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Screening of Acetic Acid Bacteria from Pineapple Waste for Bacterial Cellulose Production using Sago Liquid Waste

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Abstract

Bacterial cellulose is a biopolymer produced by fermentation process with the help of bacteria. It has numerous applications in industrial sector with its characteristic as a biodegradable and nontoxic compound in nature. The potential application of BC is limited by its production costs, because BC is produced from expensive culture media. The use of cheap carbon and nutrient sources such as sago liquid waste is an interesting strategy to overcome this limitation. The objective of this study was to obtain the AAB strain that capable to produce bacterial cellulose from sago liquid waste. Isolation of AAB strains was conducted using CARR media and the screening of BC production was performed on Hestrin-Schramm (HS) media with glucose as a carbon source. The strains of AAB then were evaluated for their cellulose-producing capability using sago liquid waste as a substrate. Thirteen strains of AAB producing BC were isolated from pineapple waste (pineapple core and peel) and seven of them were capable to produce BC using sago liquid waste substrate. One of the AAB strains produced a relatively high BC, i.e. isolate LKN6. The result of morphological and biochemical test was proven that the bacteria was *Acetobacter xylinum*. The result of this study showed that *A. xylinum* LKN6 can produce a high yield of BC, therefore this strain is potentially useful for its utilization as a starter in bacterial cellulose production.

How to Cite

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INTRODUCTION

Acetic acid bacteria (AAB) are obligately aerobic bacteria within the family of *Acetobacteraceae*, widespread in sugary, acidic and alcoholic niches (Mamlouk & Gullo, 2013). A recent classification of the acetic acid bacteria includes the genera of *Acetobacter, Acidomonas, Ameyamaea, Asaia, Gluconacetobacter, Gluconobacter, Granulibacter, Kozakia, Neoasaia, Neokomagataea, Saccharibacter, Swaminathania* and *Tanticharoenia*. (Yamada & Yukphan, 2008; Mamlouk & Gullo, 2013). AAB are generally found in nature because they can use a variety of substrates (Sharafi *et al.,* 2010) and these bacteria have been isolated from alcoholic beverage, vinegar, fruits and fruit juice, flowers, honey, sugar cane, soil and water (Sharafi *et al.,* 2010; Klawpiyapamornkun *et al.,* 2015). Pineapple waste contains a high organic acid (Hemalatha & Anbuselvi, 2013) that creates an acidic niche. Therefore, pineapple waste is a good source for isolation of AAB.

Some of the Acetic acid bacteria from genera of *Acetobacter* and *Gluconacetobacter* are known to produce cellulose in culture (Mamlouk & Gullo, 2013). The structural features and mechanical properties of bacterial cellulose differ from those of plant cellulose, due to its high purity, hydrophilicity, structure forming potential, chirality and biocompatibility offers a wide range of special applications, such as a food matrix (nata de coco), dietary fiber, as an acoustic or filter membrane, as ultra-strength paper and as reticulated fine fiber network with coating (Chawla *et al.,* 2009; Keshk, 2014).

Bacterial cellulose is produced from expensive culture media, containing glucose as a carbon source (Chawla *et al.,* 2009; Dewi, 2009) and other nutrient sources. It is resulting in a very high production cost which limits the use of this material to a very high value-added applications. The use of cheaper carbon and nutrient sources is an interesting strategy to overcome that limitation and therefore to increase the competitiveness of unique material, such as agricultural and industrial wastes. Majority of wastes end up being discarded, whereas, wastes have abundant sugars that can be converted biologically into useful products such as bacterial cellulose (Cavka *et al.,* 2013). An advantage of using agricultural or industrial residual as feedstock for the production of bacterial cellulose is the low cost of the raw material.

Sago liquid waste is very potential as a substrate to grow the BC-producing bacteria, since it has a high carbohydrate content, especially starch. However, not all AAB are able to grow in waste containing complex carbohydrates. Therefore, it is necessary to explore the strain of acetic acid bacteria that is capable to produce BC in sago liquid waste as a substrate. This study was intended to isolate acetic acid bacteria from the pineapple wastes to assess their potentiality for BC production using sago liquid waste as a substrate. The benefit of this research is to provide further information of acetic acid bacteria strains that can be utilized to produce BC from inexpensive substrates such as industrial waste, so that the utilization of BC can be widely expanded. In addition, the utilization of sago liquid waste to produce BC can be a solution to overcome the environmental pollution.

