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Utilization of Sugarcane Bagasses for Lactic Acid Production by acid Hydrolysis and Fermentation using *Lactobacillus* sp

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***Abstract*—**Sugarcane bagasses are one of the most extensively used agricultural residues. Using acid hydrolysis and fermentation, conversion of sugarcane bagasses to lactic acid was technically and economically feasible. This research was concerned with the solubility of lignin in ammonium hydroxide, acid hydrolysis and lactic acid fermentation by *Lactococcus lactis*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, and *Lactobacillus casei*. The lignin extraction results for different ammonium hydroxide concentrations showed that 10 % (v/v) NH_4OH was favorable to lignin dissolution. Acid hydrolysis can be enhanced with increasing acid concentration and reaction temperature. The optimum glucose and xylose concentrations occurred at 121 °C for 1 hour hydrolysis time in 10% sulphuric acid solution were 32 and 11 g/l, respectively. In order to investigate the significance of medium composition on lactic acid production, experiments were undertaken whereby a culture of *Lactococcus lactis* was grown under various glucose, peptone, yeast extract and xylose concentrations. The optimum medium was composed of 5 g/l glucose, 2.5 g/l xylose, 10 g/l peptone and 5 g/l yeast extract. *Lactococcus lactis* represents the most efficient for lactic acid production amongst those considered. The lactic acid fermentation by *Lactococcus lactis* after 72 hours gave the highest yield of 1.4 (g lactic acid per g reducing sugar).

***Keywords*—**sugarcane bagasses, acid hydrolysis, lactic acid, fermentation

I. Introduction

LACTIC acid is a potential chemical, which can be used in food, pharmaceutical, textile and other chemical industries [1]. The demand on lactic acid has increased because of its potential use as a monomer for production of biodegradable polymer, polylactic acid (PLA) [2]. Lactic acid can be manufactured either by chemical synthesis or by biotechnological fermentation. The production of lactic acid through biotechnological fermentation has gained interest due to a relatively inexpensive, and environmentally friendly.

Abrupt decreases in petrochemical resources have caused excessive application of lactic acid production by fermentation. Cheap raw materials such as cassava powder [3], agricultural residues [4-5], and molasses [1] are necessary for the feasible economic production of lactic acid.

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One of the most extensively used agricultural residues in Thailand is sugarcane bagasse, which is a byproduct of the sugar industry. Sugarcane bagasse consists of mainly cellulose, hemicellulose and lignin. The hydrolysis of sugarcane bagasse by acid solution gave fermentation sugars such as glucose, xylose and arabinose [5]. Lactic acid can be produced by fermentation of sugars or sugar containing sugarcane bagasse hydrolysates using Lactobacillus sp. [6].

The objectives of this study were to determine the optimum NH_4OH concentration for lignin removal from sugarcane bagasses, to determine the yield of glucose and xylose from sugarcane bagasses hydrolysate under different reaction conditions and to investigate the possibility of the lactic acid fermentation using *Lactococcus lactis*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, and *Lactobacillus casei*.

II. Experimental

A. Sugarcane bagasses

Sugarcane bagasses originating from a factory producing sugar in Thailand were dried in an oven in temperature range of 50-60 °C for 20 hours, then ground in a centrifugal mill, screened into size range of 1.5 - 3.0 mm and kept in a desiccator prior to testing. Lignin contents were determined as Klason lignin following the ASTM D-1106 standard method [7]. Lignin of sugarcane bagasse was found to be 20.88 % w/w.

B. Microorganisms and growth conditions

Lactococcus lactis (TISTR No. 1401), *Lactobacillus delbrueckii* (TISTR No. 326), *Lactobacillus plantarum* (TISTR No. 543), and *Lactobacillus casei* (TISTR No. 390) were grown separately in Lactobacillus deMan Rogosa Sharpe (MRS) medium with composition of 10 g/l peptone, 10 g/l beef extract, 5 g/l yeast extract, 20 g/l glucose, 2 g/l tri-ammonium citrate, 5 g/l sodium acetate, 2 g/l K_2HPO_4, $MgSO_4 \cdot 7H_2O$ 0.2 g/l, $MnSO_4 \cdot 4H_2O$ 0.2 g/l and Tween80 0.1 %(v/v). The MRS medium was sterilised by an autoclave at 121 °C for 15 minutes.

