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Production of Acetone-Butanol-Ethanol from Cassava Rhizome Hydrolysate by *Clostridium Saccharobutylicum* BAA 117

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Cassava rhizome, a by-product from cassava plants, consists mainly of cellulose, hemicellulose and lignin. Thermal pretreatment and alkaline thermal pretreatment were studied. Alkaline thermal pretreatment removed more lignin and hemicellulose than thermal pretreatment. Substrate from alkaline thermal pretreatment showed the maximum cellulose content (81.02%) and hence was hydrolyzed by commercial enzyme preparation (Celluclast 2 L and Novozym 188). The effects of the concentration of substrate (0.75% to 9% w/w) and of enzyme dosage (20 to 30 U/g substrate) were investigated. The highest conentraton of reducing sugar from pretreated substrate (9% w/v) and enzymatic hydrolysis (50 °C, pH 4.8, 48 h) using 30 unit of each enzyme preparation per g of substrate was 72.26 ± 2.11 g/L (89.03% yield). The hydrolysate of Cassava rhizome was used as a carbon source for acetone, butanol and ethanol (ABE) fermentation by *Clostridium saccharobutylicum* (*Cl. saccharobutylicum*) in batch culture. The effects of reducing sugar concentrations on the solvents production were investigated in the range of 40~70 g/L as well as the effects of different pH (pH 4.5-6.0). The results showed that *Cl. saccharobutylicum* BA 117 provided solvent production from cassava rhizome hydrolysate. The optimum pH for ABE production was 5.5 when the initial reducing sugar was 60 g/L. The culture produced only 7.29 g/L ABE from the hydrolysate that had not been treated with polymeric adsorbent resin. The fermentation with inhibitor removal resulted in the production of 10.57 g/L ABE as compared with 13.37 g/L ABE when glucose was used.

1. Introduction

Acetone-butanol-ethanol (ABE) fermentation has been presented as one of the most promising ways of converting biomass to valuable compounds. It has been industrially used in the past with two major substrates, i.e. molasses and grain (Ni *et al.*, 2012). The economic feasibility of the molasses-based fermentation process is decreasing, because the cost of raw material represents major cost of the total cost of final products, and other cheaper substrates are desirable (Zverlov *et al*., 2006). In Thailand, the production of food crops presents the mainstay of national agriculture. Cassava (*Manihot esculenta Crantz*) is one of the important crops. Cassava rhizome is disposed of as a waste resulting in creation of around 20–35% of the original weight as solid waste. Its accumulation is over 6 million tons a year (OAE, 2004). Most of the rhizome context is the cellulose fraction about 43-60% dry weight which is packed with hemicellulose and lignin. Lignin serves as a connection between cellulose and hemicellulose by covalent bonding, whereas hemicellulose is linked to cellulose and lignin fibre (Sun *et al*., 2004). Since the structural complexity of the lignocellulosic matrix is the barrier for acid and enzymatic hydrolysis, the pretreatment is required to remove the surrounding matrix and provide the cellulose-rich fraction (Hendriks and Zeeman, 2009). The releasable sugars obtained from this step can be used as carbon source to produce biogas, bio-fuel and other valuable products. Butanol is a superior fuel to ethanol, as it has higher energy content and can be added to gasoline in high concentration up to nearly 100% of the fuel concentration without vehicle modification (Ladisch, 1991). The best microorganisms for acetone-butanol-ethanol (ABE) production from pentose and hexose sugars obtained from hydrolysis of lignocellulosic

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biomass are the anaerobic *Clostridium* sp. In our previous work*, Cl. acetobutylicum*, *Cl. butylicum* and *Cl. saccharobutylicum* have been found to produce high total solvent from many sugar types and sugarcane juice (Satirapipathkul and Thongkha, 2012). The objective of the present study was to explore the feasibility of using cassava rhizome hydrolysate as carbon source for production of ABE by *Cl. saccharobutylicum* BAA 117. Thermal pretreatment and hot alkaline pretreatment were selected for cassava rhizome prior to enzymatic hydrolysis step. The effects of initial reducing sugar temperature and pH for solvent production by *Cl. saccharobutylicum* BAA 117 were investigated.

