J. Biol. Chem. Chron. **2019**, **5***(1)*, 123-129
ISSN (Print): 2454 – 7468
ISSN (Online): 2454 – 7476
www.eresearchco.com/jbcc/

Isolation and Characterization of Acetoacetic Bacteria from Decomposed Fruits

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DOI: http://dx.doi.org/10.33980/jbcc.2019.v05i01.020

(Received 16 Feb, 2019; Accepted 20 May, 2019; Published 22 May, 2019)

ABSTRACT: Acetic Acid Bacteria (AAB) are rod shaped, aerobic, and gram-negative bacteria. These have ability to oxidize ethanol to acetic acid and are used in vinegar production. The aim of this study was to isolate and identify the acetic acid bacteria which are capable of growing at 30-40°C, temperature generally prevalent in this area for most of the year. Microorganisms were isolated from decomposed fruits like banana, pear, and orange obtained from local vegetable market. We used specific culture media viz. isolation, enrichment and peptone agar media. The samples were inoculated in sterilized GYC standard media and then incubated at 30°C for 48 hours. Successive subculture was performed to screen out the strains. In Gram's staining, the morphology of the isolated bacteria exhibited pink and rod shaped. Various biochemical tests of the isolated strain like starch hydrolysis, methyl red, and keto genesiswere positive, while starch hydrolysis and cellulose test were negative which revealed that the strain isolated from decomposed fruits were acetic acid bacteria. The study indicated that all the strains grew well at pH 5-6 and temperature28°Cto30°C.

Keywords: Acetic acid; fruits; fermentation; temperature; isolation and characterization.

INTRODUCTION: Acetic Acid Bacteria (AAB) are rod shaped, aerobic and gram-negative bacteria. They have ability to oxidize ethanol to acetic acid. They are widely distributed in natural habitats and classified into the family acetobacteraceae. Members of this family are useful in Industrial production of vinegar.[1,2&3] In past there were two main genera-Acetobacter and Glucanobacter but at present there are twelve genera which are in the family acetobacteraceae i.e. Acetobacter, Glucanobacter, Acidomonas, Gluconaerobacter, Asaia, Kozakia, Swaminathania, Saccharibacter, Neoasaia, Granulibacter, Tanticharoenia and Ameyamaea.[4] The genus acetobacter that is primarily used in vinegar manufacturing. The main difference between *Acetobacter*and *Gluconobacter*is that *Acetobacter*can oxidizes acetate to CO_2 and H_2O, while *Gluconobacter* cannot oxidize it.

Vinegar is defined as a 4%acetic acid solution produced by the alcoholic fermentation process using sugary substances by acetic acid producing bacteria. These are very important in industrial production of vinegar. These bacteria are generally found in nature associated with some fruits such as grapes and are normally present in decomposed fruits. There are several factors that affect the growth and survival of AAB. These include ethanol concentration, acetic acid concentration, oxygen, temperature and nutrient availability are the most important factors that can affect the survival of AAB. The thermo tolerant strains were able to oxidize ethanol concentration without any appreciable lag time; they worked very rapidly with a higher fermentation rate.

Acetobacteraceti has not yet been reported as a pathogenic microbe to humans or animals. It does not produce any toxins, enzymes, or any viruses that harm any human or animal. It is ubiquitous in nature and there is contact of this bacterium on all animals on a frequent basis. *Acetobacteraceti* is known to cause rotting and browning discoloration in fruits such as apples, pears and citrus products. These bacteria can be isolated from vinegar alcoholic beverages, decomposed fruits, flowers, honey, sugarcane, fruit juice, soil and water etc.[3 & 5] There are various factors that affect the growth and survival of acetic acid bacteria like ethanol concentration, pH, oxygen, temperature and availability of nutrients, carbon source etc. De-

composed fruits are a good source for isolation of acetic acid bacteria. AAB are generally found in nature because they can use a variety of substrates.[3] These bacteria have been isolated from alcohol, vinegar, citrus fruits, sugar cane, and fruit juice.[3 & 5]

Therefore, the present study was undertaken on isolation and identification of AAB from various fruits and to shed some light on different morpho-physiological and biochemical properties of isolated.