C. Sugarcane bagasses pretreatment

Experiments on the extraction of lignin from sugarcane bagasse were carried out using solvent extraction. Sugarcane bagasse with size range between 1.5 and 3.0 mm was extracted by maceration in 5 - 12 % (v/v) NH_4OH for 12, 24, and 48 hours. Quadruple trials were conducted in all cases at 25 °C. The pulp obtained in each experiment was carried out at a liquor/solid ratio of 100:1 (ml of NH_4OH solution: g dry weight of sugarcane bagasse) in a conical flask with screw cap.

The slurries were washed with distilled water until neutral pH. The solid fraction was dried and used for further acid hydrolysis. Residual lignin contents were determined as Klason lignin.

D.Acid hydrolysis

In this step, pretreated sugarcane bagasse was hydrolyzed with dilute sulphuric acid and heat treatment. All batch experiments were carried out in a conical flask with screw cap. Dried samples, weighing 5 g each were added to 50 ml of different concentrations of sulphuric acid solution (0.25-10 % v/v). These experiments were carried out at 60, 80 and 98 °C using a water-bath shaker for 4 hours and at 121 °C using an autoclave for 1 hour. All experiments were replicated two times. The hydrolyzed solution was adjusted the pH of about 7 with NaOH, subsequently passed through a filter paper and microfiltration (Clyde filter, 0.2 μm) and diluted with sterile distilled water prior to the analysis of glucose and xylose.

E. Fermentation

All batch experiments of *Lactococcus lactis* were carried out in a conical flask with screw cap containing 90 ml of previously sterilised medium for 48 hours of fermentation time at 37 °C under static conditions. The components of the medium were glucose (2.5 - 25 g/l), peptone (3.5 - 20 g/l), yeast extract (2.5 - 15 g/l) and xylose (2.5 - 10 g/l). The 24 hrs-bacterial cell measured by a microscope counting chamber (hemocytometer) was about 4 x 10^8 cells/ml. A 10 % (v/v) inoculum of the bacteria was added to a series of flasks. The medium that contained glucose, peptone, yeast extract and xylose was chosen as an appropriate medium for the growth of *Lactococcus lactis*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, and *Lactobacillus casei*. Experimental conditions for the growth of *L. lactis* were carried out at 37 °C under static condition, whereas for the growth of *L. delbrueckii*, *L. plantarum*, and *L. casei* were set at the same temperature in a incubate shaker at 150 rpm. All fed-batch fermentations were initiated as a batch culture with initial glucose concentration of 5 g/l, peptone concentration of 5 g/l, yeast extract concentration of 5 g/l and xylose concentration of 2.5 g/l, and the mixed solutions of glucose and xylose were added into flask after 72 hours of experiment. Two flasks were removed to provide duplicate samples for each data point. The fermentation broth was passed through a microfiltration (Clyde filter, 0.2 μm) and subsequently diluted with sterile distilled water prior to the analysis of the concentration of lactic acid, glucose and xylose.

F. Analytical methods

The total reducing sugar was measured by the dinitrosalicylic acid (DNS) method [8]. Glucose and xylose concentration was determined by a high performance liquid chromatography system (Model YL 9100, YOUNG LIN INSTRUMENT Co., Ltd, Korea), equipped with a SofTA ELS detector (Model 1400, SofTA Corporation, USA). A HPLC column (Vertisep^{TM} sugar LMP) was used with deionized water as a mobile phase at a flow rate of 0.4 ml/min and column temperature was maintained at 85 °C.

