30 minutes on a thermo-shaker (40 ^{o}C, 900 r/min) and clear the sample by centrifugation at 10,000 r/min for 5 min. The following step was referred to the description in session 3.1.2.

The SBP hydrolysis was conducted in 100ml flasks with the working volume of 40 ml. The trials were divided into three catagories- (i) hydrolysis of SBP with crude extract (ii) hydrolysis of SBP with cellulase from Sigma (iii) hydrolysis of SBP with enzyme cocktail. For the enzyme cocktail tests, 1 ml of cellulase with a series of diluted concentration (10 times to 10000 times dilution) was added together with 1ml of crude extract. The incubation time was conducted at 40 ^{o}C, 110 r/min and samples were collected at 24 h and 36 h, respectively.

2.4 Yeast screening

The yeasts screened were listed in Table 1. All fermentations used YPD medium supplemented with Glu, Ara and Gal correspondingly. The carbon content was 4 g/L. Precultures were grown overnight on the YPD with glucose as the carbon source, unless stated otherwise. Yeasts were screened for Glu, Ara and Gal fermentation under microaerophilic culture conditions. Yeast strains were transferred from the petri dish to 12-mL sterile polypropylene test tubes containing 5 mL of medium. The tilted culture tubes were incubated with shaking (160 rpm) at 30 ^{o}C in an incubator/shaker. After approx 24 h, 0.2-mL aliquots were removed from the culture tubes and used to inoculate capped 12-mL test tubes containing 5 mL of medium. The culture tubes were incubated with shaking as described for 24 h before OD, pH and ethanol analysis. Experiments were conducted in duplicates.

Table 1 Yeast strains screened for glucose, arabinose and galacturonic acid utilization

Strain No.	Collection	Strains Name
1	CBS 8244	*Arxula adeninivorans*
2	CBS 463	*Pichia gvillicmondi*
3	CBS 2753	*Debarymycos sp.*

4	CBS4803	*Pixhi scolyti*
5	CBS 8468	*Candida arabinofermentans*
6	CBS 6040	*Brettanomyces naardenensis*
7	Y1	***Candida boidinii***
8	Y2	***Candida intermedia***
9	Y6	***Pichia fermentans***
10	Y7	***Pichia stipitis***
11	Y8	***Rhodutorula rubra***
12	Y9	***Saccharomyces cerevisiae***

2.5 Sixfors Run for the hydrolysis and fermentation

2.5.1 SBP hydrolysis

Hydrolysis of SBP were conducted in a SixFors multi-fermentation system (Infors HT, Bottmingen, Switzerland) with six 300 mL working volume fermenters and independent control of temperature (40 ^{o}C), and stirrer speed (250 rpm). The substrate concentrations for the Run 1 and Run 2 were 60 g/L and 20 g/L. The inoculums for each fermentor were listed in Table 2.

Table 2 Inoculum of the enzyme/enzyme cocktail into the Sixfors reactors for SBP hydrolysis

Inoculum for each reactor / Run no.	**Fermentor 1**	**Fermentor 2**	**Fermentor 3**	**Fermentor 4**	**Fermentor 5**	**Fermentor 6**
Run 2	**CE 9ml+cel2.5ml**	**CE 9ml+cel2.5ml**	**CE 9ml**	**cel 2.5ml**	**CE 9ml+cel0.25ml**	**CE 9ml+cel0.25ml**

2.5.2 Yeast fermentation

Two strains of yeasts were testified (CBS 2753 *Debarymycos sp and Y9-Saccharomyces cerevisiae*). Yeast strains were transferred from the petri dish to 250 ml sterile flasks containing 80 mL of YPD medium and grown overnight. The tilted culture tubes were incubated with shaking (160 rpm) at 30 ^{o}C in an incubator/shaker. After approx 16 h, 8-mL aliquots were used to inoculate each fermentor.

Table 3 Yeast inoculums for each reactor in the Sixfors run 2 for ethanol fermentation

Reactor no. / Yeast inoculum	Fermentor 1	Fermentor 2	Fermentor 3	Fermentor 4	Fermentor 5	Fermentor 6
Yeast Inoculum	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	*Debarymyces sp.*

2.6 Analytical methods

2.6.1 DNSA test for total reducing sugars

600 ul of the hydrolysates was added to 600 ul DNSA agent in a 2 ml reaction tube. Incubate the mixture for 15 minutes at 95 ^{o}C. After incubation, immediately add 200 ul of the Rochelle-salt to stabilize the color. Chill the sample on ice for 5 minutes and record the absorbance at 575 nm. The standard curve of glucose was employed in this study to test the total reducing sugar of the SBP hydrolysates.

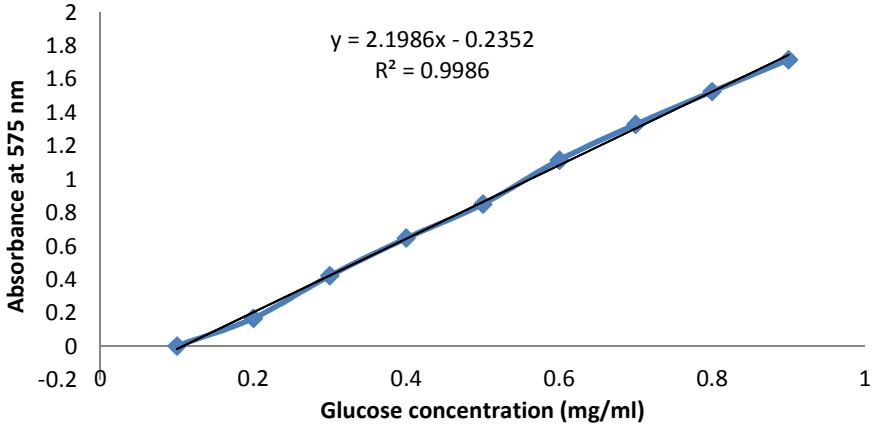


Figure 1 Standard curve of glucose

2.6.2 HPLC tests

A Dionex CarboPac PA-1 pellicular anion-exchange column (4 *250 mm) and precolumn (4 *50 mm) were used for analysis. Equipment consisted of a ThermoSeparations AS3000 autosampler, P4000

pump, Chromquest V4 data collection software, Dionex ED50 pulsed electrochemical detector with standard (not disposable) gold working electrode and pressurized postcolumn eluent container. Mobile phases were prepared in deionized water and sparged with helium as recommended in Dionex Technical Note 20 (2000) to minimize carbonate. The linear gradient used is outlined in Table 6. NaOH, 0.6 M, was added after the column and before the detector at 0.25 ml/min through a mixing tee. The retention time of the reducing sugar analyzed were demonstrated in Figure 4.

Table 4 Gradient profile used for sugar analysis

Time (min)	Flow (ml min^{-1})	Solvent (% by vol.)		
		A	B	C
0	1.1	100	0	0
7	1.1	100	0	0
16	1.1	70	30	0
18	1.1	3	82	12
21	1.1	0	0	100
23.5	1.1	0	0	100
24	1.1	0	100	0
27	1.1	0	100	0
28	1.5	100	0	0
39	1.5	100	0	0
39.5	1.1	100	0	0

Solvent A: aqueous 18 mM NaOH, B: aqueous 200 mM NaOH, C: aqueous 100 mM NaOH and 150 mM sodium acetate

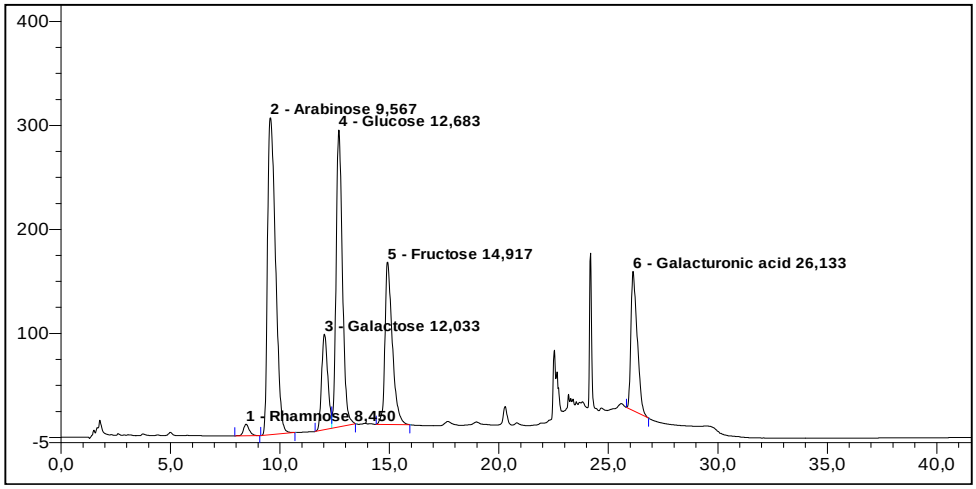


Figure 2 Retention time of the reducing sugars using the Dionex CarboPac PA-1 pellicular anion-exchange column

2.7 Statistical Analysis

All the treatments were conducted in triplicate and the differences between the treatments were

statistically analyzed by ANOVA test. The significant level was set at 0.05.