MATERIAL AND METHODS: This present study was carried out in Department of Biotechnology, Dayanand College, Hisar on Acetic Acid Bacteria. The experimental study material for the present study was different fruits like pear, banana and orange were collected from the local vegetable market. The different growth media used in the present study for isolation of acetic acid bacteria producing strains for their maintained optimization of cultural conditions and for production of acetic acid were (1) Isolation media, which contains 1% Yeast extract, 2% glucose, 0.005%, $MgSO_4.7H_2O$, 0.01% Di-Potassium hydrogen phosphate. (2) Enrichment media contains YE10gm, alcohol 30 ml, Bromocresol green 0.002gm, magnesium sulphate 0.5 gm, Dipotassium hydrogen phosphate 0.1 gm, agar 10gm per liter. Pure cultures were preserved on peptone Agar media.

At first, for the isolation of bacteria, approximately 2 g of fruits were cut and incubated in 25 ml of liquid media for isolation of AAB and incubated at 30degree Celsius for 7 days till pellicle formation takes place. After primary culture, a small pellicle was further crumbled and inoculated on solidified enrichment culture medium and incubated at 30°C for 7 days. Appearances of green zones were observed as an indication of acetobacter growth. These colonies were purified till we got the single cell colonies. then pure cultures were preserved on Peptone agar media (YE 0.5g, NaCl 1g, peptone 1g, agar 4g, per 200 ml distilled water) incubated at 30°C.

Determination of acetic acid production by isolated strains: The culture was (5 ml) mixed with 20 ml of distilled water. Three-five drops of phenolphthalein indicator were added. The solution was titrated against 0.5 N NaOH. The amount of acetic acid was produced was calculated.

Gram's staining: Gram's staining was performed for morphological characteristics and staining reaction (+ve, -ve) of isolated strain. The pure cultures of bacterial strains were put for gram staining for specific identification of the colonies. The gram staining was done in laminar air flow hood. The slides were firstly cleaned with ethanol and colonies were put on the slides with the inoculating needle and heat fixed. Then smears were stained in following steps: (a) First applied crystal violet on each slide and kept for 1 min. (b) Distilled water wash. (c) Iodine on the slides as mordant (1 min) then 95% alcohol wash (30 sec.) and then washed with distilled water. (d) Safranin was applied on the slides and then washed with distilled water and (e) air dried the slides. The entire gram staining technique was done following the Christian Gram technique (1989).[6]

Biochemical and physiological examination of the isolates:

pH variation assay: The ability of acetic acid bacteria to grow at different pH was tested in Yeast extract peptone agar medium by adjusting the pH to 5.0, 6.0, 7.0, 8.0 with NaOH and HCl.

Temperature tolerance: Temperature Tolerance was investigated by assaying the growth of bacterial cultures in Yeast extract peptone agar medium at different temperature viz. 10° C, 25° C, 35° C and 40° C.

Effect of metal salts: The isolate was tested for their sensitivity to metal by amendment of freshly prepared Yeast extract peptone agar media plates with metal salts i.e. $HgCl_2$ and $ZnSO_4$ at 1 % (w/v) concentration. Effect of metal salts was determined by observing bacterial growth after incubating the plates at 30°C for 48 hours.

Starch hydrolysis test: This test was performed to determine the capability of acetic acid bacteria to use starch as a carbon source. Bacteria was inoculated in Starch agar medium and analyzed for starch utilization. Iodine test was used to determine the capability of acetic acid bacteria for starch utilization. A drop of iodine (0.1N) was spread on 24 hours incubated culture and clear zone of inhibition was formed.

Mannitol Motility Agar test: This test was used to detect whether the inoculated organism is motile. Mannitol Motility Agar media was prepared, dispensed into test tubes in upright position and allowed to solidify. The bacterial colony was stabbed, inoculated with acetic acid bacteria and incubated at 28°C for 24 hrs. The tube was observed for growth around the stabbed area.

