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Short Communication

Butanol production from thin stillage using *Clostridium pasteurianum*

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ABSTRACT

The production of butanol from thin stillage by *Clostridium pasteurianum* DSM 525 was evaluated in the paper. At initial pH values ranging from 5.0 to 7.0 *C. pasteurianum* DSM 525 produced 6.2–7.2 g/L of butanol utilizing glycerol in thin stillage as the main carbon source, with yields of 0.32–0.44 g butanol produced/g glycerol consumed, which are higher than previously reported yields (e.g., 0.14–0.31 g butanol/g glycerol, Biebl, 2001). Lactic acid in the thin stillage acted as a buffering agent, maintaining the pH of the medium within a range of 5.7–6.1. Lactic acid was also utilized along with glycerol, enhancing butanol production (6.5 g/L butanol vs. 8.7 g/L butanol with 0 and 16 g/L lactic acid, respectively). These results demonstrate the feasibility of cost-effective butanol production using thin stillage as a nutrient-containing medium with a pH buffering capacity.

1. Introduction

Global production of ethanol, one of the most popular renewable fuels, has increased during the last decade. However, for the process of ethanol production to be cost-effective and environmentally sustainable, it is essential to consider the treatment and utilization of stillage (Wilkie et al., 2000). For each liter of ethanol produced, up to 20 L of stillage is generated after the distillation process (Van Haandel and Catunda, 1994) and the chemical oxygen demand of stillage is in a range of 30–91 g/L depending on the type of feedstock (Wilkie et al., 2000). Stillage (also referred to as whole stillage) is usually separated into a solid fraction (distillers' grains) and a liquid fraction (thin stillage) through centrifugation. The solid fraction is usually dried and mixed with the concentrated thin stillage to produce dried distillers' grains with solubles (DDGS), which is sold as animal feed (Kim et al., 2008). Some of the thin stillage is either recycled as process water or sent to wastewater treatment facilities. Recently, given the large amount of stillage generated from ethanol production facilities and the low price of DDGS, researchers have investigated the use of stillage or DDGS as a substrate for biofuel production (Ezeji and Blaschek, 2008; Davis et al., 2005). In these processes, chemical/physical pretreatment and enzymatic hydrolysis of distillers' grains are required, because carbohydrates in the distillers' grains are primarily unfermentated fiber materials that must be decomposed to fermentable sugars for biofuel production. Unlike distillers' grains and DDGS, thin stillage itself has not been widely considered as a substrate for biofuel production due to its low concentration of carbohydrates and its complex chemical compositions (Gonzalez et al., 2010). Condensed thin stillage can be added to distillers' grains to produce DDGS; however, evaporating a large amount of water from thin stillage is a costly and energy-consuming process.

In this study, butanol fermentation by *Clostridium pasteurianum* DSM 525 using thin stillage (containing glycerol) was investigated. Glycerol is a by-product of ethanol fermentation and its concentration in thin stillage ranges from 5.1 to 24.6 g/L (Dowd et al., 1994; Gonzalez et al., 2010; Kim et al., 2008). *C. pasteurianum* DSM 525 can produce butanol using glycerol as a sole source of carbon and energy (Biebl, 2001). Other organic compounds in thin stillage are lactic and acetic acids, which are mainly produced by contaminating bacteria in the ethanol fermentation process (Skinner and Leathers, 2004). Therefore, the effects of these compounds on butanol fermentation were also examined.

2. Methods

2.1. Microorganisms and media

Saccharomyces cerevisiae KCTC 7296 was purchased from the Korea Collection for Type Cultures (KCTC) and pre-cultured in a GPY medium [glucose 4% (w/v), peptone 0.5% (w/v), and yeast extract 0.5% (w/v)] at 30 °C. *C. pasteurianum* DSM 525 was purchased from DSMZ. For preparation of the pre-culture, the spore suspension was heat-shocked at 80 °C for 10 min and inoculated into an MP2 medium (pH 6.5, modified from Baer et al., 1987), which contained 30 g/L of glycerol as a carbon source and 100 mM of 2-(*N*-morpholino) ethanesulfonic acid (MES) as a pH buffering agent.