METHODS

Sample Collection

Samples of bacteria used for acetic acid bacteria (AAB) isolation was obtained from pineapple waste. Pineapple wastes (pineapple core and peel) were collected from local market in Kendari, Southeast Sulawesi, Indonesia. Samples then were stored in sterile bags at 4°C for further use.

Isolation of Acetic acid bacteria

Samples (10 g) strored in sterile plastic bags, then were homogenized using a sterile 0.85% (w/v) NaCl solution. They were serially diluted and spread on modified CARR Agar (Romero-Cortes *et al.,* 2012) (expressed as g/L): glucose, 3; $CaCO_3$, 10; bromothymol blue, 0.04; yeast extract,10; agar, 20 and ethanol 17.5 ml/L, pH 6.8. Incubation process was performed at 30°C and the growth of bacteria was observed after 48 hours. The colonies with yellow and clear zone on the agar plates were selected for further examination. Suspected AAB isolates from CARR medium were inoculated onto nutrient agar plates and were further identified by identifying the morphological characters of colonies and cells developed after 18-24 hour of incubation.

Screening of Acetic Acid Bacteria cellulose producer

Screening of potentially bio cellulose-producing bacteria was performed on Hestrin-Schramm (HS) modified test media, contained 20 g glucose, 5 g bacto peptone, 5 g yeast extract, 2.7 g Na_2HPO_4, and 1.2 g citric acid monohydrate per liter of distilled water, and it was adjusted to pH 5.5 (Son *et al.,* 2002). The AAB isolates were diluted with sterilized water and transferred into

10 ml of HS medium. The isolates then statically cultured at 30°C in a test tube for 7 days, and screened by observing BC pellicle formation. The AAB isolate that capable to produce BC were selected to determine their ability in producing bacterial cellulose in sago liquid waste.

Production of Bacterial Cellulose from Sago Liquid Waste

In this study, sago liquid waste was collected from sago processing industry in Konawe regency, Southeast Sulawesi, Indonesia. The sago liquid waste medium contained 200 g commercial sucrose, 15 g ammonium sulfate (ZA) per liter of sago liquid waste and pH was adjusted at pH 5.0 with acetic acid 1%. The medium was boiled for 15 minutes and was cooled. Sago liquid waste media was prepared in containers with the following dimension: diameter (d) = 6 cm and height (h) = 12 cm and surface area (a) = 56.4 cm^2. Then, the AAB isolates in HS medium was inoculated 5.0 % (v/v) into the sago liquid waste medium aseptically and incubated for 14 days in undisturbed condition at room temperature (28-30°C). The Bacterial Cellulose layer formed after 14 days, then it was harvested and immersed in water for 24 h with repeatedly changing the water to remove the sour odor. The wet and dry weight of BC are determined by method as described by Raghunathan (2013). The yield of the cellulose was calculated as follows (Goh *et al.,* 2012):
Yield (%) = (wet weight of bacterial cellulose (g/L)) / (sucrose concentration (g/L))

Phenotypic Characterization

The AAB isolate with the highest BC-production was selected for further identification. Identification of AAB isolate was performed by using morphological, biochemical and physiological characteristics of the pure isolates. Characterization of AAB isolate was examined according to the Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994).

RESULTS AND DISCUSSION

Isolation of Acetic Acid Bacteria (AAB) from Pineapple Waste

Acetic acid bacteria were successfully isolated from pineapple wastes (pineapple core and pineapple peel). A total of 16 isolates of AAB, 7 from pineapple core and 9 from pineapple peel (Table 1) were obtained. pH of the pineapple waste was acidic, which was in the range of 3.39 to 3.43 for pineapple core and 3.86 to 4.14 for pineapple peel so that it is good for the growth of acetic acid bacteria. Nadzirah (2013) reported that pH of the pineapple core and peel extracts were 3.37 and 3.84, respectively. Mamlouk and Gullo (2013) reported that AAB likes the environment at acidic pH, with a range of 3 to 5. The number of AAB isolate was found to be higher in pineapple peel than in pineapple core (Table 1), this may be due to the pH of the pineapple peel (pH 3.8-4.1) is more suitable for the growth of AAB rather than the pineapple core (pH 3.3-3.4). This result is consistent with study by Osborne (2010) and Raghunathan (2013), reported that the growth of AAB decreasing below pH 3.5. Ukwo and Ezeama (2011) also reported that the growth of acetic acid bacteria decreased more rapidly at pH 3.7 than at pH 4.1.