Ketogenesis: Ketogenesis from glycerol was determined by inoculating the isolates in a test tube containing YG medium (3% yeast extract; 3% glycerol) incubated at 30 ° C for 10 days and adding 8-10 drops of Fehling's solution into medium, modified from

Aydin and Akshoy (2009)**7**. The change in medium colour indicated a positive test.

Ammonia by peptone test: The test was performed to check the presence of ammonia. Prepare media having sodium chloride, peptone, potassium, nitrate. Take media in test tube. After 48 hours' inoculation done and add 1ml Nessler reagent in each of the test tubes. The change in color indicates the presence of ammonia.

Nitrate Reduction test: This test was performed to determine the ability of Acetic acid bacteria to produce nitrate reductase which can reduce nitrate to nitrite. The nitrate broth (potassium nitrate, peptone, beef extract) was inoculated with test organism and incubated at 30°C for 24 hrs. Then few drops of nitrate reagent (zinc chloride, starch, potassium iodide) and 1-2 drops of sulphuric acid (1:3; acid: water) to the culture tubes and observe for color change.

Cellulose test: Cellulose production tested on GYE medium (2%glucose; 0.5% yeast extract; 0.25% ethanol 95%) incubated at 30°C for 7 days. Cellulose test was carried out using Lugol's Iodine stain followed by 60% sulphuric acid on cellulose fiber is blue.

Fermentation of carbohydrates: Test was performed to check the effect of fermentation on growth of acetobacterium. Fresh media was prepared and placed in 3 test tube with each sugar glucose, maltose, sucrose. Then, add 1ml bromocersol purple to check the effect.

RESULTS AND DISCUSSION: Acetic acid bacteria were successfully isolated from 3 fermented fruit. All the isolates were Gram-negative, rod-shaped and catalase positive, which belong to the family of the AAB. The ability of acetic acid bacteria to oxidize acetate to CO_2 and H_2O was used to distinguish between members of the genera *Acetobacter* and *Gluconobacter*[8].

Figure 1: Gram's staining of different isolates of acetic acid bacteria.

Isolation and identification of isolates: Gram's staining of the isolates (Fig. 1) was confirmed by microscopic observations and acetic acid bacteria was found to be gram negative. All the isolates were Gram negative, rod shaped which belong to the family of the AAB. Similar results were also obtained by Lisdiyanti *et al.*[9]. Ashecraft *et al*. (2001)[10] who also isolated Acetobacter spp. and found that morphologically these are gram-negative rods and Kadere *et al*. (2008)[11] found *Acetobacteraceti* was motility positive acetic acid bacteria were found to be high in orange sample.

Table 1: Morphology study of Acetobacter.

S. No.	*Acetobacter* strain characteristics
1	Circular
2	2.5mm
3	White
4	Rod-shaped
5	Gram negative
6	Mobile

Effect of salt, pH, and temperature on acetic acid bacteria growth:

Temperature: Temperature optimization is essential for any biotechnological process as bacterial deactivation process can occur above optimum temperature. In our study, we have found that acetic acid bacteria showed good growth at room temperature and least growth 0^0 C or freezing temperature in all the three isolates (Table 2; Fig.2) That means AAB needs normal warm temperature for its growth. Talukder *et al.* (2015) reported that acetic acid bacteria capable of growing at 30-37°Cwere collected from various decomposed fruits.