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2.2. Preparation of thin stillage

Whole stillage was either prepared in the authors' laboratory or obtained from a local ethanol fermentation plant. The pre-culture of *S. cerevisiae* KCTC 7296 was inoculated (5% v/v) into a 3-L fermentor (Fermentec, Cheongwon, Korea) containing a 2-L mineral medium (pH 5.0, modified from Alfenore et al. (2002)), which contained 220 g/L of glucose as a carbon source (Y-Glu medium). Lactic acid (5 g/L) was added to Y-Glu medium (Y-Glu medium w/lactic acid) in order to simulate the contamination of lactic acid bacteria. Thin stillage using Y-Glu medium (no lactic acid) was also prepared to investigate the effect of lactic acid on butanol fermentation. Ethanol fermentation was carried out at 30 °C with shaking (300 rpm) for 84 h. Ethanol in the fermentation broth was removed using a rotary evaporator (Sunil Eyela, Korea) and the concentrate was diluted with distilled water to 89% of the initial volume of the fermentation broth. The local ethanol fermentation plant, from which the whole stillage was obtained, used tapioca and brown rice as feedstock and was operated without stillage recycling. All stillage samples were filtered through a 0.45 μm syringe filter to produce thin stillage.

2.3. Fermentation for butanol production

Butanol fermentation was performed using thin stillage prepared in the laboratory with either Y-Glu medium or Y-Glu medium w/lactic acid to investigate the effect of lactic acid in thin stillage on butanol production. For the thin stillage obtained from a local ethanol plant, 15 g/L of glycerol was added to bring the total concentration of glycerol to 20 g/L. The initial pH of the thin stillage was adjusted to 5.0, 6.0, or 7.0 with potassium hydroxide. Y-Glu medium containing 20 g/L of glycerol instead of 220 g/L of glucose (Y-Gly medium) was also prepared for butanol fermentation to compare butanol production behavior before and after ethanol fermentation.

To investigate the effects of lactic and acetic acids on butanol fermentation, 0–20 g/L of lactic acid, or 0–10 g/L of acetic acid, was added to MP2 medium and the initial pH was adjusted to 6.5 with sodium hydroxide.

Butanol fermentation was carried out in a 125-mL serum bottle containing 50 mL of medium. The medium was purged with argon gas to remove dissolved oxygen, and the serum bottle was then sealed with a septum and an aluminum crimp seal. After the serum bottle was autoclaved, *C. pasteurianum* DSM 525, grown overnight (37 °C) in MP2 medium, was inoculated (1% v/v) and incubated at 37 °C with shaking (130 rpm).

2.4. Analytical methods

The samples were analyzed as previously described (Lee et al., 2008a). The concentration of glycerol was analyzed using Free Glycerol Reagent (Sigma, USA). Lactic acid was measured by high-performance liquid chromatography (RID G1362A, 1200 series, Agilent Technologies, Germany; Aminex HPX-87 H Ion Exclusion Column 300 × 7.8 mm, Bio-Rad, USA). Butanol fermentation results are presented in the tables as the average values of duplicate experiments. The range of raw data was within 10% of the average value.

3. Results and discussion

3.1. Butanol fermentation using thin stillage as a medium

Table 1 shows the compositions of the thin stillage prepared from Y-Glu medium w/lactic acid and that obtained from a local ethanol fermentation plant. The concentration of glycerol was higher in the thin stillage prepared in the laboratory than that obtained from the ethanol plant. Factors influencing the production of glycerol during ethanol fermentation include temperature, pH, osmotic pressure, the form of nitrogen source, and yeast strain (Scanes et al., 1998). Glycerol concentration in thin stillage was reported to increase up to 40 g/L when thin stillage was recycled as make-up water for ethanol fermentation (Cheryan and Parekh, 1995). Glycerol content in the thin stillage obtained from the local ethanol plant is likely to increase through stillage recycling.

Table 1
Compositions of thin stillage prepared in the laboratory and obtained from a local ethanol fermentation plant.

