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Exploration and Characterization of Thermophilic Bacteria from the Kerinci-Jambi Geothermal Source for Alcohol Production

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Abstract: Alcohol produced by thermophilic bacteria is renewable and a safe fuel because it is mainly based on microbial fermentation. This research needs to be done to find out the potential alcohol production of thermophilic bacteria because have many advantages such as degrading a much wider range of carbohydrates, the possibility of direct alcohol recovery from the fermentation broth, tolerance to extreme conditions, and also can reduce the contamination. This research stage started with isolation using Nutrient Agar (NA) media, using the pour plate method, and incubated at a temperature of 50°C for 24 h. The isolate obtained was cultured on basal media for qualitative testing with the addition of potassium dichromate. Isolates that were indicated to be positive for alcohol-producing potential were measured for absorbance using a spectrophotometer at a wavelength of 579 nm. Seven isolates of potential alcohol producers were checked using GC-MS to determine the group of alcohol produced. From these determinations, alcohol was obtained from ethanol, 4-penten-2ol, and 2,3-butanediol.

Keywords: Alcohol, Characterization, Exploration, Geothermal, Thermophilic Bacteria

Introduction

Numerous potential bacteria and other thermophilic microorganisms can be found in Indonesia. The potential for thermophilic bacteria to be produced in many areas of life, particularly to serve industry, is significant (Agustina *et al*., 2019). Nowadays, alcohol has become part of daily activities, especially after COVID-19, which occurred in early 2020, and the use of alcohol as the main ingredient in making hand sanitizers to prevent the spread of the virus. Alcohols are hydrocarbons formed by the hydroxylation process. The first alcohol derivative in the alcohol series is methanol (methyl alcohol), which contains a single carbon atom and is represented by the chemical formula "CH_3OH". The next derivative is Ethanol, or Ethyl Alcohol (C_2H_5OH). Different names are based on the alkyl group attached to the hydroxyl functional group, such as propanol, butanol, pentanol, and others. Alcohol is a volatile, flammable, polar, colorless liquid and is a good organic solvent (Fortunato *et al*., 2021).

Apart from being needed for the manufacture of hand sanitizers, alcohol is also much needed in various other industries, such as the medical world, where it can stimulate brain activity, lower blood sugar levels continuously, and be used as an effective antiseptic that kills bacteria. One type of alcohol, such as ethanol, is used in the medical world as an ingredient for making disinfectants and antiseptics (Al Zuhri and Dona, 2021). The need for alcohol will continue, alternative production sources can be obtained from microorganisms through the isolation of thermophilic bacteria that have the potential to produce alcohol.

Thermophilic bacteria are microorganisms that can survive in extreme environments, such as environments with high temperatures, namely 45-80°C. In many cases, besides being able to adapt, thermophilic bacteria also use extreme environmental conditions to produce. Research on thermophilic bacteria obtained from hot springs is usually carried out by isolating, characterizing, and testing their enzymatic potential. This is because thermophilic bacteria can produce thermostable enzymes or heat-resistant enzymes that can be used in industry,

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waste treatment, weathering of minerals, or biotechnology studies (Mahmudah *et al*., 2016).

Previously, thermophilic bacteria capable of producing bioethanol had been found in research by Salim *et al*. (2015), who isolated six isolates of thermophilic bacteria from the Ciater hot springs. Of these six isolates, all had the potential to produce ethanol and only two had a low potential. In addition, several species were found to be able to produce ethanol, such as *Thermoanaerobacterium saccharolyticum*, *Thermoanaerobacter mathranii*, *Thermoanaerobacter ethanolicus* and *Clostridium thermocellum* (Olson *et al*., 2015), as well as several groups of Bacillus (*B. pumilus*, *B. subtilis*, *B. megaterium, B. fusiformis and B. flexus*) (Thomas, 2012), *Geobacillus thermoglusidasius* (Cripps *et al.*, 2009).