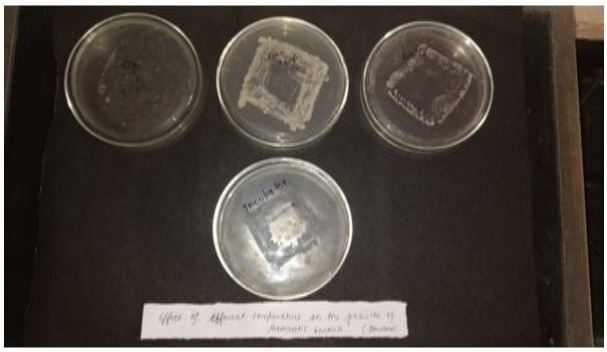

Figure 2: Effect of different temperature on the growth of acetic acid bacteria (banana).

pH: Significant differences were noticed between treatments and strains of acetic acid production is an important parameter for the growth of an organism. In our result, maximum growth of Acetic acid bacteria has been observed at pH 5 and least growth was observed at pH 8 all the three isolates. At pH 5-6 max growth was observed and at pH 8 least growth (Table 3; Fig.3) Kadere e*t al.* (2008) reported that optimal pH

growth of AAB is reported that between 4.5-7. In the present study, the optimum pH for growth and acetic acid production is between 5-7.

Figure 3: Effect of pH on growth of acetoacetic bacteria (pear).

Effect of different metal salts: Many bacteria have developed controlled system to differentiate and cope up with harmful metal ions. (Table 4; Fig.4) represents growth pattern of *Acetobacter* strains towards effect of these metal ions. Assessing the effect of metal salts on Acetic Acid Bacteria on different fruit sample, every sample shows different sensitivity to different metal ions. As like bacteria of pear sample shows more growth on $HgCl_2$ and least /no growth on $MnSo_4$ and bacteria of orange and banana sample shows more growth on $MnSo_4$ and least/no growth on $ZnSO_4$.

Figure 4: Effect of metal on the growth of acetic acid bacteria (orange).

Different sugar test: This test was performed to check the different growth pattern on different sugars. Different plates of different sugars were prepared like glucose, fructose, and maltose. Highest growth of bacteria observed on glucose as compared to other plates. All the five samples showed positive growth in case of glucose. orange showed positive growth on sucrose while banana and pear showed weak growth in case of sucrose (Table 5). So, overall glucose is the best carbon source which showed maximum positive growth of acetobacter. Kim *et al.* 2002 reported fructose was found to be the most effective carbon sources with an optimal concentration of 2%.

Table 2: Response of acetobacter to varying temperature conditions.

Temperature	Fruits		
	Orange	Pear	Banana
0^o C	-	-	-
40^o C	W	+	+
Room Temperature	+	+	+

Symbols: +, 90% or more of the bacteria shows growth; 90% or more bacteria show no growth; W, 40-45% of the bacteria shows growth on particular temperature provided.

Table 3: Response of acetobacter to different pH.

pH	Fruits		
	Orange	Pear	Banana
5	+	+	+
6	+	+	+
7	W	W	-
8	-	-	W

Symbols: +, 90% or more of the bacteria shows growth; 90% or more bacteria show no growth; W, 40-45% of the bacteria shows growth on particular pH provided.

Table 4: Metal salt tolerance of acetic acid bacteria isolated from fruits.

Metals	Fruits		
	Orange	Pear	Banana
$ZnSO_4$	-	+	W
$HgCl_2$	-	+	W
$MnS0_4$	+	-	W

Symbols: +, 90% or more of the bacteria shows growth;90% or more bacteria shows no growth; W, 40-45% of the bacteria shows growth on particular metal salt provided.

Table 5: Response of acetobacter at different sugars.

Temperature	Fruits		
	Orange	Pear	Banana
Fructose	+	+	+
Sucrose	+	W	W
Maltose	+	+	+
Glucose	++	++	+

Symbols: ++, 90% or more of the bacteria shows growth;80% or more bacteria shows growth; W shows moderate growth.

Biochemical characterization: Biochemical characterization of selected isolates was carried out on the basis of different biochemical tests (Table 6).

Starch hydrolysis test: This test was performed to determine the capability of Acetic acid bacteria to use starch as a carbon source (Fig.5). The formation of clear zones and the color of the plates changes to yellow color which indicated the test to be positive.