Table 1. Acetic acid bacteria isolated from pineapple waste

Source	Isolate code	Total of isolates
Pineapple core	LBN	7
Pineapple peel	LKN	9
Total		16

The bacteria were cultured on CARR medium. After 48 hours of incubation at 30°C, there was a presence of colonies with the zone of clearance around them and it was initiated to convert the color of the CARR medium from green to yellowish indicating that isolated strain was acetic acid bacteria (Figure 1). The change in colour of media from green to yellow due to the pH indicator in CARR media (bromothymol blue). The media will have green color in neutral pH, and if the acid was formed, the color then will be changed to yellow (Romero-Cortes *et al.,* 2012; Mukadam *et al.,* 2016). Besides that, colonies of AAB were recognized by the surrounding $CaCO_3$ clearing zones. $CaCO_3$ neutralizes AcOH generated by AAB, preventing the physiological stress and cell death (Mamlouk & Gullo, 2013).

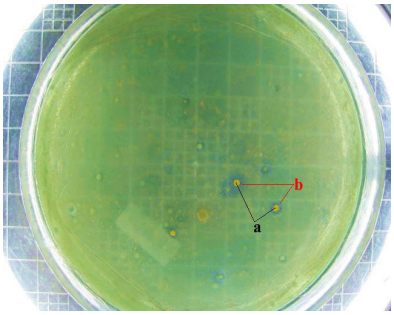

Figure 1. The Acetic Acid Bacteria colonies on CARR media. a. AAB colony, b. Clear zone

Microscopic examinations confirmed that all the isolates were Gram-negative, rod-shaped and catalase positive bacteria. Those characteristics were in accordance with the description of the family of the AAB in the Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994).

Screening of Bacterial Cellulose-Producing Acetic Acid Bacteria

Screening of BC-producing AAB was done qualitatively by using Hestrin-Schramm (HS) media. The indicator of qualitative screening for cellulose producer on HS media after seven days of fermentation were the turbidity occurred which indicated growth of isolate bacteria and pellicle formation on the surface media. These results are consistent with Ochaikul *et al.* (2013), who stated that one of the indicators of cellulose-producing bacteria is the ability of the bacterial isolate to grow and form cellulose layer on the surface of the test medium after fermentation for 7 days. The results of isolates screening of AAB to determine their capability in synthesizing bacterial cellulose are shown in Table 2.

Table 2. Isolate of acetic acid bacteria capable synthesizing bacterial cellulose on Media Hestrin-Schramm using glucose as carbon source

Isolate code	Bacterial cellulose (BC)
LKN1	+
LKN2	+
LKN3	-
LKN4	-
LKN5	+
LKN6	+
LKN7	+
LKN8	+
LKN9	+
LBN1	+
LBN2	+
LBN3	-
LBN4	+
LBN5	+
LBN6	+
LBN7	+

+ : capable to synthesize of BC;
- : unable to synthesize of BC

Table 2 showed that from 16 isolates of AAB, 13 isolates were selected as a representative of bacterial cellulose producer. Pellicle formation was proven to be a good indicator of cellulose producers and virtually all AAB cultures were capable of producing pellicles. Thirteen AAB isolates were further evaluated for cellulose production capability using sago liquid waste as the substrate.

Screening of Bacterial Cellulose producing AAB with Sago Liquid Waste as a Substrate

13 AAB isolates that was capable to produce cellulose in HS media with glucose as carbon source, were tested for their ability to produce BC with sago liquid waste as the substrate. From 13 isolates, only 7 isolates were observed to form pellicle at air-liquid interface (Figure 2), pellicle formation was proven as a good indicator of cellulose producers. The 7 isolates capable to produce BC in sago liquid waste were LBN1, LBN6, LKN1, LKN5, LKN6, LKN7, and LKN8. This result indicated that not all AAB were capable to produce BC using the substrate which contains complex sugar as a carbon source. Keshk (2014) stated that in general, glucose has been used as a carbon source for cellulose production by AAB. However, polysaccharides such as soluble starch might also be used by these bacteria as carbon sources.