3 Results and discussion

3.1 Comparison of the enzyme activities of the crude extract from *A. rofsii* and *S. rofsii*

To compare the influence of fungal co-cultivation on extracellular enzyme production, such as cellulase, arabinase and pectinase, the selected fungal species were grown for 11 day in SBP medium, respectively.

The cultivations of *S.rofsii* resulted in increased enzyme activities of pectinase (5.19 U/ml) compared to the cultivations of *A.rofsii* (1.57 U/ml), although not always for all enzymes tested (Fig. 2). The increment was confirmed to be statistically significant ($p<0.05$). Arabinase activity in the case of *S.rofsii* was 15.6% higher than that for the case of *A.rofsii*, while cellulase activities were extremely close to each other with the value of about 0.12 U/ml.

Overall, the cultivations of *S.rofsii* showed higher enzyme activities of cellulase, arabinase and pectinase than those of *A.rofsii*.

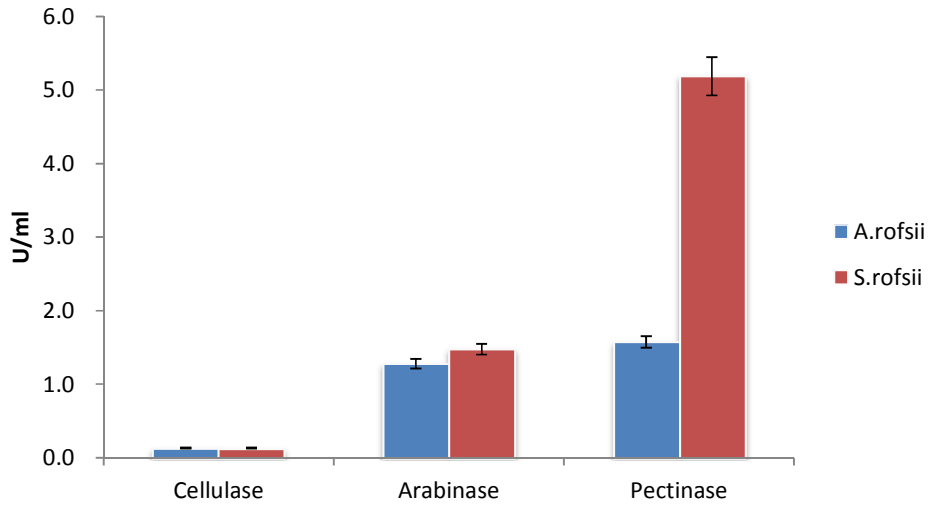


Figure 3 Enzyme activities from A.rofsii and S.rofsii after 11-day cultivation

3.2 SBP hydrolysis

3.2.1 Hydrolysis of SBP by crude extract of *S. Rofsii*

This section discussed the effects of inoculums of crude extract and the incubation time on the sugar composition of SBP hydrolysates.

3.2.1.1 Effect of the inoculums of the crude extract on SBP hydrolysis

Test of DNSA showed that the total reducing sugar liberated from SBP increased as the inoculums increased (Table 5). The highest yield of reducing sugar was 30.46 g/L when inoculated at 10 ul/ml after 7 day incubation. However, owing to the various reducing sugars in the hydrolysates, the DNSA method could only show the draft reference by calculating only from the standard curve of glucose.

Table 5 Analysis of the total reducing sugar in SBP after 7th day incubation using DNSA method

Addition of crude extract	**Dilution**	**OD**	**Total reducing sugar**
ul/ml			**g/L**
1	20	1.43	15.16
5	100	0.32	25.50
10	100	0.43	30.46

Table 6 showed the contents of different liberated reducing sugars in the 7th day culture of SBP hydrolysates at various inoculum levels from 1ul/ml to 20ul/ml. The results exhibited that for the reducing sugars analyzed (rhamnose, arabinose, galactose, glucose, fructose and galacturonic acid), the liberated contents inclined as the inoculums level increased, which demonstrated that high level of inoculums of crude enzyme extract facilitated the SBP hydrolysis. The highest contents of reducing sugars (rhamnose, arabinose, galactose, glucose, fructose and galacturonic acid) were achieved at the inoculums of 20 ul/ml with the yield of 0.337 g/l, 6.339 g/l, 1.083 g/l, 1.796 g/l, 1.704 g/l and 4.848 g/l, respectively. However, the increments of the reducing sugars contents between the inoculums of 10 ul/ml and 20 ul/ml were not statistically significant except for rhamnose but with only 2.4 % in the SBP. The results indicated that further increasing the inoculum from 20 ul/ml would not significantly benefit the SBP hydrolysis but raised the total costs for the hydrolysis.

Table 6 Hydrolysates composition after 7th day cultivation with various inoculums using HPLC method

Inoculation volume / Reducing Sugar (g/l)	1ul/ml	5ul/ml	10ul/ml	20ul/ml
Rhamnose	0.0403	0.148	0.263	0.337
Arabinose	1.881	5.084	6.976	6.339
Galactose	0.356	0.617	0.928	1.083
Glucose	1.275	1.444	1.754	1.796
Fructose	1.224	1.344	1.694	1.704.
Galacturonic acid	1.59	3.235	4.535	4.848

3.2.1.2 Effect of incubation time on the SBP hydrolysis

The compositions of SBP hydrolyzed with 10 ul/ml crude extract at different incubation times were listed in table 9. For all the reducing sugars in Table 9, more reducing sugars were liberated as the hydrolysis incubation time continued. The highest yields of reducing sugars occurred on the 7th day incubation.

Table 7 SBP hydrolysates composition at various incubation times with the inoculum of 10 ul/ml

No.	Sugar (g/l)	1st day	2nd day	3rd day	7th day
1	Rhamnose	0.123	0.161	0.251	0.263
2	Arabinose	4.012	5.501	6.270	6.976
3	Galactose	0.514	0.644	0.851	0.928
4	Glucose	1.199	1.266	1.386	1.754
5	Fructose	1.088	1.102	1.294	1.694
6	Galacturonic acid	2.733	3.294	4.034	4.535

3.2.2 Enzyme cocktail optimization

Data in Table 8 and 9 showed that the contents glucose of was around 1.7 g/L with the inoculums of 20 ul/ml, which was not reasonably high since the SBP composed of 20 % of cellulose. Meanwhile,

the ratio of glucose and fructose was approximately one, implying that the sucrose was hydrolyzed instead of cellulose. To overcome this problem, we tried to develop an enzyme cocktail consisting of crude extract and cellulase for obtaining a sufficient quantity of glucose from cellulose in the SBP.

Increase of the cellulase inoculums corresponded to the increment of the glucose liberated. The highest yield of glucose was achieved at 8.26 g/l after 36 h incubation in the case of adding 1 ml of cellulase and 1ml of crude extract together, which was 8.42 fold of that only hydrolyzed by 1ml crude extract (0.98 g/l glucose). Followed by the highest yield of glucose were the cases of adding 1ml of cellulase diluted from 10 times to 10000 times in the cocktail with the glucose content from 3.54 g/l to 1.042 g/l, respectively. Nevertheless, the contents of glucose liberated with crude extract, cocktail of 1000-time diluted cellulase and cocktail of 10000-time diluted cellulase did not significantly differ from each other, which indicate further dilution of cellulose from 100 time did not contribute to the hydrolysis of cellulose in SBP.