	Prepared from Y-Glu w/lactic acid[a,b]	Obtained from an ethanol plant
Ethanol (g/L)	0.6 ± 0.7	0.3
Glycerol (g/L)	19.0 ± 3.9	5.1
Acetic acid (g/L)	1.3 ± 0.8	1.0
2,3-Butanediol (g/L)	4.6 ± 0.9	0.4
Glucose (g/L)	0.4 ± 0.4	Not detected
Lactic acid (g/L)	4.0 ± 0.0	5.7
pH	3.7 ± 0.2	3.7

[a] Y-Glu medium with 5 g/L of lactic acid as described in Section 2.
[b] Average values and standard deviations of the results obtained from three

Table 2 shows the results of butanol fermentation when thin stillage was used as a medium. When the thin stillage was used without pH adjustment (initial pH of 3.9), *C. pasteurianum* DSM 525 did not grow. When the pH was adjusted to 5.0–7.0, *C. pasteurianum* DSM 525 grew and the butanol production was in a range of 6.2–7.2 g/L. Interestingly, the pH values of the media converged to a range of 5.7–6.1 at the end of the fermentation irrespective of the initial pH. This appears to be caused by the buffering effect of lactic acid and the consumption of lactic acid by *C. pasteurianum* DSM 525. The pH buffering effect by lactic acid is supported by the pH variation observed in the experiment with thin stillage prepared from Y-Glu medium containing no lactic acid. In this experiment, pH values of the media decreased from 5.2, 6.0, and 6.8 to 4.9, 5.1 and 5.2, respectively, during 72 h of fermentation (Fig. S1).

Butanol yields were in a range of 0.32–0.44 g butanol produced/g glycerol consumed, and increased with decreasing initial pH of thin stillage (Table 3). This is typical of butanol fermentation at low pH, as butanol-producing microorganisms mainly produce solvents rather than acids to avoid the inhibitory effect of acids at low pH (Lee et al., 2008b; Napoli et al., 2011). Butanol yields obtained in this study were higher than the value observed using MP2 medium containing no lactic acid (0.27 g butanol/g glycerol, Table 4) as well as previously reported values (e.g., 0.14–0.31 g butanol/g glycerol, Biebl, 2001). These higher yield values appear to be attributable to two factors. First, part of the lactic acid consumed by *C. pasteurianum* DSM 525 is likely to be converted to butanol. This was supported by the finding that the butanol yields (0.26–0.38 g butanol/g glycerol, Table 3) using thin stillage prepared from Y-Glu medium containing no lactic acid were lower than those using thin stillage with lactic acid. Because *C. pasteurianum* DSM 525 did not utilize lactate as a sole source of carbon and energy (data not shown), it appears that *C. pasteurianum* DSM 525 uses lactic acid as a co-substrate and glycerol as the primary substrate. Second, some unknown organic matters formed during ethanol fermentation was utilized by *C. pasteurianum* DSM 525, leading to a higher butanol production. In fact, when *C. pasteurianum* DSM 525 grew in Y-Gly medium (i.e., Y-Glu medium with glycerol instead of glucose), the butanol yields were further decreased to a range of 0.23–0.29 g butanol/g glycerol (Table 3). This result indicates that high butanol yields using thin stillage was not

Table 2
Butanol fermentation by *C. pasteurianum* DSM 525 using thin stillage at various initial pH values.

	Stillage prepared from Y-Glu w/lactic acid			Stillage obtained from an ethanol plant		
	pH 5.0[b] (67 h)[a]	pH 6.0[b] (67 h)[a]	pH 7.0[b] (67 h)[a]	pH 5.0[b] (72 h)[a]	pH 6.0[b] (48 h)[a]	pH 7.0[b] (48 h)[a]
pH						
Initial[c]	4.7	5.7	6.5	5.1	5.8	6.1
Final	5.7	5.9	6.0	6.1	6.1	6.1
Consumption						
Glycerol (g/L)	15.7	17.6	18.2	15.9	18.9	20.2
Lactic acid (g/L)	3.3	2.9	2.7	4.7	4.7	4.6
Production						
Butyric acid (g/L)	0.4	0.8	1.9	1.7	2.9	2.7
Butanol (g/L)	6.5	6.7	6.2	6.9	7.2	6.5
1,3-Propanediol (g/L)	ND[d]	ND[d]	0.5	2.7	2.5	2.5

[a] Fermentation time. Product analysis was performed at the end of fermentation.
[b] Measured before autoclaving.
[c] Measured after autoclaving.
[d] Not detected.

