Anaerobic Digestion of Glucose by *Bacillus licheniformis* and *Bacillus coagulans* at Low and High Alkalinity

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Abstract : The utilization of glucose in synthetic media at low alkalinity (300 mg $CaCO_3$/l) and high alkalinity (3,500 mg $CaCO_3$/l) by *B. licheniformis* and *B. coagulans* was studied. The initial culture pH was also maintained at neutral condition under high alkalinity. It was found that glucose utilization under high alkalinity was higher than low alkalinity. However, the buffering capacity of the low alkalinity was not effective enough to maintain pH at neutral condition if a large amount of glucose was consumed and high concentration of Volatile Fatty Acids

(VFAs) were produced, resulting in a pH drop. The alkalinity related to pH also had an effect on product formation. Ethanol was the major product at low alkalinity and low pH, whereas lactate was the major product at high alkalinity and high pH. The effect of glucose concentration on glucose utilization was also determined. Both *Bacillus* species could completely consume glucose at 1 and 5g/l, however, glucose utilization decreased when the glucose concentration was increased to 10 g/l. Higher glucose concentration shows higher toxicity to *B. coagulans* than *B. licheniformis*.

Keywords: Bacillus licheniformis, Bacillus coagulans, Alkalinity, Anaerobic digestion, Glucose utilization, Glucose concentration, pH.

Introduction

Anaerobic conversion of agricultural and municipal solid wastes is used to reduce environmental pollution and produce methane [1]. Methane production from agricultural wastes involves at least three distinct bacterial groups, fermentative/ hydrolytic, acetogenic and methanogenic bacteria, whose metabolic interactions lead to the final products of CH_4, CO_2, and microbial cells [2,3]. Methanogenesis of agricultural wastes is especially suited for an agricultural country like Thailand where the availability of such wastes is abundant. Residues from the production and processing of pineapples is one agricultural waste that has been intensively studied for biogas

production at King Mongkut's University of Technology Thonburi (KMUTT) for more than a decade. The early studies found that pineapple waste is a good substrate for methanogenesis [4]. However, the difficulty of methanogenesis from pineapple waste lies in the composition of the wastes which are mainly cellulose with a high concentration of volatile fatty acids. The reactor was also very sensitive to environmental and loading changes

Recently, the dominant fermentative bacteria, *B. licheniformis* and *B. coagulans,* were isolated from an anaerobic digester using pineapple peels at 35°C mesophilic temperature [5]. The fermentative bacteria are facultative anaerobe, hydrolytic/fermentative bacteria that have ability to degrade glucose, xylose and xylan with the optimum growth temperature at 37°C. The fermentative bacteria produce a variety of fermentative products and concentrations, mainly ethanol, acetate, propionate, lactate and butyrate from glucose concentration of 2 g/l. However, it was found that the glucose utilization and product formation was affected by the initial glucose concentration. Increasing glucose from 1 g/l to 2 g/l resulted in an accumulation of lactic acid in *B. coagulans*, whereas there was no accumulation of lactic acid in *B. licheniformis.*

In addition to glucose concentration, alkalinity of the growth medium is also an important parameter for the fermentation process. The alkalinity indicates the buffering capacity of the system as it controls pH in the anaerobic process

and consequently has an effect on glucose utilization and volatile fatty acids (VFAs) formation. Usually, the growth rates of microorganisms are optimal at pH values around neutrality and then fall quickly at high and low pH [6]. This study was thus carried out to determine the effect of low and high alkalinity and glucose concentration on glucose utilization by *B. licheniformis* and *B. coagulans* in order to enhance glucose utilization and product formation.

Materials and Methods

Microorganisms. The fermentative bacteria, *B. licheniformis* and *B. coagulans* were obtained from the Biogas Laboratory at KMUTT. The general characteristics of the fermentative bacteria are illustrated in Table 1.

Table 1. General characteristics of *B. licheniformis* and *B. coagulans* incubated at 35˚C and glucose concentration of 2 g/l [5].