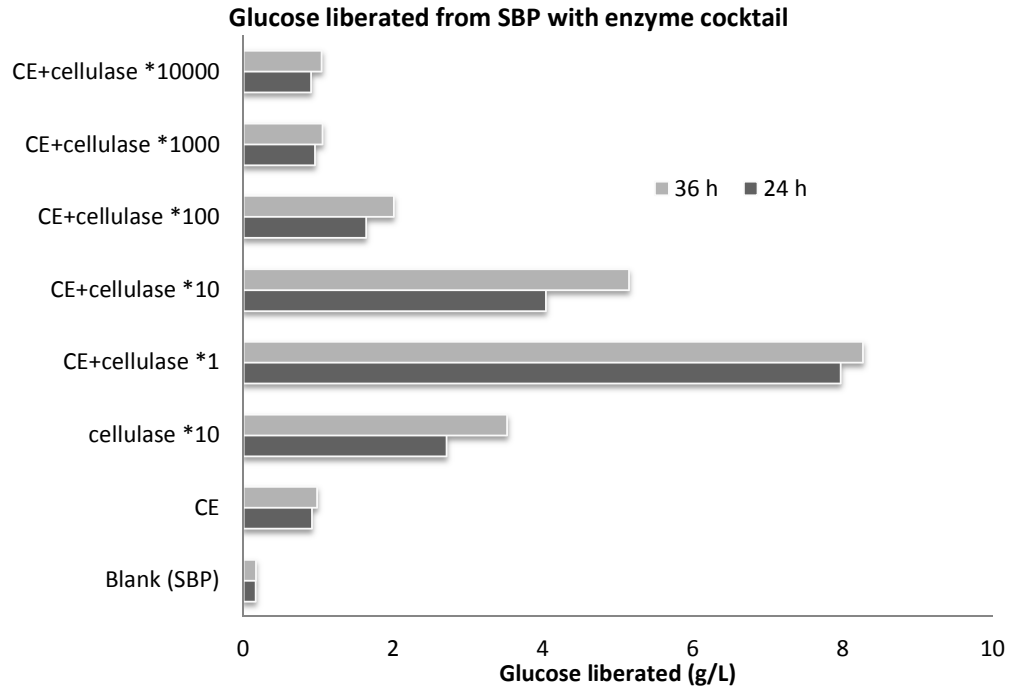


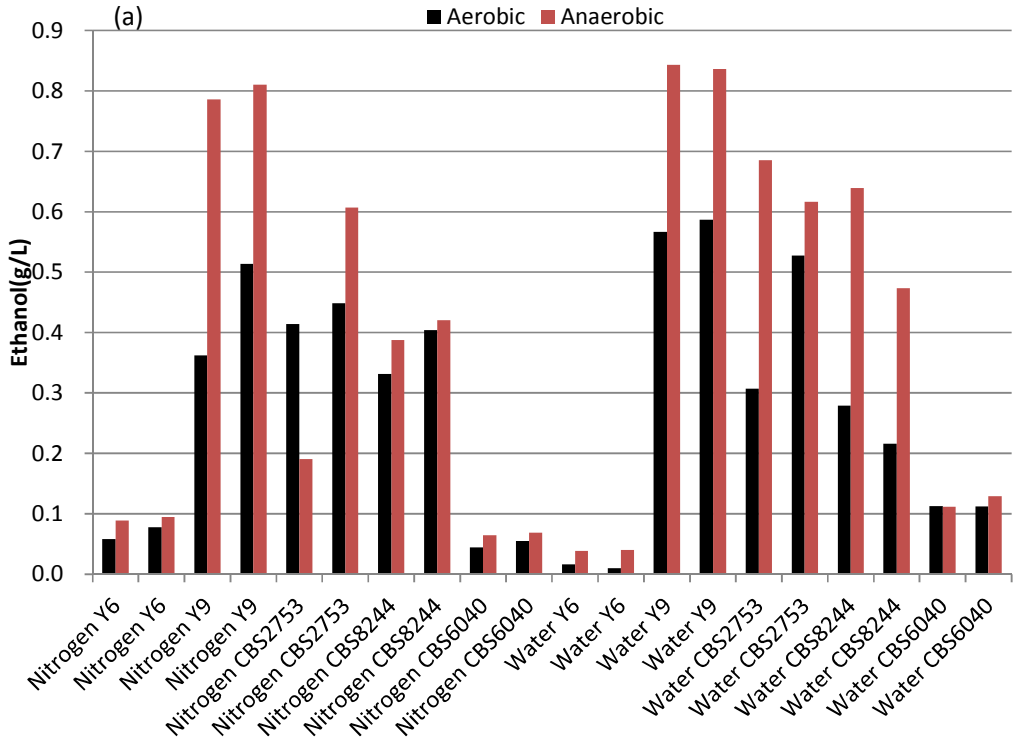
Figure 4 Performances of enzyme cocktail on the glucose liberated from the SBP (CE-crude enzyme extract; cellulase *1-cellulase was not diluted; cellulose *10, 100, 1000, 10000-cellulase was diluted by 10, 100, 1000, 10000; in all cases, the additions of crude extract and cellulase were 1,000 ul, respectively)

3.2Prescreening of the yeast strains

3.2.1 Influences of cultivation conditions on OD and Ethanol conversion efficiency

Representative fermenting microorganisms were screened to evaluate their ability to ferment sugar beet pulp when grown in aerated and anaerobic conditions, respectively (Figure 2 (a) and (b)).

For the strain of Y9, Y6, CBS 8244, CBS 2753, in both cases of medium with and without nitrogen supplement, significantly higher ethanol yield was obtained in anaerobic condition than that generated from the aerated condition. For CBS 6040, no significant difference of the ethanol content was observed between the cases of anaerobic and aerobic conditions. Similar observations were achieved for the optical density. The optical densities of both cultivations with and without nitrogen supplement in anaerobic condition were not as high as that from aerobic conditions for all the tested strain except CBS 6040. The results demonstrated that sufficient oxygen in the culture broth benefited the yeast growth whereas the overgrowth of cells seemed to a byproduct for ethanol generation.



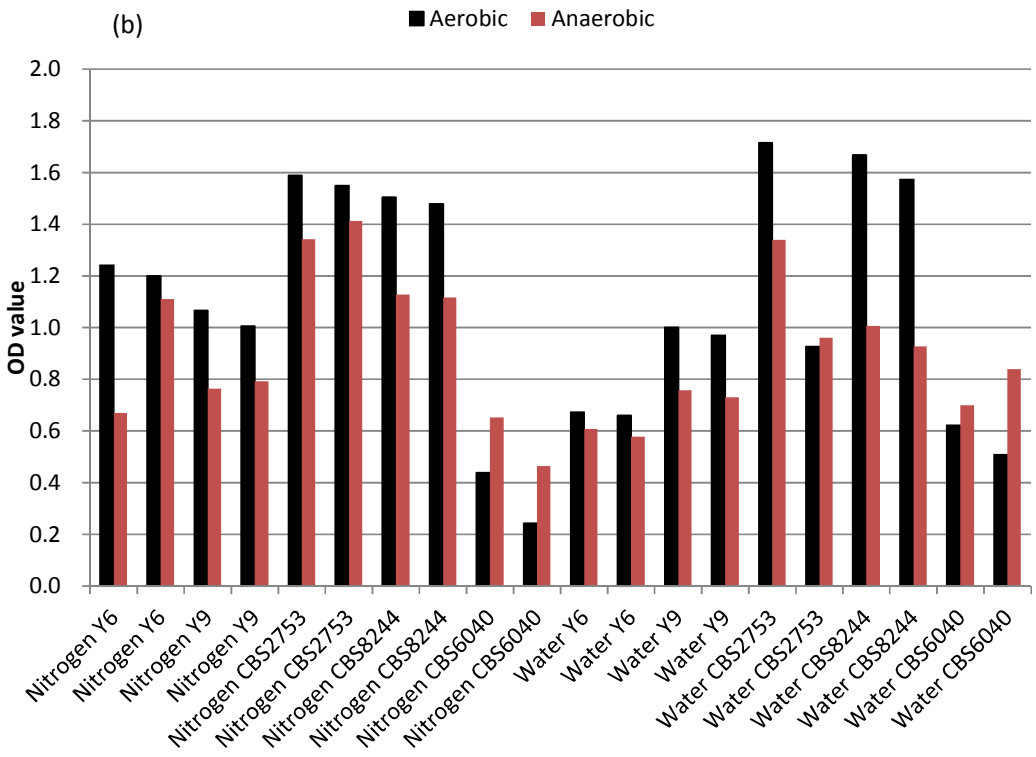


Figure 5 Profiles of (a) ethanol conversion rate and (b) OD (nitrogen + strain no.---the SBP hydrolysates was supplemented with 20g/l peptone and 15 g/l yeast extract; water + stain –equal amount of water was added to SBP hydrolysates)

3.2.2 Media influences on OD and Ethanol conversion efficiency

Besides investigating the influences of cultivation conditions, the strains were subjected to the medium with and without nitrogen supplement separately, respectively (Figure 2 a) and b)).

