2015 International Conference on Alternative Energy in Developing Countries and Emerging Economies

Ethanol Production from Reused Liquid Stillage

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Abstract

Stillage, a liquid waste remaining after ethanol distillation, was collected and characterized. The liquid stillage contained reducing sugars (fructose and glucose) and volatile fatty acids including acetic acid, propionic acid and butyric acid. 1.48 g/L of reducing sugar was found in raw stillage. Therefore, the stillage was able to be utilized as a substrate for ethanol production. The production of ethanol from stillage was done using different glucose concentrations [10, 20, 40 and control (raw material, no glucose addition)] and different microorganisms including Saccharomyces cerevisiae and Enterobacter aerogenes. In addition, the mix-culture of S. cerevisiae and E. aerogenes for ethanol production was also evaluated. The process was done under batch fermentation at 35°C, 100 rpm for 96 h. The results showed that mix-culture between S. cerevisiae and E. aerogenes yield the highest ethanol (1.762 g ethanol/ g sugar) after 72 h of cultivation using the raw stillage (non-glucose addition) as a substrate.

Keywords: Ethanol, Liquid stillage, Simultaneous Saccharification and Fermentation

1. Introduction

Bioethanol, a clean and renewable energy, is considered a new resource of fuel instead of petroleum oil [1]. Bioethanol can be produced from raw materials such as simple sugars, starch and lignocellulose. Sugar- and starch-based are the most well known substrates for ethanol production [2, 3]. The major drawback of utilization of raw materials for ethanol production is their cost and the availability of raw

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materials. Therefore, to search for novel substrate, the creation of a sustainable bioethanol industry with decreased energy consumption and a resolution of the problem of wastewater treatment are crucial. Stillage, a liquid remaining waste after ethanol distillation, has been suggested as a novel substrate for ethanol production. The pH of fresh liquid stillage was acidic (pH 3.8–4.7), and the chemical oxygen demand (COD) was 90 g/L (with dissolved solids at 55 g COD/L). The total and reducing sugar contents were 17 and 6 g/L, respectively. Suspended solids were 20–30 g/L, and the total nitrogen content was 6 g/L [4]. Due to chemical composition of the liquid stillage such as total and reducing sugar showed that the stillage can be used as a substrate for ethanol production. Therefore, the objectives of this study aimed to determine and characterize pineapple-ethanol stillage. Moreover, the stillage was re-utilized as a substrate for ethanol production. In addition, the optimal conditions for ethanol production including glucose concentration and type of microorganisms were also evaluated.

2. Research methodology

2.1 Strain and medium

Saccharomyces cerevisiae TISTR 5048 and Enterobacter aerogenes TISTR 1468 were obtained from the Thailand Institute of Scientific and Technology Research (TISTR) and used throughout this study. The culture of S. cerevisiae was maintained on MPDY slant (malt extract 0.3%, peptone 0.5%, yeast extract 0.3% and dextrose 2%) and agar (1.5%) at 4°C [1]. E. aerogenes was cultivated on Luria-Bertani (LB) agar (peptone 0.5%, beef extract 0.3% and agar 1.5%). Each inoculum was prepared by growing the cell in 25 mL of the sterile liquid medium in 100 mL conical flask on a rotary shaker (100 rpm) for 48 h [5].

2.2 Pineapple peel pretreatment

Pineapple peel was obtained from locally canned pineapple in Chumphon, Thailand. It was dried and milled to a particle size of 40 BS (British standard) mesh in an apex mill. The pineapple peel was firstly reduced to a small size followed by pretreatment to increase enzymatic hydrolysis. Therefore, a pretreatment process was necessary. For the pretreatment process, 20 g of dry feedstock was placed in a 250 mL plastic centrifuge tube, and then mixed with 200 mL of distilled water. The sample was incubated in a water bath (100°C, 240 min). Afterward, the pretreated pineapple peel was collected and dried in an oven (60°C, 72 h) before used [5].

2.3 The production of ethanol from pretreated pineapple peel

Pretreated pineapple peel was utilized as a substrate for ethanol production by E. aerogenes. 6% (w/v) of pineapple peel with the addition of nutrient broth medium (250 mL) and was sterilized. Afterward, 10% (v/v) of inoculum and 0.012% (w/v) cellulase was added. The culture was incubated up to 4 days under a rotary shaker (120 rpm). The experiments were maintained at 35°C (pH 7). Afterwards, ethanol was recovered and distilled. The liquid stillage was collected and determined for reducing sugar and volatile acid concentration. Thereafter, the stillage was re-utilized for ethanol fermentation [5].

