

Isolation and Characterization of Acetic Acid Bacteria from Pineapple, Sugarcane, Apple, Grape, Pomegranate, and Papaya Fruit

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ABSTRACT

The present research was carried out with the aim of isolating acetic acid bacteria (AAB) from pineapple, sugarcane, apple, grape, pomegranate, and papaya fruits. The characteristics of acetic acid bacterial isolates were identified by morphological and biochemical tests. Among the different fruits, pineapple possessed the maximum (38.46%) AAB producers, followed by sugarcane (22.90%), grape (18.29%), apple (14.63%), papaya (4.34%) and pomegranate (1.38%). Reference strain *Acetobacter pasteurianus* (DSM-2324) was collected from Deutsche Sammlung Von Microorganismen und Zellkulturen (DSMZ), Germany. Strains of *Acetobacter pasteurianus* were identified through morphological and biochemical characteristics. The isolated strains (A) were characterized and compared with the reference strain (B). Different levels of ethanol (1%, 2%, 5%, and 10%), glucose (10%, 20%, 25%, and 30%) and temperature (25 °C, 30 °C, 34 °C, and 37 °C) were used in standard medium to observe the growth characteristics of strain A and B. Both the strains revealed similar trends grown on ethanol, glucose and temperature and showed better growth at 2% ethanol, 20% glucose and 30 °C temperature. The members of the genus *Acetobacter* and *Gluconobacter* are confirmed with this research.

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1. INTRODUCTION

Vinegar is a sharp, sour liquid usually used as a condiment and in food preservation. It is produced by fermenting substances containing sugars and carbohydrates in fruits and vegetables, which turn into ethanol alcohol and then acetic acid [1]. The variation of vinegar available all over the world is due to raw materials used, such as grapes, rice, malt, apples, different berries, grains, whey and honey [2]. Fruits are regarded as one of nature's greatest gifts to humans from ancient times as a source of energy when they are hungry. In Bangladesh, plenty of different fruits are produced in different seasons. During 2017–2018, the total amount of fruits produced in Bangladesh was about 3833 thousand metric tons [3]. Huge amounts of fruits are wasted annually that are unable to utilize the excess. Although there are currently certain ways of direct fruit consumption, such as jams, fruit concentrates, juices, nectars, purees, etc., a lot of fruit is still left in the

fields that are rotten or picked to be disposed of as waste [4]. The second and third-quality fruits and their wastes can be utilized for vinegar production. In a wide range, fresh fruit wastes to produce vinegar cannot be collected for commercial production. Market policy and awareness of people inhibit the commercial production of vinegar from fruit wastes. Wasted pineapple peel may be turned into vinegar, a beneficial product that reduces pollution in the environment, preserves important nutrients in our food and lowers the cost of creating processed meals. Vinegar from fruit wastes is a new concept in Bangladesh, although it is widely practised in developed countries. All vinegars undergo a fermentation process known as alcoholic fermentation (AF) that produces ethanol, carbon dioxide (CO₂), and cellular energy from carbohydrates like glucose, fructose, and sucrose. Yeast is mostly responsible for this process. *Saccharomyces cerevisiae* is the most common species of yeast [5]. While AF in the production of vinegar, non-*Saccharomyces* organisms were additionally



identified [6]. Heterofermentative metabolic pathways can provide ethanol to microorganisms such as lactic acid bacteria [7], [8]. In the oxidation of ethanol, known as acetification, the sugar is transformed into ethanol; AAB performs the second bioprocess, which is highly dependent on oxygen supply. Acetic acid bacteria (AAB) are the microorganisms that are involved in acetic fermentation.

Bangladesh is a tropical country with abundant microbiological resources and fruit biodiversity. The main goals of this study were to isolate and identify AAB from a variety of fruits such as pineapple, sugarcane, apple, grape and papaya and to find strains that yielded significant amounts of acetic acid. Additionally, isolated AAB in the laboratory were compared with the reference strain *Acetobacter pasteurianus* DSM 2324.

2. MATERIALS AND METHODS

2.1. Collection of Fruits Samples and Reference Strain

Samples of ripened pineapple, apple, grape, papaya, sugarcane, and pomegranate fruits were gathered at a nearby fruit market, allowed to stand for a few days, and thereafter aseptically crushed and placed in bottles to be incubated at 30 °C for seven days. *Acetobacter pasteurianus* (DSM-2324) was collected from Deutsche Sammlung Von Microorganism and Zellkulturen (DSMZ), Germany. The strain was activated on GYC medium. The strain was activated and analyzed in comparison with the isolated strain from local fruits.

