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Potential Isolates Characterization of Thermophilic Bacteria from Hot Springs and Waste Agricultural Production in Subang District Area

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Abstract. The characterization of local thermophilic bacteria originated from hot spring and agricultural waste production have been done. The purposes of this research were to study the morphology colony of isolates, cellulolytic activity, potency to produce ethanol, and other characteristics of thermophilic bacteria. The method used was involved morphology isolates characterization, cellulolytic activity testing, trials ethanol production in an aerobic and anaerobic condition, and characterization of fermented media covered pH, the decreasing of sugar, and titrated acid. The isolates used in this research were coded by B1, B2, B3, C2, C5, N1 and J2. The result of this research was known the isolates characteristic covered form, size, color and margin. Cellulolytic activity test showed that isolate of B2, C2, and C5 was positive when tested by congo red dye and only B2 isolate was positive when tested by I-KI 3% dye. Aerobic and anaerobic fermentation did not show the capabilities of isolates to produce ethanol. The characteristic of anaerobic fermented media shown the decrease of pH number from 7.16 to about 4.43 to 4.49, the decrease of reduced sugar to about 1.02 to 24.43%, the remaining glucose, and xylose in the medium respectively were about 3.45 to 4.07% and 3.40 to 3.99%. The titrated acid as lactic acid is about 0.16 to 0.18%.

Keywords: Thermophilic bacteria, bioethanol, fermentation, cellulolytic.

1. Introduction

Indonesia has a lot of potential thermophilic microorganisms including bacteria. Thermophilic bacteria have an important potential to be developed in various fields of life, especially to support the industry. Thermophilic bacteria can be classified into two groups: obligative and facultative thermophilic bacteria. Obligative thermophilic bacteria can survive at temperatures above 50 °C, while the facultative thermophilic bacteria can grow at a temperature range between 50 – 66 °C or at temperatures lower than 38 °C. Some obligate thermophilic bacteria can grow at a temperature of 77 °C. The habitat of thermophilic bacteria can be found in a high-temperature environment such as hot springs, ground that is always exposed to sunlight and soil fermented compost [1].

The study of the source and the potential of thermophilic bacteria is getting a lot done. Thermophilic Microbes can be used as a source of enzymes and better than animal and plant [2]. Thermophilic bacteria's have the ability to survive in high temperatures caused the bacteria are able to produce enzymes that more stable at high temperatures (thermostable). Nowadays the use of thermostable enzymes become very important as in biotechnology and industrial applications [3].



Increasing the reaction temperature will generally be able to increase the speed of a reaction. Increased reaction temperatures above 10 °C minimum will shift the reaction rate by two times. Additionally, the rising temperatures will inhibit other microbes to live so that the contamination of other microbes can be reduced [4]. Sutiarniharja (2008) also mentions the use of thermostable enzymes that can save the cost of production because this enzyme has a longer shelf life and activity remains optimal at high temperatures [5].

Thermophilic bacteria can be taken or isolated from various potential sources such as hot springs and wastes of industrial or production processes. Microorganisms can be at the hot springs caused by environmental conditions that are supported by biotic and abiotic factors. Biotic conditions in the hot springs can be used as an energy source while abiotic factors also affect the existence of thermophilic bacteria such as alkaline pH caused by high mineral content, causing a high diversity of microorganisms [6]. In the production of waste both liquid and solid wastes generated in addition to indeed have a sufficiently high temperature, normally there will be also a fermentation process that can increase the temperature. The high temperature of the waste will make the microorganisms that grow well adapted.

The purpose of this study was to Determine colony morphology of isolates, cellulolytic activity, the potential to produce ethanol, and other characteristics of thermophilic bacteria originating from the hot springs in the tourist area of Ciater and Batu Kapur and from waste processing production of pineapple and guava in the Subang District, West Java, Indonesia.

2. Experimental method

Materials used in this study were isolated of local thermophilic microorganism obtained from hot springs of Batukapur Subang (B) is (B1, B2, B3), hot springs of Ciater Subang (C) is (C2, C5), waste of guava production (J), i.e. (J2), and waste processing production of pineapple (N), i.e. (N1). Others materials used were tryptone broth, NaCl, yeast extract, KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, glucose, xylose, peptone broth, distilled water, filter paper, and dinitrosalicylic acid. The tools used were the analytical balance, vertical laminar flow, autoclaves, incubators, centrifuges and HPLC Waters e 2695.