No matter at aerated or anaerobic cultivations, optical density measurements and visual observations indicated that the higher cell mass growth was achieved by the culture medium with nitrogen supplement for Y6 than that from the medium with SBP hydrolysates only. Oppositely, nitrogen additions did not seem to facilitate cell growth of CBS 6040 since the average OD in the nitrogen supplemented medium at aerobic and anaerobic were 0.34 and 0.558, which were 66.0% and 37.9% lower than that from the medium without any additional supplement. No significant differences of optical densities were observed for the strains of Y9, CBS 2753 and CBS 8244 between the cases of medium with and without nitrogen supplement.

In summary, the highest yield of ethanol (0.84 g/l) was achieved by Y9 in the medium with only SBP hydrolysates under anaerobic cultivation, followed by CBS 2753 generating 0.66 g/l ethanol.

3.2.3 HPLC tests for sugar utilization

Table 8 and 9 showed the utilization efficiency of the reducing sugar liberated from SBP by strains of Y9 and CBS 2753 under anaerobic and aerobic conditions, respectively. Compared to other strains, these two strains (Y9 and CBS 2753) demonstrated highest reducing sugar consumptions (arabinose, galacturonic acid and glucose) among the tested five strains (the detailed data for the other strains was listed in table 11 and 12 in Appendix).

Under aerobic conditions, both strains of Y9 and CBS 2753 exhibited significantly higher consumption of fructose and galactose when cultivated in the SBP hydrolysates medium than in the nitrogen supplemented medium (Table 4). Similar observations for arabinose, fructose and galactose were illustrated under anaerobic cultivations (Table 5). One thing that needs to be mentioned, arabinose was completely consumed (100 %) by the two strains under anaerobic condition with no nitrogen supplement. For arabinose consumption under aerobic condition, Y9 and CBS 2753 cultivated in the SBP hydrolysates represented 49.4 % and 52.18 % of arabinose consumption while only 37.08 % and 48.72 % in the cultivation supplemented with nitrogen were achieved separately. For galacturonic acid in the cases of medium with or without nitrogen supplement, higher galacturonic acid was consumed under aerated condition than that of the anaerobic condition for both strains. Under aerated conditions, 61.48 % of the galacturonic acid was utilized for Y9 in the SBP hydrolysates medium, which was 8.05% higher than that from the nitrogen supplemented medium. For CBS 2753, no significant differences of galacturonic acid utilization percentage being 59.90 % and 61.56% respectively were displayed in both medium conditions (Table 4).

For glucose, it was totally utilized by both strains in all the cases except for CBS 2753 cultivated aeratedly in the nitrogen supplemented medium.

Based on the above analysis, the strains of Y9 and CBS 2753 were selected as the candidates for the following fermentation study.

Table 8 Utilization percentage of the reducing sugar liberated from SBP under aerobic condition for the candidate of Y9 and CBS 2753

Strain	Arabinose %	Galacturonic acid %	Frucose %	Galactose%	Glucose %
Nitrogen Y9	49.04%	56.53%	58.83%	97.46%	100.00%
Nitrogen CBS2753	52.81%	61.56%	64.15%	100.00%	100.00%
Water Y9	37.08%	61.48%	83.30%	100.00%	100.00%
Water CBS2753	48.72%	59.90%	91.22%	100.00%	100.00%

Table 9 Utilization percentage of the reducing sugar liberated from SBP under anaerobic condition for the candidate of Y9 and CBS 2753

Strain	Arabinose %	Galacturonic acid %	Frucose %	Galactose%	Glucose %
Nitrogen Y9	54.04%	25.53%	45.47%	86.84%	100.00%
Nitrogen CBS2753	56.42%	57.66%	36.24%	100.00%	98.42%
Water Y9	42.01%	34.79%	81.09%	100.00%	100.00%
Water CBS2753	44.83%	28.49%	91.62%	100.00%	100.00%

*Nitrogen + strain means 20g/l peptone and 15 g/l yeast extract were added to SBP hydrolysates. Water + strain means equal volume of water was added to SBP hydrolysates.

3.3 Hydrolysis and fermentation in Sixfors run

3.3.1 pH profiles of the SBP hydrolysis

Similar observation was obtained to the Run 1. Overall, pH values in all cases of incoculum exhibited a declining trend except fermentor 1 with the inoculums of CE 9ml+cel2.5ml of which pH increased slightly after 102.6 h hydrolysis. The pH values in fermentors 2, 3, 6 became steady after 54.3 hour hydrolysis, which were in consistent with the galactuonic acid curves that the increment of the production occurred before 54.4 hour (Fig.6). The solution inoculated with cel 2.5ml exhibited

stable pH value during the entire period compared to other treatment, which could be explained by the least galacturonic acid liberated.

Table 10 pH profiles of the hydrolysis process in the Run2

Date	Sampling Time	Time (h)	CE 9ml+cel2.5ml	CE 9ml+cel2.5ml	CE 9ml	cel 2.5ml	CE 9ml+cel0.25ml
5.15	7:15 AM	0	4.85	4.81	4.81	4.87	4.8
5.16	10:35 AM	26.3	4.72	4.63	4.65	4.85	4.63
	2:00 PM	29.8	4.72	4.62	4.64	4.85	4.62
5.17	10:10 AM	50.3	4.73	4.59	4.61	4.88	4.6
	2:00 PM	54.3	4.74	4.59	4.61	4.86	4.59
5.18	10:00 AM	74.3	4.76	4.57	4.59	4.87	4.58
5.19	2:10 PM	78.3	4.79	4.55	4.58	4.87	4.57
5.2	2:37 PM	102.6	4.8	4.54	4.57	4.87	4.57
5.21	9:45 AM	123.6	4.8	4.54	4.56	4.87	4.56
5.22	10:13 AM	147.2	4.83	4.53	4.56	4.87	4.56