2.2. Selection of Bacteria Isolates and Culture Purification

Spread plate technique, as described by Harley and Prescott in 2008, was followed. Diluted samples of 0.1 ml were transferred on Green yellow color (GYC) agar plates with a sterile bent rod inside the laminar flow under sterile condition. Then, all the GYC plates were incubated at 30 °C for 48 hours. Colonies with a distinct halo on GYC have been selected with a high possibility of isolating dominant species. After that, applying the streak plate technique, the selected isolates were subcultured [9].

2.3. Morphological Characteristics of Acetic Acid Bacterial Isolates

AAB identification was carried out using standard morphological and cultural testing. The culture isolates were morphologically examined using the protocol outlined in [10]. Following a 48-hour incubation period, morphological features (color, size, shape, and elevation) were examined. Gram's staining was carried out using the method outlined in [10]–[12].

2.4. Biochemical Characterization of Acetic Acid Bacterial Isolates

The catalase and oxidase tests were carried out to distinguish the microorganisms. In the catalase test, the appearance of a bubble within a few seconds indicated a positive result, whereas its absence indicated a negative result [13]. The oxidase test results were obtained by recording the blue color development on the bacterial

colony swab using the procedure outlined in [14]. On Carr medium, *Acetobacter* and *Gluconobacter* were differentiated from one another in the presence of bromocresol green. Whereas *Gluconobacter* makes the medium yellow, *Acetobacter* first turns it yellow and then green. According to [15], the ability of *Acetobacter* and *Gluconobacter* to produce acid from calcium carbonate is apart from the *Acetobacteriaceae* family.

2.5. Growth of Culture Isolates (Both A and B) on Different Concentrations of Ethanol and Glucose

Different levels of ethanol (1%, 2%, 5%, and 10%) and D-glucose (10%, 20%, 25%, and 30%) were used in GYC medium to observe on the growth of culture isolates incubated at 30 °C for 48 hours.

2.6. Growth of Culture Isolates (Both A and B) at Different Temperatures and Nutrient Agar Medium

GYC medium plates were inoculated using the streak plate method and incubated for 48 hours at different temperatures (20 °C, 25 °C, 30 °C, and 37 °C). Culture isolates were observed for 48 hours, and the results have been recorded [16]. On nutritional agar medium, the isolate was cultivated at the highest possible temperature.

3. RESULTS AND DISCUSSION

3.1. Isolation of AAB

Acetobacter spp. oxidizes glucose and produces acid, which react with CaCO_3 present in the media and dissolve it. Colonies with a distinct halo on GYC medium were chosen with a high possibility of isolating dominant species [17]. Thus, the AAB (acetic acid bacteria) was confirmed by the clear zone found in the plate. Colonies were continuously sub-cultured on the surface of GYC medium until a pure culture growth was obtained. Isolation of AAB (acetic acid bacteria) from different fruits is presented in Fig. 1.

Among the different natural sources, pineapple source possessed the maximum AAB (acetic acid bacteria) producers, followed by sugarcane, grape, apple, papaya and pomegranate. Therefore, the least producers were recorded with pomegranate. The highest AAB (acetic acid bacteria) producers were pineapple (38.46%), followed by sugarcane (22.90%), grape (18.29%), apple (14.63%), papaya (4.34%) and pomegranate (1.38%). The variation might result from differences in how sugar and alcohol are used in the fermentation medium and how well AAB can tolerate acetic acid [18]. Investigated 99 acetic acid bacteria isolates from 18 different fruit varieties and 4 fermented fruit juices using sterile distilled water as an enrichment medium that was enhanced with 4.00% ethanol (v/v) The genus *Acetobacter* was found to have eighty-nine isolates. Additionally, [19] showed that out of thirteen fruits, eleven pineapple fruits contained 10 identified *Acetobacter* sp. The results of the current experiment were comparable to those of the previous one. According to the findings, pineapple is the fruit that is most ideal for isolating acetic acid bacteria (AAB).

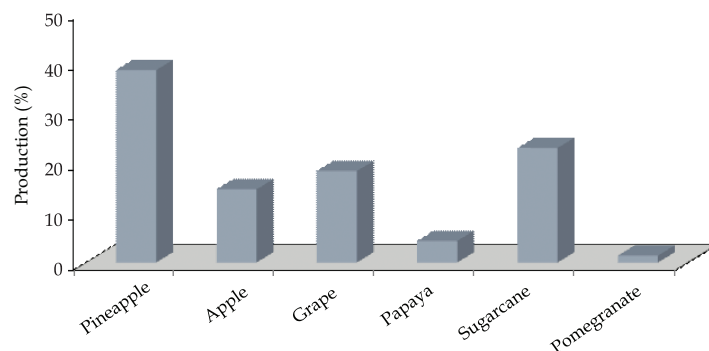


Fig. 1. Isolation of acetic acid bacteria (AAB) from different fruit sources.