III. Results and Discussion

A. Sugarcane bagasses pretreatment

The ammonium hydroxide treatment had a significant effect on lignin removal from sugarcane bagasse. The residual lignin contents after extraction by 5-12 % NH_4OH were shown in table I. A significantly lower value of residual lignin contents implies higher lignin removal from sugarcane bagasse. Residual lignin contents also decreased with increasing NH_4OH concentrations, reaching minimum value of 0.1107 g lignin per g sugarcane bagasse when 10% NH_4OH were used for 48 hours. The highest lignin removal was calculated to be about 47 %. An increase in reaction time had a positive effect on the lignin removal (table 1), whilst the reducing sugar released also increased. For 5% NH_4OH treatment, the reducing sugar concentrations in the soluble fraction were 0.015, 0.016 and 0.021 g per liter after 12, 24 and 48 hours, respectively. In addition the reducing sugar concentrations in the soluble fraction were 0.017, 0.022, 0.024 and 0.025 g per liter when 6%, 8%, 10% and 12% NH_4OH were used for 48 hours, respectively. This finding can therefore confirm that the procedure for sugarcane bagasses pretreatment by NH_4OH lies in maximizing lignin removal with respect to a lower value of reducing sugar released in the soluble fraction. Thus, 10% NH_4OH treatment for 48 hours was chosen as an appropriate process for sugarcane bagasses pretreatment.

TABLE I
Residual Lignin Contents And Lignin Removal At Different NH_4OH Concentrations And Reaction Time

NH_4OH (% v/v)	Reaction time (hours)	Residual lignin contents (g lignin/ g fiber)	lignin removal (%)
5	12	0.1808	13.4
5	24	0.1751	16.1
5	48	0.1570	24.8
6	48	0.1541	26.2
8	48	0.1281	38.7
10	48	0.1107	47.0
12	48	0.1198	42.6

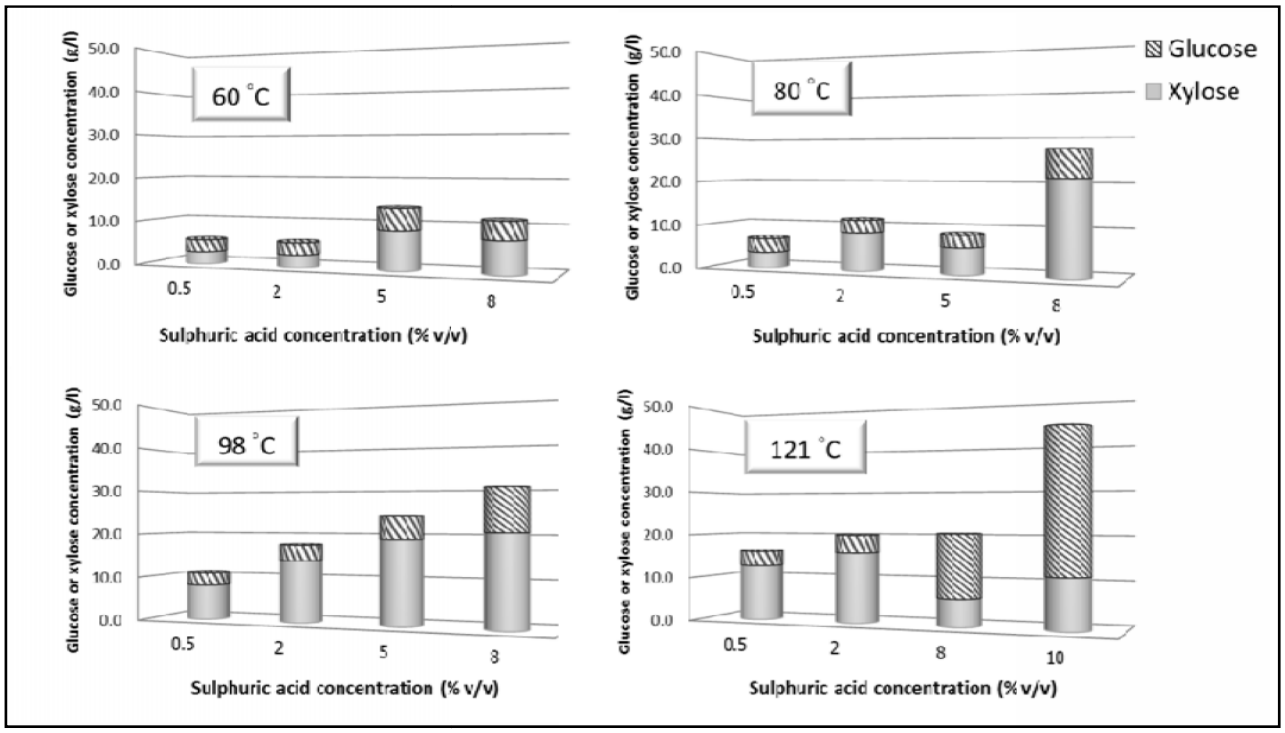

Fig. 1 Glucose and xylose contents of sugarcane bagasse hydrolysed at different temperatures and concentrations of sulphuric acid solutions