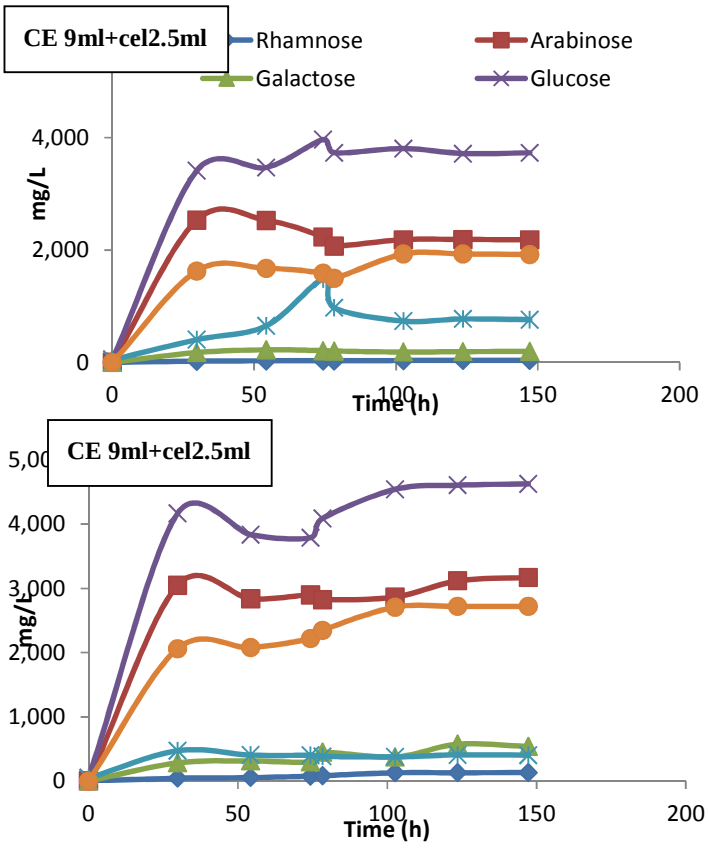
3.3.2 Reducing sugar liberated

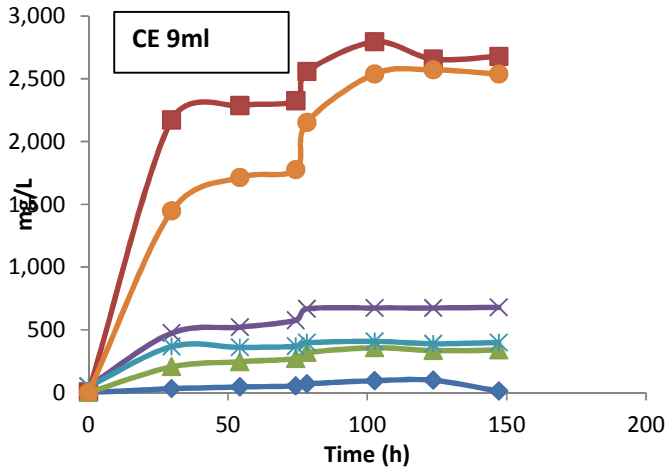
For all the reducing sugars in Fig. 4, more reducing sugars were liberated as the hydrolysis incubation time continued. No significant differences of the total reducing sugar was observed between the cases of the inoculum with CE 15ml+cel2.5ml and CE 15ml+cel2.5ml (Table 6). 4.15 g/L and 3.6 g/L glucose on average was liberated with 2.5ml cellulase and 0.25 ml cellulase respectively. The compositions of SBP hydrolyzed with various enzyme preparations at different incubation times were listed in Fig. 6. Yields of arabinose and galacturonic acid increased fast during the cultivations of 0h -60h when the inoculums contained CE 9ml. The liberated contents of glucose inclined to highest around the cultivation of 40h when the inoculums level of cellulase was 2.5 ml while the highest yield of glucose occurred at around the cultivation of 80h with 0.25ml cellulose inoculated.

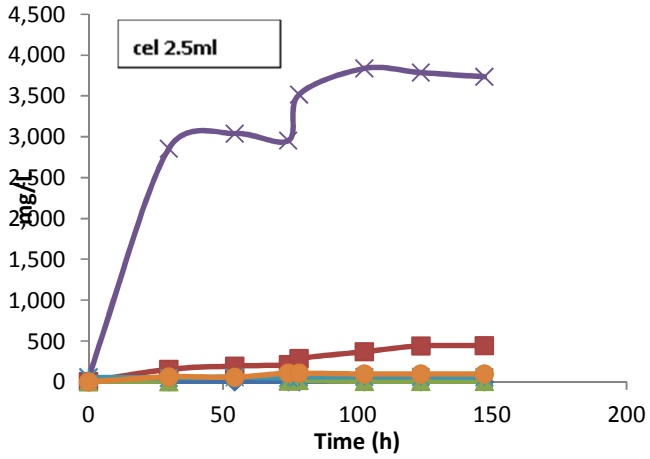
Table 11 Reducing sugar composition and total reducing sugar produced (Unit: mg/L)

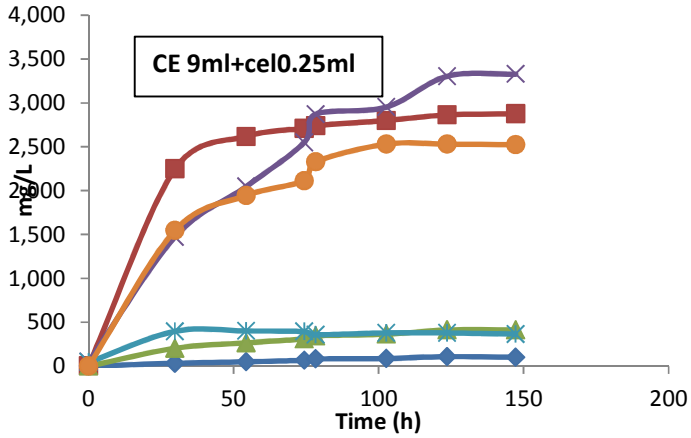
	Rhamnose	Arabinose	Galactose	Glucose	Fructose	Galacturonic acid	Total reducing

							sugar
CE 9ml+cel2.5ml	37.12	2182.508	196.646	3727.967	758.592	1918.208	6902.833
CE 9ml+cel2.5ml	131.154	3170.898	538.337	4628.839	403.392	2720.592	8872.62
CE 9ml	147.2	11.547	2678.257	340.347	678.984	399.200	3856.335
cel 2.5ml	1.508	442.883	3.887	3734.962	55.454	96.043	4238.694
CE 9ml+cel0.25ml	101.691	2875.367	412.784	3327.990	365.600	2524.147	7083.432
CE 9ml+cel0.25ml	100.015	2654.899	380.926	3920.591	352.888	2231.608	7409.319




CE 9ml
3,000
2,500
2,000
1,500
1,000
500
0
mg/L
0
50
100
150
200
Time (h)


cel 2.5ml
4,500
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
mg/L
0
50
100
150
200
Time (h)


CE 9ml+cel0.25ml
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
mg/L
0
50
100
150
200
Time (h)

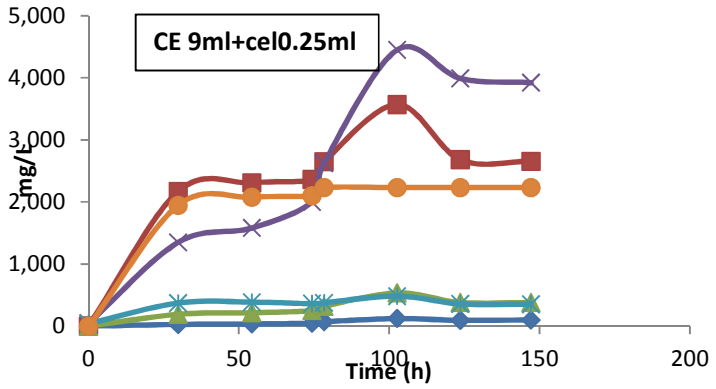


Figure 6 Reducing sugar profiles of the hydrolysis at different incubation time

3.3.3 Hydrolysis efficiency by Calculating Dry mass of SBP analysis

The case of CE9ml+cel2.5ml resulted in the highest hydrolysis percentage of 63.17%, followed by the average of 60.3% obtained by CE9ml+cel0.25ml. The results confirmed that the combination of crude extract from *S.rofsii* and cellulase resulted in increased reducing sugar liberation compared to the single incubation supplying with either CE or cellulase.

