Original Research Article

CHARACTERIZATION OF ACETIC ACID BACTERIA ISOLATED FROM SOME SELECTED WASTE PROTEIN SOURCE

ABSTRACT

Background: AABs are an essential and diverse group of bacteria involved in producing fermented foods and beverages, primarily known for their production of acetic acid (ethanoic acid) used in vinegar making.
Aim: This study aims to isolate Acetic Acid Bacteria from protein waste and quantify the acetic acid produced; it is research carried out as a means of protein waste management/recycling to conserve the environment/society at large while producing the value-added product; acetic acid from waste protein sources (fish, milk, meat, egg, soy milk, beans and unpasteurized milk).
Methodology: The samples were homogenized in sterile distilled water, followed by serial dilution and inoculation on GYC agar using the spread plate method with an inoculum size of 0.1 ml. The plates were incubated at 30^{o}C for 48 hours. Several biochemical tests, including oxidase, catalase, and Gram stain, were done on the isolates. The acetic acid production rate was estimated using the YGEA medium.
Results: Twenty-five (25) (white, creamy and yellow pale-coloured) well-isolated colonies were selected to be analyzed. Out of 25 isolates, fourteen (14) suspected acetic acid bacteria because they produced clear zones around their colonies in the selective GYC culture medium. Of the 14 analyzed isolates, six (6) were Gram-negative rods. Presumptive identification of the acetic acid bacterial isolates included *Acetobacter aceti* and *Gluconobacter oxydans*. Morphologically identified and biochemically confirmed acetic acid bacterial isolates were then further checked for their abilities to produce acetic acid by titration in YGEA (Yeast, Glucose, Ethanol, Acetic acid) medium. Acetic acid production rate varied among the isolates. Among three (3) acetic acid-producing strains, isolates from fish 2 showed the highest acetic acid production (24.34 g/100ml) rate at 37^{o}C, while those from milk 1 and meat 2 showed 23.96 g/100ml and 23.46 g/100ml, respectively.
Conclusion: This study is based on the importance of AAB in converting low-value protein waste to value-added products such as acetic acid, ascorbic acid, cellulose and others. This is due to their ability to use protein waste as a substrate.

Keywords: *Acetobacter aceti, Acid Bacteria, Gluconobacter oxydans, Protein Waste*

ABBREVIATION
AAB: Acetic acid bacteria
GYC: Glucose yeast calcium carbonate
YGEA: Yeast glucose ethanol acetic acid

1. INTRODUCTION

Louis Pasteur first described acetic acid bacteria (AAB) as vinegar bacteria. They constitute a diverse group of bacteria known for their importance in producing fermented foods and beverages, especially in the production of acetic acid (ethanoic acid) in vinegar making [1]. AAB are obligatory aerobes usually found in sugary, acidic and alcoholic niches belonging to the Alphaproteobacteria class, Rhodospirillales order, and Acetobacteraceae family [2]. They are known for oxidizing carbohydrates, alcohols and sugar alcohols (polyols) into their corresponding organic acids, aldehydes or ketones [3]; this process is known

as oxidative fermentation. Acetic acid bacteria are motile, ellipsoidal to rod-shaped gram-negative bacteria occurring singly, pairs or chains. They move with the aid of either polar or peritrichous flagella and do not form endospores as defensive resistance. They are obligate aerobes with oxygen as the terminal electron acceptor in the electron transport chain. They grow at an optimum pH of 5-6.5 but can also grow at lower pH values between 3-4. They are catalase positive and oxidase negative with sizes ranging from 0.4-1 µm wide and 0.8-4.5 µm long. They are mesophiles having an optimum growth temperature between 25 and 300C. They produce different polysaccharides and pigments in solid cultures [4,5].

An important characteristic that makes AAB desired industrially is its ability to oxidize ethanol to acetic acid. Two membrane-bound dehydrogenases catalyze this process; pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase (ADH) and membrane-bound aldehyde dehydrogenase (ALDH), located near ADH [6,7]. On acetic acid production, some genera of AAB further oxidize acetic acid to CO_2 and H_2O. This property is known as over-oxidation of ethanol and is customarily used to distinguish Gluconobacter, which does not have the capability. Over-oxidation depends on the medium composition, mainly when bacteria use ethanol [8].