Research conducted by Hitschler *et al*. (2021) also found species from the genus *Thermoanaerobacter*, which is a group of thermophilic chemoorganotrophic sugar-utilizing microorganisms, can produce various kinds of fermentation products such as acetate, lactate, CO_2, H_2, and ethanol. In many species of *Thermoanaerobacter*, ethanol is the main product. In particular, the species from the subgroup (clade 1) were described as highly efficient in the formation of ethanol from sugar but less efficient than *Zymomonas mobilis*.

Other studies show that growing cellulite bacteria at temperatures >70°C allows the conversion of lignocellulosic material into ethanol. Seven new strains of highly thermophilic anaerobic cellulolytic bacteria of the genus *Caldicellulosiruptor* and eight strains of highly thermophilic xylanolytic/saccharolytic bacteria from the genus *Thermoanaerobacterium* that were isolated from environmental samples showed stable growth. rapid at 72°C, extensive lignocellulose degradation, and high-yield ethanol production in pre-processing cellulose and lignocellulose biomass (Svetlitchnyi *et al*., 2013). In the field of biotechnology, research related to the potential of thermophilic bacteria has increased due to their ability to produce ethanol from a variety of substrates and the ability of some species to break down biopolymers such as cellulose, almonds, and hemicelluloses, including xylene. Microorganisms are classified according to optimal growth Temperatures (T Opt) and Maximums (T Max). Moderate Thermophiles have (T Opt) values ranging from 50-64°C, extreme thermophiles between 65 and 79°C, and hyperthermophiles grow optimally at temperatures above 80°C (Zeldes *et al.*, 2015).

Since 2008, more than 300 species of thermophilic anaerobic bacteria have been isolated and described in their habitats, including geothermal areas, deep ocean wells, river sediments, and artificial habitats such as self-heated compost piles, solid municipal waste or wastewater, oil pools, and thermally processed food. It suggests that thermophilic anaerobes are everywhere. The most well-known thermophilic species are either compulsory or optional anaerobes due to the low oxygen concentration of the native geothermal environment of the thermophilic bacteria. The physiology and production potential of ethanol species in most genera have not been extensively studied (Scully and Orlygsson, 2019).

One of the geothermal sources in Indonesia that is a habitat for thermophilic bacteria is in Jambi Province, specifically in Kerinci, at the hot springs of the Medang River. Zuhri *et al*. (2013) reported the results of their research using geothermal sources for the production of alkaline protease from *Bacillus* sp. M1.2.3 and optimizing its growth by providing carbon and nitrogen compounds to thermophilic microorganism cells at a temperature and pH of 50-78°C and pH 8.45-8.71. Based on the existing facts, research was carried out to screen thermophilic bacteria from the same hot springs (Kerinci-Jambi) to see their potential as alcohol producers.

Materials and Methods

Preparation of Tools and Materials

Prepared all tools to be used in the research such as petri dish, test tube, Erlenmeyer, Eppendorf tube, microtip, Nutrient Agar (NA) media for bacterial isolation, and basal media for alcohol screening. After all the media were prepared, sterilization used autoclaves 121° and 15 lbs as well as all other experimental supporting tools such as test tube shelves, ose needles, bunsen, and micropipettes. All the media that have been sterilized are stored in the refrigerator.

Sample Collecting and Measurement of the Physical and Chemical Properties of Water

Hot water samples were taken using purposive sampling at locations (geothermal source) with a temperature of 45-80°C. Measurement of the physical and chemical properties of water. Water samples are taken at five points using sample bottles, placed in a thermal container, and taken directly to the laboratory.

Isolation of Thermophilic Bacteria

Bacterial isolation was carried out using the pour plate method on the NA media. NA media was made by dissolving NA media synthetic (20 g/ 2% v/v) into 1000 mL of distilled water. Cultures are incubated at 50°C for 24-48 h. The purification of bacteria is carried out on colonies growing on NA media. Cultures were grown on NA media using the quadrant streak plate method. Single colonies were inoculated on culture slants then labeled and stored at 50°C in an incubator (Muqarramah *et al*., 2023).