2.4 Component analysis of liquid stillage from distillation process

The liquid stillage was collected and determined for total solids (TS), volatile solids (VS), total soluble solids (TSS) and chemical oxygen demand (COD) contents. All parameters were measured according to the APHA standard methods for the examination of water and wastewater [6].
The qualitative analysis of reducing sugars was analyzed by high-performance liquid chromatography, with Zorbex reverse-phase column and acetonitrile-H2O was utilized as a mobile phase. However, volatile fatty acids were determined by a gas chromatography-Flame ionization detector with HP-inn wax column.

2.5 The production of ethanol from liquid stillage

250 mL of liquid stillage addition with a different glucose concentrations [10, 20, 40 g/L and control, no glucose addition] was used as a substrate for the second fermentation. In addition, different microorganisms including *S. cerevisiae*, *E. aerogenes* and the mix-culture of *S.cerevisiae* and *E. aerogenes* were also investigated. Ten percent of inoculum was loaded into each sample and all experiments were operated at 35°C (120 rpm) for 96 hours. The samples were collected every 24 h to determined reducing sugar concentration and the production of ethanol.

2.6 Analytical methods

Reducing sugar was analyzed by the dinitrosalicylic acid (DNS) method. 1 mL of sample was added to DNS solution 2.0 mL. The mixture was incubated at 100°C, 5 min precisely. After that, the reaction was terminated in cold water for 5 min and 2.0 mL distilled water was added. The reducing sugar was measured at 540 nm using glucose as a standard. [7].

Ethanol was determined by dichromate reagent method. 2 mL of sample was added to tributylphosphate (TBP) solution 2.0 mL, on a rotary shaker for 30 min for stratification. Afterwards, 1.5 mL of supernatant solution was collected and 1.5 mL dichromate reagent was added. The experiment was done on a rotary shaker for 30 min. The ethanol (bottom-phase solution) was collected and measured at 595 nm using ethanol as a standard [8].

3. Result and Discussion

3.1 The production of ethanol from pineapple peel

The production of ethanol from pineapple peel using *E. aerogenes* by simultaneous saccharification and fermentation (SSF) was first determined. The process was maintained at 35°C with an agitation speed at 120 rpm. The maximum reducing sugar production (4.15 g/L) was obtained after 36 h of incubation and the maximum ethanol yield (1.67%, 1.42 g ethanol/g sugar) was achieved after 72 h. Afterwards, ethanol was recovered by the distillation method. The pineapple-ethanol stillage was collected for characterization and re-utilized for ethanol production.

3.2 The characterization of liquid stillage

Liquid stillage, a waste-effluent after ethanol, was first characterized. The liquid stillage was an acidic (pH 4.3-4.8). The characteristic of liquid stillage after ethanol recovery was shown in Table 1.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liquid stillage (mg/L)</th>
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<tbody>
<tr>
<td>Total solids (TS)</td>
<td>17,307</td>
</tr>
<tr>
<td>Volatile solids (VS)</td>
<td>14,400</td>
</tr>
<tr>
<td>Total soluble solids (TSS)</td>
<td>9.0</td>
</tr>
<tr>
<td>Chemical oxygen demand (COD)</td>
<td>20,900</td>
</tr>
</tbody>
</table>

Table 1. The characteristic of liquid stillage after ethanol recovery
Andalib et al. [9] also reported the characteristic of thin stillage from ethanol industry using corn as a substrate. However, the COD, TS, VS and TSS were 10-times higher than our study. The data indicated that the characteristic of stillage was dependent on substrate use for ethanol production. The sugar and volatile fatty acid contents in pineapple-ethanol stillage were also determined. The stillage contained reducing sugars (fructose and glucose) and volatile fatty acids including acetic acid, propionic acid and butyric acid. 1.48 mg/L of reducing sugar was found in raw liquid stillage. The presence of sugar and volatile fatty acid analyzed by HPLC and GC were presented in Fig. 1 and 2.

The presence of sugar and volatile fatty acid in stillage (this study) was similar to the sugar analysis of corn-ethanol stillage determined by Mitra et al. [10] and Eskicioglu et al. [11]. Therefore, the liquid stillage was suitable to utilize as a substrate for ethanol production due to the presence of sugars and volatile acids. In addition, the stillage, waste from ethanol production, can be a serious source of water pollution. Since the bioethanol production technology available as of 2000 inevitably results in the production of 11.1 - 16.4 L of ethanol fermentation waste (i.e. ethanol stillage) for every 1 L of ethanol production, the total estimation of the production of ethanol stillage would be approx. 179.82 - 264.06 million kL in Brazil (2005), 172.05 - 252.65 million kL in USA (2005) and 66.6 - 97.8 million kL in Japan (2030). The stillage can also be a valuable resource from which to recover useful products such as...
fertilizer, animal feed, or methane gas. Selecting the most appropriate stillage management is a matter of trade-offs between energy, economic, and environmental considerations. One can understand that the studies on the utilization of ethanol stillage are urgently needed [12]. However, little data of the re-utilization of stillage for ethanol production has been reported.