Parameters	Fermentative bacteria	
	B. licheniformis	*B. coagulans*
1. Source of isolation	Mesophilic anaerobic digester of pineapple peels	Mesophilic anaerobic digester of pineapple peels
2. Original selective carbon source	Xylose	Xylose
3. Morphology	Gram positive, rod shape, and spore forming	Gram positive, rod shape, and spore forming
4. O_2 requirement	Facultative	Facultative
5. Ability to utilize various carbon sources	Glucose, xylose, xylan, starch and gelatin	Glucose, xylose, xylan, starch and cellulose
6. Intermediate products	Ethanol, acetate, propionate and lactate	Ethanol, acetate, propionate, lactate and butyrate

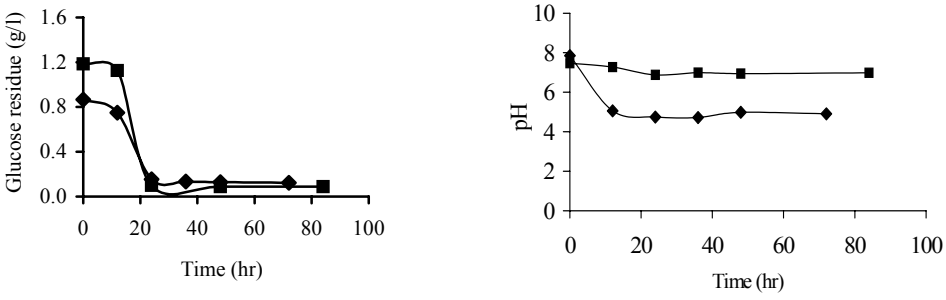
Media and culture conditions. The medium used in this study contains the following compositions (per litre): KH_2PO_4, 0.4 g; K_2HPO_3, 0.4 g; $MgCl_2$, 0.1 g; NH_4Cl, 1 g; yeast extract, 2 g; L-cysteine-HCl, 0.5 g; $Na_2S.9H_2O$, 0.5 g; mineral solution, 10 ml; and vitamin solution, 10 ml [7]. To determine the glucose utilization at low and high alkalinity by *B. licheniformis* and *B. coagulans*, the initial pH of the culture medium was adjusted to 7.0-7.2 using HCl and/or NaOH. Glucose was used as the sole carbon source at concentrations of 1, 5 and 10 g/l. For the experiment with high alkalinity (3,500 mg $CaCO_3$/l), $NaHCO_3$ was added into the culture medium at the concentration of 5 g/l, whereas no $NaHCO_3$ was added into the culture media in the experiment with low alkalinity (300 mg $CaCO_3$/l). To determine the effect of glucose concentration on glucose utilization by *B. licheniformis* and *B. coagulans*, glucose concentrations of 5 and 10 g/l were used with the addition of $NaHCO_3$ at concentration of 5 g/l. The initial pH was also adjusted to 7.0-7.2 by using HCl and/or NaOH. The experiments were carried out by incubating 10 ml (9.0 mg VSS) of *B. licheniformis* or *B. coagulans* into 90 ml of the culture medium in 100-ml vial bottles. The vial bottles then were closed with rubber-stoppers and sealed with aluminum caps before being incubated at 37°C. The samples were taken periodically until glucose was completely utilized or until glucose utilization had ceased.

Analytical techniques. Growth of the bacteria was determined by measuring the volatile suspended solids (VSS),

while glucose utilization and lactic acid production were analyzed by using a Glucose-Lactate Analyzer (YSI 2300STAT PLUS). Methane and carbon dioxide were analyzed by using gas chromatography (GC) with a thermal conductivity detector (Shimadzu model GC 9A), while ethanol and volatile fatty acids were analyzed by using GC with a flame ionization detector (Shimadzu model GC 14B).