Production and Screening of Alcohol

Production of alcohol by thermophilic bacteria is carried out by culturing isolates in alcohol-producing media. The alcohol-producing media was made by dissolving KH_2PO_4 (3 g/ 0.3% v/v), K_2HPO_4 (3 g/ 0.3% v/v), $MgSO_4$ (5g/ 0.5% v/v), NaCl (5 g/ 0.5% v/v) and peptone (10 g/ 1% v/v) into 900 mL of distilled water and glucose (20 g/ 2% v/v) into 100 mL. Alcohol fermentation was carried out by inoculating 5 mL of isolate inoculum in an Erlenmeyer containing 95 mL of alcohol production media, then the culture was incubated at 50°C, agitation 150 rpm for 24 h on an incubator shaker. Screening for alcohol produced by thermophilic bacteria is carried out with supernatant obtained was centrifuged for bacterial culture. Next, the determination was carried out; the alcohol-producing bacteria were indicated as positive in the qualitative test, where the supernatant changed color from yellow or orange to greenish blue with the addition of potassium dichromate reagent. A quantitative assay was done by spectrophotometer (thermoscientific GENEYSY 15 UV-vis) at a wavelength of 579 nm. The determination of the alcohol produced by bacteria was carried out using GC-MS (Thermoscientific trace 1310) to obtain the types of alcohol produced, such as ethanol, propanol, and butanol (Simatupang *et al*., 2019).

Results

Observation Result

The hot water samples in this study were taken from geothermal sources, namely hot springs located on the Medang River, Kerinci, Jambi Province, Indonesia which geographically is located at 02° 00' 34.75" south latitude and 101° 24' 52.00" north latitude at an altitude of 824 m above the surface of the sea. The sampling location is shown in the following Fig. 1.

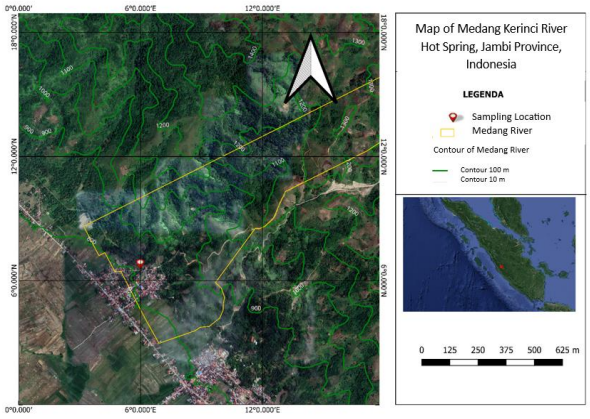

Fig. 1: Map of the location of geothermal sources

A sampling of water samples starts measuring the temperature and pH with a glass alcohol thermometer and digital pH meter, it was found that the Madang River hot spring has a temperature ranging from 68-79°C with a pH of 7.6-7.9 (Fig. 2). This was obtained when making observations, which can be seen in Table 1.

Around this hot spring, there is also various vegetation, including moss, grass, and various other plants, and various remains of dead organisms were also found, such as leaves, wooden twigs, dead insects, and rocks (Fig. 2). In this study, the incubation temperature used was 50°C, according to research by Kurniawan (2017).

The results of the isolation of thermophilic bacteria samples are given in Fig. 3. Thermophilic bacteria were isolated using Nutrient Agar media, which contains beef extract, peptone, and agar that bacteria need to grow and reproduce. After isolation at an incubation temperature of 50°C for 24 h, 29 thermophilic bacterial colonies were obtained.