TABLE I: COLONY CHARACTERISTICS OF THE STRAIN

Color	Shape	Size	Elevation	Surface
Pale to off white and moist	Circular	Less than 3 mm in diameter	Convex	Smooth

TABLE II: MORPHOLOGICAL AND STAINING PROPERTIES OF ACETIC ACID BACTERIA

Shape	Arrangement	Gram's staining properties	Color (after gram's staining)
Small rods and cocci	Single, in pair and in short chain	Gram (–)ve	Pink and red

3.2. Identification of AAB (Acetic Acid Bacteria) by Morphological and Colony Characteristics

After inoculating the cultures on the standard medium of GYC agar plates, the colonies were observed after an incubation period of 48 hours. The cultural and morphological characteristics of colonies are presented in Table I. The colonies were circular in shape, and their size varied from 1 mm to 3 mm with convex elevation. The colours of the colonies were pale to off-white. The surfaces of the colonies were smooth and moist. Staining characteristics of strain A (strain isolated from fruits in laboratory) and strain B (reference strain DSM-2324) recorded after gram's staining are presented in Table II. Similar results were also obtained by [14], [16], [20]–[22]. who also found that *Acetobacter* sp. are morphologically gram negative rods.

3.3. Identification of AAB (Acetic Acid Bacteria) by Biochemical Tests

For the identification of AAB (acetic acid bacteria), the broth was further analyzed on the basis of biochemical and enzymatic tests (Catalase and Oxidasetest) as recommended by [23], [24]. All the isolated strains were positive for the acid and gas production test (Table III). All the isolated strains were positive for the mobility test in the hanging drop slide. That means all the isolates have peritrichous flagella. It is reported [15] that the ability to produce acid from calcium carbonate allows *Acetobacter* and *Gluconobacter* to be differentiated from the *Acetobacteriaceae* family.

3.4. Distinguishing Between *Acetobacter* and *Gluconobacter*

The two genera of acetic acid bacteria, *Acetobacter* and *Gluconobacter*, have been distinguished from one another on Carr medium when bromocresol green was present. Whereas *Gluconobacter* turns the medium yellow, *Acetobacter* first turns it green [15]. In Carr media, all isolated strain exhibited positive results (Fig. 2). The medium's color changes from green to yellow.

3.5. Effect of Ethanol and Glucose on the Growth of Strain A and Strain B

Different levels of ethanol (Figs. 2A, 2C) and glucose (Figs. 2B, 2D) were used in a standard medium to observe the effect of those concentrations on the growth of strain A (Figs. 2A, 2B) and Strain B (Figs. 2C, 2D). From the values of OD (Optical density), it was found that both strains grow well at 2% ethanol and 20% glucose than others. Both strains show similar trends in that they grow faster in 2% ethanol and 20% glucose concentration. In 1% and 5% ethanol concentration, they grow well, but in 10% ethanol concentration, they cannot grow. References [16], [23] also observed similar results that no growth occurs when 10% alcohol was added. They recommended that the level of alcohol tolerance (maximum 5%) for identification of AAB. Reference [15] recommended that the optimum condition of a medium composed of 3% ethanol concentration, which more or less matches with the present experiment, but [25] stated that the concentration of ethanol did not have an effect on the acetic acid bacterial

TABLE III: BIOCHEMICAL IDENTIFICATION OF THE ISOLATES

Sl. no.	Isolates	Catalase test	Oxidase test	Acid and gas production	Mobility	Colonies appearance
1	Strain A	+	–	+	+	White with granulated surfaces
2	Strain B	+	–	+	+	with clear zones around colonies

Note: Strain A (strain isolated from fruits in laboratory) and strain B (Reference strain DSM-2324).