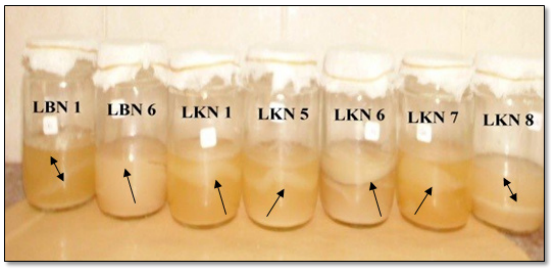

Figure 2. Pellicle formed at air liquid interface by the AAB isolates in sago liquid waste media. The arrows show the pellicle

The ability of 7 AAB isolates to produce BC in sago liquid waste medium were examined based on a wet and dry weight of pellicle cellulose formed on surface media and the yield of bacterial cellulose in sago liquid waste substrate. Production of bacterial cellulose from seven AAB isolates is shown in Table 3.

Table 3 shows that the yield of cellulose pellicle that was produced by 7 isolates in sago liquid waste substrate after 14 day of fermentation are in range of 23.16 to 62.73%, with a wet weight ranged from 46.32 to 125.45 g/L and dry weight ranged from 0.73 to 4.12 g/L. The isolate of LKN6 isolated from pineapple peel, is the highest bacterial cellulose producer in sago liquid

waste substrate with wet and dry weight of cellulose pellicle and yield of BC, are 125.45 g/L, 4.12 g/L, and 62.73%, respectively. Result of this study showed that LKN6 isolate can be used as a starter for bacterial cellulose production with industrial waste containing polysaccharides as a substrate.

Table 3. Bacterial cellulose produced by isolated strains of acetic acid bacteria in sago liquid waste media after 14 days of fermentation

Isolate Code	Bacterial cellulose production		
	Wet weight (g/L)	Dry weight (g/L)	Yield (%)
LBN 1	46.32	0.73	23.16
LBN 6	60.57	1.27	30.29
LKN 1	58.63	1.14	29.32
LKN 5	73.47	1.52	36.74
LKN 6	125.45	4.12	62.73
LKN 7	98.75	2.22	49.38
LKN 8	58.24	1.08	29.12

Several studies on cellulose production have been reported. Goh *et al.* (2012) reported that AAB that produced the highest yield (66.7%) of bacterial cellulose was in black tea broth (kombucha) in 8 days. Abdelhady *et al.* (2015) mentioned that *Acetobacter xylinum* (= *Gluconacetobacter xylinus*) ATCC 10245 gave 3.63 g/L of dry weight with a yield of dry BC weight of 24.20% when cultivated in glucose-ethanol acetic acid (GAM) medium using starch as a carbon source under static condition for 7 days. It is well known that *Acetobacter xylinum*, a Gram-negative acetic acid bacterium, has long been used as a model organism for the study of BC biosynthesis, since it can utilize a wide range of substrates such as 5- or 6-carbon monosaccharides (e.g. D-glucose, D-fructose and D-xylose), oligosaccharides (e.g. sucrose) (Chawla *et al.,* 2009), polysaccharides (e.g. starch) (Abdelhady *et al.,* 2015), sugar alcohols (e.g.glycerol, D-mannitol and D-sorbitol) (Esa *et al.* 2014; Abdelhady *et al.* 2015) and industrial wastes including sugar cane molasses (Esa et al, 2014), coconut water, pineapple water (Kongruang, 2008) and hydrolyzed konjac powder (Hong & Qiu, 2008) to generate high amounts of cellulose.

Identification of the best bacterial cellulose producer

Morphological and biochemical characteristics of the LKN6 isolate were examined and compared with *A. xylinum* in the Bergey's manual of determinative of Bacteriology. The isolate LKN6 was a Gram-negative, rod-shaped or short rod, 0.6-0.8×1.0-3.0 μm bacteria and occurred singly or in pairs (Table 4). The morphological results obtained are congruent with Son *et al.* (2002); Dewi (2009) and Mohammad *et al.* (2014). The isolate LKN6 showed catalase positive reactions, motile and growth at pH 3.0-7.0 (Table 4). These characteristics are congruent with Suwanposri *et al.* (2013), reported that all the BC-producing isolates isolated from tropical fruits in Thailand showed catalase-positive reaction and growth at pH 3.0-7.0.