B. Acid hydrolysis of pretreated sugarcane bagasses

Glucose and xylose of the soluble fraction of pretreated sugarcane bagasses hydrolyzed at different hydrolysis temperatures and sulphuric acid concentrations are shown in figure 1. The results for different hydrolysis temperatures (i.e. 60, 80 and 98 °C) showed that xylose was the main product, especially when sulphuric acid concentration increased. The results show a good agreement with works of Laopaiboon *et al*., 2010. These authors also reported that glucose and xylose concentrations were 1.2 and 8.4 g/l, respectively, when the sugarcane bagasses were hydrolyzed by 5% H_2SO_4 for 1 hour hydrolysis time at 120 °C. However, the hydrolysis temperature of 121 °C showed a different trend to the other hydrolysis temperatures. As sulphuric acid concentration increased, an increase in glucose concentration was observed. This led to the maximum glucose concentration of 32 g/l which was comparatively very high compared to low xylose concentration of only 11 g/l. Since the xylose content was high but glucose content was low, it appears that this difference was significant and not preferential yield of lactic acid fermentation was occurring under these conditions (table 2). The increase in xylose concentration did not enhance the yield of lactic acid production. This finding can therefore confirm that using 10% H_2SO_4 for 1 hour hydrolysis time at 121 °C was chosen as an appropriate condition for acid hydrolysis.

C. Effect of glucose, peptone, yeast extract and xylose concentrations on lactic acid production

Glucose and xylose of the soluble fraction of The fundamental elements for cell growth and cell biomass are carbon, oxygen, hydrogen, nitrogen, phosphorus, sulphur and magnesium. Organic carbon may have some benefit for the bacteria if there is any toxic metal in the solution, for example yeast extract at low concentration has been shown to diminish toxicity to bacteria [9]. The lactic acid production by batch culture of bacteria depends on various microbial and process parameters, including medium composition. In order to investigate the significance of glucose, peptone, yeast extract and xylose concentrations on lactic acid production, experiments were undertaken whereby a culture of *Lactococcus lactis* was grown under various glucose, peptone, yeast extract and xylose concentrations. Table 2 shows the yield and concentration of lactic acid production for the various medium compositions. The lactic acid concentration increased with the increase of initial glucose concentration, but then remained roughly constant. This was probably due to bacterial growth inhibition under these high glucose concentrations. The results gave the optimal lactic acid concentration of 8.2 g/l, reaching maximum yield value of 1.9 when the components of this medium were 5 g/l glucose, 10 g/l peptone and 5 g/l yeast extract.

TABLE II
EFFECT OF GLUCOSE, PEPTONE, YEAST EXTRACT AND XYLOSE CONCENTRATIONS ON LACTIC ACID PRODUCED IN 48 HRS-FERMENTATION BY BATCH CULTURE OF *LACTOCOCCUS LACTIS*

Medium composition (g/l)				Lactic acid production	
Glucose	Peptone	Yeast extract	Xylose	Yield (g lactic acid / g reducing sugar)	concentration (g/l)
2.5	10.0	5.0	0	2.36	5.9
3.5				1.69	6.8
5.0				1.18	8.2
10.0				0.59	8.6
15.0				0.39	8.6
25.0				0.24	8.7
5.0	3.5	5.0	0	1.46	7.3
	5.0			1.68	8.4
	10.0			1.64	8.2
	15.0			1.72	8.6
	20.0			1.64	8.2
5.0	5.0	2.5	0	1.50	7.5
		5.0		1.68	8.4
		10.0		1.56	7.8
		15.0		1.54	7.7
5.0	5.0	5.0	0	1.90	8.4
			2.5	0.97	7.3
			3.5	0.85	7.2
			5.0	0.72	7.2
			10.0	0.48	7.2