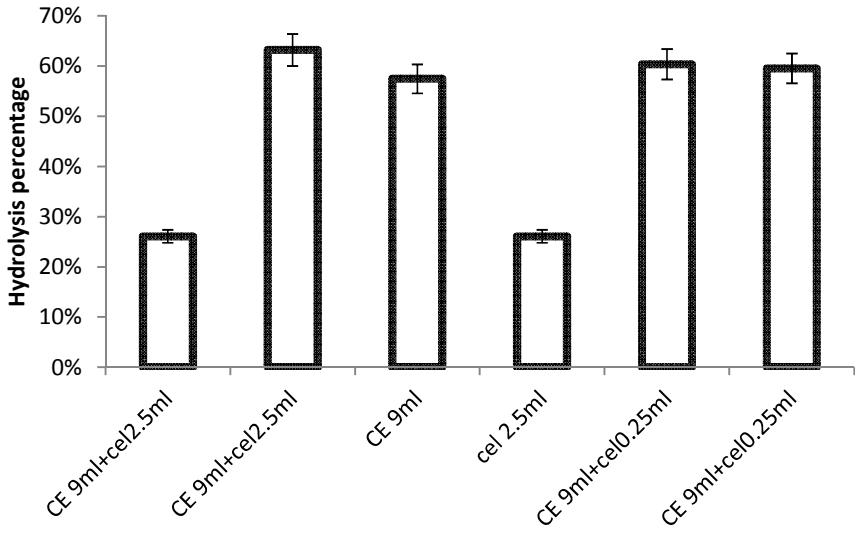


Figure 7**.** Hydrolysis percentage based on the dry mass after hydrolysis

3.3.4 Ethanol fermentation-pH profile

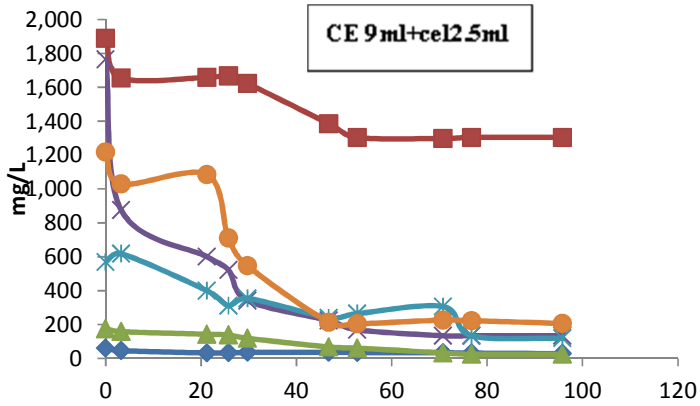
pH profiles during the ethanol fermentation process showed the declining trend in all the cases except for fermentor 1 with the inoculums of CE 9ml+cel2.5ml of which pH increased from 6.33 to 6.58 after 70.75 h fermentation.

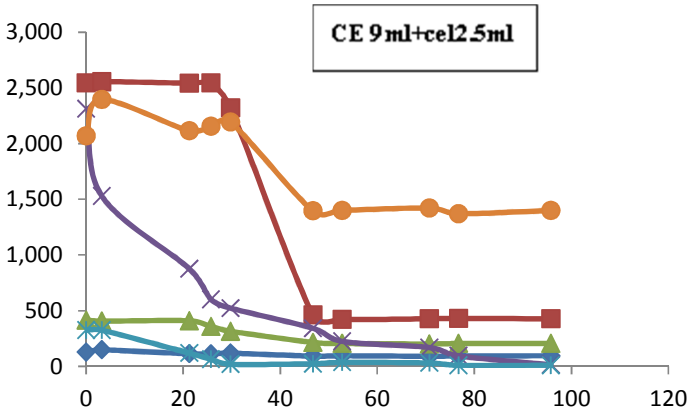
Table 12 pH profiles of the fermentation process

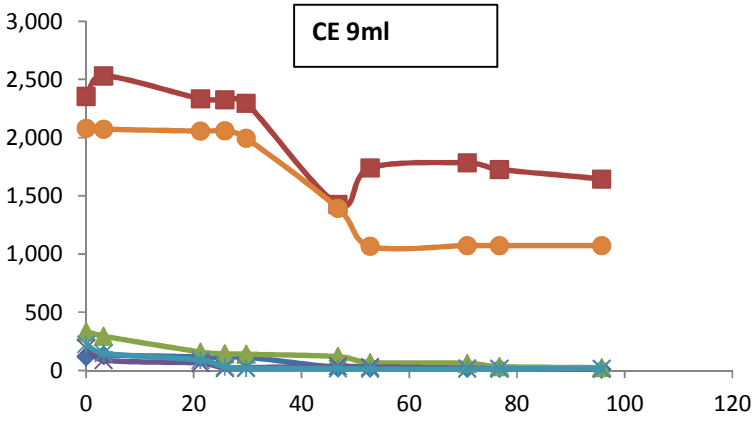
Date	Sampling Time	Time (h)	CE 9ml+cel2.5ml	CE 9ml+cel2.5ml	CE 9ml	cel 2.5ml	CE 9ml+cel0.25ml	CE 9ml+cel0.25
5.22	11:30 AM	0	6.86	6.94	6.98	6.94	6.93	6.93
	2:45 PM	3.25	6.74	6.8	6.82	6.76	6.79	6.76
5.23	8:45 AM	21.25	6.23	6.08	6.29	6.11	6.11	6.18
	1:15 PM	25.75	6.33	6.07	6.29	6.11	6.1	6.18
	5:20 PM	29.75	6.4	6.07	6.28	6.1	6.09	6.18
5.26	10:10 AM	46.75	6.55	6.06	6.27	6.07	6.07	6.17
	4:20 PM	52.75	6.58	6.06	6.27	6.05	6.08	6.17
5.17	11:00 AM	70.75	6.65	6.06	6.26	6.03	6.07	6.19
	5:00 PM	76.75	6.63	6.06	6.26	6.03	6.07	6.19
5.28	12:00 PM	95.75	6.58	6.06	6.25	6.01	6.07	6.2

3.3.5 Ethanol fermentation-reducing sugar utilization profile

The utilization of the reducing sugars in the SBP hydrolysates by various enzyme combinations were plotted verse time (Fig.8). In the cases of CE 9ml+cel 0.25 ml inoculated with *Saccharomyces cerevisiae and Debarymtcos,* glucose was utilized fastest by yeast followed by galacturonic acid and arabinose. Galacturonic acid concentration decreased from 2.4 g/L to 1 g/L and 2.6 g/L to 1 g/L respectively. For arabinose, the content decreased from 2.83 g/L to 1.83 g/L and 3.0 g/L to 2.18 correspondingly after fermentation. The results indicated that the arabinose and galacturonic acid could be metabolized by both yeasts to some extent. Similar observation was obtained in the cases of CE 9ml+cel 2.5ml by *Saccharomyces cerevisiae.* The declining changes of the contents of arabinose and galacturonic acid were about 1.4 g/L and 1 g/L, respectively.


CE 9ml+cel2.5ml
mg/L
2,000
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0
0
20
40
60
80
100
120


