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Isolation and identification of acetic acid producer from mixed culture of soil and banana stem waste in anaerobic condition.

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Abstract

Anaerobic fermentation is a feasible option and a low-cost process for production of acetic acid. However, if the material used for production is hardly degradable such as lignocelluloses waste, the process does need the bacteria that are versatile in order to produce a high amount of yield and degrade combination of substrate. Banana stem waste is lignocellulosic waste, and it contained high amount of cellulose that can be used as a substrate for acetic acid production. Meanwhile, mixed culture from soil did contain high amount and variety species of bacteria. In order to increase the yield and find the suitable bacteria for the process, the pure strain needs to be obtained. Therefore, isolation and identification of bacteria need to be done. Isolation and identification of acetic acid producer from mixed culture of soil and banana stem waste was done. Isolation was done by aseptically serial diluted. Culture from each dilution will be cultured anaerobically towards selective medium that consists of glucose. 15 strains from 57 strains isolated able to utilize glucose to produce acetic acid. From 15 strains, one strain able to produce acetic acid up to 1.8 g/l and can degrade lignin. This strain was chosen to be identified using gram stain, biochemical test using BIOLOG kit. From the identification results, the strain was known as *Enterobacter Soli*. This strain can degrade a variety of substrate, including lignin, cellulose and almost all types of reducing sugar (C5-C6) and produce a high amount of acetic acid by using complex raw material as lignocellulosic waste.

Keywords: Acetic Acid, Mixed Culture, Banana Stem Waste, *Enterobacter*

1. Introduction

Earth's surface consists of a variety of soil. Acetic acid producer (AAP) had to live in the soil long before the plants, and animals appeared. This means that their multiplicity growth has become a huge diversity which includes of some of the best properties of microorganisms. Besides, because the abundant sources of soil, AAP can be obtained directly without cost. Moreover, because of their diversity growth in soil, the AAP from soil is higher resistant compare to other types of AAP. The AAP able to operate in a large range of temperature and pH ranged. Thus, the AAP from soil are capable of be used in several types of operation, which will directly reduce a cost of the operation and energy utilization. Meanwhile, banana stem waste (BSW) is lignocelluloses waste, which consists of lignin, cellulose and hemicellulose. It consists of 15.42% lignin, 53.45% cellulose and 28.56% hemicellulose [1]. This data shows that the cellulose content that can be recovered from banana stem is high. As a result, banana stem waste is a very excellent substrate for a variety of microbes to growth.

Acetic acid is widely used as the chemical reagent in chemical industry and additives in food industry. It is also one of the important intermediate substrate used in bio gas production. The annual global demand of acetic acid is around 6.5 million tonnes of which approximately 1.5 million tonnes are produced by recycling [2]. The rest is

manufactured from biological sources and petrochemical feed stocks by carbonylation of methanol, liquid phase oxidation on n-butane, and direct oxidation of acetaldehyde also known as Wacker process. Major disadvantages of the synthetic processes are a requirement of high temperature and pressure, a good agitation, high cost of catalyst, and the dependence on non-renewable sources of a raw material such as crude oil, and also. It exposed to the threat of an explosion [3]. Hence, production of acetic acid using a biological process becomes a more interesting alternative.

The biological process via fermentation could cut the high cost since it uses renewable sources of raw material and low energy utilization. There are two types of the fermentation process which is with oxygen (aerobic fermentation) and without oxygen (anaerobic fermentation) [4]. In aerobic fermentation, two stages are needed, which are conversion of reducing sugar into ethanol followed by ethanol oxidation into acetic acid. So, it needs two reactors that will increase the cost. Another one is anaerobic fermentation able to convert reducing sugar directly to acetic acid in a single step. In anaerobic fermentation, the condition during the fermentation process should be strictly to oxygen supply. The advantages by using anaerobic fermentation is could reduce time required, give higher yield and could eliminate separate reactors for the different stages of acetic acid production.