2.1. Morphology Characterization colony

Isolate scratched in agar media and incubated at 50 °C, and colony growth was seen in the form of shapes, sizes, colors, and edges.

2.2. Cellulolytic activity

Bacterial isolates were inoculated on a medium containing substrate of carboxymethyl cellulose (CMC), which had previously been inoculated on media LB. After inoculated on CMC media then incubated for 48 hours at a temperature of 50 °C. Colonies that grew drip with congo red solution or I-KI 3% for ten minutes and rinsed using 1% NaCl. The positive results are shown by the formation of a clear zone in the area around the colony.

2.3. Fermentation Media

The medium used is composed of three kinds of media Luria Broth, Seed culture, and fermentation media. In Luria Broth media as much as 0.75 g tryptone broth, 0.375 g NaCl, and 0.375 g yeast extract, dissolved in 75 ml of distilled water, then stir until homogeneous and then poured into a test tube 7 respectively of 10 ml, then sterilized.

Seed culture media made of 0.1875 g yeast extract, 0.075 g KH_2PO_4 , 0.0225 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.75 g glucose, 3.75 g xylose, and 0.1875 peptone broth dissolved in 75 ml of distilled water, then stir until homogeneous and then poured into a test tube (10 ml), then sterilized.

Fermentation media bolted from 1.875 g yeast extract, 0.75 g KH_2PO_4 , 0.225 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 37.5 g of glucose, 37.5 g xylose, and 1,875 peptone broth dissolved in 750 ml of distilled water, stir until homogeneous and then poured 100 ml of the media into 250 ml Erlenmeyer. The sterilization is carried out using an autoclave at 121°C for 15 minutes.

2.4. Making a thermophilic starter

Isolate thermophilic bacteria inoculated in Luria broth media, and incubated at 50 °C for 24 hours, after it was taken 4% (v/v) and then centrifuged for 10 minutes at a speed of 3000 rpm, The bacterial pellets were taken and inoculated on the Seed culture medium and incubated at 50 °C. The incubation time is determined by the growth curve of each isolate namely B1, B2, B3, N1 for 24 hours, J2 for 36 hours, C5 for 42 hours and C2 for 54 hours.

2.5. Fermentation process

The fermentation process carried out in two ways: aerobic and anaerobic. A total of 10% (v/v) thermophilic starter media were taken and centrifuged for 10 min at 3000 rpm, and the pellet was taken and inoculated into the

fermentation medium, then incubated at 50 °C. Alcoholic fermentation trials carried out in aerobic conditions for 5 days. In anaerobic fermentation, trials carried out for 7 days. In the aerobic test, sample analysis is stopped until HPLC assay to determine the ability of the alcohol production. While on samples of anaerobic fermentation then carried out a further test which includes parameters of pH, a concentration of reducing sugar, titrated acid total assumed as lactic acid, ethanol concentration, and residues of glucose and xylose. The concentration of ethanol, glucose, and xylose residues were analyzed using HPLC. Total acid for reducing sugar analysis was carried out by the method of Badan Standardisasi Nasional (1992) [7].

3. Result and discussion

3.1. Morphology Characterization colony

Isolates used in this study was isolated that has the ability to grow on the minimum alcohol content of up to 6%. The characteristics of each isolate can be seen in Table 1.

Table 1. Characteristics of isolates from various sources

Isolates	Morphology colonies			
	Shape	Size	Color	Edges
B ₁	Round	Large	Beige	Filamentous
B ₂	Flower	Medium	Beige	-
B ₃	Round	Large	Beige	Undulate
C ₂	Round	Medium	Beige	Filamentous
C ₅	Irregular	Medium	Beige	Filamentous
N ₁	Stem cells	Large	Beige	Stringy
J ₂	Round	-	White	-

3.2. Cellulolytic Activity

Testing the ability of cellulolytic activity was carried out to determine the ability of bacterial isolates to degrade cellulose. So that it can be used in the process of lignocellulose hydrolysis. Qualitative test results on cellulolytic activity using two different types of dyes namely congo red dye, and I-KI 3% dye showed that the coloring by red congo contained 3 positive isolates and 3% I-KI staining with one positive isolate.

