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Bioethanol production from glucose by thermophilic microbes from Ciater hot springs

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Abstract

Bioethanol has been considered as one of the alternative energy resources for fossil fuel substitute. Second generation of bioethanol production usually uses lignocellulosic material as its raw material which conducted at high temperature range (70-80^{o}C). In this case the thermophilic microbe is needed for fermentation process in order to minimize the use of energy. This paper will discuss the results of the study on bioethanol production from glucose by using thermophilic microbes isolated from local source namely from Ciater hot springs in Subang District, Indonesia. In this study six thermophilic isolates (C1, C2, C3, C4, C5 and C6) were tested their capability in producing ethanol in the fermentation medium containing 5% glucose substrate for 5 days incubation. To determine the activity of isolates in ferment substrate is done by measuring the concentration of glucose and ethanol produced using a spectrophotometer. Isolates tested (C1,C2,C3,C4 and C5) could reduce glucose concentration from 1.1up to 1.7 % in the fermentation medium. The ethanol produced was tested qualitatively by reacting the samples with $K_2Cr_2O_7$inacidic conditions by observing its color change from yellow-orange to green-blue. The presence of ethanol indicatedby the decrease of OD's sample. This study showed that all isolates have the ability to produce ethanol. However, there are 2 isolates potentially produce ethanol that isolates C3 and C5 are characterized by low absorbance after adding potassium dichromate ($K_2Cr_2O_7$).

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Keywords: thermophilic microbes, bioethanol, hot springs

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1. Introduction

Bioethanol has been considered as one of the alternative energy resources for fossil fuel substitute. The advantages of bioethanol as fuel are: its raw material is renewable , more environmentally friendly, has better characteristic to increase combustion efficiency[3], reducing CO_2 emission [5] and its raw material can be found abundantly in Indonesia for example agricultural wastes. In Indonesia bioethanol usually commercially produced by using starch based materials which is so called the first generation bioethanol and hence, will compete with the goverment program on the food supply issues. Therefore an effort to develop the production technology of second generation bioethanol with lignocellulosic base needs to be improved.

Second-generation bioethanol production involves several stages of the process namely: pretreatment , saccharification or hydrolysis of cellulose into simple sugars , and fermentation of sugar into ethanol and then followed by purification by distillation process[4]. Generally, this second generation of bioethanol production process requires the extensive physical or enzymatic pretreatment which is relatively expensive that complicates this process to be developed commercially[4]. In practice, the production of bioethanol is still using the fermentation process of glucose from starch material that uses *Sacharomyces cerevisiae* and *Zymomonas mobilis*[6]. Both types of these microbes have several weaknesses, such as: the use of limited substrate (ie.glucose) ,the limited temperature growth for the mesophilic microbes (30-37^{o}C) so that the purification process (distillation) will need extra energy because the process takes place at the temperature range of 70-80^{o}C[5]. The use of thermophilic microbes is expected overcome the limitations that are owned by mesophilic microbes .

Compared to mesophilic microbes the use of thermophilic microbes have many advantages in second generation ethanol production such as[8]: it can degrade a much wider range of carbohydrates, no requirement of extensive mixing, cooling or heating of the fermentation vessel to cultivate them, posibility in direct ethanol recovery from the fermentation broth by in situ vacuum distillation, have a broad range of operating temperatures, tolerate to extremes of pH and salt concentrations during fermentation process as well as less risk. Besides, this process has easier mixing operations at elevated temperatures due to reduced viscosity and increased substrate loadings[8]. Utilization of thermophilic microbes also can reduce the contamination that is the problem in ethanol production process which using yeast, because the optimum temperature higher than microorganisms contaminants[15].

This paper will discuss the results of ethanol fermentation process using several local thermophilic microbes isolated from Ciater hot springs.