In the single step, anaerobic fermentation of BSW, mixed culture of soil can be uses to produce acetic acid. The BSW contained high amount of cellulose that can be used as a substrate to produce acetic acid. Although BSW contained high amount of cellulose, lignin content in BSW structure needed to break down before it can be obtained. Not all of AAP available in mixed culture of soil can degrade lignin. Most of the AAP relies on other bacteria that able to degrade lignin in order to use cellulose. Besides, the amount of acetic acid produce is limited due to different type of AAP in the soil have a different optimum range of temperature and substrate to be use in order to produce acetic acid. Therefore, it is a need to find the AAP that able to produce high yield but able to act as lignin degrader while producing acetic acid. The purpose of this research is to find the best pure strain isolated from soil and banana stem waste, which can be used to produce acetic acid that able to degrade lignin.

2 Materials and methods

Feedstock Materials

Banana stems from waste was used as feedstock in the experiments. The banana stem waste (BSW) first has to be washed to remove the soil. After it is washed clean, the banana stem waste is then dried at ambient temperatures between one to two weeks. These were cut into small pieces (5 cm) and mixed with distilled water.

Mixed Culture and Inoculums

Banana stem sludge (BSS) contained of soil, and water was the mixed culture inoculum source in this study. Both BSS and BSW for this study were collected from banana plantation in Kuantan, Pahang. 3 kg BSS was collected from banana plantation soil and placed in 2 l container. 1000 g/l of BSW was then added into the container as a substrate for the BSS. The acclimatization process between the BSS and the BSW takes three-month periods before the isolation and identification process run.

Isolation of bacteria

Isolation process to find potential microbe was run on selective medium, 15 strains from 57 strains found can produce acetic acid. Isolation is done by aseptically serial diluted and culture from each dilution will be cultured on Petri's plat. The isolated single colony will be subcultured on Petri's plat. This pure culture will also be cultured on inclined agar and in nutrient broth as a culture stock and kept under 4^oC. Only one strain was chosen to be identified using gram stain analysis and biochemical test using BIOLOG kit based on the ability to degrade lignin and high yield of acetic acid.

Experimental Set-up

Experiment was done at 30^oC in anaerobic condition. The fermentation process was run until 72 hours. A medium for growth and cultivation of isolated strain is a modification of some medium composition from earlier research. The media contained of 5 g/l glucose, 5 g/l peptone, 3 g/l meat extracts, 2 g/l KH_2PO_4, 0.5 g/l $MgCl_2$ and $NH_4Cl_2H_2O$ were prepared by heating before sterilization to help driven off dissolved oxygen. Initial microbe concentration was 10% v/v. Next; nitrogen was purged for 10 minutes to maintain anaerobic conditions in the reactor. One best strain that able to produce acetic acid from a medium provided was chosen to be test with lignin degradation.

Analytical Method

Sample was periodically taken by syringe every six hours, oxidized by exposure to air to stop the reaction, and stored at -20°C prior to analysis by HPLC. Acetic acid concentration was measured by HPLC. HPLC system equipped with a diode array detector and an Agilent Zorbaq Sb-Aq C18 analytical column with corresponding guard column. The column temperature was maintained at 35^o C. Phosphate buffer 20mm was used as mobile phase at a flow rate of 0.8 ml/min, the volume of the injection loop was 5 µl [4] .

3 Results and Discussion

Fermentation was run for 72 hours before the acetic acid yield was checked by using High-Performance Liquid Chromatography (HPLC). The experiment was repeated three times before the average amount of acetic acid and pH value acquired. Below was the result of acetic acid concentration and pH according to their strains.

Table 1: Acetic acid yield from 15 isolated strains from banana stem waste and mixed culture of soil used in fermentation using the selective medium.