On staining by congo red positive isolates were isolate of C₂, C₅, and B₂ while the staining by I-KI 3% was isolate of B₂. Pictures of the test results can be seen in Figure 1.

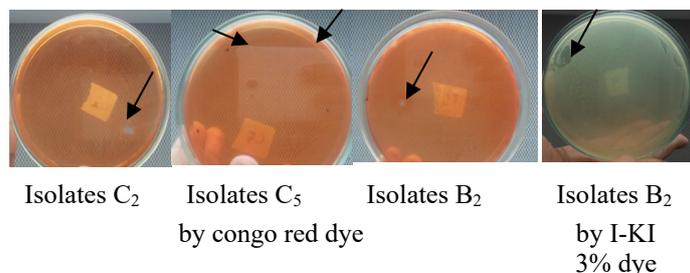


Figure 1. Isolate thermophilic bacteria capable of producing a clear zone

The formation of a clear zone as shown in figure 1 show that isolates bacteria sourced from hot spring have cellulolytic activity. Establishment of a clear zone around the colony of bacteria means that the isolates of bacteria actively degraded the cellulose derived from carboxymethyl cellulose (CMC) [8].

The existence of cellulolytic activity indicates that the bacteria are able to produce the cellulase enzyme. Previous studies showed that some isolates of thermophilic bacteria capable of producing cellulase enzymes. Isolates of *Bacillus pumilus* isolated from hot springs in the area of Sikumbang-Central Java can produce cellulase enzymes with the optimum temperature of 45 °C, pH 5.0 with an incubation time of 3 days with the substrate concentration of 1.0% (w/v) [9]. Bacterial isolate (S2A) taken from hot springs Kili Ketek Solok which has 97% similarity with *Anoxybacillus flavithermus* AE3 strain capable of producing extracellular cellulase enzymes [10]. Most cellulase enzymes are produced at high temperatures and have optimum activity at high temperatures so that the cellulose enzyme can be produced by thermophilic bacteria [11].

3.3. Bioethanol Ability Test

Bioethanol ability test of bacterial isolates carried out through aerobic and anaerobic fermentation. The fermented medium then examined by using HPLC and the results show the whole isolate was not able to produce bioethanol. The data can be seen in Table 2.

Table 2. Ability Test Bioethanol In Aerobic and Anaerobic

Isolate Thermophilic Bacteria	Bioethanol Production capabilities	
	Aerobic fermentation	Anaerobic fermentation
B ₁	-	-
B ₂	-	-
B ₃	-	-
C ₂	-	-
C ₅	-	-
N ₁	-	-
J ₂	-	-

In a previous study conducted, some isolates from Ciater hot springs were tested resistance to ethanol, and the results were all resistant or able to survive on ethanol levels of up to 6 % [12]. However, after the fermentation either aerobic or anaerobic isolates cannot produce ethanol (Table 2). In contrast to research conducted by Martosuyono and Misgiyarta (2000) which states that the thermophilic bacteria (*Bacillus caldxylophilus* and *Geobacillus thermoleovorans*) in aerobic fermentation conditions, can produce ethanol with the amount of ethanol obtained is very small with a concentration of 0.2 to 1.9 g/l, while anaerobic testing shows that bacteria are able to produce higher ethanol concentrations ranging between 2.5 - 3.7 g/l. The highest yield is indicated by the bacterium *Geobacillus thermoleovorans* [13].

The research was supported by a statement of Riyanti (2011) which states thermophilic bacteria can produce ethanol because they have ethanol-producing genes are Alcohol dehydrogenase (ADH) as found in bacteria *Geobacillus thermoglucosidasius*, *Geobacillus stearothermophilus*, *Bacillus Sulfolobus* and *Thermoanaerobacter ethanolic* [14]. Some of the isolates used in this study are supposed there may be capable of producing ethanol when performed on appropriate conditions. This is due to the alcohol fermentation process can be influenced by many factors. The factors that influence the success or failure of ethanol fermentation include inoculum physiological conditions include pH, temperature, growth factor, alcohol. The quality of growth substrate consisting of a carbon source, a source of nitrogen, oxygen, and CO₂ also affect the results obtained as well as the efficiency of fermentation. Inoculum physiological conditions depending on environmental factors, the presence of microbial contaminants will greatly affect the metabolites products produced and inhibited the fermentation process [15].