Table 1: Observation results

Observation parameters	Observation result
GPS	02° 00’ 34.75” LS and 101° 24’ 52.00” LU
Water temperature	68-79 °C
pH	7.6-7.9
Turbidity	Clear
Precipitate	None
Environmental temperature	32° C
Altitude	811.4-849.5 m

Fig. 2: Sampling of water sample from hot spring

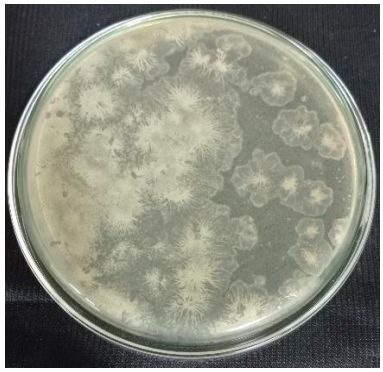

Fig. 3: Colonies of thermophilic bacteria on NA media

Alcohol Screening

The alcohol screening results are shown in Tables 2-4 as well as in Figs. 4-5.

Qualitative testing was carried out on 29 thermophilic bacterial isolates, to find out which thermophilic bacterial isolates have the potential to produce alcohol. The isolate obtained was then cultured in basal media, namely alcohol-producing media, with the addition of glucose as a carbon source for the fermentation process and incubated for 24 h at a temperature of 50°C. In qualitative testing, supernatant fermentation products reacted with potassium dichromate will change color from orange becomes bluish green in the supernatant indicated to contain an ethanol group. The qualitative test results can be seen in Fig. 4.

A total of twenty-nine isolates, seven isolates of thermophilic bacteria were indicated can produce alcohol, nine thermophilic bacteria isolates did not show color changes and the rest did not show significant color changes.

Table 2: Qualitative alcohol test results

		Dichromate test	
No.	Isolate code	Before	After
1	SM 111	Orange	Orange
2	SM 121	Orange	Brownish yellow
3	SM 122	Orange	Orange
4	SM 123	Orange	Green
5	SM 124	Orange	Orange
6	SM 211	Orange	Brown
7	SM 221	Orange	Yellowish green
8	SM 311	Orange	Brown
9	SM 312	Orange	Orange
10	SM 313	Orange	Green
11	SM 321	Orange	Brownish yellow
12	SM 322	Orange	Yellow
13	SM 323	Orange	Yellow
14	SM 411	Orange	Orange
15	SM 412	Orange	Yellow
16	SM 413	Orange	Yellow
17	SM 421	Orange	Brownish yellow
18	SM 422	Orange	Brown
19	SM 423	Orange	Orange
20	SM 511	Orange	Orange
21	SM 512	Orange	Orange
22	SM 513	Orange	Brown
23	SM 521	Orange	Brownish yellow
24	SM 522	Orange	Orange
25	SM 523	Orange	Green
26	SM 514	Orange	Green
27	SM 515	Orange	Green
28	SM 524	Orange	Green
29	SM 525	Orange	Green

Table 3: Potential isolates from quantitative test results

No	Isolate Code	Absorbance Value	Qualitative Description
1	SM 123	0.036	Green
2	SM 313	0.042	Green
3	SM 523	0.033	Green
4	SM 514	0.264	Green
5	SM 515	0.260	Green
6	SM 524	0.258	Green
7	SM 525	0.259	Green

Table 4: Bioactive compound of alcohol fermentation of isolate SM-313 by GC-MS analysis

No.	Peak name	Rel. Area %	Rel. Height
1	Ethanol	24.38	16.84
2	Propanol,2-methyl-	0.90	1.05
3	Ethyl acetate	0.27	0.59
4	Allyl ethyl ether	2.05	1.37
5	4-Penten-2-ol	0.50	0.52
6	Acetoin	0.28	0.36
7	Acetic acid	15.37	18.27
8	Propanoic acid, 2-methyl-	22.03	28.00
9	2,3-Butanediol	34.12	33.00

(a) (b)