Strain	Amount of Acetic Acid in g/l	pH
A4	1.57	5.57
B1	1.98	5.55
B9	1.16	4.87
A6	2.90	3.63
A7	0.51	4.79
A7c	1.83	5.68
B8	1.73	5.09
A6c	1.55	5.21
A2	1.83	5.55
B9c	1.49	4.87
BL1	0.81	5.14
A1	0.32	4.75
AL1	0.28	5.34
B4	0.26	4.95
A12	0.55	4.70

From the above table, the microbes that could produce acetic acid more than 1.0g/l were A2, A4, A6, A6c, A7c, B1, B8, B9 and B9c. The hypothesis of the acid base is the lower the pH, the more acidic the product is. From these nine microbes, A6 can produce a highest amount of acetic acid, which was 2.90g/l with pH 3.63. Most of the microbes can produce an amount of acetic acid which the value of pH showed below than 6. These may be caused of production of another acid that may alter the pH, value, whether increased or de-creased it.

From these nine microbes, only five best microbes were chosen to undergo further test using kraft lignin. Those strains were B8 , A6, B1, A7c, A2. It is found out, that only one microbe able to degrade kraft lignin. B8 was the only strain that can degrade lignin and chose to undergo biochemical tests. From the biolog test, the system identified the bacteria as Enterobacter cowanii. However, the results show there is a possibility the strain is new. But, the strain is not novel. The bacteria have similarities to Enterobacter Soli identified by Manter et al. (2011). This bacterium is also facultative anaerobic, can degrade lignin and have similar positive results in biochemical test. The bacteria show positive reaction with glucose, mannitol, sorbitol, saccharose, rhamnose, melibiose, amygdalin and arabinose, and negative towards inositol.

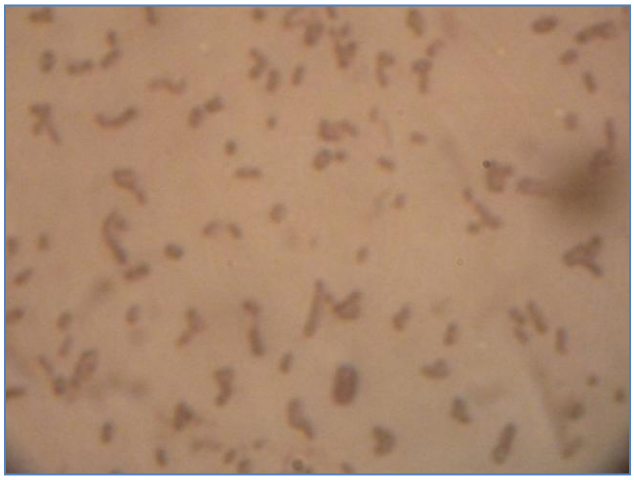

Figure 1. The view of *Enterobacter soli* sp. under the microscope

From the view under the microscope, the colour of the cell was red, which mean it was gram negative. Cells are motile, rods 1.0-2.0 µm in length and ~0.5 µm wide. The colony of the *Enterobacter soli* sp. nov looks circular, raised, entire and cream in color. Younger colonies are more translucent and older colonie more opaques. The unique characteristic of this strain compared to other *Enterobacter* was facultative anaerobe, which could degrade lignin and can survive with or without oxygen.

Generally, most *Enterobacter* is found to be very similar to each other and sharing many biochemical and physiological properties. However, these bacteria can be differentiating using a method to ferment reducing sugar. Mixed acid fermenter metabolizes glucose anaerobically to produce acetic acid, lactic acid, succinic acid, formic acid, ethanol, CO_2 and H_2. The pH of the medium will be lowered due to a large amount of acid produced. This family also needs simple nutritional requirements and are facultative anaerobes that ferment glucose to be acid under anaerobic conditions. The major biochemical test used to determine *Enterobacteriaceae* family is using Voges-Proskauer fermentation reaction, Phenylalanine deaminase activity, Indole production from tryptophan and Citrate utilization as carbon sources [6]. Below is the comparison of biochemical characteristic between B8 with other *Enterobacter* species.

Table 2: Result from biochemical test and comparison with other *Enterobacter* species.