3.3 The production of ethanol from liquid stillage

The maximum ethanol production of ethanol from liquid stillage was 2.62% with ethanol yield at 1.76 g ethanol/g sugar. The highest production was obtained from mix-culture of *E. aerogenes* and *S. cerevisiae* using raw stillage as substrate (non-glucose addition) after 72 h of fermentation (Fig. 3.)

![Graph showing the production of ethanol from liquid stillage](image)

Fig 3. The production of reducing sugar (▲) and ethanol (●) from pineapple-ethanol stillage with non-glucose addition from mix-culture of *Saccharomyces cerevisiae* and *Enterobacter aerogenes*.

Co-cultures may be promising in fermentation of pentose and hexoses. The most well-known of co-culture for ethanol production such co-culture fermentation involving *S. cerevisiae* and *Zymomonas mobilis*. Ndaba et al. [13] revealed that the highest ethanol yield of 0.5 g/g from sweet sorghum bagasse was obtained from co-culture of *S. cerevisiae* and *Z. mobilis* with the concentration of 10 g/L for and 5 g/L, respectively. Interestingly *S. cerevisiae* and *Z. mobilis* worked better when they used in the same broth, since an even higher ethanol concentration of 9.30 g/L was obtained. The increase in ethanol concentration is due to high competition of *S. cerevisiae* and *Z. mobilis* for the same source of substrate, this led to a very rapid conversion of sugar to ethanol. The other successful experiment to increase bioethanol production using mix-culture was also reported by Park et al. [14]. They produce ethanol from cellulose using co-culture of *Acremonium cellulolyticus* and *S. cerevisiae*. In a single reaction, *A. cellulolyticus* was firstly produced cellulase. Subsequently, the produced cellulase saccharifies the cellulose, and then liberated reducing sugars are converted to ethanol by *S. cerevisiae*. The ethanol concentration and yield were 9.5-35.1 g/L with their yields of 0.12-0.19 (g/g), respectively. Interestingly, the ethanol yield from our study was 10-times higher. However, no data has investigated the interaction between the interaction between *S. cerevisiae* and *E. aerogenes*.

The ethanol production from other samples was in the range of 3.30-1.98% with the ethanol yield at 0.08-0.06 g ethanol/ g sugar. The productions of ethanol from liquid stillage in addition with 40 g/L glucose yield the highest ethanol 3.30%. However, the lowest ethanol yield (0.06 g ethanol/ g sugar) was obtained (Fig. 4a). The result found that decreased of glucose concentration (20-10 g/L) was increased
ethanol yield. Only 0.08 and 0.13 g ethanol / g sugar were obtained from the stillage with 20 g/L and 10 g/L, respectively (Fig 4b, c)

![Diagram](image)

Fig 4. The production of reducing sugar and ethanol from liquid stillage (a) 10 g/L of glucose, (b) 20 g/L of glucose and (c) 40 g/L of glucose from *Enterobacter aerogenes* ( ), *Saccharomyces cerevisiae* ( ) and mix-culture ( ).

4. Conclusion

A simple and realistic process for re-utilization of pineapple-ethanol stillage was presented in this study. This process can be used and inserted into conventional processes as suggested in Fig. 5. Raw stillage showed the possible ability to be utilized as a substrate for ethanol production due to the presence of reducing sugar (1.48 g/L) and volatile fatty acid (acetic and propionic acid). Raw stillage with no glucose addition gave the highest ethanol yield (1.762 g ethanol/g sugar) after 72 h using mix-culture of *E. aerogenes* and *S. cerevisiae*. 
ACKNOWLEDGEMENT

The author would like to thank Research and Development Institute Thaksin University, The Graduate School Thaksin University and the Department of Chemistry, Faculty of Science, Thaksin University for their financial support. Finally, I would like to thanks Dr. Christopher Joseph Forti (English adviser and English proof-reader, Thaksin University) for useful comments and suggestions on the language and structure of our manuscript.

References

[14] Park YE, Naruse K, Kato T. One-pot bioethanol production from cellulose by co-culture of Acremonium cellulolyticus and Saccharomyces cerevisiae. Biotechnology for Biofuels 2012; p. 5:64