Fig. 4: (a) Before adding dichromate; (b) After adding dichromate

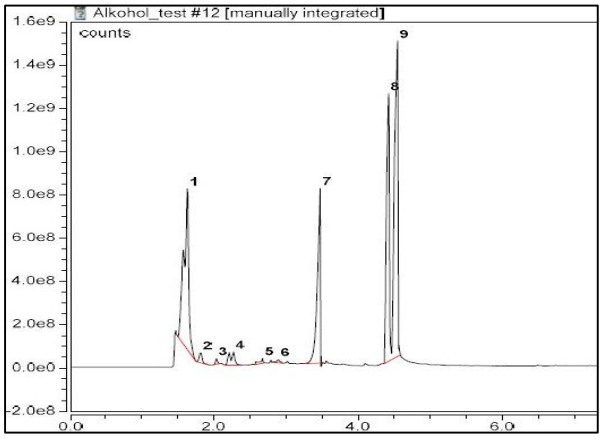

Fig. 5: Electropherogram of GC-MS analysis of alcohol fermentation from SM-313 isolate

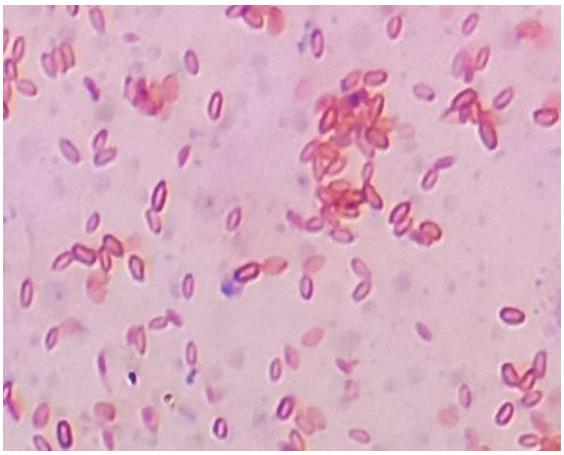

Fig. 6: Gram staining of SM-313 isolate

A total of seven indicated alcohol-producing potential isolates all showed a change in color from orange to green in a qualitative test using potassium dichromate with a range of absorption values between 0.033 and 0.264.
The GC-MS results on the SM-313 showed several peaks, including alcohol groups such as ethanol, 4 penten-2-ol, and 2, 3-butanediol (the highest peak). Based on Table 4; 2, 3 butanediol was obtained from alcoholic fermentation as highest as 34.12%.

The results of Gram staining of potential alcohol-producing thermophilic bacterial isolates are indicated in Fig. 6.

Gram staining carried out on isolate SM-313 showed that the isolate was gram-negative. Gram staining aims to group bacteria into two large groups, namely Gram-positive bacteria and Gram-negative bacteria. Gram-positive bacteria will be purple and Gram-negative bacteria will be red. Gram-positive bacteria can retain the crystal violet dye even when washed with alcohol, while Gram-negative bacteria will lose the crystal violet dye after being washed with alcohol so that the color that appears is a rival color, namely safranin, which is red (Tripathi and Sapra, 2023).

Discussion

The fermentation period was complete and the isolated supernatant was taken using the centrifugation method at a speed of 5,000 rpm for 10 min. From the results of qualitative tests carried out with the addition of potassium dichromate, 29 isolates were obtained, seven of which had the potential to produce alcohol, as indicated by a color change from orange to green (Table 2).

Qualitative test results were also obtained by Simatupang *et al*. (2019), who found that several isolates experienced a color change from orange to green to bluish-green from the isolation of potential ethanol-producing bacteria from the Bali arak industry in Karangasem-Bali. As a result of these qualitative observations, there were five colors in the final result: Orange (showing no color change), greenish orange, green, bluish green, and blue. These four color changes indicate that the supernatant has undergone a chemical reaction. This chemical reaction occurs because, in the potassium dichromate reagent, the carbon chain binds to the -OH group in the supernatant resulting from bacterial fermentation and the reduction reaction of ethanol by potassium dichromate in acidic conditions which changes the color of the substance from orange to green or blue. After obtaining the potential alcohol-producing isolate, the absorbance was then measured using a spectrophotometer with a wavelength of 579 nm (Table 3).