Table 3
Butanol yield according to the type of medium and initial pH.

	Butanol yield (g butanol produced/g glycerol consumed)			
	Stillage prepared from Y-Glu w/lactic acid	Stillage obtained from an ethanol plant	Stillage prepared from Y-Glu (no lactic acid)	Y-Gly[a]
pH 5.0	0.42	0.44	0.38	0.28
pH 6.0	0.38	0.38	0.34	0.29
pH 7.0	0.34	0.32	0.26	0.24

[a] The Y-Gly medium is the Y-Glu medium with glycerol instead of glucose as described in Section 2.

Table 4
Effect of lactic acid on butanol fermentation by *C. pasteurianum* DSM 525. Fermentation was carried out in MP2 medium for 41 h.

Lactic acid added (g/L)	0 (control)	4	8	12	16	20
Maximum OD_{600}	11.8	9.5	8.4	6.6	5.6	0.9
Glycerol consumed (g/L)	24.0	24.1	24.0	23.5	24.0	8.3
Lactic acid consumed (g/L)	–	2.5	4.4	7.0	9.3	2.0
Butanol (g/L)	6.5	6.7	6.9	8.0	8.7	0.5
Butanol yield (g/g)[a]	0.27	0.28	0.29	0.34	0.36	0.06
Final pH	5.7	5.9	6.1	6.3	6.5	5.8

[a] g butanol produced/g glycerol consumed.

attributed to the nutrients in Y-Glu medium but to unknown organic matters in thin stillage generated after ethanol fermentation. Acetic acid and 2,3-butanediol made no contribution to butanol yield, because these compounds were not consumed during the butanol fermentation by *C. pasteurianum* DSM 525.

The use of thin stillage for butanol fermentation is expected to provide significant advantages: first, on the basis of successful butanol fermentation using thin stillage demonstrated in this study, thin stillage has potential as a medium providing all the nutrients necessary for butanol fermentation. The addition of carbon sources (i.e., glycerol) or other nutrients might be necessary depending on the composition of thin stillage. Second, the buffering capacity of thin stillage is beneficial, because the amount of base titrant during butanol fermentation can be reduced significantly. Note that pH control was not even necessary in this study once the initial pH of the medium was adjusted to 5.0–7.0.

3.2. Effects of lactic acid and acetic acid on butanol fermentation

Table 4 shows the effect of lactic acid on butanol fermentation by *C. pasteurianum* DSM 525 in MP2 medium. As the amount of lactic acid added was increased, the maximum cell density decreased. The presence of 20 g/L of lactic acid inhibited cell growth significantly. The final butanol concentration and the yield increased with the initial lactic acid concentration up to 16 g/L (Table 4). The addition of lactic acid also increased the final pH compared with that of the control. Note that beneficial effects of lactic acid on butanol production, butanol yield, and the buffering capacity of the medium were also observed in the results obtained with thin stillage (Tables 2 and 3). When acetic acid was added to MP2 medium from 0 g/L to 10 g/L, butanol production was slightly decreased (7.5 ± 0.1 g/L and 6.5 ± 0.2 g/L butanol with 0 g/L and 10 g/L acetic acid, respectively). The consumption of acetic acid was not observed. Because the concentrations of lactic acid and acetic acid in thin stillage are generally reported to be below 16 g/L and 10 g/L, respectively (Dowd et al., 1994; Kim et al., 2008), it is expected that the inhibitions caused by these acids would be insignificant in butanol fermentation by *C. pasteurianum* DSM 525.

4. Conclusions

Clostridium pasteurianum DSM 525 produced 6.2–7.2 g/L of butanol from thin stillage containing 20 g/L of glycerol at initial pH values ranging from 5.0 to 7.0. Lactic acid and acetic acid did not significantly inhibit butanol production up to the concentrations of 16 g/L and 10 g/L, respectively. In addition to glycerol, lactic acid was utilized by *C. pasteurianum* DSM 525 and thus increased butanol production. Lactic acid also acted as a buffering agent, maintaining the pH of the medium within a range of 5.7–6.1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.01.046.

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