CE 9ml+cel2.5ml
3,000
2,500
2,000
1,500
1,000
500
0
0
20
40
60
80
100
120


CE 9ml
3,000
2,500
2,000
1,500
1,000
500
0
0
20
40
60
80
100
120

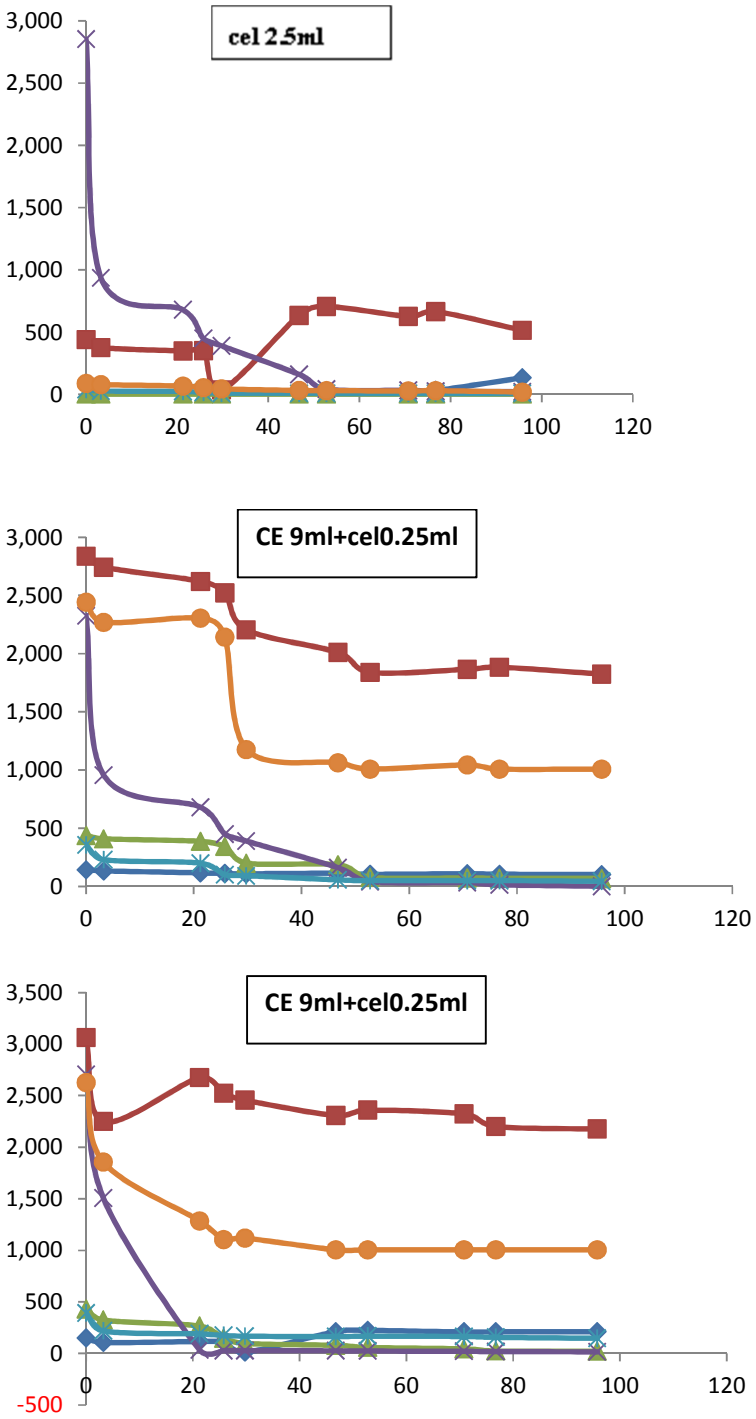


Figure 8 Fermentation profiles of sugars at various inoculum

3.3.6 Ethanol fermentation-ethanol yield profile

The highest ethanol yield (2.0 g/L) was achieved in the case of CE 9ml+cel0.25ml. No significant difference was observed between the case of CE 9ml+cel0.25ml and CE 9ml+cel2.5ml, indicating that further increment of cellulase addition from 0.25 ml to 2.5 ml did not benefit ethanol yield. The incubations hydrolyzed with only CE had the lowest ethanol yield of 0.57 g/L occurring after 26 h cultivation. The reason was that arabinose and galacturonic acid were the main carbon sources released, which were not favored to yeasts compared to glucose. The discussion could be verified by the demonstration of the ethanol over glucose yield (Fig.10). The case of cel 2.5ml had maximum ethanol production efficiency of 0.50 g/g sugar used, which was 98% of the theoretical yield. Overall, higher ethanol over reducing sugar yield was observed for the inoculums of CE 9ml+cel0.25ml than that of CE 9ml+cel2.5ml. And 32.2% increase of higher ethanol over reducing sugar yield was observed for *Debarymycos sp.* than *Saccharomyces cerevisiae* when the inoculums were the same (CE 9ml+cel0.25ml). However, the statement that *Debarymycos sp.* performed better fermentation properties than *Saccharomyces cerevisiae* on SBP hydrolysates could not be conclusive due to the variance of the reducing sugar compositions.

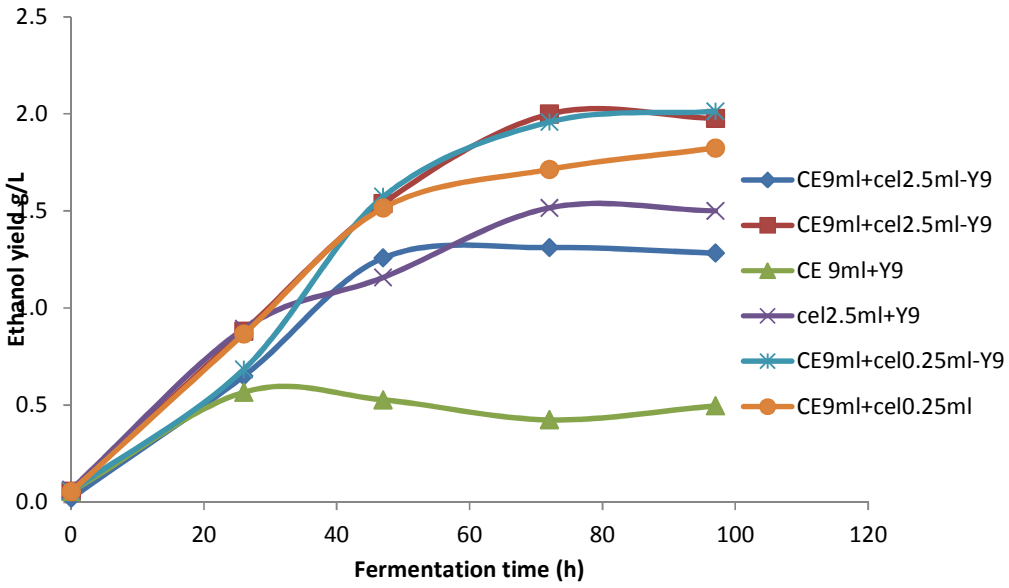


Figure 9 Ethanol yields at various inoculums

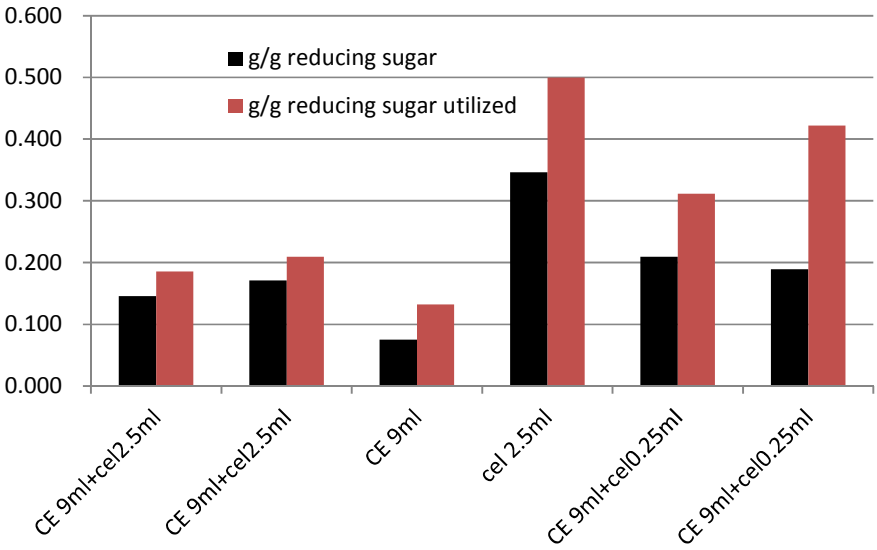


Figure 10 Fermentation efficiencies of ethanol over the reducing sugars yield in different SBP hydrolysates

3.3.7 Ethanol fermentation-yeast cell profile

In all cases, the concentrations of yeast strains reached 10^7 after 26 hour cultivation. The final overall cell concentration in the cultivation with *Debarymycos sp.* was 7.9 *10^7 CFU/ml, higher than the concentration achieved in the cultivation with *Saccharomyces cerevisiae* (3.4*10^7) after 72 hour

cultivation with the same enzyme inoculum of CE 9ml+cel 0.25ml. The result implied that the carbon sources were more utilized for cell mass accumulation for *Debarymycos sp.* although the same quantity level was achieved both strains.