The primary metabolite of ethanol production is influenced by the growth of microbial cells used. Nutrients are used to the life and growth of cells, including growth factors such as vitamins and minerals. Nutrients needed to form and develop energy cell components. Components containing organic carbon source is used as an energy source for microbes and uses mostly organic components containing protein as a nitrogen source or organic nitrogen source [16].

3.4. Change of pH

The degree of acidity (pH) is one of several important factors that can affect the fermentation process, while the pH test results in fermentation media are as follows :

Table 3. Media Isolates pH levels before and after Fermentation

Media Isolates	pH Before Fermentation	pH After Fermentation
B ₁	7.16	4.49
B ₂	7.16	4.44
B ₃	7.16	4.45
C ₂	7.16	4.43
C ₅	7.16	4.43
N ₁	7.16	4.48
J ₂	7.16	4.49

Table 3 showed a significant difference between the pH of media before and after fermentation. From the data, it is known that the pH of media before fermentation was 7.16 and after fermentation pH ranged from 4.43 to 4.49. A decrease in pH caused by the fermentation which can produce acidic metabolites. Some acidic metabolites such as ethanol, acetic acid, levulinic acid, and formic acid by the conditions of the media [17].

In the microbial fermentation required media with optimal pH conditions, the pH value of the media is very influential on the microbial growth, good pH values maximum of about pH 6.5-7.5, while a pH below 5.0 or

above 8.5 bacteria will not grow well except acetic acid bacteria [16]. The degree of acidity (pH) optimum for the fermentation process ranges from 4-5, at a pH below 3 fermentation process will decelerate [17].

3.5. Reducing sugar content

Measurement reducing sugar aim to find out how much sugar is in use when the fermentation. These sugars can serve as a source of energy that will be converted into a variety of compounds, such as ethanol, acetic acid, and lactic acid. Sugar is in use on media that is the sugar glucose and xylose sugar. The results in the can are as follows :

Table 4. Total Sugar Reduction

Sample	Sugar concentration (%)		
	DNS	HPLC	
		Glucose	Xylose
Before Fermentation	9.5792	± 5.00	± 5.00
B ₁	7.2393	3.7248	3.6660
B ₂	9.4817	3.6705	3.6759
B ₃	8.4580	4.0767	3.9995
C ₂	8.4824	3.4523	3.3975
C ₅	8.1411	3.5335	3.5926
N ₁	7.6780	3.5341	3.4271
J ₂	7.7268	3.5980	3.5690

In Table 4 it can be seen that the initial sugar concentration of the media is tested using the dinitrosalicylic acid (DNS) method that is as much as 9.5793%, which is almost the same as the composition of the sugar added into the media of 5% glucose and 5% xylose. There is a decrease in sugar levels after media fermented, despite the relative decline slightly but this proves that the media added isolates had the fermentation process in which sugars that exist in the media converted into ATP and generate other compounds that make sugar levels lower.

While the sugar levels are measured using HPLC method both glucose and xylose in each medium is 3.45 to 4.07 % and 3.40 to 3.99%, this is possible because the DNS method of measurement carried out on the overall total amount of sugar, whereas the HPLC method measuring total sugars glucose and xylose separately, so the total is divided into two. The decrease in sugar xylose and glucose as xylose and glucose is a monosaccharide included in the group, reducing sugar, wherein the fermentation of xylose and glucose can be converted into hydrogen [18].

Reducing sugars during fermentation will increase after fermentation lasts for three days, after which the reducing sugar will decrease. Fermentation time will affect the content of reducing sugar which on the first day will be increased as much as 7.9% to 16% on the third day [19].

As a pentose sugar xylose being very abundant as hydrolysis products which when fermented lignocellulosic materials can produce bioethanol. While still showing glucose fermentation product of lactic acid as the main products, followed by acetate and ethanol [13].