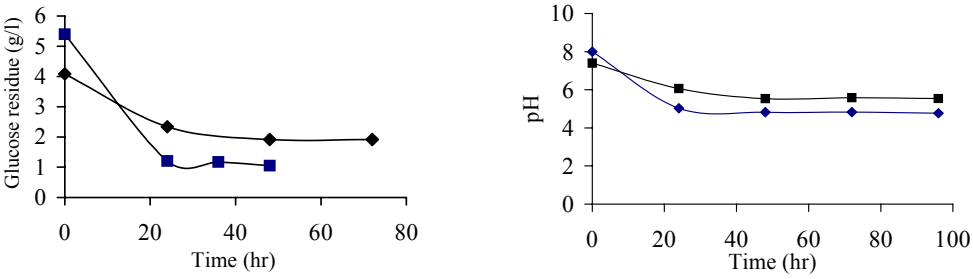
Results and Discussion

Effect of low and high alkalinity on glucose utilization. It was found that the culture with high alkalinity could maintain culture pH at neutral conditions and could utilize a larger amount of glucose than the culture with low alkalinity (Figures 1 and 2). However, the buffering capacity of the low alkalinity was not sufficient to keep pH at neutral conditions until the completion of fermentation if a high concentration of glucose was consumed and a large amount of acids were produced. Figure 1 shows that under low alkalinity, pH of the culture decreased rapidly from 7 to 5 within 12-24 hours with an increase in glucose utilization, whereas the pH was maintained at neutral condition in the culture with high alkalinity. Even though approximately 80% of the initial glucose was utilized at the end of the fermentation, culture with high alkalinity showed a faster glucose utilization rate (0.08 g/min) than the culture with low alkalinity (0.05 g/min) (Figure 1).



(a) (b)

Figure 1. Profile of glucose utilization (a) and culture pH (b) of *B.licheniformis* at 1g/l glucose with low alkalinity (◆) and high alkalinity (■).



(a) (b)

Figure 2. Profile of glucose utilization (a) and culture pH (b) of *B. licheniformis* at glucose concentration of 5 g/l with low alkalinity (◆) and high alkalinity (■).

The profile of the culture pH was not much different when glucose was increased to 5 g/l however, the culture with high alkalinity could maintain higher pH than low alkalinity (Figure 2). This indicated that under the availability of large amounts of glucose, *B. licheniformis* utilized more glucose and produced large amounts of volatile fatty acids beyond the buffering capacity of $NaHCO_3$ to neutralize. Consequently, the pH in the culture with high alkalinity dropped to almost the same levels as

the culture with low alkalinity. However, *B. licheniformis* utilized more glucose at a faster rate under high alkalinity (0.17 g/min) than low alkalinity (0.07 g/min) at 5 g/l glucose (Figure 2). The results were the same with *B. coagulans* (data not shown). The products derived from both *B. licheniformis* and *B. coagulans* were ethanol, acetic acid, and lactic acid. However, lactic acid was the major acid produced during fermentation, thus the reduction of culture pH could be mainly attributed to this accumulation of lactic acid (Figure 3).

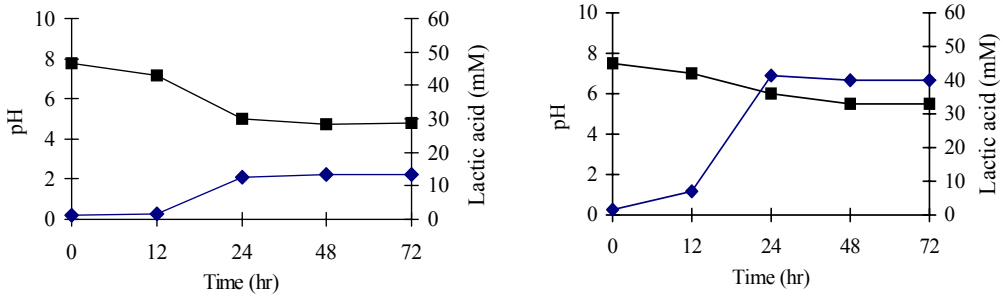


Figure 3. Profile of lactic acid formation (◆) and culture pH (■) of *B. coagulans* during utilization of 5 g/l glucose at low alkalinity (a) and high alkalinity (b).