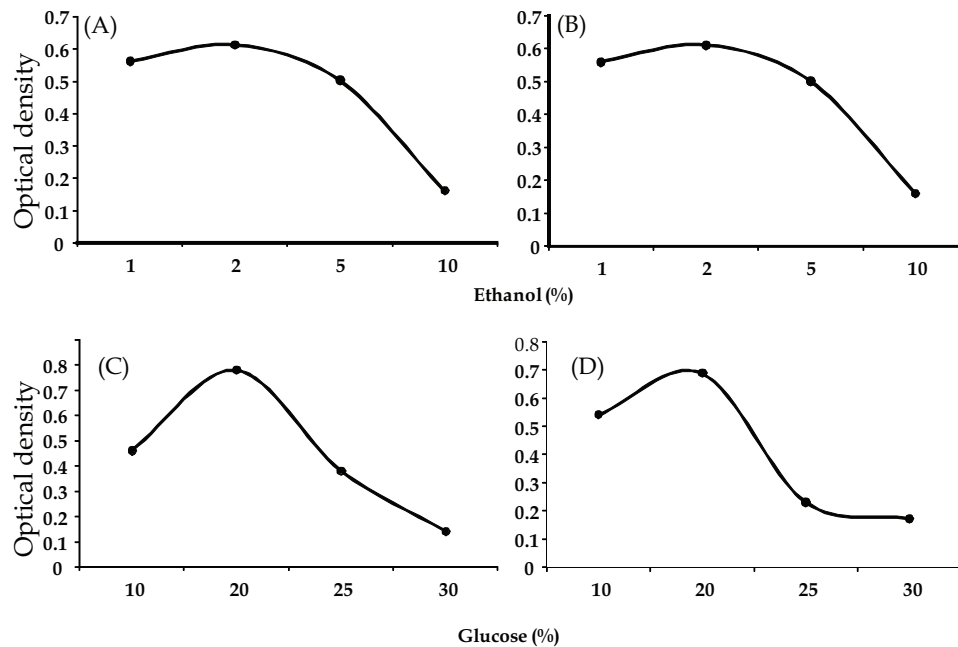


Fig. 2. Optical density of strain A (A, C); at different levels of ethanol (A, B); and glucose (C, D).

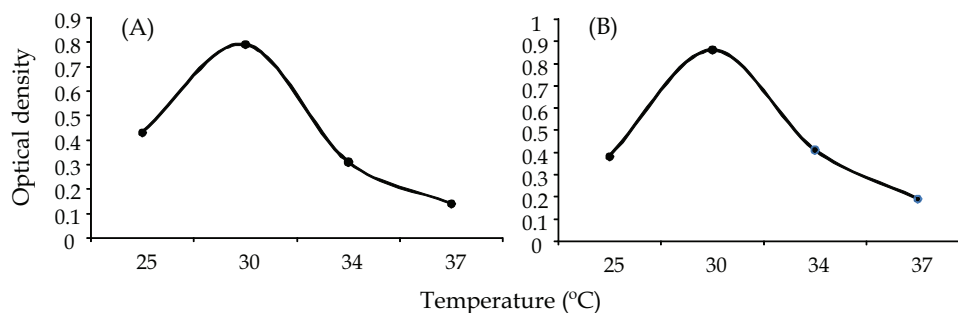


Fig. 3. Optical density of strain A (A) and strain B (B) at different temperatures.

growth. They also reported that the glucose tolerance of the strain of *Acetobacter* sp. showed that the growth of acetic acid bacteria was important in a juice with a high concentration of sugar. Both strains exhibit similar results on different glucose concentrations.

3.6. Effect of Different Temperature on the Growth of Strain A and Strain B

Acetic acid bacterial cultural isolates were characterized by incubating them at different temperatures, i.e., 25 °C, 30 °C, 34 °C, and 37 °C. After 48 hours of incubation, a large number of growths were found at 30 °C, while moderate growth was found at 25 °C and 34 °C. No growth was found at 37 °C. From the results of optical density, it is clear that the strain grows more at a temperature of 30 °C (Fig. 3).

The growth was confirmed by the clear area found on the plates. The clear area indicates more acetic acid bacterial culture growth. Reference [16] found that the acetic acid bacterial cultures could grow at 28 °C and 34 °C but could not grow at 37 °C. Reference [15] stated that the thermotolerant strains were able to oxidize ethanol at high temperatures (38 °C–40 °C) and ethanol concentrations (up to 9%) without any appreciable lag time; they worked rapidly with a higher fermentation rate, whereas

mesophilic strains were unable to do this. Reference [26] Two strains were selected for their ability to grow at 40 °C and 45 °C and proposed to produce artisanal spirit vinegar. Pure culture of vinegar (Both Strain A and Strain B) did not show growth on nutrient agar plates. The results obtained are similar to the findings of [23], which stated that in the media without a carbon source for growth, e.g., yeast extract broth, peptone broth or nutrient agar, no growth of AAB (acetic acid bacteria) occurs.

4. CONCLUSION

Fruits such as pineapple, sugarcane, apple, grape, and papaya can be used to identify and isolate acetic acid bacteria, or AAB. Pineapple appeared to be the best fruit sample for isolating acetic acid bacteria based on the results of six fruit samples. Based on their ability to produce acid from calcium carbonate, *Acetobacter* and *Gluconobacter* were distinguished from the *Acetobacteriaceae* family. They might be distinct from one another using Carr media. Whereas *Gluconobacter* turns the media yellow, *Acetobacter* turns it yellow and eventually green. It is also evident from the findings that strains A and B show similar trends at various temperatures and ethanol and glucose concentrations. This research confirms the

existence of representatives of the genus *Acetobacter* and *Gluconobacter*.

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CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

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