2. Materials and methods

2.1 Bacterial strain and Culture conditions

Cl. saccharobutylicum BAA 117 was used for ABE fermentation. The strain was maintained as spores in Reinforced Clostridial Medium (Oxoid Limited, Hampshire, England) at 4°C. The Oxiod RCM has the following typical formula (per liter): “Lab-Lemco” powder, 10.0 g; yeast extract, 3.0 g; peptone , 10.0 g; glucose, 5.0 g; soluble starch, 1.0 g; sodium chloride, 5.0 g; sodium acetate,3.0 g; cysteine hydrochloride, 0.5 g (Huang *et al*., 2004). For seed culture preparation, 1 ml of spore suspension was transferred into 9 ml of RCM medium and subjected to heat shock at 70-80 ^{o}C for 10 min. The spore culture was cultivated under anaerobic conditions at 35 ^{o}C for 24 h. The anaerobic condition was produced and maintained using nitrogen gas feeding.

The medium for the pre-cultures was prepared. The composition of pre-culture medium (per liter) was: glucose, 20 g; Yeast extract, 8.41 g; KH_2PO_4, 0.5 g; CH_3COONH_4, 3 g; $MgSO_4.7H_2O$, 0.3 g and $FeSO_4.7H_2O$, 0.01 g. The pre-culture medium was sterilized at 121 °C for 15 min. 10% (v/v) of cultures were transferred to anaerobic flask containing 60 mL of pre-culture medium and incubated under anaerobic condition at 35 ^{o}C for 24 h.

The fermentation studies were carried out in 1L laboratory fermenter (Mituwa, KMJ 2-3, Japan) with a 400 ml working volume of cassava hydrolysate medium. The composition of fermentation medium (per liter): reducing sugar, 40 - 70 g; Yeast extract, 6 g; CH_3COONH_4, 3 g; KH_2PO_4, 0.5 g; $MgSO_4.7H_2O$, 0.2 g and $FeSO_4.7H_2O$, 0.01 g. The pH of medium was adjusted to 8.0 prior to sterilization (121 °C for 15 min). Before inoculation, the medium was sparged with nitrogen gas to maintain strict anaerobic condition. The fermenter was inoculated with 10% (v/v) of a 24 h old flask culture. The temperature was 35 ^{o}C and the agitation was maintained at approximately 50 rpm. In the controlling pH, the pH examined was ranging from 4.5 to 6.0. For inhibitor removal, the hydrolysate was treated by using a nonionic polymeric adsorbent resin (Amberlite XAD-4, Sigma Chemicals St. Louis, MO) before sterilization. During fermentation, all samples were periodically withdrawn for sugar and ABE measurement.

2.3 Pretreatment and hydrolysis of cassava rhizome

Oven-dried cassava rhizome was cut to 80 mesh size in a Wiley Mill and treated with 4% (w/v) NaOH solution at 100 °C for 30 min. After filtration, the residue was washed with tap water until the solution remains at pH 7. The pretreated sample was dried and cut to 50 mesh size by pulverizer. A weighed amount of pretreated sample in a 0.05 M citrate buffer (pH 4.8) was placed in 1L Erlenmeyer flask and an enzyme solution was added to a final volume of 600 mL. The hydrolysis was performed using commercial enzyme solutions of Celluclast 2L and Novozym 188 (Novo Nordisk A/S, Bagesvaerd, Denmark). Each enzyme was diluted for use at different concentration. The hydrolysis experiments were carried out on a shaker at 100 rpm and at 50°C for 48 h. The hydrolysate was separated by centrifugation at 10,000 rpm for 10 min and analyzed for reducing sugar. All experiments were performed in triplicate.

2.4 Analysis method

The reducing sugar content was analyzed by the 3, 5-dinitrosalicylic acid (DNS) method. Glucose was assayed by Glucose Analyzer (YSI model 27). The composition of cassava samples were analyzed for cellulose, hemicellulose, and lignin fraction (Van *et a*l., 1991). Filter paper activity and cellobiase activity were determined according to Mandels *et al.* (1976). Solvent and acids were analyzed by gas chromatography (Shimadzu, GC 7AG). Cell concentration was estimated by optical density using a predetermined correlation between optical density at 540 nm wavelength and cell dry weight. Three parallel samples were used for all analysis.