2. Materials and Methods

2.1 Thermophilic Microbes

Six isolates of thermophilic microbes were isolated from Ciater hot springs, with the code of C1, C2, C3, C4, C5 and C6. Each of isolates were grown in Luria Agar slant (containing of 10 g L^{-1} tryptone, 5 g L^{-1}

Yeast Extract, 5 g L^{-1} NaCl, and 15 g L^{-1} agar) as stock culture.

2.2 The Growth Curve

The growth curve was made by growing 1 ose of isolates into 15 ml LB media and incubated for 24 hours at 50°C. This culture was transferred into the erlenmeyer flask (250 mL) containing 135 mL LB (consisted of 10 g L^{-1} tryptone (BD), 5 g L^{-1} Yeast Extract (Merck) and 5 g L^{-1} NaCl (Merck)) and incubated at waterbath shaker (Memmert, Germany) with temperature of 50°C and agitated at 90 rpm for 66 hours. The profile of cell growth was observed every 2 hours during 12 hours and every 6 hours until the growth of isolates reached the stationary phase. It was observed by optical density (OD) measurement using spectrophotometer (Genesys 10 UV, USA) with wavelength (λ) 600 nm[13] and Total Plate Count (TPC) analysis every 6 hours (duplo replication for each isolates).

2.3 Fermentation Process

Inoculum preparation was done by transfering isolates from slant agar into the test tube containing 7.5 mL of seed culture medium and grown at 50°C, with the time of incubation based on the growth curve (see Fig. 1). The growth culture was performed in the erlenmeyer flask (250 mL) containing 150 mL of fermentation medium. The seed culture and fermentation medium containing per liter : 5% glucose (Merck) as a substrate, 0.25% yeast extract (Merck), 0.25% peptone (Merck), 0.03% $MgSO_4.7H_2O$ (Merck) and 0.1% KH_2PO_4(Merck)[12]. Initial pH of the medium was 5.84. Fermentation process was performed at waterbath shaker for 5 days, at temperature of 50°C and agitation at 90 rpm. Sampling of the process was carried out every day to measure the optical density (OD), pH, glucose concentration and qualitative ethanol analysis.

2.4 Analytical Methods

Analysis of fermentation product such as OD, pH, glucose concentration and qualitative ethanol were done every day (mono replication for each samples). The optical density was observed by turbidimetry using spectrophotometer with wavelength (λ) 600 nm. pH was measured by using pH meter (Mettler Toledo). Measurement of glucose concentration and qualitative ethanol by spectrophotometer. Samples for glucose concentration and qualitative ethanol analysis must be separated between pellet and supernatant by using a centrifuge (Gemmy Industrial corp., Taiwan) which operated at 4000 rpm, for 20 minutes. The supernatant was used for analyzing of glucose concentration and ethanol produced. Glucose concentration was measured using Nelson-Somogyi method[10]. The ethanol produced was measured by qualitative method using potassium dichromate ($K_2Cr_2O_7$, Merck) in acidic conditions (potassium dichromate dissolved by using 6N H_2SO_4)[9].

3. Result and Discussion

Microbial inoculum prepared by growing microbes and incubated for a certain time. From the growth curve at Fig.1 was known that each isolates thermophiles has a different time to reach stationary phase. Incubation time required each isolates to reach the stationary phase are as follows isolates C1 was 48 hours, C2

was 54 hours, C3 was 42 hours, C4 was 36 hours, C5 was 42 hours and C6 was 60 hours. Generally, isolates has a long lag phase, between 6-12 hours and as well as its log phase. It's maybe caused by the adaptation phase of the isolates in the environmental conditions as well as the nutrients contained in their growth media. From observations of the growth curve and the results of Total Plate Count it was obtained that the exact time for each isolates when used as inoculum occured at the end of the log phase (see Fig.1), because at that time they had reached the highest colonies number (see Table. 1). Inoculum must be containing microorganisms that healthy and active so that the lag phase of fermentation can be shortened[7]. In addition, at the end of the log phase the growth will be slow, but the metabolism ability will increase[2].