Furthermore, the increase in peptone concentration did not enhance the yield of lactic acid production. The yield values remained roughly constant at value of 1.9 when the peptone concentration was increased from 5 to 20 g/l. Similarly, there was no significant difference in yield of lactic acid production when yeast extract concentrations were in the range of 5 and 15 g/l. However, yield of lactic acid production decreased with increasing xylose concentration, i.e. 0.97, 0.85, 0.72, 0.48. This was probably due to bacterial growth inhibition under these conditions. Although it has been found that acid hydrolysis of sugarcane bagasse show a high yield of glucose in hydrolyzed solution there is also quite a high yield of xylose, which as mentioned previously reduces the yield of lactic acid fermentation. However, the downstream glucose purification unit increased costs. This is deemed to be the distinct advantage of using original hydrolyzed solution. Experiments were undertaken whereby the culture of *L. lactis*, *L. delbrueckii*, *L. plantarum*, and *L. casei* were grown in the presence of xylose concentration of 2.5 g/l.

D.Effect of bacteria strains on lactic acid production

The efficiency of bacterial fermentation of sugar depends upon a number of different factors. *Lactococcus lactis* is one of the dominant bacteria used in lactic acid production. Many researchers have studied either the pure strain obtained from a collection company. Lactic acid bacteria were found to be influenced by various parameters such as medium composition, temperature, pH and other factors.

Lactic acid bacteria are mesophiles having optimum temperatures for growth at around 37 °C. *Lactococcus lactis* [5, 10], *Lactobacillus delbrueckii* [11-15], *Lactobacillus plantarum* [15-17] and *Lactobacillus casei* [11, 18] were chosen as the base strains because they are recognized as being responsible for the lactic acid production. Overall, the lactic acid concentration increased as the fermentation time increases (figure 2). The maximum lactic acid concentration was found to be 11 g/l when using *L. lactis*. This high lactic acid concentration was presumably because the cell bacteria of *L. lactis* were observed to grow better than those of *L. delbrueckii*, *L. plantarum* and *L. casei*. The lactic acid concentrations obtained from the experiments after the addition of sugar solution (72 hours of fermentation time) increased slightly to 10.6 g/l and remained roughly constant until the end of experiments. Therefore, an increase in sugar concentration has only a slight positive influence on lactic acid production. This low lactic acid concentration is likely to be due to the inhibition effects of acidity, since it would appear that when sugar was added to a series of flasks after 72 hours of fermentation, bacterial growth was inhibited, especially *L. lactis.* The lactic acid fermentation is critically dependent on successful bacterial growth under optimum conditions. Table 3 shows the comparison of lactic acid results of some independent researchers. Although, lactic acid production by *L. lactis* from this work was lower in terms of concentration, the maximum of yield was 1.4, compared to 0.4 for the work of Laopaiboon *et al*., [5].