3. Results and discussion

3.1 Chemical composition of Cassava rhizome

Thermal pretreatment and alkaline thermal pretreatment were performed to convert lignocellulosic material from its native form into a form which yields the cellulose-rich fraction. The composition of the Cassava rhizome before and after different treatment is presented in Table 1. In comparison with the cellulose content in untreated material, it revealed that the higher cellulose content was obtained after both pretreatments. Both pretreatments removed lignin and hemicellulose and produce higher cellulose content, ranging from 72.63 % to 81.02%. Alkaline thermal pretreatment removed more lignin and hemicellulose than thermal pretreatment. This pretreatment method provided the maximum content of cellulose-rich fraction.

Table 1 Comparison of main components of cassava rhizome (% w/w) after different pretreatment

	Cellulose	Lignin	Hemicellulose
Control	63.13±0.46	25.80±0.34	11.07±2.89
100 ^{o}C, 30 min	72.63±0.98	21.76±2.33	6.14±1.73
4% NaoH, 100 ^{o}C, 30 min	81.02±3.19	13.26±0.76	3.72±0.34

3.2 Hydrolysis of treated cassava rhizome

3.2.1 Effects of substrate concentration

Enzymatic hydrolysis of alkaline thermal pretreated substrate by commercial enzymes was studied. The effect of the concentration of substrate (0.75% to 9% w/v) was investigated. The hydrolysis process by using 20 unit of each enzyme preparation per g of substrate was performed at 50 °C, pH 4.8 for 48 h. Figure 1 showed hydrolysis yield and reducing sugar concentration of enzymatic hydrolysis at different substrate concentration. The maximum hydrolysis yield (85.71%) was obtained with the lowest substrate concentration (0.75%). The highest reducing sugar concentration (64.18 g/L) was obtained at 9% substrate concentration. With increasing substrate concentration, the reducing sugar concentration increased but the hydrolysis yield decreased. This may be due to product inhibition (Wen et al., 2004), decreasing reactivity of the substrate (Sinitsyn et al., 1991) and enzyme inactivation (Reese, 1980).

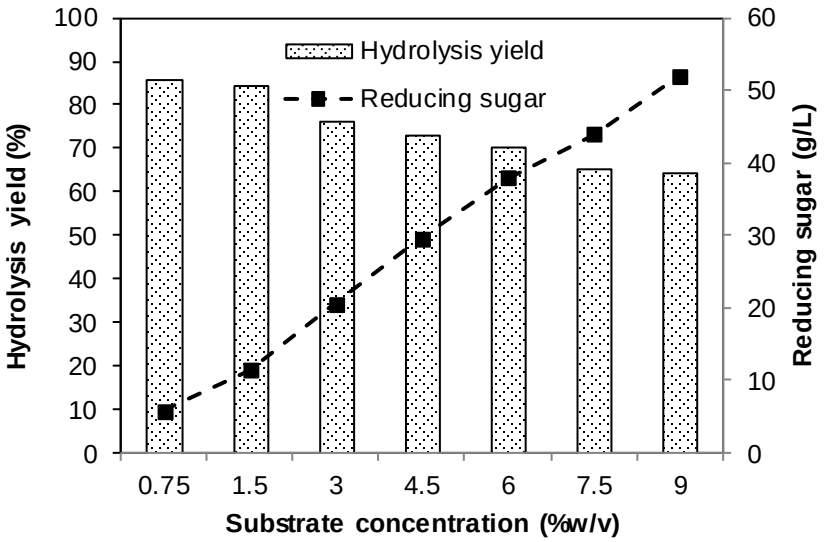

Figure 1: Effects of substrate concentration on the enzymatic hydrolysis at fixed enzyme dosage (20 U/g substrate; 20 U/g substrate).