Table 1. Incubation time and TPC value each of isolates as an inoculum

Isolates	∑colony/ml x 10^6 (hours)											
	0	6	12	18	24	30	36	42	48	54	60	66
C1	0,04	105	104	115	113	114	122	132	133	3,88	-	-
C2	0,3	118	153	190	244	237	255	230	315	484	480	40
C3	0,26	151	122	180	168	193	191	250	112	58,8	-	-
C4	0,4	111	106	116	164	158	213	84	63	60	-	-
C5	0,28	145	151	168	159	160	192	217	166	164	-	-
C6	0,06	1,25	7,3	10,8	15,8	143	179	166	161	312	445	28

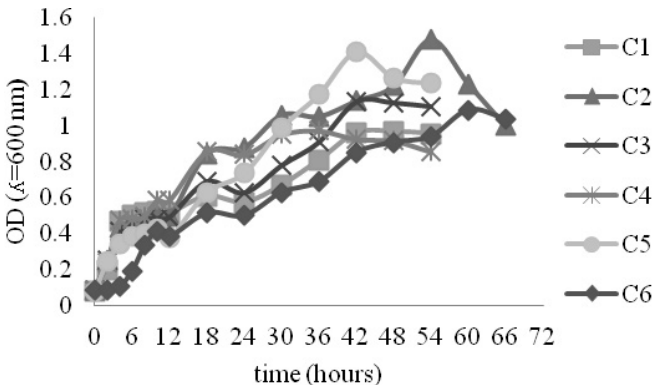

Fig. 1. The growth curve of thermophilic isolates

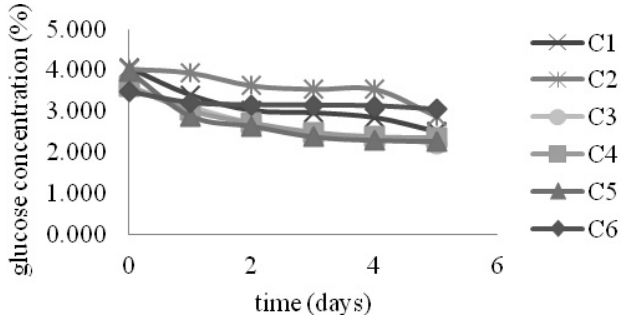

Fig. 2. Glucose concentration during fermentation

The optical density (OD) increased during fermentation process (except C6 isolate) (see Fig. 3). Increased optical density is inversely proportional to the concentration of glucose in the fermentation medium (see Fig. 2). It was occured because of each isolates utilized the nutrition (glucose) in the fermentation medium. All of isolates (except C6) utilized almost half of the concentration of glucose(± 1.7 %) in the fermentation medium. Whereas, C6 unable to utilize nutrients, in this case it could only use about 0.4 % of the glucose in the medium. So that the OD measurement of the cells tend not to increased (tend to remain constant).

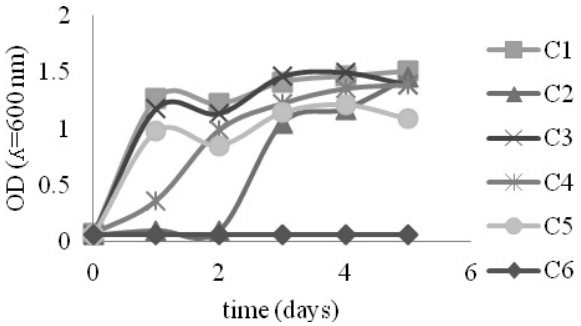

Fig. 3. Optical density during fermentation

During fermentation process, pH medium changed (Fig. 4) and tends to slightly decreased. It occurred because during fermentation the microorganism not only produced ethanol but also produced other organic acids that affected the pH value of the fermentation medium. In other study, ethanol fermentation using *Thermoanaerobacter* BG1L1 with corn stover hydrolyzate as a substrate showed that increasing the ethanol content is directly proportional to the amount of acetic acid produced so that it will affected pH of the medium[1]. Besides that, pH decrease was assumed to be correlated to the nitrogen consumption that produces H^+ions which were released in solution[11].