The above results showed that the alkalinity of the culture medium is important to the fermentation process since it governs the carbonic acid equilibrium and determines the buffering capacity of the fermentation system, hence the value and stability of the pH over the natural range. Tables 2 and 3 show that when pH was maintained at neutral conditions, more glucose was consumed by *B. licheniformis* and *B. coagulans*. In

the presence of 5 g/l glucose with low alkalinity, *B. licheniformis* consumed only 53% of the glucose at 48 hours and 59% at 96 hours, whereas it consumed more than 80% under high alkalinity. In the latter case, the pH of the culture was 5.6, compared to 4.8 of the former case. Similar results were observed with *B. coagulans* (Table 3). Several previous studies from the literature showed a product inhibition on glucose utilization [6, 8]. However, in this case product inhibition was not the major factor since at low pH, lactic acid concentration was still lower than at high pH, thus pH should be regarded as the major inhibitory effect on glucose utilization. For many organic acid fermentation processes, the non-dissociated acids were found to be stronger inhibitors than the dissociated acids, which were influenced by the medium pH. Monot *et al.* (1984) found that the growth of *Clostridium thermoaceticum* on glucose was inhibited by the non-dissociated acetic acid at pH below 6 [9]. Additionally, a decrease in culture pH was accompanied by an increase in non-dissociated acids, which are very toxic to fermentative bacteria [9, 10]. McInerney and Bryant (1981) reported that a decrease in culture pH may shift the fermentative pathway to produce more electron sink products, such as ethanol and lactic acid, that are very toxic to fermentative bacteria upon their accumulation at high concentration [3].

Table 2. pH, glucose utilization and fermentative products of *B. licheniformis* at 1 and 5 g/l glucose and incubated at low and high alkalinity for 48 hours.

Condition	pH	Glucose utilization (%)	Fermentative products (mM)		
			Ethanol	Acetic acid	Lactic acid
1 g/l glucose with low alkalinity	4.97	85	94.46	4.08	3.35
1 g/l glucose with high alkalinity	6.94	92	34.25	3.37	12.21
5 g/l glucose with low alkalinity	4.82	53	84.16	2.59	8.40
5 g/l glucose with high alkalinity	5.58	81	21.39	4.80	42.53

Table 3. pH, glucose utilization and fermentative products of *B. coagulans* at 1 and 5 g/l glucose and incubated at low and high alkalinity for 48 hours.

Condition	pH	Glucose utilization (%)	Fermentative products (mM)		
			Ethanol	Acetic acid	Lactic acid
1 g/l glucose wit low alkalinity	4.88	79	38.76	3.54	11.81
1 g/l glucose with high alkalinity	7.53	86	57.43	3.07	11.68
5 g/l glucose with low alkalinity	4.74	23	65.29	3.20	13.39
5 g/l glucose with high alkalinity	5.60	92	71.02	5.38	17.46

Effect of low and high alkalinity on product formation.

Tables 4 and 5 show glucose utilization, culture pH, and product formation of *B. licheniformis* and *B. coagulans*, respectively. The major products of *B. licheniformis* and *B. coagulans* were ethanol, acetic acid and lactic acid. It was found that the culture with high alkalinity could neutralize the acid production better than the culture with low alkalinity. Thus it could maintain a higher pH and create more suitable environment for the *Bacillus* to utilize greater amounts of glucose and produce higher products than the culture with low alkalinity, especially at 5 g/l glucose. There was a shift in product profile if the culture pH

was low and the most striking effect was on ethanol and lactic acid. There was a higher volume of ethanol at low pH, while a larger amount of lactic acid resulted at high pH. This finding agrees with Gomez and Viniegra-Gonzalez (1981) [11] who reported that ethanol and lactic acid were the main products produced at pH 4.5 and 6.5, respectively. In addition, Neish and Blackwood (1951) [12] reported that a higher pH level resulted in the production of a smaller amount of ethanol and higher amounts of acetate, glycerol and lactate. It was found that as the fermentation progressed, lactic acid production decreased gradually with an increase in acetic acids at 1 g/l glucose in the culture of *B. licheniformis*. This indicates that lactic acid is the intermediate product, which was produced and further degraded to acetic acid by *B. licheniformis*.