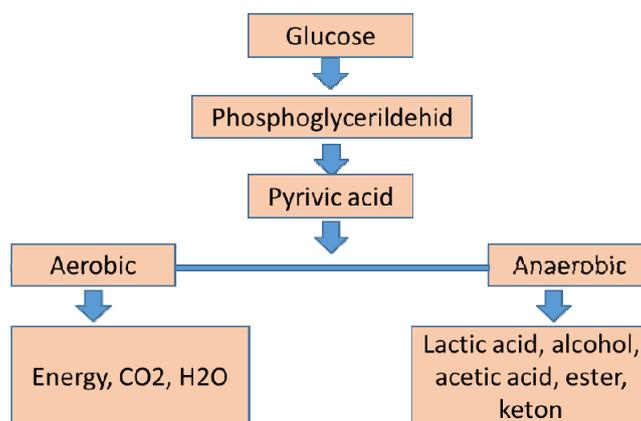


Figure 2. Reaction formation of glucose into other compounds [19].

In contrast to the sample being tested even though it contains xylose and glucose but not producing ethanol, this is possible because the isolate cannot convert the sugars into ethanol but other compounds.

3.6. Test of Titrated Acid (Lactic Acid)

Lactic acid is one of the organic acids that are important in the industry, especially the food industry. In testing the Total titratable acidity can result in the following :

Table 5. Total titratable acidity for lactic acid

Sample	Total acid (%)
Before fermentation	0.1429
B ₁	0.1606
B ₂	0.1606
B ₃	0.1874
C ₂	0.1787
C ₅	0.1693
J ₂	0.1696
N ₁	0.1606

Based on Table 5 it can be seen that at the time of fermentation, isolates tested can produce acids that are assumed as lactic acid, the highest total acid produced by B3 isolate as many as 0.1874% and the lowest produced by isolates B1, B2 and N1 as much as 0.16065%. Although the results can be relatively small, this proves that the tested isolates thermophilic bacteria can produce acid and assumed as lactic acid.

The results showed that thermophilic bacteria could produce lactic acid is supported by research conducted by Nurwati and Refdinal [20] which states that the test of glucose fermentation in bacteria thermophilic *Caldicellusiruptor kristjanssonii* produce end products such as acetic acid, lactic acid, ethanol, and formic acid. This was confirmed by research conducted by Rahmawati [21] which states that carbohydrates and proteins in whey fermented by thermophilic bacteria produce lactic acid. The bacteria will use the nutrients contained in the ingredients to turn it into acid.

The acid is fermented to form lactic acid bacteria to convert glucose into lactic acid. According to Hernawati [22] that the lactic acid fermentation of glucose is converted to pyruvic acid and pyruvic acid molecules result glycolysis accept electrons and hydrogen from NADH. Transfer of electrons and hydrogen to produce NAD back. At the same time, pyruvic acid is converted to lactic acid in large quantities.

There are several types of bacteria called lactic acid bacteria are able to convert glucose into lactic acid. *Streptococcus lactis* is one example of the bacteria capable of causing sour milk and break down glucose to lactic acid [22]. The mechanism that is one molecule of glucose is converted to two molecules of pyruvic acid, and pyruvic acid molecules result from glycolysis accept electrons and hydrogen from NADH. Transfer of electrons and hydrogen to produce NAD back and pyruvic acid is converted to lactic acid [22].

4. Conclusion

This research can be concluded that: Isolate tested had an average colony morphology round, medium-sized to large, cream and white colours and had stringly edge line of undulated, filamentous and entire. From all isolates tested, three isolates have cellulolytic activity when tested using a solution of congo red dye, i.e. C2, C5, and B2 and one isolate when tested using a solution of I-KI 3 %, i.e. B2. The characteristic of anaerobic fermented media shown the decrease of pH number from 7.16 to about 4.43 to 4.49, the decrease of reduced sugar to about 1.02 to 24.43%, the remaining glucose, and xylose in the medium respectively were about 3.45 to 4.07% and 3.40 to 3.99%. The titrated total acidity as lactic acid about 0,16 to 0,18%.

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