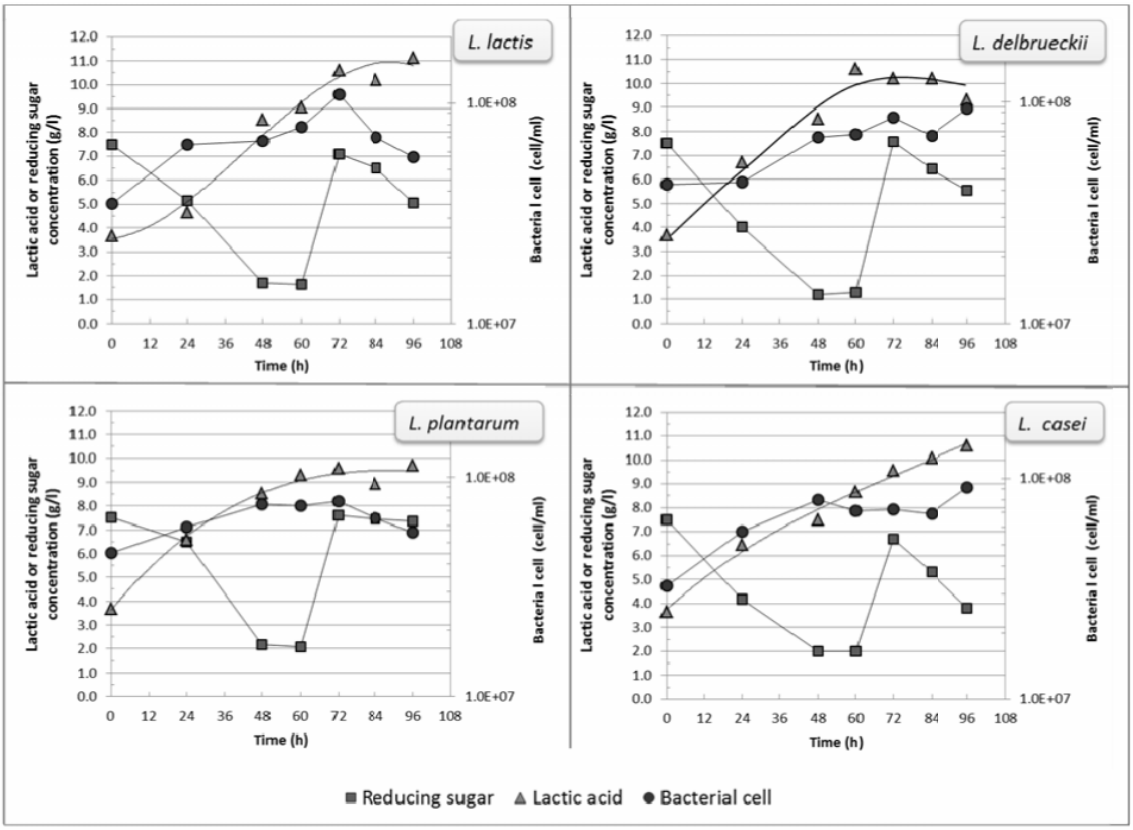

Fig. 2 Time course of lactic acid production by *L. lactis*, *L. delbrueckii*, *L. plantarum*, and *L. casei* in fed batch fermentation. The fermentation was carried out in a medium containing glucose (5 g/l), peptone (5 g/l), yeast extract (5 g/l) and xylose (2.5 g/l)

TABLE III
COMPARISON OF LACTIC ACID RESULTS OF SOME INDEPENDENT RESEARCHERS

Microorganism	Medium	Fermentation time (h)	Lactic acid production		Ref.
			Yield (g lactic acid / g reducing sugar consumed)	Concentration (g/l)	
Lactococcus lactis IO-1	5 g/l yeast extract, 5 g/l peptone, 5 g/l NaCl, 30 g/l xylose	40	0.42	12.7	Laopaiboon *et al.*, [5]
Mixed culture of *Lactobacillus casei* and *Lactobacillus delbrueckii*	150 g/l Cassava bagasse, 12.5 ml/l enzyme, 7.5 g/l yeast extract, 3 g/l NH_4Cl, $CaCO_3$	60	-	78.9	John *et al.*, [11]
Lactobacillus delbrueckii	72 g/l glucose, 15 g/l yeast extract, 5 g/l brain heart infusion, 5 g/l tryptone, 5 g/l tween80	24	0.72	52	Michelson et al., [12]
Lactobacillus casei	140 g/l glucose, 25% (w/w) NH_4OH	24	0.8	112.5	Ding and Tan [18]
Lactococcus lactis	5 g/l glucose, 2.5 g/l xylose, 5 g/l yeast extract, 5 g/l peptone	72	1.4	10.6	This work

IV. Conclusion

The data presented in this work supports the use of sugarcane bagasses for hydrolysis to obtain valuable fermentation products. Lignin extraction by NH_4OH was an essential pretreatment process prior to the acid hydrolysis. Maximum glucose yield of 32 g/l was obtained when sugarcane bagasses was hydrolyzed by 10% (v/v) sulphuric acid solution at 121 $^{\circ}$C. Medium composition of glucose (5 g/l), xylose (2.5 g/l), peptone (10 g/l) and yeast extract (5 g/l) were found to be a potential substrate for lactic acid fermentation. *Lactococcus lactis* was shown to be capable of producing higher yield of lactic acid.

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