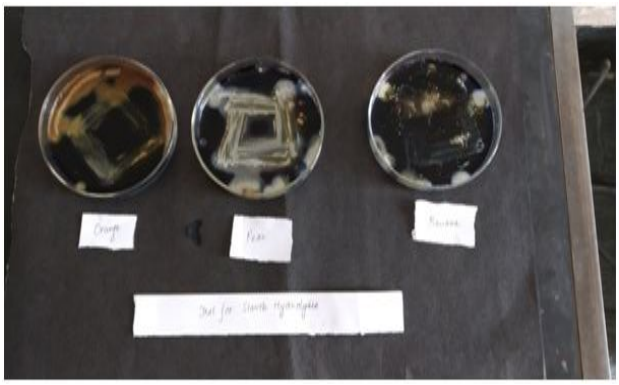

Figure 5: Effect of starch hydrolysis on the growth of acetic acid bacteria.

Methyl red test: The change in the color of test samples different from control indicates the test to be positive.

Mannitol mobility test: This test was performed to detect the Acetic Acid Bacteria isolated is motile or not. After incubation the growth of the Acetic Acid Bacteria was observed in test sample indicated the test to be positive while there was no growth observed in control test tube (Fig. 6).

Figure 6: Effect of mannitol motility agar test on growth of acetic acid bacteria.

Production of ammonia by peptone test: This test was performed to check whether it produces ammonia or not. The color of the media had changed from orange to brown, which indicates the presence of ammonia.

Fermentation of carbohydrates: This test was performed to determine the production of acid as a metabolic waste that is capable of metabolizing the constituent substrate. After incubation, there was no fermentation observed into control whereas the sugars which got fermented shows change in color are glucose and dextrose whereas mannitol, sucrose, maltose, inositol and lactose shows no change in color and did not get fermented.

Nitrate reduction test: This test determines the ability of the Agrobacteria to produce nitrate reductase which converts nitrate to nitrite (Fig.7). No change in color is observed in control while color changes from yellow to orange in sample containing test tube indicating test to be positive.

Figure 7: Nitrate reductase test.

Ketogenesis test: This test was used to detect the presence of acetic acid bacteria. The color of the media has changed from orange to coppery brown indicated the presence of acetic acid bacteria (Fig.8).

Figure 8: Effect of ketogenesis on the growth of acetic acid bacteria.

Lactose assay: This test shows the ability of the Agrobacteria to utilize lactose as a sole carbon source. After incubation, growth is observed on plates indicating the test to be positive.

Cellulose test: This test was performed to check the production of cellulose. We prepared a yeast medium having ethanol and culture for 2-3 days, after that, we

added Lugol's Iodine stain followed by 60% H_2 So_4 then added cellulose fiber blue. We observed that not any kind of change would occur in the media, so the test showed –ve results.

Table 6: Effect of various biochemical tests.

Test	Result	Characterization
Nitrate Reduction Test	+ve	Brick Red color observed
Agar Peptone Test for different sugar	+ve	Microbial Growth observed
Methyl Red Test	+ve	Change in color observed
Ammonia Production Test	+ve	Initial color-Orange
		Final color-Brown
Ketogenesis Test	+ve	Color changed
Lactose Assay	+ve	Growth of microbes due to lactose utilization
MM Agar Test	+ve	Microbes are motile and penetrate inside the agar media
Cellulose test	-ve	
Iodine Test	+ve	Clear zones observed
Starch Hydrolysis Test	-ve	Clear zone of inhibition

Estimation of acetic acid: This test was performed to check the effect on the growth of acetic acid bacteria (Fig.9). We add 5 ml of the culture in 20 ml distilled water with 3-5 drops of phenolphthalein which is further titrated with 0.5 NaOH. Acetic acid was produced and estimated at Acetic acid=vol. of NaOH (0.03) (20).

Figure 9: Estimation of acetic acid.

CONCLUSION: In the present study, an isolation and characterization method of acetic acid producing bacteria has been standardized and can be utilized at commercial scale for the production of vinegar for fruits waste.

ACKNOWLEDGEMENT: The critical editing of the manuscript by Dr. Sumit Jangra is highly acknowledged.

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