Effect of glucose concentration. The profile of glucose utilization by *B. licheniformis* and *B. coagulans* are shown in Figure 4. It was found that *B. coagulans* had a very long lag phase when glucose was increased to 10 g/l. However, there was a rapid increase in glucose utilization after the lag phase. On the other hand, glucose utilization by *B. licheniformis* commenced gradually after the start of fermentation, with a lag phase of only 12 hours. In comparison to 5 g/l, glucose utilization by *B. licheniformis* and *B. coagulans* at 10 g/l glucose decreased where only 39% and 57% of the initial glucose was utilized by *B. licheniformis* and *B. coagulans*, respectively, after 144 hours of fermentation (Tables 4 and 5). The ineffective

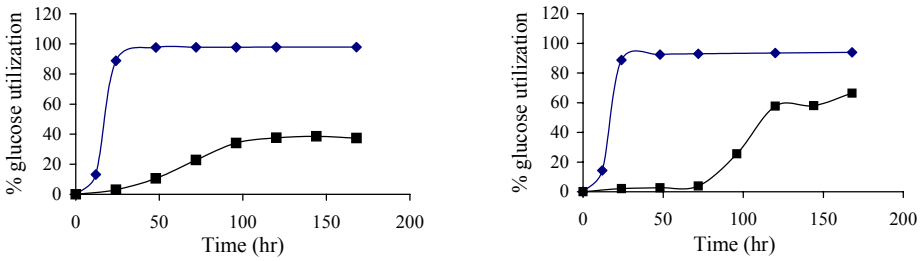
glucose utilization at high glucose concentration was not due to the culture pH, since the pH of both cultures was almost the same. Product inhibition was also ruled out since product formation at glucose concentration of 10 g/l was lower than at 5 g/l. Thus glucose concentration would be the major inhibitor of glucose utilization at 10 g/l glucose. Even though *B. coagulans* consumed a larger amount of glucose than *B. licheniformis* at the end of fermentation, the product yield was lower than *B. licheniformis* (Table 6). This indicates that *B. coagulans* used most of the glucose to produce cells rather than produce products, since the *B. coagulans* culture had a higher bacterial cell count and CO_2 than the *B. licheniformis* culture (Figures 5 and 6) [5]. The influence of initial glucose concentration was also considered by Monot, *et al.* (1984) [9]. They reported that batch fermentation at an optimal pH of 4.8 and with an initial glucose concentration of 7 g/l resulted in very low glucose utilization and low product formation. Only 30% of the initial glucose had been utilized after 50 hours of fermentation when glucose was increased to 60 g/l.

Table 4. Fermentative products of *B. licheniformis* after incubation at 1 and 5 g/l glucose under low and high alkalinity for 48 hours.

Glucose Concentration	Glucose utilization (%)	Culture pH	Fermentative products (mM)		
			Ethanol	Acetic acid	Lactic acid
1 g/l	a) Low alkalinity				
	13 (12 hr)	5.06	76.52	3.38	9.58
	82 (24 hr)	4.74	103.50	3.04	7.48
	85 (48 hr)	4.97	94.46	4.08	3.35
	b) High alkalinity				
	5 (12 hr)	7.27	22.45	0.64	0.39
	92 (24 hr)	6.88	38.89	3.56	14.31
	92 (48 hr)	6.94	34.25	3.37	12.21
5 g/l	a) Low alkalinity				
	43 (24 hr)	5.03	73.35	2.79	10.50
	53 (48 hr)	4.82	84.16	2.59	8.40
	59 (96 hr)	4.77	122.97	4.79	7.22
	b) High alkalinity				
	62 (18 hr)	6.06	14.49	1.10	32.55
	78 (24 hr)	5.57	31.30	2.57	42.53
	81 (48 hr)	5.58	21.39	4.80	42.53

Table 5. Fermentative products of *B. coagulans* after incubation at 1 and 5 g/l glucose under low and high alkalinity for 48 hours.