The qualitative test results were also carried out using GC-MS to determine the group of alcohol it contained by analyzing the peaks formed. The GC-MS results (Fig. 5). show that the isolate with code SM-313 contains several alcohol groups, such as ethanol at peak 1,4 penten-2-ol at peak 5, and 2,3-butanediol at peak 9 which is also the highest peak and highest yield (Table 4). As well as several acid groups such as ethyl acetate and acetic acid. A flammable, colorless chemical component, ethanol is also known as "ethyl alcohol" or "grade alcohol," and it is one of the alcohols that is most frequently included in alcoholic beverages. It is frequently referred to as alcohol. C_2H_6O, sometimes written as EtOH or C_2H_5OH, is its molecular formula (Alam and Tanveer, 2020). In this case, ethanol is produced from the fermentation process of glucose, which is added to the basal media by bacteria. The simple sugar molecules that are produced during hydrolysis by the action of microorganisms can be changed into bioethanol (Fig. 7). H_2O and CO_2 are the products of fermentation, while bioethanol is the primary end product (Rasul *et al*., 2019).

The development of liquid transportation fuels from renewable sources, ethanol is a key aim. Alcohol dehydrogenase E (AdhE) or pyruvate decarboxylase can create it biologically from pyruvate or acetyl-CoA, respectively. AdhE, a bifunctional enzyme with both acetaldehyde dehydrogenase and alcohol dehydrogenase functions, is used by thermophilic bacteria. Although the function of AdhA in the manufacture of ethanol is often unclear, many of these organisms also include a distinct Alcohol dehydrogenase (AdhA) that produces ethanol from acetaldehyde (Keller *et al*., 2017).

Ethanol can also be used as an alternative fuel because it can be manufactured from agricultural products like molasses, sugar, and corn. Ethanol is less harmful than other alcoholic fuels and incomplete oxidation of ethanol results in less harmful by-products (Rasul *et al*., 2019). Currently, ethanol has many applications such as bioethanol has begun to replace gasoline since it releases fewer hazardous chemicals, including nitric oxide and carbon monoxide, as well as less CO_2. Burning at low temperatures is conceivable because oxygen exists in molecular structures (Rodionova *et al*., 2017).