Aside from the use of AAB in vinegar making and fermented foods, they are harnessed industrially to produce relevant compounds, primarily ascorbic acid (vitamin C) and miglitol (antidiabetic drug) [9]. They also have an essential role in cocoa fermentation [10]. Also, species of the genera Acetobacter and Gluconacetobacter are used in agriculture, where nitrogen fixation is attributed to strains of G. diazotrophicus. Furthermore, some genera of AAB are known for their production of exopolysaccharides (EPS) and bacteria cellulose. The absence of hemicellulose and lignin in bacteria cellulose offers an advantage over conventional cellulose [11]. Gluconic acid, sorbose and ascorbic acid are also products of AAB metabolism. Due to the importance of AAB metabolic products, many kinds of research have been carried out. However, most researchers focus on their isolation from sugary substrates and alcohol. In contrast, this study emphasizes identifying acetic acid bacteria isolated from waste protein waste as waste management. In addition to waste management, this study aims to isolate AAB from protein wastes like milk, fish, egg and meat, estimate the amount of acetic acid produced, and show the transformation of waste (protein waste) to wealth (acetic acid).

2. MATERIALS AND METHODS

2.1. Study area

Umuahia is the capital city of Abia State in South-Eastern Nigeria, coordinating latitude 5^{o} 32'N and longitude 7^{o} 29'E on the Northern and Eastern hemispheres respectively, located along the railroad that lies between Port-Harcourt to the South and Enugu city to its North.

2.2. Media Used

Glucose yeast extract calcium carbonate medium (GYC), peptone water, yeast glucose ethanol acetic acid medium, triple sugar iron agar (TSI), Simmon citrate agar, nutrient agar. All media were prepared according to manufacturers' instructions.

2.3. Sample Description and Sampling Procedure

The samples used are waste protein products (fish, milk, meat, egg, soy milk, beans and unpasteurized milk). Two different samples of each protein waste listed in the bracket were collected randomly from different areas in Umudike, Umuahia. Each sample was collected aseptically and handled carefully to ensure no change between sampling and analysis. The seven samples were taken to the laboratory, where the culture was done immediately.

2.4. Preparation of Samples and Isolation of Acetic Acid Bacteria

In the isolation of acetic acid bacteria (AAB), each sample (1 ml/ 1g) was suspended in 9 ml sterilized distilled water and mixed thoroughly. Serial dilutions (10^{-3} & 10^{-7}) were prepared, and 0.1ml of each diluted sample was spread on glucose yeast extract $CaCO_3$ (GYC) medium plates. All plates were incubated aerobically for 2- 5 days at 30°C, and colonies with clear zone were isolated.

2.5. Purification and Maintenance of the Microbial Isolates

The streak plate method obtained pure cultures of the AAB isolates by sub-culturing in fresh GYC medium. The final cultures containing discrete colonies were maintained by transferring onto McCartney bottles containing agar slants, and they were stored in the refrigerator at 4^{o}C for further analysis.

2.6. Identification of AAB Using Biochemical Tests

Acetic acid bacteria species, after isolation, were identified based on microbiological, cultural and biochemical properties as described by Cheesbrough [12]. Biochemical tests include Gram's Staining, catalase test, oxidase test (cytochrome oxidase test), sugar fermentation test, indole test, citrate utilization test, and hydrogen sulfide test.

2.7. Endorespore Staining

A smear of the organism was made on the slide, then saturated with malachite green stain solution and steamed for 5 minutes keeping blotting paper moist and adding more dye as required. Then the slide was washed under the tap and then counterstained with 0.5 safranin for 30 seconds, washed again and blotted dry and then observed under the microscope. Endospores stain bright green while vegetative cells stain pink.

2.8. Acetic Acid Production

The selected colonies from GYC were transferred to peptone water until OD_{600} of 0.5 was achieved. Inoculum size of 4% from the preceding peptone water was cultured in yeast glucose ethanol acetic acid medium for acetic acid production. The production medium was aerated, and samples were taken at 48 hours intervals.

2.8.1. Estimation of acetic acid: 5ml of the culture was mixed with 20ml of distilled water. 3-5 drops of phenolphthalein were added. The solution was titrated against 0.05N NaOH. The amount (g) of acetic acid produced in 100ml of the medium was calculated using the formula:

Acetic acid (g/100ml) = volume of NaOH (ml) used in titration * 0.03 * 20

3. RESULTS

3.1 Macroscopic Characteristics of Isolates

The cultural characteristics observed on the GYC medium is shown in Table 1. Different colonies were observed with different shapes as below.