3.2.2 Effect of enzyme dosage

Hydrolysis experiments were performed with 90 g/L substrate and different dosages of enzymes at pH 4.8 and 50 °C. The result is shown in Table 2. The result revealed that as the enzyme concentration was increased, reducing sugar concentration and hydrolysis yield increased. The optimal enzyme

concentration of each enzyme was 30 unit per gram of substrate. The maximum hydrolysis yield was 89.03 % and the reducing sugar yield was 72.26 g/L.

Table 2 Effects of enzyme dosage on the enzymatic hydrolysis at fixed substrate concentration (9% w/v)

Enzyme dosage (u/g substrate)	Reducing sugar (g/L)	Hydrolysis (%)
20	52.09 ± 1.25	64.18
25	63.72 ± 3.04	78.51
30	72.26 ± 2.11	89.03

3.3 Effect of pH on ABE fermentation

Controlling pH affected solvent production from cassava rhizome hydrolysate by *Cl. saccharobutylicum* BAA 117. The result is presented in Table 3. Although the fermentation was run for 96 h, the culture stopped solvent production within 72 h leaving about 3.72- 7.60 g/L reducing sugar. The ABE production at pH 4.5, 5.0, 5.5 and 6.0 was 2.58, 5.40, 7.29 and 4.04 g/L, respectively. The fermentations that were controlled at pH 5.5 produced the highest solvent production. Solvent concentrations of 7.29 g/L (0.29 g/L acetone, 1.83 g/L butanol, and 5.17 g/L ethanol) were obtained from 54.23 g/L reducing sugar of cassava rhizome hydrolysate after 72 hr. The maximum cell concentration that was achieved during the fermentation was 2.84 ± 0.31 g/L. Control batch fermentation at pH 5.5 was performed using 60 g/L glucose as the carbon source. During 96 h of fermentation 13.37 g/L ABE was produced of which 10.43 g/L was butanol (data not shown). The results suggested that presence of some inhibition substances in cassava rhizome hydrolysate was the reason for low ABE production (Soni *et al.*, 1982).

Table 3: Product profile of Cl. saccharobutylicum BAA 117 in batch fermentations at various pH

	pH 4.5	pH 5.0	pH 5.5	pH 6.0
Acetone (g /L)	0.43	0.26	0.29	0.18
Butanol (g /L)	0.84	0.41	1.83	1.07
Ethanol (g /L)	1.31	4.21	5.17	2.79
Total solvent (g /L)	2.58	5.40	7.29	4.04
Acetic acid (g /L)	5.11	5.32	6.48	6.07
Butyric acid (g /L)	6.07	4.84	7.93	6.92
Sugar utilized (g /L)	56.28	52.40	54.23	53.77

3.3 Effect of initial reducing sugar on ABE fermentation

Enzymatic hydrolysate of cassava rhizome with a reducing sugar concentration of 40 g/L or 70 g/L was used for the ABE fermentation. The inhibitory substances in the hydrolysate were removed by using resin Amberlite XAD-4 which adsorbs furfural and hydroxymethyl furfural (HMF) (Weil *et al*., 2002). The result of the ABE production at pH 5.5 is shown in Figure 2. Cell mass and products are shown in Figure 3. Solvent production in each batch fermentation provided higher butanol and total solvent concentration. The maximum ABE concentration was obtained after 96 h using hydrolysate with 60 g/L reducing sugar. Solvent concentrations of 10.57 g/L (1.58 g/L acetone, 7.35 g/L butanol, and 1.64 g/L ethanol) were obtained from 57.63 g/L reducing sugar of Cassava rhizome hydrolysate after 96 hr. The maximum cell concentration that was achieved during the fermentation was 3.62 ± 0.77 g/L. At lower sugar concentration (50 g/L), the culture provided 8.35 g/l ABE. At higher sugar concentration (70 g/l), the culture provided lower solvent production.