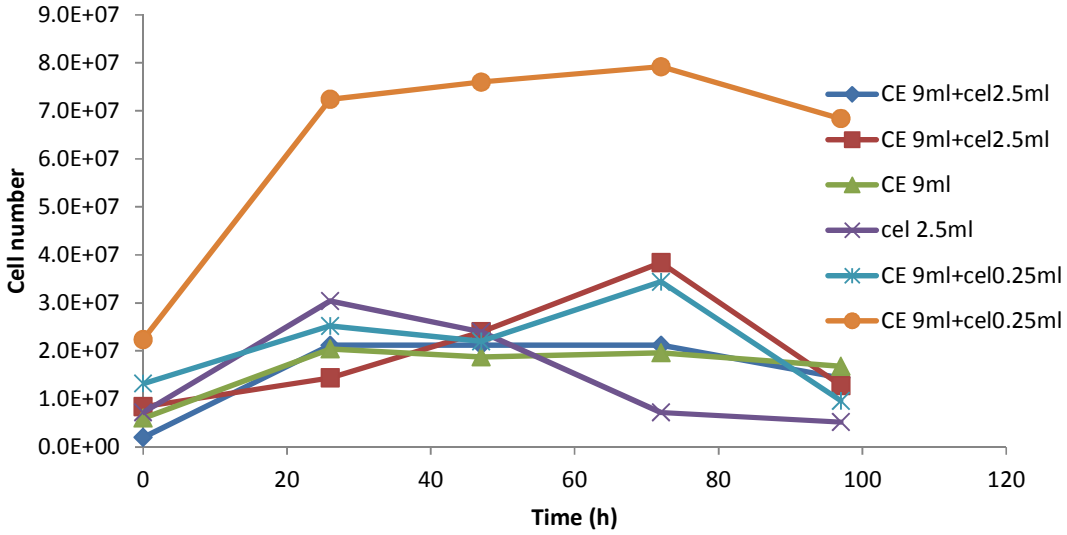


Figure 11 Concentrations of yeast cells when cultivated in different SBP hydrolysates

4.Conclusions

In summary, the highest yield of ethanol (0.84 g/l) was achieved by Y9 in the medium with only SBP hydrolysates under anaerobic cultivation, followed by CBS 2753 generating 0.66 g/l ethanol. Compared to other strains, these two strains (Y9 and CBS 2753) demonstrated highest reducing sugar consumptions (arabinose, galacturonic acid and glucose) among the tested five strains.

SBP hydrolysis with crude extract

The results of the compositions of SBP hydrolyzed with 10 ul/ml crude extract at different incubation times showed that more reducing sugars were liberated as the hydrolysis incubation time continued. The highest yields of reducing sugars occurred on the 7^{th} day incubation.

The test regarding the contents of different liberated reducing sugars in the 7^{th} day culture of SBP hydrolysates at various inoculum levels from 1ul/ml to 20ul/ml exhibited that for the reducing sugars analyzed (rhamnose, arabinose, galactose, glucose, fructose and galacturonic acid), the liberated contents inclined as the inoculums level increased, which demonstrated that high level of inoculums of crude enzyme extract facilitated the SBP hydrolysis. The highest contents of reducing sugars (rhamnose, arabinose, galactose, glucose, fructose and galacturonic acid) were achieved at the

inoculums of 20 ul/ml with the yield of 0.337 g/l, 6.339 g/l, 1.083 g/l, 1.796 g/l, 1.704 g/l and 4.848 g/l, respectively. However, the increments of the reducing sugars contents between the inoculums of 10 ul/ml and 20 ul/ml were not statistically significant except for rhamnose but with only 2.4 % in the SBP.

Enzyme cocktail for the efficient release of glucose from cellulose

The cellulolytic activity of the celllase and crude extract at 40 ^{o}C and 50 ^{o}C were analyzed by DNSA method. The results were demonstrated that the enzymatic activity of the cellulase from sigma was 25.64 U/ml at 40 ^{o}C while 27.12 U/ml was obtained at 50 ^{o}C. For the crude extract, 2.24 U/ml was obtained at 40 ^{o}C, which was 8.89% higher than that assayed at 50 ^{o}C. Statistical analysis (data now shown) proved that no significant differences of the enzymatic activities of cellulase and crude extract were observed between the temperature of 40 ^{o}C and 50 ^{o}C ($p<0.05$).

The experiment on the performance of the enzyme cocktail on hydrolyzing cellulose in SBP showed that increase of the cellulase inoculums corresponded to the increment of the glucose liberated. The highest yield of glucose was achieved at 8.26 g/l after 36 h incubation in the case of adding 1 ml of cellulase and 1ml of crude extract together, which was 8.42 fold of that only hydrolyzed by 1ml crude extract (0.98 g/l glucose). Nevertheless, the contents of glucose liberated with crude extract, cocktail of 1000-time diluted cellulase and cocktail of 10000-time diluted cellulase did not significantly differ from each other, which indicate further dilution of cellulose from 100 time did not contribute to the hydrolysis of cellulose in SBP.

Future plan

(1) The following study will examine other sources of cellulase and further optimize the cocktail performance.
(2) The ethanol fermentation with the SBP hydrolysates in the reactor could be set up next week. Anaerobic, aerobic and microaerophilic conditions could be employed to investigate the optimum ethanol fermentation. The growth curve, pH, ethanol production and carbon source consumption will be analyzed during the entire incubation time.
(3) Since the crude extract harvested from *M. rofsii* did not show high cellulase activity, another fungus was considered to investigate its performance of hydrolyzing SBP or the co-cultivation of these two fungi.

(4) Co-cultivation of fungus and yeasts were conducted by streaking them in the same plates. The results could be obtained next week.

Sixfors Overall, pH values in all cases of incoculum exhibited a declining trend except fermentor 1 with the inoculums of CE 9ml+cel2.5ml of which pH increased slightly after 102.6 h hydrolysis.

No significant differences of the total reducing sugar were observed between the cases of the inoculum with CE 15ml+cel2.5ml and CE 15ml+cel2.5ml. 4.15 g/L and 3.6 g/L glucose on average was liberated with 2.5ml cellulase and 0.25 ml cellulase respectively. Yields of arabinose and galacturonic acid increased fast during the cultivations of 0h -60h when the inoculums contained CE 9ml.

The results confirmed that the combination of crude extract from *S.rofsii* and cellulase resulted in increased reducing sugar liberation compared to the single incubation supplying with either CE or cellulase.

pH profiles during the ethanol fermentation process showed the declining trend in all the cases except for fermentor 1 with the inoculums of CE 9ml+cel2.5ml of which pH increased from 6.33 to 6.58 after 70.75 h fermentation.

In the cases of CE 9ml+cel 0.25 ml inoculated with *Saccharomyces cerevisiae and Debarymtcos*, glucose was utilized fastest by yeast followed by galacturonic acid and arabinose. The results indicated that the arabinose and galacturonic acid could be metabolized by both yeasts to some extent.

The highest ethanol yield (2.0 g/L) was achieved in the case of CE 9ml+cel0.25ml. Further increment of cellulase addition from 0.25 ml to 2.5 ml did not benefit ethanol yield. Overall, higher ethanol over reducing sugar yield was observed for the inoculums of CE 9ml+cel0.25ml than that of CE 9ml+cel2.5ml. And 32.2% increase of higher ethanol over reducing sugar yield was observed for *Debarymycos sp.* than *Saccharomyces cerevisiae* when the inoculums were the same (CE 9ml+cel0.25ml). However, the statement that *Debarymycos sp.* performed better fermentation

properties than *Saccharomyces cerevisiae* on SBP hydrolysates could not be conclusive due to the variance of the reducing sugar compositions.

In all cases, the concentrations of yeast strains reached 10^7 after 26 hour cultivation. The final overall cell concentration in the cultivation with *Debarymycos sp.* was 7.9 *10^7 CFU/ml, higher than the concentration achieved in the cultivation with *Saccharomyces cerevisiae* (3.4*10^7) after 72 hour cultivation with the same enzyme inoculum of CE 9ml+cel0.25ml. The result implied that the carbon sources were more utilized for cell mass accumulation for *Debarymycos sp.* although the same quantity level was achieved both strains.

References

Babalola, O.O., 2007. Pectinase and cellulase enhance the control of Abutilon theophrasti by Colletotrichum coccodes. *Biocontrol Science and Technology*, 17, 53-61.

Chanliaud, E., De Silva, J., Strongitharm, B., Jeronimidis, G., Gidley, M.J., 2004. Mechanical effects of plant cell wall enzymes on cellulose/xyloglucan composites. *Plant Journal*, 38, 27-37.

Doran, J.B., Cripe, J., Sutton, M., Foster, B., 2000. Fermentations of pectin-rich biomass with recombinant bacteria to produce fuel ethanol. *Applied Biochemistry and Biotechnology*, 84-6, 141-152.

Foster, B.L., Dale, B.E., Doran-Peterson, J.B., 2001. Enzymatic hydrolysis of ammonia-treated sugar beet pulp. *Applied Biochemistry and Biotechnology,* 91-3, 269-282.