Table 1: Cultural appearance of colonies.

Sample	Appearance on agar
Raw milk 1	Large creamy/ milk coloured colonies were observed.
Raw milk 2	Creamy colonies with a clear zone around them.
Beans 1	Creamy mucoid colonies with clustered growth.
Beans 2	No growth appeared.
Meat 1	Creamy large elevated colonies with a clear zone around the colonies.
Meat 2	Large creamy convex colonies observed.
Milk 1	Creamy large elevated colonies were observed.
Milk 2	Creamy elevated colonies were observed.
Fish 1	Creamy mucoid colonies with clustered growth.
Fish 2	Tiny creamy colonies and yellow colonies were observed.
Egg 1	Large creamy elevated colonies appeared on plates.
Egg 2	Yellow colonies and creamy colonies observed.
Soy milk 1	Creamy large elevated colonies with a smooth edge were observed.
Soy milk 2	No growth observed.

3.2 Growth of Colonies Per Time

Table 2 shows the time for visible growth to be noticed on each media plate. There was no growth on all plates till after 48 hours. However, colonies appeared at 72 hours on some medium plates while there was no growth on two plates (Beans 2 and Soy milk 2) even after 144 hours.

Table 2: Growth of colonies per time

Sample No	Growth of Colonies/ Time					
	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs
Raw milk 1	No	No	Yes	Yes	Yes	Yes
Raw milk 2	No	No	Yes	Yes	Yes	Yes
Beans 1	No	No	Yes	Yes	Yes	Yes
Beans 2	No	No	No	No	No	No
Meat 1	No	No	Yes	Yes	Yes	Yes
Meat 2	No	No	Yes	Yes	Yes	Yes
Milk 1	No	No	Yes	Yes	Yes	Yes
Milk 2	No	No	Yes	Yes	Yes	Yes
Fish 1	No	No	Yes	Yes	Yes	Yes
Fish 2	No	No	Yes	Yes	Yes	Yes
Egg 1	No	No	Yes	Yes	Yes	Yes
Egg 2	No	No	No	Yes	Yes	Yes
Soy milk 1	No	No	No	Yes	Yes	Yes
Soy milk 2	No	No	No	No	No	No

3.3 Biochemical Characterization Tests for AAB

Isolates with a clear zone around their colonies were presumed to be AAB and subjected to further biochemical tests for confirmation. Table 3. shows the reaction of selected isolates to biochemical tests, including catalase test, oxidase test, Gram stain, H2S test, Gas production, Indole, and Citrate test.

Table 3: Characterization tests for acetic acid bacteria (AAB)

Sample	Catalase	Oxidase	Gram stain	Cell morphology	H_2S	Gas	Indole	Citrate
Raw milk 1	+	-	+	Cocci	-	-	-	+
Raw milk 2	+	+	-	rod	-	-	-	-
Beans 1	-	-	+	rod	-	-	-	-
Meat 1	+	-	+	Rod	+	-	-	+
Meat 2	+	-	-	rod	-	+	-	+
Milk 1	+	-	-	Rod	-	+	-	+
Milk 2	+	-	+	cocci	+	-	-	+
Fish 1	+	+	-	Rod	-	+	-	+
Fish 2	+	-	-	rod	-	-	-	+
Egg 1	+	+	-	Rod	+	-	-	+
Egg 2	+	-	+	Cocci	-	-	-	-
Soy milk 1	+	-	+	rod	-	-	-	-

+ = positive, - = negative.

3.4 Estimation of Acetic Acid Production.

Isolates whose biochemical characteristics agree with Bergey's manual of determinative bacteriology were cultured using Yeast glucose ethanol acetic acid (YGEA), and the amount of acetic acid produced by each isolate was estimated by titration. Tables 4, 5, and 6 shows the amount of acetic acid produced in g/100ml

Table 4: Estimation of acetic acid produced in milk 1

Initial (ml)	Final (ml)	(ml)
0.00	40.30	40.30
0.00	39.70	39.70
0.00	39.80	39.80

40.30 + 39.70 + 39.80 = 119.80; 119.80/3 = 39.93

Acetic acid (g/100ml) = volume of NaOH (ml) used in titration * 0.03 * 20

Acetic acid (g/100ml) = 39.93*0.03*20 = 23.96

Table 5: Estimation of acetic acid produced in fish 2.