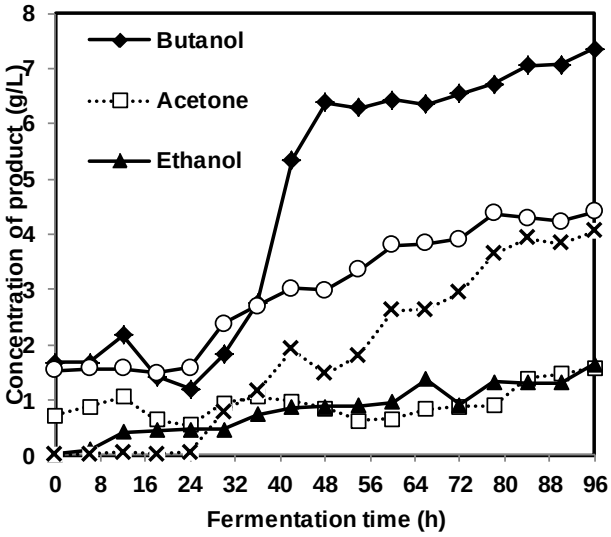

Figure 2: Production of ABE after inhibitor removal by Cl. saccharobutylicum BAA 117 at 35 ^{o}C , pH 5.5 and reducing sugar concentration 60 g/L.

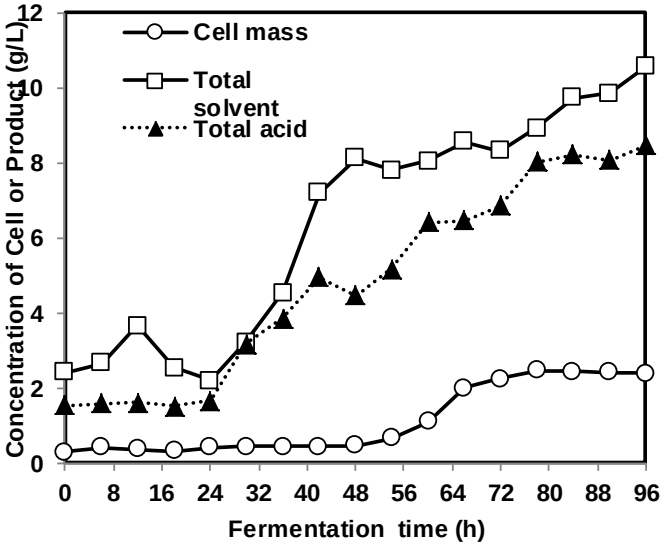

Figure 3: Cell mass and products after inhibitor removal of Cl. saccharobutylicum BAA 117 at 35 ^{o}C , pH 5.5 and reducing sugar concentration 60 g/L.

Table 3: Product profile of Cl. saccharobutylicum BAA 117 in batch fermentations at various reducing sugar content

	40 g/L	50 g/L	60 g/L	70 g/L
Acetone (g /L)	0.31	1.24	1.58	0.97
Butanol (g /L)	3.16	4.53	7.35	4.31
Ethanol (g /L)	3.96	2.58	1.64	3.22
Total solvent (g /L)	7.43	8.35	10.57	8.50
Acetic acid (g /L)	8.84	7.94	4.41	6.82
Butyric acid (g /L)	5.65	6.72	4.06	6.44
Sugar utilized (g /L)	34.59	42.65	57.63	56.11

3. Conclusion

In this work, we have shown that hot alkaline pretreatment is suitable for Cassava rhizome. The optimal condition of enzymatic hydrolysis was 30% (w//v) treated material with 30: 30 unit Filter paper activity and cellobiase at 50 °C, pH 4.8 with 0.05M citrate buffer. Enzymatic hydrolysate of cassava rhizome could be inhibitory to the fermentation by *Cl. saccharobutylicum* BAA 117. The inhibitory substance removal was an important step to promote cell growth and production yield of butanol. The hydrolysate after treatment with Amberlite XAD-4 resin did not show any signs of cell inhibition and resulted in the production of 10.57 g/L ABE from 57.63 g/L total sugars used. The results also demonstrated the importance of optimization of fermentation condition from cassava rhizome hydrolysate. The optimum pH for ABE production was 5.5 when the initial reducing sugar was 60 g/L and 35 ^{o}C.

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