Glucose concentration	Glucose utilization (%)	Culture pH	Fermentative products (mM)		
			Ethanol	Acetic acid	Lactic acid
1 g/l	a) Low alkalinity				
	53 (12 hr)	5.97	102.14	2.75	6.83
	79 (24 hr)	5.06	51.63	3.89	12.34
	79 (48 hr)	4.88	38.76	3.54	11.81
	b) High alkalinity				
	86 (12hr)	7.36	43.48	3.40	9.78
	86 (24 hr)	7.81	63.75	3.12	8.93
	86 (48 hr)	7.53	57.43	3.07	11.68
5 g/l	a) Low alkalinity				
	14 (24 hr)	5.00	52.80	3.23	12.07
	23 (48 hr)	4.74	65.29	3.20	13.39
	26 (72 hr)	4.81	56.36	3.80	13.39
	b) High alkalinity				
	64 (24 hr)	6.27	43.66	4.04	13.39
	92 (48 hr)	5.60	71.02	5.38	17.46
	92 (72 hr)	5.45	43.40	5.31	19.03

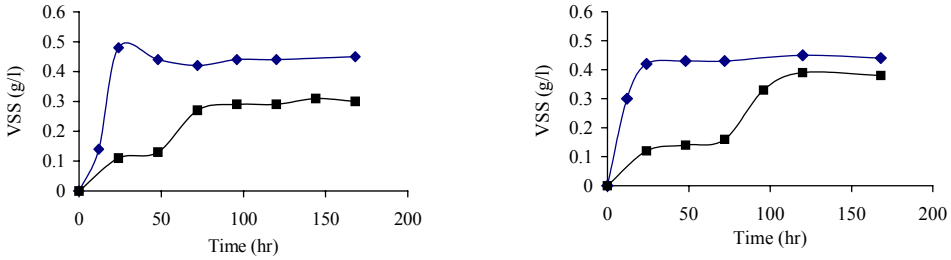


(a) (b)

Figure 4. Glucose utilization by *B. licheniformis* (a) and *B. coagulans* (b) at glucose concentrations of 5 g/l (◆) and 10 g/l (■).

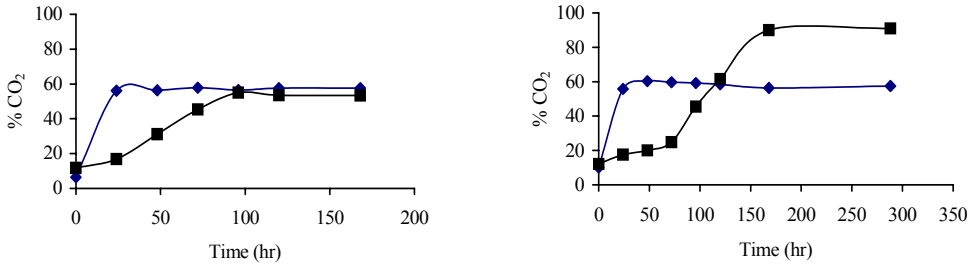
Table 6. Culture pH, VSS, CO_2 and product yield of *B. licheniformis* and *B. coagulans* after fermentation for 48 hours.

Starting glucose	Bacillus species	pH	VSS (g/l)	CO_2 (%)	Fermentative products (mM)/glucose utilized (g)		
					Ethanol	Acetic acid	Lactic acid
5 g/l	*B. licheniformis*	5.56	0.44	57.63	3.37	0.40	8.73
	B. coagulans	5.49	0.45	58.43	2.75	0.29	7.17
10 g/l	*B. licheniformis*	5.59	0.31	53.34	9.86	0.16	7.24
	B. coagulans	5.12	0.40	82.96	4.16	1.10	4.72



(a) (b)

Figure 5. Growth of *B.licheniformis* (a) and *B. coagulans* (b) at glucose concentration of 5 g/l (◆) and 10 g/l (■).



(a) (b)

Figure 6. Profile of CO_2 of *B. licheniformis* (a) and *B. coagulans* (b) at glucose concentrations of 5 g/l (◆) and 10 g/l (■).

Conclusion

In summary, the pH of the growth medium is an important parameter to many fermentation processes. This study found that the alkalinity of the culture medium has strong effect on the culture pH, glucose utilization and product formation. Under high alkalinity, acid production was neutralized, thus the culture pH was controlled close to neutral condition, creating suitable conditions for *Bacillus* to utilize more glucose and produce a higher volume of products than the culture with low alkalinity. The initial glucose concentration was also found to play an important role in glucose utilization by the *Bacilli*. Glucose utilization decreased when the glucose concentration was increased to 10 g/l and a longer lag phase was found in the culture of *B. coagulans* than with *B. licheniformis*.

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