Initial (ml)	Final (ml)	(ml)
0.00	40.00	40.00
0.00	41.30	41.30
0.00	40.40	40.40

40.00 + 41.30 + 40.40 = 121.70; 121.70/3 = 40.57

Acetic acid (g/100ml) = 40.57*0.03*20 = 24.34

Table 6: Estimation of acetic acid produced in meat 2

Initial (ml)	Final (ml)	(ml)

0.00	39.00	39.00
0.00	39.50	39.50
0.00	38.80	38.80

39.00 + 39.50 + 38.80 = 117.30; 117.30/3 = 39.10

Acetic acid (g/100ml) = 39.10*0.03*20 = 23.46

4. DISCUSSION

Just as AAB is commonly isolated from a diversity of AAB in different fruits, vegetables, soil, water, and carbohydrate sources [13, 14], this study shows that protein-rich foods harbours AAB and can be used as a substrate to produce other value-added products. In the present study, acetic acid bacteria that comprise a large group of obligate aerobic Gram-negative bacteria commonly found in association with various sugary materials were isolated and characterized from the various protein wastes in Umudike, Umuahia. For this reason, various protein waste samples were targeted. A total of 25 (white, creamy and yellow pale coloured) colonies on GYC agar plates were derived from protein waste samples collected from various markets of Umudike (Table 1). Out of 25 isolated colonies, 14 suspected acetic acid bacteria were screened based on their morphological, cultural and biochemical properties. The fourteen strains were primarily screened and detected as acetic acid bacteria because they were small, white, raised and produced clear zones around their colonies in the selective GYC culture medium. The property of clear zones around their colonies agrees with the findings of Trcek [15] and Assietta *et al.* [16]. However, because clear zone can be produced by other strains such as lactic acid bacteria [14], further biochemical tests were carried out to confirm the presence of AAB. The theory of using $CaCO_3$ in the media was that if the grown bacteria on the media are acetic acid bacteria, the AAB will produce acetic acid to react with $CaCO_3$ to form Calcium acetate ($Ca(CH_3COO)_2$), water and carbon dioxide. The calcium acetate is more soluble in water than $CaCO_3$ with a solubility of 34.7 g/100 mL (20 °C), which made the clear zones around AAB colonies [17].

Examination using Gram staining revealed that 6 isolates were Gram-negative rods. Following Gram stain, biochemical tests such as oxidase, catalase, Indole, and hydrogen sulfide were performed using the selected 6 Gram-negative bacterial isolates according to the standard guideline of Bergey's manual of

determinative bacteriology [18]. Several biochemical characterizations, including negative oxidase and positive catalase tests (Table 3), suggested that our isolates from 3 samples (milk 1, fish 2 and meat 2) are acetic acid bacteria. These characteristics agree with those of Gullo M. and Giudici, P. [19], Beheshti and Shafiei [20], Farzana *et al.* [21] and Zahoo *et al.* [22].

Morphologically identified and biochemically confirmed acetic acid bacterial isolates were then further checked for their abilities to produce acetic acid by titration in YGEA (Yeast, Glucose, Ethanol, Acetic acid) medium. Acetic acid production rate varied among the isolates at different incubation periods. Three acetic acid-producing strains isolated from fish 2 showed the highest acetic acid production (24.34 g/100ml) rate at 37˚C (Table 5). In contrast, those from milk 1 and meat 2 showed 23.96 g/100ml and 23.46 g/100ml, respectively this is similar to the findings of Lin *et al.* [23] and Beheshti and Shafiei [20] but contrary to research done by Farzana *et al.* [21], this could be because they used a different substrate (decomposed Fruits) in their study.

5. CONCLUSION

Most previous studies show that acetic acid bacteria are commonly associated with various kinds of sugary materials and alcohol. However, AABs were isolated and characterized from waste protein foods such as fish, meat, and milk in this study. This shows that these protein waste sources can serve as a substrate for AAB growth. In these findings, these wastes can be converted to wealth by applying AAB to convert many low-cost protein wastes to high-value products, including acetic acid, bacterial cellulose, vitamin C and other exopolysaccharides, industrially important. This is also important in waste management and environmental conservation.

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