Reaction	B8 / Soli	*Aero-genes*	*Asbu-riae*	*Cloa-cae*	*Co-wanii*
o-Nitrophenyl-*β*-galactosidase	+	-	+	+	+
Arginine dihydro-lase	+	-	+	+	-
Lysine decarbox-ylase	-	+	-	+	-
Ornithine decar-boxylase	+	+	+	+	-
Citrate utilization	+	+	+	+	+
H2S production	-	-	-	-	-
Urease	-	-	-	-	-
Tryptophan dea-minase	+	-	+	-	-
Voges-Proskauer reaction	+	+	+	+	+
Glucose	+	+	+	+	+
Mannitol	+	+	+	+	+
Inositol	-	+	-	-	-
Sorbitol	+	+	+	+	+
Rhamnose	+	+	-	+	+
Saccharose	+	+	+	+	+
Melibiose	+	+	-	+	+
Amygdalin	+	+	**ND**	+	+
Arabinose	+	+	+	+	+

This study; symbols are as follows; + , positive reaction; - , negative reaction. ND = No data

From the biochemical test, the B8 can be indicated as Enterobacter soli sp. nov. This species are able to degrade a variety of carbon sugar including glucose, mannitol, sorbitol, rhamnose, saccharose, melibiose, amygdalin and arabinose. Furthermore, this species can undergo delignification itself without rely on other strains to break down lignin, especially in complex structure as lignocellulose. Therefore, it is huge advantaged when using this species as AAP in production of acetic acid using banana stem waste (lignocellulosic waste). The cellulose and hemicellulose contained in lignocellulose can be easily be obtained as a substrate to produce acetic acid. Moreover, this strain can degrade cellulose and hemicellulose into reducing sugar (C5-C6) before used for acetic acid is production.

If the comparison is made based on carbon sources (glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, melibiose, amygdalin and arabinose) from a biochemical test available in the well, the results show that this strain, *Enterobacter Cowanii* and *Enterobacter cloacae* share the same results [8]. The results show the same results because of common physiology among these species. These species are gram-negative species, facultative anaerobe and rod-shaped bacteria. Besides, *Enterobacter cloacae* can also be found from soil same as *Enterobacter soli*. Meanwhile, *Enterobacter cowanii* is new species and there is no information of this species is available in soil and mostly the species is found as clinical strain. The *Enterobacter Soli* can be differed from *Enterobacter Cowanii* and *Enterobacter Cloacae* based on negative results on Arginine, Ornithine, Lysine and Tryptophan.

In 2008, Sandra et al., have done a research on fermentative hydrogen production by a microbial consortium. From the research, it was indicated that the microbial consortium presented, which was *Enterobacter cloacae* was recognized as volatile acid producers. The research was carried out in batch reactors under anaerobic conditions because to verify the efficiencies of sucrose conversion to H_2. At the end of the research, the result showed that the intermediary products were acetic acid, butyric acid, methanol and ethanol. Furthermore, *Enterobacter Soli*, *Enterobacter Asburiae* and *Enterobacter Aerogenes* can also produce H_2 and produce acids as intermediary products. Those strains share some similarities to in terms of product formation from fermentation [5].

It can be concluded that acetic acid producer, B8 is an *Enterobacter soli* sp. nov. This strain is able to produce a high amount of acetic acid from lignocellulosic waste and easily be obtained from soil. The following table is the scientific classification of *Enterobacter soli*.

Table 3: Result from biochemical test and comparison with other *Enterobacter* species.

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	Enterobacter
Species	*Enterobacter soli*

4 Conclusion

This study demonstrates that there are so many types of bacteria can be found in soil that can produce acetic acid. Besides, there is a strain that is a multi-task strain which means, the strain can utilize every substrate available and produce acetic acid. *Enterobacter Soli* is a new strain available from soil and able to degrade lignocellulose and produce high amount acetic acid from it. This strain can be used as a microbe source to degrade banana stem waste in anaerobic condition to produce acetic acid.

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