The overoxidation of ethanol and ketogenesis of glycerol for isolate LKN6 and reference strain were positive (Table 4). Son *et al.* (2002) also reported that *A. xylinum* KJ-1 have a positive reaction for ketogenic activity towards glycerol. Table 4 also shows that there are 3 distinct characters between local strain LKN6 with *A. xylinum* reference strain: growth using ethanol as a carbon source, ethanol tolerance and growing ability at a glucose concentration of 30%. The difference characters indicated that the local strain LKN6 is a member of the *A. xylinum* species with specific characters that distinguish it from the reference strain. Several previous studies have also reported that some strains of *A. xylinum* species have characteristics of ethanol and glucose tolerant (Chaves-Pacheco *et al.,* 2005) and are capable using ethanol as a carbon source (Abdelhady et al.,2015). According to the study by Chaves-Pacheco *et al.* (2005), ethanol and glucose (up to 50%) can improve the growth and cellulose synthesis in *Gluconacetobacter xylinum* (formerly *Acetobacter xylinum*). Mohammad *et al.* (2014), also reported that some of *Acetobacter* strains (*A. xylinum* BRC5, *Acetobacter* sp. V6, and *Acetobacter* sp. A9) showed growth in media containing ethanol. Besides that, Abdelhady *et al.* (2015) also reported that *A. xylinum* ATCC 10245 is able to grow and produce bacterial cellulose using ethanol as a carbon source. Previous research showed that some strains of *A. xylinum* are capable of using ethanol as a carbon source and ethanol tolerant, therefore the LKN6 isolate was estimated as a member of ethanol tolerant *A. xylinum*. The characteristics of LKN6 isolate which are ethanol and sugar tolerant indicated that isolate LKN6 is able to produce acetic acid. Konate *et al.* (2014) stated that the AAB that are tolerant to high ethanol and sugar concentrations have the ability to produce acetic acid.

Consequently, those characteristics were appropriate with the description of the *A. xylinum*

in the Bergey's manual of determinative bacteriology, suggesting that isolate LKN6 should belong to a group of *A. xylinum*, but the further molecular analysis is yet required.

Table 4. Characteristics of isolate LKN6 compared with the reference strain, *Acetobacter xylinum*

Characteristics	Isolate LKN6	*Acetobacter xylinum*[1]
Morphological tests		
Shape	Rod	Ellipsoidal to rod shape
Size	0.6-0.8×1.0-3.0 µm	0.6-0.8×1.0-3.0 µm
Arrangement of cell	Singly, in pairs	Singly, in pairs, in chains
Gram stain	Negative	Negative
Motility	+	+
Biochemical tests		
Catalase	+	+
Gelatin liquefaction	-	-
Overoxidation of ethanol	+	+
Ketogenesis from glycerol	+	+
Growth in the presence of 10% ethanol	+	-
Growth in the presence of 30% D-glucose	+	-
Growth in carbon sources		
Ethanol	+	-
Sodium Acetate	-	-
Methanol	-	-
Dulcitol	-	-
Growth in L-amino acids in the presence of D-mannitol as the carbon source		
L-glycine	-	-
L-tryptophan	-	-
L-glutamine	-	-
L-asparagine	-	-
Acid production from :		
Glucose	+	+
Sucrose	+	+
Fructose	-	-
Lactose	-	-
Physiological		
Growth at pH 3-7	+	+

[1]Bergey's Manual of Determinative Bacteriology

In this study, it obtained a local AAB strain showing high BC production from sago liquid waste substrate. This bacterial strain potential for the improvement of cellulose synthesis with future implications of bioengineering to produce cellulose on an industrial scale.

CONCLUSION

The study concluded that 7 acetic acid bacteria isolated from pineapple waste were capable to produced bacterial cellulose using sago liquid waste substrate. The isolate LKN6 isolated from pineapple peel was selected as the highest cellulose producing bacteria in sago liquid waste substrate with a yield of bacterial cellulose of 62.73% and was identified as *Acetobacter xylinum.* Therefore, *Acetobacter xylinum* LKN6 is a potential starter for bacterial cellulose production.

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