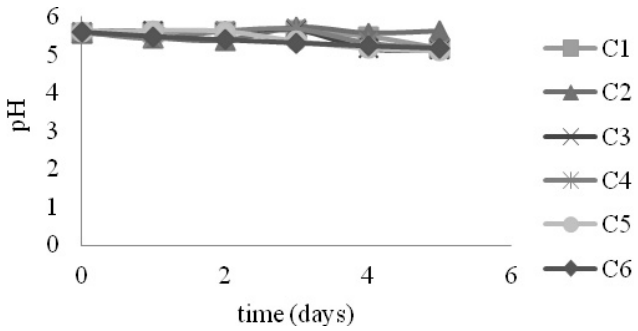

Fig. 4. pH during fermentation

Analysis of ethanol in this study conducted qualitatively by reacting the sample (supernatant that has been separated from the pellets) with potassium dichromate at acid condition. Samples containing ethanol, when added with potassium dichromate will change color from yellow-orange to green-blue. This color change is caused due to the reduction reaction of ethanol by potassium dichromate in acidic conditions which produce blue-green color of the ionCr^{3+}[9]. Fig. 5 shows the equation of this reaction .

$$CH_3CH_2OH + K_2Cr_2O_7 + H_2SO_4 \rightarrow CH_3COOH + Cr_2(SO_4)_3 + K_2SO_4 + H_2O$$

Fig. 5 Reaction for ethanol reduction-oxidation by potassium dichromate

To prove the presence of ethanol in the sample, the sample was reacted with potassium dichromate and measured its absorbance. The color change that occured was compared with the color of potassium dichromate solution without sample addition (blanko = absorbance 0.575) and ethanol p.a as control (absorbance = 0.108) that measured the absorbance at a wavelength of 480 nm[9]. The absorbance values of the samples can be seen in Fig. 6 below. The lower of the absorbance shows the greater ethanol content was caused the chromium ion was reducted by ethanol[14].

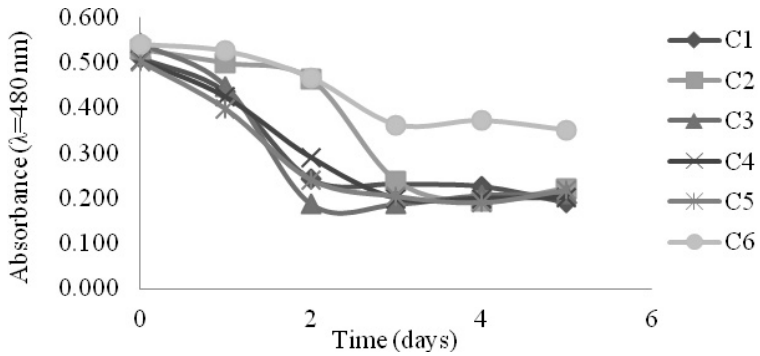

Fig. 6 Absorbance of the samples during the analysis qualitatively ethanol

Fig. 6 shows the absorbance of the sample for 5 days of incubation. Absorbance of each sample was generally declining from day to day. However, a significant decrease in absorbance occurred in isolates C3

from 0.542 to 0.187 at days 3 and C5 from 0.502 to 0.189 at days 4. Another study conducted by Pinata and Nawfa, absorbance of the sample reported that ethanol produced from the fermentation process by *Zymomonas mobilis* decreased from 0.180 to 0.070[9].

4. Conclusion

This study showed that all isolates have the ability to produce ethanol but there are 2 isolates potentially produce ethanol that isolates C3 and C5 are characterized by low absorbance after adding potassium dichromate ($K_2Cr_2O_7$). More research is still needed to determine the concentration of ethanol produced by these isolates and also molecular identification of isolate to determine the type of C3 and C5 isolates until the species level identified.

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