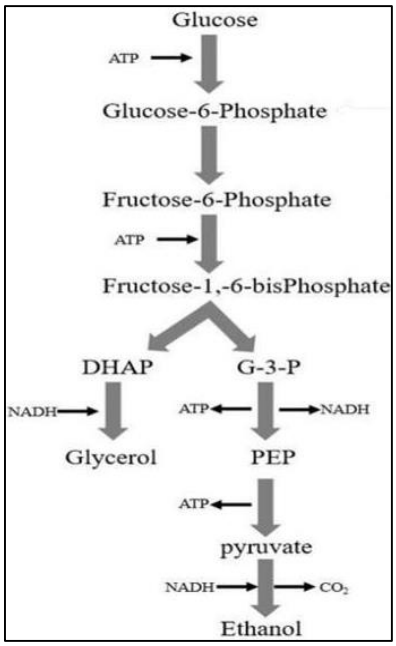

Fig. 7: Pathway of ethanol biosynthesis from glucose

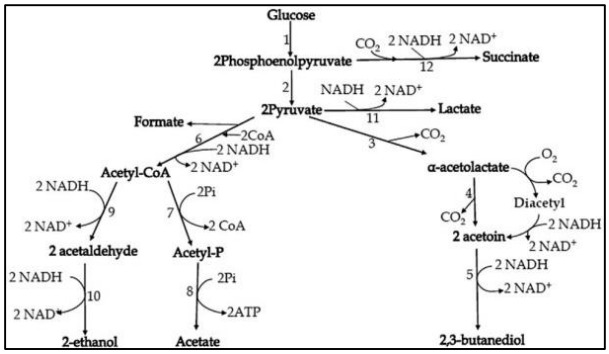

Fig. 8: The metabolic pathway for 2,3 butanediol synthesis in bacteria

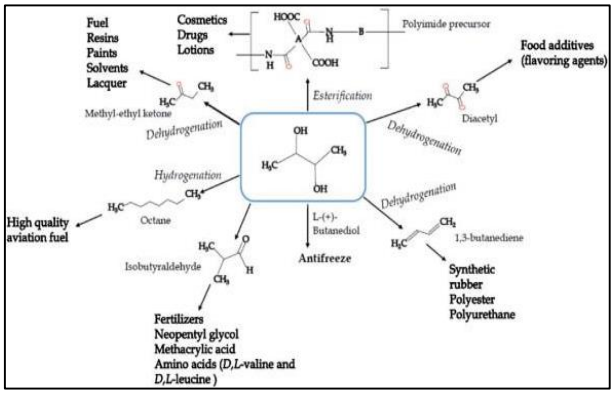

Fig. 9: Some important derivation of 2,3 butanediols and their potential application

The 2-3-butanediol produced by the SM-313 showed the highest peak in the GC-MS test, which means that the SM-313 isolate has the potential to produce alcohol from the 2.3-BD group with relative concentrations based on area and height of 34.12 and 33.00%.

A mixed-acid fermentation pathway is used to produce 2,3-butanediol, and depending on the microbe and fermentation techniques, several other end products are typically produced as well (Ye, 2015). Shi *et al*. (2014) Fig. 5 depicts the metabolic process by which glucose is converted in bacteria into 2,3-BD. The creation of 2,3-BD from pyruvate involves three primary enzymes, acetolactate synthase, decarboxylase, and butanediol dehydrogenase (Ji *et al*., 2011).

Metabolism for the biosynthesis of 2,3-BD from glucose in bacteria, the pathway is shown in Fig. 8. The three main enzymes that play a role are α-acetolactate synthase, α-acetolactate decarboxylase, and butanediol dehydrogenase involved in the production of 2,3-BD from pyruvate (Ji *et al*., 2011). In *Klebsiella* sp. bacteria, these main enzymes are encoded by the budB, budA, and budC genes (Bialkowska, 2016).

Figure 9 shows derivatives of 2.3 BD that can be used in various industrial fields. The dehydrogenation process of 2,3 butanediol will produce 3 molecules, namely the first molecule: Methyl-ethyl ketone which is used for fuel, resin, paint, solvent, and lacquer, the second molecule is 1,3-butanediene which is useful for synthetic rubber, polyester, and polyurethane. Meanwhile, the third molecule is diacetyl which is a flavoring agent in food additives. The esterification process will produce polyimide precursors which are used in cosmetics, drugs, and lotions. The Hydrogenation process produces octane as a High-quality aviation fuel. Other derivatives in the form of isobutyraldehyde molecules are useful for fertilizers, neopentyl glycol, methacrylic acid, and amino acids (D, L-valine, and D, L-leucine. L-(+)-butanediol is also a derivative of 2,3 butanediol which is useful for antifreeze (Bialkowska, 2016; Hakizimana *et al*., 2020).

Conclusion

Based on the results and discussions carried out on research to screen thermophilic bacteria for potential alcohol production, it can be concluded that in the hot springs, there are thermophilic bacteria that have the potential to produce alcohol, with a total of 29 isolates, 7 isolates were found to be potential producers of alcohol and based on the results of GC-MS isolate SM-313 produces alcohol such as ethanol, 4-penten-2-ol, and 2, 3 butanediol.

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Author's Contributions

Anthoni Agustien: Conceived the original idea, wrote the manuscript, designed the study, reviewed and approved the manuscript and coordinated research.

Weni Cahyati: Collected data in the field and laboratory, data analysis, and experimental development.

Yetria Rilda: Supervised laboratory work and designed the research, methodology, and analysis.

Ethics

This article is entirely original and includes never-before-seen content. The corresponding author attests that the work has been read and approved by all other authors and that there are no ethical omissions.

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